PROINFLAMMATORY CYTOKINES AND ELASTASE-α-1-ANTITRYPSIN IN ARGENTINE HEMORRHAGIC FEVER


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Abstract. Argentine hemorrhagic fever (AHF) is a disease caused by Junin virus. In the acute phase, patients present hematologic and neurologic involvement with high levels of interferon-α and tumor necrosis factor-α (TNF-α). Nineteen patients with a confirmed diagnosis of AHF were studied: six severe, four moderate, and nine mild cases. Serum levels of interleukin-6 (IL-6), IL-6 soluble receptor (IL-6sR), IL-8, IL-10, and elastase-α-1-antitrypsin complex (E-α1AT) were assayed by ELISAs. Levels of IL-6, IL-8, and IL-10 were high in nine, 12, and 13 patients, respectively, while levels of IL-6sR were high in two patients and low in one patient. Seven patients had increased levels of E-α1AT. Significant correlations were found between levels of both IL-8 and IL-10 with those of TNF-α as well as between IL-8 and E-α1AT. These data demonstrate activation of pro-inflammatory and anti-inflammatory cytokine pathways, and statistical analysis showed differences among the clinical forms of illness. This study shows that IL-8 plays an essential role in neutrophil activation in AHF patients as demonstrated in other infectious diseases.

Junin virus, a member of the arenaviridae group, is the etiologic agent of Argentine hemorrhagic fever (AHF). This disease mainly affects young farmers in a fertile, rural region known as the humid pampas in Argentina.1 The signs and symptoms of AHF are malaise, fever, hemorrhagic diathesis, and central nervous system (CNS) involvement. A mortality rate of 20% was reduced to less than 1% by infusion of immune plasma obtained from convalescent patients when it was administered within eight days after the onset of fever.2

The hematologic profile is characterized by leukopenia, thrombocytopenia, and abnormalities of the hemostatic system, including increased levels of the early indicators of blood coagulation (thrombin-antithrombin complex and pro-thrombin fragment 1 + 2) and fibrinolysis activation (tissue plasminogen activator, plasminogen activator inhibitory, and D-dimer).3-7 Neurologic involvement includes symptoms such as tongue tremor in those with the mild form of this disease, hyporeflexia or areflexia and mental confusion in patients with the moderate form, and areflexia, muscular hypotonia, ataxia, seizures, and coma in those with the severe form.

Levis and others detected high levels of circulating interferon-α in serum samples taken before treatment, which abruptly returned to normal values after infusion of immune plasma form patients who recovered.8,9 We have previously shown that increased levels of tumor necrosis factor-α (TNF-α) in AHF patients were related to disease severity.10 In contrast, circulating levels of interleukin-1β (IL-1β) were not detected in these patients.

Tumor necrosis factor-α induces the synthesis of IL-6 and IL-8,11-13 Tumor necrosis factor-α and IL-6 were found to be related to the activation of blood coagulation, and IL-6 is a well-known inducer of acute-phase proteins.14-16 The signal transduction associated with IL-6 can be triggered by this cytokine complexed to its soluble receptor (IL-6sR), as well as to the one anchored to the cytoplasmic membrane.17 Interleukin-8 is a chemokine from the C-X-C subfamily with potent neutrophil chemotactic and activating activity.18 Lysozomal degranulation by polymorphonuclear leukocytes can be verified by the detection of circulating neutrophil-derived elastase complexed to its main plasma inhibitor, α1-antitrypsin complex (E-α1AT).19 Interleukin-10 is a potent modulator of monocyte/macrophage function and can inhibit the production of numerous pro-inflammatory cytokines, including TNF-α, IL-1, IL-6, and IL-8, thus acting as a counterbalance to the inflammatory state triggered by infections.20-22

To improve our understanding of the pathophysiology of AHF, we designed a study in a group of patients with this disease to determine if circulating plasma levels of the pro-inflammatory cytokines IL-6 and IL-8, the anti-inflammatory cytokine IL-10, and IL-6sR were enhanced and if neutrophil activation occurred in the acute phase of this illness.

PATIENTS, MATERIALS, AND METHODS

Patients. We studied 19 patients with AHF admitted to the Instituto Nacional de Enfermedades Virales Hemorrágicas (INEVH) in Pergamino, Argentina. The study was approved by the Institutional Ethics Committee of INEVH. Written informed consent was obtained from all patients or their parents. Diagnosis was confirmed by the appearance of anti-Junin virus antibodies or by isolation of Junin virus from patients who died. Patients were grouped according to their clinical picture and the final outcome into nine mild cases (MiCF), four moderate cases (MoCF), and six severe cases (SCF). Patients with mild disease had fever during the first week of illness, and the only sign of CNS involvement was tongue tremor. Those with moderate disease had fever until the second week of the disease with signs of CNS involvement including hyporeflexia or areflexia, mental confusion, somnolency, or both, and discrete ataxia. Severe cases had obvious signs and symptoms of CNS involvement, including severe muscular hypotonia, areflexia, ataxia, convulsions, and coma. Hemorrhagic manifestations were mild, i.e., petechiae, ecchymosis, hematomas, and gingival bleeding. Fever indicated the first day of the disease. Patients were admitted to the hospital within 6–9 days of its onset. Three patients with SCF died of shock syndrome within the first two days of admission to the hospital; the other three sur-
vived. Patients with MoCF and MiCF recovered within 2–3 weeks.

Blood collection. The first blood sample was taken on admission before treatment with immune plasma, and the second one was taken on day 30, while in remission. Blood was collected into tubes without anticoagulant for cytokine assays and into tubes containing 0.066 M EDTA for the E-α1AT assay. Platelet-poor plasma was prepared by centrifuging blood samples at 2,500 × g for 20 min at room temperature. Aliquots of sera and plasma were then stored at −70°C until tested.

Interleukin-6, IL-8, IL-10, IL-6sR, and E-α1AT assays. Levels of IL-6, IL-8, IL-10, IL-6sR, and E-α1AT were measured by ELISAs. The ELISA for IL-6 has been described elsewhere.23 The ELISAs for IL-8, IL-10, and IL-6sR were carried out using commercial ELISA kits (R & D Systems, Minneapolis, MN). The assay for E-α1AT was performed using antibodies described previously.19 The lower limits of detection were 0.7 pg/ml for IL-6, 10 pg/ml for IL-8, 2 pg/ml for IL-10, 0.14 ng/ml for IL-6sR, and 1.0 ng/ml for E-α1AT. Normal values were < 7.5 pg/ml for IL-6, < 31.2 pg/ml for IL-8, < 31.2 pg/ml for IL-10, between 15 and 40 ng/ml for IL-6sR, and < 43 ng/ml for E-α1AT.

Tumor necrosis factor-α assay. Levels of TNF-α were measured with an ELISA (Sigma, St. Louis, MO) to correlate the results with those of the cytokines IL-6, IL-8, and IL-10. Levels less than 15.6 pg/ml were considered normal values.

Statistical analysis. Data are presented as median values and ranges. Spearman’s rank test was used for calculation of correlation coefficient between the variables. To establish differences between the levels of a single variable among the three clinical forms of the illness, Kruskal-Wallis one-way nonparametric analysis of variance was used. The Mann-Whitney Wilcoxon rank sum test with an α descent modification was used to compare data of the various clinical forms of the disease.24

RESULTS

Interleukin-6. As shown in Figure 1, all six patients with SCF had increased levels of IL-6 (median = 381.2 pg/ml, range = 12.9–48,046). Levels of IL-6 were elevated in one patient with MoCF (48.7 pg/ml) and in two patients with MiCF (both = 12.5 pg/ml). The difference between the levels of IL-6 in the patients with SCF and those with MiCF was significant (P = 0.007).

Interleukin-8. Serum levels of IL-8 were markedly increased in five of six patients with SCF (308.5 pg/ml, 10.5–26,610). Increased levels of IL-8 were also found in all four patients with MoCF (258.3 pg/ml, 40.7–716) and in three patients with MiCF (14.2 pg/ml, 10–320) as shown in Figure 2. Significant differences were found between IL-8 levels in the patients with SCF and those with MiCF (P = 0.011).

Interleukin-10. Levels of circulating IL-10 were elevated in five of six patients with SCF (44.5 pg/ml, 7.7–87.8). Three of four patients with MoCF had increased IL-10 levels (22.1 pg/ml, 9.6–42.5) and five of nine patients with MiCF also had high levels of this cytokine (12.1 pg/ml, 5.2–21.2) (Figure 3).

Interleukin-6 soluble receptor. Only two patients had elevated levels of IL-6sR, one with SCF (66.9 ng/ml) and another with MoCF (53.8 ng/ml). Nevertheless, in a larger group of AHF seven patients (30%) of 26 had elevated levels of IL-6sR. Only one patient with SCF had a low level of IL-6sR (6.98 ng/ml), which coincided with the highest levels of the pro-inflammatory cytokines IL-6 and IL-8.
**FIGURE 3.** Interleukin-10 (IL-10) levels in serum samples from patients with Argentine hemorrhagic fever grouped according to the clinical picture. SCF = severe clinical form; MoCF = moderate clinical form; MiCF = mild clinical form. Open diamonds = fatal cases; solid diamonds = patients who recovered from the disease. The gray bar indicates the normal range.

**FIGURE 4.** Levels of elastase-$\alpha$-1-antitrypsin complex (E-alpha1AT) in serum samples from patients in the acute phase of Argentine hemorrhagic fever grouped according to the clinical picture. SCF = severe clinical form; MoCF = moderate clinical form; MiCF = mild clinical form. Open diamonds = fatal cases; solid diamonds = patients who recovered from the disease. The gray bar indicates the normal range.

**Elastase-$\alpha$-1-antitrypsin complex.** This was assayed in only two patients with SCF and three with MoCF, but in all nine patients with MiCF. As shown in Figure 4, the highest E-$\alpha$1AT levels were observed in the patients with SCF (334 and 919 ng/ml); levels were also increased in the patients with MoCF (64, 78.6, and 90.4 ng/ml) and in two of the nine patients with MiCF (53.5 and 55.8 ng/ml). Significant differences were found in the levels of E-$\alpha$1AT between patients with MoCF and those with MiCF ($P < 0.004$).

**Levels of serum parameters on remission.** Circulating levels of IL-8 and E-$\alpha$1AT returned to normal values on day 30 in all patients who recovered. However, one patient with SCF who survived had elevated levels of IL-6 (15.8 pg/ml) and IL-10 (14.6 pg/ml) at recovery. One patient with MiCF had a high level of IL-6 (14.6 pg/ml) and one patient with MoCF had a high level of IL-10 (24.2 pg/ml). The remaining patients had normal levels of IL-6 and IL-10 on day 30 at recovery.

**Tumor necrosis factor-$\alpha$.** Levels of TNF-$\alpha$ measured on admission were elevated in all three patients with SCF evaluated (252, 90, and 48.1 pg/ml), in one of three patients with MoCF (17.3 pg/ml), and in one of nine patients with MiCF (30.7 pg/ml). These data are consistent with those previously reported.\textsuperscript{10}

**Correlations.** Significant correlations were found between levels of TNF-$\alpha$ and those of IL-8 and IL-10 ($r = 0.78$, $P < 0.001$ and $r = 0.87$, $P < 0.001$, respectively). Levels of IL-10 also correlated with those of IL-6 ($r = 0.62$, $P < 0.005$) and IL-8 ($r = 0.55$, $P < 0.025$). Levels of IL-8 also correlated with those of E-$\alpha$1AT ($r = 0.82$, $P < 0.001$).

**DISCUSSION**

The pro-inflammatory cytokines TNF-$\alpha$, IL-10, IL-6, and IL-8 play a key role in the pathogenesis of infectious diseases. Interleukin-6 plays a major role in host defense mechanisms, including immune responses, acute-phase reactions, and hematopoiesis.\textsuperscript{25-27} In disease conditions associated with increased TNF-$\alpha$ levels such as sepsis and following infusion of bacterial endotoxin or TNF-$\alpha$ in healthy subjects, enhanced synthesis of IL-6, IL-8, and IL-10 has been observed.\textsuperscript{28-34} Moreover, Linderholm and others reported increased plasma levels of TNF, IL-6, and the anti-inflammatory cytokine IL-10 in patients with hemorrhagic fever caused by the Puumala hantavirus in Europe.\textsuperscript{35}

The patients with AHF in this study had increased serum levels of IL-6, IL-8, and IL-10 at the onset of disease. These levels were related to the severity of illness. Consistent with published data, this group also had elevated serum levels of TNF-$\alpha$.\textsuperscript{10} These data, together with the significant correlations found between the levels of the cytokines and TNF-$\alpha$, substantiate the activation of pro-inflammatory and anti-inflammatory cytokine pathways in these patients. In addition, levels of two acute-phase proteins, fibrinogen and von Willebrand factor, have also been reported to be increased in patients with AHF.\textsuperscript{34}

It has been reported that pathologic states with elevated levels of IL-6 might also be associated with increased production of IL-6sR.\textsuperscript{36} The high levels of soluble receptor found in some patients with AHF suggests a potentiation of IL-6 activity. This hypothesis is based on the fact that IL-6sR may form biologically active complexes with IL-6.\textsuperscript{37}

Interleukin-8 shows pro-inflammatory effects by acting as a chemoattractant factor and causing leukocyte infiltration. \textit{In vivo}, levels of this cytokine were found to be increased in most patients with gram-positive or gram-negative sepsis.\textsuperscript{32,38} Moreover, IL-8 plays an important etiologic role in acute respiratory distress syndrome, which is common in
patients with AHF with a fatal outcome.\textsuperscript{39} Interleukin-8 causes neutrophil mobilization, adhesion, and activation with subsequent granule content release, microvascular injury, and vascular permeability. Leukocyte activation can be confirmed by the detection of E-\alpha-LAT. The existence of this complex in plasma was demonstrated by cross-immunoelectrophoresis in a few patients with AHF (Heller MV, Universidad de Buenos Aires, unpublished data). We measured plasma levels of E-\alpha-LAT with a sensitive ELISA as a marker of in vivo neutrophil activation. Complex levels were higher in the moderate and severe clinical forms of AHF than in the mild clinical form. The significant correlation between levels of E-\alpha-LAT and IL-8 ($r = 0.82$, $P < 0.001$) supports the contention that this cytokine is implicated in polymorphonuclear leukocyte activation.

Interleukin-10, which was shown to inhibit the production of pro-inflammatory cytokines, was also increased in patients with AHF, suggesting a regulatory mechanism against inflammation. These studies were further supported by the finding that the increase in levels of pro-inflammatory cytokines induced by endotoxin in monkeys was prevented by recombinant IL-10.\textsuperscript{40} We have also hypothesized that the activation of coagulation, fibrinolysis, and/or complement systems in patients with AHF is partially due to proteolytic activity by proteases released from neutrophils.\textsuperscript{41} An alternative pathway of blood coagulation and fibrinolysis activation would be triggered by pro-inflammatory cytokines. The complexity of the clinical and pathophysiologic picture in patients with AHF and the difficulty in analyzing each pathway independently make it difficult to analyze this issue more thoroughly.

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