Elevated Plasma Levels of Tumor Necrosis Factor (TNF)-α, Soluble TNF Receptors, Interleukin (IL)-6, and IL-10 in Patients with Hemorrhagic Fever with Renal Syndrome

Mats Linderholm, Clas Ahlm, Bo Settergren, Anders Waage, and Arne Tärnvik

Plasma levels of cytokines were measured by EIA in 15 subjects hospitalized with nephropathia epidemica, a European form of hantavirus-induced hemorrhagic fever with renal syndrome. Concentrations of tumor necrosis factor (TNF)-α and interleukin (IL)-6 were increased in all patients at admission, and the concentration of IL-10 was increased in most. TNF-α concentrations were still increased 1 week after onset of disease; levels of IL-6 and IL-10 were normalized. TNF-α was undetectable by the WEHI cell assay in serum samples obtained throughout the acute phase of disease. Serum levels of the two soluble TNF receptors p55 and p75 correlated with levels of the cytokine, indicating that receptor binding may be the reason for lack of bioactivity in vitro. TNF-α is known to induce pathophysiologic and clinical changes similar to those seen in nephropathia epidemica and in diseases caused by other hantaviruses.

From rodent reservoirs, hantaviruses are spread by respiratory transmission to humans. Two main forms of febrile illness are recognized: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS).

HFRS, which is endemic in Europe and Asia, is caused by several hantaviruses [1]. Each virus has a specific rodent reservoir and a characteristic geographic distribution, and there is also a relation between viral agent and severity of disease.

Puumala virus is the etiology of nephropathia epidemica (NE), an HFRS in Europe [2]. The course of NE is generally milder than the Asian forms of HFRS, and the mortality rate is <0.5% [3-5]. Predominant symptoms are fever, malaise, headache, and back pain. Thrombocytopenia, proteinuria, hematuria, oliguria followed by polyuria, and increased serum creatinine concentration are frequent findings. On occasion, the course is more fulminant, with hypotension, vascular hyperpermeability, and disseminated intravascular coagulation [6-9]. Such cases may be clinically indistinguishable from those associated with more virulent hantaviruses, such as Hantaan virus.

HPS is a recently recognized fulminant disease causing a high rate of mortality and occurring in the United States [10, 11]. There is rapid development of hypotension, thrombocytopenia, and noncardiogenic pulmonary edema with the disease, and the etiologic agent, presently named Sin Nombre virus, is closely related to Puumala virus.

Mechanisms behind the development of hantavirus-induced symptoms and lesions are largely unknown. As in the pathogenesis of septic shock and other fulminant febrile conditions, cytokines may be involved. In this study, we measured concentrations of several cytokines in plasma samples obtained at various intervals after onset of NE.

Materials and Methods

Subjects. Fifteen NE patients (9 men, 6 women; 24-73 years old, median, 48) hospitalized within 4 days after onset of disease (i.e., onset of fever) were consecutively included in the study from March 1992 to April 1993.

One patient had uncomplicated Crohn’s disease. Two patients had been under long-term treatment, 1 with antiepileptic and the other with antihypertensive medication. The remaining 12 patients were previously healthy. No patient received corticosteroids or other immunomodulatory drugs.

Clinical data. Blood pressure was recorded on admission and at a 3-month follow-up. Mean blood pressure was defined as [(2 x diastolic blood pressure) + systolic blood pressure]/3; normal value is 70-109 [12]. During the acute phase of disease, blood samples were obtained daily for routine hematology and chemistry at the clinical laboratory at Umeå University.

Microbiology. Serum samples were assayed for IgG and IgM antibodies to Puumala virus by an immunofluorescent antibody technique [4]. Complement fixation tests were done on paired sera to exclude other viral diseases (adenovirus, respiratory syncytial virus, influenza A and B, and parainfluenza) or bacterial diseases (Mycoplasma pneumoniae and Chlamydia psittaci). Blood, urine, nasopharyngeal, and throat specimens were obtained for bacterial cultures from most patients.

Cytokine assays. Venous blood was collected daily during hospitalization and once at 3 months. Blood was drawn in tubes with...
and without EDTA and centrifuged at 500 g for 10 min. Aliquots of plasma or serum were stored at −70°C and thawed immediately before assay. Cytokines were measured by ELISA (Medgenix Diagnostics, Fleurus, Belgium) in plasma samples, according to the manufacturer’s instructions. Detection limits were as follows: tumor necrosis factor (TNF)-α, 3 ng/L; interleukin (IL)-1β, 2 ng/L; IL-2, 0.1 IU/mL; IL-4, 2 ng/L; IL-6, 2 ng/L; IL-8, 0.7 ng/L; IL-10, 1 ng/L; interferon (IFN)-γ, 0.03 IU/mL; granulocyte-macrophage colony-stimulating factor (GM-CSF), 3 ng/L.

Bioassay of TNF-α in serum was done using WEHI 164 cells as previously described [13].

**Assay of soluble TNF receptors (sTNFR).** Immunoassays were used to analyze sTNFR p55 and p75 in serum as previously described by Liabakk et al. [14]. In brief, immunoplates (Nunc, Roskilde, Denmark) were coated with 100-μL portions of monoclonal antibody IV4E [14] or 3H5 [15] at 10 μg/mL for 12 h at 4°C.

After being blocked with 0.5% RIA-grade bovine serum albumin (Sigma, St. Louis) in PBS (PBS-BSA) (200 μL/well) for 1 h at 37°C, the plates were washed three times (100 μL/well) with PBS-BSA (PBS-BSA-T). Samples diluted in PBS-BSA-T were added to the plates in aliquots of 50 μL/well. Recombinant human sTNFR p55 and p75 served as standards.

The plates were incubated overnight at 4°C and washed. Bound TNF-R was detected by the addition of digoxigenin-labeled recombinant TNF (Genentech, South San Francisco) in PBS-BSA-T (50 ng/mL, 50 μL/well) for 1 h at 37°C. For digoxigenin labeling, a labeling kit (Dig-antibody; Boehringer Mannheim, Ingelheim, Germany) was used according to the manufacturer’s instructions. After being washed, peroxidase-labeled antidigoxigenin Fab fragments (Boehringer Mannheim) diluted 1:2000 in 0.1 M TRIS and 0.1 M NaCl (pH 7.5; 50 μL/well) were added and incubated for 30 min at 37°C. After another washing step, the assays were developed using o-phenylenediamine (Dako, Glostrup, Denmark) as substrate (50 μL/well), and the reaction was stopped with 2 M H2SO4 (50 μL/well). A490 was measured by use of a microplate reader (model 3550-UV; Bio-Rad, Richmond, CA). The technique allowed reliable detection of free and reversibly occupied sTNFR. Detection limits of both assays were 150–300 pg/mL, and the intra- and interassay variations were <10%.

**Statistical analyses.** StatView SE + Graphics (v. 1.03; Abacus Concepts, Berkeley, CA) software was used. Spearman’s rank correlation coefficient and single regression were used to correlate clinical data with cytokine levels. For paired comparison of data from assays done at different time intervals, Wilcoxon’s rank sum test was used.

**Results**

**Clinical data.** All 15 patients had symptoms typical of NE (i.e., fever, abdominal pain, and headache). The median duration of fever was 6 days (range, 3–9). Each case was serologically confirmed by the demonstration of specific IgM and IgG antibodies against Puumala virus. Additional viral and bacterial serology and bacterial cultures were negative, except for 1 patient who had asymptomatic bacteriuria. No therapy, apart from supportive care, was given. All patients survived.

In 14 patients at admission, the mean value (± SE) of the mean blood pressure was 92 ± 3 mm Hg (range, 68–107) compared with 100 ± 3 mm Hg (range, 80–120) at 3 months (P = .009, Wilcoxon rank sum test). Urinalysis revealed proteinuria in all patients and hematuria in 14 of 15 patients. The median maximum white blood cell count was 13.0 × 10^9/L (range, 6.3–19.8). The nadir of platelet counts occurred on days 3–5, with a minimum value of 49 × 10^9/L (range, 27–123). The median maximum serum concentrations of C-reactive protein and creatinine were 79 mg/L (range, 18–178) and 271 μmol/L (range, 78–1013), respectively. Serum creatinine was maximally increased on days 5–10 after onset of NE.

**Cytokine levels.** In general, increased plasma levels of TNF-α, IL-6, and IL-10 were demonstrated by ELISA during the acute phase of NE. The plasma concentration of TNF-α was elevated in all 15 patients (figure 1). The maximum value, noted on days 3–5, was 114 ng/L (range, 45–281). On day 8, concentrations were still significantly higher (P = .012) than those determined at 3 months.

Besides being immunoassayed for TNF-α, serum samples were also consistently subjected to the WEHI cell test. In no sample was in vitro bioactive TNF-α detectable. Also, all samples were examined for the presence of two sTNFR, p55 (sTNFR-I) and p75 (sTNFR-II). Increased levels were found during the first week of hospitalization (figure 1). The maximum level of TNF-α correlated with the maximum level of sTNFR-I (r = .89; P = .001) and sTNFR-II (r = .52; P = .05) (figure 2).

Plasma levels of IL-6 were increased in all patients; the median value of the maximum concentration was 58 ng/L (range, 11–402; figure 1). IL-10 concentrations were increased in samples from 13 of 15 patients, with a median maximum concentration of 25 ng/L (range, 0–223). The highest values of IL-6 and IL-10 were found in the first samples assayed, indicating that the peak may have occurred before hospital admission. Plasma levels of both cytokines rapidly decreased during the first few days of hospitalization.

Cytokine responses other than those for TNF-α, IL-6, and IL-10 were generally weak and transient and occurred in only a few patients (figure 1). IL-1β was detected in plasma from 1 of 15 patients in acute-phase NE. IFN-γ was demonstrable on days 3–4 of disease in plasma of 7 of 15 patients; however, more than minute levels were found in only 2 of them (5.5 and 16.8 IU/mL, respectively). Minute amounts of IL-8 were found in plasma from 6 of 7 patients, with peak levels occurring 2–4 days after onset of NE. There was a correlation between the initial levels of IL-8 and TNF-α (r = .96, P = .019), which is in accordance with results indicating that TNF-α may induce IL-8 production [16].

GM-CSF occurred at low levels in 1 sample only from 3 of 7 patients (5, 16, and 21 ng/L, respectively). IL-2 was detectable or low in all but 1 of 7 patients. In the remaining patient, the association to NE was unclear because IL-2 was also detected at the same level at the 3-month follow-up. IL-4 was...
Figure 1. Plasma levels of cytokines and serum levels of sTNFR at various times after onset of nephropathia epidemica. Data are mean ± SE. *P < .05, **P < .01 vs. 3-month follow-up (Wilcoxon rank sum test). Nos. of patients are in parentheses. IFN = interferon; GM-CSF = granulocyte-macrophage colony-stimulating factor.
either not detected at all or was found in minute amounts in the 7 patients.

Cytokine levels in relation to clinical data. One of several biologic effects of TNF-α observed in humans is a relative hypotension [17]. In the present subjects, the initial level of TNF-α was inversely correlated with the ratios of the mean blood pressure recorded on admission and at 3 months \( r = -0.69, P = 0.007 \), single regression; figure 3). Maximum levels of TNF-α and IL-6 were correlated with the maximal serum concentrations of creatinine \( r = -0.61 \) and \( -0.60, P = 0.022 \) and \( 0.026 \), respectively, Spearman’s rank correlation). There was no significant correlation between maximum levels of TNF-α and minimum platelet counts \( r = -0.49, P = 0.070 \).

The possibility that TNF-α might accumulate during NE due to impaired renal function was considered. In mice, bilateral nephrectomy has resulted in prolonged clearance of immuno-logically detectable TNF [18]. Kinetics of TNF-α were, however, quite similar in NE patients with maximal serum creatinine values \( \geq 200 \mu{\text{mol/L}} \) and in those with values \(< 200 \mu{\text{mol/L}} \) (figure 4), indicating that renal dysfunction did not by itself explain the high levels of TNF-α. Taking into consideration that the half-life of the cytokine after intravenous injection in humans may be as short as 14–18 min [19], an increased or protracted (or both) synthesis seemed more probable. Protracted increased levels of TNF-α have been demonstrated during the course of acute hepatitis [20], a viral disease in which renal function is not supposed to be impaired.

Discussion

Increased plasma levels of immunoreactive TNF-α were demonstrated in all patients during acute-phase NE. Maximum levels were seen on days 3–5 after onset of disease and were still increased on day 8. Of eight other cytokines assayed, only IL-6 and IL-10 occurred in more than minute amounts. It should be recalled, however, that most patients had been ill for 1 or a few days when hospitalized, and a diverse cytokine response may well have occurred during that time. In fact, an increased expression of IL-2 receptors on peripheral blood T cells has
been found during the first week of NE infection (unpublished data).

An involvement of IL-6 and IL-10 is in accordance with a well recognized interregulation of these cytokines with TNF-α. TNF-α and IL-10 are believed to form an autoregulatory loop, in which TNF is an inducer of IL-10, and IL-10 is a down-regulator of TNF [21]. TNF-α induces IL-6 [22].

TNF-α was demonstrable by immunoassay but not by cytotoxic assay. This disparity has been a usual finding in studies of febrile disease [23, 24]. TNF-α is a short-ranged mediator with local effects. In the circulation, it seems to be bound to the extracellular components of TNF-R shed during acute disease [25]. In line with this, we found a correlation between plasma levels of TNF and sTNFR. It is unknown whether TNF is bound in an ultimately inactive form or if it is just stabilized by the soluble receptors and afforded a sustained biologic activity in vivo [24, 26]. The latter possibility was lent support by the fact that in a clinical study of malaria, no activity of TNF-α could be detected in the WEHI cell bioassay, despite an ability of a monoclonal TNF-α antibody to mitigate symptoms [27].

When intravenously injected into cancer patients, TNF-α causes several changes reminiscent of clinical characteristics seen in NE. Fever, chills, headache, myalgia, and a relative hypotension [17] have been observed, signs which are characteristic of NE and which all occurred in patients in the present study. In addition, weight gain has been observed after intravenous administration of TNF-α [17] and in acute-phase NE.

The most prominent adverse hematologic effect of TNF-α infusion in cancer patients is thrombocytopenia [28], which is characteristic of NE and which was consistently observed in our subjects. TNF-α has been ascribed the role of a mediator of lipolysis [29]: Infusion in cancer patients results in decreased serum levels of high-density lipoprotein and a reciprocal increase of triglycerides [17]. This pattern has been described also in NE [5].

In a rat model, injection of high doses of TNF-α caused acute tubular necrosis [30], a change found also in severe human cases of NE and believed to be a primary lesion leading to decreased glomerular filtration [31]. In other animal experiments, TNF-α injection has caused increased vascular permeability in the lungs [32] and other organs [33, 34], whereas no such effect was induced by any of several other cytokines [33].

Increased vascular permeability seems to be an important event in the development of HFRS [31]. In NE, interstitial pulmonary infiltrates and pleural effusions are common findings [3, 35], which may at least partly depend on vascular hyperpermeability.

Although several signs and symptoms of NE can be mimicked by infusion of TNF-α in humans and experimental animals, the pathophysiologic involvement of the cytokine remains to be proven. If TNF-α is indeed involved, its effects would be expected to vary widely due to interactions by several determinants. Some cytokines, for example, IL-1 and IFN-γ, are known to potentiate the biologic effects of TNF-α [33, 36], whereas others, such as IL-10, are effective down-regulators [37]. As indicated by several studies [26], including the present one, the biologic activity of TNF-α may also be modulated by sTNFR.

TNF-α levels have been reported to be increased in patients with the Korean hemorrhagic fever [38] and may also be increased in other diseases caused by hantaviruses. This may be true also for HPS. The Sin Nombre virus of HPS is closely related to Puumala virus [10], and HPS shares important clinical features, such as increased vascular permeability, hypotension, and thrombocytopenia. Pulmonary symptoms, which are typical to HPS, may occur also in fulminant cases of NE [6, 7]. Increased levels of TNF-α have been found in severe cases of Dengue [39] and Argentine [40] hemorrhagic fevers, which both bear some resemblance in clinical expression to hantavirus disease.

If cytokines, TNF-α in particular, can be shown to be among the determinants of the outcome of hantavirus disease, this should be a clue in the search for treatment of fulminant cases. Work to find inhibitors of TNF-α is in progress [41, 42]. As a basis of future trials, it seems worthwhile to assay TNF-α and other cytokines in various forms of HFRS and in HPS.

In conclusion, this study demonstrated protracted increased plasma levels of TNF-α during acute-phase NE. No activity was demonstrable by the WEHI bioassay. TNF-α levels correlated well with levels of sTNFR, indicating that receptor binding may be the reason for lack of bioactivity in vitro. TNF-α may be pathophysiologically relevant because it is known to possess several activities mimicking clinical signs and symptoms of the disease.

Acknowledgments

We thank Marie Sorensen and Aina Hansson for skillful technical assistance.

References