Brief Definitive Report

THE COMPLEX PATTERN OF CYTOKINES IN SERUM FROM PATIENTS WITH MENINGOCOCCAL SEPTIC SHOCK

Association between Interleukin 6, Interleukin 1, and Fatal Outcome

By ANDERS WAAGE,* PETTER BRANDTZAEG,‡ ALFRED HALSTENSEN,§ PETTER KIERULF,‖ AND TERJE ESPEVIK*

*From the *Cell Research Laboratory, Institute of Cancer Research, University of Trondheim, 7006 Trondheim; the ‡Department of Infectious Diseases, Ullevål University Hospital, 0407 Oslo 4; the §Medical Department B, University of Bergen, 5021 Bergen; and the ‖Central Laboratory, Ullevål University Hospital, 0407 Oslo 4, Norway*

TNF-α/cachectin, released by monocytes/macrophages in response to bacterial endotoxin (LPS), has been implicated as a principal mediator in endotoxic shock in experimental animals (1). We have previously demonstrated a strong association between the presence of TNF-α in serum and septic shock, and we have shown that patients with serum concentrations of TNF-α exceeding 140 pg/ml invariably died (2).

IL-6 (also called IFN-β2, 26-kD protein, B cell stimulatory factor 2, and hybridoma growth factor) stimulates growth and differentiation of various lymphoid cells (3), and induces the production of acute-phase proteins in cultured liver cells (4). Patients receiving renal transplants have elevated serum levels of IL-6 (5) and TNF (6). The biological activities of IL-6 are not yet completely uncovered, but appear to overlap with those of TNF-α and another cytokine, IL-1 (4, 7).

In contrast to the marked lethality associated with TNF-α in septic shock, TNF-α is tolerated when administered to humans in clinical trials. Furthermore, the lethal effect of TNF-α in mice is potentiated by IL-1 (8) and by LPS (9). This suggests that the deleterious effect associated with TNF-α in human septic shock is not caused by TNF-α alone, but by TNF-α in cooperation with other factors.

In this study we demonstrate that IL-6 and IL-1 are released into serum, and coexist with TNF-α and LPS in the systemic circulation during the initial phase of meningococcal septic shock. High serum levels of IL-6 were associated with fatal outcome, and IL-1 was exclusively detected in patients who had high serum levels of IL-6, TNF-α, LPS, and a rapid fatal outcome.

Materials and Methods

Patients. We studied 79 patients (43 male, 36 female, aged 1–93 yr) admitted to the university hospitals in Bergen, Oslo (Ullevål) and Trondheim during 1981–86. The clinical diagnosis of meningococcal disease was confirmed bacteriologically (72 patients) or serologically. Blood was drawn on admission to hospital, and, from 9 patients with septic shock, also at intervals during the initial 72 h of hospitalization. Serum/plasma samples were stored at −70°C.

Patients were considered to have septic shock if systolic blood pressure ≤70 mm Hg (patients ≤12 yr old), or ≤100 mm Hg (>12 yr old). Patients with blood pressure above these

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limits, and positive blood cultures were considered to have bacteriaemia. Meningitis was defined as $>10^8$ cells/liter cerebrospinal fluid, or back rigidity.

Assay for IL-6. IL-6 was determined by the IL-6-dependent mouse hybridoma cell line B13.29 clone B9, as described (10). Serial dilutions of the test sample were incubated for 72 h with IL-6-dependent cells. Viability was measured in a colorimetric assay with a tetrazolium salt (Sigma Chemical Co., St. Louis, MO), as described (11). rIL-6 (12) was included as a standard. The detection limit of the assay was 15–20 pg rIL-6/ml serum. An antiserum to rIL-6 neutralized ~400 ng rIL-6/ml antiserum (gift from Dr. W. Fiers, University of Ghent, Belgium). The IL-6 activity in the samples was completely neutralized by this antiserum.

Assay for IL-1 and IL-2. IL-1 was determined by an assay as described by Conlon (13) with modifications. The subclone B of the LBRM-33-1A5 cells produces IL-2 upon activation with IL-1 and PHA-P. The cells were incubated with mitomycin C (50 μg/ml) (Sigma Chemical Co.) for 30 min, and washed three times. 100 μl of the LBRM-33-1A5 cells (5×10^5 cells/ml) was added to each well of a 96-well microplate with PHA-P (1:2,000) (Difco Laboratories, Detroit, MI) and serial dilutions of test sample. After 24 h, IL-2-dependent HT-2 cells were added to each well (10^4 cells/well), and cell proliferation was determined 24 h later by the incorporation of [^{3}H]thymidine (Amersham Inc., Amersham, UK) (1 μCi/well). Purified natural human IL-1 (Genzyme Corp., Boston, MA) was included as a standard (units as specified by Genzyme Corp.). The sensitivity of the assay was 0.02 U/ml serum. Antiserum against rIL-1α and rIL-1β had previously been raised in sheep (gift from Dr. A. Shaw, Glaxo, Geneva, Switzerland), and neutralized at least 20 μg/ml of rIL-1α (Glaxo) and at least 2 μg/ml of rIL-1β (Glaxo). The IL-1 activity in the patient samples was completely neutralized by a mixture of antiserum to rIL-1α and antiserum to rIL-1β.

IL-2 activity was determined by adding dilutions of test samples and rIL-2 (Genzyme) to the HT-2 cells in a 96-well microplate. After 24 h, viability was measured in a colorimetric assay with a tetrazolium salt (11). The detection limit of the assay was about 6 U/ml serum.

Assays for TNF-α and TNF-β. TNF-α was determined by its cytotoxic effect on the fibrosarcoma cell line WEHI 164 clone 13, as described (14). rTNF-α (Biogen, Cambridge, MA and BASF/Knoll, Ludwigshafen, FRG) were included as a standard. The detection limit of the assay was 2–3 pg rTNF-α/ml serum. An antiserum to rTNF (neutralizing capacity, 600 ng rTNF-α/ml) (14) completely neutralized the TNF-α activity in the serum samples.

The WEHI 164 clone 13 cell line is also sensitive to rTNF-β. The addition of a polyclonal antiserum against rTNF-β (Biogen) had no effect on the cytotoxicity in the serum samples.

Assay for LPS. LPS was determined in heparinized plasma by the limulus amebocyte lysate test, as described (15). LPS from *Escherichia coli* (B 5505; Mallinckrodt Inc., St. Louis, MO) were included as a standard. The detection limit of the assay was 25–30 pg/ml plasma.

Assay for IFN-γ. IFN-γ was determined by ELISA as described elsewhere (16), and the detection limit of rIFN-γ (Genetech Inc., South San Francisco, CA) was 0.4 ng/ml serum.

Results

IL-6 in Serum. IL-6 was detected in 69 of the 79 admission serum samples (Fig. 1 *a*). The median serum level of IL-6 was ~1,000 times higher in patients with septic shock (189 ng/ml) than in patients with meningitis (0.2 ng/ml), bacteriaemia (0.2 ng/ml), or combined septic shock and meningitis (0.2 ng/ml). Serum levels in 15 healthy controls were below 0.05 ng/ml (data not shown). 11 of 21 patients with IL-6 levels >3.0 ng/ml died, whereas all 58 patients with levels at ≤3.0 ng/ml, survived. All four patients with serum IL-6 levels >750 ng/ml died (Fig. 1 *b*).

IL-1 in Serum. IL-1 was detected in 3 of 20 serum samples tested. These three patients had septic shock and the most rapid fatal courses of the patients who died (Table I).

Levels of Cytokines and LPS in Individual Patients. Admission plasma/serum levels of LPS, TNF-α, IL-1, and IL-6 were recorded in 10 patients with septic shock (Table I). Patients with high levels of LPS seemed to have high levels of TNF-α ($r = 0.87$, $p = 0.001$), IL-6 ($r = 0.94$, $p = 0.0002$), presence of IL-1 in serum ($r = 0.86$, $p =$

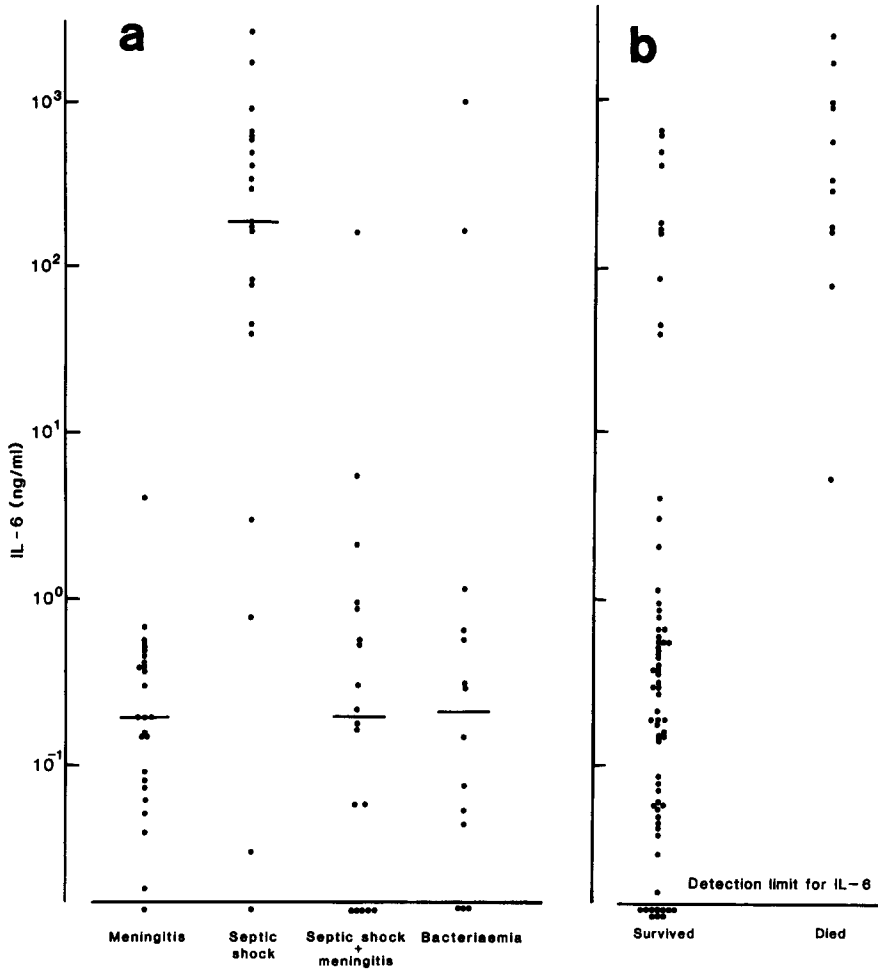

FIGURE 1. Serum levels of IL-6 on admission in patients with meningococcal disease (*a*), and relation between serum levels of IL-6 and outcome (*b*). Horizontal bars denote median values.

0.006), and fatal outcomes. TNF-α in serum in the remaining 69 patients has previously been reported (2), and was included in the calculation of the positive correlation ($r = 0.62$, $p = 0.0001$) between the levels of TNF-α and IL-6 in admission serum samples. The data indicate that the induction of IL-6, IL-1, and TNF-α in human septic shock is related, and that the common activation of these cytokines is associated with fatal outcome.

IL-2, IFN-γ, and TNF-β were not detected in any of the samples.

Kinetics of IL-6 and TNF-α. The kinetics of production and elimination of IL-6 and TNF-α during septic shock were studied in samples consecutively drawn from nine of the patients included in Table I (data from five patients are shown in Fig. 2). The peak concentration of TNF-α occurred before (eight patients), or shortly after (one patient) admission to hospital, whereas the peak concentration of IL-6 was detected 1–4 h after admission in five of eight patients (four patients shown).

TABLE I

Plasma/Serum Levels of LPS and Cytokines in Patients with Meningococcal Septic Shock

Died (hours after admission)	LPS	TNF-α	IL-1	IL-6
	ng/ml	*pg/ml*	*U/ml*	*ng/ml*
1/3	300	30,000	4.6	2,400
6	170	600	0.05	1,731
9	12.0	78	0.15	918
39	10.5	300	<0.02	300
Survived	3.8	65	<0.02	666
Survived	2.8	7	ND	81
Survived	2.8	33	<0.02	189
Survived	2.7	33	<0.02	165
29	1.8	22	ND	ND
Survived	0.8	13	<0.02	660

Plasma or serum were collected on admission to hospital.

The results show that TNF-α is released into serum before IL-6 in septic shock, and that the levels of IL-6 are $\sim 10^4$ times higher than those of TNF-α.

TNF-α was eliminated from the circulation at a constant rate in five of nine patients (four patients shown in Fig. 2); the half-life of TNF-α was calculated to be 70 ± 11 min (mean ± SD, n = 5). In the remaining four patients, additional TNF-α was released into the circulation after an initial decline of the serum concentration (one patient shown). Detectable amounts of TNF-α were present in serum for 3–5 h after admission in five patients, and for up to 18 h in four patients. IL-6 was

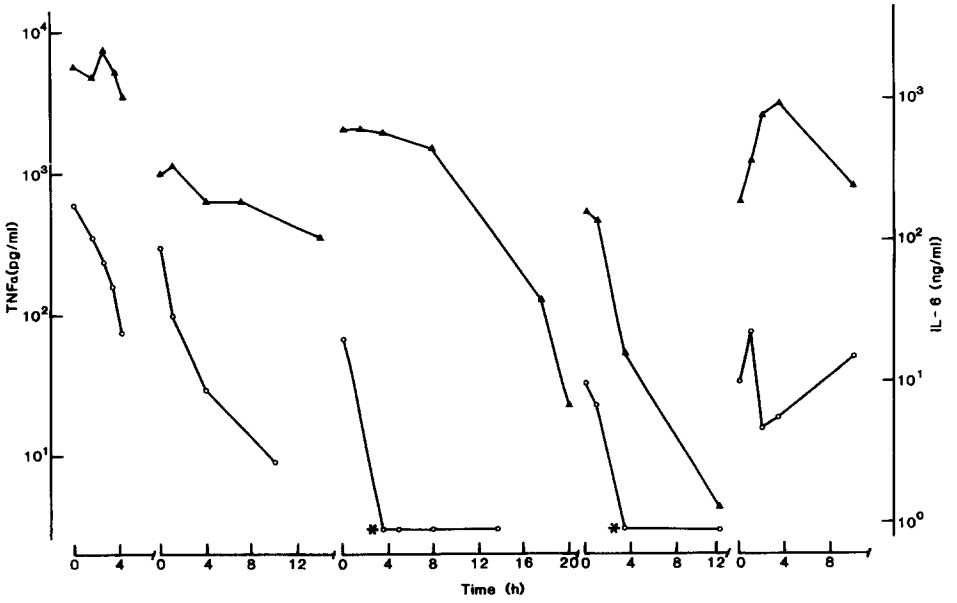

FIGURE 2. Production and elimination of IL-6 (▲) and TNF-α (○) in meningococcal septic shock. Asterisk indicates detection limit for TNF-α; 0 is point of time for admission to hospital.

eliminated from the circulation at a constant rate in seven of eight patients. The half-life of IL-6 was calculated to be 103 ± 37 min (mean ± SD, n = 6, the half-life in one patient in Fig. 2 was 8 h, and was excluded from this calculation). Detectable amounts of IL-6 were present in serum for up to 36 h.

Discussion

The study demonstrates that IL-6 and IL-1 are released into serum in patients with meningococcal septic shock, and that high levels of IL-6 and presence of IL-1 are associated with fatal outcome.

Evidence has been given that TNF-α is implicated as a mediator in septic shock (1), whereas the role of IL-6 in septic shock is so far unclear. However, the relation between levels of IL-6 and fatal outcome found in the present study is similar to that previously demonstrated for TNF-α (2), and opens the possibility that IL-6, either by itself, or by interactions with TNF-α, IL-1, or LPS, is involved in the pathogenesis of the shock.

Recent studies have shown that IL-1 (8) and LPS (9) potentiate the lethal effect of TNF-α in mice. The three patients with high levels of LPS, TNF-α, IL-6, and detectable levels of IL-1 in serum, rapidly died, and emphasize that combinations of LPS and cytokines may be particularly potent in producing lethal shock also in humans.

The relation between the levels of IL-6, IL-1, and TNF-α demonstrated in this study, indicates that the induction mechanisms of these cytokines in septic shock are related. In experimental animals, injection of LPS induces release of TNF-α and IL-6 after 1–2 h, whereas injection of TNF-α induces release of IL-6 after ~7 h (Shalaby, R., and A. Waage, manuscript in preparation). Both TNF-α and IL-1 induces the release of IL-6 by endothelial cells (Shalaby, R., A. Waage, and T. Espevik, submitted for publication). In human septic shock, there may consequently be multiple routes of induction of IL-6. The time interval between the release of TNF-α and IL-6 demonstrated in our study, suggests that TNF-α is a stimulator of the IL-6 production in human septic shock.

Our study demonstrates that a complex pattern of cytokines exists in the circulation during the initial phase of septic shock, and emphasizes that the pathogenesis of the shock should be analyzed not only with respect to TNF-α, but also in terms of interactions between IL-6, IL-1, TNF-α, and LPS.

Summary

Serum samples from patients with meningococcal disease were examined for the presence of IL-6, TNF-α, and LPS. Median serum concentration of IL-6 was 1,000 times higher in patients with septic shock (189 ng/ml) than in patients with bacteriaemia, meningitis, or combined septic shock and meningitis. 11 of 21 patients with serum levels >3.0 ng/ml died, whereas all 58 patients with serum levels at ≤3.0 ng/ml, survived. All four patients with serum IL-6 levels >750 ng/ml, died. IL-1 was detected in serum from three patients who also had high serum levels of IL-6, TNF-α, and LPS, and rapidly fatal courses. IL-6 appeared to be released into serum later than TNF-α, and was detected in serum for up to 36 h. The half-life of IL-6 and TNF-α was calculated to be 103 ± 27 min and 70 ± 11 min, respectively. These data indicate that a complex pattern of cytokines exists in serum from patients with meningococcal septic shock, and that the release of IL-6 and IL-1, in addition to TNF-α, is associated with fatal outcome.

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