LOCAL PRODUCTION OF TUMOR NECROSIS FACTOR α, INTERLEUKIN 1, AND INTERLEUKIN 6 IN MENINGOCOCCAL MENINGITIS

Relation to the Inflammatory Response

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Experimental studies have demonstrated that TNF-α/cachectin is an important mediator in septic shock (1–3), and that IL-1 synergizes with TNF-α in producing lung tissue damage and lethal shock (4, 5). Furthermore, previous studies have shown that the systemic release of TNF-α (6), IL-1 (7, 8), and IL-6 (8) is associated with septic shock and fatal outcome in patients with meningococcal disease.

However, many of the activities of TNF-α, IL-1, and IL-6 indicate a local rather than a systemic production and function of these cytokines. TNF-α and IL-1 may recruit granulocytes to the site of inflammation by margination of granulocytes in the blood stream (9, 10) and chemotactic effect (11, 12). TNF-α induces the production of toxic oxygen radicals (13) and augments the phagocytosis by neutrophils (14), which may be important in the inflammatory response. IL-6 is locally produced in arthritis (15) and in bacterial meningitis (16), and induces growth of B lymphocytes (17). Furthermore, IL-6 shares many of its biological activities with TNF-α and IL-1 (18), and probably, all three cytokines act in a network of factors directing the inflammatory reaction.

Meningococcal disease includes a variety of clinical syndromes of which the dominant manifestations are local meningitis, and bacteremia with or without septic shock. Whereas the roles of TNF-α and in part IL-1 in septic shock have been clarified, their functions in meningococcal meningitis remain unclear.

In this study we demonstrate that TNF-α, IL-1, and IL-6 can be detected in cerebrospinal fluid (CF)[1] in patients with meningococcal meningitis, and that meningococcal LPS or viable meningococci trigger the sequential release of TNF-α, IL-1, and IL-6 activities in CF in rabbits before infiltration of leukocytes. The subarachnoid space and systemic circulation are functionally separate compartments with respect to production of TNF-α, IL-1, and IL-6.

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[1] *Abbreviation used in this paper:* CF, cerebrospinal fluid.

Materials and Methods

Patients. CF was obtained from 32 men and 28 women on admission to the university hospitals in Bergen, Oslo (Ullevål), and Trondheim during 1982–87. The age range was 2–93 yr; 63% of the patients were 10–20 yr old. The clinical diagnosis of meningococcal disease was confirmed bacteriologically (56 patients) or serologically. Patients were considered to have bacteremia if blood cultures were positive, meningitis if CF contained $>10^8$ cells/liter, and septic shock if systolic blood pressure was ≤70 mm Hg (≤12 yr old), or ≤100 mm Hg (>12 yr old).

The patients had these manifestations: meningitis (n = 33), septic shock (n = 9), septic shock and meningitis (n = 11), and bacteremia without meningitis or shock (n = 7).

CF was also obtained from 91 patients with nonbacterial neurological diseases, including cerebrovascular disease, brain tumor, disseminated sclerosis, back pain, and headache. All samples of CF were stored at −70°C until they were assayed for cytokines and LPS.

Assays for TNF-α, IL-1, and IL-6. TNF-α was determined by its cytotoxic effect on the mouse fibrosarcoma cell line WEHI 164 clone 13, essentially as previously described (19). Dilutions of human rTNF-α (Genentech Inc., South San Francisco, CA; supplied by Dr. G. Adolf, Boehringer Ingelheim, Vienna, Austria) were included as a standard. The detection limit of the assay was 2–3 pg of rTNF-α/ml CF. All samples were assayed the same assay day. The addition of a rabbit antiserum to rTNF-α (19) (neutralizing capacity, 600 ng rTNF-α/ml) completely neutralized the cytotoxicity in the patient samples. TNF-α activity in samples of CF from rabbits was completely neutralized by a goat antiserum to rTNF-α (kindly provided by Dr. J. Mathison, Research Institute of Scripps Clinic, La Jolla, CA). This antiserum has previously been demonstrated to neutralize rabbit TNF-α (20).

IL-1 was determined in a two step assay using the T cell lines NOB 1 (21), which produces IL-2 upon exposure to IL-1, and HT 2 (22), which requires IL-2 to grow. Triplicate test samples were fourfold serially diluted in 96-well microtiter plates (100 μl/well), and 100 μl of a NOB 1 cell suspension (2×10^6 cells/ml) was added to each well. Medium consisted of RPMI 1640 (Gibco Laboratories, Grand Island, NY), 10% FCS (Hyclone Laboratorium Inc., Logan, UT), 2 mmol/liter glutamine, and 30 μg/ml of gentamicin. After 24 h, the plates were centrifuged, and 100 μl of the supernatant was added to HT 2 cells (100 μl/well, 1.5×10^5 cells/ml) in a microtiter plate. After 24 h, cell growth was estimated by adding a tetrazolium salt, as described (23). rIL-1β (24) (kindly provided by Dr. A. Shaw, Glaxo Institute for Molecular Biology, Geneva, Switzerland) was included as a standard, and the detection limit of the assay was ~30 pg of rIL-1β/ml CF. Antiserum to rIL-1α and rIL-1β have previously been raised in sheep, and neutralized 20 μg of rIL-1α/ml and 2 μg of rIL-1β/ml (gift from Dr. A. Shaw). A mixture of these antisera was added to positive patient samples, and it neutralized completely the IL-1 activity.

IL-6 was determined by the IL-6-dependent mouse hybridoma cell line B9, (kindly provided by Dr. L. Aarden, University of Amsterdam, The Netherlands) as previously described (25). In short, serial dilutions of triplicate test samples were incubated for 72 h with B9 cells. Dilutions of rIL-6 (26) were included as a standard. Cell growth was measured as described (23). The detection limit of the assay was ~15 pg of rIL-6/ml CF. All samples were assayed the same assay day. A rabbit antiserum to rIL-6 (gift from Dr. W. Fiers, University of Gent, Belgium) neutralized 400 ng of rIL-6/ml when tested in the B9 cell assay. This antiserum completely neutralized the growth stimulatory effect of the patient samples.

Assay for LPS. LPS was measured with the chromogenic Limulus amebocyte lysate test, as described in detail (27). The detection limit of the assay was ~25 pg/ml (LPS from *Escherichia coli* 055B5; Mallinckrodt, St. Louis, MO).

Protein, Glucose, Leukocytes. Samples of human CF were analyzed by routine methods used in the clinical laboratories. Protein levels in rabbit CF were determined by Bio-Rad protein assay (Bio-Rad Laboratories GmbH, München, FRG), and leukocytes were counted with Coulter Counter. Smears were evaluated by May-Grünwald/Giemsa and esterase staining.

Animal Experiments. Chinchilla rabbits (Chb:cH; Dr. K. Thomae GmbH, Biberach a.d. Riss, FRG) weighing 2.7–3.3 kg were anesthetized with fentanyl/fluanison (Hypnorm, Janssen, Brüssel, Belgium) and midozolam (Dormicum, Hoffmann-LaRoche, Basel, Switzerland). The

cisterna magnum was punctured by a 25-gauge needle, and 300 μl of CF was withdrawn and replaced by saline containing 1.5 μg/ml of LPS extracted from *N. meningitidis* (gift from K. Bryhn, National Institute of Public Health, Oslo, Norway) or 10^9 CFU/ml of viable meningococci (strain 44/76; National Institute of Public Health, Oslo). Samples of CF were obtained by repeated punctures of cisterna magnum and centrifugation, and supernatants were stored at −20°C. Samples were discarded if contamination of whole blood, as estimated by optical reading at 450 nm, was above 1/75. In control experiments, contamination below this limit did not influence the activity of TNF-α, IL-1, and IL-6 or the leukocyte count. Samples of CF that were assayed for protein contained whole blood <1/300.

Statistical Analysis. The significance of differences in proportions of samples positive for TNF-α or IL-1 was determined by χ^2 test or Fisher's exact test. The significance of differences in concentrations of IL-6 was determined by Mann-Whitney rank sum test. All tests were two-sided; p values of <0.05 were considered to be statistically significant.

Results

TNF-α, IL-1, and IL-6 in CF. TNF-α was detected in CF in 24 of 44 (55%) of the patients with meningitis, and 3 of 16 (19%) of the patients with septic shock/bacteremia (Fig. 1). This difference was statistically significant (p = 0.03). Furthermore, patients with meningitis had higher concentrations of TNF-α than patients with septic shock/bacteremia (range 0.003–31 ng/ml versus 0.003–0.9 ng/ml, respectively).

IL-1 was detected in CF in 21 of 42 patients (50%) in patients with meningitis,

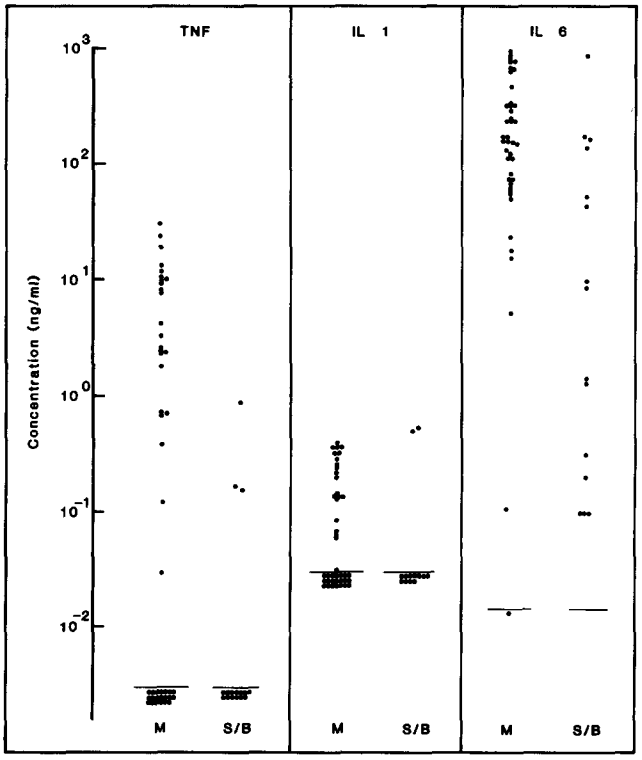

FIGURE 1. Concentrations of TNF-α, IL-1, and IL-6 in samples of CF obtained from patients with meningococcal disease. M denotes patients with meningitis, and patients with meningitis and septic shock/bacteremia; S/B denotes patients with septic shock or bacteremia (without meningitis). Each dot represents mean of triplicate determination of the sample from one patient. The horizontal bars denote the detection limit of the assays (~0.003 ng/ml of rTNF-α, 0.03 ng/ml or rIL-1β, and 0.015 ng/ml of rIL-6).

and 2 of 13 patients (15%) with septic shock/bacteremia ($p = 0.05$). Patients with septic shock/bacteremia had the two highest concentrations of IL-1.

IL-6 was detected in 41 of 42 samples (98%) from patients with meningitis and in all 15 samples from patients with septic shock/bacteremia. However, the median values in the two groups were 154 ng/ml (range 0.015–920) and 42 ng/ml (range 0.1–807), respectively. This difference was statistically significant ($p = 0.001$).

In 91 patients with nonbacterial neurological diseases, TNF-α and IL-1 were not detected in CF. IL-6 was detected in CF from six of these patients, but the highest concentration was only 0.07 ng/ml (data not shown).

These data show that TNF-α, IL-1, and IL-6 can be detected in the CF in patients with meningococcal disease, and that patients with meningitis more frequently have TNF-α and IL-1 and higher concentrations of IL-6 in the CF than patients with septic shock/bacteremia.

Cytokines in Relation to Inflammation Parameters. The LPS concentration, the protein concentration, and leukocyte count in the CFs and the blood/CF glucose were determined (data not shown), and we analyzed the correlations between these parameters and the levels of cytokines in the CF (Table I). The levels of TNF-α, IL-1, and IL-6 correlated with each other by significantly positive ($p < 0.01$) correlation coefficients between 0.34 and 0.54. High concentration of LPS and low CF/blood glucose are considered to be indicators of high number of bacteria present in the CF. The CF/blood glucose inversely correlated with the three cytokines ($r = -0.34$ to -0.67, $p < 0.01$), whereas only the TNF-α level positively correlated with the LPS level ($r = 0.91$, $p < 0.001$). Leakage of serum proteins and migration of leukocytes into the CF compartment are characteristics of the inflamed meninges. The level of all the cytokines correlated with the protein concentration in the CFs ($r = 0.34$–0.62, $p < 0.01$), but only the level of IL-6 correlated with the cell count ($r = 0.37$, $p < 0.01$).

Experimental Meningitis in Rabbits. On admission to the hospital, patients were in different stages of the development of the inflammatory reaction of the meninges. To better understand the data obtained in these patients, a rabbit model was developed to study the kinetics of production and elimination of TNF-α, IL-1, and IL-6, infiltration of leukocytes and increase of protein in the CF. Meningococcal LPS or viable meningococci were injected into the subarachnoid space, and thereafter, consecutive samples of CF were drawn, and analyzed for the relevant parameters. Results from one rabbit are shown in Fig. 2, and demonstrate that TNF-α, IL-1, and IL-6

TABLE I

Correlations between Cytokine Levels in CF and Other Inflammatory Parameters

	IL-1	IL-6	LPS	Blood/CF glucose	Protein	Leukocyte count
				r		
TNF-α	0.34*	0.38*	0.91‡	−0.42*	0.43‡	0.01
IL-1	—	0.54‡	0.04	−0.34*	0.34*	0.16
IL-6	—	—	0.18	−0.67‡	0.62‡	0.37*

* $p < 0.01$.
‡ $p < 0.001$.

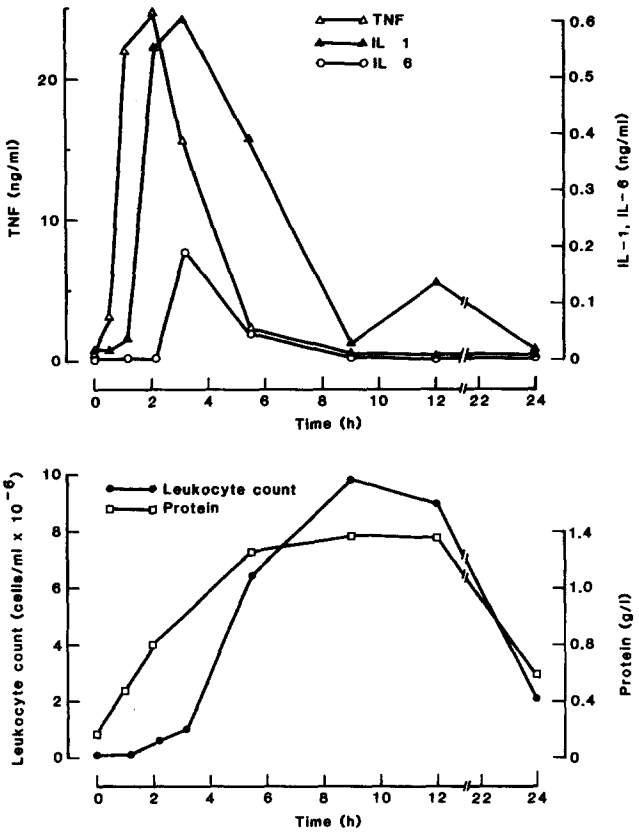

FIGURE 2. TNF-α, IL-1, IL-6 (*top*), protein, and leukocyte count (*bottom*) in CF from a rabbit with experimental meningitis. Meningococcal LPS (0.5 μg) was injected into the subarachnoid space at time point 0. Data from one rabbit are shown. Similar results were obtained in 10 independent experiments with LPS doses varying from 0.001 to 1 μg. Injection of LPS-free saline did not induce detectable levels of TNF-α, IL-1, and IL-6.

activities were detected respectively 1/2, 1, and 3 h after injection of LPS. A small increase of leukocytes was observed after 2 and 3 h, but the main infiltration of cells started after 4 h. The protein concentration was elevated 1 h after injection of LPS, continued to rise for 6 h, and elevated levels persisted for at least 12 h.

The sequential appearance of TNF-α, IL-1, and IL-6 bioactivity followed by migration of leukocytes was reproduced in 10 rabbit experiments. In some of the rabbits, high leukocyte counts persisted for >24 h. Differential counts consistently showed that >95% of the cells in the CF were neutrophils within 8 h after injection; thereafter there was an increase in the number of mononuclear cells to 20–30% after 14 h.

The point of time for appearance of IL-1 activity varied by 1–3 h, and a corresponding variation was observed for IL-6. In some rabbits near maximal activities of IL-1 and IL-6 persisted for several h. There was no difference in the kinetics curves of the cytokines, or the sequence of their release whether LPS or viable meningococci were injected (data not shown). TNF-α, IL-1, or IL-6 bioactivity was not detected after injection of LPS-free saline into the subarachnoid space.

These data show that LPS or viable meningococci trigger the sequential release of TNF-α, IL-1, and IL-6 activities in the CF before infiltration of leukocytes.

Induction of Cytokines in the Subarachnoid Space and in the Systemic Circulation. When LPS was injected into the subarachnoid space, TNF-α activity was detected in serum

at concentrations <1/1,000 of those in the CF (Fig. 3, *right*). IL-1 and IL-6 activities were not detected in serum. Conversely, when LPS was injected intravenously, TNF-α activity was detected in the CF at concentrations <1/100 of those in serum (Fig. 3, *left*), whereas IL-1 and IL-6 activities were not detected in the CF. TNF-α activity in CF after intravenous injection of LPS might have been caused by contamination of serum in the course of the sampling procedure. There was no cellular reaction in CF after intravenous injection of LPS. The kinetics of production and clearance of TNF-α was similar in the circulation and the subarachnoid space after injection of LPS in the respective compartments.

The data demonstrate that the subarachnoid space and the circulation are separate compartments with respect to production of TNF-α, IL-1, and IL-6.

Discussion

This study demonstrates that TNF-α, IL-1, and IL-6 are released into the subarachnoid space in patients with meningococcal meningitis, and that the subarachnoid space and systemic circulation are distinct compartments with respect to the production of TNF-α, IL-1, and IL-6. In rabbits, TNF-α activity was detected in the CF already 30 min after the injection of LPS, whereas IL-1 and IL-6 activity were released later than TNF-α, but before the presence of leukocytes. This indicates that the cytokines are released from cells normally present in the CF compartment, and not by leukocytes migrating from the systemic circulation.

We have previously observed that patients with meningococcal septic shock and TNF-α serum levels >140 pg/ml, invariably died (6), and that the presence of both TNF-α and IL-1 in the circulation is particularly potent in inducing lethal shock (5, 8). In contrast, the TNF-α level in CF was >140 pg/ml in all 14 patients with meningitis (without septic shock/bacteremia), and these patients also had detectable levels of IL-1 in the CF. However, none of these patients died, and obviously, TNF-α and IL-1 induce entirely different reactions when present in the CF compartment and systemic circulation.

The rabbit model proved to be well suited to monitor the cytokine response in meningitis. The sequential release of TNF-α and IL-6 has also been demonstrated

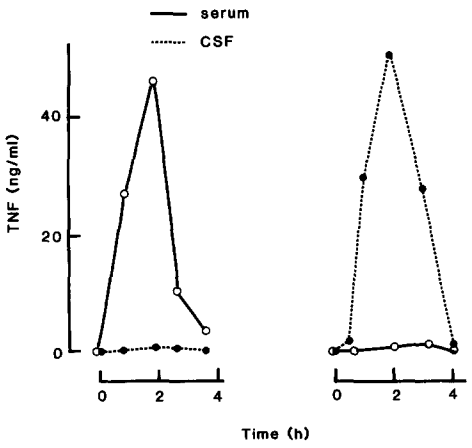

FIGURE 3. TNF-α in serum and CF after injection of meningococcal LPS in the systemic circulation (*left*) and in the subarachnoid space (*right*). 5 μg of LPS was injected intravenously, and 0.5 μg was injected into the subarachnoid space. Results from one rabbit are shown in each panel. Each point on the curves represents mean of triplicate determination.

in the systemic circulation in humans (8, 28). In the present study we were also able to consistently determine the release of IL-1 activity after the release of TNF-α, and clearly, the sequential releases of TNF-α, IL-1, and IL-6 activities are initial events in meningococcal meningitis in rabbits.

The analysis of the relation between the various parameters of inflammation in humans, is confused by two conditions: (*a*) the patients had a variable preadmission history of meningococcal disease, and the samples of CF were thus taken at different time points in relation to the induction of cytokines, and (*b*) the kinetics of production of TNF-α, IL-1, and IL-6 in CF in meningococcal meningitis in humans is unknown. However, the intermediate positive correlation coefficients found for the relations between TNF-α, IL-1, and IL-6 (Table I) are consistent with an overlapping presence of TNF-α, IL-1, and IL-6 in the CF, and a sequential release as demonstrated in the rabbit. Furthermore, the concentration of protein positively correlated with all the cytokines, whereas the leukocyte count positively correlated only with the level of IL-6. This is consistent with an early and persistent increase in the protein concentration and a later increase in IL-6 level and leukocyte count. Our data thus indicate that the sequence of initial events in the inflammatory response in meningococcal meningitis is similar in humans and rabbits.

In conclusion, this study establishes that TNF-α, IL-1, and IL-6 are released into the subarachnoid space in meningococcal meningitis, and demonstrates that a sequential release of TNF-α, IL-1, and IL-6 activities precedes the cellular infiltration in experimental meningitis. The results further demonstrate the versatile role of TNF-α, IL-1, and IL-6 in meningococcal disease. When present in the circulation, the cytokines may be implicated in the complex pathogenesis of septic shock, whereas their functions in the CF compartment, even at very high concentrations, probably are confined to the local inflammatory reaction (29).

Summary

We examined the cerebrospinal fluid (CF) taken on admission from 60 patients with infections caused by *Neisseria meningitidis* for presence of TNF-α, IL-1, and IL-6. TNF-α was detected in CF in 55 and 19% ($p = 0.03$), IL-1 in 50 and 15% ($p = 0.05$), and IL-6 in 98 and 100% of patients with meningitis and septic shock/bacteremia, respectively. The median IL-6 concentration in CF in patients with meningitis was 154 ng/ml, and in patients with septic shock/bacteremia it was 42 ng/ml ($p = 0.001$).

The level of LPS in CF correlated with the level of TNF-α ($r = 0.91$, $p < 0.001$), but not with the level of IL-1 and IL-6. CF levels of TNF-α, IL-1, and IL-6 correlated with each other ($r = 0.34$–0.54, $p < 0.01$), with the protein concentration ($r = 0.34$–0.62, $p < 0.01$) and inversely with the CF/blood glucose ratio ($r = -0.34$ to -0.67, $p < 0.01$). Only the IL-6 level correlated with the leukocyte count ($r = 0.37$, $p < 0.01$).

In rabbits TNF-α, IL-1, and IL-6 activities sequentially appeared in CF within 3 h of injection of meningococcal LPS or viable meningococci, whereas the main infiltration of granulocytes started after 4 h. TNF-α was detected in serum at concentrations <1/1,000 of those in CF after administration of LPS into the subarachnoid space, and conversely, TNF-α was detected in CF at concentrations 1/100 of those in serum after intravenous injection of LPS.

The results demonstrate that TNF-α, IL-1, and IL-6 are sequentially produced in the initial phase of the local inflammatory response caused by meningococci, and that the subarachnoid space and systemic circulation are separate compartments with respect to production of TNF-α, IL-1, and IL-6.

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