

Net Inflammatory Capacity of Human Septic Shock Plasma Evaluated by a Monocyte-based Target Cell Assay: Identification of Interleukin-10 as a Major Functional Deactivator of Human Monocytes

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Summary

We have developed a functional assay to study the inflammatory capacity of plasma collected from patients with severe gram-negative septic shock. In this assay, elutriation-purified, cryo-preserved human monocytes from one healthy donor are combined with plasma from patients with severe persistent septic shock for 5 h. Subsequently, the plasma is removed, medium added, and procoagulant activity (PCA) and secretion of tumor necrosis factor α (TNF- α) and interleukin 6 (IL-6) measured after 18-h incubation. Plasma from 10 patients (6 died) infected with *Neisseria meningitidis* previously shown to contain high levels of native lipopolysaccharide (LPS) (median 2,700 pg/ml), TNF- α , IL-6, IL-8, and complement activation products, had a low net spontaneous inflammatory capacity on the monocytes. The median levels of PCA, TNF- α , and IL-6 were 5, 0, and 4%, respectively, of the monocyte activities induced by normal plasma boosted with purified *N. meningitidis* (Nm)-LPS (2,500 pg/ml; net LPS-boosted capacity, 100%). The levels of PCA, TNF- α , and IL-6 obtained with plasma from shock patients were not different from those induced by plasma from 10 meningococcal patients without shock or with plasma from healthy persons. Boosting shock plasma with 2,500 pg/ml Nm-LPS had little effect on the monocyte activities since the median values of PCA, TNF- α , and IL-6 revealed a minimal increase from 5, 0, and 4% to 9, 2, and 6%, respectively. The shock plasmas revealed a strong LPS-inhibitory capacity that was largely absent in plasmas from 10 meningococcal patients without shock since the median levels of PCA, TNF- α , and IL-6 increased from 5, 0, and 0% to 135, 51, and 73%, respectively, after boosting with 2,500 pg/ml Nm-LPS. The LPS-inhibitory capacity was closely associated with the levels of IL-10. The median levels of IL-10 were 19,000 pg/ml in nine shock patients vs. 22 pg/ml in nine nonshock patients with systemic meningococcal disease. Removal of native IL-10 by immunoprecipitation restored the capacity of plasmas to induce monocyte activation either by native LPS or by boosting with Nm-LPS. IL-4 and TGF- β were not detected in shock plasmas. In 24 patients with detectable meningococcal LPS (>10 pg/ml, 0.1 endotoxin units/ml), the levels of IL-10 were correlated to the levels of LPS ($r = 0.79$, $P < 0.001$). IL-10 declined from initiation of antibiotic therapy and paralleled the levels of native LPS. Decreasing levels of IL-10 in serially collected shock plasmas were directly related to increasing monocyte responsiveness after Nm-LPS boosting. These results suggest that IL-10 plays a major role in containing activation of monocytes and possibly other LPS-responsive cells during overwhelming meningococcemia.

Systemic meningococcal disease (SMD)¹ has been a model infection to elucidate the biological effects of LPS in humans. It is the only gram-negative infection so far stud-

ied that reveals a dose-dependent association between plasma levels of LPS and mortality due to circulatory collapse (1, 2). The circulating levels of native *Neisseria meningitidis* (Nm)-

¹Abbreviations used in this paper: LAL, limulus amoebocyte lysate; Nm-LPS, purified LPS extracted from *Neisseria meningitidis*; PCA, procoagulant activity; SMD, systemic meningococcal disease.

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LPS are, furthermore, closely associated with the levels of various inflammatory mediators that cooperatively induce septic shock and multiple organ failure (2–4). SMD was the first human infection in which bioactive TNF- α was identified as an important mediator of septic shock (5). Shortly after this discovery, a network of proinflammatory cytokines comprising IL-1, -6, -8, and leukemia inhibiting factor (LIF; 6–10) was identified. The effects of the proinflammatory cytokines are counterbalanced by several cytokine-neutralizing principles such as IL-1 receptor antagonist (IL-1ra), soluble (s)TNFR p55 and sTNFR p75, and IL-10, all secreted in a tightly coordinated manner (4, 10, 11). Several other intravascular effector systems, including the complement, the kallikrein-kinin, and the coagulation and fibrinolytic systems, are activated concomitantly with the cytokine network during fulminant meningococemia, revealing the very complex pathogenesis of human septic shock (3, 12–14).

With our current knowledge, one would assume that the combined effects of exceptionally high levels of LPS, bioactive proinflammatory cytokines, and complement activation products in plasma from patients with fulminant meningococemia would exert a profound proinflammatory effect on human monocytes and other LPS-sensitive target cells (15). One study has shown that monocytes isolated from patients with fulminant meningococcal septicemia displayed increased synthesis of tissue factor (TF) that correlated with disease severity (16). PBMC collected from SMD patients without shock and studied *ex vivo* suggested, however, that spontaneous and LPS-stimulated proinflammatory cytokine production was downregulated and differed markedly from the production of IL-1ra during the first days of the disease (17). The latter observations are in line with studies of PBMC collected from other categories of septic shock patients, indicating a dissociation between the plasma levels of cytokines and the cytokine production in PBMC (18, 19). PBMC isolated during experimental human endotoxemia also reveal a reduced responsiveness to LPS stimulation (20).

With the multitude of potential mediators involved in the pathogenesis of septic shock as well as naturally occurring neutralizing principles of these mediators, it is presently impossible to predict the net inflammatory effect of septic shock plasma on various target cells. Consequently, we have developed a functional assay to study the effect of shock plasma on LPS-responsive human cells. Normal human monocytes, representing key target cells for LPS-induced activities, were combined with plasma collected from patients with fulminant meningococcal septic shock. The capacity to activate monocytes was compared with the results obtained with normal plasma boosted with purified Nm-LPS and with plasmas collected from patients with SMD who did not develop septic shock. The results indicate that shock plasmas contain high LPS-inhibitory capacity that effectively counteracts the cooperative action of native meningococcal LPS and proinflammatory components that are induced by LPS. IL-10 appears to account for much of this antiinflammatory activity.

Materials and Methods

Clinical Definitions. SMD was present if *N. meningitidis* was cultivated from blood and/or cerebrospinal fluid (CSF) or confirmed by serology (21). Shock patients were those with persistent hypoperfusion due to bacterial infection, with an initial systolic blood pressure of <85 mm Hg in adults (≥ 12 yr) and <70 mm Hg in children (<12 yr) that required fluid therapy and treatment with vasoactive drugs for at least 24 h or until death. Nonshock patients were SMD patients without persistent hypoperfusion. Clinically, the nonshock patients presented with either meningococcal meningitis or mild systemic meningococemia.

Patient. Heparin plasmas from 10 patients (6 mo–20 yr-old) with shock were studied. Six patients died owing to the circulatory collapse and one suffered severe sequelae due to disseminated thrombosis. *N. meningitidis* was isolated from blood cultures in nine patients and SMD was diagnosed by serological tests in one patient. Plasmas from 10 less severely affected SMD patients (15–41-yr-old) without septic shock were used as a control group. SMD was confirmed by positive culture(s) of blood and/or CSF in nine patients and by serology in one patient.

The levels of IL-10 were determined in heparin plasma from 9 of the 10 patients with and without meningococcal septic shock. Samples were lacking for one patient in each group. Subsequently, IL-10 levels were measured in samples from 7 additional patients with meningococcal shock and in 16 samples from meningococcal patients without shock. Quantitative studies of various inflammatory mediators have previously been published on the same group of patients (1, 3, 7, 12–14).

Nm-LPS. LPS was extracted from *N. meningitidis* prototype strain 44/76 (B:15:P1.16: L,3,7,9) belonging to the ET5 complex by genotyping (22).

Patient Plasma. Blood was collected in LPS-free (1), heparinized (15 U/ml) vacuum tubes and immediately centrifuged (1,400 g, 10 min, 20°C); plasma was pipetted off, aliquoted, and stored at -70°C in Cryotubes (Nunc, Roskilde, Denmark) tested to be LPS-free. The samples used in this assay had previously been thawed.

The initial plasma samples from both shock and nonshock patients were collected within 6 h of hospital admission. Samples were collected serially from many patients with shock in 2–12-h intervals.

Control Plasma. Heparinized blood was collected from one healthy adult and processed as above. An LPS-positive control was prepared by adding 2,500 pg Nm-LPS to 1 ml heparin plasma and vigorously mixed at room temperature (20°C). Plain heparin plasma was used as negative control.

Plasma Endotoxin Assay. Native meningococcal LPS in heparin plasma was quantified by the limulus amoebocyte lysate (LAL) assay, as previously described in detail (1, 23). The lower detection limit was 4 pg/ml (23). 100 pg of the LPS standard equalled 1 endotoxin unit (EU). The high levels of neisserial lipid A have previously been verified by gas chromatography and mass spectrometry in plasma from several patients (22).

Acid-treated FCS. FCS (Flow Laboratories, Irvine, Scotland) tested to contain <100 pg/ml of LPS was prepared as described previously (24), aliquoted, and stored at -20°C.

Cell Culture Fluid (Acid-treated-FCS-RPMI). 19,300 μ l RPMI and 25 mM Hepes buffer with L-glutamine (GIBCO, Paisley, Scotland, UK) was supplemented with 500 μ l acid-treated (AT)-FCS and 200 μ l penicillin/streptomycin solution (Sigma Chemical Co., St. Louis), mixed, and stored at 4°C.

Glucose Phosphate Buffer. The buffer contained 0.14 M NaCl,

0.04 M KCl, 0.001 M Na₂HPO₄, and 0.011 M glucose, adjusted to pH 7.4 with 1 N HCl.

Preparation of PBMC. PBMC were prepared from one healthy donor by density gradient centrifugation and further purification of monocytes by centrifugal elutriation, as described (24, 25). Cryopreservation and thawing of these cells were done according to procedures described in detail elsewhere (24).

Assaying the Net Inflammatory Capacity of Plasma. Monocytes (5×10^4 suspended in 80 μ l FCS-RPMI/well) were seeded in microtiter plates (Dynatech; Ludwigsburg, Germany). 120 μ l patient plasma, positive and negative control plasmas, was added to duplicate wells, the plate sealed off and incubated for 5 h (37°C, 5% CO₂). The plate was then centrifuged (47 g, 5 min, 20°C), and the supernatant gently removed and discarded. The wells were washed once with glucose phosphate buffer (GPB), centrifuged as above, the supernatant discarded, and 200 μ l AT-FCS-RPMI added to each well. The plate was sealed off and incubated for 18 h (37°C, 5% CO₂) before the supernatants were pipetted off and stored at -70°C until assayed for TNF- α and IL-6. The cells were harvested and stored in 75 μ l barbital buffer (26) at -70°C until assayed for procoagulant activity (PCA)(25).

PCA Clot Assay. This assay was performed essentially according to the method of Jungi (27) as modified by Osnes et al. (25).

Immunoassay. Immunoassays of human IL-4, TGF- β , TNF- α , IL-6 (R & D Systems, Abingdon, UK), and IL-10 (Biosource, Camarillo, CA) were performed according to the manufacturers' instructions.

Recombinant Human IL-10. (1–2 $\times 10^6$ U/mg rhIL-10, >97% pure) was purchased from R & D Systems. 5 μ g rhIL-10 was dissolved in 2 ml PBS containing 0.2% (wt/vol) BSA (Sigma Chemical Co.), final concentration 2.5 ng/ μ l or 2.5–5 U/ μ l.

Polyclonal Goat Anti-human rIL-10 Antibodies. 1 mg/ml of these antibodies was purchased from R & D Systems.

The Effect of Boosting LPS-positive Control Plasma with rhIL-10. 2 U rhIL-10 was added to each well with the positive control plasma and PCA monitored. The PCA was 6% of the positive control and equal to the negative control, indicating the profound inhibitory effect of IL-10 on monocyte activation in our assay.

Immunoprecipitation of rhIL-10 in Control Plasma. 600 μ l heparinized control plasma was boosted with 4 μ l (10–20 U) rhIL-10 and vortexed. The level of rhIL-10 was shown to be >500 pg/ml. 50 μ l (50 μ g) of polyclonal goat anti-human IL-10 was added; the sample was first incubated for 1 h at 37°C with gentle agitation, and again for 18 h at 4°C, and then centrifuged (3,000 g, 30 min, 4°C) (28). The supernatant of the immunoprecipitated plasma was pipetted off and shown to contain <15 pg/ml rhIL-10. When the supernatant was boosted with 2,500 pg/ml Nm-LPS, the monocyte activities were restored to close to normal values, i.e., 71% PCA, 99% TNF, and 72% IL-6, respectively. To control for possible unspecific monocyte activation by remaining immune complexes, the IL-10-depleted plasma was added to the target monocytes and monitored for PCA, TNF- α , and IL-6. The levels of PCA (4%), TNF- α (3%), and IL-6 (1%) were not different from the negative control, indicating that remaining immune complexes did not stimulate the monocytes.

Immunoprecipitation of Native IL-10 in Patient Plasma. 50 μ l of goat anti-human rIL-10 was added to 600 μ l patient plasma, the samples mixed, incubated, and centrifuged as above. The effect of the immunoprecipitation was evaluated in plasma from four patients with shock that had initially contained 44,600, 24,400, 11,100, and 1,940 pg/ml of IL-10. The levels were reduced by $\geq 99.5\%$.

Statistics. The differences between groups were calculated with the Mann-Whitney U test. The correlation coefficient was calculated with the Spearman rank test. *P* values <0.05 were considered statistically significant.

Results

Levels of Native Nm-LPS in Patient and Control Plasma. The median level of native Nm-LPS in plasmas from 10 patients with severe persistent septic shock was 2,700 pg/ml (range 50,000–1,100 pg/ml) by LAL assay. One patient sample with 50,000 pg/ml was diluted 1:5 with normal plasma before assaying. When applied to the target monocytes, the median in-well concentration of native Nm-LPS was 320 pg (range 1,260–130 pg).

Plasma from 3 of 10 nonshock patients contained 110, 20, and 13 pg/ml native LPS, respectively, whereas samples from 7 nonshock patients were without detectable LPS (<4 pg/ml) by LAL assay. The control plasma contained 2,500 pg/ml purified Nm-LPS, equivalent to an in-well concentration of 300 pg, which was verified by LAL assay.

Net Spontaneous Inflammatory Capacity (PCA, TNF- α , IL-6) Induced by Plasma from Shock Patients. The median level of monocyte-generated PCA for the whole group was 9% of the positive control plasma (range 0–22%; Fig. 1 A). The median level of secreted TNF- α was 0% (range 0–5%) and IL-6 was 4% (range 0–29%), respectively, of the positive control plasma (Fig. 1 A). These levels were not significantly different from those obtained with the negative control plasma that averaged 2, 2, and 0%, respectively, for PCA, TNF- α , and IL-6.

The results indicate that plasmas from patients with severe, persistent, and often fatal septic shock induced little or no activation of normal human monocytes as monitored by PCA, TNF- α , and IL-6.

Net Spontaneous Inflammatory Capacity of Plasma from Nonshock Patients. Samples from 10 SMD patients without septic shock were studied. The median levels of monocyte-generated PCA were 5% (range 0–12%), 1% TNF- α (range 0–2%), and 0% IL-6 (range 0–1%) of the activities induced by the positive control plasma (Fig. 1 C). One sample was missing in the analysis of TNF- α and IL-6. The levels of PCA and TNF- α were not significantly different from those obtained with shock plasmas or with the negative control (*P* ≥ 0.32), whereas the levels of IL-6 were lower in nonshock than shock plasma (*P* = 0.03).

Net Inflammatory Capacity of Plasma from Shock Patients Boosted with Purified Nm-LPS. Samples from 7 of the 10 shock patients previously studied were assayed. Each sample was boosted with 2,500 pg/ml Nm-LPS giving the same in-well concentration (300 pg/well) of purified Nm-LPS as in the positive control plasma. The median monocyte-generated activities were: 9% PCA (range 3–43%), 2% TNF- α (range 0–5%), and 6% IL-6 (range 0–11%), respectively, compared with the positive control (Fig. 1 B). The value for IL-6 was missing for one patient. Purified Nm-LPS added to shock plasma thus induced a minimal increase of PCA and no increase in TNF- α and IL-6 levels.

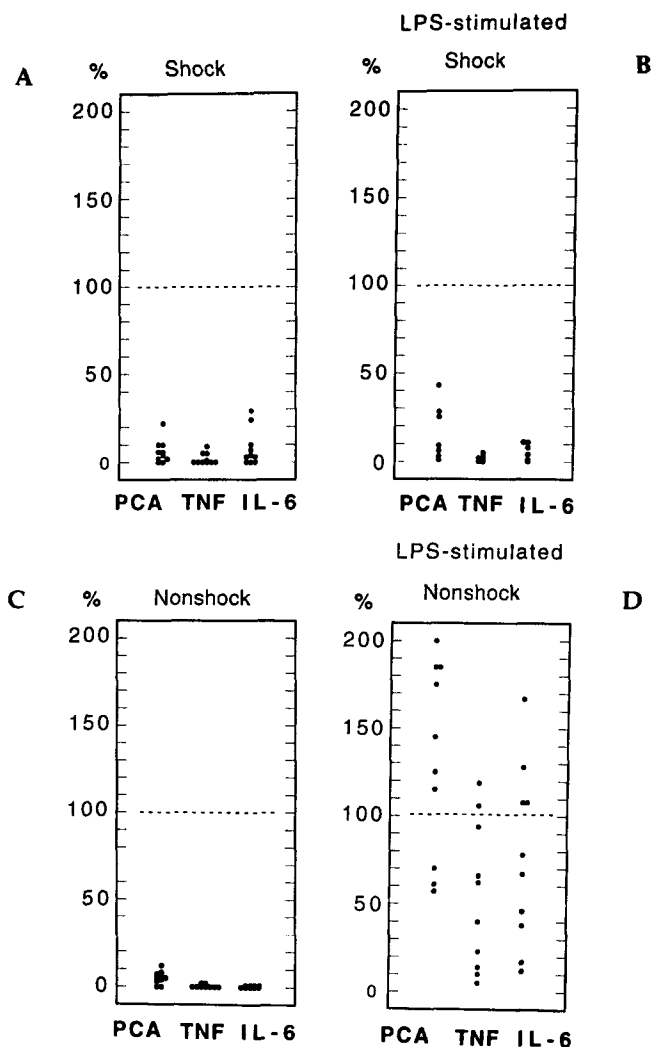


Figure 1. Net spontaneous (A and C) and LPS-boosted (B and D) inflammatory capacity of plasmas from 20 patients with SMD, 10 patients with shock, and 10 patients without shock. The values are given as percent activity (100%) induced by the positive control, i.e., plasma, from a healthy donor boosted with 2,500 pg purified Nm-LPS per ml.

Net Inflammatory Capacity of Plasma from Nonshock Patients Boosted with Purified Nm-LPS. Plasmas from the 10 nonshock patients were boosted with 2,500 pg/ml Nm-LPS (300 pg Nm-LPS/well) as above. The median monocyte-generated activities were: 135% PCA (range 57–200%), 51% TNF- α (range 5–119%), and 73% IL-6 (range 12–167%) of the positive control (Fig. 1 D). The levels of PCA, TNF- α , and IL-6 were significantly higher ($P \leq 0.001$) than observed with shock plasma boosted with Nm-LPS. PCA was significantly more upregulated than TNF- α ($P = 0.007$) and IL-6 ($P = 0.03$), whereas no significant difference existed between TNF- α and IL-6 ($P = 0.23$). The results indicated that plasma from nonshock patients with SMD differed markedly from shock plasmas in the ability to induce monocyte activation after boosting with Nm-LPS.

Net Spontaneous Inflammatory Capacity in Sequentially Collected Samples from Shock Patients. Previous studies have shown that native Nm-LPS declines with $t_{1/2}$ varying from 1 to 3 h after initiation of antibiotic and fluid therapy (1). In serially collected plasma samples from three patients with initially high levels of native LPS, the spontaneously generated levels of PCA, TNF- α , and IL-6 were $\leq 29\%$ of the positive control as long as LPS was detectable by LAL assay (Table 1).

The LPS-inhibitory Capacity of Patient Plasma. The difference in levels of PCA, TNF- α , and IL-6 between the LPS-boosted and native plasma was defined as the LPS-inhibitory capacity of a patient plasma. A small difference indicated a large LPS-inhibitory capacity. In patients with shock, no significant differences existed ($P > 0.05$ for all three parameters) between LPS-boosted and unboosted plasmas. Thus, the LPS-inhibitory capacity was high (Fig. 1, A and C). In plasmas from nonshock patients, highly significant differences existed between LPS-boosted and native plasmas ($P \leq 0.001$), indicating that the LPS-inhibitory capacity was significantly lower than observed in shock plasmas (Fig. 1, B and D).

Changes in the LPS-inhibitory Capacity of Shock Plasma Over Time. Serially collected plasmas from four patients that initially contained 14,000, 10,500, 3,800, and 2,500

Table 1. The Relation between Native Meningococcal LPS, IL-10, and Net Spontaneous Inflammatory Capacity

No.	h	Native LPS	Native IL-10	IL-10 present			IL-10 removed		
				PCA	TNF	IL-6	PCA	TNF	IL-6
	*	pg/ml		%			%		
1.1	1	10,500 [‡]	40,000 [§]	27	16	8	>254	127	142
1.2	8	700	7,000	4	1	5	26	9	10
1.3	11	400	5,000	1	8	3	11	11	3
1.4	15	200	2,800	3	6	1	10	13	2
1.5	28	<25	170	2	5	0	4	3	0
2.1	11	2,700	—	3	1	0	228	186	74
2.2	15	190	1,800	2	7	0	11	9	3
2.3	82	<25	66	1	7	0	1	2	0
3.1	6	2,600	16,500	4	1	29	51	22	33
3.2	19	380	410	2	4	7	4	2	4
4.1	6	1,000	1,170	4	2	5	147	50	83
5.1	8	120	14,000	2	5	1	4	3	1

The net spontaneous inflammatory capacity (PCA, TNF- α , IL-6) of sequentially collected plasmas ($n = 12$) from five patients with meningococcal shock (1–5) before and after immunoprecipitation of IL-10. The values are given as percent monocyte activity induced by the positive control, i.e., plasma, from a healthy donor boosted 2,500 pg purified Nm-LPS per ml.

*Hours after antibiotic therapy was initiated.

[‡]Levels of Nm-LPS generated in vivo in patient samples as determined by LAL assay.

[§]Levels of IL-10 generated in vivo as determined by ELISA.

pg/ml of native meningococcal LPS, respectively, were studied. The LPS-inhibitory capacity was tested using paired samples of native shock plasma and shock plasma boosted with purified Nm-LPS (2,500 pg/ml). The LPS-inhibitory capacity changed markedly during the first 24 h as indicated in Fig. 2, A–D. The levels of PCA and IL-6 in LPS-boosted plasmas increased from <10% to >100% of the positive control 15–20 h after initiation of the anti-shock therapy. In three of four patients the levels of TNF- α in LPS-boosted plasmas were less upregulated than were the levels of PCA and IL-6, indicating a complex and fine-tuned regulation of different monocyte activities.

Quantitation of IL-4, IL-10, and TGF- β in Patient Blood Samples. IL-4 (detection limit, 4 pg/ml) and TGF- β (detection limit, 31 pg/ml) were not detected in any of the plasma samples with high LPS-inhibitory capacity. The levels of IL-10 were initially determined in 9 of the 10 samples from patients with and without shock. The median level of IL-10 in the shock plasmas was 19,000 pg/ml (range 1,800–111,000 pg/ml) vs. 22 pg/ml (range <15–4,400 pg/ml) among 9 of 10 plasmas from nonshock patients. One sample for IL-10 analysis was lacking in each patient group.

Subsequently, IL-10 was determined in plasma from 23

additional patients with SMD, 7 with shock and 16 without shock. Among 16 shock patients, IL-10 was present in all and the median level was 13,300 pg/ml (range 1,800–111,000 pg/ml; Fig. 3). Of 25 patients without shock, IL-10 was present in 17 (68%; Fig. 3). The median level was 44 pg/ml (range <15–4,400 pg/ml). The difference in levels of IL-10 was highly significant between shock and non-shock patients ($P = 0.0001$; Fig. 3). The levels of IL-10 were significantly correlated with the levels of native LPS ($r = 0.79$, $P = 0.0002$, $n = 24$).

Disappearance of the LPS-inhibitory Capacity Versus Declining Levels of IL-10 in Plasma. An inverse relation existed between declining levels of IL-10 and increasing monocyte reactivity after stimulation with Nm-LPS (2,500 pg/ml) in serially collected shock plasmas (Fig. 2, A–D). Levels of native IL-10 >1,000 pg/ml were associated with significant LPS-inhibitory capacity. Levels of IL-10 <1,000 pg/ml were associated with reduced LPS-inhibitory capacity, i.e., increasing monocyte reactivity when stimulated with Nm-LPS (Fig. 2, A–D). The levels of PCA and IL-6 increased to >150% of the positive control when monocytes were stimulated with shock plasma containing <1,000 pg/ml of IL-10 boosted with Nm-LPS (Fig. 2, A–D). The levels of IL-10 declined from the initiation of antibiotic and anti-

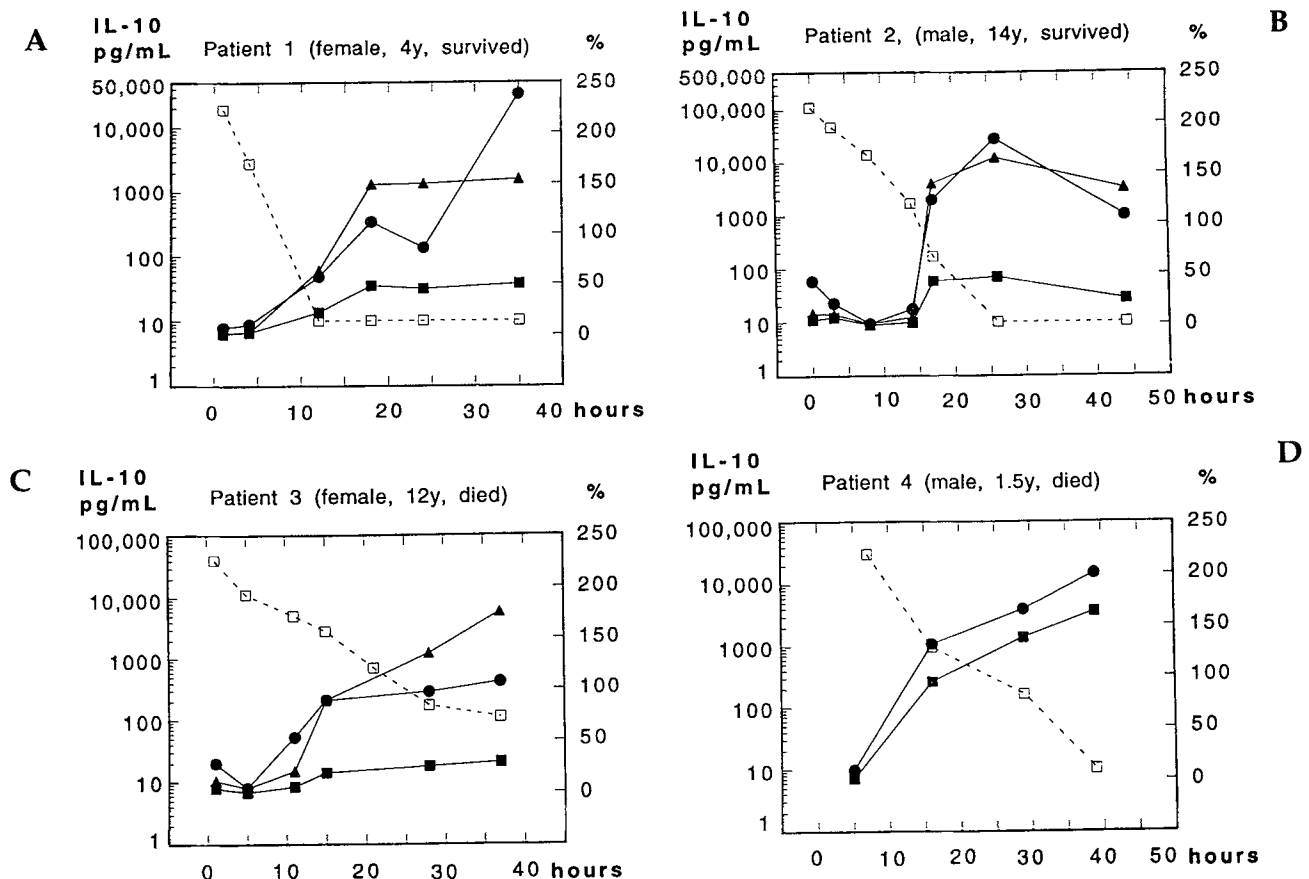
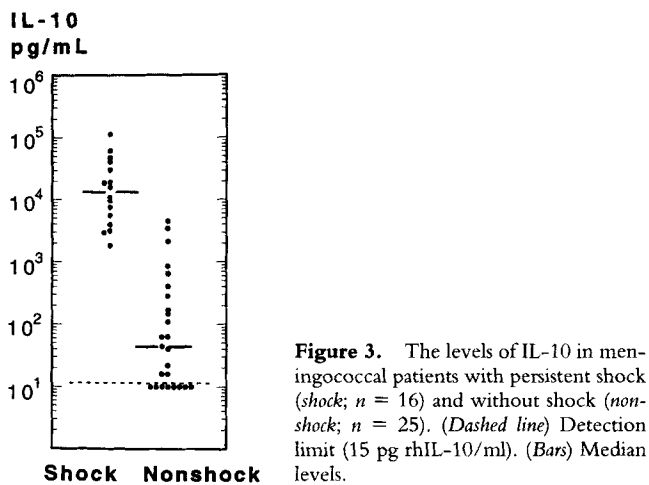


Figure 2. The levels of monocyte-generated PCA (dosed circles) and secreted TNF- α (dosed squares), and IL-6 (dosed triangles) elicited by plasma from four different shock patients collected serially and boosted with 2,500 pg/ml of purified Nm-LPS. The values are given as percent positive control (healthy donor plasma boosted with 2,500 pg/ml Nm-LPS). (Dashed line) Levels of IL-10.

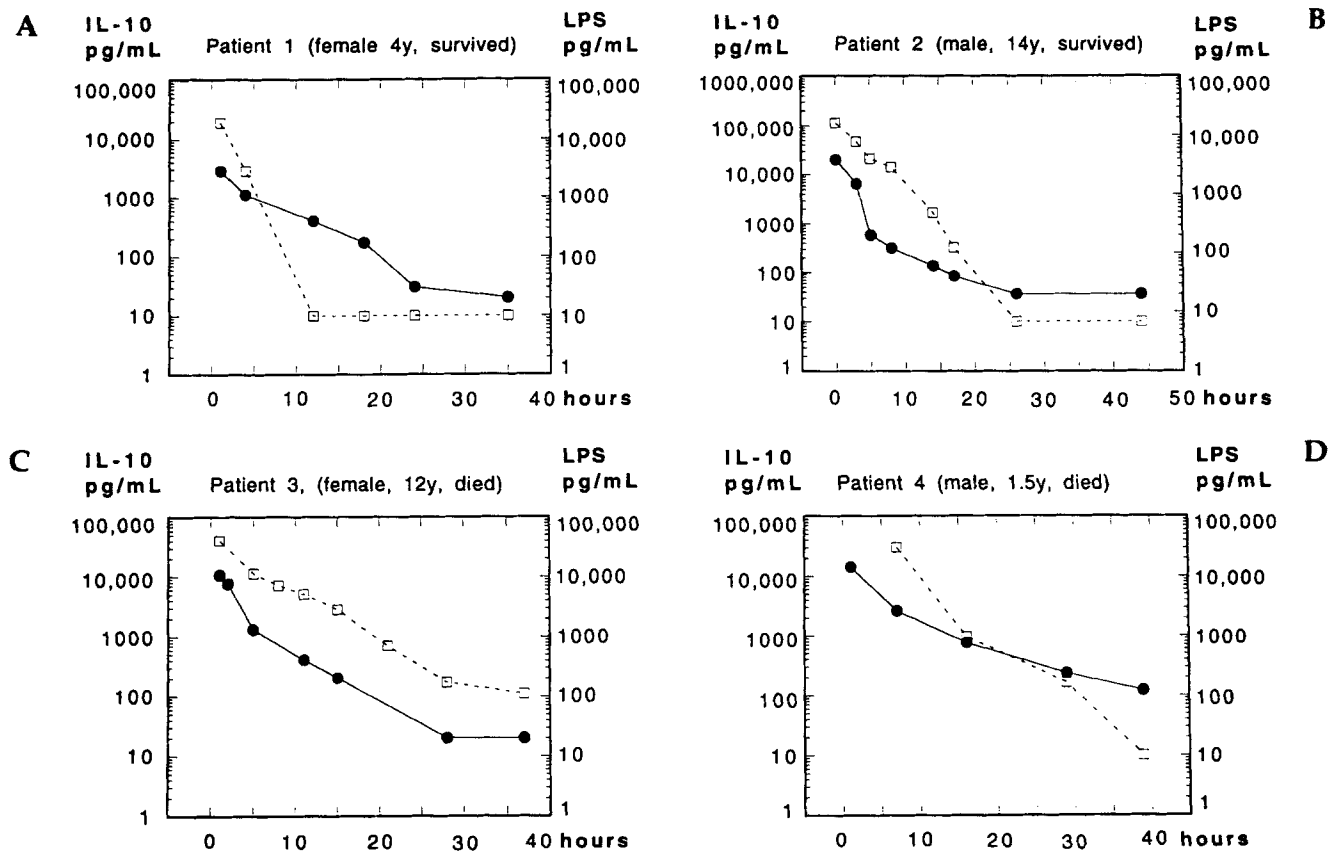


shock therapy and paralleled the declining levels of native Nm-LPS (Fig. 4, A–D).

The Net Inflammatory Capacity of Shock Plasmas Depleted of IL-10. 12 plasma samples from 5 shock patients collected serially during the acute stage of the disease were immunodepleted of IL-10 (Table 1). 10 of 12 plasmas contained native meningococcal LPS ranging from 10,500 to 120 pg/ml by LAL assay. LPS was <25 pg/ml in two samples. 4 of 12

samples contained native LPS concentrations of >700 pg/ml. Before IL-10 depletion, all plasmas showed low net spontaneous inflammatory capacity (Table 1). Removal of IL-10 by immunoprecipitation increased the spontaneous net inflammatory capacity dramatically in samples containing >700 pg/ml of native meningococcal LPS (Table 1). IL-10-depleted plasmas with native LPS <700 pg/ml had little or no effect on the monocytes (Table 1). After immunodepletion, there was a close correlation between the LAL-detected LPS activity in the IL-10-depleted plasmas and their capacity to induce PCA ($r = 0.91$, $P = 0.006$), TNF- α ($r = 0.81$, $P = 0.02$), and IL-6 ($r = 0.92$, $P = 0.006$) in the target monocytes.

The Net Inflammatory Capacity of Shock Plasmas Depleted of IL-10 and Boosted with Nm-LPS. Boosting IL-10-depleted patient plasmas with the standard amount of Nm-LPS (2,500 pg/ml) restored the capacity to stimulate monocytes. This was particularly evident for plasmas with native LPS <700 pg/ml since the median levels of PCA, TNF- α , and IL-6 increased from 7, 6, and 3%, respectively, before LPS stimulation, to 118, 91, and 120% after LPS stimulation. When Nm-LPS was added to the four IL-10-depleted plasmas with native LPS >700 pg/ml, the median levels of PCA, TNF- α , and IL-6 went from 188, 89, and 79% to 206, 107, and 131%, respectively.



Discussion

We have developed a functional assay to study the impact of highly pathological plasma, collected from patients with severe gram-negative septic shock on normal human monocytes. A large number of experiments were performed under strictly standardized conditions by combining purified, cryopreserved, and functionally active human monocytes from a healthy donor with pathological plasmas from different patients. The assay system was constructed to monitor three different monocyte functions that represent activation of “immediate early genes” after stimulation with LPS. These functions are mediated by CD14 and are closely coordinated in their expression (25). This approach also gave us the possibility to study the net effect of various pro- and antiinflammatory components generated during overwhelming gram-negative sepsis. Furthermore, the method made it possible to study the effect of native LPS generated during severe gram-negative bacteremia vs. the purified LPS usually employed in experiments.

Surprisingly, plasma collected from severely ill septic shock patients with exceptionally high levels of native meningococcal LPS and bioactive proinflammatory cytokines did not activate the monocytes. When the samples were boosted with purified Nm-LPS, they remained partly or completely inactive, suggesting that inhibitory components were present in the plasmas that effectively antagonized the proinflammatory components, in particular LPS. This LPS-inhibitory capacity declined gradually after antibiotic and antishock treatment was initiated, but was present during the whole elimination phase of native LPS. Thus, minimal or no spontaneous activation of the donor monocytes occurred in sequentially collected samples.

When purified Nm-LPS was added to plasma samples collected 12–36 h after hospital admission, increasing monocyte responsiveness was present, reflecting reduced inhibition of the monocytes. The PCA and IL-6 responses gradually surpassed the response obtained with Nm-LPS added to normal donor plasma, indicating the presence of proinflammatory components that acted synergistically with purified LPS. This rapid shift in the inhibitory capacity of shock plasma over time suggested that the inhibitory principle(s) disappeared with a kinetics similar to that of various cytokines, i.e., TNF- α , IL-6, IL-8, and LIF in fulminant meningococcal septicemia (7–9, 11).

The inhibition of Nm-LPS-induced monocyte activity was absent or only weakly present in plasma collected upon hospital admission from patients with SMD who did not develop severe septic shock. The latter group is characterized by “low” (<700 pg/ml) or undetectable levels of native meningococcal LPS and low levels or absence of bioactive TNF- α , IL-6, and complement activation products upon admission (1–5, 7, 8, 12–14). The acute phase response is massive, upon admission, in the latter group of patients as reflected by high levels of C-reactive protein and fibrinogen. In patients with fulminant meningococcal septicemia, the acute phase response is less pronounced be-

cause of the shorter duration of symptoms before hospital entrance. The behavior of the inhibitory principle(s) in sequentially collected shock samples and among the different clinical categories was clearly different from the behavior of common acute phase reactants.

TGF- β , IL-4, and IL-10 are all cytokines with antiinflammatory properties that may downregulate monocyte functions (29–36). The patient plasmas were screened for the presence of any of the three. High levels of IL-10 were present in all shock plasmas. In plasmas from SMD patients without shock, the levels of IL-10 were several magnitudes lower or absent, i.e., <15 pg/ml. IL-4 and TGF- β were not detected in any plasmas with high graded inhibitory capacity. The levels of IL-10 declined, furthermore, in parallel with the reduction of the inhibitory capacity of serially collected shock plasma.

Removal of IL-10 by immunoprecipitation restored the capacity of shock plasma to react with purified Nm-LPS. After removal of *in vivo*-generated IL-10, several plasmas with high levels of native meningococcal LPS, i.e., >700 pg/ml, induced PCA, TNF- α , and IL-6 in the target monocytes without being boosted with purified LPS. We assume that native LPS together with proinflammatory plasma components induced this activity. In patients with low levels of both native LPS (\leq 700 pg/ml or 7 EU/ml) and proinflammatory cytokines, minimal or no upregulation of monocyte activities occurred after removal of *in vivo*-generated IL-10, indicating that the procedure per se, i.e., formation of immune complexes, did not activate the cells.

These results demonstrate that IL-10 is abundantly present in plasma from patients with meningococcal septic shock, an observation that is in accordance with that of other research groups (10, 37). The plasma levels of IL-10 were closely associated with the circulating level of native LPS, as has previously been documented for several proinflammatory cytokines (3, 4, 7). IL-10 appears, furthermore, to play an important functional role in deactivating monocytes and possibly also tissue macrophages *in vivo*. Generation of IL-10 in animal experiments has been related to the development of endotoxin tolerance (38). Our observations suggest that IL-10 might play an important functional role in downregulating the response of human monocyte to LPS in human bacteremia and endotoxemia, explaining the deactivated state of PBMC during septic shock and experimental human endotoxemia (18–20). In our patients, it represented an important antiinflammatory cytokine with a strong impact on the functional state of the monocytes. The antiinflammatory effect of IL-10 alone was apparently stronger than the collective action of bioactive native LPS, TNF- α , IL-6, IL-8, complement activation products, and other proinflammatory components in plasma in determining the functional state of monocytes. These observations suggest that IL-10 plays a key role in modulating the intravascular inflammatory response in overwhelming sepsis rendering plasma antiinflammatory as concerns monocyte

activation. IL-10 appears to represent an important link in a negative feedback loop to contain the effect of excessively produced proinflammatory cytokines.

We have previously observed that isolated monocytes collected from two patients with fulminant meningococemia with massive coagulopathy were in a deactivated state. These monocytes, which were collected 12 h after hospital admission and purified by elutriation, did not secrete TNF- α , IL-6, or express TF spontaneously as determined by flow cytometry and in a microclot assay of lysed cells. The cells lacked mRNA for TNF- α and IL-6, whereas mRNA for IL-8 was present. They were unable to respond to purified Nm-LPS (Brandtzaeg, P., unpublished results).

IL-10 was present in all patients with fulminant meningococcal septicemia and in two thirds of all patients with mild systemic meningococemia. The median level in the former group was 300 times higher than was the median level in patients without persistent septic shock. IL-10 is elicited during experimental human endotoxemia and the levels are attenuated by a fragment of bactericidal permeability increasing protein that blocks the biological effect of lipid A (39). It is secreted by monocytes, and by tissue macrophages including the large population of Kupffer cells in the liver. Animal experiments have shown that lethality increases if the action of IL-10 is blocked (40, 41). Increasing severity, as reflected by increasingly higher levels of native LPS upon admission, was associated with a dose-dependent increase in IL-10 levels in our patients. This observation is in line with previous results linking increasing bacterial proliferation, and consequently, increasing levels of native LPS to a dose-dependent increase in various cytokines in fulminant meningococcal infections (3, 42). The levels of IL-10 declined immediately after the bacterial proliferation and production of LPS was terminated by penicillin treatment. The declining levels of IL-10 paralleled the levels of native meningococcal LPS closely. The cells that produce IL-10 appear to recognize the absolute levels of native LPS and produce IL-10 accordingly. In patients developing fulminant septicemia, the production of IL-10 appears to increase until antibiotic therapy is initiated. When the microbial growth is terminated and circulating LPS decline due to elimination, the production of IL-10 is gradually downregulated, reducing the circulating plasma levels to a few picograms per milliliter within 10–40 h. IL-10 clearly circulates in a biologically active form as was demonstrated by immunoprecipitation experiments.

Although IL-10 appears to be a key antiinflammatory cytokine that accounts for much of the inhibitory activity in septic shock plasma, it is presumably not the only antiinflammatory cytokine elicited. Increased levels of IL-1ra have previously been documented in meningococcal septic

shock plasma (4). Experience, so far, has shown that a complex cytokine network exists, comprising molecules with overlapping activities. This is also the case for downregulation of cytokine action (43). Presumably, there are more antiinflammatory principles that have yet to be identified. Some of our observations indicated that LPS-inhibitory capacity cannot completely be explained by IL-10 alone. In certain plasmas from nonshock patients, stimulation with Nm-LPS had little impact on TNF- α and IL-6 production despite undetectable levels of IL-10. In plasmas from two of four patients examined serially, LPS-inhibitory capacity as concerns PCA, reached its maximum 5–7 h after admission when the IL-10 levels had declined to 30–40% of the admission levels (Fig. 2, B and C). This may indicate that the complex balance between pro- and antiinflammatory principles in plasma had changed over time. The changing balance may also have a different impact on the different monocyte functions since PCA appeared to be more sensitive to the changes than secretion of TNF- α and IL-6.

In our assay system, samples containing native LPS >700 ng/liter revealed a significantly higher monocyte activity after removal of IL-10 than did samples with lower levels of LPS. The latter samples induced virtually no monocyte activity despite removal of IL-10. This may primarily relate to the sensitivity of the assay system, but could also partly be explained by the interaction of native meningococcal LPS with various LPS-neutralizing buffer systems in plasma, primarily lipoproteins (44, 45). These systems may prevent cell activation by low levels of LPS but not the excessively high levels observed in fulminant meningococcal septic shock.

The results of this study suggest that the antiinflammatory principles in septic shock plasma have a stronger impact on the activation state of human monocytes than do the proinflammatory principles. Certain fundamental questions arise from these observations. Are LPS-responsive cells other than monocytes also deactivated by septic shock plasma? In particular, are neutrophils impaired in their phagocytic function by IL-10 and possibly other antiinflammatory cytokines? What is the effect of shock plasma on endothelial cells, smooth muscle cells, and platelets? To answer these questions, the functional role of IL-10 on other LPS-responsive cells has to be elucidated. Assays built according to the same principles as the one used in this study may help to disclose the net effect of septic shock plasma on important target cells that contribute to the development of septic shock. We believe that this assay combined with immunoprecipitation can also be used to study the functional role of other plasma components that are assumed to play an important role in regulating cell activation during intravascular inflammation.

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