

Is Circulating Endotoxin the Trigger for the Systemic Inflammatory Response Syndrome Seen After Injury?

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Objective

Patients with severe traumatic or burn injury and a mouse model of burn injury were studied early after injury to determine the relation of plasma endotoxin (lipopolysaccharide [LPS]) to the production of proinflammatory cytokines and subsequent resistance to infection.

Summary Background Data

Elevated levels of plasma LPS have been reported in patients after serious injury. It has been suggested that circulating LPS may be a trigger for increased proinflammatory cytokine production and may play a role in the septic syndromes seen in a substantial portion of such patients. Yet, despite multiple reports of leakage of LPS from the gut and bacterial translocation after injury in animal models, there is little direct evidence linking circulating LPS with production of inflammatory mediators.

Methods

The authors studied serial samples of peripheral blood from 10 patients with 25% to 50% surface area burns and 8 trauma patients (Injury Severity Score, 25–57). Patients were compared with 18 healthy volunteers. The study was focused on the first 10 days after injury before the onset of sepsis or the systemic inflammatory response syndrome. Plasma samples were assayed for LPS, and adherent cells from the blood were studied for basal and LPS-stimulated production of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6). The correlation of increased plasma LPS with TNF- α production was studied as was the association of increased plasma LPS and increased TNF- α production with subsequent septic complications. We also studied a mouse model of 25% burn injury. Burn mice were compared with sham burn control subjects. Plasma samples were assayed at serial intervals for LPS, and adherent cells from the spleens were studied for basal- and LPS-stimulated production of TNF- α , IL-1 β , and IL-6. Expression of the messenger RNAs for IL-1 β and TNF- α also was measured. The relation of increased TNF- α production with mortality from a septic challenge, cecal ligation and puncture (CLP), was determined. Finally, the effect of administration of LPS to normal mice on subsequent mortality after CLP and on TNF- α production was studied.

Results

Elevated plasma LPS (>1 pg/mL) was seen in 11 of the 18 patients within 10 days of injury and in no normal control subjects. In this period, patients as compared with control subjects showed increased stimulated production of TNF- α , IL-1 β , and IL-6. Increased TNF- α production was not correlated with elevated plasma LPS in the same patients. Neither increased plasma LPS nor increased TNF- α production early after injury was correlated with subsequent development of systemic inflammatory response syndrome or sepsis in the patients. Burn mice, as compared with sham burn control subjects, showed elevated plasma LPS levels chiefly in the first 3 days after injury. Increased stimulated production of proinflammatory cytokines by adherent splenocytes from the burn mice also was seen at multiple intervals after injury and did not correlate with mortality from CLP. Increased production of TNF- α and IL-1 β was associated with increased expression of messenger RNAs for these cytokines. Finally, two doses of 1 ng LPS administered 24 hours apart to normal mice had no effect on mortality from CLP performed 7 days later nor on the production of TNF- α at the time of CLP.

Conclusions

These findings call into question the idea that circulating LPS is the trigger for increased proinflammatory cytokine production, systemic inflammatory response syndrome, and septic complications in injured patients.

It has been apparent for many years that patients who sustain major thermal or traumatic injury and who survive initial resuscitation are at substantial risk for subsequent complications, including systemic sepsis, which, in turn, may lead to the failure of multiple organ systems and ultimately to death.¹ It recently has become clear that the sepsis syndrome may occur in some patients without evidence of invasive bacterial or fungal infection, and the clinical picture manifest in these individuals has been redefined as the systemic inflammatory response syndrome (SIRS).² Systemic inflammatory response syndrome in relevant animal models and in some clinical studies appears to be mediated by excessive production of proinflammatory cytokines and other mediators of inflammation, including eicosanoids. A number of laboratories have therefore attempted to determine what the trigger is for this excessive mediator production and how to control it. Maejima et al.³ a decade ago showed conclusively that serious injury led to disruption of the gut barrier function and translocation of enteric bacteria into the mesenteric lymph nodes, liver, and other organs. These findings appeared to provide an answer to the question of what triggered the excessive production of cytokines and prostanoids⁴ because bacterial products, particularly

gram-negative endotoxins, are known to be potent activators of both macrophages and granulocytes.

Nevertheless, there remain important unanswered questions concerning the relation of presumed bacterial translocation and/or endotoxin leak from the gut and the end stage of multiple organ failure after severe injury.^{5,6} Evidence of endotoxin in the plasma may be found early after burn injury, for example, but ordinarily remains undetectable thereafter unless or until invasive bacterial infection ensues.⁶ Munster et al.⁶ have shown in a series of patients with major burns that neutralizing endotoxin with low doses of polymyxin eliminated endotoxin from the plasma of their burn patients but had no evident effect on clinical outcome. Roumen et al.⁷ found that increased intestinal permeability measured by the lactulose-mannitol excretion ratio in patients after major surgery, and serious injury bore no relation to systemic sepsis or organ failure. Conversely, enteral feeding, which is known to restore gut barrier function, has improved survival in some animal models of sepsis and injury⁸ and has influenced favorably the clinical course of trauma and burn patients in reported studies.^{9,10}

In the past, determination of the relation between circulating bacterial endotoxin and inflammatory mediator production after injury was complicated further by questions concerning the accuracy and sensitivity of the available bioassays for endotoxin in body fluids, particularly plasma. However, recently, techniques such as that described by Ditter et al.¹¹ have overcome many of these difficulties and have made accurate measurements of endotoxin in circulating plasma in humans and animals locally feasible.

The purpose of the current investigation was to deter-

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mine the relation between circulating plasma endotoxin after serious injury and the production of inflammatory mediators in patients and an animal model and to relate these findings to subsequent episodes of sepsis in the patient population and to diminished resistance to infection in the animal model.

MATERIALS AND METHODS

Patient Population

We studied 18 adult patients admitted to the Burn-Trauma Intensive Care Unit of the Brigham and Women's Hospital. Ten of these individuals had sustained burns of 25% to 50% body surface area and 8 were victims of accidental trauma (Injury Severity Score, 25–57). One of the latter patients had minor burn injury as well. There were 16 men and 2 women ranging in age from 18 to 56 years. Eighteen healthy human volunteers, 12 men and 6 women, aged 22 to 57, served as control subjects. Patients were studied chiefly during the first 10 days after injury before the onset of SIRS or sepsis in this group. They were monitored clinically until discharge for evidence of SIRS or sepsis. Systemic inflammatory response syndrome was defined by published criteria,² and sepsis was defined as SIRS with evidence of invasive infection by an identified organism or organisms.

Blood was drawn from human subjects only after informed consent was obtained using a form approved by the Brigham and Women's Hospital Committee for the Protection of Human Subjects from Research Risks and in accordance with the National Institutes of Health guidelines. We drew samples of venous blood from patients at intervals of 1 to 10 days after injury on 124 occasions. Small samples of blood (range, 2–5 mL) were placed in endotoxin-free tubes with lithium heparin (Kabi tubes; Pharmacia, Piscataway, NJ) for plasma separation for endotoxin determinations. Larger blood samples (no more than 30 mL) were drawn for collection of peripheral blood mononuclear cells. Blood was drawn simultaneously from a control subject on each occasion.

Separation of Peripheral Blood Mononuclear Cells

As previously described,¹² peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation of heparinized blood on Ficoll-Hypaque (Pharmacia, Piscataway, NJ) for 35 minutes at 400 g. The interface cells were collected; washed three times in complete medium consisting of minimal essential medium with antibiotics (penicillin, streptomycin, and fungizone) 2 mmol/L glutamine, 1% HEPES buffer, and 5% fetal bovine serum (FBS) (all reagents from GIBCO, Grand Island, NY); and

counted with trypan blue for viability and Turk's stain for observation of morphology. The cells were always more than 95% viability. Because patient's interface cells sometimes had increased contamination with myeloid cells, the percentage of mononuclear cells was estimated by Turk's stain, and cell counts were adjusted accordingly so that the total number of mononuclear cells per well was similar in each experiment. Cytospin slides of each PBMC preparation were stained with Wright's stain for a differential count performed later to verify findings with the Turk's stain.

Human Adherent Cell Production of Cytokines

Two hundred microliters PBMC at 5×10^6 cells/mL of complete medium was allowed to adhere to each well of flat-bottomed 96-well tissue culture plates (Nunc, Roskilde, Denmark) for 1 hour at 37 C in 5% carbon dioxide (CO₂) and 95% air. Nonadherent cells were removed by washing the plates three times in RPMI-1640 medium with additions as above but without FBS, and the adherent cells were cultured for 24 hours in 200 μ L of RPMI medium in the presence of 0.3 μ g/well lipopolysaccharide (LPS, from *Escherichia coli* 055:B5, Difco, Detroit, MI) or with no additions. The supernatants were removed at 24 hours, and similar wells pooled and frozen at -20 C until tested.

Mouse Burn Model

Eight-week old male A/J mice obtained from Jackson Laboratories (Bar Harbor, ME) were acclimatized for 1 week in the animal facilities at the Brigham and Women's Hospital. Animals were maintained with water and mouse food ad libitum. Care of the animals and all procedures performed were in accordance with National Institutes of Health guidelines and with the permission of Harvard Medical School Standing Committee on Animals. Mice undergoing burn or sham burn injury as described previously¹² were studied in groups of 5 to 10. After induction of anesthesia with intraperitoneal pentobarbital (66 μ g/g body weight), all animals were shaved over the dorsum and placed in a specially constructed plastic mold that exposed approximately 25% of their total body surface area. The mold containing the animal was immersed in water for 9 seconds. Animals undergoing sham burn injury were immersed in thermoneutral water, whereas those undergoing burn injury were immersed for 9 seconds in water at 90 C. The latter treatment resulted in a histologically proved full-thickness burn of the exposed dorsum. Animals were dried immediately and resuscitated with 1 mL 0.9% sterile pyrogen-free saline intraperitoneally.

Cecal Ligation and Puncture

Animals were anesthetized as described above, and the abdomen was shaved and opened in the midline. The cecum was delivered and ligated at its base with a 4–0 silk ligature. The cecum was punctured twice with a 25-gauge needle and then was replaced in the abdomen, and the wound was closed with 5–0 nylon suture. Animals again were resuscitated with 1 mL 0.9% saline intraperitoneally.

Isolation and Preparation of Splenocytes

Sham burn and burn mice in groups of 5 to 10 were killed in a CO₂ chamber at serial intervals after injury. At the time of sacrifice, all animals were bled by cardiac puncture using sterile technique to obtain plasma for endotoxin measurements. Spleens were removed and minced and the cells counted as described previously.¹² The cells were resuspended in RPMI-1640 medium containing glutamine, Hepes buffer, 2-mercaptoethanol, antibiotics, and 5% FBS (all reagents from GIBCO, Grand Island, NY). Spleen cells at a final concentration of 1×10^7 cells/mL were allowed to adhere to the bottoms of the wells in 96-well plastic tissue culture plates for 1 hour at 37 C, 5% CO₂. Nonadherent cells were removed by washing three times with RPMI complete medium without FBS. Adherent cells then were cultured in 225 μ L RPMI complete medium without FBS containing 110 ng/mL LPS (*E. coli* 026:B6, Difco) or no additions. The supernatants were removed at 24 hours, and similar wells were pooled and frozen at –20 C until tested.

Measurement of Endotoxin

Endotoxin was measured by a kinetic turbidimetric limulus lysate assay¹¹ in plasma samples that had been diluted 1:5 or 1:10 in endotoxin-free water (Associates of Cape Cod, Woods Hole, MA) and heat inactivated for 30 minutes at 80 C. Estimation of endotoxin was made by spiking samples of plasma with serial dilutions of an endotoxin standard (Associates of Cape Cod) and estimating endotoxin concentration using limulus lysate at 37 C in a Thermomax enzyme-linked immunosorbent assay (ELISA) reader as described by Ditter et al.¹¹ Sensitivity of the assay was 0.1 pg/mL. Computer software (Pyrokin) was provided to us by Drs. Renate Urbascheck and Peter Becker with permission from the German government.

Interleukin-6 Bioassay

This assay uses the IL-6-dependent cell line B9 (provided by Dr. Steve Clark at Genetics Institute, Cambridge, Massachusetts, with permission from Dr. Lucien Aarden

of the Netherlands Red Cross). As described previously,¹³ IL-6 concentration was determined from a standard curve using recombinant IL-6 and compared with a standard monocyte supernatant stimulated with LPS and known to contain IL-1, TNF, and IL-6. Results were expressed as units per milliliter.

Interleukin-1 Bioassay

Supernatants were tested for IL-1 using a CTLL-2/NOB-1 bioassay.¹³ Briefly, 1×10^5 NOB-1 cells and 5×10^3 CTLL-2 cells were added in 100 μ L medium per well to 96-well plates as above. Supernatants were added to wells in serial dilutions from 1:2 to 1:128. Plates were incubated for 20 hours at 37 C in the presence of 5% CO₂, pulsed with tritiated thymidine (³HTdr; New England Nuclear, Boston, MA), 3.7×10^4 becquerel per well, and the incorporation of ³HTdr was determined using a liquid scintillation counter (LKB Instruments, Gaithersburg, MD). Interleukin-1 then was calculated using probit analysis by a program (provided by Brian Davis, Immunex, Seattle, WA).

Enzyme-Linked Immunosorbent Assay for Human Tumor Necrosis Factor- α

In this assay, a polyclonal goat antihuman TNF- α antibody (Rand D Systems, Minneapolis, MN) diluted 1:1500 in 0.1 M carbonate buffer, pH 9.6, was added to 96-well ELISA plates (Nunc Maxisorp, Roskilde, Denmark). The plates were incubated overnight at 4 C and then washed three times in washing solution, phosphate-buffered saline plus 0.05% Tween-20. Active sites on the plates were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline for 1 hour at room temperature, and the plates were washed again four times in washing solution. The samples and standards (diluted in medium corresponding to the samples) were added in 50 μ L volumes to each well. The standards were prepared from serial dilutions beginning with 10 μ L of a stock solution at 10,000 pg/ μ L TNF- α (Rand D Systems) in 10 mL medium. The plates were incubated 60 minutes at 37 C in 5% CO₂, washed four times and 50 μ L of the second antibody, polyclonal rabbit antihuman TNF- α (Genzyme, Cambridge, MA) diluted 1:800 in 0.1% BSA in wash solution, was added to each well. Plates were incubated for 60 minutes at room temperature and washed four times as before. Fifty microliters of goat antirabbit immunoglobulin conjugated with alkaline phosphatase (Boehringer–Mannheim, Indianapolis, IN) diluted 1:1500 in wash solution with 0.1% BSA was added to each well, and the plates again were incubated for 1 hour at room temperature. Plates again were washed four times in wash solution and the substrate paranitrophenylphosphate diso-

dium, 1 mg/mL in substrate diluent (pH 9.8, 0.05M sodium carbonate buffer with 1 mM magnesium chloride) was added 100 μ L per well, and plates were incubated at room temperature until acceptable color development occurred. The results were read at 405 nm in an ELISA reader (UV Max; Molecular Devices, Palo Alto, CA). Data were obtained from standard curves generated by the ELISA reader software (SoftMax; Molecular Devices). The immunoassay was tested for sensitivity and specificity using recombinant cytokines obtained from the Biological Response Modifiers Program, National Institutes of Health.

Mouse Tumor Necrosis Factor- α Enzyme-Linked Immunosorbent Assay

This assay, as described previously,¹³ was similar to the human assay and used a hamster antimouse TNF- α monoclonal antibody and a rabbit polyclonal antibody (Genzyme, Cambridge, MA) with goat antirabbit immunoglobulin conjugated with alkaline phosphatase (Boehringer-Mannheim).

Enzyme-Linked Immunosorbent Assay for Human Interleukin-1 β

This assay was similar to the above ELISA's and used mouse monoclonal antibodies raised against two epitopes of human IL-1 β (donated by Dr. John Kenney of Syntex, Palo Alto, CA). The second antibody was biotinylated with avidin-conjugated alkaline phosphatase (Sigma Chemical, St. Louis, MO) used to react with the substrate.

RNA Extraction and Northern Blotting

In some experiments, mouse splenocytes 1×10^6 cells were lysed in guanidium isothiocyanate and 2-mercaptoethanol. Total RNA was obtained by phenol-chloroform extraction.¹² For Northern blotting, equal quantities of RNA (5 μ g/lane) were fractionated by electrophoresis on a 1% agarose-formaldehyde gel, transferred to nylon membranes, and fixed by ultraviolet crosslinking, as described previously. Membranes then were hybridized for 18 hours at 42 C with a $\{\alpha\text{-}^{32}\text{P}\text{dCTP}\}$ (New England Nuclear Research Products, Boston, MA), labeled cDNA probe. Human and murine β -actin, TNF- α , and IL-1 β probes were purchased from the American Type Culture Collection (Rockville, MD). Probes were radiolabeled using a random primer DNA labeling kit (Boehringer-Mannheim). After hybridization, the blots were washed and autoradiographed. Blots were stripped at 80 C using a 1% glycerol solution (Sigma Chemical) and probed again with a radiolabeled cDNA probe for β -actin. Signals were quantitated using a laser densitometer (Model

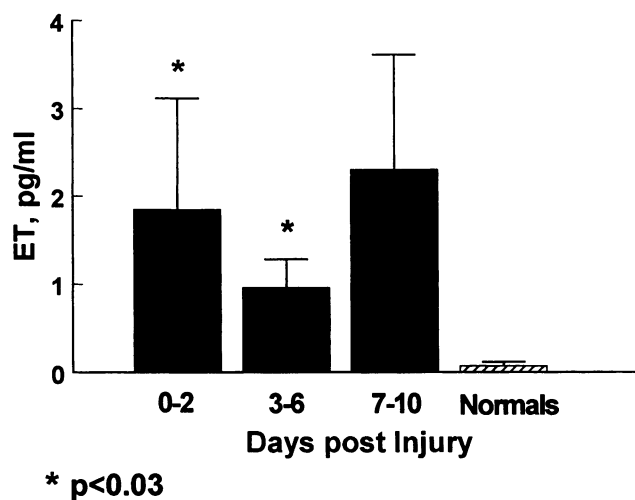


Figure 1. Plasma endotoxin levels in the patient population (mean \pm standard error). It is apparent that endotoxin levels were elevated significantly in the patients as compared with those of normal control subjects at two intervals in the 10-day study period.

300 series; Molecular Dynamics, Waltham, MA) and were standardized to the β -actin message.

Statistical Analysis

Patients and normal subjects and burn and control mice were compared regarding cytokine production by the Mann-Whitney U test. Association of endotoxin or cytokine production with later clinical events was determined by Fisher's exact test and the correlation between circulating endotoxin and cytokine production by Spearman's rank correlation. These statistical analyses were done using the statistical program InStat 2 (Graph Pad Software, Carlsbad, CA). Survival of mice after CLP was compared by the Wilcoxon-Gehan test using the statistical package STATA (Los Angeles, CA). The level of significance was considered to be $p \leq 0.05$.

RESULTS

Plasma endotoxin was determined on 72 occasions in the 18 patients during the 10-day study period. The assay, when tested against an endotoxin standard, was accurate to levels of 0.1 pg/mL. Because no normal control subject had a circulating endotoxin concentration greater than 1 pg/mL, this arbitrarily was set as the lower limit of endotoxin positivity for purposes of this report. Eleven of the 18 patients had detectable endotoxin 1 pg/mL or greater (range, 0–27 pg/mL) in the circulating plasma in the first 10 days after injury. As shown in Figure 1, plasma endotoxin elevation was detected in the patients throughout the study period, and mean endotoxin in the patient

group at the 0- to 2- and 3- to 6-day intervals significantly was greater than that seen in normal control subjects (mean < 1.0 pg/mL). Patients who became septic later in their hospital course frequently had high levels of circulating plasma endotoxin at the time of sepsis, sometimes reaching the 200 pg/mL range. There appeared to be no difference between burn patients and trauma patients with respect to early detection of plasma endotoxin in this study.

Adherent Cell Production of Proinflammatory Cytokines

Adherent cells from the PBMC from the 18 study patients on 31 occasions and from 7 normal individuals on 43 occasions were studied for basal and LPS-stimulated production of TNF- α , IL-1 β , and IL-6. Basal production of the three cytokines was uniformly low. Stimulated production of all three cytokines was increased in the patient population early after injury as noted in Figure 2. It is apparent that this increase statistically was significant largely in the first 3 to 4 days after injury, except for IL-6, where the increase was of somewhat longer duration. Surprisingly, detection of endotoxin in the plasma in the study period had a negative correlation with increased adherent cell-stimulated TNF- α production in the same patients ($r = -0.47, p < 0.05$).

Circulating plasma TNF- α was measured in the blood samples from both patients (93 occasions) and normal control subjects (14 occasions) during the 10-day study period. In the patients, values ranged from 0 to 84 pg/mL and in the control subjects, values ranged from 0 to 88 pg/mL. The mean for the patients was 14.0 pg/mL and for the control subjects was 28.6 pg/mL. In the first 10 days after injury, no patient showed elevation of plasma TNF- α more than two standard deviations above the mean for the normal population.

Correlation of Circulating Endotoxin and Tumor Necrosis Factor- α Production with Clinical Course

As listed in Tables 1 and 2, neither the detection of endotoxin 1 pg/mL or greater in the plasma in the first 10 days after injury nor increased TNF- α production (defined as more than two standard deviations above the mean of the normal control subjects) by adherent cells from the circulating blood appeared to be predictive of subsequent episodes of SIRS or documented sepsis in the patient population. Sepsis or SIRS developed in 10 of the 18 patients at some time during their hospital course. There were two patient deaths. One patient died of a myocardial infarction that did not appear to be related to

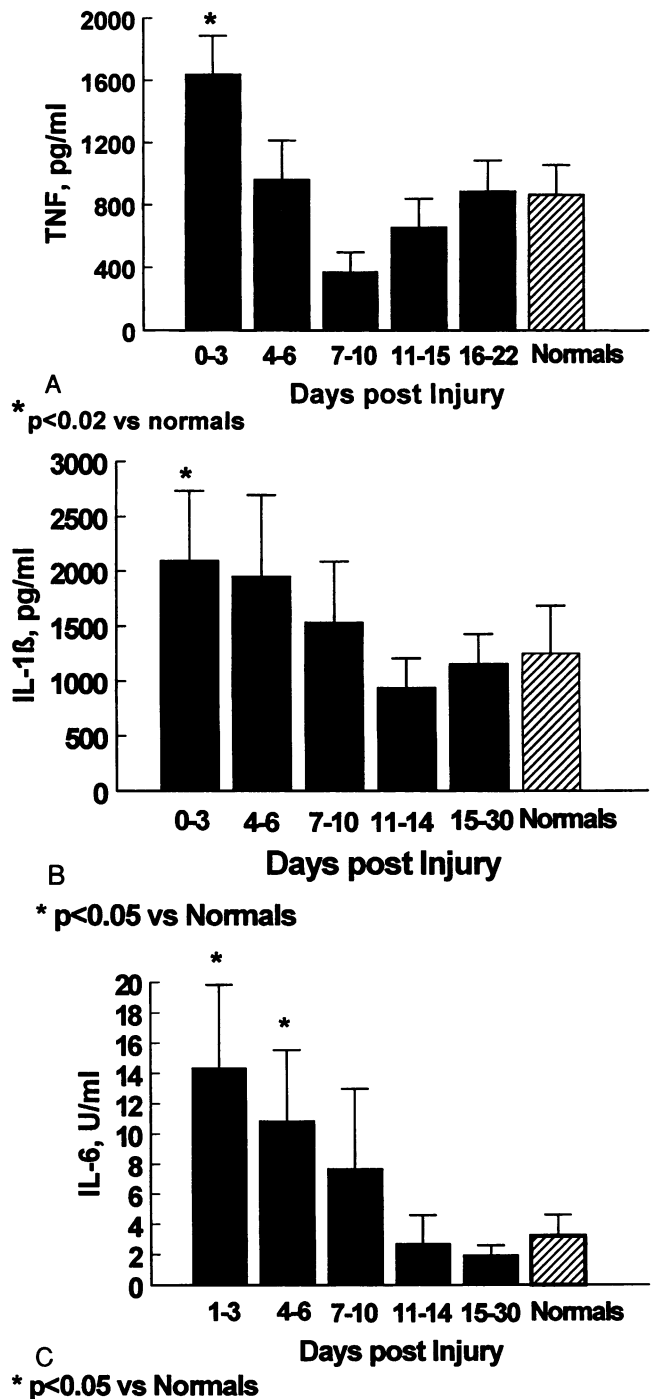


Figure 2. (A) Lipopolysaccharide-stimulated production of tumor necrosis factor- α (TNF- α) by adherent cells from the peripheral blood mononuclear cells (PBMCs) of the study patients (mean \pm standard error). It is apparent that patient TNF- α production was elevated significantly above that of normal control subjects only early after injury. (B) Interleukin-1 β (IL-1 β) production by adherent cells from the PBMC of the study patients (mean \pm standard error). Again, production of IL-1 β in the patients was significantly elevated only early after injury. (C) Interleukin-6 production by adherent cells from PBMC of the study patients (mean \pm standard error). Elevated production of interleukin-6 in the patients was somewhat more protracted than production of TNF- α and IL-1 β .

Table 1. RELATIONSHIP OF PLASMA ENDOTOXIN TO SUBSEQUENT SIRS OR SEPSIS

	SIRS or Sepsis	No SIRS or Sepsis	Total
Plasma endotoxin (+)	6 (33)	5 (28)	11 (62)
Plasma endotoxin (-)	4 (22)	3 (16)	7 (38)
Total	10 (56)	8 (44)	18 (100)

Values are no. (%); p = 1.00.
SIRS = systemic inflammatory response syndrome.

sepsis or SIRS and another patient died of sepsis and multiple organ failure.

Mouse Plasma Endotoxin

To study the issues raised by these human experiments more systematically, we turned to the mouse burn model. Burn and sham burn mice in groups of five were killed at 12-hour intervals for the first 24 hours after burn injury and daily thereafter. Their plasma was assayed for endotoxin. It is apparent from Figure 3 that elevated plasma endotoxin was seen chiefly early in the first 2 to 3 days after injury, sometimes at rather high levels. Thereafter, only occasional animals showed detectable low levels of circulating endotoxin. These results resembled those found in the patients studied above.

Mouse Cytokine Production

In a second experiment, burn and sham burn mice in groups of 10 were killed at intervals after burn injury, and adherent cells from their spleens were tested for basal and LPS-stimulated production of inflammatory cytokines. Plasma samples were tested for TNF- α . As shown in Figures 4 and 5, adherent

Table 2. RELATIONSHIP OF INCREASED TNF PRODUCTION TO SUBSEQUENT SIRS OR SEPSIS

	SIRS or Sepsis	No SIRS or Sepsis	Total
Increased TNF	4 (25)	5 (31)	9 (56)
No increased TNF	5 (31)	2 (13)	7 (44)
Total	9 (56)	7 (44)	16 (100)

Values are no. (%); p = 0.36
TNF = tumor necrosis factor; SIRS = systemic inflammatory response syndrome.

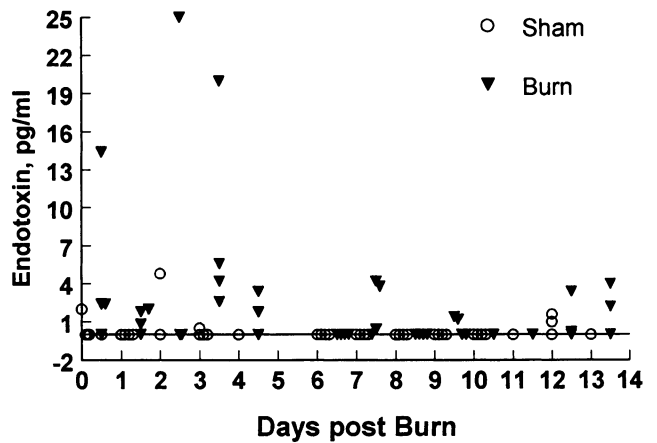


Figure 3. Plasma endotoxin levels in burn and sham burn mice measured at serial intervals after injury. Elevated plasma endotoxin was seen chiefly early, 2 to 3 days postinjury, in the burn mice.

splenocyte production of TNF- α , IL-1 β , and IL-6 after LPS stimulation was increased in the postinjury period in the burn as opposed to that of the sham burn animals. These increases in stimulated cytokine production persisted for 21 days or longer, far beyond the time when elevated plasma endotoxin was seen in the previous experiment. Basal production of inflammatory cytokines was uniformly low, and no animals showed elevated plasma TNF- α during the entire study period.

Mouse Cytokine Message Expression

As shown in Figures 6 and 7, the elevations in stimulated production of TNF- α and IL-1 β by splenocytes from

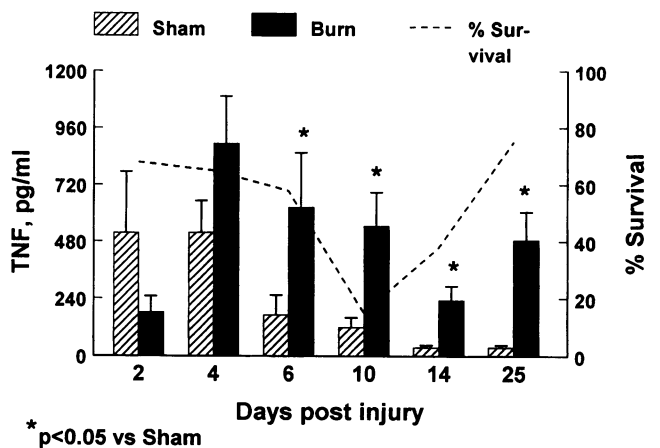


Figure 4. Lipopolysaccharides stimulate tumor necrosis factor- α (TNF- α) production by adherent splenocytes from burn and sham burn mice at serial intervals after injury (mean \pm standard error). It is apparent that increased TNF- α production in the burn mice persisted for the entire study period. The survival of burn mice after septic challenge in the form of cecal ligation and puncture also is plotted. It is clear that mortality from cecal ligation and puncture returns to control levels, whereas TNF- α production still is elevated.

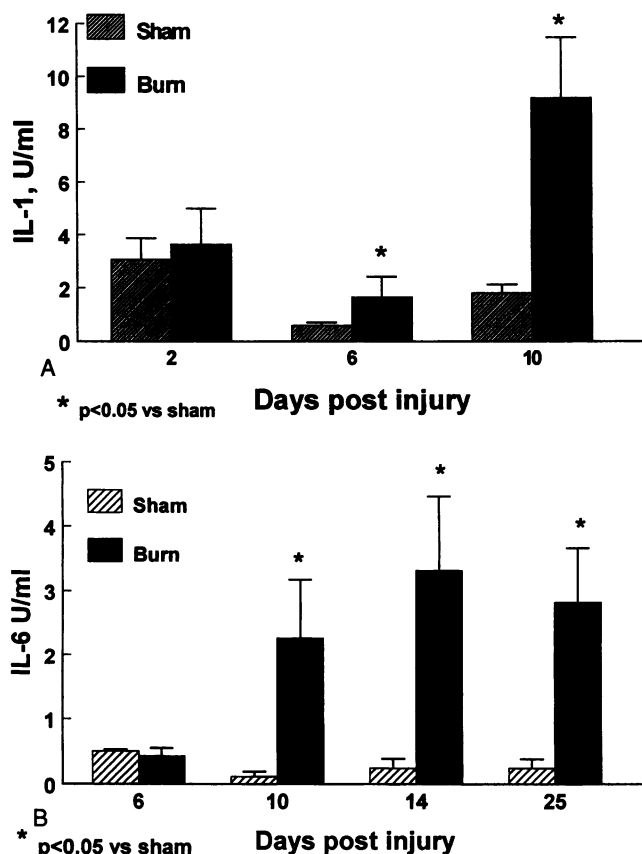


Figure 5. (A) Interleukin-1 β production by adherent splenocytes from burn and sham burn mice at serial intervals after injury (mean \pm standard error). Interleukin-1 β production is increased in the burn mice in similar fashion to tumor necrosis factor- α . (B) Interleukin-6 production by adherent splenocytes from burn and sham burn mice (mean \pm standard error). Again, interleukin-6 production is increased in the burn animals at serial intervals after injury.

burn mice reported above also were accompanied by elevated expression by splenocytes of the messenger RNAs for these cytokines. These findings suggest, but do not prove, that burn injury increased transcription of the genes for these two proinflammatory cytokines. With respect to IL-6, the results were much less clear-cut, because there often was considerable basal expression of IL-6 message (results not shown).

Correlation Between Inflammatory Cytokine Production and Resistance to Infection in the Mouse Burn Model

As we have reported previously,¹³ the mouse burn model is an example of the two-hit phenomenon in that after burn injury, the mortality from a septic challenge in the form of cecal ligation and puncture increases progressively until approximately 10 days after injury at which time the mortality gradually decreases, reaching control

levels by days 21 through 28. In Figure 4, the mortality from CLP in the burn mouse model is plotted together with sequential stimulated production of TNF- α . It is apparent that markedly increased production of TNF- α persists long after the maximum mortality from CLP, which reaches control levels while TNF- α secretion still is elevated.

Effect of Endotoxin Infusion on Mortality from CLP and on Cytokine Production

As noted above, the maximum mortality from CLP in the mouse burn model is at approximately 10 days after burn injury. We have now shown that there is an early and transient elevation in plasma endotoxin in mice after burn injury that is maximal at approximately day 2 after injury and usually is not evident in most animals after day 3. To determine what effect of this early spike in plasma endotoxin might have on later mortality from CLP, we administered 1 ng endotoxin intraperitoneally to a group of 10 mice on 2 occasions 24 hours apart. This dose of endotoxin was shown to produce measurable plasma endotoxin in the 10 to 40 pg/mL range. A group of 10 mice who received an equal volume of saline intraperitoneally served as control subjects.

On day 7 after endotoxin infusion, a time corresponding to day 10 after burn injury, both groups of mice were subjected to CLP. The mortality of the two groups is shown in Figure 8. It is apparent that endotoxin infusion did not increase mortality from CLP performed 7 days later. If anything, the survival of the endotoxin-treated group was superior to that of the control animals.

In a final experiment, groups of 10 mice each received 2 doses of endotoxin or saline 24 hours apart. Both groups of animals were killed at day 7 after the last dose of endotoxin, and their spleens were harvested and the adherent cells were cultured *in vitro* with and without LPS stimulation. The culture supernatants were assayed for TNF- α production. The mean stimulated TNF- α production by the control group was 526 \pm 121 pg/mL as compared with 513 \pm 115 pg/mL by the LPS-treated group. The difference was clearly not significant. Basal TNF- α production also was similar in both groups, 76 \pm 9 pg/mL for the control subjects and 60 \pm 8 pg/mL for the LPS group.

DISCUSSION

Munster et al.⁶ have reported previously that patients with serious burn injury frequently have measurable levels of plasma endotoxin early after injury, presumably from leakage from the gut or from bacteria translocated from the gut. The current observations confirm these findings and show that patients, after severe traumatic

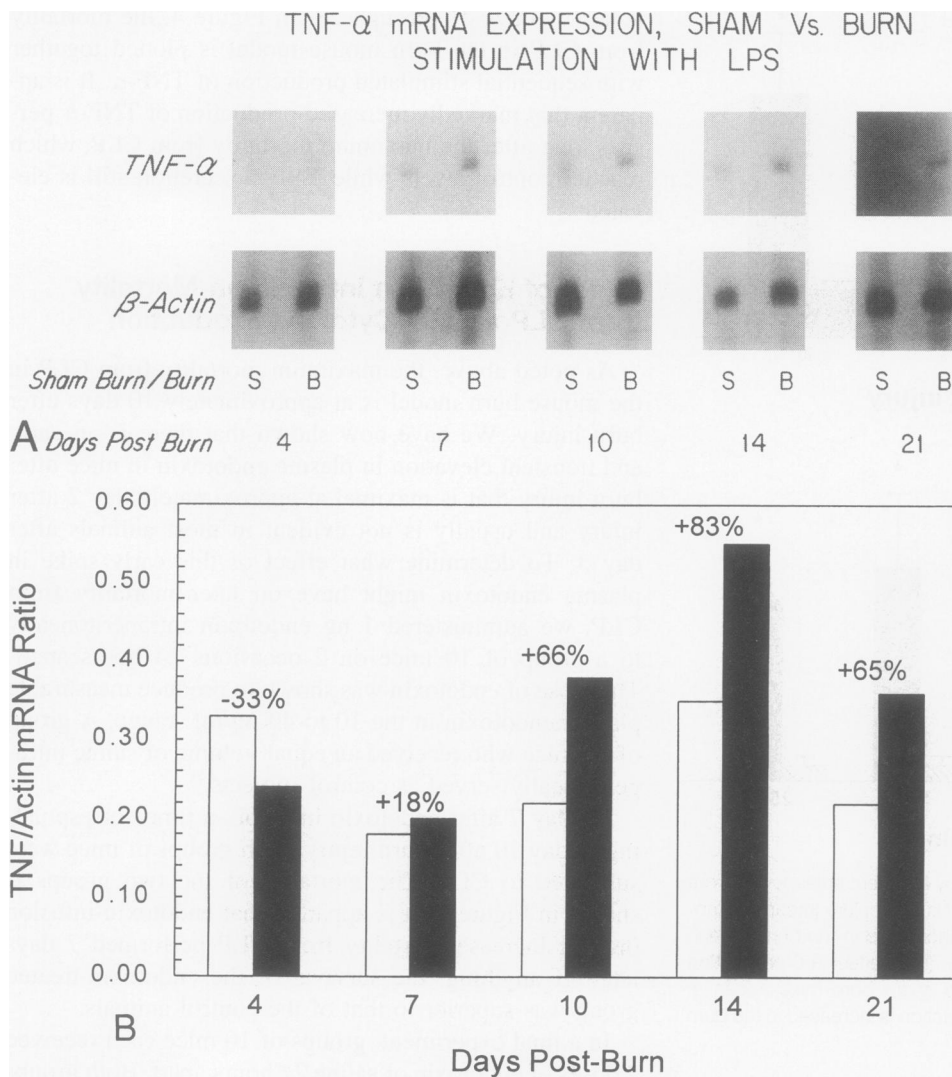


Figure 6. (A) Northern blots of tumor necrosis factor- α (TNF- α) message in lipopolysaccharide (LPS)-stimulated and -unstimulated adherent splenocytes from burn and sham burn mice at serial intervals after injury. The β -actin message is shown as a control. It is apparent that tumor necrosis factor- α (TNF- α) message is increased in the LPS-treated burn mouse splenocytes as compared with sham burn splenocytes at multiple times after injury. (B) The TNF- α message in LPS-stimulated burn and sham burn splenocytes at serial intervals after burn injury normalized to the β -actin message by laser densitometry. Again, increased TNF- α message is expressed in the burn splenocytes at multiple times after injury.

injury, also may have detectable plasma endotoxin early after injury. Munster et al.⁶ prospectively treated some of their burn patients with low doses of polymyxin to bind the circulating endotoxin and found that such treatment had no evident effect on the clinical course of the treated patients as compared with those untreated. The current results parallel the findings of Munster et al.⁶ in that the presence of circulating plasma endotoxin early after injury did not appear to predict later clinical events, such as SIRS, sepsis, or death in our group of 18 burn and trauma patients.

Excessive production of proinflammatory cytokines has long been considered a predictable sequela of serious injury, including burn injury.¹⁻³ Because it has been well established by many investigators that after serious injury in animal models, there is translocation of gut bacteria to lymph nodes and neighboring organs and leak of endotoxin from the gut,^{1,3,14} many investigators have thought

that endotoxin, a known stimulus for proinflammatory cytokine production by cells of the monocyte-macrophage lineage and other cell types, might be the trigger for increased proinflammatory cytokine production after injury. In the current experiments, however, we surprisingly found that circulating plasma endotoxin detected in the first 10 days after injury had a negative correlation with the stimulated production of proinflammatory cytokines by adherent cells from the PBMC of the patients studied. Unstimulated production of proinflammatory cytokines was low in all instances, and no patient in the 10-day study period had circulating levels of TNF- α significantly higher than those detected in simultaneously studied normal control individuals. The unexpected negative association between circulating plasma endotoxin and stimulated proinflammatory cytokine production in our patients is, however, in concert with previous findings that administration of low doses of endotoxin intravenously

IL-1 β mRNA EXPRESSION, SHAM Vs. BURN STIMULATION WITH LPS

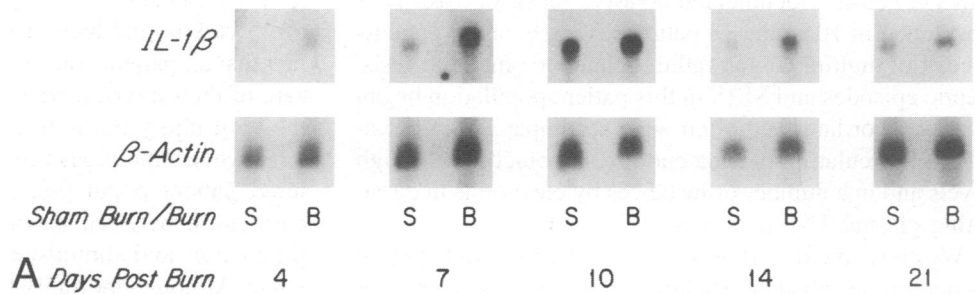


Figure 7. (A) Northern blots of interleukin-1 β (IL-1 β) message expressed in lipopolysaccharide (LPS)-stimulated and -unstimulated adherent splenocytes from burn and sham burn mice at serial intervals after injury. The β -actin message again is shown as a control. It is apparent that there is increased expression of IL-1 β message in the LPS-treated burn mouse splenocytes as compared with that of sham burn at multiple times after injury. (B) The IL-1 β message expression in LPS-stimulated burn and sham burn splenocytes normalized to β -actin message by laser densitometry. There is increased expression of IL-1 β message in burn splenocytes at multiple times after injury.

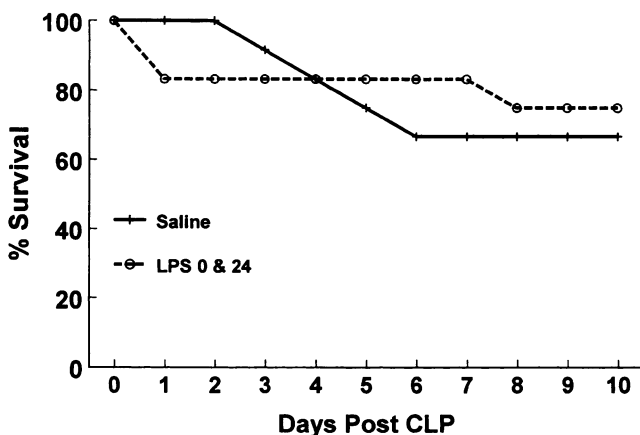
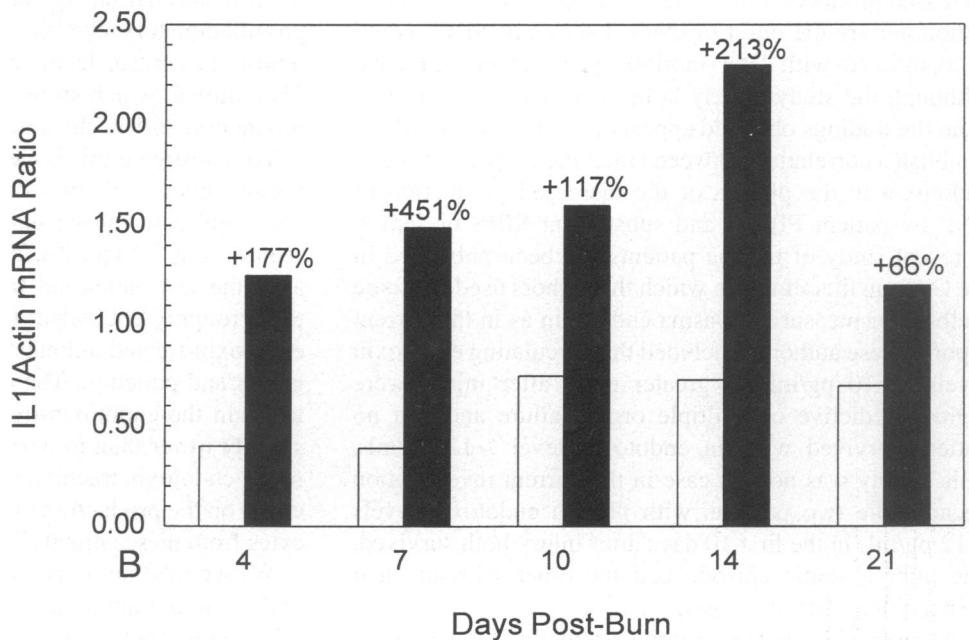


Figure 8. Survival of mice treated with 1 ng lipopolysaccharide on days 8 and 7 before cecal ligation and puncture. The survival of saline-injected control subjects also is shown. It is apparent that two doses of endotoxin had no deleterious effect on survival after cecal ligation and puncture performed 7 days after the second dose.

to normal volunteers resulted in a paradoxical decreased production of proinflammatory cytokines by the PBMCs from these individuals early after endotoxin exposure.¹⁵ This finding was thought to represent a temporary tachyphylaxis of cells after an initial endotoxin stimulus or possibly a version of endotoxin “tolerance” or hyporesponsiveness. Hyporesponsiveness to a secondary endotoxin challenge has been studied in *in vitro* systems.¹⁶ This effect has been shown not to be caused by inability of cells to bind LPS,^{16,17} but is associated with a change in the transcription factor NF κ B. After LPS challenge, NF κ B is expressed in the nucleus chiefly as a possibly suppressive p50 homodimer rather than the customary p65–50 heterodimer.¹⁷

Increased production of proinflammatory cytokines by the adherent PBMCs of the patients in the current study chiefly was seen early after injury and had returned to relatively normal levels by days 5 to 7. Moreover, increased production of TNF- α , defined as more than two

standard deviations above the mean for the normal control subjects, early after injury, was not predictive of later SIRS or sepsis. Documented invasive sepsis or SIRS later developed in 10 of the 18 patients studied, and 2 patients died, 1 of multiple organ failure related to ongoing sepsis. Septic episodes and SIRS in this patient population began on day 10 or later and often were accompanied by elevations of circulating plasma endotoxin, sometimes to high levels and in a number of instances by elevations in circulating plasma TNF- α as well.

We chose the first 10 days after injury as a study period to determine whether detection of plasma endotoxin or increased production of proinflammatory cytokines in this period, before the onset of clinical sepsis or SIRS, could be correlated with later morbid events in our patients. Although the study clearly is limited in size, the answer from the findings obtained appears to be no. We could not establish a correlation between either the early presence of endotoxin in the plasma or the increased production of TNF by patient PBMC and subsequent SIRS or sepsis. A recent study of trauma patients has been published in the German literature¹⁸ in which the authors used the same method for measuring plasma endotoxin as in the current report. These authors concluded that circulating endotoxin levels of 10 pg/mL or greater early after injury were highly predictive of multiple organ failure and that no patient survived with an endotoxin level >12 pg/mL. This clearly was not the case in the current investigation because the two patients with plasma endotoxin levels >12 pg/mL in the first 10 days after injury both survived, one after a septic episode and the other without clear evidence of SIRS or sepsis.

To study this question more systematically, we turned to a mouse burn model, which we previously had shown resembles serious injury in the human with respect to increased production of proinflammatory cytokines after injury and diminished resistance to a septic challenge, which reaches a nadir at day 10 after injury and then gradually returns to normal.^{12,13} We could detect elevations in plasma endotoxin early after burn injury peaking on days 2 to 3 and thereafter returning to baseline levels in most animals. In the mouse model, there apparently was a decrease in the production of TNF- α by adherent splenocytes early after injury followed by a subsequent increase that persisted for 25 days. This persistence of increased stimulated proinflammatory cytokine production in the mouse model was accompanied by increased expression of messenger RNA for both TNF- α and IL-1 β in the splenocytes of the same animals. This prolonged production of proinflammatory cytokines long after injury in the mouse model is at variance with the findings in the patients studied, including the burn patients who were indistinguishable from the trauma patients in showing an early increase in proinflammatory cytokine production

followed by a prompt decline to normal levels within the first week after injury. Why the mouse model differed from the humans with respect to persistent proinflammatory cytokine production is unclear, but may reflect the fact that all patients received prompt surgical and medical care of their injuries, whereas no effort was made to treat the burn injury in the mice.

However, the mouse burn model did resemble the injured patient population in that there was no apparent correlation between elevated proinflammatory cytokine production and diminished resistance to a septic challenge. Although mortality from CLP in this model is maximal at approximately day 10 when the production of proinflammatory cytokines is high, mortality from CLP returns to control levels of 15% to 20% by days 21 to 25, a time at which stimulated proinflammatory cytokine production still is elevated markedly.

To investigate this issue further, we treated a group of normal mice with two doses of endotoxin, which had been shown to induce plasma levels equivalent to those seen on days 2 and 3 early after burn injury. On day 7 after the last endotoxin treatment, a time that appeared to be roughly equivalent to day 10 after burn injury, the endotoxin-treated animals were challenged with cecal ligation and puncture. The mortality from this septic challenge in the endotoxin-treated animals was, if anything, slightly lower than in untreated control animals. Furthermore, endotoxin treatment appeared to have no significant effect on the production of TNF- α by the adherent splenocytes from these animals 7 days after endotoxin treatment.

We would, thus, conclude that circulating plasma endotoxin in both injured humans and in animal model of burn injury commonly is seen early after injury. However, in humans, the detection of plasma endotoxin is not associated with increased production of TNF- α in the same individuals nor does it appear to be predictive of later septic events or mortality. In the mouse burn model, plasma endotoxin again is seen early after injury, but increased production of proinflammatory cytokines persists long after endotoxin levels return to baseline in most animals studied. As in the patient population, elevations of proinflammatory cytokine production do not appear to correlate with decreased resistance to infection, and an elevation in plasma endotoxin in uninjured animals does not result in a later increase in mortality from a septic challenge or in a persistent increase in proinflammatory cytokine production. These findings call into question the idea that circulating plasma endotoxin is the trigger for increased proinflammatory cytokine production, SIRS, and septic complications in injured patients. However, the current studies clearly cannot rule out continual priming of circulating PBMC in injured patients for increased proinflammatory cytokine production by small quantities of endotoxin encountered in the gut lymphoid tissue or

in the sinusoids of the liver where translocated bacteria may be found. Such primed PBMC might, in turn, play an important role in subsequent SIRS or septic syndromes.

Acknowledgments

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Discussion

DR. WILLIAM G. CHEADLE (Louisville, Kentucky): Dr. Griffen, Dr. Copeland, Members, and Guests. I really enjoyed this piece of work, and it is another fine contribution by Dr. Mannick’s laboratory. I think we are all aware that the most common cause of late death after major burns or trauma is sepsis and organ failure; however, the mechanisms of immune suppression leading to these poor clinical outcomes have been quite elusive.

It is tempting to lay the blame on endotoxin because its administration reproduces the septic state; however, as Dr. Mannick has shown nicely, levels are certainly much lower in burn and trauma patients and in the clinically relevant experimental models than the doses that have been used to produce sepsis in volunteers and other experimental models. And, in fact, anti-lipopolysaccharide therapy has failed in clinical trials.

It was also interesting to note that splenocyte tumor necrosis factor (TNF) production bore little relation to mortality in their 2-hit experimental model, and in fact, others have shown anti-TNF treatment actually increases mortality in the cecal ligation and puncture model.

So, I have the following three questions. Trauma and burns are certainly two different kinds of stresses, and I wondered if there were any particular differences in the patterns of lipopolysaccharide levels and cytokine production that might be noted in between these two particular patient groups.

Secondly, in the burn model, have you had the chance to add a little *Pseudomonas* or *Staph aureus* to the wound to sort out whether the burn stress itself or the associated bacteria might be responsible or contribute to the LPS levels that you saw early on in that particular model?

Finally, Dr. Mannick and his colleagues have been leaders in the field in dissecting out the immune suppression seen after a variety forms of stress, most notably trauma and burns. And they have shown in recent work that production of anti-inflammatory cytokines, particularly by T lymphocytes and T Helper 2 cells, may be responsible for such immune suppression seen early after a burn or a trauma.

I would like to know if you could speculate a little on the potential role of these cytokines, namely the interleukin-4 and interleukin-10, on the potential therapeutic role in suppression of the inflammatory response and maybe the treatment of organ failure that we see after injury.

I have certainly enjoyed following your work over the years, and I look forward to your comments and to your future studies in this area. Thank you.

DR. BASIL A. PRUITT, JR. (San Antonio, Texas): Dr. Griffen, Dr. Copeland, Fellows, and Guests. I rise to compliment Dr. Mannick and his associates on another interesting study in their