

ANTIBODIES TO CACHECTIN/TUMOR NECROSIS FACTOR
REDUCE INTERLEUKIN 1 β AND INTERLEUKIN 6
APPEARANCE DURING LETHAL BACTEREMIA

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The cytokine cachectin/tumor necrosis factor (cachectin/TNF) plays a pivotal role in the pathophysiologic consequences of severe infection, and is a mediator of fatal bacteremic shock (reviewed in references 1, 2). Infusions of recombinant cachectin/TNF induce cardiovascular shock, hemorrhagic necrosis in tissues, and metabolic derangements similar to both experimental endotoxemia and clinical septic shock (3, 4). Anti-cachectin/TNF antibodies protect mice (5) and rabbits (6) from otherwise lethal endotoxemia, and prevent the development of lethal septic shock syndrome during overwhelming experimental bacteremia in baboons (7). Moreover, high concentrations of circulating cachectin/TNF correlate with lethal outcome in critically ill patients with meningococcal infection (8), infectious purpura (9), or burn injury-associated septicemia (10).

Two other cytokines, IL-1 and IL-6, have also been implicated in the pathophysiology of injury and infection (11-13). IL-1 and IL-6 can be detected in the circulation of patients with active infection or endotoxemia (14-16), and administration of IL-1 or IL-6 induces some of the characteristic physiological derangements associated with injury (17, 18), including fever and acute-phase protein responses. The recent availability of sensitive and specific assays for IL-1 and IL-6 prompted us to measure the concentrations of these cytokines in stored plasma samples obtained in a previously reported series of lethal, experimental bacteremic challenges in baboons (7). The current report demonstrates that gram-negative bacteremia induces rapid and marked increases in circulating IL-1 β and IL-6. Moreover, passive immunization against cachectin/TNF attenuates the appearance of IL-1 β and IL-6, suggesting that cachectin/TNF is an essential stimulus for the release of these other cytokines during septic shock syndrome.

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Materials and Methods

Experimental Protocol. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the New York Hospital-Cornell Medical Center (NYH-CMC). Female *Papio anubis* baboons (Charles River Primate Center, Port Jefferson, NY), free of infections and parasites, were housed in the animal care facility of the NYH-CMC. After a 12-h overnight fast, general anesthesia (sodium pentobarbital) was administered to the animals and maintained throughout the study period as previously described (7).

All animals received an intra-aortic infusion of *Escherichia coli* 086:B7 ($1.2 \pm 0.3 \times 10^{11}$ live bacteria/kg body weight) over 30 min (19). Individual baboons were randomized to one of three pretreatment groups: (a) animals receiving anti-cachectin/TNF antibodies (10 mg/kg) 1 hour before the bacterial infusion (Ab -1 h), $n = 3$; (b) animals receiving anti-cachectin/TNF antibodies (10 mg/kg) 2 h before the bacterial infusion (Ab -2 h), $n = 3$; and (c) control animals receiving saline infusions 1 h ($n = 3$) or 2 h ($n = 3$) before bacterial infusion. Arterial blood samples collected before and periodically for 8 h after bacterial challenge were assayed for cytokine levels and metabolic substrate concentrations. Plasma samples had been collected in heparinized tubes, centrifuged at 4°C, frozen immediately, and stored at -70°C until analysis.

Cachectin/TNF Antibody. Anti-cachectin/TNF mAbs were prepared as previously described (7). F(ab')₂ fragments were prepared by pepsin digestion of IgG (20), and purified by Mono Q (Pharmacia Fine Chemicals, Piscataway, NJ) column chromatography. Anti-cachectin/TNF antibodies at a concentration of 10 µg/ml neutralized at least 50 ng/ml of recombinant human cachectin/TNF (10^8 U/mg) as assayed by L929 cell cytotoxicity assay (21). Antibodies were diluted in 0.9% saline and infused over a period of 30 min. The LPS content of the antibody preparation was <0.25 ng/mg as assayed by the Limulus amoebocyte lysate test.

IL-1 Assay. Concentrations of IL-1α and IL-1β in thawed plasma samples were assayed by two-site ELISA using mAbs specific for each protein (22). The lower limit for detecting IL-1α is 36 pg/ml while that for IL-1β is 15 pg/ml. Baboon plasma does not inhibit the detection of exogenously added IL-1 in either assay.

IL-6 Assay. IL-6 activity in plasma samples was measured by a B.9 hybridoma proliferation assay (23). Briefly, serial dilutions of thawed plasma were incubated in triplicate with 2,000 B.9 cells for 84 h in 96-well microtiter plates. MTT (3-[4,5-dimethyl-thiazol-2-yl]2,5-diphenyltetrazolium bromide) (300 µg/ml) was added; 3 h later the supernatant was removed and the cells were lysed with isopropanol-0.004 N HCl. Cell proliferation was estimated colorimetrically in an ELISA plate reader (570, 690 nm). One B.9 hybridoma growth unit was defined as the quantity of diluted plasma required to produce one-half maximal proliferation. Values for each sample were calculated by interpolation from three or four dilutions. Recombinant human cachectin/TNF and recombinant human IL-1β have no proliferative effects in this assay. B.9 cell proliferation was not affected by as much as 10 µg/ml cachectin/TNF antibody in vitro. Inhibition by antiserum specific for human IL-6 verified specificity of this assay. The lower limit for detecting IL-6 by this assay is 50 U/ml.

Metabolic Parameters. Plasma glucose concentrations were determined using an automated glucose analyzer (No. 23A; Yellow Springs Instrument Co., Yellow Springs, Ohio) using the glucose oxidase reaction (24). Plasma triglyceride concentrations were determined enzymatically (25).

Statistical Analysis. All data are expressed as means \pm SEM. Two-way ANOVA and Newman-Kuels' tests were used in statistical analysis.

Results and Discussion

We have previously reported that the appearance of circulating cachectin/TNF in this primate model of septic shock is monophasic, peaking 1.5 h after bacterial challenge (7). The results of the current report demonstrate that two other cytokines, IL-1β and IL-6 also exhibit distinct and reproducible patterns of appearance during infection. Circulating IL-1β, appearing slightly later than cachectin/TNF, was detectable by 2 h and peaked 3 h after the bacterial infusion (Fig. 1). Circulating IL-6,

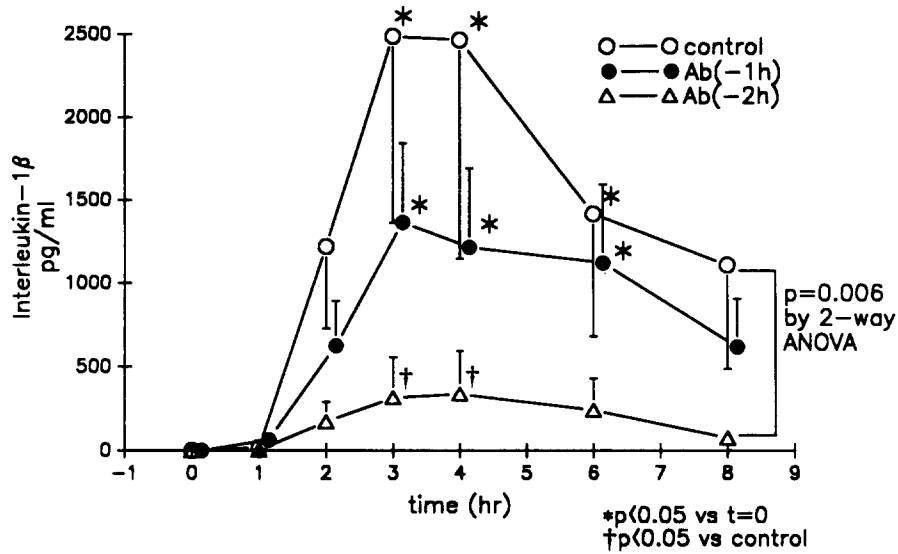


FIGURE 1. Circulating IL-1 β levels during experimental bacteremia. Assay for IL-1 β was performed on plasma obtained before ($t = 0$) and after *E. coli* infusions. Animals immunized against cachectin/TNF received anti-cachectin/TNF mAbs 1 h [Ab(-1 h)] or 2 h [Ab(-2 h)] before bacterial challenge.

appearing even later, was detectable within 3 h, and continued to rise throughout the 8-h study period (Fig. 2).

In cultured macrophages and other cells, and in experimental animals, exposure to recombinant cachectin/TNF induces the biosynthesis of IL-1 and IL-6 (11, 13,

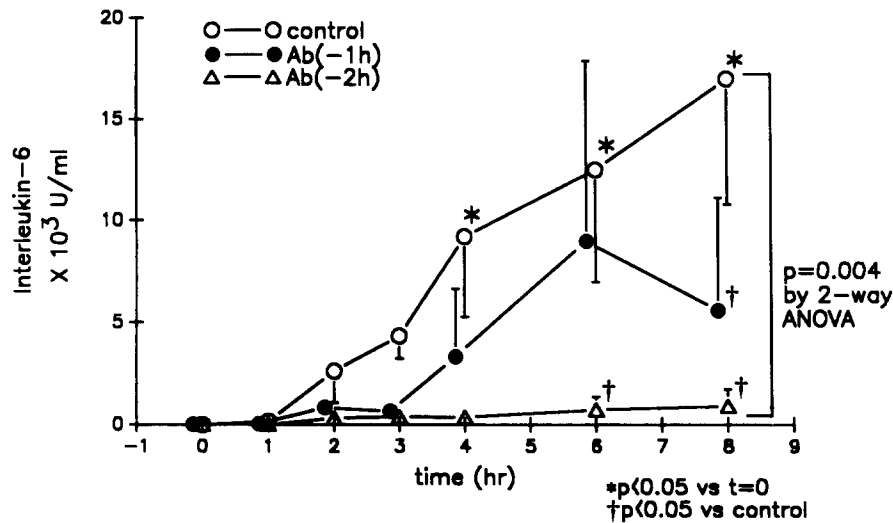


FIGURE 2. Circulating IL-6 levels during experimental bacteremia. Assay for IL-6 was performed on plasma obtained before ($t = 0$) and after *E. coli* infusions. Experimental animals were immunized against cachectin/TNF as described in Fig. 1.

26), and these factors have been implicated as secondary mediators of the pathophysiological effects of cachectin/TNF. The present data indicate that during overwhelming infection, cachectin/TNF secretion preceded the production of IL-1 β , and IL-6, and was a necessary stimulus for their release in vivo. Pretreatment with anti-cachectin/TNF antibodies significantly attenuated the circulating IL-1 β response ($p < 0.006$) (Fig. 1). Comparisons between peak IL-1 β levels were particularly noteworthy (control: 2.5 ± 1.1 ng/ml; Ab (-1 h): 1.4 ± 0.5 ; Ab (-2 h): 0.3 ± 0.2). Pretreatment with anti-cachectin/TNF antibodies also attenuated the IL-6 response to bacterial challenge ($p < 0.004$) ($t = 8$; control: $17 \pm 6 \times 10^3$ U/ml; Ab (-1 h): $6 \pm 6 \times 10^3$; Ab (-2 h): $0.9 \pm 0.8 \times 10^3$) (Fig. 2). Considering that bacterial LPS is itself a potent stimulus for IL-1 and IL-6 release in vitro (12, 13), the observation that specific neutralization of cachectin/TNF significantly reduced appearance of IL-1 β and IL-6 during overwhelming gram-negative bacteremia is striking.

No detectable IL-1 α was found in the circulation at any time point in any group despite the production of large quantities of IL-1 β . This is consistent with the observation that IL-1 β is the predominate form of IL-1 produced by activated monocytes (27). Since IL-1 and cachectin/TNF can induce the biosynthesis of IL-6, and the peak IL-1 level in the circulation precedes that of IL-6, it cannot be determined from the present study whether the attenuation of IL-6 is a direct or indirect effect of neutralizing cachectin/TNF. Peak IL-6 levels correlate well with peak IL-1 β responses in the current experiment ($r = 0.87$, data not shown), so it is possible that the reduced appearance of IL-6 was due in part to reduction of the earlier IL-1 response. Further studies with antibodies against IL-1 β may resolve the question.

The overwhelming dose of live *E. coli* used in the current experiment caused a rapidly fatal syndrome in nonimmunized control animals that is likely attributable to tissue effects of cachectin/TNF. Previously we reported that the earlier (-2 h) antibody treatment was more effective in preventing septic shock and mortality (7). We now report that anti-cachectin/TNF antibodies given 2 h before bacterial challenge are significantly more effective in attenuating IL-1 β and IL-6 appearance than antibodies administered only 1 h before *E. coli*. 2-h pretreatment was also more effective in reducing hypoglycemia and hypertriglyceridemia, which are characteristic metabolic sequelae of severe infections (Fig. 3). Improved biologic outcome might well reflect superior tissue distribution and penetration with earlier antibody administration. Inability to completely prevent mortality, cytokine production, and metabolic derangements in the -1 h group might be attributed to incomplete neutralization of the large amounts of cachectin/TNF produced and acting in tissues in response to this overwhelming bacteremia. With less severe bacteremic challenge, improved survival and hemodynamic stability are observed when anti-cachectin/TNF antibodies are administered even 30 min after infusion of lesser quantities of bacteria (28).

That cachectin/TNF elicits IL-1 β and IL-6 release in infection further emphasizes the pivotal role of cachectin/TNF as a mediator of septicemia and septic shock syndrome. The lethality of cachectin/TNF is synergistically enhanced by even low concentrations of IL-1 (29), so that stimulation of IL-1 biosynthesis by cachectin/TNF serves to amplify cachectin/TNF-mediated shock and tissue injury. Further studies are necessary to determine the precise molecular and cellular mechanisms of cachectin/TNF-mediated cytokine release. The current data nevertheless demonstrate that cachectin/TNF is a major determinant in the complex pathogenesis of

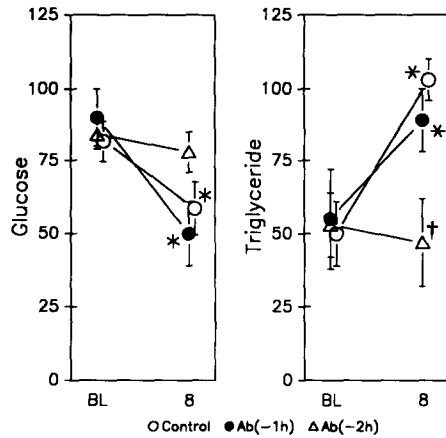


FIGURE 3. Anti-cachectin/TNF antibodies prevent the hypoglycemia and hypertriglyceridemia of bacteremia. Arterial glucose and triglyceride levels were measured at baseline (BL, before bacterial infusion) and 8 h after *E. coli*. * $p < 0.05$ vs. $t = 0$, † $p < 0.05$ vs. control.

septic shock syndrome, not only through its direct toxicity, but also as a proximal mediator capable of inducing a cascade of other humoral factors.

Summary

Cytokines secreted in response to invading micro-organisms are important mediators of detrimental hemodynamic and metabolic changes in the host. To test whether cachectin/TNF plays a role in triggering release of other cytokines in the setting of infection, anesthetized baboons were passively immunized against systemic cachectin/TNF before infusion of a LD₁₀₀ dose of live *Escherichia coli*. Bacteremia led to significant increases in circulating levels of cachectin/TNF, IL-1 β , and IL-6. Although bacterial endotoxin/lipopolysaccharide is a potent stimulus for the synthesis and release of IL-1 and IL-6 in vitro, specific neutralization of cachectin/TNF in vivo with mAb pretreatment significantly attenuated both the IL-1 β and the IL-6 responses despite fulminant overwhelming bacteremia. These data suggest that cachectin/TNF is essential for the initiation or amplification of IL-1 and IL-6 release during lethal gram-negative septic shock syndrome.

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