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

By YUMAN FONG,* KEVIN J. TRACEY,*‡ LYLE L. MOLDAWER,* DAVID G. HESSE,* KIRK B. MANOGUE,‡ JOHN S. KENNEY,§ ANNETTE T. LEE,‡ GEORGE C. KUO,‖ ANTHONY C. ALLISON,§ STEPHEN F. LOWRY,* AND ANTHONY CERAMI‡

*From *The Laboratory of Surgical Metabolism, The Department of Surgery, New York Hospital-Cornell Medical Center, New York, New York 10021; the ‡Laboratory of Medical Biochemistry, The Rockefeller University, New York, New York 10021; the §Department of Immunology, Syntex Research, Palo Alto, California 94303; and the ‖Chiron Research Laboratories, Emeryville, California 94608*

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 cachetin/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-1β and IL-6. Moreover, passive immunization against cachectin/TNF attenuates the appearance of IL-1β and IL-6, suggesting that cachectin/TNF is an essential stimulus for the release of these other cytokines during septic shock syndrome.

This work was supported by National Institutes of Health grants GM-34695, KO4GM-00505, GM-40586, and AI-21359 and by a Clinical Fellowship from the American Cancer Society to Y. Fong.

Address correspondence to Dr. Yuman Fong, Laboratory of Surgical Metabolism, F-2016, New York Hospital-Cornell Medical Center, 525 East 68th Street, New York, NY 10021.

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 amebocyte 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 cachetin/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 ± 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,

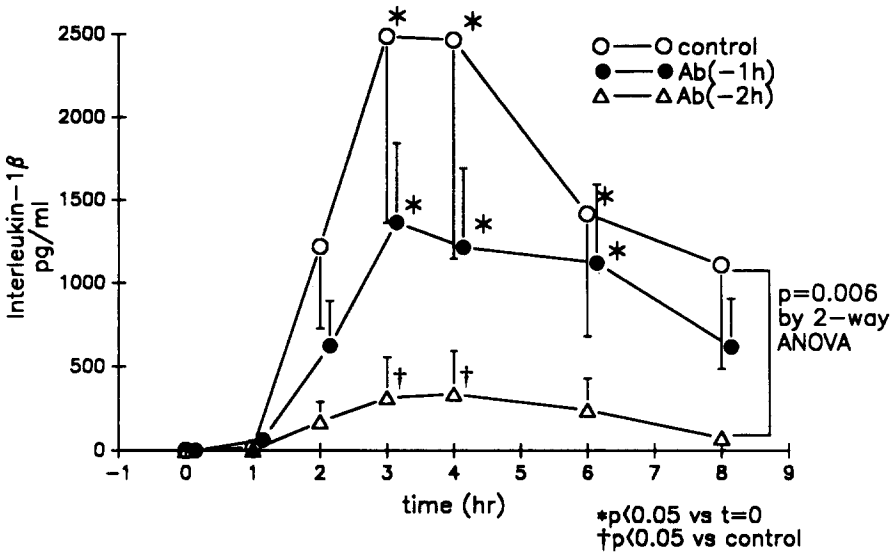

FIGURE 1. Circulating IL-1β levels during experimental bacteremia. Assay for IL-1β was performed on plasma obtained before (t = 0) and after *E. coli* infusions. Animals immunized against cachectin/TNF received anti-cachectin/TNF mAbs 1 h [Ab(-1 h)] or 2 h [Ab(-2 h)] before bacterial challenge.

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,

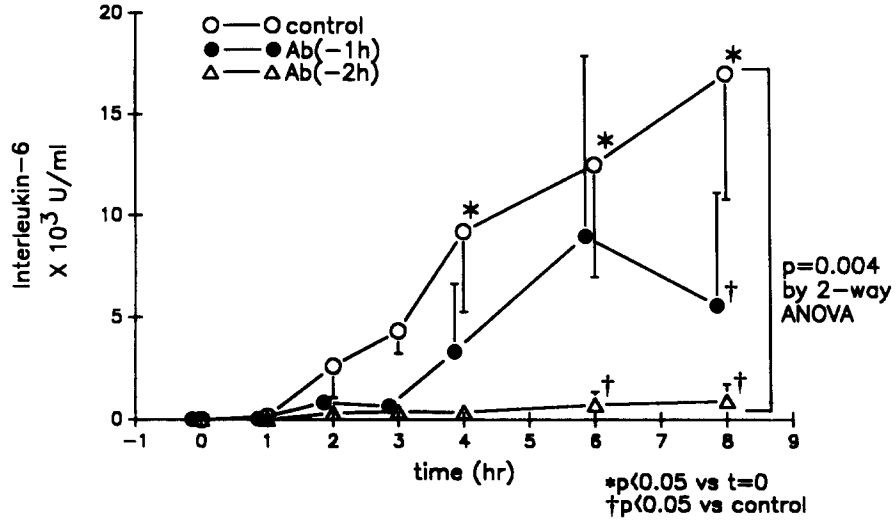

FIGURE 2. Circulating IL-6 levels during experimental bacteremia. Assay for IL-6 was performed on plasma obtained before (t = 0) and after *E. coli* infusions. Experimental animals were immunized against cachectin/TNF as described in Fig. 1.

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-1β, and IL-6, and was a necessary stimulus for their release in vivo. Pretreatment with anti-cachectin/TNF antibodies significantly attenuated the circulating IL-1β response ($p < 0.006$) (Fig. 1). Comparisons between peak IL-1β 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 ± 6 × 10^3 U/ml; Ab (−1 h): 6 ± 6 × 10^3; Ab (−2 h): 0.9 ± 0.8 × 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-1β and IL-6 during overwhelming gram-negative bacteremia is striking.

No detectable IL-1α was found in the circulation at any time point in any group despite the production of large quantities of IL-1β. This is consistent with the observation that IL-1β 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-1β 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-1β 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-1β 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-1β 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

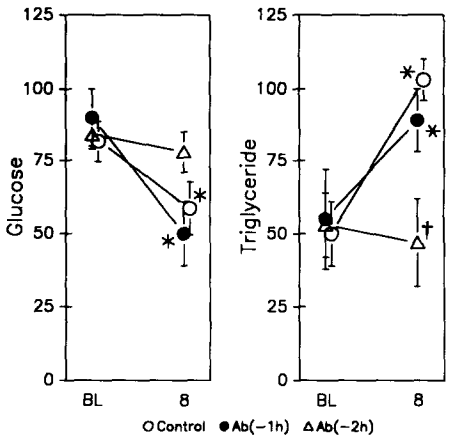

FIGURE 3. Anti-cachectin/TNF antibodies prevent the hypoglycemia and hypertriglyceridemia of bacteremia. Arterial glucose and triglyceride levels were measured at baseline (BL, before bacterial infusion) and 8 h after *E. coli*. $^{*}p < 0.05$ vs. $t = 0$, $^{+}p < 0.05$ vs. control.

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 LD_{100} dose of live *Escherichia coli*. Bacteremia led to significant increases in circulating levels of cachectin/TNF, IL-1β, 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-1β 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.

Received for publication 29 June 1989.

References

1. Fong, Y., K. J. Tracey, S. F. Lowry, and A. Cerami. 1989. Biology of cachectin. *In* Cytokines; Macrophage-derived Cell Regulatory Factors. C. Sorg, editor. Vol. 1. Karger, 74–88.
2. Tracey, K. J., H. Vlassara, and A. Cerami. 1989. Cachectin/tumour necrosis factor. *Lancet.* i:1122.
3. Tracey, K. J., B. Beutler, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. J. Hariri, T. J. Fahey, A. Zentella, J. D. Albert, G. T. Shires, and A. Cerami. 1986. Shock and tissue injury induced by recombinant cachectin. *Science (Wash. DC).* 470:234.
4. Tracey, K. J., S. F. Lowry, T. J. Fahey, Y. Fong, D. G. Hesse, B. Beutler, K. Manogue, S. E. Calvano, H. Wei, J. D. Albert, A. Cerami, and G. T. Shires. 1987. Cachectin/tumor necrosis factor induces lethal septic shock and stress hormone responses in the dog. *Surg. Gynecol. Obstet.* 164:415.

5. Beutler, G., I. W. Milsark, and A. Cerami. 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from the lethal effect of endotoxin. *Science (Wash. DC).* 1985, 229:869.
6. Mathison, J. C., E. Wolfson, and R. J. Ulevitch. 1988. Participation of tumor necrosis factor in the mediation of gram negative bacterial lipopolysaccharide-induced injury in rabbits. *J. Clin. Invest.* 81:1925.
7. Tracey, K. J., Y. Fong, D. G. Hesse, K. R. Manogue, A. T. Lee, G. C. Kuo, S. F. Lowry, and A. Cerami. 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature (Lond.).* 330:662.
8. Waage, A., A. Halstensen, and T. Espevik. 1987. Association between tumor necrosis factor in serum and fatal outcome in patients with meningococcal disease. *Lancet.* i:355.
9. Girardin, E., G. E. Grau, J. M. Dayer, P. Roux-Lombard, and P. H. Lambert. 1988. Tumor necrosis factor and interleukin-1 in the serum of children with severe infectious purpura. *N. Engl. J. Med.* 319:397.
10. Marano, M. A., Y. Fong, L. L. Moldawer, H. Wei, S. E. Calvano, K. J. Tracey, P. S. Barie, K. Manogue, A. Cerami, G. T. Shires, and S. F. Lowry. 1989. Serum cachectin/TNF in critically ill burn patients correlates with infection and mortality. *Surg. Gynecol. Obstet.* In press.
11. Dinarello, C. A. 1989. Interleukin-1 and its biologically related cytokines. *Adv. Immunol.* 44:153.
12. Le, J., and J. Vilcek. 1987. Tumor necrosis factor and interleukin-1: cytokines with multiple overlapping biological activities. *Lab. Invest.* 56:234.
13. Sehgal, P. B., G. Gruninger, G. Tosoto, editors. 1989. Regulation of the acute phase and immune responses: interleukin-6. *Ann. NY Acad. Sci.* 557:1.
14. Waage, A., P. Brandtzaeg, A. Halstensen, P. Kierulf, and T. Espevik. 1989. The complex pattern of cytokines in serum from patients with meningococcal septic shock. *J. Exp. Med.* 169:333.
15. Helfgott, D. C., S. B. Tatter, U. Santhanam, R. H. Clarick, N. Bhardwaj, L. T. May, and P. B. Sehgal. 1989. Multiple forms of IFN-beta 2/IL-6 in serum and body fluids during acute bacterial infection. *J. Immunol.* 142:948.
16. Fong, Y., L. L. Moldawer, M. Marano, H. Wei, S. B. Tatter, R. H. Clarick, U. Santhanam, L. May, P. B. Sehgal, and S. F. Lowry. 1989. Endotoxemia elicits increased circulating β_2-interferon/interleukin-6 in man. *J. Immunol.* 142:2321.
17. Fong, Y., L. L. Moldawer, M. Marano, K. Manogue, K. J. Tracey, H. Wei, G. Kuo, D. A. Fischman, A. Cerami, and S. F. Lowry. 1989. Cachectin/TNF or IL-1α induces cachexia with redistribution of body proteins. *Am. J. Physiol.* 256:R659.
18. Ramadori, G., J. Van Damme, H. Reider, and K. H. Meyer zum Buschenfelde. 1988. Interleukin 6, the third mediator of acute-phase reaction, modulates hepatic protein synthesis in human and mouse. Comparison with interleukin 1 beta and tumor necrosis factor-alpha. *Eur. J. Immunol.* 18:1259.
19. Pool, J. L., S. E. Owen, F. K. Meyers, J. J. Coalson, D. D. Holmes, C. A. Guenter, and L. B. Hinshaw. 1971. Response of the subhuman primate in gram-negative septicemia induced by live *Escherichia coli. Surg. Gynecol. Obstet.* 132:469.
20. Parham, P. 1983. On the fragmentation of monoclonal IgG1, IgG2a, and IgG2b from BALB/c mice. *J. Immunol.* 131:2895.
21. Aggarwal, B. B., W. J. Kohr, P. E. Hass, B. Moffat, S. A. Spencer, W. J. Henzel, T. S. Bringmam, G. E. Nedwin, D. V. Gowddel, and R. N. Harkins. 1985. Human tumor necrosis factor. Production, purification, and characterization. *J. Biol. Chem.* 260:2345.
22. Kenney, J. S., M. P. Masada, E. M. Eugui, B. M. Delustro, M. A. Mulkins, and A. C. Allison. 1987. Monoclonal antibodies to human recombinant interleukin 1 (IL-1)beta: quantitation of IL-1 beta and inhibition of biological activity. *J. Immunol.* 138:4236.

23. Aarden, L. A., E. R. De Groot, O. L. Schaap, and P. J. Landsdorp. 1987. Production of hybridoma growth factor by human monocytes. *Eur. J. Immunol.* 17:1411.
24. Huggett, A. S., and D. A. Nixon. 1957. Use of glucose oxidase, peroxidase, and o-dianisidine in determination of blood and urinary glucose. *Lancet.* ii:368.
25. McGowan, M. W., J. D. Artiss, D. R. Strandbergh, and B. Zak. 1983. A peroxidase-coupled method for the colorimetric determination of serum triglycerides. *Clin. Chem.* 29:538.
26. Dinarello, C. A., J. G. Cannon, S. M. Wolff, H. A. Bernheim, B. Beutler, A. Cerami, I. S. Figari, M. A. Palladino, and J. V. O'Connor. 1986. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. *J. Exp. Med.* 163:1433.
27. March, C. L., B. Mosley, A. Larsen, D. P. Cerretti, G. Braedt, V. Price, S. Gillis, C. S. Henny, S. R. Kronheim, K. Grabstein, P. J. Conlon, T. P. Hopp, and D. Cosman. 1985. Cloning, sequence, and expression of two distinct human interleukin-1 complementary DNAs. *Nature (Lond.).* 315:641.
28. Hinshaw, L., P. Olson, and G. Kuo. 1989. Efficacy of post-treatment with anti-TNF monoclonal antibody in preventing the pathophysiology and lethally of sepsis in the baboon. *Circ. Shock.* 27:362.
29. Waage, A., and T. Espevik. 1988. Interleukin 1 potentiates the lethal effect of tumor necrosis factor-α/cachectin in mice. *J. Exp. Med.* 167:1987.