Administration of Bacterial Lipopolysaccharide Elicits Circulating Tumor Necrosis Factor-Alpha in Neonatal Calves

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The presence of tumor necrosis factor-alpha (TNF- α) during endotoxemia in ruminants has not been reported previously. In this study, we detected the *in vivo* release of bovine TNF- α by using WEHI-164 murine fibrosarcoma cells as targets in an 18-h cytotoxicity assay. Treatment of the WEHI-164 cells with 1 μ g of actinomycin D (dactinomycin) enhanced approximately twofold the susceptibility of the cells to TNF- α activity. TNF- α activity in sera from neonatal calves injected intravenously with 2.7 μ g of *Escherichia coli* lipopolysaccharide (LPS) increased rapidly within the first 2 h postinjection and then declined until it was undetectable by 4 h postinjection. Sera taken before LPS administration had no TNF- α activity. LPS (10 μ g/ml) and fetal, newborn, and pooled adult bovine sera alone and in combination had no direct cytotoxic effects on WEHI-164 cells. TNF- α cytotoxic activity is probably not due to the presence of interleukin-1 (IL-1), alpha interferon, or gamma interferon in the sera since recombinant human IL-1, natural bovine IL-1, and recombinant bovine alpha and gamma interferons had no direct cytotoxic effects on WEHI-164 cells. A monoclonal antibody that neutralizes recombinant human TNF- α significantly reduced the cytotoxic activity of sera from LPS-injected calves.

Systemic infection with gram-negative bacteria can lead to endotoxin shock and, in some cases, death. This phenomenon has been reproduced in laboratory animals by intravenous (*i.v.*) administration of endotoxin or lipopolysaccharide (LPS) (8, 9). Manifestations of the physiological response of LPS injection include fever, systemic hypotension, decreased cardiac output, disseminated intravascular coagulation, tissue necrosis, and the induction of many inflammatory mediators (11). This last group includes tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, alpha interferon (IFN- α), and eicosanoids (11).

TNF-alpha (TNF- α) is a 17-kilodalton protein that is secreted from activated monocytes and macrophages in response to a number of stimuli, including LPS (2). TNF possesses a wide range of biological activities, including induction of fever; direct activation of neutrophils, T cells, and macrophages; direct tumor cell cytotoxicity; cachexia; induction of other mediators (e.g., IL-1, IL-6, and TNF itself); and suppression of lipoprotein lipase (5). Many of these activities parallel those that follow administration of LPS. Administration of recombinant TNF- α has been shown to induce a shocklike state in mice, rabbits, dogs, sheep, and humans (4, 6, 9, 10, 12, 13). Furthermore, TNF has been detected in the sera of mice, rabbits, baboons, and humans *in vivo* in response to LPS administration (2, 3, 6, 8). Serum TNF levels in these species rise within the first 1 h after LPS administration, peak between 1 and 2 h, and decline to undetectable levels shortly thereafter (2, 3, 8).

Calves are extremely sensitive to administration of LPS *in vivo* (7). The clinical signs of endotoxin-induced shock in calves are similar to those observed upon LPS or TNF administration in other animal models (6, 7, 13). Little is known about the release of inflammatory mediators in cattle in response to LPS-induced shock. In this study, we examined the production of TNF by calves after administration of bacterial LPS. Calves displayed significant levels of TNF

activity in serum for the first 2 h after *i.v.* injection of LPS. Bovine TNF activity in the WEHI-164 cell assay was neutralized by the addition of a monoclonal antibody (MAb) directed against recombinant human TNF- α (rHuTNF- α).

MATERIALS AND METHODS

Animals and animal preparation. Four Holstein calves between 2 and 4 days old were studied. The calves ranged in weight from 40.4 to 48.6 kg. These colostrum-deprived calves were obtained from local farms within 6 h of birth. Upon admission to the School of Veterinary Medicine, each calf was weighed on an electronic scale. Each calf was allowed to suckle 2 to 3 liters of pooled bovine colostrum and was then fed bovine milk replacer at a rate of 10% of body weight divided between two feedings per day. Physical examination of each calf was made upon admission, and fecal samples were submitted for the *Escherichia coli* K99 screening test. At 24 h after colostrum consumption, a zinc sulfate turbidity test was performed on the serum of each calf. Only those calves which appeared normal on physical examination, had a positive zinc sulfate turbidity test, and were negative for the K99 antigen test were used in the study. At 1 h after feeding, when the calves were 30 to 36 h old, a polyethylene 14-gauge catheter was placed into each jugular vein by using aseptic technique. The calves were allowed to rest for 1 h to recover from the trauma of catheterization. Blood sampling and volume replacement with lactated Ringer solution were performed through the left jugular vein. Prior to the administration of a sublethal dose of LPS via the right jugular vein, a blood sample was withdrawn; this sample was designated the time-zero sample. Purified *E. coli* LPS (type O55:B5; Sigma Chemical Co., St. Louis, Mo.) reconstituted in 0.9% sterile NaCl was infused over a period of 3 h as follows: 1.5 μ g/kg of body weight per h for 30 min, followed by 0.5 μ g/kg of body weight per h for 2.5 h. Blood was withdrawn at various intervals over a 24-h period. Blood samples were allowed to

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clot, and serum was collected and stored at -70°C until it was assayed for TNF activity.

Cytokines. rHuTNF- α (lot NP200B; specific activity, 2.2×10^7 U/mg) was a kind gift from A. Creasey, Cetus Corp., Emeryville, Calif. Recombinant human IL-1 α (rHuIL-1 α) (lot IL-1 1/87; specific activity, 2.5×10^9 U/mg) was generously provided by A. Stern and P. T. Lomedico, Hoffmann-La Roche Inc., Nutley, N.J. Recombinant bovine IFN- α (rBoIFN- α_1) (lot 1942-33; specific activity, 8×10^6 U/mg) and rBoIFN- γ (lot 3229-38; specific activity, 1.8×10^5 U/mg) were provided by M. Shepherd, Genentech, South San Francisco, Calif., and R. Steiger, CIBA-GEIGY, Basel, Switzerland, respectively.

Cell lines. WEHI-164 murine sarcoma cells (ATCC CRL 1751) were obtained from the American Type Culture Collection, Rockville, Md. The cells were maintained in Dulbecco modified Eagle tissue culture medium (DMEM) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% fetal bovine serum (lot 38P4084; GIBCO). Murine hybridoma cells (cell line B154.2.1) that secrete anti-rHuTNF- α antibodies (immunoglobulin G1) were a kind gift from Giorgio Trinchieri, Wistar Institute, Philadelphia, Pa., and were maintained in DMEM supplemented with 5% fetal bovine serum. A control MAb directed against influenza hemagglutinin (immunoglobulin G1) was generously provided by V. Hinshaw, University of Wisconsin, Madison.

Assay for TNF- α . WEHI-164 cells were grown to confluency in DMEM containing 5% fetal bovine serum (GIBCO), 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 2 mM L-glutamine, 100 U of penicillin G (GIBCO) per ml, and 100 μg of streptomycin (GIBCO) per ml at 37°C in an atmosphere containing 5% CO_2 . The cells were gently detached with a sterile cell scraper (American Scientific Products, McGaw Park, Ill.) and washed in fresh DMEM. The cells were seeded into 96-well plates (Costar, Cambridge, Mass.) at a rate of 10^5 cells per well in 0.2-ml portions of fresh DMEM and allowed to adhere for 4 to 6 h at 39°C in an atmosphere containing 5% CO_2 . The medium was then aspirated, and 100 μl (1 μg) of actinomycin D (dactinomycin) sulfate (Sigma) was added to each well. Serum samples to be tested were diluted in complete DMEM, and 100 μl of each dilution was added to triplicate or quadruplicate wells. For antibody neutralization, serum samples were diluted similarly and added to wells in the presence or absence of various amounts of MAb. rHuTNF- α (1, 10, and 100 pg per well) and DMEM were included as positive and negative controls, respectively, in each plate. The plates were then incubated at 39°C in an atmosphere containing 5% CO_2 for 18 h. The culture supernatants were removed, and the monolayers were washed twice with warm (37°C) phosphate-buffered saline (pH 7.2). The monolayers were fixed with 40% formaldehyde and stained for 10 min with a 0.5% solution of crystal violet in 25% ethanol-0.85% NaCl. After this the stained cells were washed twice with a gentle stream of tap water and allowed to dry. The stained monolayers were solubilized by adding 100 μl of a 0.5% sodium dodecyl sulfate-0.05 M acetic acid solution. The optical density (OD) of each well was read with a microplate reader (Dynatech, Inc., Chantilly, Va.) at a wavelength of 550 nm. Data were expressed as percentages of cytotoxicity compared with the negative control (DMEM alone) and were calculated as follows: $[(\text{OD of negative control} - \text{OD of test well}) / \text{OD of negative control}] \times 100$. The standard errors of the mean were routinely less than 10% of the mean within a given set of replicate wells.

Production and purification of anti-TNF- α . Hybridoma

FIG. 1. TNF activity in sera from calves after i.v. administration of $2.7 \mu\text{g}$ of *E. coli* LPS per kg. Serum samples were diluted to final concentrations of 10% (●), 1% (○), and 0.1% (△) in DMEM. Data are expressed as the mean $\pm$ standard error of the mean percentages of cytotoxicity for sera from four calves that were tested independently.

cells (B154.2.1) were grown in DMEM supplemented with 5% fetal bovine serum. Five *nu/nu* BALB/c mice were treated with 1 ml of 2,6,10,14-tetramethyl-pentadecane (Pristane; Sigma) 2 weeks prior to intraperitoneal injection of 2×10^6 hybridoma cells. Ascites fluid was collected and centrifuged to remove the particulate debris. The samples were delipidated by using a lipid-clearing solution (Beckman Instruments, Inc., Fullerton, Calif.). The MAbs were purified by using high-performance anion-exchange liquid chromatography (ABX 300; Beckman). The MAb was eluted from the column with a 0 to 400 mM gradient of sodium acetate buffer. Fractions were screened for the presence of murine immunoglobulin G by using a MAb isotyping kit (HyClone Laboratories, Logan, Utah). Fractions that were positive as determined by an enzyme-linked immunosorbent assay were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to verify the purification of the antibody. Fractions that contained the MAb were dialyzed extensively against phosphate-buffered saline, concentrated by using an ultrafiltrator (molecular weight cutoff, 50,000; Amicon Corp., Danvers, Mass.), and stored as portions at -70°C .

RESULTS

Detection of the in vivo release of TNF- α . Calves were bled prior to and at selected times (1 to 24 h) after i.v. administration of LPS. There was no significant TNF activity in the sera of these animals before they received LPS (Fig. 1, time zero). There was a significant increase in TNF activity at 1 h after LPS treatment (Fig. 1). Activity was detected at final concentrations of 10, 1, and 0.1% serum. Serum TNF activity peaked between 1 and 2 h after LPS treatment and subsequently fell to undetectable levels by 4 h after injection. Examination of sera at later times failed to show a second peak of cytotoxic activity. This pattern was seen at each of the three serum concentrations (0.1, 1.0, 10%) tested.

Direct effects of serum and LPS on WEHI-164 cells. To eliminate the possibility that the concentrations of bovine sera tested were nonspecifically cytotoxic for WEHI-164 cells, samples of normal bovine sera were tested for their direct effects on WEHI-164 cells. No cytotoxic activity was

FIG. 2. Bovine sera and LPS do not have direct nonspecific cytotoxic effects on WEHI-164 cells. Fetal bovine serum (FBS), newborn bovine serum (NBS), and pooled adult bovine serum (PAS) were diluted to final concentrations of 10% in DMEM. LPS was diluted to a final concentration of 10 μ g/ml in DMEM. rHuTNF- α was tested at concentrations of 1, 10, and 100 pg per well in DMEM as a positive control for cytotoxicity. Data expressed are the mean $\pm$ standard error of the mean percentages of cytotoxicity for three independent experiments.

observed when fetal, newborn, and pooled adult bovine sera were tested at final concentrations of 10% (Fig. 2). To eliminate the possibility that residual LPS in the serum could be mediating the cytotoxic effects, *E. coli* LPS was tested for direct effects on the WEHI-164 cells. LPS at a concentration of 10 μ g/ml had no cytotoxic effects on the WEHI-164 cells (Fig. 2). Samples containing 10% fetal or pooled adult bovine serum that were spiked with 10 μ g of LPS per ml similarly displayed no cytotoxic effects on WEHI-164 cells (data not shown).

Direct effects of rHuIL-1 α , rBoIFN- α_1 , and rBoIFN- γ . The cytotoxicity for WEHI-164 cells of other cytokines (e.g., IL-1, IFN- α , and IFN- γ) that might be released during an LPS-initiated inflammatory response was investigated. rHuIL-1 α (100 pg to 1 μ g), partially purified natural bovine IL-1, rBoIFN- α_1 (10 to 1,000 U), and rBoIFN- γ (1 to 100 U) had no direct cytotoxic effects on the WEHI-164 cells (Table 1). In addition, pooled adult bovine serum spiked with rHuIL-1 α , rBoIFN- α , or rBoIFN- γ had no significant cytotoxic effects on the WEHI-164 cells (data not shown).

Abrogation of cytotoxic activity by anti-rHuTNF- α . A MAb against rHuTNF- α was used to show that the serum cytotoxic activity was due to TNF- α . Treatment of positive serum samples with this anti-TNF- α MAb significantly reduced their cytotoxic activity as detected by the WEHI-164 assay (Fig. 3). We verified the effectiveness of this MAb by showing that 15 μ g of MAb completely abrogated the cytotoxic activity of 100 pg of rHuTNF- α (92.3% $\pm$ 0.6% cytotoxicity for the control compared with 6.0% $\pm$ 1.0% cytotoxicity for treatment with 15 μ g of antibody). A control MAb of the same isotype directed against an irrelevant determinant (influenza hemagglutinin) had no effect on the cytotoxicity of positive serum samples, nor did the anti-TNF- α MAb have any cytotoxic effects on WEHI-164 cells (data not shown).

DISCUSSION

The results of this study demonstrate the *in vivo* release of detectable levels of TNF- α in sera of calves after *i.v.*

administration of LPS. TNF activity was detected by lysis of murine WEHI-164 cells. The L929 murine fibroblast cell line, which is often used for detection of murine and human TNFs, was only slightly susceptible to TNF activity in bovine serum. This finding is similar to the results which we have obtained with culture supernatants from LPS-stimulated bovine monocytes, which exhibited no cytotoxicity for L929 cells but had substantial cytotoxic activity for WEHI-164 cells (manuscript submitted). In this study, TNF activity was observed at concentrations of serum as low as 0.1%. The time course of TNF release that we observed was similar to that reported previously for mice and humans

TABLE 1. rHuIL-1 α , rBoIFN- γ , and rBoIFN- α_1 have no direct cytotoxic effects on WEHI-164 cells

Cytokine	Dose (per well)	% of cytotoxicity ^a
rHuTNF- α	100 pg	86.8 $\pm$ 2.5
	10 pg	77.9 $\pm$ 0.5
	1 pg	59.3 $\pm$ 3.9
rHuIL-1 α	1,000 ng	12.9 $\pm$ 3.7
	100 ng	7.4 $\pm$ 4.9
	10 ng	6.6 $\pm$ 4.5
	1 ng	13.1 $\pm$ 3.5
	0.1 ng	7.4 $\pm$ 3.3
rBoIFN- α_1	1,000 U	6.7 $\pm$ 1.7
	100 U	2.5 $\pm$ 4.6
	10 U	5.9 $\pm$ 2.9
rBoIFN- γ	100 U	10.2 $\pm$ 4.5
	10 U	12.0 $\pm$ 3.8
	1 U	7.8 $\pm$ 1.8

^a Various concentrations of rHuTNF- α , rHuIL-1 α , rBoIFN- α_1 , and rBoIFN- γ were added to actinomycin D-treated WEHI-164 cells, and the preparations were incubated for 18 h at 39°C. Percentages of cytotoxicity were determined as described in Materials and Methods. Data are expressed as the means $\pm$ standard errors of the mean percentages of cytotoxicity for three independent experiments.

FIG. 3. Neutralization of the cytotoxic activity of bovine serum TNF by a MAB directed against rHuTNF- α . Bovine serum samples were diluted to a final concentration of 1% in DMEM. Various doses of MAB were added to each well along with the serum sample. A control MAB of the same isotype (immunoglobulin G1, specific for influenza hemagglutinin) had no effect. Data expressed are the means $\pm$ standard errors of the means for one representative experiment.

injected with LPS (2, 6, 8). Cytotoxic activity rapidly increased in sera within the first 1 to 2 h after i.v. administration of LPS and then subsequently declined to undetectable levels by 4 h after LPS treatment. Cytotoxic activity was not due to nonspecific cytotoxicity of bovine sera for WEHI-164 cells, nor was it likely to result from residual LPS in the sera of LPS-treated animals. Other cytokines that might be released during an LPS-initiated inflammatory response (e.g., IL-1, IFN- α , and IFN- γ) were shown to be not cytotoxic for WEHI-164 cells. Convincing evidence that the cytotoxic activity was due to TNF- α was obtained by neutralizing TNF activity in serum samples with a MAB directed against rHuTNF- α .

To the best of our knowledge, this is the first report of the production of bovine TNF. Previous studies of other ruminant species, specifically sheep, have shown that recombinant TNF- α can mimic endotoxemia (4). Our data demonstrate that calves are capable of responding to LPS administration by producing significant levels of TNF in serum. It has been demonstrated that calves are physiologically extremely sensitive to LPS administration (7). It is probable that the rapid induction of TNF- α during LPS-induced shock plays some part in the outcome of endotoxemia in calves. This is similar to other animal and human models of LPS-induced shock in which TNF- α has been shown to mediate many of the physiological responses that are observed (1, 6, 9, 12).

The results of this study provide important new information regarding the pathogenesis of endotoxemia in calves. Our results demonstrate that significant quantities of TNF- α are released in vivo shortly after LPS administration. Because of the diverse biological activities of TNF, systemic release of TNF could be a critical mediator in the bovine response to bacterial LPS. Further studies are needed to determine whether significant circulating levels of TNF- α are released during naturally occurring gram-negative septicemia in cattle.

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ADDENDUM IN PROOF

While this paper was in press, Peel et al. reported a similar TNF response in 2- to 3-week-old calves injected i.v. with *E. coli* LPS (J. E. Peel, M.-J. Voirol, C. Kolly, D. Gobet, and S. Martinod, *Infect. Immun.* 58:439-442, 1990).

LITERATURE CITED

- Beutler, B., I. W. Milsark, and A. Cerami. 1985. Passive immunization against cachectin/tumor necrosis factor (TNF) protects mice from the lethal effect of endotoxin. *Science* 229:869-871.
- Carswell, E. A., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA* 72:3666-3670.
- Hesse, D. G., K. J. Tracey, Y. Fong, K. R. Manogue, M. A. Palladino, Jr., A. Cerami, G. T. Shires, and S. F. Lowry. 1988. Cytokine appearance in human endotoxemia and primate bacteremia. *Surg. Gynecol. Obstet.* 166:147-153.
- Johnson, J., B. Myerick, G. Jesmok, and K. L. Brigham. 1989. Human recombinant tumor necrosis factor alpha infusion mimics endotoxemia in awake sheep. *J. Appl. Physiol.* 66:1448-1454.
- Le, J., and J. Vilcek. 1987. Biology of disease. Tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. *Lab. Invest.* 56:234-248.
- 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.* 81:1925-1937.
- Michaels, F. H., and K. L. Banks. 1988. Contribution of various host factors to resistance to experimentally induced bacterial endotoxemia in calves. *Am. J. Vet. Res.* 49:557-562.
- Michie, H. R., K. R. Manogue, D. R. Spriggs, A. Revhaug, S. O'Dwyer, C. A. Dinarello, A. Cerami, S. M. Wolff, and D. W. Wilmore. 1988. Detection of circulating tumor necrosis factor after endotoxin administration. *New Engl. J. Med.* 318:1481-1486.
- Natanson, C., P. W. Eichenholz, R. L. Danner, P. Q. Eichacker, W. D. Hoffman, G. C. Kuo, S. M. Banks, T. J. MacVittie, and J. E. Parrillo. 1989. Endotoxin and tumor necrosis factor challenges in dogs simulate the cardiovascular profile of human septic shock. *J. Exp. Med.* 169:823-832.
- Remick, D. G., R. G. Kunkel, J. W. Larrick, and S. L. Kunkel. 1987. Acute *in vivo* effects of human recombinant tumor necrosis factor. *Lab. Invest.* 56:583-590.
- Rosenblum, M. G., and N. J. Donato. 1989. Tumor necrosis factor- α : a multifaceted peptide hormone. *Crit. Rev. Immunol.* 9:21-44.
- Tracey, K. J., B. Beutler, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. J. Hariri, T. J. Fahey III, A. Zentella, J. D. Albert, G. T. Shires, and A. Cerami. 1986. Shock and tissue injury induced by recombinant human cachectin. *Science* 234:40-44.
- Warren, R. S., H. F. Starnes, Jr., J. L. Gabilove, H. F. Oettgen, and M. F. Brennan. 1987. The acute metabolic effects of tumor necrosis factor administration in humans. *Arch. Surg.* 122:1396-1400.