

## ENDOTOXIN AND TUMOR NECROSIS FACTOR CHALLENGES IN DOGS SIMULATE THE CARDIOVASCULAR PROFILE OF HUMAN SEPTIC SHOCK

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Endotoxin, an LPS found in the outer membrane of Gram-negative bacteria, has been considered by many to be the principal toxin involved in the pathogenesis of Gram-negative septic shock (1). However, it is now clear that endotoxin may cause most (if not all) of its biological effects via the release of host factors (2-5). Several studies have suggested that TNF, a cytokine produced by macrophages during septic shock, is one of the endogenous mediators that causes cardiovascular injury and death (2-5).

Previous studies examining the effects of endotoxin and TNF in animal models have used a number of techniques to study cardiovascular function during the first 1-12 h after challenge. Most of these investigations have shown evidence of myocardial depression (2-11). However, recent studies of human and animal septic shock have demonstrated that cardiovascular function continues to change over a period of several days. Typically, the left ventricular ejection fraction (LVEF)<sup>1</sup> falls to a nadir 2-3 d after the onset of hypotension. This progressive decrease in LVEF is associated with LV dilatation and maintenance of normal or increased cardiac output. In survivors, these cardiovascular changes return to normal in 7-10 d (12-20).

The present study shows that dogs given a single intravenous bolus of TNF produce all the same complex cardiovascular changes over 7-10 d as those seen in septic shock over the same period. Furthermore, endotoxin without any other bacterial products can also produce the same serial changes in cardiovascular function seen in human septic shock.

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Part of the funding for this research was provided by the Armed Forces Radiobiologic Research Institute under task number 4441-00082. Address correspondence to Charles Natanson, Critical Care Medicine Department, Building 10, Room 10-D-48, National Institutes of Health, Bethesda, MD 20892.

The opinions and assertions contained herein are the private ones of the authors, and are not to be construed as official or reflecting the views of the armed forces. The experiments reported herein were conducted according to the principles set forth in "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, Department of Health, Education, and Welfare Publication No. 74-23 (National Institutes of Health).

<sup>1</sup> *Abbreviations used in this paper:* b.w., body weight; CI, cardiac index; EDVI, end diastolic volume index; ESVI, end systolic volume index; LVEF, left ventricular ejection fraction; MAP, mean arterial pressure; SVRI, systemic vascular resistance index.

## Materials and Methods

**Experimental Design.** The protocol used in the present study has been previously described (17, 18). 1 wk before challenge, we performed baseline hemodynamic evaluations and laboratory blood tests. On day 0, a thrombin-fibrin clot containing endotoxin (15 mg/kg body weight [b.w.]) was surgically implanted (as previously described) into the peritoneum of eight purebred male beagles weighing 10–12 kg (17, 18). The endotoxin (Sigma Chemical Co., St. Louis, MO) was phenol extracted from *Escherichia coli* 26:B6 (Lot No. 93F-4041). On day 0, another group of 16 purebred male beagles weighing 10–12 kg were infused with TNF. Eight dogs received active TNF (60  $\mu$ g/kg b.w.) and six animals received heat-inactivated TNF (60  $\mu$ g/kg b.w.). Recombinant TNF was provided by the Chiron Corporation (Emeryville, CA) and was expressed in a yeast system with purity >98% as judged by SDS-PAGE (21). The protein concentration was 12 mg/ml as determined by Bradford protein assay with sp act of  $2-4 \times 10^7$  U/ng in the L929 cell killing assay (22, 23). The endotoxin content of the TNF solution was 25 ng/mg protein as assayed by the limulus amebocyte lysate test (20). Control TNF was denatured by boiling for 30 min in endotoxin-free test tubes. Individual dosages of active or heat-inactivated TNF were mixed with 200 cc of PBS in bags previously treated with 3 cc of dog plasma to coat the administration tubing. The TNF solution was then infused into dogs through a central intravenous catheter using a control pump for 1 h.

Endotoxin and TNF were given to dogs by different methods to simulate more closely their role in human infection. Endotoxin was placed in an intraperitoneal clot (to simulate a peritoneal abscess) as a nidus of infection releasing toxin slowly over several days. The TNF was given as a sustained infusion for 1 h to reproduce the rapid increase in blood levels that occurs in human and animal sepsis (3–5).

The same hemodynamic evaluations performed at baseline were repeated on days 1, 2, and 10 after challenge with endotoxin or TNF. At each time point, intravascular catheters were inserted using only subcutaneous 1% lidocaine to obtain blood for laboratory analysis. In addition, hemodynamic data were determined in conscious non-sedated dogs simultaneously using pulmonary and femoral artery catheters and radionuclide cineangiography of the left ventricle. Animals then received an intravascular volume infusion (80 ml/kg b.w. lactated ringers for 45 min), after which simultaneous hemodynamic and radionuclide studies were repeated. All intravascular catheters were removed each day after completing hemodynamic evaluations.

Hemodynamic changes after endotoxin and TNF challenges in the present study were compared with those of dogs previously described (17, 18). In previous studies, 39 animals received challenges of viable *E. coli* in a peritoneal clot of various bacterial doses (7, 14, or  $30 \times 10^9$  CFU/kg b.w.), and 14 control dogs received surgical implants of a sterile peritoneal clot.

**Physiologic Measurements and Hemodynamic Calculations.** All intravascular catheter hemodynamics and radionuclide cineangiogram measurements were made using techniques previously described (17, 18). Hemodynamic data were indexed by animal body weight in kilograms. Hemodynamic values were calculated according to standard formulas previously described (17, 18).

**Statistical Methods.** Mean values were compared with an analysis of variance and significance adjusted with a Tukey Test (Figs. 1–4). The time courses of a hemodynamic variable (e.g., EF) were summarized at each of four time points (baseline, days 1, 2, and 10 after challenge) by assigning ranks to the corresponding mean values of the variable. Patterns of hemodynamic responses to each challenge were assessed with the Kendall coefficient for concordance among the groups of dogs receiving endotoxin, TNF, and three different doses of viable *E. coli* (24). A coefficient of concordance value of 1 indicated complete agreement among the assigned ranks and a value of 0 indicated maximal disagreement. The shaded areas in Figs. 1–4 represent the mean value  $\pm$  SEM for 100 normal dogs; the respective SE was adjusted to the size of the comparison group.

## Results

**Blood Cultures and Clinical Manifestations.** All dogs treated with endotoxin or TNF had sterile blood cultures for the first 2 d after treatment and at baseline and recovery

(day 10). For 2 d after challenge, all dogs given endotoxin or TNF were febrile and had signs of septic shock (i.e., weakness and lethargy). One of the eight dogs treated with endotoxin and one of the eight dogs treated with TNF died 2 d after challenge. By days 7-10, the seven surviving dogs in both groups were afebrile and appeared healthy. As previously reported, dogs treated with viable *E. coli* had a similar time course of illness. Control dogs treated with sterile clots or heat-inactivated TNF had sterile blood cultures, and were afebrile and healthy throughout the study (17, 18).

**Hemodynamics in Dogs Treated with Endotoxin.** From baseline to days 1 and 2, dogs challenged with endotoxin had a significant decrease ( $p < 0.05$ ) in mean value for mean arterial pressure (MAP). By day 10, mean MAP returned toward baseline values. At each time point (baseline, days 1, 2, and recovery), volume loading increased ( $p < 0.05$ ) mean MAP similarly (Fig. 1 A). From baseline to days 1, 2, and 10, the mean value for cardiac index (CI) did not change significantly (Fig. 1 B).

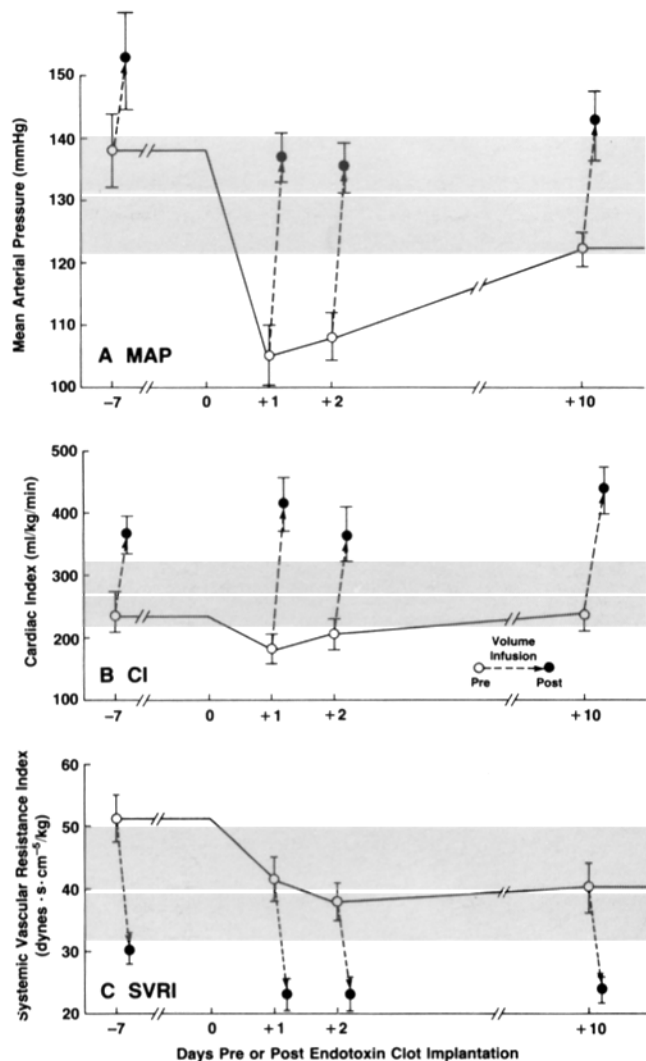


FIGURE 1. Mean  $\pm$  SE changes in MAP (A), CI (B), and SVRI (C) in dogs treated with endotoxin. The solid lines connect the mean hemodynamic values pre-volume infusion on serial days and the dotted arrows connect mean hemodynamic values for the pre- and post-volume infusion each day. The shaded areas represent the "normal" range and are the mean  $\pm$  SE values obtained from 100 other normal dogs standardized for comparison.

At each time point, volume loading increased ( $p < 0.05$ ) mean CI similarly. From baseline to days 1, 2, and 10, systemic vascular resistance index (SVRI) did not change significantly. At each time point, volume loading also decreased ( $p < 0.05$ ) SVRI similarly (Fig. 1 C).

In dogs challenged with endotoxin, there was a small, insignificant decrease in LVEF from baseline to day 1 (Fig. 2 A). By day 2, however, LVEF decreased significantly ( $p < 0.05$ ). By day 10, mean LVEF returned toward baseline values. At each time point, volume loading increased mean LVEF similarly ( $\sim 5\%$ ). On day 2 after volume infusion, mean LVEF was still markedly depressed. From baseline to day 1, end diastolic volume index (EDVI) decreased ( $p < 0.05$ ) and end systolic volume index (ESVI) did not change significantly (Fig. 2, B and C). From day 1 to day 2, EDVI and ESVI increased ( $p < 0.05$ ) in size. Volume loading did not

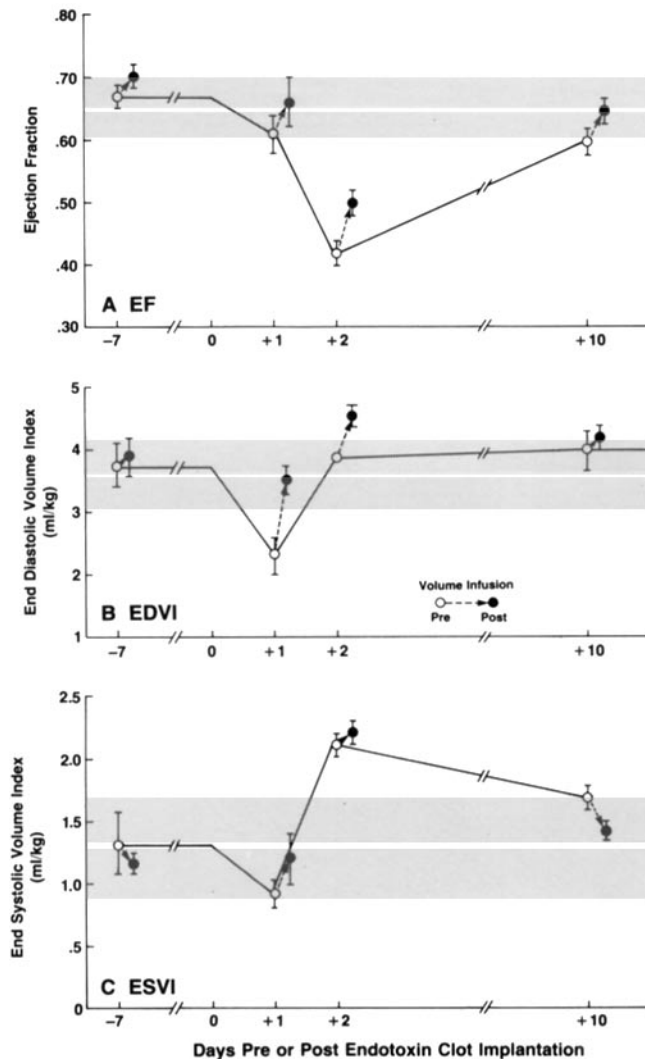


FIGURE 2. Mean  $\pm$  SE changes in EF (A), EDVI (B), and ESVI (C) in dogs treated with endotoxin. The format is the same as Fig. 1.

significantly affect ESVI at any time point. In response to volume loading, mean EDVI showed no significant change at baseline or recovery. On days 1 and 2, however, volume infusion increased EDVI significantly ( $p < 0.05$ ). By day 10, these changes in LV size had decreased but had not yet returned to baseline values.

**Hemodynamics (MAP, CI, SVRI, EF, EDVI, ESVI) in Dogs Treated with TNF.** From baseline to days 1 and 2, dogs challenged with TNF had a significant decrease ( $p < 0.05$ ) in mean value for MAP (Fig. 3 A). By day 10, mean MAP returned toward baseline values. At each time point (baseline, days 1 and 2, and recovery), volume loading increased mean MAP similarly. From baseline to days 1, 2, and 10, the mean value for CI did not change significantly (Fig. 3 B). At each time point, volume loading increased ( $p < 0.05$ ) mean CI similarly. From baseline to days 1, 2, and 10, SVRI did not change significantly. At every time point, however, volume loading decreased ( $p < 0.05$ ) SVRI similarly (Fig. 3 C).

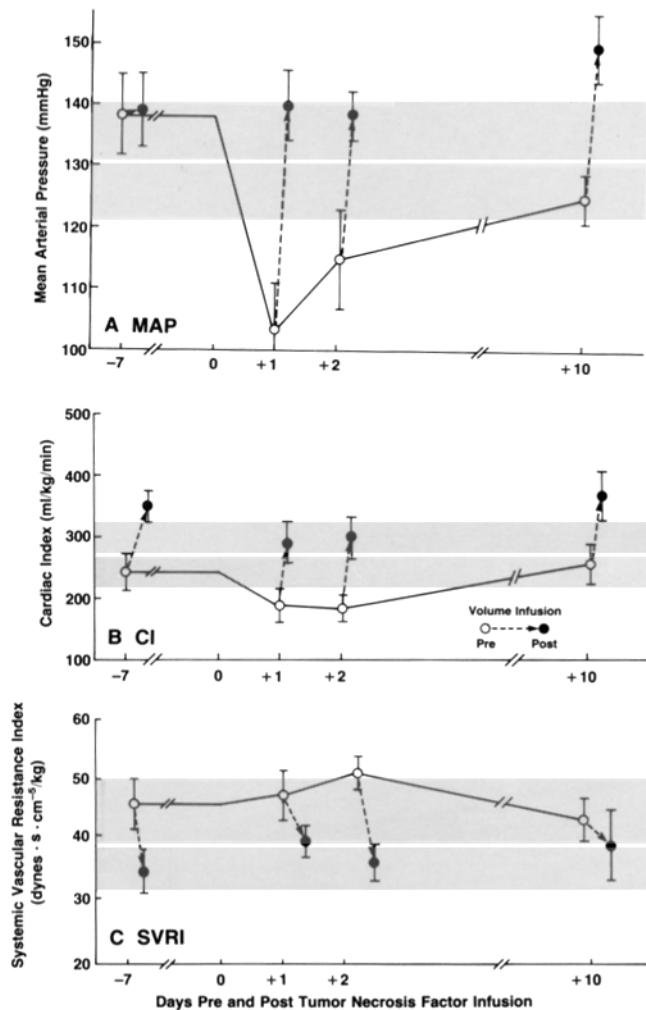


FIGURE 3. Mean  $\pm$  SE changes in MAP (A), CI (B), and SVRI (C) in dogs treated with TNF. The format is the same as Fig. 1.

From baseline to days 1 and 2, dogs challenged with TNF had a significant decrease in LVEF ( $p < 0.05$ ). By day 10, mean LVEF returned toward baseline values (Fig. 4 A). At each time point, volume loading increased mean LVEF similarly ( $\sim 4\%$ ). On days 1 and 2 after volume infusion, mean LVEF still remained markedly depressed. From baseline to days 1 and 2, EDVI and ESVI did not change significantly (Fig. 4, B and C). Volume loading did not significantly affect ESVI at any time point. Volume loading on day 2 significantly increased ( $p < 0.05$ ) LV size (EDVI).

*Comparison of Serial Hemodynamics with Endotoxin, TNF, and Viable E. coli.* Serial

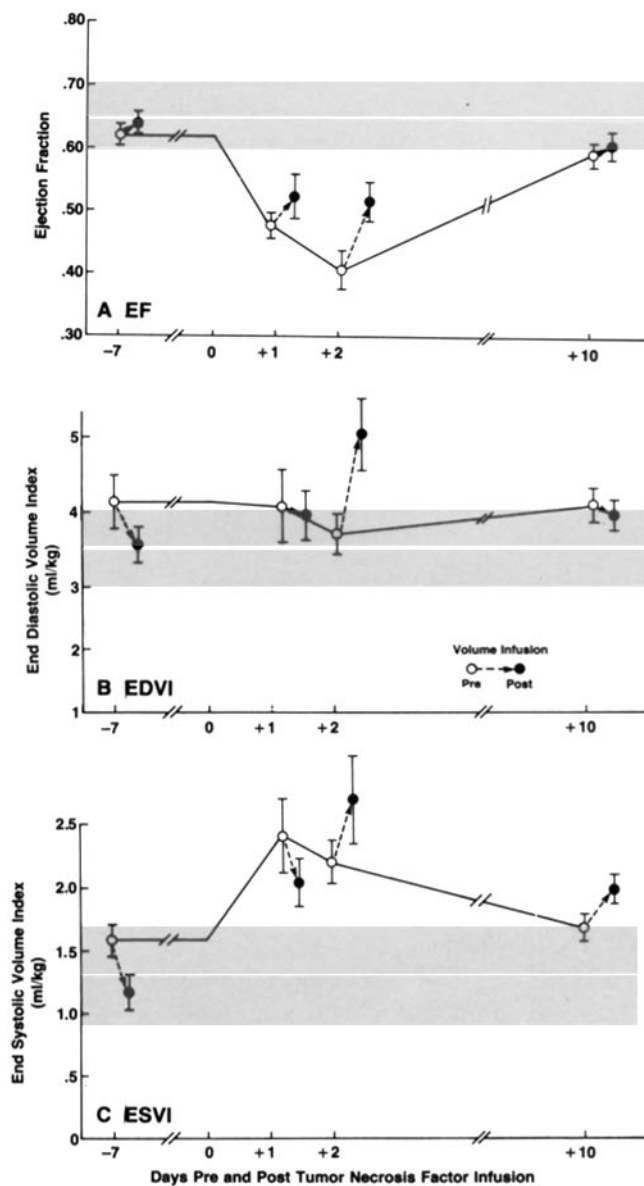


FIGURE 4. Mean  $\pm$  SE changes in EF (A), EDVI (B) and ESVI (C) in dogs treated with TNF. The format is the same as Fig. 1.

mean changes in hemodynamic parameters were compared in dogs challenged with viable *E. coli* (previously described), endotoxin, or TNF (17–20). To determine if changes in hemodynamic parameters were similar for different types of challenges, we calculated the coefficient of concordance (see Materials and Methods). The coefficient of concordance determined whether the hemodynamic responses of animals in these different groups occurred at similar time points.

When comparing the pattern of hemodynamic changes of all the groups of dogs treated with *E. coli*, endotoxin, or TNF, we noted an overall positive concordance for all the hemodynamic parameters studied ( $p < 0.05$ ). The individual concordance for the serial changes in hemodynamics were (see Materials and Methods): EF, 0.94 ( $p < 0.005$ ); MAP, 0.85 ( $p < 0.03$ ); CI, 0.81 ( $p < 0.02$ ); stroke volume index, 0.73 ( $p < 0.03$ ); ESVI, 0.55 ( $p < 0.08$ ); and EDVI, 0.07 ( $p = \text{ns}$ ). As previously described, control dogs treated with heat-inactivated TNF or sterile clots had no significant changes in hemodynamic parameters compared with those at baseline (17–20).

**Laboratory Values.** Laboratory results from baseline, days 1 and 2, and recovery showed that pH and  $p\text{O}_2$  were normal (laboratory data not shown). Hemoglobin, sodium, potassium, bicarbonate, chloride, glucose, creatinine, blood urea nitrogen, and calcium values were within normal range and could not have affected cardiovascular function.

### Discussion

When a fibrin clot containing endotoxin is implanted intraperitoneally or when TNF is infused intravenously, the hemodynamic profile after volume infusion is decreased LVEF, increased left ventricular size, with normal or high CI, and normal or low systemic vascular resistance (a “hyperdynamic” response). The endotoxin-induced or TNF-induced decrease in EF was greatest at 2–3 days after challenge. In surviving animals, this decrease in EF reversed after 10 d. These serial cardiovascular changes are identical to those seen in dogs receiving surgical implants of viable *E. coli* in a fibrin clot and are very similar to the cardiovascular profile seen in humans with septic shock (12, 13, 17–20). The finding that either endotoxin or TNF (without viable bacteria or other bacterial cell components) induced this characteristic cardiovascular response supports the hypothesis that either endotoxin or TNF are mediators of shock caused by sepsis.

While hemodynamic data from dogs treated with endotoxin and TNF followed a similar time course over 10 d, there were some notable but not significant differences in LV volumes (EDVI and ESVI) on day 1. These differences may have been caused by effects of the method of administration or by specific mediator properties. Animals treated with intraperitoneal endotoxin developed peritonitis, which may have lowered effective circulating volume by shifting fluid into the abdomen. This action may explain the lower EDVI and ESVI seen on day 1 in endotoxin-treated animals. Differences in hemodynamics may also have been caused by the effects of different mediators. Endotoxin may have produced hemodynamic compromise via TNF release; however, endotoxin may have also caused the release of other endogenous substances with their own hemodynamic effects. Thus, the exact hemodynamic sequelae would result from the complex cardiovascular interactions among a variety of substances (mediators) and physiologic conditions (peritonitis).

In previous studies, we have examined whether endotoxin is the universal or sole

mediator of the cardiovascular changes observed during septic shock (19, 20). In one study, we implanted *Staphylococcus aureus* (a Gram-positive microorganism without endotoxin) into animals intraperitoneally, and measured serial hemodynamic changes and serial endotoxin levels using a sensitive chromogenic limulus lysate assay. *S. aureus* produced hemodynamic changes identical to Gram-negative bacteria with no detectable endotoxemia, thus documenting that endotoxin is not the only bacterial mediator of septic shock. Findings from the present study combined with those from this previous study demonstrate that endotoxin can produce all of the major cardiovascular abnormalities observed in septic shock, even though it is not the only mediator of this syndrome.

The finding that structurally and functionally distinct bacteria result in similar hemodynamic patterns suggests that they cause this cardiovascular dysfunction via a final common pathway of injury (25, 26). Recent studies suggest that endogenous cytokines released from monocytes, such as TNF or IL-1, may be part of this common pathway. TNF is a potent inducer of acute shock in animals, and anti-TNF antibodies can protect against lethal intravenous bacterial and endotoxin infusions (2-5). IL-1 can also produce hypotension, and both IL-1 and TNF can act synergistically to produce hypotension (27). This present study demonstrates that a single intravenous injection of TNF in dogs produces many of the complex hemodynamic changes seen during human septic shock over a 7-10-d period. The findings from this study support the hypothesis that different bacterial toxins can stimulate the release of endogenous mediators, which then act by a common pathway to produce a similar pattern of cardiovascular injury during septic shock (2-5, 17-20, 25, 26).

This study demonstrates that either endotoxin (a component of the outer membrane of Gram-negative bacteria) or TNF (a cytokine released from macrophages) can reproduce all the complex serial hemodynamic changes seen in the 7-10 d after onset of sepsis. These changes were analogous to those previously documented in human and animal bacterial sepsis (i.e., acute hypotension followed by a falling LVEF over 2-3 d; and, with adequate fluid resuscitation, high or normal cardiac output and low or normal systemic vascular resistances) (12-20). In surviving animals, these changes returned to normal in 7-10 d. This study confirms that endotoxin, although not necessary to produce septic shock, it is probably one of several important bacterial toxins that may induce septic shock. This study also supports the hypothesis that endogenous mediators (such as TNF) respond to bacterial products to induce the cardiovascular abnormalities of septic shock.

### Summary

Survivors of both human and animal bacterial shock develop a characteristic pattern of progressive changes in cardiovascular function over a period of 7-10 d. In this present study, we examined whether endotoxin (a product of Gram-negative bacteria) or TNF (a cytokine released from macrophages) could reproduce the same complex cardiovascular changes observed in septic shock over a period of 7-10 d. To test this hypothesis, we implanted a thrombin-fibrin clot containing purified endotoxin from *E. coli* into the peritoneal cavity of eight dogs, and infused TNF into eight different dogs. Over the next 10 d, serial simultaneous heart scans and thermodilution cardiac outputs were performed in these awake nonsedated animals. By day 2 after challenge with either endotoxin or TNF, animals developed a decrease



( $p < 0.05$ ) in both mean arterial pressure and left ventricular ejection fraction. With fluid resuscitation, animals manifested left ventricular dilatation (increased [ $p < 0.05$ ] end diastolic volume index), increased or normal cardiac index, and decreased or normal systemic vascular resistance index. In surviving animals, these changes returned to normal with 7–10 d. The time course of these changes was concordant ( $p < 0.05$ ) with that previously described in a canine model of septic shock using viable bacteria. During the 10-d study, control animals receiving sterile clots or heat-inactivated TNF had not significant changes in hemodynamics. The results from this canine model demonstrate that either endotoxin or TNF alone can produce many of the same hemodynamic abnormalities seen in human septic shock and in a canine septic shock model induced by live bacteria. These findings support the hypothesis that the action of endogenous mediators (TNF) responding to bacterial products (endotoxin) is the common pathway that produces the serial cardiovascular changes found in septic shock.

We thank Gary L. Akin, Mike E. Flynn, Steven Richmond, John Stewart, John K. Warrenfelt, Nelson L. Flemming, Denise M. Ratica, Jim Foster, and Kevin Peart for their technical support during this study; Major James E. Hall, Major Jerome Sauber, Major James Rogers, and Captain Gordon Rahmus for veterinary care and surgical procedures; Dr. David W. Alling for reviewing the statistical analysis; Lee Hoffman for editorial assistance; and Kathy Kiefer for preparation of this manuscript.

Received for publication 17 August 1988 and in revised form 4 November 1988.

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