

## Macrophage-Conditioned Medium and Interleukin 1 Suppress Vascular Contractility

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Isolated rat aortas, after incubation in medium conditioned by endotoxin-stimulated peritoneal macrophages, exhibited diminished contraction to norepinephrine (maximal contraction: control medium =  $713 \pm 37$  (SE) mg tension/mg tissue; medium conditioned by macrophages =  $437 \pm 38$ ,  $P < .001$ ). Medium containing endotoxin alone or medium conditioned by nonstimulated macrophages had no effect on aortic tissue response to norepinephrine. Stimulation of peritoneal macrophages in vivo by sterile silica particles also induced diminished contractile responses to norepinephrine by subsequently isolated aortas. Incubation of rat aortas with human monocyte-derived interleukin 1 or recombinant human tumor necrosis factor resulted in diminished aortic contraction and sensitivity to norepinephrine, and gel filtration of medium conditioned by endotoxin-stimulated macrophages yielded suppressive activity at a molecular weight equivalent to interleukin 1 and tumor necrosis factor. The data suggest that mononuclear phagocytes may contribute to altered vascular function in sepsis via the release and vascular modulatory effects of interleukin 1 and tumor necrosis factor.

Key words: rat, aorta, sepsis, tumor necrosis factor, vascular contraction, (copy), (5)

### INTRODUCTION

Septic patients often display hyperdynamic cardiovascular activity that is characterized by an elevated cardiac output and a low systemic vascular resistance [1]. Reduced vascular resistance occurs despite elevated circulating catecholamine levels and is not corrected by administration of exogenous catecholamines [2,3]. Subnormal arterial blood pressure responses to the pressor actions of norepinephrine (NE) and angiotensin II have also been described in rats with peritoneal sepsis [4]. The diminished response to pressor agents persists when aortas from endotoxin-treated or septic rats are removed and tested in

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in vitro; aortas from endotoxin-treated rats are hyporesponsive to both NE and the prostaglandin endoperoxide analog U-46619 [5], and aortas from septic rats are also hyporesponsive to NE [6]. Effects on vascular contractility by treatment, in vivo, with endotoxin may be via a mediator(s) rather than by a direct effect on vascular tissue because exposure of isolated vascular tissue to endotoxin at concentrations greater than those measured in experimental bacteremia or sepsis [7,8] causes no change in contractile responses to NE or epinephrine [9,10].

Macrophages release numerous inflammatory mediators upon exposure to bacteria or bacterial products. Two mediators, interleukin 1 (IL 1) and tumor necrosis factor (TNF, or cachectin), have multiple influences on vascular endothelial and smooth muscle cell function. IL 1 induces expression of endothelial cell surface proteins which may contribute to enhancement of tissue factor procoagulant activity [11], plasminogen activator inhibitor [12], and endothelial cell adhesiveness for lymphocytes [13]. IL 1 also enhances biosynthesis of vasoactive prostanoids by endothelial and smooth muscle cells [14,15]. IL 1 itself is synthesized and released by endothelial and smooth muscle cells in response to endotoxin or TNF [16,17]. TNF has effects on endothelial cells similar to those of IL 1 [see ref. 18 for review]; TNF also causes distinct changes in the morphology of cultured endothelial cells and inhibits proliferation of cultured endothelial and aortic smooth muscle cells [19]. The present study was undertaken to investigate whether macrophage products, in vitro, induce diminished aortic tissue function similar to that observed in sepsis.

## MATERIALS AND METHODS

### Cell Culture

Male Sprague-Dawley rats were injected intraperitoneally with 25 ml of 3% thioglycollate broth, and peritoneal macrophages were harvested 4 days later. After attachment to culture plates and washing, adherent cells ( $2.7 \times 10^6$  cells/ml) were cultured for 24 hr in Dulbecco's Minimum Essential Medium (DMEM) with 1.8% heat-inactivated fetal calf serum and 0.1  $\mu$ g/ml endotoxin (Difco, serotype 026:B6). Other macrophages received DMEM without endotoxin, and DMEM with and without endotoxin was placed in empty wells for control media. The cell viability and proportion of adherent cells that were macrophages were both tested (>95%) by trypan blue and nonspecific esterase staining.

### Rat Aortic Ring Bioassay

Rats were killed by decapitation; the thoracic aortas were rapidly excised, the adventitia was removed, and each aorta was sectioned into 4 rings (3.5 mm in length). Aortic rings were incubated by placing a ring and 1.5 ml of medium in dialysis tubing (Spectrum, 3.5-kd cutoff) and dialyzing against Krebs' Ringer bicarbonate (KRB) buffer at 37°C for 6 hr. The millimolar composition of KRB (pH 7.4 while continually bubbled with 95% O<sub>2</sub> - 5% CO<sub>2</sub>) was 118 NaCl, 4.7 KCl, 1.3 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub> · 7H<sub>2</sub>O, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 11.7 glucose. After incubation, rings were mounted between stainless-steel hooks, with one fixed in a jacketed organ bath (10 ml) and the other attached to an isometric force transducer (Kulite Semiconductor BG-10). Contractile responses were recorded on a Gould Brush 2400 chart recorder. Rings were kept at 37°C in KRB and continually gassed. A resting tension of 2.5g was applied, and the rings were allowed to equilibrate for 30-40 min. Rings were then treated with (1) norepinephrine (NE, 10<sup>-7</sup> M) with application of acetylcholine (10<sup>-6</sup> M) during maximal contraction to confirm that the endothelium was present [20], (2) an additional conditioning dose of NE (10<sup>-7</sup> M), and (3) a stepwise cu-



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mulative addition of NE ( $10^{-10}$ – $3 \times 10^{-5}$  M). The rings were flushed between each treatment until a stable baseline tension was attained. Rings were removed after completion of the protocol, blotted on tissue paper, and weighed.

#### Stimulation of Macrophages In Vivo

Silica (0.05 g of 0.014- $\mu$ m particles plus 0.22 g of 1–5- $\mu$ m particles) was suspended in 10 ml Hank's balanced salt solution (HBSS) and autoclaved. Rats were injected intraperitoneally with 10 ml of this suspension or HBSS (control) and killed 24–48 hr postinjection. Peritoneal fluid and blood were collected at death and cultured on blood-agar plates. Thoracic aortas were removed and aortic ring contraction to NE was measured as detailed above.

#### Chromatography of Macrophage-Conditioned Medium

Proteins in medium conditioned by endotoxin-stimulated macrophages were absorbed onto hydroxyapatite columns (1  $\times$  4 cm preequilibrated with 0.001 M NaCl, unbuffered) and eluted with a stepwise sequence of 0.005 M MgCl<sub>2</sub>, 1.0 M MgCl<sub>2</sub>, and 0.3 M sodium phosphate buffer [21]. Fractions that suppressed aortic ring contraction (1.0 M MgCl<sub>2</sub> and 0.3 M PO<sub>4</sub>, data not shown) were combined, dialyzed against deionized H<sub>2</sub>O, and dried. The residue was dissolved in KRB, placed on a calibrated Sephacryl S-200 column (1.5  $\times$  90 cm), and eluted with KRB. Fractions (1.2 ml) were collected and frozen until bioassayed for aortic suppressive activity as detailed above.

#### Incubation of Aortic Rings With IL 1 and TNF

Aortic rings from normal rats were incubated with purified, human monocyte-derived IL 1 and with recombinant human TNF $\alpha$  for 5 hr and contractile responses to NE were determined as detailed above. The incubation media contained 5% heat-inactivated fetal calf serum in KRB and 100 U/ml IL 1 or 10,000 U/ml TNF. One unit of IL 1 is defined as the amount required to double the proliferative response of thymocytes stimulated with 1  $\mu$ g/ml phytohemagglutinin alone. One unit of TNF is defined as the amount required to reduce survival of L-929 cells by 50% in the presence of 1  $\mu$ g/ml actinomycin-D.

#### IL 1 Assay

IL 1 activity in medium conditioned by endotoxin-stimulated macrophages and in fractions of conditioned medium was measured by T.L. Gerrard of the Food and Drug Administration, Bethesda, MD, using a phytohemagglutinin-stimulated murine thymocyte assay. Units of activity are defined as detailed above.

#### Analysis of Data

Contractile performance by aortic rings was characterized by integrating the tension developed by rings in response to sequential doses of NE (i.e., mg tension/mg tissue vs.  $\ln$  [NE] M). EC<sub>50</sub> values (concentration of NE causing a half-maximal contraction) were calculated by linear regression after logit-log transformation of dose responses. Tests for differences between EC<sub>50</sub> values were based on mean log values [22]. Effects of treatments on aortic contraction were tested in a pairwise fashion, with 2 rings per rat incubated in medium conditioned by macrophages or media with IL 1 or TNF added, and 2 rings incubated in control media. Responses of duplicate rings were averaged prior to statistical analysis. T-tests for paired comparisons were used to test for significant differences in aortic contraction and sensitivity to NE. The influence of silica treatment on aortic contraction

was tested by independent t-tests. Bonferroni corrections were applied to any multiple comparisons [23]. A probability of .05 or less was accepted as significant. Data are presented as mean  $\pm$  SE.

### Materials

Human ultrapure monocyte-derived IL 1 was obtained from Genzyme, Boston, MA, and is stated by the supplier to be pyrogen-free and to contain less than 1 U/ml interferon and less than 1% interleukin 2. Recombinant human tumor necrosis factor (alpha) was also obtained from Genzyme. DMEM and HBSS were obtained from Gibco Laboratories, Grand Island, NY. The remaining materials were purchased from Sigma, St. Louis, MO.

## RESULTS

### Effect of Medium Conditioned by Macrophages on Aortic Ring Function

Incubation of isolated aortic rings in medium conditioned by nonstimulated macrophages or containing only endotoxin had no effect on subsequently measured aortic ring contractile responses, while medium with the 2 treatments combined (macrophages stimulated with 0.1  $\mu$ g/ml endotoxin) caused marked decreases in aortic ring sensitivity (reflected by an increase in  $EC_{50}$  values) and contraction to NE (Fig. 1A, Table I). Comparison of mean  $EC_{50}$  values or mean integrated dose responses for rings incubated in medium conditioned by endotoxin-stimulated macrophages vs. means for rings incubated in control medium yielded significant differences in each case ( $P < .001$ , independent t-test). IL 1 activity in three random samples of medium conditioned by endotoxin-stimulated macrophages ranged from 63 to 441 U/ml.

### Effect of Intraperitoneal Silica on Aortic Ring Function

Aortas removed from rats treated with silica displayed diminished contractile performance similar to that of rings incubated in medium conditioned by endotoxin-stimulated macrophages. The reduced response by aortic rings from silica-treated rats also included decreases in sensitivity and force of contraction to NE (Fig. 1B, Table II). All peritoneal exudate and blood cultures were negative for the presence of bacteria.

TABLE I. Paired Comparisons of Mean Integrated Dose and  $EC_{50}$  Responses to Norepinephrine by Incubated Rat Aortic Rings

Medium (control)	Medium + endotoxin	Medium + macrophage	Medium + macrophage + endotoxin	N pairs	P
5,372 $\pm$ 374 <sup>a</sup>	4,856 $\pm$ 322			6	NS
-7.41 $\pm$ .12 <sup>b</sup>	-7.23 $\pm$ .06				NS
4,716 $\pm$ 410		4,406 $\pm$ 628		6	NS
-7.66 $\pm$ .12		-7.22 $\pm$ .21			NS
	3,687 $\pm$ 195		1,836 $\pm$ 387	6	<.01
	-7.34 $\pm$ .08		-6.52 $\pm$ .17		<.01
		4,298 $\pm$ 413	2,100 $\pm$ 221	7	<.001
		-7.05 $\pm$ .14	-6.46 $\pm$ .06		<.01

<sup>a</sup>Mean integrated dose response (mg tension/mg tissue vs. ln [NE] M).

<sup>b</sup>Mean log  $EC_{50}$  (M).

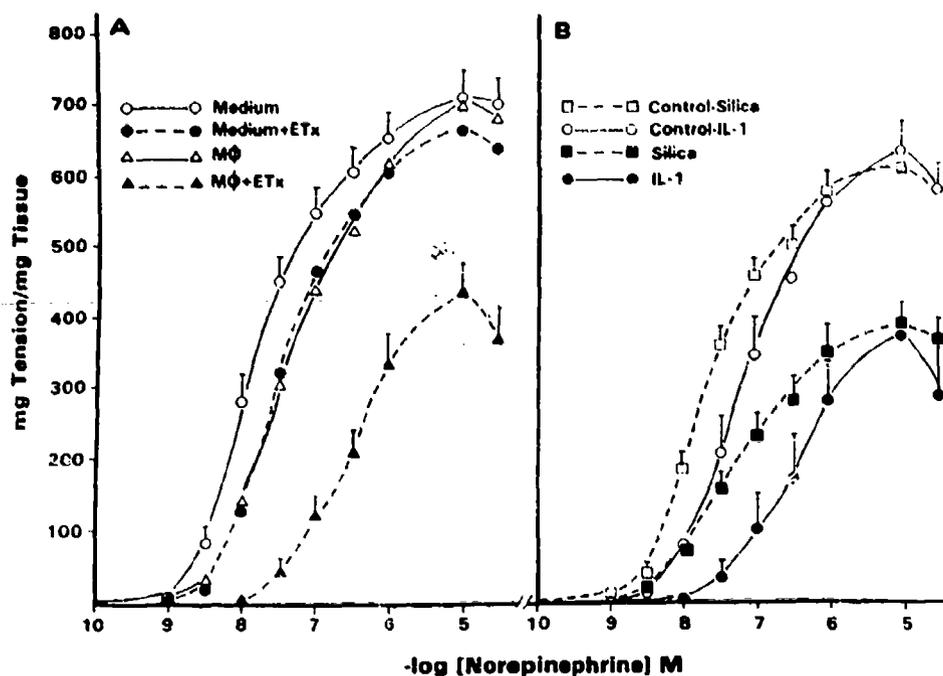


Fig. 1. **A:** Contractile responses to NE by aortic rings after incubation in medium conditioned by macrophages. Aortic rings from normal rats were incubated in control medium or medium containing 0.1  $\mu\text{g/ml}$  of endotoxin (ETx),  $N = 12$ ; aortic rings were also incubated in medium conditioned by nonstimulated macrophages (m $\Phi$ ) and in medium conditioned by endotoxin (0.1  $\mu\text{g/ml}$ )-stimulated macrophages (m $\Phi$  + ETx),  $N = 13$ . Each point plotted is pooled from paired comparisons and represents the mean  $\pm$  SE. Some error bars are omitted for clarity but are similar to those shown. **B:** Effects of treatment with sterile silica particles *in vivo* and of incubation with IL 1 on contractile responses to NE by isolated aortic rings. Contractile responses by aortic rings isolated from normal rats injected with silica intraperitoneally (silica) were compared to contractile responses by rings isolated from control rats injected with vehicle (control-silica),  $N = 7$ . Effects of IL 1 treatment were examined by preparing 4 rings per normal rat; 2 rings were incubated with 100 U/ml IL 1 (IL 1), and 2 rings were incubated in control buffer (control-IL 1). The duplicate responses were averaged, and the mean responses for 8 rats were plotted.

### Effect of IL 1 and TNF on Aortic Ring Function

Exposure of aortas to purified human monocyte-derived IL 1 at a concentration (100 U/ml) approximating that measured in medium conditioned by endotoxin-stimulated macrophages resulted in significant decreases in aortic performance. The diminished responses included significantly increased  $EC_{50}$  values and decreased contraction to NE (Fig. 1B, Table II). Attempts to induce aortic ring contractile defects by incubation with less IL 1 (1–10 U/ml) produced small and generally nonsignificant decreases in ring contractile function (data not shown).

Incubation of aortic rings with TNF (10,000 U/ml) resulted in significantly increased  $EC_{50}$  values and a small but significant decrease in integrated dose responses (Table II). The significant decrease in integrated dose responses was primarily attributable to diminished sensitivity to NE; contractions by aortic rings treated with TNF and by control rings at maximal stimulatory doses of NE were not significantly different in magnitude. (At  $10^{-6}$

TABLE II. Mean Integrated Dose and EC<sub>50</sub> Responses to Norepinephrine by Aortic Rings From Silica-Treated Rats and by Rings Incubated in IL 1 and TNF $\alpha$ 

Control	Treated	N	P
Silica			
4184 $\pm$ 179 <sup>a</sup>	2346 $\pm$ 222	7 <sup>c</sup>	<.001
-7.49 $\pm$ .04 <sup>b</sup>	-7.17 $\pm$ .08		<.005
IL 1	(100 U/ml)		
3654 $\pm$ 327	1659 $\pm$ 375	8 <sup>d</sup>	<.001
-7.02 $\pm$ .12	-6.48 $\pm$ .13		<.005
TNF	(10,000 U/ml)		
5756 $\pm$ 301	5070 $\pm$ 225	8 <sup>e</sup>	<.05
-7.52 $\pm$ .07	-7.30 $\pm$ .08		<.01

<sup>a</sup>Mean integrated dose response (mg tension/mg tissue vs.  $\ln$  [NE] M).

<sup>b</sup>Mean log EC<sub>50</sub> (M).

<sup>c</sup>Per group.

<sup>d</sup>Pairs.

<sup>e</sup>Pairs.

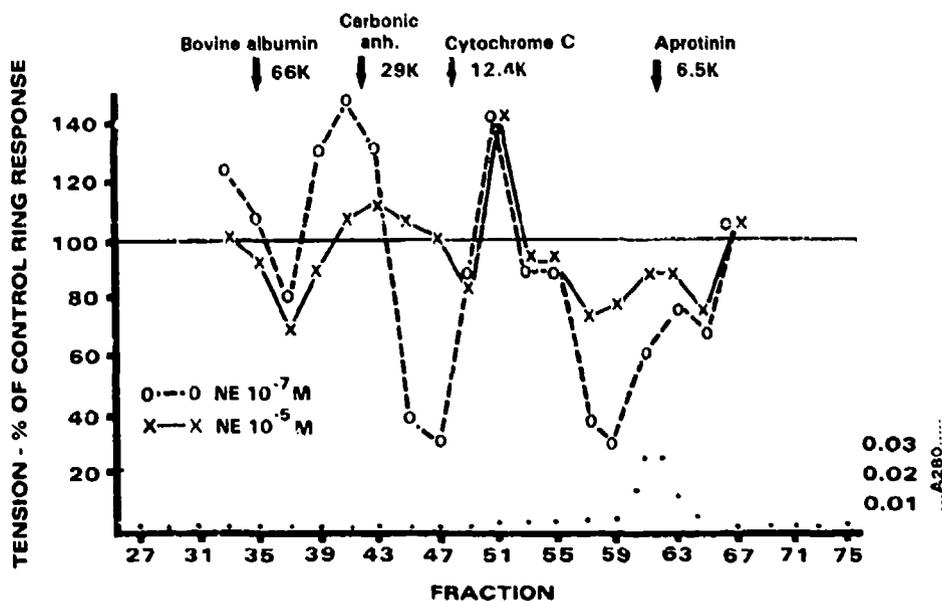


Fig. 2. Distribution, by molecular weight, of vascular suppressive activity in medium conditioned by endotoxin-stimulated macrophages. Medium was fractionated on a Sephacryl S-200 column and tested for suppressive activity by incubating 3 of 4 aortic rings per rat in successive fractions and expressing contractile responses to NE ( $10^{-7}$  M and  $10^{-5}$  M) as a percentage of the fourth, control ring. The figure is representative of the pattern of suppressive activity in 3 replicates of the experiment.

M NE TNF-treated rings exhibited  $715 \pm 36$  mg tension/mg tissue vs. control ring performance of  $766 \pm 40$ ; at  $10^{-5}$  M NE contractile performances were  $797 \pm 34$  vs.  $823 \pm 42$ .)

### Molecular Weight of Suppressive Macrophage Product(s)

Gel filtration of medium conditioned by endotoxin-stimulated macrophages yielded fractions that suppressed aortic ring contraction at  $10^{-7}$  M NE, while having little effect at the maximal ( $10^{-5}$  M) dose (Fig. 2). This suggests that changes in sensitivity to NE are apparent before changes in maximal contractile performance. The suppressive fractions eluted in molecular weight ranges of approximately 16 and 7 kd. Fraction 47 (16 kd) contained 77 U/ml IL 1 activity, whereas fractions 63 and 67, serving as controls, showed no activity.

### DISCUSSION

Aortic rings from normal rats manifest impaired contractile performance after incubation in medium conditioned by endotoxin-stimulated macrophages, after incubation in medium containing IL 1, or after peritoneal macrophages are stimulated *in vivo* by silica. The sensitivity to NE ( $EC_{50}$ ) and maximum tension generated after stimulation by NE are both adversely affected. IL 1 could be a mediator for the suppressive effects of activated macrophages on vascular function because (1) purified monocyte-derived IL 1, at a concentration equivalent to that measured in medium conditioned by endotoxin-stimulated peritoneal macrophages, induces defects in contractile performance similar to those caused by the conditioned medium (Fig. 1), and (2) gel filtration of medium conditioned by endotoxin-stimulated macrophages yields fractions, at the molecular weight of IL 1 (16 kd), that suppress aortic ring contraction (Fig. 2). IL 1 has been demonstrated to fragment into an active peptide of 4.2 kd [24]; it is possible that the second peak of suppressive activity in Figure 2 (at approximately 7 kd) is the same fragment within the error of resolution of molecular weight on this gel.

Tumor necrosis factor is another monokine which shares molecular weight range and many biological activities with IL 1; in addition, infusion of recombinant TNF (cachectin) into rats causes shock and tissue injury [25]. These observations suggest that TNF could be another agent released by activated macrophages that mediates suppression of vascular contraction. In the current experiments, aortic rings were incubated with a concentration of recombinant TNF 10–100-fold greater than concentrations that caused significant alterations in endothelial cell morphology and smooth muscle cell growth in culture [19]. The concentration of purified IL 1 in which rings were incubated was also approximately 10–100-fold greater than concentrations that caused significant changes in endothelial and smooth muscle cell function in culture [12,15]. Because aortic rings incubated with TNF exhibited decrements in contractile performance much smaller than those measured in rings incubated with IL 1 (Table II), TNF may not be as effective as IL 1 in suppressing vascular contractile function.

Microvascular responses, *in vivo*, to NE or epinephrine (EPI) after endotoxin treatment have been investigated by others and generally exhibit an initial, prolonged period of intense vasoconstriction followed by hyposensitivity to catecholamines and vasodilation. In rabbits, injection of a mixture of endotoxin and EPI intradermally resulted in intense vasoconstriction followed 4–6 hr later by formation of hemorrhagic lesions with greatly dilated superficial skin vessels at the site of injection [26]. Intravenous doses of endotoxin greater than 250  $\mu$ g, in rats, caused an initial enhancement of mesoappendix microvascular response to EPI, followed by a hyporeactive state 60 or more min after injection of the endotoxin [27]. These observations, in view of the current data, suggest that the hypores-

ponsive, vasodilatory state which occurs in the microvasculature after stimulation by endotoxin could be mediated by IL 1 and perhaps TNF and that the monokines may have a role in limiting excessive vasoconstriction.

Peritoneal inflammation caused by treatment of rats, *in vivo*, with sterile silica particles results in diminished aortic contractile performance very similar to that seen in rats made septic by cecal ligation and puncture [6]. Because all peritoneal and blood cultures from silica-treated rats were negative, this implies that at least some of the defective vascular responses observed in sepsis may be caused by mediators elicited from mononuclear phagocytes by endotoxin or other bacterial components rather than by direct toxic effects of endotoxin on the vasculature. Macrophage products may contribute to a generalized auto-destructive inflammation in a multiple organ failure syndrome in which no nidus of infection or positive blood culture is observed, as has been described by Goris et al. [28]. Significant decreases in effective hepatic and renal tissue perfusion have been described in septic rats [29], and peripheral vascular failure, which may result in impaired effective organ perfusion, has been observed to be closely associated with mortality in human septic shock [3]. We suggest that mononuclear phagocytes may play an important role in altered vascular function in sepsis.

## CONCLUSIONS

Rat peritoneal macrophages, when stimulated *in vitro* by endotoxin, release an agent(s) that suppresses the contractile performance of isolated rat aortic tissue incubated in the macrophage-conditioned medium. The suppressive agent has a molecular weight approximating that of interleukin 1 and tumor necrosis factor, and incubation of isolated rat aortas in purified interleukin 1 mimics the suppressive effects of macrophage-conditioned medium on aortic contraction. Recombinant tumor necrosis factor also caused a small, but significant, suppression of vascular contraction. Stimulation of rat peritoneal macrophages, *in vitro*, with sterile silica particles results in defects in aortic contraction similar to those observed in sepsis and similar to those observed after incubation of aortas in macrophage-conditioned medium. The data suggest that mononuclear phagocytes may contribute to altered vascular function in sepsis and that the effect could be mediated via release of the inflammatory monokine interleukin 1, and to a lesser extent, via the release of tumor necrosis factor.

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The experiments described in this paper were performed in adherence to the NIH guidelines for the use of experimental animals.