Tumor Necrosis Factor α and Interleukin 1β Are Responsible for In Vitro Myocardial Cell Depression Induced by Human Septic Shock Serum

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Summary

Previous studies have demonstrated the presence of myocardial depression in clinical and experimental septic shock. This depression is associated with the presence of a circulating myocardial depressant substance with physical characteristics consistent with cytokines. The present study utilized an in vitro myocardial cell assay to examine the role of various human recombinant cytokines, including tumor necrosis factor (TNF)α and interleukin (IL)1β, in depression of cardiac myocyte contractile function induced by serum from humans with septic shock. The extent and velocity of electrically paced rat cardiac myocytes in tissue culture was quantified by a closed loop video tracking system. Individually, TNF-α and IL-1β each caused significant concentration-dependent depression of maximum extent and peak velocity of myocyte shortening in vitro. In combination, TNF-α and IL-1β induced depression of myocardial cell contractility at substantially lower concentrations consistent with a synergistic effect. Using immunoabsorption, removal of both TNF-α and IL-1β (but not either alone) from the serum of five patients with acute septic shock and marked reversible myocardial depression resulted in elimination of serum myocardial depressant activity. IL-2, -4, -6, -8, -10, and interferon γ failed to cause significant cardiac myocyte depression over a wide range of concentrations. These data demonstrate that TNF-α and IL-1β cause depression of myocardial cell contraction in vitro and suggest that these two cytokines act synergistically to cause sepsis-associated myocardial depression in humans.

Despite therapy with appropriate antibiotics and intensive supportive care, septic shock remains a serious disorder with significant morbidity and mortality. The typical human cardiovascular response to septic shock is characterized by hypotension, decreased systemic vascular resistance, and elevated cardiac index. In addition, myocardial depression manifested by reversible biventricular dilation and reduction of ejection fraction has been shown to be common in human septic shock (1–3).

A previously described in vitro model of myocardial cell performance utilizes spontaneously beating rat cardiac myocytes in culture (4–6). This system allows assessment of myocardial cell performance (i.e., contraction) independent of changes in preload, afterload, and heart rate. In this system, serum from patients with acute septic shock produced in vitro depression of cardiac myocyte contractile function (decreased maximum extent and peak velocity of shortening) that correlated quantitatively and temporally with the depression of ventricular ejection fraction seen in the same patients in vivo. This suggested the presence of a potentially pathophysiologically relevant myocardial depressant substance or substances (5, 6). The identity or identities of

the specific molecule(s) responsible for this myocardial depression have not been determined.

A number of exogenous and endogenous mediators have been implicated in the pathogenesis of septic shock including endotoxin, complement components (particularly C5a), histamine, kinins, prostaglandins, leukotrienes, endorphins and cytokines (7, 8). Potentially, any of these could have myocardial depressant effects. However, recent studies have suggested that the myocardial depressant substance (or substances) of septic shock possesses physical properties and a molecular mass (10–30 kD) consistent with cytokines (4, 9). Cytokines that may potentially contribute to septic myocardial depression include TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, and IFN-γ. Each is known to be involved in inflammatory states, and serum levels of most are elevated in endotoxic and/or septic shock.

The hypothesis of this study was that one or more of these factors may be a myocardial depressant and may account for some or all of the cardiac depression associated with human septic shock. This study was designed to evaluate whether these factors could act as direct myocardial depressant substances in an in vitro model of myocardial

function and whether elimination of these substances from human septic serum would attenuate its in vitro myocardial depressant activity.

Materials and Methods

The methods employed were a modification of those previously described (4–6). The basic myocyte assay is designed to measure the depression or enhancement of contractility of beating cardiac myocytes in cell culture caused by agents that are introduced into the growth medium. Spontaneously beating newborn rat myocardial cells were established using a modification of the technique described by Harary and Farley (10). Using sterile technique, hearts from 2-d-old Lewis rat pups were removed, pooled, and minced into small blocks. The cells were disaggregated with 0.15% bovine pancreatic trypsin (Sigma Chemical Co., St. Louis, MO) in a modified HBSS (potassium, calcium, and magnesium free; GIBCO BRL, Gaithersburg, MD). Suspensions were centrifuged at 1,500 rpm (500 g) for 15 min and plated into 35 × 10 mm petri dishes at a density of 300,000 cells/ml. Plating media (standard control media) consisted of 25% Hepes-buffered Medium 199 and 10% heat-inactivated newborn calf serum (both from GIBCO BRL) diluted in a balanced salt solution and supplemented with glutamine, penicillin, and streptomycin (all from Sigma Chemical Co.). Cells were incubated at 37°C in 5% CO_2 and growth media was changed every 48 h. After plating, cells became confluent and began spontaneously beating within 4 d. Latex microbeads were introduced into the culture after 4 d and affixed themselves to cell membranes. Beating cells were used for the assay between 5 and 10 d after plating.

All pipettes, plates, and other equipment used for preparation, culture, or testing of cardiac myocytes were endotoxin tested and disposable. All liquid media contained <1 pg/ml endotoxin with the exception of newborn calf serum which contained 0.48 ng/ml endotoxin. All recombinant cytokines contained <50 pg endotoxin per μg cytokine. Culture media, cytokine solutions, and other test solutions were tested for endotoxin content using a quantitative, chromogenic Limulus amebocyte lysate assay (Whittaker M.A. Bioproducts, Walkersville, MD).

The extent and velocity of myocardial cell shortening during cell contraction was assayed by a modification of previously described techniques (4–6). The petri dish containing beating myocardial cells was fastened to the heated (37°C) stage of an inverted-optics, phase-contrast microscope that had an attached video camera. A television monitor displayed the image of the target cells. A custom-built electronic tracking system was used to quantitate the movement of a latex bead selected from the many beads attached to the membranes of beating myocytes. The typical maximum initial extent of rhythmic displacement of the bead was between 3 and 10 μm, depending on the length of the myocyte to which it was adherent. The tracking system produced analog signals that were relayed to an intervening electronic instrument (which derived contraction velocity from rate of change of bead displacement) and a two-channel strip chart recorder that printed an analog recording of extent and velocity of bead displacement. To ensure a fixed contraction frequency, a custom-built alternating current electrical pulse generator was used to pace myocytes (12 V, maximum 40 mA, 0.7–7-ms pulse duration). The minimum current and pulse duration required to effectively pace cardiac myocytes was used for each experiment.

Each individual assay was performed as follows. Plates were removed from the incubator and fresh growth media applied. Plates were mounted on the microscope stage and myocytes were paced to 60 contractions per min. An appropriate bead was located and the extent and velocity of myocyte shortening measured for 5 min (baseline contractility). If extent of cell contraction was stable (maximum 2.5% variation over 5 min), a test or control solution was added. Subsequently, measurements of maximum extent and peak velocity of myocyte shortening and velocity were obtained every 5 min for 30 min. By comparing the maximum extent and peak velocity of shortening at each 5-min interval to the baseline value, changes were referenced to initial contractility.

Five groups of experiments were performed. (a) Human recombinant TNF-α and IL-1β were initially assayed (each along with controls) in order to evaluate whether either cytokine could individually produce concentration-dependent depression of cardiac myocyte shortening. The concentrations tested were: for TNF-α (Sigma Chemical Co.), 0 (control), 0.0125, 0.05, 0.2, 0.8, 3.2, 12.5, 25, 50, and 100 ng/ml (n = 8 each concentration); for IL-1β (Endogen, Inc., Boston, MA), 0 (control), 2, 8, 32, 125, 500, and 1,000 ng/ml (n = 8 each concentration). Test media consisted of standard culture media (as previously described) with the addition of the specified cytokine concentrations.

Reversibility of depression induced by TNF-α (50 ng/ml) and IL-1β (500 ng/ml) was individually determined by repeated washout with standard control media (n = 8 each group). 5 min after washing, maximum extent and peak velocity of shortening was determined and compared to the level of depression of similarly washed cardiac myocytes exposed only to control media.

In addition, cardiac myocytes were separated from nonmyocytes by centrifugation through a Percoll step gradient (Sigma Chemical, Co.) resulting in a >95–98% pure cardiac myocyte culture (viable cardiac myocytes/all viable cells). Myocyte purity was determined 48 h after plating by a combination of trypan blue exclusion to differentiate viable and nonviable cells followed by differentiation of myocytes from nonmyocytes by cell contraction in response to an electrical pulse. TNF-α (50 ng/ml) and IL-1β (500 ng/ml) were individually tested for effects on cardiac myocyte maximum extent and peak velocity of shortening using purified cardiac myocyte cultures (n = 8 each group).

(b) In a second group of experiments, combinations of TNF-α and IL-1β were evaluated at lower concentrations to evaluate the possible additive or synergistic effects of these cytokines. In addition to a control solution without either cytokine, paired test concentrations of TNF-α/IL-1β (ng/ml) included (a) 0.003: 0.125; (b) 0.0125:0.5; (c) 0.05:2; (d) 0.2:8 (n = 8 each group). Test media consisted of standard culture media as described with the addition of the specified cytokine concentrations.

(c) To determine the specific roles of TNF-α and IL-1β in cardiac myocyte depression induced by human septic serum, these cytokines were removed from serum by immunoabsorption. Serum from five human survivors with acute septic shock associated with marked, reversible depression of left ventricular ejection fraction (mean ± standard deviation left ventricular ejection fraction 26 ± 7% during septic shock, 55 ± 7% at recovery; mean absolute decrease 28 ± 10%) was studied. Each serum sample had also been previously documented to contain myocardial depressant activity. Serum was treated by exposing it overnight (16 h) at 4°C to agarose beads bonded to specific rabbit-derived anti-human cytokine polyclonal antibodies (Endogen, Inc.). Serum was treated with antibodies against recombinant human TNF-α, recombinant human IL-1β, both, or neither (native antibodies derived from rabbits before immunization with recombinant human TNF-α or IL-1β). Each serum sample had TNF-α and IL-1β concentrations determined by ELISA (TNF-α by T Cell Sci-

ences, Inc., Boston, MA; IL-1β by Citron Biotechnologies, Pine Brook, NJ) (11). The lower limit of detection in these assays was 20 pg/ml.

Insufficient serum remained after performing the contractility assay to test cytokine concentrations after treatment. However, effectiveness of the cytokine immunoabsorption process was confirmed by adding TNF-α (50 ng/ml) or IL-1β (500 ng/ml) to standard media (with 10% serum), similarly treating that serum to remove TNF-α or IL-1β using specific polyclonal antibodies, measuring remaining TNF-α or IL-1β by ELISA, and confirming elimination of depressant activity in the cardiac myocyte contractility assay.

Test solutions were created by mixing standard plating media without serum with the resultant (antibody-treated) human septic serum at a concentration of 10%. Four groups (and a control group) were tested to determine serum-induced changes in maximum extent and peak velocity of cardiac myocyte shortening. The control solution consisted of 10% neonatal bovine calf serum (standard plating media) and was common to all experiments (nonseptic control). The test solutions consisted of 10% native (septic control), anti-TNF-α, anti-IL-1β, or anti-TNF-α and anti-IL-1β treated serum ($n = 8$ each group). Additional control solutions ($n = 8$ each group) consisting of 10% newborn calf serum treated with antibodies against recombinant human TNF-α, recombinant human IL-1β, both, or neither (native antibodies derived from rabbits before immunization with recombinant human TNF-α or IL-1β).

(d) Six other human recombinant cytokines were also initially assayed (each along with controls) in order to evaluate whether any could individually produce concentration-dependent depression of cardiac myocyte shortening. The concentrations tested were: IL-2, 0 (control), 1.6, 8, 40, 200, and 1,000 ng/ml ($n = 8$ each concentration); IL-4, 0 (control), 1.6, 8, 40, 200, and 1,000 ng/ml ($n = 6$ each concentration); IL-6, 0 (control), 0.5, 5, 20, 80, 320, 1,250, and 3,000 ng/ml ($n = 8$ each concentration); IL-8, 0 (control), 1.6, 8, 40, 200, and 1,000 ng/ml ($n = 7$ each concentration); IL-10, 0 (control), 1.6, 8, 40, 200, and 1,000 ng/ml ($n = 6$ each concentration); and IFN-γ, 0 (control), 4, 16, 64, 250, and 1,000 ng/ml ($n = 7$ each concentration; all from Endogen, Inc.).

Where known, cytokine concentrations were selected based on a range from one order of magnitude below to two orders of magnitude above the serum cytokine concentrations documented during human septic shock. Test media consisted of standard culture media including 10% neonatal calf serum with the addition of the specified cytokine concentrations.

(e) To study the potential role of endotoxin in cytokine and septic shock serum-mediated cardiac myocyte depression, selected test solutions were depleted of endotoxin by overnight co-incubation with silica bead-bonded endotoxin neutralizing protein derived from horseshoe crab amebocytes (Associates of Cape Cod, Inc., Falmouth, MA) and then assayed for myocardial depressant activity as previously described. Test media included solutions containing TNF-α (50 ng/ml), IL-1β (500 ng/ml), TNF-α (0.05 ng/ml) with IL-1β (2 ng/ml), or 10% serum from one of the five patients with acute septic shock as well as controls containing 10% neonatal calf serum without cytokines or septic serum ($n = 9$ each group).

Statistical Analysis. For cytokine testing, data at all concentrations and time points were pooled and analyzed by multivariate regression to determine whether a significant relationship existed between cytokine concentration or time and degree of cardiac myocyte depression.

Data for the change in maximum extent and peak velocity of

cardiac myocyte shortening (percent change from baseline) were plotted as a function of time for each control and test solution concentration. Linear regression analysis was employed to fit a line for the each resulting plot. For cytokines that were demonstrated to exert depressant activity, the slopes of these lines were compared to that for the control solution by a two-tailed Student's t test in order to determine at what concentration these slopes became significantly different (i.e., at what concentration significant depression of cardiac myocyte contractility occurred). In this manner, increased depressant activity was indicated by a more negative value for slope of the regression line. Similarly, if any factor caused significant depression, a two-tailed Student's t test was used to determine at what time point significant depression began.

Data for the comparison of the effects of TNF-α and IL-1β on purified and unpurified cardiac myocytes were similarly pooled for each group. Regression-derived slopes were determined and compared to slopes for control media via a two-tailed Student's t test. A Bonferonni adjustment for multiple comparisons was applied so that each comparison was considered significant only if $p \leq 0.025$.

Similarly, for analysis of antibody-treated septic serum and for endotoxin-removal experiments, the slope of the plot for the control media was compared to each treatment group by a two-tailed Student's t test. A Bonferonni adjustment for multiple comparisons was again made in both sets of experiments so that each comparison was considered significant only if $p \leq 0.0125$.

Results

Effects of TNF-α or IL-1β. Both TNF-α and IL-1β individually exhibited concentration-dependent depression of cardiac myocyte contractility.

TNF-α-induced depression of maximum extent of cardiac myocyte shortening began at a concentration of 0.8 ng/ml ($p < 0.05$) and progressed through concentrations of 3.2 ($p < 0.02$), 12.5 ($p < 0.01$), 25 ($p < 0.01$), 50 ($p < 0.001$), and 100 ng/ml ($p < 0.001$). Fig. 1 A shows cardiac myocyte depression as a function of time for the control media without TNF-α, 0.8 ng/ml TNF-α, and 100 ng/ml TNF-α. The slopes of the lines fit to the data at each concentration were plotted against the log of the concentrations (Fig. 1 D). A highly significant relationship ($p < 0.0001$) existed, suggesting that increased depression of maximum extent of myocyte shortening occurred with increasing concentrations of TNF-α. Parallel and similarly significant changes in peak velocity of myocyte shortening were also demonstrated.

Significant depression occurred by 10 min ($p < 0.02$) with progressively more depression to 30 min ($p < 0.001$) for both changes in maximum extent and peak velocity of cardiac myocyte shortening. Overall, TNF-α-induced cardiac myocyte depression was significantly time dependent ($p < 0.0001$).

IL-1β-induced depression of maximum extent of cardiac myocyte shortening began at a concentration of 32 ng/ml ($p < 0.05$) and progressed through concentrations of 128 ($p < 0.01$), 500 ($p < 0.01$), and 1,000 ng/ml ($p < 0.001$). Figure 1 B shows cardiac myocyte depression as a function of time for the control media without IL-1β, IL-1β 32 ng/ml, and 1,000 ng/ml IL-1β. The slopes of the lines fit to the
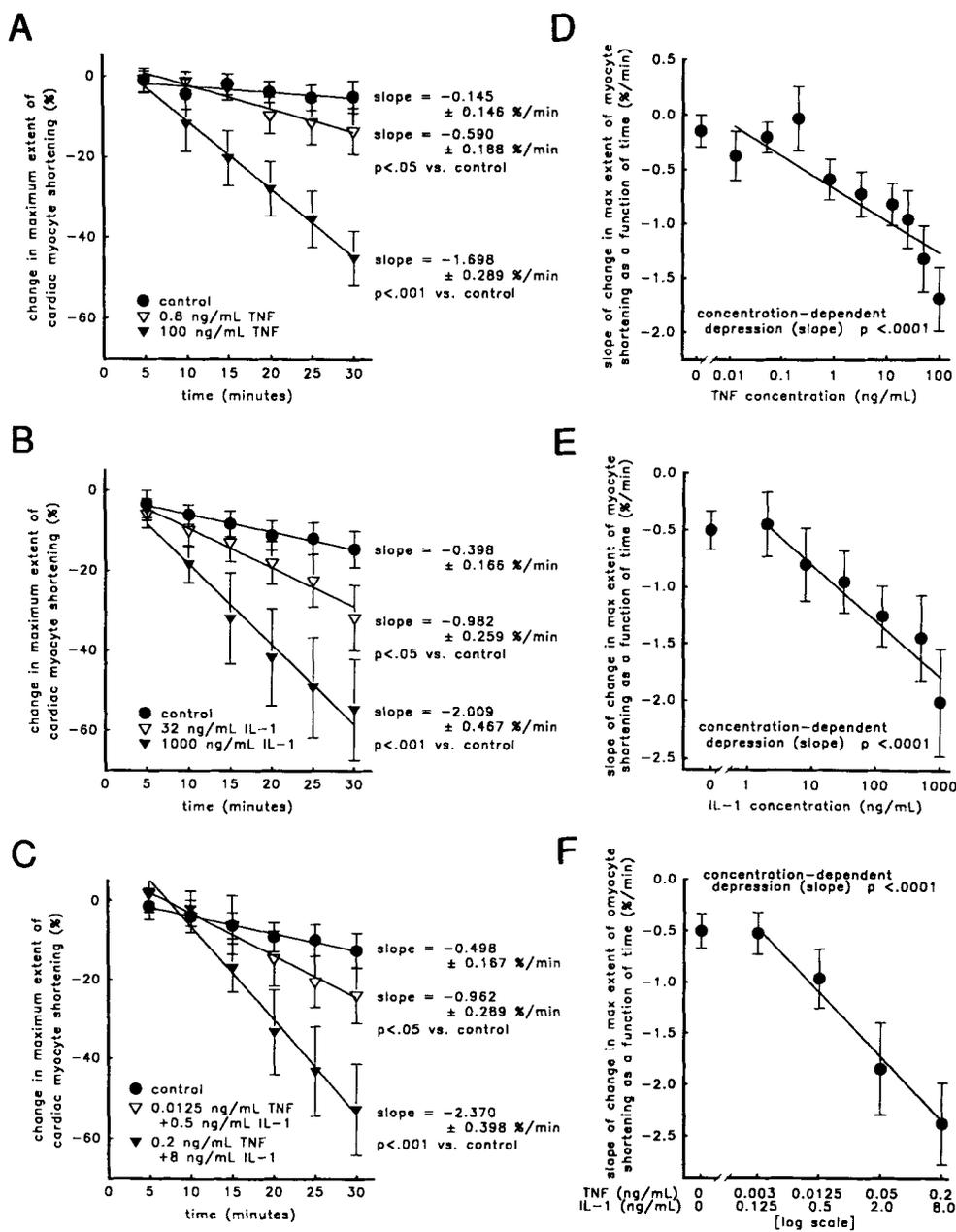
Figure 1. The change in maximum extent of myocardial cell shortening after exposure to control and the specified concentrations of TNF-α (*A*), IL-1β (*B*), or TNF-α + IL-1β (*C*). *p* value refers to the difference in slope between specified concentrations of cytokines and control media without cytokine. The slopes representing rate of decrease of maximum extent of myocardial cell shortening in response to different cytokine concentrations tested are shown in *D*, *E*, and *F*. In each case slopes become progressively more negative to the maximum cytokine concentrations tested. *p* value refers to difference from 0 slope. A significant *p* value indicates the existence of a concentration-dependent relationship. Error bars, SEM.

data at each concentration were plotted against the log of the concentrations (Fig. 1 *E*). As with TNF-α, a highly significant relationship (*p* <0.0001) existed, suggesting that increased depression of maximum extent of myocyte shortening occurred with increasing concentrations of IL-1β. Data for changes in peak velocity were similar. Also similar to TNF-α, significant depression of both maximum extent and peak velocity of cardiac myocyte shortening occurred by 10 min (*p* <0.02) and increased through 30 min (*p* <0.001). Depression was significantly time dependent (*p* <0.0001) in addition to being concentration dependent for both maximum extent and peak velocity of cardiac myocyte shortening.

50 ng/ml TNF-α and 500 ng/ml IL-1β each also produced significant depression of both maximum extent and

peak velocity of shortening (*p* <0.01 each) when applied to highly purified cardiac myocyte tissue cultures (>95–98% purity versus normal 75–80%).

Reversal of 50 ng/ml TNF-α or 500 ng/ml IL-1β induced depression of maximum extent (and peak velocity) of cardiac myocyte contractility could be produced by washing the cell monolayer with standard growth media without cytokine (Fig. 2). Contractile function of cardiac myocytes reverted to baseline within 5 min of washing the cells with cytokine-free media.

Trypan blue supravital staining failed to demonstrate loss of cell viability within the cardiac myocyte culture. No increase in supernatant lactate dehydrogenase was noted before and after exposure of cardiac myocytes to TNF-α or IL-1β.

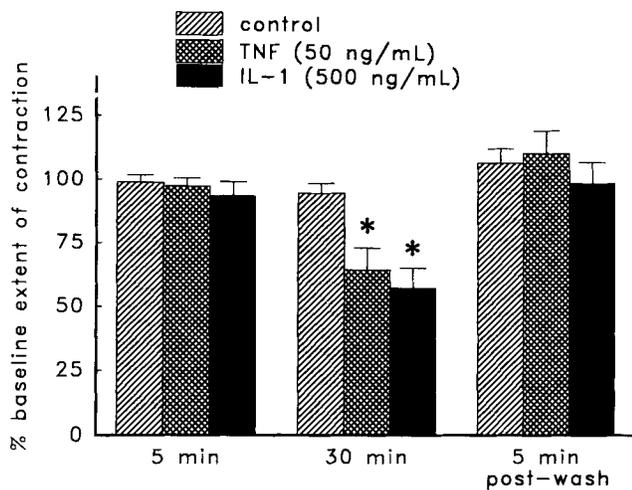*Effects of TNF-α and IL-1β in Combination.* Significant de-

Figure 2. Reversibility of cardiac myocyte depresssion induced by TNF-α or IL-1β. At 5 min after introduction of TNF-α or IL-1β, myocyte shortening was similar to control media-exposed cells. 30 min after exposure to TNF-α or IL-1β, cardiac myocyte shortening was significantly decreased compared to controls. 5 min after washing with control media, shortening of TNF-α– and IL-1β–exposed cardiac myocytes was again comparable to similarly washed controls. (*) $p < 0.01$ vs. control media cells at the same time point.

pression of maximum extent of cardiac myocyte shortening was present starting at a concentration of 0.0125 ng/ml TNF-α: 0.5 ng/ml IL-1β ($p < 0.05$) with increasing degrees of depression with increasing concentrations through to 0.2 ng/ml TNF-α: 8 ng/ml IL-1β ($p < 0.001$; Fig. 1 C). A highly significant relationship ($p < 0.0001$) between TNF-α/IL-1β concentration and depression of maximum extent of cardiac myocyte shortening existed (Fig. 1 F).

Changes in peak velocity of cardiac myocyte shortening were once again entirely parallel except that significant depression was noted beginning with the lowest concentration tested, 0.003 ng/ml TNF-α with 0.125 ng/ml IL-1β ($p < 0.05$), and progressed through higher concentrations to 0.2 ng/ml TNF-α and 8 ng/ml IL-1β ($p < 0.001$). A highly significant relationship between concentration of TNF-α/IL-1β and degree of depression of peak velocity of cardiac myocyte shortening also existed ($p < 0.0001$). As with TNF-α and IL-1β individually, depression of maximum extent and peak velocity of shortening was first apparent by 10 min ($p < 0.05$), progressed through 30 min ($p < 0.001$), and was, overall, time dependent ($p < 0.0001$) in addition to being concentration dependent.

Immunoabsorption of TNF-α and IL-1β from Human Septic Serum. Five samples of serum drawn from patients with acute septic shock were studied. Measurable TNF-α concentrations were present in each sample tested before treatment with anticytokine antibodies. The mean TNF-α concentration of the samples was 72 ± 26 pg/ml (mean ± standard deviation). Similarly, IL-1β was detectable in untreated serum samples with a mean concentration of 167 ± 71 pg/ml. Although insufficient serum remained after immunoabsorption for direct demonstration of cytokine removal from test (clinical) serum specimens, efficacy of im-

munoabsorption of TNF-α and IL-1β was confirmed by 98–99.5% cytokine elimination from standard media with 10% neonatal bovine serum to which human recombinant TNF-α (50 ng/ml) and IL-1β (500 ng/ml) had been added. In addition, such serum was rendered devoid of depressant activity in the described cardiac myocyte contractility assay.

In each case (Fig. 3, A–E), sera treated with preimmune rabbit antibodies (septic control) resulted in significant depression (minimum $p < 0.01$) compared to the 10% nonseptic neonatal bovine serum (nonseptic control). Treatment with anti-TNF-α or anti-IL-1β antibody alone also resulted in significantly more depression (minimum $p < 0.05$, $p < 0.01$ in four of five septic sera) than 10% nonseptic neonatal bovine serum. Immunoabsorption of both TNF-α and IL-1β from human septic sera resulted in loss of depressant activity compared to preimmune antibody-treated sera (minimum $p < 0.01$) for all patient sera (i.e., depression similar to nonseptic control). Similar results were found for changes in peak velocity of shortening in each case.

Additional control solutions consisting of 10% newborn calf serum treated with antibodies against recombinant human TNF-α, recombinant human IL-1β, both, or neither (native antibodies derived from rabbits before immunization with recombinant human TNF-α or IL-1β) failed to affect maximum extent or peak velocity of cardiac myocyte shortening compared to media containing untreated 10% newborn calf serum (data not shown).

Effect of IL-2, -4, -6, -8, -10, and IFN-γ. For IL-2 (Fig. 4 A), -4 (Fig. 4 B), -6 (Fig. 4 C), -8 (Fig. 4 D), -10 (Fig. 4 E), and IFN-γ (Fig. 4 F), concentration-dependent depression of maximum extent of cardiac myocyte shortening was absent over the concentration range tested. Similar results were obtained for changes in peak velocity of cardiac myocyte shortening for each cytokine tested.

Effect of Endotoxin Removal from Test Solutions. Each test solution from which endotoxin was removed continued to demonstrate highly significant depression of maximum extent (and peak velocity) of cardiac myocyte shortening: TNF-α, $p < 0.005$; IL-1β, $p < 0.001$; TNF-α and IL-1β, $p < 0.01$; and 10% septic serum, $p < 0.001$. Similar p values were generated for peak velocity data.

Endotoxin concentration in serum-free media was consistently found to be < 5 pg/ml. Media containing 10% neonatal calf serum (including those with cytokines) demonstrated endotoxin concentrations of between 40 and 65 pg/ml. The patient serum samples employed in this study have been evaluated previously as part of another study (12). In aggregate, these samples were shown to contain 440 ± 120 pg/ml (mean ± SEM) endotoxin. All test solutions treated with endotoxin neutralizing protein had endotoxin concentrations of < 5 pg/ml.

Discussion

The major finding of this study is that a synergistic interaction of TNF-α and IL-1β may be responsible for the myocardial depressant activity of serum from patients with acute septic shock. This finding is supported by the experi-
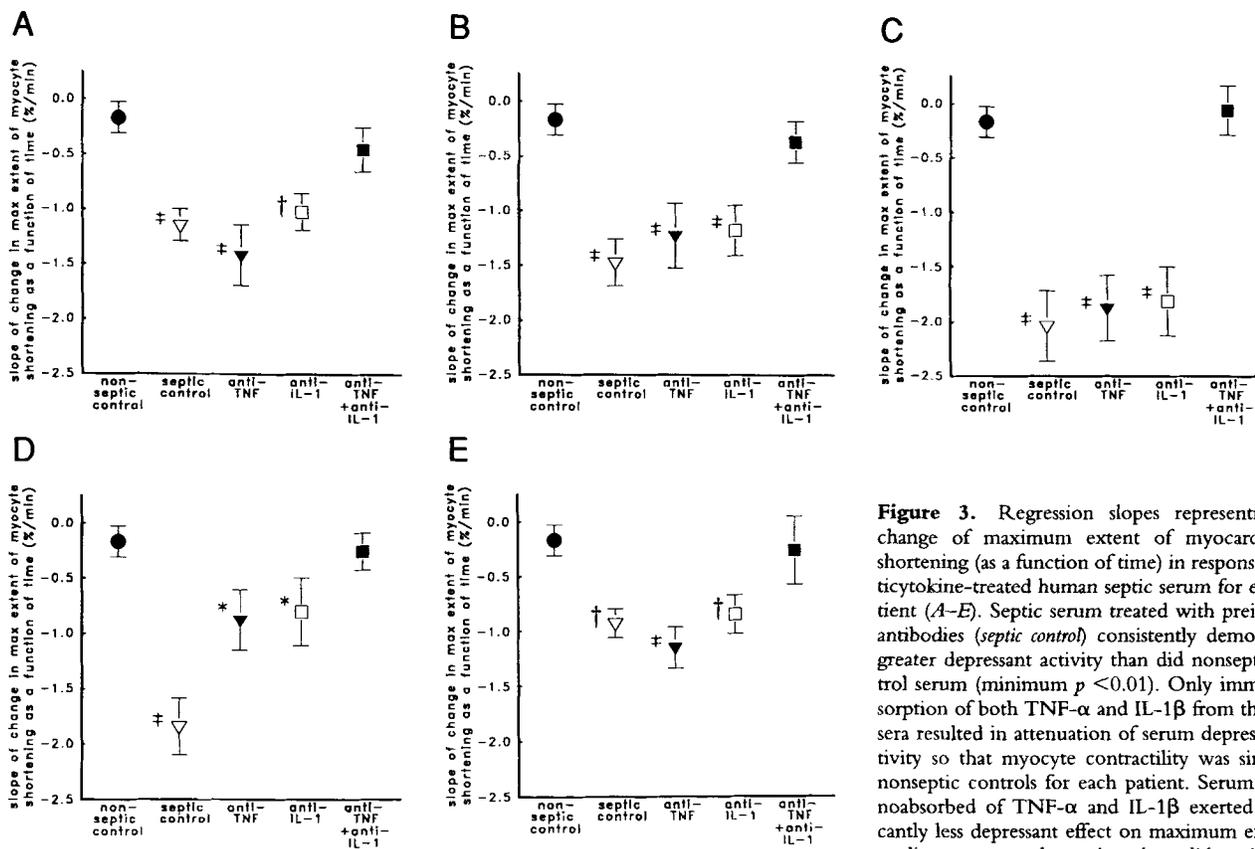
Figure 3. Regression slopes representing the change of maximum extent of myocardial cell shortening (as a function of time) in response to anticytokine-treated human septic serum for each patient (A–E). Septic serum treated with preimmune antibodies (septic control) consistently demonstrated greater depressant activity than did nonseptic control serum (minimum p <0.01). Only immunoabsorption of both TNF-α and IL-1β from the septic sera resulted in attenuation of serum depressant activity so that myocyte contractility was similar to nonseptic controls for each patient. Serum immunoabsorbed of TNF-α and IL-1β exerted significantly less depressant effect on maximum extent of cardiac myocyte shortening than did preimmune antibody treated (septic control) samples (minimum p <0.01). A Bonferonni adjustment for multiple comparisons was made so that each comparison was considered significant only if p ≤0.0125. (*) p <0.05; (†) p <0.01; (‡) p <0.001 vs. nonseptic control. Error bars, SEM.

mental data which demonstrate that immunoabsorption of both TNF-α and IL-1β abrogates in vitro myocardial depressant activity of serum from humans with acute septic shock associated with clinical myocardial depression (decreased left ventricular ejection fraction). This conclusion is strengthened by additional data which show that, whereas TNF-α and IL-1β independently produce a concentration-dependent depression of cardiac myocyte contractility, the same cytokines in combination produce similar depression at far lower concentrations (comparable to those found in human septic serum).

The primary evidence of the importance of an interaction of TNF-α and IL-1β during human septic shock is provided by experiments involving immunoabsorption of TNF-α, IL-1β, or both from human septic serum. This serum was drawn from patients with acute septic shock and marked reversible myocardial depression as evidenced by a decreased ejection fraction (26 ± 7% during acute septic shock, 55 ± 7% after recovery) and had been shown to possess a substantial degree of myocardial depressant activity in this in vitro cardiac myocyte assay. Immunoabsorption of either TNF-α or IL-1β from this serum resulted in relatively little attenuation of myocardial depressant activity in comparison to preimmune, antibody-treated septic serum (septic control). However, immunoabsorption of both TNF-α and IL-1β from the serum resulted in virtual elim-

ination of depressant activity. This result was seen in each of the five individual patient sera tested and was statistically highly significant.

Synergism between TNF-α and IL-1β in the context of this study implies that complete elimination of either should result in loss of serum myocardial depressant activity. There are at least two possible reasons for the limited attenuation of serum myocardial depressant activity seen in this study when only TNF-α or IL-1β was immunoabsorbed. As we have shown, immunoabsorption is not entirely effective in cytokine removal. The small amounts of TNF-α or IL-1β that remain may be sufficient to sustain synergy. However, when both cytokines are targetted, total TNF-α and IL-1β may drop below the threshold required for expression of synergistic activity. Alternately, the generation of septic serum-induced cardiac myocyte dysfunction may be more complex than a limited interaction between TNF-α and IL-1β. Although other cytokines have no demonstrable depressant activity of their own, they may produce a synergistic effect so that removal of only TNF-α or IL-1β is inadequate to eliminate serum depressant activity.

Further support for the synergistic role of TNF-α and IL-1β in septic myocardial depression comes from our experiments which show that, in combination, TNF-α and IL-1β cause cardiac myocyte depression at concentrations that are incompatible with merely additive depressant ac-
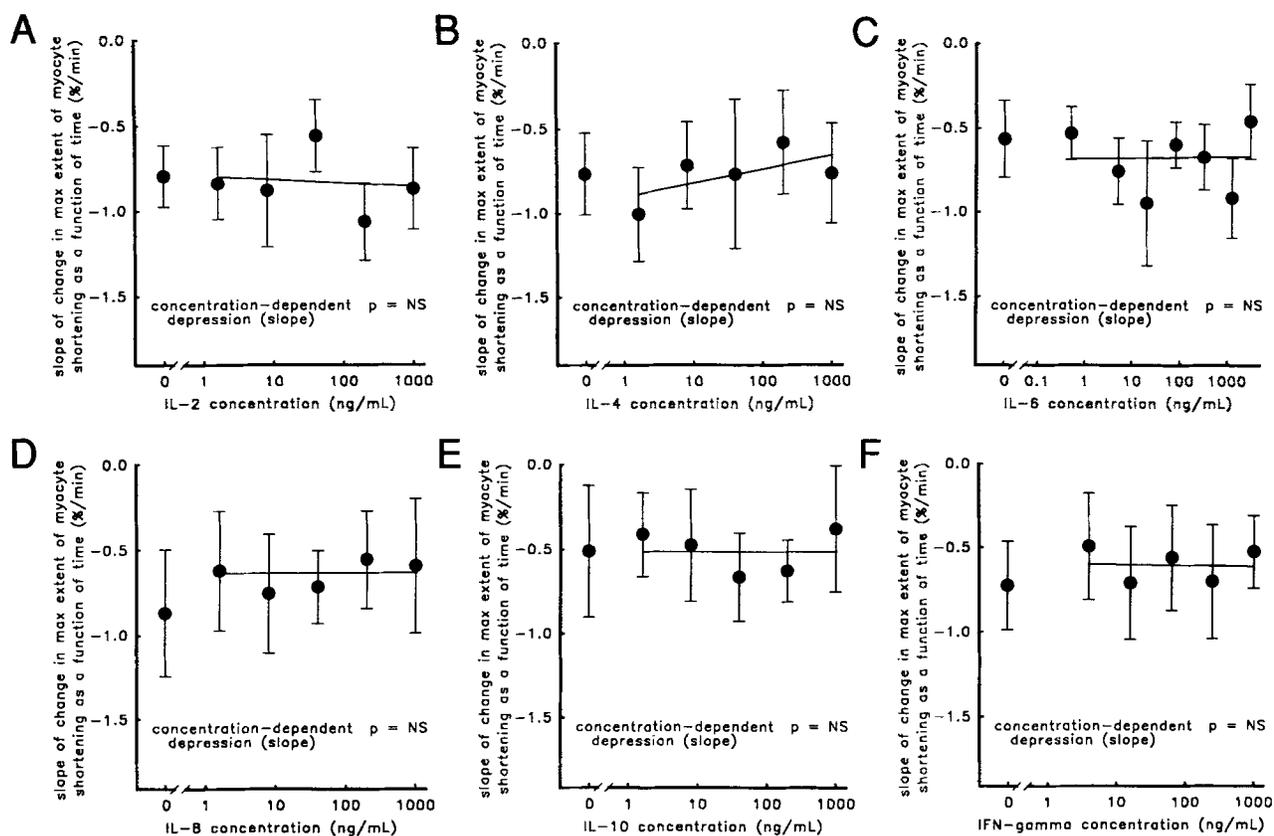
954   TNF and IL-1 Cause Septic Serum-induced Myocardial Cell Depression

Figure 4. Slopes representing rate of change of maximum extent of myocardial cell shortening in response to the specified concentrations of (A) IL-2, (B) IL-4, (C) IL-6, (D) IL-8, (E) IL-10, and (F) IFN-γ. None of these cytokines resulted in significant concentration-dependent decrease in maximum extent of cardiac myocyte shortening. Error bars represent SEM of the calculated slopes.

tivity. Since concentrations of 0.003–0.0125 ng/ml TNF-α and 0.125–0.5 ng/ml IL-1β together produce depression of maximum extent and peak velocity of cardiac myocyte shortening, whereas 64–256-fold higher concentrations are required to produce depression individually, synergism of their depressant activity appears likely. Of note is the fact that these concentrations of TNF-α and IL-1β are two to three orders of magnitude smaller than those previously documented to cause depression of myocardial tissue and fall well within the range commonly seen in patients with severe sepsis and septic shock (11, 13–17).

A number of studies have inferred a potential role for TNF-α and IL-1β in inflammatory myocardial depression by demonstrating that cytokine-containing supernatants of activated macrophages exhibit myocardial depressant activity (decreased myocardial sensitivity to catecholamines; 18, 19). However, cytokine concentrations in such supernatants are typically extremely high compared to levels circulating during sepsis. These studies have not demonstrated any form of a synergistic action between cytokines in the generation of myocardial depressant activity.

Several previous studies have shown that a combination of the two cytokines, TNF-α and IL-1β, exerts synergistic hemodynamic effects in a number of in vivo models of sepsis and septic shock (20–22). Weinberg et al. (20) have shown that doses of LPS, TNF-α, and IL-1β too low to

cause a hemodynamic disturbance individually can act synergistically to produce hypotension in unanesthetized rabbits. Okusawa et al. (21) have shown that low doses of IL-1β in combination with TNF-α produced a shocklike state in rabbits. Waage and Espevik (22) demonstrated that IL-1β synergistically potentiated the lethal effect of TNF-α administered to mice.

Whereas TNF-α and IL-1β, in combination, cause in vitro cardiac myocyte depression at concentrations well within the serum range documented in human septic shock, individually each can also cause cardiac myocyte depression. TNF-α begins to cause significant concentration-dependent depression of cardiac myocyte contraction (both maximum extent and peak velocity of shortening) beginning at concentrations within the range documented during septic shock (0.2–0.8 ng/ml). This concentration, though, is clearly infrequently documented during clinical septic shock. Similarly, cardiac myocyte depression induced by IL-1β begins at concentrations 4–10 times higher (8–32 ng/ml) than those typically documented during human septic shock. Again, only rare patients reach such serum levels during clinical septic shock.

Myocardial depression in septic shock has been well characterized in both spontaneous human septic shock and in experimental animal models of septic shock (1–3, 23, 24). Available evidence suggests that myocardial dysfunction in

septic shock is mediated by a circulating myocardial depressant substance or substances (4, 6, 7, 9, 25). Early studies of a myocardial depressant factor associated with endotoxic and septic shock (26, 27) focused on a small (<1 kD) peptide of pancreatic origin. Later studies by other groups implicated at least two distinct factors of unknown chemical composition with differing molecular masses (<1 kD and 1–10 kD; 28) or a heat-stable, lipid-soluble estrogenic compound of <1 kD (29, 30). None of these substances was definitively identified or isolated.

The most recent studies attempting to characterize the circulating myocardial depressant substances in human septic shock (4) and canine endotoxic shock (9) have implicated a heat-labile, proteinase-sensitive molecule or molecules ranging from 10 to 30 kD. Such physical properties exclude prostaglandins and leukotrienes but are compatible with most known cytokines.

The human recombinant cytokines used in this study are those that have been suggested to play a potential role in sepsis and septic shock in animals or humans. Each, to a varying extent, possesses biologic activity in rat tissues. At one extreme, TNF-α, IL-1β, and IL-6 are generally acknowledged to exert a broad range of biologic effects with substantial cross-species activity. At the other, IFN-γ and IL-4 exert a narrow range of activity and are, with occasional exceptions, highly species specific. Other cytokines utilized in this study have intermediate ranges of bioactivity and cross-species specificity. Of the cytokines studied, TNF-α, IL-1β, IL-2, and IL-6 have previously been suggested to cause depression of contractility in some studies of in vitro myocardial tissue (16, 31, 32).

In contrast to our findings that support a rapid (<10 min) onset of cardiac myocyte depression by TNF-α, IL-1β, and human septic serum, a number of recent studies have implicated a later onset, cytokine-mediated β-adrenergic signal transduction defect (18, 33) and/or stimulation of an inducible nitric oxide synthetase (19, 34–36) in septic and inflammatory myocardial depression. Depression of myocardial tissue contractility in these studies is delayed (hours to days) relative to the early depressant activity in our model. Similarly, evidence of relatively delayed (days) depression of myocardial contractility in animal models of septic shock (23, 37) is parallelled by data that show that infusion of endotoxin or TNF can result in early (<1 h) depression of cardiac function (38–40). Therefore, it seems likely that different processes may be responsible for early (14, 16) and late (34–36) cytokine-induced septic myocardial dysfunction. Such a dual or biphasic, cytokine-driven mechanism of inflammatory cardiac dysfunction is consistent with our observations that the degree of septic serum-induced cardiac myocyte depression (occurring within min of exposure) significantly correlates with the amount of clinical myocardial depression as measured by decrease in ejection fraction (which typically occurs 2–3 d after the onset of sepsis; 2, 6).

The specific intracellular mechanisms by which cytokines cause depression of cardiac myocyte contractility in this model have not been addressed in this study. However,

a number of inferences can be made. First, the effect does not appear to be related to direct cytotoxicity since, in the present study, lactate dehydrogenase levels are not increased, supravital staining is not decreased, and most importantly, the cardiac myocyte depressant effect induced by TNF-α and IL-1β is reversed within minutes after removal of cytokines. Second, since depression was also produced by application of TNF-α or IL-1β to >95–98% purified myocyte cultures, it is unlikely that cytokine-induced production of secondary mediators from cardiac nonmyocytes (such as fibroblasts, endothelial cells, or dendritic cells) plays a significant role. Third, since depression occurs and reverses within 5–10 min of introduction or removal of cytokine or septic serum, de novo protein synthesis cannot be involved.

A significant amount of endotoxin is present in most of the test solutions used in this study. Endotoxin has been suggested by one group to have a potential role in suppression of the beating rate of neonatal rat cardiac myocytes via coinduction (with IL-1β) of an inducible nitric oxide synthetase (41). Others have suggested that a similar inducible nitric oxide synthetase dependent mechanism may also explain delayed onset depression of myocardial tissue exposed to IL-1β or activated macrophage products (19, 34–36). Such a mechanism, whether endotoxin or cytokine driven, is unlikely to be relevant in these observations since the time frame of the depressant response (<10 min after the introduction of cytokines) is far too rapid to be explained by any form of de novo protein synthesis. However, the same authors have correctly pointed out that evidence is accumulating that nitric oxide has been shown to be produced by endotoxin-stimulated constitutive nitric oxide synthetase in endothelial cells (42). They have raised the possibility that endotoxin contamination, rather than cytokine effects, may account for myocardial tissue depression in some studies (16). Although endotoxin is clearly present in this model, it is unlikely to be the sole cause of depression since control samples (which contain identical amounts of endotoxin) would then also be expected to express similar depressant activity. In addition, we have demonstrated that endotoxin concentrations as high as 1 μg/ml in this assay fail to produce depression of cardiac myocyte contractility (43). Finally, the possibility that endotoxin contributes significantly to TNF-α, IL-1β, or human septic serum-induced cardiac myocyte depression is strongly refuted by the demonstration that test solutions depleted of endotoxin continue to exhibit significant myocardial depressant activity.

There exist two limitations to this model. First, the neonatal rat cardiac myocytes utilized have the disadvantage of not being terminally differentiated as are adult myocytes. Their characteristics change as they age. They exhibit metabolic differences from adult cells including significant differences in membrane receptors (44). It can be argued that phenomena observed in neonatal rat myocytes may have limited relevance to human adult cardiac pathophysiology. However, we have already shown a significant relationship between septic serum-induced in vitro cardiac depression in this model and in vivo cardiac depression (decreased

ejection fraction) in the septic shock patients from whom the serum is obtained (4, 6). This argues in favor of the potential clinical relevance of this phenomenon. The use of serum-based media, although a necessary component of this study, also introduces additional variables. Apart from the cytokines of interest, serum contains a variety of poorly understood, unidentified factors that can affect myocyte metabolism. However, the use of controls using the same serum for comparison purposes compensates for this variable.

The results of this study strongly suggest that a synergistic interaction of TNF-α and IL-1β may be at least partially responsible for septic myocardial depression in vivo. Each cytokine is individually capable of myocardial depressant activity at concentrations somewhat higher than those typically found during human septic shock. In combination, however, concentrations of TNF-α and IL-1β well within the range documented during clinical septic shock appear sufficient to cause myocyte depression, whereas elimination of TNF-α and IL-1β from human septic serum abrogates its depressant activity. These observations suggest that the search for a single myocardial depressant substance in sepsis and shock may be fruitless because myocardial depression in such conditions may be the result of interaction of more than one factor.

References

1. Parker, M.M., K.E. McCarthy, F.P. Ognibene, and J.E. Parrillo. 1990. Right ventricular dysfunction and dilatation, similar to left ventricular changes, characterize the cardiac depression of septic shock in humans. *Chest.* 97:126–131.
2. Parker, M.M., J.H. Shelhamer, S.L. Bacharach, M.V. Green, C. Natanson, T.M. Frederick, B.A. Damske, and J.E. Parrillo. 1984. Profound but reversible myocardial depression in patients with septic shock. *Ann. Intern. Med.* 100:483–490.
3. Ellrodt, A.G., M.S. Riedinger, A. Kimchi, D.S. Berman, J. Maddahi, H.J.C. Swan, and G.H. Murata. 1985. Left ventricular performance in septic shock: reversible segmental and global abnormalities. *Am. Heart J.* 110:402–409.
4. Reilly, J.M., R.E. Cunnion, C. Burch-Whitman, M.M. Parker, J.H. Shelhamer, and J.E. Parrillo. 1989. A circulating myocardial depressant substance is associated with cardiac dysfunction and peripheral hypoperfusion (lactic acidemia) in patients with septic shock. *Chest.* 95:1072–1080.
5. Schuette, W.H., C. Burch, P.O. Roach, and J.E. Parrillo. 1987. Closed loop television tracking of beating heart cells in vitro. *Cytometry.* 8:101–103.
6. Parrillo, J.E., C. Burch, J.H. Shelhamer, M.M. Parker, C. Natanson, and W. Schuette. 1985. A circulating myocardial depressant substance in humans with septic shock. Septic shock patients with a reduced ejection fraction have a circulating factor that depresses in vitro myocardial cell performance. *J. Clin. Invest.* 76:1539–1553.
7. Parrillo, J.E., M.M. Parker, C. Natanson, A.F. Suffredini, R.L. Danner, R.E. Cunnion, and F.P. Ognibene. 1990. Septic shock in humans. Advances in the understanding of pathogenesis, cardiovascular dysfunction, and therapy. *Ann. Intern. Med.* 113:227–242.
8. Parrillo, J.E. 1993. Pathogenetic mechanisms of septic shock. *N. Engl. J. Med.* 328:1471–1477.

9. Jha, P., H. Jacobs, D. Bose, R. Wang, J. Yang, R.B. Light, and S. Mink. 1993. Effects of *E. coli* sepsis and myocardial depressant factor on interval-force relations in dog ventricle. *Am. J. Physiol.* 264:H1402–H1410.
10. Harary, I., and B. Farley. 1960. In-vitro studies of single isolated beating rat heart cells. *Science (Wash. DC).* 131:1674–1675.
11. Casey, L.C., R.A. Balk, and R.C. Bone. 1993. Plasma cytokine and endotoxin levels correlate with survival in patients with the sepsis syndrome. *Ann. Intern. Med.* 119:771–778.
12. Danner, R.L., R.J. Elin, J.M. Hosseini, R.A. Wesley, J.M. Reilly, and J.E. Parrillo. 1991. Endotoxemia in human septic shock. *Chest.* 99:169–175.
13. Hospenud, J.D. 1993. The effects of interleukin 1 on myocardial function and metabolism. *Clin. Immunol. Immunopathol.* 68:175–180.
14. Yokoyama, T., L. Vaca, R.D. Rossen, W. Durante, P. Hazarika, and D.L. Mann. 1993. Cellular basis for the negative inotropic effects of tumor necrosis factor-α in the adult mammalian heart. *J. Clin. Invest.* 92:2303–2312.
15. 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.* 169:333–338.
16. Finkel, M.S., C.V. Oddis, T.D. Jacobs, S.C. Watkins, B.G. Hattler, and R.L. Simmons. 1992. Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science (Wash. DC).* 257:387–389.
17. Girardin, E., G.E. Grau, J.M. Dayer, P. Roux-Lombard, and P.H. Lambert. 1989. Plasma tumor necrosis factor and interleukin-1 in the serum of children with severe infectious purpura. *N. Engl. J. Med.* 319:397–400.
18. Gulick, T., M.K. Chung, S.J. Pieper, L.G. Lange, and G.F. Schreiner. 1989. Interleukin 1 and tumor necrosis factor in-

hibit cardiac myocyte adrenergic responsiveness. *Proc. Natl. Acad. Sci. USA.* 86:6753–6757.

19. Balligand, J., D. Ungureanu, R.A. Kelly, L. Kobzik, D. Pimental, T. Michel, and T.W. Smith. 1993. Abnormal contractile function due to induction of nitric oxide synthesis in rat cardiac myocytes follows exposure to activated macrophage-conditioned medium. *J. Clin. Invest.* 91:2314–2319.

20. Weinberg, J.R., P. Boyle, A. Meager, and A. Guz. 1992. Lipopolysaccharide, tumor necrosis factor, and interleukin-1 interact to cause hypotension. *J. Lab. Clin. Med.* 120:205–211.

21. Okusawa, S., J.A. Gelfand, T. Ikejima, R.J. Connolly, and C.A. Dinarello. 1988. Interleukin-1 induces a shock like state in rabbits. *J. Clin. Invest.* 81:1162–1172.

22. Waage, A., and T. Espevik. 1988. Interleukin-1 potentiates the lethal effect of tumor necrosis factor α/cachectin in mice. *J. Exp. Med.* 167:1987–1992.

23. Natanson, C., M.P. Fink, H.K. Ballantyne, T.J. MacVittie, J.J. Conklin, and J.E. Parrillo. 1986. Gram-negative bacteremia produces both severe systolic and diastolic cardiac dysfunction in a canine model that simulates human septic shock. *J. Clin. Invest.* 78:259–270.

24. Goldfarb, R.D., L.M. Nightingale, P. Kish, P.B. Weber, and D.J. Loegering. 1986. Left ventricular function during lethal and sublethal endotoxemia in swine. *Am. J. Physiol.* 251:H364–H373.

25. Lefer, A.M., and J. Martin. 1970. Origin of myocardial depressant factor in shock. *Am. J. Physiol.* 218:1423–1427.

26. Wangensteen, S.L., W.T. Geissenger, W.L. Lovett, T.M. Glenn, and A.M. Lefer. 1971. Relationship between splanchnic blood flow and a myocardial depressant factor in endotoxin shock. *Surgery (St. Louis).* 69:410–418.

27. Lovett, W.L., S.L. Wangensteen, T.M. Glenn, and A.M. Lefer. 1971. Presence of a myocardial depressant factor in patients with circulatory shock. *Surgery (St. Louis).* 70:223–231.

28. McConn, R., J.K. Greineder, F. Wasserman, and G.H.A. Clowes. 1979. Is there a humoral factor that depresses ventricular function in sepsis? *Circ. Shock.* 1:9–22.

29. Carli, A., M.C. Auclair, C. Vernimmen, and P. Jourdon. 1979. Reversal by calcium of rat heart cell dysfunction induced by human sera in septic shock. *Circ. Shock.* 6:147–157.

30. Benassayag, C., M.C. Christeff, M.C. Auclair, C. Vernimmen, C. Carli-Vielle, E. Nunez, and A. Carli. 1984. Early released lipid-soluble cardiodepressant factor and elevated oestrogenic substances in human septic shock. *Eur. J. Clin. Invest.* 14:288–294.

31. Kinugawa, K., T. Takahashi, O. Kohmoto, A. Yao, T. Aoyagi, S. Monomura, Y. Hirata, and T. Serizawa. 1994. Nitric oxide-mediated effects of interleukin-6 on $[Ca^{2+}]_i$ and cell contraction in cultured chick ventricular myocytes. *Circ. Res.* 75:285–295.

32. Weisensee, D., J. Bereiter-Hahn, W. Schoeppe, and I. Low-Friedrich. 1993. Effects of cytokines on the contractility of cultured cardiac myocytes. *Int. J. Immunopharmacol.* 15:581–587.

33. Chung, M.K., T.S. Gulick, R.E. Rotondo, G.F. Schreiner, and L.G. Lange. 1990. Mechanism of cytokine inhibition of beta-adrenergic agonist stimulation of cyclic AMP in rat cardiac myoctyes: impairment of signal transduction. *Circ. Res.* 67:753–763.

34. Schulz, R., E. Nava, and S. Moncada. 1992. Induction and potential biological relevance of a $Ca(^{2+})$-independent nitric oxide synthase in the myocardium. *Br. J. Pharmacol.* 105:575–580.

35. Schulz, R., D.L. Panas, S. Moncada, P.M. Olley, and G.D. Lopaschuk. 1992. Depression of cardiac function in cytokine treated hearts is diminished by inhibition of nitric oxide synthesis and abolished by dexamethasone. *Circulation.* 86:1295. (Abstr.)

36. Evans, H.G., M.J. Lewis, and A.M. Shah. 1993. Interleukin-1 beta modulates myocardial contraction via dexamethasone sensitive production of nitric oxide. *Cardiovasc. Res.* 27:1486–1490.

37. Natanson, C., R.L. Danner, M.P. Fink, T.J. MacVittie, R.I. Walker, J.J. Conklin, and J.E. Parrillo. 1988. Cardiovascular performance with *E. coli* challenges in a canine model of human sepsis. *Am. J. Physiol.* 254:H558–H569.

38. Eichenholz, P.W., P.Q. Eichacker, W.D. Hoffman, S.M. Banks, J.E. Parrillo, R.L. Danner, and C. Natanson. 1992. Tumor necrosis factor challenges in canines: patterns of cardiovascular dysfunction. *Am. J. Physiol.* 263:H668–H675.

39. 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.

40. Walley, K.R., P.C. Hebert, Y. Wakai, P.G. Wilcox, J.D. Road, and D.J. Cooper. 1994. Decrease in left ventricular contractility after tumor necrosis factor-alpha infusion in dogs. *J. Appl. Physiol.* 76:1060–1067.

41. Roberts, A.B., Y. Vodovotz, N.S. Roche, M.B. Sporn, and C.F. Nathan. 1992. Role of nitric oxide in antagonistic effects of transforming growth factor-beta and interleukin-1 beta on the beating rate of cultured cardiac myocytes. *Mol. Endocrinol.* 6:1921–1930.

42. Salvemini, D., R. Korbut, E. Anggard, and J.R. Vane. 1990. Immediate release of a nitric oxide–like factor from bovine aortic endothelial cells by *Escherichia coli* lipopolysaccharide. *Proc. Natl. Acad. Sci. USA.* 87:2593–2597.

43. Kumar, A., R. Kosuri, P. Kandula, L. Lakshminarayanan, E. Bunnell, L. Dee, J. Olson, E. Uretz, and J.E. Parrillo. 1994. Interleukin-1 beta but not endotoxin, interferon-gamma, or interleukin-6 depresses myocardial cell contractility in-vitro. *Clin. Res.* 42:168a. (Abstr.)

44. Stemmer, P., P.L. Wisler, and A.M. Watanabe. 1991. Isolated myocytes in experimental cardiology. *In* The Heart and Cardiovascular System. Scientific Foundations. H.A. Fozzard, E. Haber, R.B. Jennings, A.M. Katz, and H.E. Morgan, editors. Raven Press, New York. 387–404.