The cytokine tumor necrosis factor-α (TNFα), a product of activated macrophages, is increased in the serum of patients with severe congestive heart failure (CHF). In addition to its local cellular effects on immune activation and coagulation, TNFα appears to play an important role in the regulation of nitric oxide metabolism in leukocytes, vascular endothelial cells, and vascular smooth muscle cells. TNFα has been reported to decrease mRNA for the constitutive nitric oxide synthase in vascular endothelial cells, increase expression of the inducible form of nitric oxide synthase in macrophages, vascular endothelial cells, and vascular smooth muscle cells, and increase vascular smooth muscle production of superoxide anion, a substance known to decrease the half-life of nitric oxide. Previous studies demonstrated that TNFα may either stimulate or inhibit endothelium-dependent, nitric oxide-mediated vasodilation under varying experimental conditions.

Abnormalities in nitric oxide-mediated vasodilation have been reported in patients with CHF. The regional vasodilatory responses to intra-arterial administration of acetylcholine and nitroglycerin are decreased in the peripheral circulation of patients with CHF when compared with age-matched normal subjects. The vasodilatory action of acetylcholine is dependent on the vascular endothelial production of nitric oxide from the amino acid precursor L-arginine by the constitutive form of nitric oxide synthase. The vasodilatory action of nitroglycerin is dependent on the conversion of nitroglycerin to nitric oxide by a sulfhydryl-requiring biotransformation process localized to the vascular smooth muscle cell membrane. Whether increased concentrations of TNFα can affect nitric oxide–mediated vasodilation in patients with CHF has not been studied previously.

Accordingly, the present study was undertaken to measure serum TNFα concentrations and forearm blood flow responses to administration of acetylcholine and nitroglycerin in patients with CHF and normal subjects.
subjects. In addition, because cytokines are most often activated as a group of interrelated signals, serum concentrations of additional cytokines including interleukin-1 (IL-1, α and β), interleukin-2 (IL-2), and interleukin-6 (IL-6) were measured in patients with CHF and age-matched normal subjects.

Methods

Study Population

Fourteen men and 3 women with idiopathic dilated cardiomyopathy were studied (age ranged from 34 to 68 years and averaged 58±11 years). Mean left ventricular ejection fraction determined by radionuclide angiography was 19.5±7.3%. All patients had stable symptoms of CHF of at least 3 months' duration. According to the criteria of the New York Heart Association, 14 patients were in functional class III and 3 were in functional class II. Therapy for CHF consisted of furosemide and angiotensin-converting enzyme inhibitors in all patients, digoxin in 10 patients, and long-acting oral nitrate preparations in 9 patients. Cardiovascular medications were withheld for at least 24 hours before the study. No patients had clinical or laboratory evidence of coronary artery disease, peripheral vascular disease, hypertension, or diabetes mellitus. No patients had serum sodium <136 mEq/L, serum creatinine >2.0 mg/dL, or serum cholesterol >240 mg/dL.

Eleven men and 6 women without evidence of cardiovascular or inflammatory disease as determined by history and physical examination served as normal controls. The mean age of these normal subjects was similar to the mean age of the patients with CHF (54±12 versus 58±11 years, respectively, P=NS). Normal subjects did not have clinical or laboratory evidence of diabetes mellitus, hypercholesterolemia, or hypertension and were not taking any medication. The study was approved by the Ethical Review Board of the Albert Einstein College of Medicine and Montefiore Medical Center. All patients and normal subjects gave written informed consent before the study.

Cytokine Measurements

Thirty milliliters of venous blood was obtained from an indwelling antecubital polyethylene catheter for determination of serum cytokine concentrations in patients with CHF and normal subjects. Serum was separated from blood elements by centrifugation, and aliquots were stored at −20°C. Concentrations of TNFα, IL-1 (α and β), IL-2, and IL-6 were measured with commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) (pg/mL). The limit of detection for the assays was 20 pg/mL. All samples were run in duplicate; the average value of the two measurements is reported.

Forearm Blood Flow Measurements

Forearm blood flow (mL/min per 100 mL of forearm volume) was determined in 17 patients with CHF and 7 age-matched normal subjects with strain-gauge venous occlusion plethysmography as previously described in detail. Briefly, with the arm resting comfortably 10 cm above the right atrium, a mercury-in-silastic strain gauge was placed around the widest portion of the upper third of the forearm. The strain gauge was electrically coupled to a plethysmograph (Parks Electronics) calibrated to measure percent change in volume. The plethysmographic tracings of forearm blood flow were recorded on photographic paper for analysis (Electronics for Medicine, model VR6). For each measurement, forearm venous blood flow was occluded just proximal to the elbow with the rapid inflation of a blood pressure cuff to 40 mm Hg (Hokanson Instruments, model E20). A wrist cuff was inflated to suprasystolic pressures 1 minute before and during each measurement to exclude hand circulation from the blood flow determination. The venous occluding cuff was rapidly inflated for 5 seconds at 15-second intervals; five plethysmographic measurements were averaged for determination of forearm blood flow at rest and during administration of acetylcholine and nitroglycerin. To assess maximal vasodilatory reserve to a metabolic stimulus, peak reactive hyperemic blood flow was determined after 5 minutes of brachial artery occlusion as the highest forearm blood flow measured within 10 seconds after release of the arterial occluding pressure cuff.

Drug Administration

All drugs were prepared on the day of the study in 5% dextrose in water solution and administered directly into the brachial artery at a rate of 1 mL/min. Acetylcholine was administered to achieve regional forearm blood concentrations of 10⁻⁷ mol/L and 10⁻⁶ mol/L. Nitroglycerin was administered to achieve regional forearm blood concentrations of 10⁻⁷ mol/L and 10⁻⁶ mol/L. Each dose of acetylcholine and nitroglycerin was administered sequentially as continuous 2-minute infusions in 17 patients with CHF and 7 normal subjects.

Study Protocol

All studies were conducted in a quiet, temperature-controlled room with the subjects resting in the supine position. In all patients with CHF and in 7 normal subjects, a 20-gauge angiocath was inserted under local anesthesia into the brachial artery of the nondominant forearm for regional administration of acetylcholine and nitroglycerin. A 20-gauge angiocath was also inserted into the antecubital vein of the contralateral arm for venous blood sampling. After 30 minutes of supine rest, 30 mL of blood was obtained from the venous catheter for determination of serum cytokine concentrations. Forearm blood flow was then measured before and during administration of graded concentrations of acetylcholine and nitroglycerin and after 5 minutes of brachial artery occlusion.

The remaining 10 normal subjects did not undergo brachial artery catheterization; a 20-gauge angiocath was inserted in the antecubital vein for blood sampling. After 30 minutes of supine rest, 30 mL of blood was collected for determination of serum cytokine concentrations.

Data Analysis

All values are stated as mean±SEM. Serum concentrations of TNFα, IL-1, IL-2, and IL-6 were compared with patients with CHF and normal subjects with the Student's t test for unpaired observations. Forearm blood flows of patients with and without detectable concentrations of TNFα and normal subjects were compared with a repeated-measures ANOVA model. Forearm blood flows at rest and during administration of acetylcholine were correlated to serum cytokine concentrations with simple linear regression (least-squares method). A two-tailed probability value of <.05 was considered statistically significant.

Results

Serum concentrations of TNFα were above the detection limits of the assay in 10 patients with CHF with a mean serum concentration of 39.4±3.8 pg/mL; the remaining 7 patients had undetectable serum concentrations of TNFα (<20 pg/mL, Fig 1A). Forearm blood flow responses to administration of acetylcholine and nitroglycerin were greater in the 10 patients with detectable concentrations of TNFα than in the 7 patients without detectable serum TNFα (Fig 2). In the 10 patients with detectable TNFα, forearm blood flow responses to administration of acetylcholine and nitroglycerin were highly correlated with the serum concentrations of TNFα (Fig 3, A through D). Forearm blood flows at rest and in response to 5 minutes of brachial
Mean systemic arterial pressures were similar in the 10 patients with detectable concentrations of TNFα and the 7 patients without detectable serum TNFα (84.9±5.7 versus 81.6±3.5 mm Hg). Mean age, functional class, and left ventricular ejection fraction also were similar in patients with and without detectable serum TNFα. In 1 of the 17 normal subjects, the serum concentration of TNFα was just above the detection limits of the assay (21.9 pg/mL). Forearm blood flow responses to regional administration of graded concentrations of acetylcholine and nitroglycerin in 7 normal subjects were significantly greater than in patients with CHF and detectable concentrations of TNFα (Fig 2).

Serum concentrations of IL-2 were above the detection limits of the assay in 14 of the patients with CHF, with a mean serum concentration of 112±19 pg/mL; the remaining 3 patients had undetectable serum concentrations of IL-2 (<20 pg/mL, Fig 1B) and undetectable serum concentrations of TNFα. Serum concentrations of IL-2 and TNFα did not significantly correlate in the 10 patients in whom both cytokines were detected (r=.13). In the 14 patients with detectable serum IL-2, serum concentrations of IL-2 did not correlate with forearm blood flows at rest in response to administration of graded concentrations of acetylcholine and nitroglycerin and after 5 minutes of brachial artery occlusion (r<.20, P>.10 for all correlations). IL-2 was not detected in the serum of the normal subjects. Serum concentrations of IL-1 and IL-6 were below the detection limits of the assays both in patients with CHF and in normal subjects.

**Discussion**

The present data confirm previous reports that serum concentrations of TNFα are increased in patients with CHF when compared with those of age-matched normal subjects. In addition, the data indicate that the serum concentrations of IL-2 are increased in patients with CHF, whereas serum concentrations of IL-1 and IL-6 are not. This study also demonstrates that increased serum concentrations of TNFα are highly correlated with forearm blood flow responses to administration of acetylcholine and nitroglycerin in the brachial artery.

Whereas the vasodilatory responses to administration of acetylcholine and nitroglycerin were positively corre-
related to serum concentrations of TNFα, the peak hyperemic response to brachial artery occlusion for 5 minutes was not related to serum concentrations of TNFα. The limitations of venous occlusion plethysmography as a technique to measure absolute limb blood flow are recognized.17 However, this technique is well accepted to determine the vasodilatory responses to different pharmacological interventions in patients with CHF and normal subjects.18 Our data suggest that TNFα may specifically enhance cGMP-dependent vasodilation in patients with CHF, possibly by altering nitric oxide metabolism in different cell types. In the hypotensive state associated with endotoxia and septic shock, TNFα and other cytokines induce peripheral vasodilation by stimulating production of an inducible form of nitric oxide synthase in endothelial cells, vascular smooth muscle cells, and activated macrophages.19-21 In the absence of endotoxin, high concentrations of TNFα have been demonstrated to directly decrease levels of mRNA for the constitutive form of nitric oxide synthase in vascular endothelial cells and to increase expression of the inducible form of nitric oxide synthase in vascular endothelial cells, vascular smooth muscle cells, and macrophages.5,6,22-24 Serum concentrations of TNFα were lower in our patients with CHF than those reported in patients with septic shock and in normal subjects after injection of endotoxin.25,26 Nevertheless, the moderate increase in TNFα serum concentration noted in our patients may be sufficient to activate the inducible form of nitric oxide synthase. Activation of the inducible form of nitric oxide synthase did not alter forearm blood flow at rest but clearly potentiated the vascular effects resulting from either stimulation of the constitutive form of nitric oxide synthase by acetylcholine or direct release of nitric oxide by nitroglycerin.

Additive effects of combined stimulation of the constitutive and inducible forms of nitric oxide synthase on nitric oxide–mediated production of cGMP were previously demonstrated in cultured vascular endothelial cells.19,27 Likewise, additive effects in response to simultaneous administration of endothelium-dependent and -independent nitric oxide–mediated vasodilators were demonstrated in the forearm circulation of patients with CHF.28

In the present study, serum TNFα concentration was measured by ELISA. Fifty-eight percent of our patients had increased serum TNFα concentration. This percentage is comparable to that noted with the measurement of TNFα in patients with CHF by cytotoxic radioimmunoassay.1,2 The observation that serum IL-2 concentration is increased in patients with CHF has pathophysiological implications. Previously, serum concentrations of soluble IL-2 receptors and soluble CD8 have been found to be elevated in patients with CHF.29 These data, along with our findings of increased serum levels of IL-2 in patients with CHF, suggest an immunological component to this disease process.

TNF and IL-1 are synthesized principally by activated macrophages, and their expression is often closely linked. Why only serum TNFα concentration was increased in our patients cannot be readily explained. IL-2 is exclusively synthesized by activated T cells. Thus, cellular components of the immune system appear to be activated in patients with CHF.

Although the correlation between increased serum TNFα concentration and the vasodilatory responses to
acetylcholine and nitroglycerin was highly significant in 10 patients with CHF and increased serum TNFα concentration, this observation remains to be confirmed in a larger patient population. The relation between TNFα and vasodilatory responses to acetylcholine and nitroglycerin suggests that both the inducible and constitutive forms of nitric oxide synthase are involved in the regulation of peripheral vasomotor tone in patients with CHF. Increased serum TNFα concentration may partially compensate for the previously reported decreased vasodilatory response to activation of the constitutive form of nitric oxide synthase in patients with CHF.

Acknowledgments

The authors appreciate the technical expertise of Michelle Guida. This work was funded in part by a grant-in-aid from the American Heart Association, New York City Affiliate.

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_Circulation._ 1994;90:12-16
doi: 10.1161/01.CIR.90.1.12

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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