EXPERIMENTAL STUDY

Carvedilol Increases the Production of Interleukin-12 and Interferon-γ and Improves the Survival of Mice Infected With the Encephalomyocarditis Virus

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OBJECTIVES
This study was designed to examine the effects of carvedilol in a murine model of viral myocarditis induced by encephalomyocarditis virus (EMCV) infection.

BACKGROUND
Cytokines play an important role in the pathophysiology of viral myocarditis. Catecholamines influence the production of cytokines via β-adrenergic receptors, suggesting that β-adrenergic blockers could modulate the production of cytokines and exert a therapeutic effect in viral myocarditis by blocking the β-stimulating action of endogenous catecholamines. In clinical trials, the third-generation, nonselective β-blocker carvedilol was the first among several β-blockers to reduce mortality in heart failure. However, the effects of carvedilol in acute viral myocarditis and on cytokine production are unknown.

METHODS
This study compared the effects of carvedilol, the selective β1-blocker metoprolol, and the nonselective β-blocker propranolol in a murine model of viral myocarditis induced by EMCV.

RESULTS
Carvedilol improved the 14-day survival of the animals, attenuated myocardial lesions on day 7, and increased myocardial levels of interleukin (IL)-12 and interferon (IFN)-γ, whereas reducing myocardial virus replication. Propranolol also attenuated myocardial lesions, but to a lesser extent, and increased IL-12 and IFN-γ levels. Metoprolol had no effect in this model. Encephalomyocarditis virus infection increased plasma catecholamine levels.

CONCLUSIONS
These results suggest that by blocking the β2-stimulating effects of catecholamines, carvedilol exerts some of its beneficial effects by increasing the production of IL-12 and IFN-γ. Carvedilol may be effective in patients with viral myocarditis by boosting IL-12 and IFN-γ production. (J Am Coll Cardiol 2003;41:340–5) © 2003 by the American College of Cardiology Foundation

Viral infection of the myocardium produces an intense inflammation, which can cause acute heart failure in humans and animals. Encephalomyocarditis virus (EMCV) infection in DBA/2 mice causes acute myocarditis (1,2) and induces several types of cytokines (3), as well as neuroendocrine hormones. Recent reports have emphasized the importance of cytokines in the pathophysiology of viral myocarditis (3–5). The roles of individual cytokines have also been studied. The administration of interleukin (IL)-12 (4) or interferon (IFN)-γ (5) markedly improved survival, attenuated myocardial lesions, and reduced myocardial viral replication in this EMCV-induced myocarditis model.

In recent years, it has become clear that neuroendocrine hormones participate in the cytokine network. Catecholamines and several adrenergic agonists influence the production of cytokines via β-adrenergic receptors, which increase intracellular cyclic adenosine monophosphate levels (6–8). Therefore, we hypothesized that, when administered in our model, β-adrenergic antagonism may block the effects of sympathetic stimulation on the production of cytokines and modulate their production.

Beta-adrenergic blockers are now on the forefront in the treatment of chronic heart failure (9–12). Carvedilol is a vasodilating, nonselective β-blocker with antioxidant properties (9,13,14). In large clinical trials, carvedilol reduced the risk of death in chronic heart failure and offered a high degree of cardiac protection in humans (9). However, its effects in viral myocarditis and on cytokine production are unknown. These experiments were performed to compare the effects of carvedilol, the selective β1-blocker metoprolol, and the nonselective β-blocker propranolol in a murine model of viral myocarditis induced by EMCV infection.

METHODS

Pharmaceuticals. Carvedilol was synthesized by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). Metoprolol and propranolol were synthesized by Sigma Chemical Company (St. Louis, Missouri). They were mixed in phosphate-buffered saline containing 1% methylcellulose.

Experimental infection. Four-week-old inbred male DBA/2 mice were inoculated intraperitoneally with 0.1 ml of the M variant of EMCV diluted in Eagle’s modified...
Abbreviations and Acronyms

- **BW** = body weight
- **EMCV** = encephalomyocarditis virus
- **HW** = heart weight
- **IFN** = interferon
- **IL** = interleukin
- **TNF** = tumor necrosis factor

essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) to a concentration of 100 plaque-forming units (pfu)/ml. The day of virus inoculation was defined as day 0.

**Measurement of plasma epinephrine and norepinephrine levels.** Blood was sampled from the animals’ tails. Plasma was collected from uninfected control mice and infected mice on day 7 (n = 5 in each). The plasma specimens were stored at −80°C until measurement of epinephrine or norepinephrine levels by radioenzymatic assay, as described previously (15).

**Treatment protocols. PROTOCOL 1: DOSE-RELATED EFFECTS OF CARVEDILOL.** Carvedilol was administered in a dose of 3 or 10 mg/kg per day for 7 or 14 consecutive days, whereas control mice received the vehicle only.

**PROTOCOL 2: COMPARISON OF THE EFFECTS OF CARVEDILOL VERSUS METOPROLOL AND PROPRANOLOL.** Carvedilol was administered in a dose of 10 mg/kg per day, and metoprolol or propranolol was administered in a dose of 30 mg/kg per day for 7 or 14 consecutive days, whereas control mice received the vehicle only. The β1-blocking effect in the metoprolol or propranolol group was nearly equal to that in the carvedilol group, whereas the β2-blocking effect in the propranolol group was about three times larger than that in the carvedilol group (16,17).

All drugs were administered orally once daily for 7 or 14 consecutive days, starting on day 0.

**Survival experiments.** Survival was measured over a 14-day period.

**Histologic examination.** The hearts were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. The extent of cellular infiltration and myocardial necrosis was blindly graded by two observers and scored as follows: 0 = no lesions; 1+ = lesions involving <25% of the myocardium; 2+ = lesions involving 25% to 50%; 3+ = lesions involving 50% to 75%; and 4+ = lesions involving 75% to 100%. Scores assigned by the two observers were averaged.

**Assay of cytokine levels in the heart.** Cytokine levels in the heart were measured as previously described (18). Cytokine levels were measured with various enzyme-linked immunosorben assay kits manufactured by Genzyme Co. (Cambridge, Massachusetts) for IL-6, IL-10, IL-12, and tumor necrosis factor-α (TNF-α); BioSource International (Camarillo, California) for IL-1-β; and Endogen, Inc. (Cambridge, Massachusetts) for IFN-γ. The sensitivity of the kit is 5 pg/ml for IL-6; 15 pg/ml for IL-10, IL-12, TNF-α, and IFN-γ; and 7 pg/ml for IL-1-β. Cytokine levels are expressed as pg/mg of heart. The sensitivity of IL-12 was 0.3 pg/mg of heart.

**Assay of myocardial virus concentration.** The myocardial virus concentration was assayed by the FL (human amnion cells)-plaque assay (19). The myocardial virus concentration was expressed as log pfu/mg of heart. The sensitivity of this method is −1.0 log pfu/mg of heart.

**Statistical analysis.** Survival was analyzed by the Kaplan-Meier method. Statistical comparisons of plasma catecholamine levels, heart weight to body weight ratios (HW/BW, an index of the severity of congestive heart failure), histologic scores, and cytokine levels were performed by analysis of variance with Bonferroni’s multiple comparisons correction. Measurements of myocardial virus concentration were tested by the Kruskal-Wallis test. Data are expressed as the mean value ± SE. A p value <0.05 was considered statistically significant.

**RESULTS**

**Plasma epinephrine and norepinephrine levels.** Plasma epinephrine levels were significantly higher in the EMCV-infected group than in the control group (6.22 ± 1.44 vs. 0.11 ± 0.04 ng/ml; n = 5 in each; p < 0.01). Likewise, plasma norepinephrine levels were significantly higher in the test group than in the control group (1.83 ± 0.43 vs. 0.23 ± 0.08 ng/ml; n = 5 in each; p < 0.01).

**Dose-related effects of carvedilol. Survival rate.** The 14-day survival rate increased in a dose-dependent manner and was significantly higher in the 10-mg/kg carvedilol group (85.0%) than in the control group (45.0%; n = 20 in each; p < 0.05) (Fig. 1a). The 14-day survival rate in the 3-mg/kg carvedilol group was 70.0% (14/20).

**Myocardial histology and HW/BW ratio on day 7.** The HW/BW ratio and pathologic score decreased in a dose-dependent manner and were significantly lower in the low- and high-dose carvedilol group than in the control group (Table 1).

**Cytokine levels in the heart on day 7.** Figure 1b illustrates the dose-dependent increase in IFN-γ levels, reaching statistical significance in the comparison between the high-dose carvedilol group and the control group (p < 0.05). On day 7, compared with an infected control value of 21.7 ± 5.2 pg/mg of heart, carvedilol had increased IFN-γ levels by 151.8 ± 37.0% in the low-dose carvedilol group and by 225.8 ± 40.0% in the high-dose carvedilol group (n = 5 in each). No such effects were measured on IL-1-β, IL-6, or TNF-α production (data not shown).

**Myocardial virus concentration on day 7.** Figure 1c shows that, on day 7, the myocardial virus concentration decreased in a dose-dependent manner. Myocardial virus concentration was 1.6 ± 0.1 log pfu/mg heart in the high-dose carvedilol group; 2.0 ± 0.3 log pfu/mg heart in the low-dose carvedilol group; and 2.5 ± 0.2 log pfu/mg heart in the test group than in the control group (1.83 ± 0.43 vs. 0.23 ± 0.08 ng/ml; n = 5 in each; p < 0.01). Likewise, plasma norepinephrine levels were significantly higher in the test group than in the control group (1.83 ± 0.43 vs. 0.23 ± 0.08 ng/ml; n = 5 in each; p < 0.01).
Comparison of the effects of carvedilol versus metoprolol and propranolol. SURVIVAL RATE. The 14-day survival rate was significantly higher in the carvedilol group (85%) than in the control (40%), propranolol (50%), and metoprolol (35%) groups (n = 20 in each; p < 0.05) (Fig. 2a).

MYOCARDIAL HISTOLOGY AND HW/BW RATIO ON DAY 7. The HW/BW ratio was significantly lower in the carvedilol group than in the metoprolol, propranolol, and control groups. The pathologic scores were significantly lower in the carvedilol group than in the metoprolol and control groups. The scores were lower in the propranolol treatment group, and myocardial necrosis was significantly less in the propranolol group than in the control group (Table 2).

CYTOKINE LEVELS IN THE HEART ON DAY 7. Levels of IFN-γ were significantly increased by carvedilol, unchanged by metoprolol (p < 0.05 vs. control and metoprolol groups), and slightly increased by propranolol (Fig. 2b). Levels of IL-12 were significantly increased by carvedilol (p < 0.01 vs. control and metoprolol groups) and propranolol (p < 0.05 vs. control and metoprolol groups), but not by metoprolol (Fig. 2c). Levels of IL-10 were unchanged by these three β-blockers (data not shown). Compared with a control value of 20.2 ± 4.8 pg/mg of heart, IFN-γ levels were 212.4 ± 27.2% in the carvedilol group, 137.5 ± 16.6% in the propranolol group, and 106.0 ± 17.4% in the metoprolol group (n = 5 in each). Levels of IL-12, compared with a control value of 5.1 ± 2.0 pg/mg of heart, were 275.0 ± 28.8%, 210.8 ± 36.2%, and 115.8 ± 39.1% in the carvedilol, propranolol, and metoprolol, respectively (n = 5 in each).

MYOCARDIAL VIRUS CONCENTRATION ON DAY 7. Figure 2d shows that, on day 7, the myocardial virus concentration of 1.5 ± 0.3 log pfu/mg heart in the carvedilol group was significantly lower (p < 0.05) than the concentrations of 2.5 ± 0.2 log pfu/mg heart in the metoprolol group and 2.5 ± 0.2 log pfu/mg heart in the control group, whereas the concentration in the propranolol group was only slightly lower than that in the control group (2.3 ± 0.2; n = 5 in each).

DISCUSSION

Carvedilol, cytokine production, and β-antagonism. β2-adrenergic agonists inhibit IL-12 and IFN-γ production in vitro (6–8). We have studied the effects of β-blockers and epinephrine on the production of IFN-γ in human peripheral blood mononuclear cells stimulated by concanavalin A (submitted manuscript). The production of IFN-γ was significantly suppressed by epinephrine. Both carvedilol and propranolol completely blocked these suppressive effects of epinephrine. However, the selective β1-blocker metoprolol had little effect. These results suggest that carvedilol blocked the effects of epinephrine by blocking β2-adrenergic stimulation. In this study, carvedilol improved survival, attenuated myocardial lesions, and raised IL-12 and IFN-γ levels, along with a decrease in the amount of myocardial virus.
Figure 2. Comparisons of the effects of carvedilol versus metoprolol and propranolol (mean ± SE). (a) Survival rate (n = 20 in each group). Circles = control group; squares = 30-mg/kg propranolol treatment group; diamonds = 30-mg/kg metoprolol treatment group; triangles = 10-mg/kg carvedilol treatment group. *p < 0.05 versus control, propranolol, and metoprolol groups. (b) Levels of IFN-γ in the heart on day 7 (n = 5 in each group). *p < 0.05 versus control and metoprolol groups. (c) Levels of IL-12 in the heart on day 7 (n = 5 in each group). *p < 0.05 and #p < 0.01 versus control and metoprolol groups. (d) Myocardial virus concentration on day 7 (n = 5 in each group). *p < 0.05 versus control and metoprolol groups. Carv = carvedilol; Met = metoprolol; Prop = propranolol.

Table 2. Effects of Carvedilol, Metoprolol, and Propranolol on HW/BW and Myocardial Histology on Day 7

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<thead>
<tr>
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<th>HW/BW (10⁻³)</th>
<th>Histologic Score</th>
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<tr>
<td></td>
<td></td>
<td>Necrosis</td>
<td>Infiltration</td>
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<tr>
<td>Control</td>
<td>7.8 ± 0.6</td>
<td>2.2 ± 0.1</td>
<td>2.0 ± 0.2</td>
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<tr>
<td>Metoprolol (30 mg/kg)</td>
<td>8.3 ± 0.6</td>
<td>2.4 ± 0.6</td>
<td>2.3 ± 0.5</td>
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<tr>
<td>Propranolol (30 mg/kg)</td>
<td>7.9 ± 0.3</td>
<td>1.3 ± 0.3*</td>
<td>1.6 ± 0.2</td>
<td></td>
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<tr>
<td>Carvedilol (10 mg/kg)</td>
<td>6.2 ± 0.7†</td>
<td>1.1 ± 0.1†</td>
<td>1.1 ± 0.2†</td>
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*p < 0.05 vs. control and metoprolol groups. †p < 0.01 vs. control and metoprolol groups. Data are presented as the mean value ± SE. Each group contained five mice.

HW/BW = heart weight to body weight ratio.
Propranolol, to a lesser degree, also attenuated the extent of myocardial lesions, raised IL-12 levels, and modestly increased IFN-γ, although the increase was not statistically significantly. Metoprolol, a selective β1-blocker, in contrast to the nonselective β-blockers carvedilol and propranolol, had no effect in this model. These results suggest that carvedilol exerts its therapeutic effects in part by an increase in IL-12 and IFN-γ production by blocking β2-adrenergic stimulation, and that endogenous catecholamines may regulate IL-12 and IFN-γ production in part by β2- instead of β1-adrenergic receptors in this model of viral myocarditis.

**Carvedilol, cytokine production, and antioxidant property.** It is noteworthy that carvedilol caused a direct and significant increase in concanavalin A–induced IFN-γ production, an effect not observed with propranolol or metoprolol (submitted manuscript). In this study, propranolol was less effective in increasing the production of IL-12 and IFN-γ, despite its administration in doses equivalent to a β2-blocking potency three times that of carvedilol (17). This suggests the participation of mechanisms other than β-adrenergic blockade. Carvedilol is an antioxidant and free radical scavenger (14), which inhibits the production of oxidized low-density lipoproteins (20) and the generation of oxygen radicals by neutrophils (21). Oxidized low-density lipoproteins are strong inhibitors of IL-12 and IFN-γ production (22). In lymphocytes, lipid peroxide is produced by stimulation of concanavalin A (23), and oxygen radicals play an important pathogenetic role in this model (24). Lipid peroxide and oxygen radicals suppress the production of IFN-γ and IL-12, and carvedilol may inhibit the suppressive effects of these oxidants. We have also studied the effects of carvedilol on nonstimulated peripheral blood mononuclear cells and found no IFN-γ production by carvedilol, which may not be a direct inducer of IFN-γ. Collectively, these results suggest that carvedilol increases the production of IFN-γ and IL-12 by blocking β2-receptors and antioxidant effects.

**Carvedilol and α1-blocking effects.** Besides being an antioxidant, carvedilol has α1-adrenergic receptor blocking properties (13). The effects of α1-adrenergic receptors on IL-12 and IFN-γ production have not been described. Until proven otherwise, the increase in IFN-γ production cannot be attributed the α1-blocking property of carvedilol. However, in view of our findings, in this same model in which there was an attenuation of myocardial lesions on day 14 after EMCV inoculation by the α1-blocker bunazosin (25), the α1-blocking property of carvedilol may have contributed to the therapeutic effects observed in this study.

**β-blockers and large clinical trials.** In recent randomized clinical trials, carvedilol, metoprolol, and bisoprolol (a selective β1-blocker) improved survival in patients with chronic heart failure (9–11). The results of the metoprolol and bisoprolol trials are not relevant to the observations made in this study. There are several causes of dilated cardiomyopathy, including viral infection, genetic factors, and immunologic dysfunction (26). However, at present, patients with dilated cardiomyopathy are not classified on the basis of these mechanisms of disease. The results of our experiments may not be directly relevant to the outcomes of clinical trials. Carvedilol and nonselective β-blockers without intrinsic sympathomimetic activity may be more protective than selective β1-blockers in chronic heart failure caused by viral myocarditis. In fact, in our earlier studies, nonselective β-blockers without intrinsic sympathomimetic activity were more cardioprotective than selective β1-blockers or nonselective β-blockers with intrinsic sympathomimetic activity (27) (unpublished data). In addition, bucindolol, a nonselective β-blocker with an α1-adrenergic receptor blocking effect, did not confer a survival benefit in patients with chronic heart failure (12). However, bucindolol has an agonist–like binding property to the β-receptors. This property, including stimulation of β2-receptors, may be one explanation for its neutral effects in clinical trials.

**Conclusions.** In this model of viral myocarditis, carvedilol, compared with metoprolol and propranolol, conferred the greatest therapeutic benefit by upregulating the production of IL-12 and IFN-γ and by decreasing the virus load.

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**REFERENCES**