EXPERIMENTAL STUDIES

Pimobendan Inhibits the Production of Proinflammatory Cytokines and Gene Expression of Inducible Nitric Oxide Synthase in a Murine Model of Viral Myocarditis

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OBJECTIVES	This study was designed to examine the effects of pimobendan in a murine model of viral myocarditis in relation to proinflammatory cytokine production and nitric oxide (NO) synthesis by inducible NO synthase (iNOS) in the heart.
BACKGROUND	Pimobendan has been recently confirmed to improve both acute and chronic heart failure. Since the modulation of myocardial necrosis and contractile dysfunction by various proinflammatory cytokines may be partially mediated by the production of nitric oxide, the effects of pimobendan on the production of proinflammatory cytokines and NO were investigated in an animal model of viral myocarditis involving heart failure.
METHODS	DBA/2 mice were inoculated with the encephalomyocarditis virus. To observe its effect on survival up to 14 days, pimobendan (0.1 mg/kg or 1 mg/kg) or vehicles were given from the day of virus inoculation (day 0) orally once daily. The effects of pimobendan on histological changes, cytokine production, NO production and iNOS gene expression in the heart were studied in mice treated either with pimobendan, 1 mg/kg or with vehicles only, and sacrificed seven days after virus inoculation.
RESULTS	The survival of mice improved in a dose-dependent fashion such that a significant difference ($p < 0.02$) was found between the higher-dose pimobendan group (20 of 30 [66.7%]) and the control group (11 of 30 [36.7%]). Histological scores for cellular infiltration (1.1 ± 0.1 vs. 2.0 ± 0.0, $p < 0.001$), intracardiac tumor necrosis factor (TNF)-α (18.2 ± 1.8 vs. 35.8 ± 4.2 pg/mg heart, $p < 0.001$) and interleukin (IL)-1β (9.3 ± 1.2 vs. 26.6 ± 7.1 pg/mg heart, $p < 0.01$) were significantly lower in the mice given pimobendan versus those of the control mice. Interleukin-6 levels (7.1 ± 0.8 vs. 9.2 ± 1.9 pg/mg heart) were also lower in the mice treated with pimobendan. Furthermore, intracardiac NO production was significantly ($p < 0.001$) less in the pimobendan group (0.165 ± 0.004 nmol/mg heart) than in the control group (0.291 ± 0.051 nmol/mg heart), and intracardiac iNOS gene expression in the mice given pimobendan was 74% lower than it was in the control animals ($p < 0.01$).
CONCLUSIONS	These findings suggest that the beneficial effects of pimobendan in viral myocarditis are partially mediated by the inhibition of both proinflammatory cytokine production and NO synthesis by iNOS. (J Am Coll Cardiol 1999;33:1400–7) © 1999 by the American College of Cardiology

Pimobendan, a phosphodiesterase (PDE) III inhibitor with additional calcium-sensitizing properties, has recently been confirmed to be effective in both acute and chronic heart failure (1–3). Pimobendan is known to acutely improve hemodynamics, increasing cardiac output and stroke volume while reducing ventricular filling pressures and peripheral resistance in patients suffering from severe congestive heart failure (4). Despite the demonstration of favorable short-term hemodynamic effects, recent multicenter trials of several PDE III inhibitors have yielded disappointing results. Not only did the PDE III inhibitors amrinone, milrinone and enoximone fail to demonstrate a significant improvement in chronic heart failure, but they increased the morbidity and mortality of patients with severe chronic

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Abbreviations and Acronyms

cAMP	=	cyclic adenosine monophosphate
EMC	=	encephalomyocarditis
iNOS	=	inducible nitric oxide synthase
IL	=	interleukin
LPS	=	lipopolysaccharide
mRNA	=	messenger RNA
NO	=	nitric oxide
PDE	=	phosphodiesterase
TNF	=	tumor necrosis factor

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heart failure (5–8). On the other hand, a multicenter trial found that pimobendan chronically increased exercise duration, peak oxygen uptake and quality of life in patients with severe congestive heart failure concomitantly treated with digoxin, diuretics and angiotensin-converting enzyme inhibitors (9,10). Pimobendan and other PDE III inhibitors seem to improve left ventricular performance by exerting positively inotropic as well as vasodilating effects. However, in comparison with other PDE III inhibitors, pimobendan possesses the unique additional property of increasing the affinity of cardiac contractile proteins for calcium which adds to its inotropic activity (11,12). Thus far, the advantage of pimobendan over the other PDE III inhibitors in treatment of chronic heart failure has been attributed to this calcium-sensitizing property.

Recent studies have revealed the roles played by various cytokines in diverse cardiovascular disorders (13). The levels of circulating tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and other proinflammatory cytokines have been found to be elevated in patients with myocarditis and acute myocardial infarction (14–17). Furthermore, circulating TNF-alpha has been reported to be increased in patients with chronic congestive heart failure, including in the context of myocarditis, transplant rejection and dilated cardiomyopathy (18–20).

In a murine model of encephalomyocarditis (EMC) virus-induced myocarditis, the majority of mice die of congestive heart failure due to severe myocarditis in the acute stage (21). The severity in the course of the disease appears to correlate with the degree of intracardiac production of proinflammatory cytokines (22). Recent studies have shown that several proinflammatory cytokines cause myocardial contractile dysfunction and that their negative inotropic effects may be mediated by nitric oxide (NO) (23,24). We previously demonstrated that pimobendan strongly inhibited the production of NO in cultured macrophages stimulated by lipopolysaccharide (LPS) (25). Thus, in the present study, we examined the effects of pimobendan in a murine model of viral myocarditis induced by EMC virus in relation to proinflammatory cytokine production and gene expression of inducible nitric oxide synthase (iNOS) in the heart.

METHODS

Viral infection. Four-week-old inbred male DBA/2 mice were inoculated intraperitoneally with 0.1 mL of the M (myocardiotrophic) variant of EMC virus diluted in Eagle's minimal essential medium to a concentration of 10 plaque-forming units/mL. The day of virus inoculation was defined as day 0 in all subsequent studies.

Drug preparation. Pimobendan (UD-CG 115 BS) was supplied by Nippon Boehringer Ingelheim Co., Ltd., Kawanishi, Japan. The drug was prepared as an oral suspension in 0.25% methylcellulose solution in concentrations of 120 μg/mL and 12 μg/mL, and stored at 4°C.

Protocol 1—survival studies. Since, in this model, most mice die of congestive heart failure within 14 days after EMC virus inoculation (21), the survival was observed up to 14 days in this study. Pimobendan was administered in doses of 0.1 mg/kg or 1 mg/kg daily for 14 days from the day of EMC virus inoculation while control mice received vehicles only. Thirty mice were randomly assigned to each group.

Protocol 2—histochemical and pathological studies. Since we had demonstrated in earlier studies that the expression of intracardiac TNF-α messenger RNA (mRNA) is maximal at seven days after virus inoculation in the same murine model, the effects of pimobendan on the production of TNF-α, other proinflammatory cytokines (IL-1β and IL-6) and NO in the heart was examined on day seven. On day 0 the mice were randomly assigned to receive pimobendan in a dose of 1 mg/kg orally once daily (n = 7 or 5) or vehicles only (n = 5). The animals were sacrificed by cervical dislocation under general anesthesia, seven days after EMC virus inoculation. Following measurements of body and heart weights, the hearts were divided into two sections along their short axis at the midleft ventricular level. The apical sections were used to determine intracardiac levels of cytokines and NO and the basal sections were used for histological examination. For the measurements of iNOS gene expression, the mice were randomly assigned to receive pimobendan in a dose of 1 mg/kg orally once daily (n = 6) or vehicles only (n = 5), and the hearts were harvested in the same manner.

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

Assay of intracardiac cytokines. Frozen sections of hearts were mixed with 1.5 ml of PBS/0.05% NaN3 and homogenized with an ultrasonic liquid processor (ASTRASON™ Model XL2020, Misnox Inc., Farmingdale, New York). Supernatants containing cytosolic protein were used to detect the intracardiac cytokines with commercially available enzyme-linked immunosorbent assay (ELISA) kits (TNF-α and IL-6: GENZYME Co., Cambridge, Massachusetts; IL-1β: BioSource International Co., Camarillo, California). The procedures were performed according to the manufacturer's recommendations. The detection limits of TNF-α, IL-1β and IL-6 assays were 15 pg/mL, 7 pg/mL and 5 pg/mL, respectively. Final results were expressed as pg/mg of heart tissue.

Measurement of intracardiac NO production. Since nitrite/nitrate are endproducts of NO, their production in heart tissue was calculated from the levels of intracardiac nitrite/nitrate measured by the Griess reaction (26,27). Frozen sections of hearts were homogenized with 1.5 ml of PBS as described above. After centrifugation, the supernatant was mixed with nitrate reductase (0.25 U/ml) and incubated for 15 min at 37°C in the presence of 5 mM NADPH. Fifty μl of the samples were incubated with 25 μl of 1% sulfanilamide and 25 μl of 0.1% N-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid for 10 min. The absorbance at 540 nm (OD_{540}) was measured with a microplate reader (Multiskan BICHROMATIC, Labsystems, Finland). Nitrite/nitrate concentrations were calculated by comparison with OD_{540} of standard solutions of sodium nitrite prepared in PBS. The final results were expressed as nmol/mg of heart tissue.

Measurement of iNOS mRNA levels using the competitive reverse-transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted from the hearts by the guanidinium thiocyanate-phenol-chloroform-isoamylalcohol procedure. The RNA concentration was measured spectrophotochemically. Five μg of total RNA was subjected to first strand cDNA synthesis in a 40 μL reaction mixture using murine leukemia virus reverse transcriptase (Gibco BRL, Rockville, Maryland). The reaction mixture was incubated at 37°C for 60 min, heated to 70°C for 5 min to denature the reverse-transcriptase then cooled on ice for 3 min. Sixty μL of water was added to each sample.

Quantitative competitive PCR was developed as described previously (28). Using a commercially available kit (PCR MIMIC™ Construction Kit, CLONETECH Laboratories Inc., Palo Alto, California), nonhomologous DNA fragment with iNOS or glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene-specific primer templates was constructed to be 148 or 184 nucleotides shorter than each PCR product generated from native iNOS or G3PDH cDNA, respectively. The primer sites for PCR were located at nucleotides 2943 to 2967, and 3416 to 3440 of iNOS cDNA and 51 to 75, and 816 to 840 of G3PDH cDNA.

The sense primer for iNOS was 5′ CCC TTC CGA AGT TTC TGG CAG CAG C 3′ and the antisense primer was 5′ GGC TGT CAG AGC CTC GTG GCT TTG G 3′. The sense primer for G3PDH was 5′ TGA AGG TCG GTG TGA ACG GAT TTG G 3′ and the antisense primer was 5′ TCA GAT GCC TGC TTC ACC ACC TTC T 3′. One μL of the samples and 1 μL of two-fold serial dilutions of the nonhomologous DNA fragment was added to 23 μL of the PCR master mix, which contained thermostable Taq polymerase (PERKIN ELMER Japan Co., Tokyo, Japan), the gene-specific primers, dNTP, PCR buffer and [α-^{32}P]-dCTP. The two-fold serial dilutions of constructed DNA, for example, were 1.0, 5.0×10^{-1}, 2.5×10^{-1}, 1.25×10^{-1}, 6.25×10^{-2} and 3.125×10^{-2} attomoles/μL (1 attomole $= 6 \times 10^{5}$ molecules). After having heated the PCR mixture to 95°C for nine min to activate thermostable Taq polymerase, PCR was performed for 35 cycles, each consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. Ten μL of each PCR product was separated by electrophoresis on a 5% polyacrylamide gel. The gels were dried, autoradiographed with Fuji imaging plates (Fuji Photo Film Co., Ltd., Tokyo, Japan) and analyzed with the use of a Fujix BAS 2000 image analyzer (Fuji Photo Film Co., Ltd.). The log of the ratio of iNOS or G3PDH target radioactivity to the nonhomologous DNA fragment radioactivity was plotted against the log of the number of the nonhomologous DNA fragment molecules added to each PCR reaction tube. The number of iNOS or G3PDH cDNA was calculated from these plots. The values obtained were expressed as attomoles/μg of total RNA. Each value of iNOS cDNA was divided by that of the corresponding G3PDH cDNA to be normalized. Finally, iNOS cDNA levels, which were found to be equivalent to iNOS mRNA levels, were expressed as percentages of G3PDH cDNA levels.

Statistical analysis. Survival was analyzed by the Kaplan-Meier method. The χ-square test was used for the final survival rates. Student t test was used for cytokine levels, nitrite/nitrate concentration levels, iNOS mRNA levels, body weight, heart weight, heart weight to body weight ratio and histological scores. Data are expressed as mean $\pm$ SEM. Statistical significance was defined as $p < 0.05$.

RESULTS

Survival of the animals. Survival rates up to one week showed little difference among the three groups of animals. However, after day seven, the survival of mice treated with pimobendan began to improve in a dose-dependent manner (Fig. 1). The final survival rates on day 14 were 36.6% in the control group, 53.3% in the pimobendan 0.1 mg/kg group and 66.7% in the pimobendan 1 mg/kg group. The difference between the control group and the higher-dose pimobendan group was statistically significant ($p < 0.02$). Thus, pimobendan exerted beneficial effects on survival in this murine model of EMC virus-induced myocarditis.
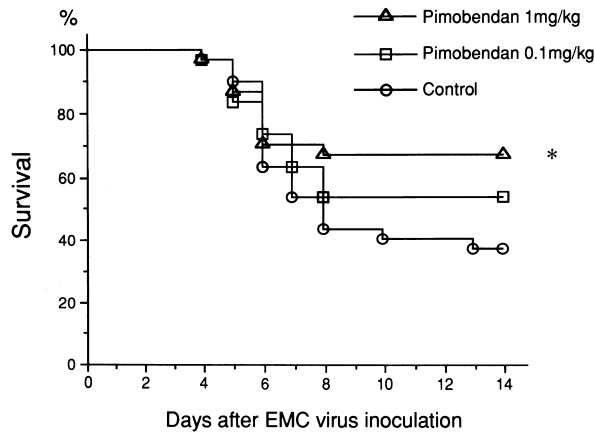
Figure 1. Effect of pimobendan on survival rate after EMC virus inoculation. Beyond seven days, the survival rate was improved in a dose-dependent manner. Treatment with pimobendan, 1 mg/kg/day, significantly reduced mortality on day 14. * = p < 0.02 versus control. Each group consists of thirty mice.

Histopathology of the heart on day seven. Figure 2 illustrates the histopathological changes. Histopathological scoring of the myocardium showed that the myocardial cellular infiltration of the pimobendan 1 mg/kg group was significantly reduced compared with the control group (p < 0.001) while no statistically significant difference was found in myocardial necrosis between the two groups (Table 1). There were no significant differences in the heart weight to body weight ratio between the control group and the pimobendan 1 mg/kg group.

Intracardiac cytokines. Figure 3 shows the proinflammatory cytokine protein levels in the whole heart homogenates of the control group and the pimobendan 1 mg/kg group. The intracardiac TNF-α level of the pimobendan 1 mg/kg group (18.0 ± 1.8 pg/mg heart, n = 7) was significantly less (p < 0.001) than that of the control group (35.8 ± 4.2 pg/mg heart, n = 5). The intracardiac IL-1β level of the pimobendan 1 mg/kg group (9.3 ± 1.2 pg/mg heart, n = 7) was also significantly less (p < 0.01) than that of the control group (26.6 ± 7.1 pg/mg heart, n = 5). Although the intracardiac IL-6 level in the pimobendan 1 mg/kg group (7.1 ± 0.8 pg/mg heart, n = 7) was also less than that in the control group (9.2 ± 1.9 pg/mg heart, n = 5), the difference did not reach statistical significance (p = 0.23).

Intracardiac NO production. The nitrite/nitrate concentrations in the hearts are shown in Figure 4. The intracardiac nitrite/nitrate concentrations in the control group (n =
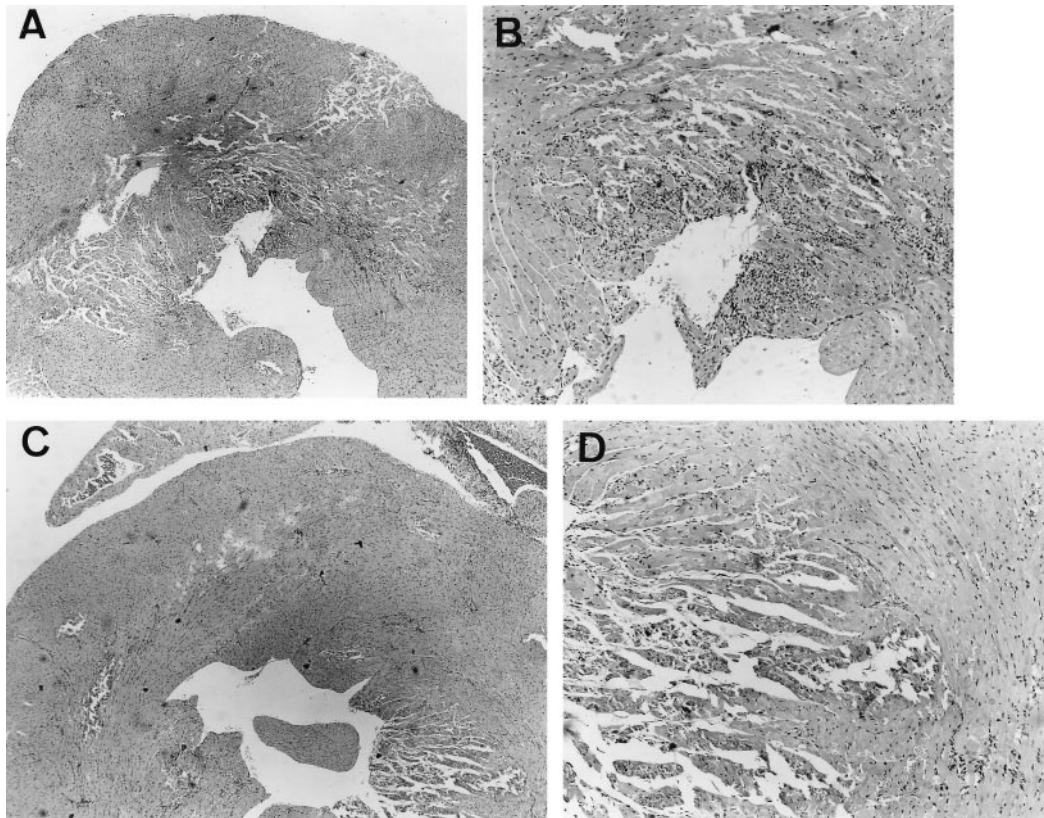
Figure 2. Typical histopathologic changes found seven days after EMC virus inoculation. **A** and **B:** Heart from a control mouse. **C** and **D:** Heart from a mouse treated with pimobendan 1 mg/kg. Extensive myocardial necrosis and cellular infiltration are found in the control preparation. In the mouse treated with pimobendan, less myocardial cellular infiltration exists despite an equivalent amount of myocardial necrosis. Hematoxylin and eosin stain—original magnification: **A** and **C,** × 40, reduced by 65%; **B** and **D,** × 200, reduced by 65%.
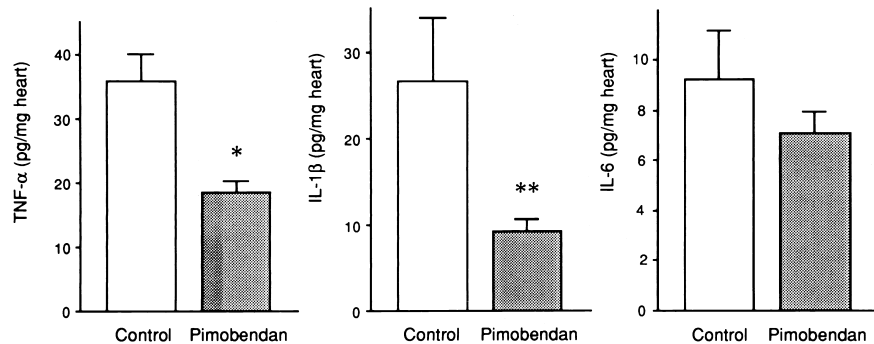
Figure 3. Effect of pimobendan on intracardiac production of TNF-α, IL-1β, and IL-6 measured on day seven after EMC virus inoculation. The intracardiac TNF-α and IL-1β level in animals treated with pimobendan 1 mg/kg (n = 7) were significantly lower than those in the control group (n = 5). * = p < 0.001 versus control. ** = p < 0.01 versus control. The intracardiac IL-6 level in the animals treated with pimobendan 1 mg/kg was also less than in the control group, but the difference did not reach statistical significance.

5) and the pimobendan 1 mg/kg group (n = 5) were 0.291 nmol/mg heart and 0.165 nmol/mg heart, respectively. The intracardiac nitrite/nitrate concentration in the pimobendan 1 mg/kg group was 43 percent lower than that in the control group (p < 0.001).

Intracardiac iNOS gene expression. Figures 5A and 5B are representative autoradiographs and plots of their densitometrical results. PCR of the nonhomologous DNA fragment with iNOS or G3PDH primer sites yielded a product which was 148 or 184 nucleotides shorter than the product derived from the native cDNA, respectively. The log of the ratio of iNOS or G3PDH PCR product to the nonhomologous DNA fragment was plotted against the log of the number of the nonhomologous DNA fragment molecules added. The number of iNOS or G3PDH cDNA molecules was obtained from the point where log (iNOS or G3PDH/ the nonhomologous DNA fragment) was equal to zero. The percentages of iNOS mRNA levels against G3PDH mRNA levels in the control group (n = 5) and the pimobendan 1 mg/kg group (n = 6) were 59.5 ± 11.6% and 15.4 ± 4.4%, respectively. Thus, iNOS mRNA expression

in the pimobendan 1 mg/kg group was 74 percent lower than that in the control group (p < 0.01, Fig. 5C).

DISCUSSION

Pimobendan is a PDE III inhibitor with additional calcium-sensitizing properties. Recent trials have shown that pimobendan is effective not only in acute but also in chronic heart failure (3,4). Pimobendan improves hemodynamics acutely, increasing cardiac output and stroke volume and reducing ventricular filling pressures and peripheral resistance in the failing heart (4) while chronically it increases exercise duration, peak oxygen uptake and quality of life in patients with severe chronic congestive heart failure (9,10). On the other hand, several PDE III inhibitors other than pimobendan and vesnarinone have been ineffective in the treatment of chronic heart failure (5–8). A recent series of investigations from our laboratory suggest that vesnarinone has immunomodulating properties and that it exerts its beneficial effect in part via such properties. Indeed, vesnarinone lowered the mortality both in a murine model of viral myocarditis (29) and in a murine model of lethal endotoxemia (30).

We have previously studied the effects of PDE III inhibitors, pimobendan, vesnarinone and amrinone on the production of NO in cultured macrophages stimulated by LPS (25). Pimobendan most strongly inhibited NO production. Thus, in the present study, the effects of pimobendan were investigated in a murine model of viral myocarditis in the perspective of the production of proinflammatory cytokines and iNOS gene expression in the heart. The doses of pimobendan chosen for the experiments were 1 mg/kg/day and 0.1 mg/kg/day. A previous pharmacokinetic study in rats who had received a comparable oral dose of pimobendan (31) suggests that its plasma and heart tissue concentrations in the present study were close to the concentrations found effective in our previous in vitro studies (25,32). A dose of 1 mg/kg/day in the present study is approximately ten times higher than the dose used in the treatment of chronic heart failure in humans and thus, may
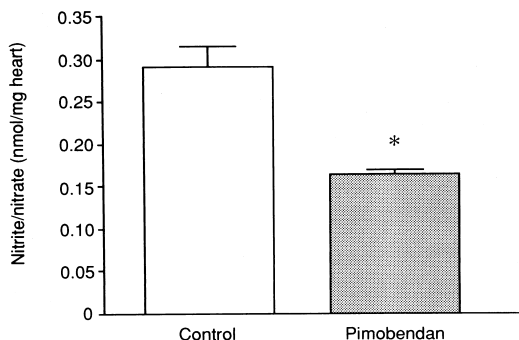
Figure 4. Effect of pimobendan on intracardiac NO production. The concentrations of nitrite/nitrate, stable endproducts of NO, in the pimobendan 1 mg/kg group (n = 5) were 43% lower than in the control group (n = 5). * = p < 0.01 versus control.
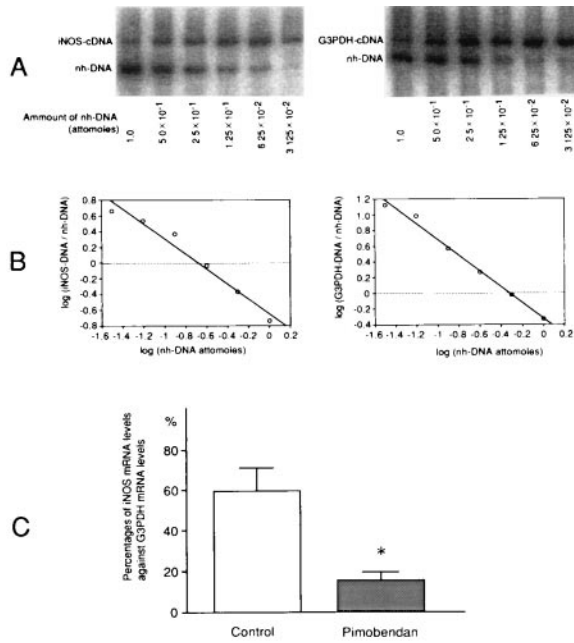
Figure 5. Quantification of iNOS mRNA in the hearts using RT-PCR. **A:** Total RNA was extracted from the hearts in the pimobendan 1 mg/kg group and the control group. Each PCR product was separated by electrophoresis on a 5% polyacrylamide gel. The gels were dried and autoradiographed. nh-DNA = nonhomologous DNA. **B:** The log of the ratio of iNOS or G3PDH target radioactivity to the nh-DNA fragment radioactivity was plotted against the log of the number of the nh-DNA fragment molecules added to each PCR reaction. The number of iNOS or G3PDH cDNA molecules was obtained from the point where log (iNOS or G3PDH/the nh-DNA fragment) was equal to zero. **C:** The number of iNOS or G3PDH cDNA was expressed as attomoles/μg of total RNA. The iNOS gene expression in the pimobendan 1 mg/kg group was 74% lower than that of the control group. * = p < 0.01 versus control.

seem to have been inappropriately high. However, on the basis of body surface area, a dose of 1 mg/kg in mice is equivalent to 0.08 mg/kg in humans, which is within the range used clinically (33). Pimobendan improved the 14-day survival of the animals in a dose-dependent manner. On histological examination, myocardial cellular infiltration was significantly reduced in mice given pimobendan in a dose of 1 mg/kg, and myocardial necrosis tended to be reduced. Since both cytokines and NO are known to act mainly in an autocrine or paracrine fashion, it is important to measure their production at the level of a particular tissue. The productions of TNF-α and IL-1β in the heart tissue were reduced significantly in the pimobendan 1 mg/kg group, and IL-6 showed a similar tendency. Furthermore, intracardiac NO production and iNOS gene expression were also significantly reduced by pimobendan.

Proinflammatory cytokines and NO in myocarditis and the failing heart. Several clinical studies have described the participation of proinflammatory cytokines in the pathogenesis of cardiac diseases. The levels of circulating proinflam-

matory cytokines such as TNF-α, IL-1 and IL-6 are elevated in patients with myocarditis (14). It has also been reported that levels of circulating IL-1, IL-6 and IL-8 are elevated in patients with acute myocardial infarction (15–17). A recent report from our laboratory has shown that, in the same murine model of viral myocarditis, the intracardiac expression of TNF-α, IL-1β, IFN-γ and IL-2 genes was increased at three days and reached maximal levels at seven days after virus inoculation (22). The degree of their expression correlated with the severity in the course of the disease, suggesting that the overproduction of these proinflammatory cytokines by viral infection may aggravate the disease. This is supported by the recent reports that the overexpression of TNF-α in the heart caused severe myocarditis and cardiomegaly in transgenic mice (34) and that IL-1β as well as TNF-α alone promoted coxsackie virus B3-induced myocarditis in resistant mice (35). We previously showed that treatment with anti-TNF-α antibody had improved both mortality and myocardial damage in the acute stage of this myocarditis model (36), indicating that the overproduction of TNF-α aggravates viral myocarditis.

Some experimental studies have indicated that these proinflammatory cytokines may contribute to myocardial contractile dysfunction. Infusion of TNF-α reversibly impaired left ventricular systolic and diastolic function in conscious dogs (37). Tumor necrosis factor-α, IL-6 and IL-2 inhibited contractility of isolated hamster papillary muscles in a concentration-dependent and reversible manner (23). Tumor necrosis factor-α exerted a direct negative inotropic effect in both the human (38) and feline (39) left ventricle and in isolated cardiac myocytes. Interleukin-1β also depressed myocardial contractility in the isolated perfused rat heart (40). In addition to cardiac contractile dysfunction, both TNF-α and IL-1β are known to cause neonatal rat cardiac myocyte hypertrophy in vitro (41,42). Overexpression of both IL-6 and IL-6 receptor also caused ventricular myocardial hypertrophy in the transgenic mice (43). Myocardial hypertrophy is considered to compensate the failing heart at first but to be profoundly related to ventricular remodeling leading to heart failure.

Although most of the signaling pathways of cardiac contractile dysfunction related to proinflammatory cytokines are still unclear, recent evidence suggests that the direct negative inotropic effect of these cytokines may be mediated by NO (23,44,45). Some experimental studies have shown that IL-1β and IFN-γ induce iNOS in both adult rat ventricular myocytes (46) and intracardiac microvascular endothelial cells (47), suggesting that the overproduction of NO in myocytes and endothelial cells by proinflammatory cytokines may depress myocyte contractility in an autocrine or paracrine fashion.

Inhibition of NO production in myocarditis. We have shown here that both NO production and the expression of iNOS gene in the heart were also significantly decreased by pimobendan. We have previously reported the effect of NO

inhibition in the same model (48). Immunohistochemical studies showed that macrophages and endothelium-like cells stained positively for iNOS protein in the myocardium of virus-inoculated mice but negatively in uninfected mice. Furthermore, L-NMMA, an inhibitor of NOS, decreased both cellular infiltration and myocardial necrosis significantly. These results indicate a beneficial effect of NOS inhibition in this model. This is supported by another recent study in which the inhibition of NO synthesis by a relatively specific iNOS inhibitor decreased myocardial inflammation and necrosis in a rat autoimmune myocarditis (49). Opposite observations have been reported of the promotion of viral replication and myocardial damage (50) or of increased mortality (51) in murine coxsackie virus B3-induced myocarditis by NOS inhibition. Although these reports appear to contradict our results, it is likely that different degrees of NO inhibition cause different responses: excessive inhibition of NO may fail to suppress viral replication, resulting in aggravation of myocarditis, whereas more moderate inhibition of NO may salvage myocardial damage and contractile dysfunction. The degree of NO inhibition by pimobendan might be appropriate to improve the disease in the present model.

Mechanism of action of pimobendan. The mechanism by which pimobendan reduces the production of proinflammatory cytokines and iNOS gene expression in the heart is unclear. In our previous study, various PDE inhibitors differentially inhibited the production of cytokines by human peripheral blood mononuclear cells stimulated by LPS (31). Interestingly, most PDE inhibitors inhibited cytokine production, though not 8 Br-cAMP. In addition, we studied the effects of various PDE III inhibitors on the production of NO in LPS-activated macrophages (25). Although pimobendan strongly inhibited NO production, 8 Br-cAMP did not suppress, but increased, NO production. Thus, the inhibitory effects of pimobendan on the production of proinflammatory cytokines and iNOS gene expression cannot be explained by an increase in cAMP.

In the earlier in vitro study, pimobendan decreased the production of TNF-α and IL-6 and increased IL-1β production, whereas, in the present study, IL-1β production and TNF-α were both significantly reduced by pimobendan. Since different stimuli cause different patterns of cytokine induction in vitro and in vivo, it is hypothesized that there may be differences in the mode of cytokine induction between the in vitro model and the present in vivo model where viral infection is the main cause of the disease.

Conclusions. In conclusion, this study showed a therapeutic effect of pimobendan in the acute stage of viral myocarditis, mediated in part by inhibition of the inflammatory response, of the production of proinflammatory cytokines and of the synthesis of NO by iNOS. Pimobendan may be of great value in the treatment not only of viral myocarditis with heart failure but also of other cardiac diseases in which the overproduction of proinflammatory cytokines and NO play a significant pathogenetic role.

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