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Nitric Oxide and Murine Coxsackievirus B3 Myocarditis: Aggravation of Myocarditis by Inhibition of Nitric Oxide Synthase

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Objectives. **This study sought to investigate the effects of nitric oxide inhibition in a murine model of coxsackievirus B3 myocarditis.**

Background. **Little is known about the contribution of nitric oxide to the pathophysiology of myocarditis.**

Methods. **Antiviral activity was tested in vitro using nitric oxide inhibition by treatment with activated macrophages of N^G-nitro-L-arginine methyl ester. In the in vivo experiments, N^G-nitro-L-arginine methyl ester and N^G-nitro-D-arginine methyl ester (both at 100 μg/ml) were administered to C3H/He mice early (days 0 to 14) and late (days 14 to 35) after infection with coxsackievirus B3.**

Results. **In the in vitro experiments with interferon-gamma- and lipopolysaccharide-induced activated murine macrophages, treatment with the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester, but not its inactive enantiomer N^G-nitro-D-arginine methyl ester, restored coxsackievirus B3 titers. In the in vivo experiments in the early treatment group, myocardial virus titers were higher in N^G-nitro-L-arginine methyl ester-treated than infected untreated animals, and both inflammatory cell infiltration and necrosis were more severe. In the late treatment group, more severe necrosis and more dense myocardial and perivascular fibrosis were observed in N^G-nitro-L-arginine methyl ester-treated than in infected untreated animals. N^G-Nitro-D-arginine methyl ester administration was ineffective.**

Conclusions. **Nitric oxide inhibition increases myocardial virus titers, resulting in the aggravation of cardiac pathology in the early stage of coxsackievirus B3 myocarditis. In the late stage, it induces more severe cardiomyopathic lesions. Nitric oxide plays a defensive role in the pathogenesis of coxsackievirus B3 myocarditis.**

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Coxsackievirus B3 is an enterovirus that can cause acute myocarditis in humans and mice (1–3). T cells and cytokines, especially interleukin-2 and interferons, are involved in the pathogenesis of acute coxsackievirus B3 myocarditis (4–8).

Nitric oxide is a gaseous free-radical molecule that has been shown (9–13) to be a mediator of vital physiologic functions. Recent evidence has suggested (14) that nonspecific immunity is associated with the induction of nitric oxide synthase. Many cell types are capable of producing nitric oxide through the enzymatic conversion of L-arginine to L-citrulline by nitric oxide synthase (9–13). In inflamed tissue, inducible nitric oxide synthase is expressed by macrophage production in large quantities of nitric oxide and low pulsatile production of nitric oxide through constituent nitric oxide synthase from the endothelium. In addition, a potential dual role of nitric oxide has been reported (14).

In the present study, we investigated the effects of inhibition of nitric oxide synthase on coxsackievirus B3 replication with an in vitro viral sensitivity assay. This investigation was followed by an in vivo experiment in mice with coxsackievirus B3 myocarditis treated with nitric oxide synthase inhibitors.

Methods

We performed not only in vitro experiments, in which murine macrophages were treated with the inhibition of nitric oxide synthase, but also in vivo experiments, in which coxsackievirus B3-infected mice were treated with the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester and its inactive analogue N^G-nitro-D-arginine methyl ester, and survival rates and histopathologic findings were examined.

In Vitro Study

The in vitro viral sensitivity protocol was performed according to recently published methods (15,16) with minor modifi-

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cations. After confirming that macrophages, on stimulation with interferon (IFN)-gamma and lipopolysaccharide, decrease coxsackievirus B3 titers, we determined whether the presence of N^G-nitro-L-arginine methyl ester reverses this effect, resulting in an increase in viral titers.

Virus and cells. The murine macrophage-like cell line RAW264.7 (17) (TIB71, American Type Culture Collection) and VERO African green monkey kidney cells were used. RAW264.7 cells are murine macrophages transformed with the Abelson leukemia virus that produce nitric oxide when stimulated with IFN-gamma or lipopolysaccharide, or both (17).

Coxsackievirus B3 (myocarditic Nancy strain; American Type Culture Collection) was used. Virus stock had a titer $>10^9$ plaque-forming units/0.1 ml. Virus suspensions were stored at −80°C until use (4–8).

Procedures. Commercially available N^G-nitro-L-arginine methyl ester and the inactive enantiomer N^G-nitro-D-arginine methyl ester (Research Biochemicals International) were used. RAW264.7 cells were inoculated into six-well plates (3×10^5/well) and incubated at 37°C for 24 h. The cells were activated with INF-gamma (50 U/ml) and lipopolysaccharide (50 ng/ml). Two concentrations (270 and 2,700 μmol/liter) of N^G-nitro-L-arginine methyl ester or N^G-nitro-D-arginine methyl ester were then added immediately to these cells. Six hours later, treated cells were infected with coxsackievirus B3 at a multiplicity of infection of 5 plaque-forming units/cell. Twenty-four hours later, the infected cells were treated by freezing and thawing. The virus titers in the supernatants were determined by a previously described plaque assay method (4–8).

In Vivo Study

Infection protocol. Two-week old male, inbred, certified virus-free C_3H/He mice (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were used. The animals were inoculated intraperitoneally with 0.1 ml of virus suspension containing 10^3 plaque-forming units. All animals were cared for in accordance with the institutional policies and guidelines of Toyama Medical and Pharmaceutical University.

Treatment protocol. C_3H/He mice were infected with coxsackievirus B3, and N^G-nitro-L-arginine methyl ester and N^G-nitro-D-arginine methyl ester were administered orally by means of the drinking water (100 μg/ml), beginning immediately after virus inoculation until day 14 (experiment I: N^G-nitro-L-arginine methyl ester, n = 53; N^G-nitro-D-arginine methyl ester, n = 47) and from day 14 until day 35 (experiment II: N^G-nitro-L-arginine methyl ester, n = 16; N^G-nitro-D-arginine methyl ester, n = 16). Control infected and untreated mice (experiment I, n = 42; experiment II, n = 16) were also prepared. The mice consumed N^G-nitro-L-arginine methyl ester or N^G-nitro-D-arginine methyl ester ad libitum, and daily fluid consumption was monitored; there were five animals per cage, and all were uniformly treated. Estimated dose (42 mg/kg body weight per day) was based on fluid consumption and drug concentration. This oral route of administration, in the dose range studied, has been shown (14) to produce systemic inhibition of nitric oxide synthase.

In experiment I, six mice on day 4 and 13 mice on day 7 in each of the three groups were killed for evaluation of early cardiac pathologic and virologic analysis. Thus, 34 mice in the N^G-nitro-L-arginine methyl ester group, 28 mice in N^G-nitro-D-arginine methyl ester group and 23 mice in the control group were used for survival analysis. Before starting experiment II, an additional five mice from each of the three groups were killed to confirm the homogeneity of the groups. Mice were observed daily, and complete autopsy studies were performed in those found dead.

In parallel with experiment I, additional control groups of uninfected mice (five treated with N^G-nitro-L-arginine methyl ester, five treated with N^G-nitro-D-arginine methyl ester, five untreated) were also studied for 14 days.

Virus titers of heart. For infectivity assays, hearts were removed aseptically, weighed and homogenized in 2 ml of Eagle's minimum essential medium. Plaque assays of the supernatants were performed (4,5).

Pathologic analysis. The hearts were processed by standard methods and stained with hematoxylin-eosin and Mallory-Azan to identify repairing fibrosis and elastic van Gieson to evaluate the remodeling of intramyocardial arteries. Myocardial sections were graded by two of the authors (Y.H., C.K.) who had no knowledge of the respective treatment groups on a scale of 1+ (mild) to 5+ (very severe) for severity of cellular infiltration, necrosis, calcification, or interstitial and perivascular fibrosis (7,8). Architectural changes in coronary vasculature were also noted.

Statistics. One-way analysis of variance with multiple sample comparisons (Bonferroni) was used to evaluate differences in the virus titers of the hearts and pathologic scores. The Kaplan-Meier method was used to determine the differences in survival rates (18). Results are reported as mean value ± SD.

Results

In Vitro Study

Effects of N^G-nitro-L-arginine methyl ester and N^G-nitro-D-arginine methyl ester on coxsackievirus B3 replication in macrophages (Fig. 1). In this experiment, nitric oxide synthase inhibition was used to determine whether nitric oxide or other reactive nitrogen intermediates contributed to the inhibition of coxsackievirus B3 replication in macrophages. As shown in Figure 1, on stimulation with INF-gamma and lipopolysaccharide, macrophages decreased virus titers, and treatment with N^G-nitro-L-arginine methyl ester, but not N^G-nitro-D-arginine methyl ester, substantially reduced the ability of INF-gamma- and lipopolysaccharide-stimulated macrophages to inhibit coxsackievirus B3 replication. Thus, treatment with N^G-nitro-L-arginine methyl ester restored coxsackievirus B3 titers in activated macrophages.

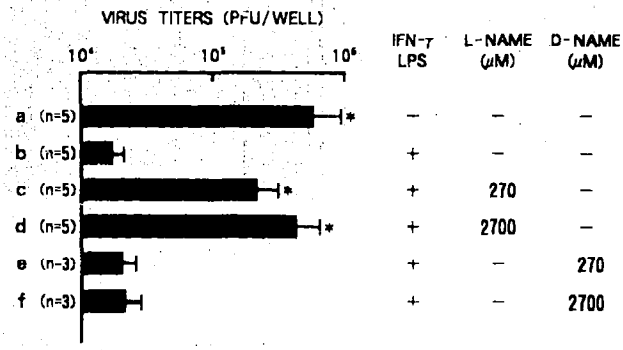

Figure 1. Viral sensitivity of N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-D-arginine methyl ester (D-NAME). Inhibitors of nitric oxide synthase reversed the antiviral effects of interferon-gamma (IFN-γ)- and lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages inoculated with coxsackievirus B3. Confluent monolayers of RAW264.7 macrophages were activated by INF-gamma (50 U/ml) and lipopolysaccharide (50 ng/ml). Then, two concentrations (270 and 2,700 μmol/liter) of N^G-nitro-L-arginine methyl ester or N^G-nitro-D-arginine methyl ester were added immediately to the cells. Six hours later, these cells were inoculated with coxsackievirus B3, at a multiplicity of infection of 5 plaque-forming units (PFU)/cell. Viral titers were determined 24 h after infection. N^G-Nitro-L-arginine methyl ester **(bars c and d)**, but not N^G-nitro-D-arginine methyl ester **(bars e and f)**, substantially reduced the ability of activated macrophages to inhibit coxsackievirus B3 replication **(bar b)**. **Bar a** = control (i.e., titer of coxsackievirus B3 alone). $p < 0.01$ for bar a versus bars b, c and f; bar b versus bars c and d; bar c versus bars e and f; bar d versus bars e and f. *$p < 0.01$ versus bar b (see text for details).

Table 1. Cardiac Pathology in Experiments I and II

	Control Group	L-NAME Group	D-NAME Group
Experiment I			
Day 4			
Infiltration	0.9 ± 0.4	1.8 ± 0.4*	1.3 ± 0.5
Necrosis	0.5 ± 0.6	1.2 ± 0.7	0.8 ± 0.6
Calcification	0.5 ± 0.5	1.2 ± 0.6	0.7 ± 0.5
	(n = 6)	(n = 6)	(n = 6)
Day 7			
Infiltration	2.6 ± 0.7	3.7 ± 0.9*	3.3 ± 0.9
Necrosis	2.2 ± 0.8	3.2 ± 0.8*	2.7 ± 0.8
Calcification	2.0 ± 0.4	2.9 ± 0.6*	2.5 ± 0.6
Fibrosis	0.3 ± 0.3	0.5 ± 0.4	0.4 ± 0.3
	(n = 13)	(n = 13)	(n = 13)
Day 14			
Infiltration	2.7 ± 0.8	3.5 ± 1.1*	3.0 ± 0.7
Necrosis	2.3 ± 0.6	3.1 ± 0.8*	2.8 ± 0.8
Calcification	2.1 ± 0.7	2.8 ± 0.6*	2.3 ± 0.7
Fibrosis	1.5 ± 0.6	2.3 ± 0.4*	1.8 ± 0.6
	(n = 23)	(n = 34)	(n = 28)
Experiment II			
Day 35			
Infiltration	0.6 ± 0.5	2.0 ± 0.7†	1.2 ± 0.6
Necrosis	0.4 ± 0.7	1.8 ± 0.8†	1.0 ± 0.6
Calcification	0.5 ± 0.6	1.6 ± 0.5†	1.0 ± 0.7
Fibrosis	0.6 ± 0.6	1.9 ± 0.5†	1.1 ± 0.6
	(n = 16)	(n = 16)‡	(n = 16)

*$p < 0.05$, †$p < 0.01$ versus control. ‡Hearts of two mice that died on days 16 and 17 were included. Data presented are mean value ± SD. D-NAME = N^G-nitro-D-arginine methyl ester; L-NAME = N^G-nitro-L-arginine methyl ester.

In Vivo Study

Experiment I: effects of nitric oxide inhibition on acute viremic stage. *Survival rate.* Three mice in the control group, 6 in the N^G-nitro-D-arginine methyl ester group and 14 in the N^G-nitro-L-arginine methyl ester group had died by day 14, corresponding to survival rates on day 14 of 87.0% (20 of 23), 78.6% (22 of 28) and 58.8% (20 of 34), respectively. The difference between the control group and the N^G-nitro-L-arginine methyl ester-treated group was significant ($p < 0.05$ by the Kaplan-Meier method).

Myocardial virus titers. Myocardial virus titers on day 4 ($4.4 \pm 3.5 \times 10^3$ plaque-forming units/mg tissue, n = 6) and day 7 ($1.0 \pm 0.9 \times 10^4$, n = 13) were significantly higher ($p < 0.05$) in the N^G-nitro-L-arginine methyl ester group, but not in the N^G-nitro-D-arginine methyl ester group (day 4: $2.2 \pm 1.2 \times 10^3$, n = 6; day 7: $4.5 \pm 3.8 \times 10^3$, n = 13), than in the control group (day 4: $9.0 \pm 4.8 \times 10^2$, n = 6; day 7: $3.3 \pm 3.4 \times 10^3$, n = 13). No virus was isolated from the hearts on day 14 in either group (each n = 4).

Pathologic analysis (Table 1). Cellular infiltration was more severe on days 4, 7 and 14 in the N^G-nitro-L-arginine methyl ester group than in the control group. Myocardial necrosis and calcification on days 7 and 14, but not on day 4, were more severe in the N^G-nitro-L-arginine methyl ester group than in the control group. Myocardial fibrosis on day 14, but not on day 7, was more severe in the N^G-nitro-L-arginine methyl ester group than in the control group. Myocardial fibrosis on day 4 was not evaluated. Multifocal lesions of thin perivascular fibrosis adjacent to necrotic myocytes were noted in all three groups. However, there were no significant pathologic abnormalities in the coronary vasculature among the three groups. N^G-nitro-D-arginine methyl ester administration was ineffective.

Uninfected groups. No mice died by day 14 in any of the groups. Pathologic examination revealed no abnormalities in the myocardium in any group.

Experiment II: effects of nitric oxide inhibition on chronic aviremic stage. *Survival rate.* Only two mice (2 [12.5%] of 16) in the N^G-nitro-L-arginine methyl ester group had died by day 35. No mice in the N^G-nitro-D-arginine methyl ester or the control group had died. Thus, the survival rates were not different among the three groups.

Pathologic analysis (Fig. 2, Table 1). Infiltration, necrosis, calcification and interstitial fibrosis were more severe in the N^G-nitro-L-arginine methyl ester group, but not in the N^G-nitro-D-arginine methyl ester group, than in the control group. In the N^G-nitro-L-arginine methyl ester group, perivascular fibrosis was more dense, and medial swelling was thicker, especially in the arteries adjacent to necrotic areas, than in the other two groups. Accordingly, vascular structural remodeling in myocarditis by N^G-nitro-L-arginine methyl ester treatment was considered significant.

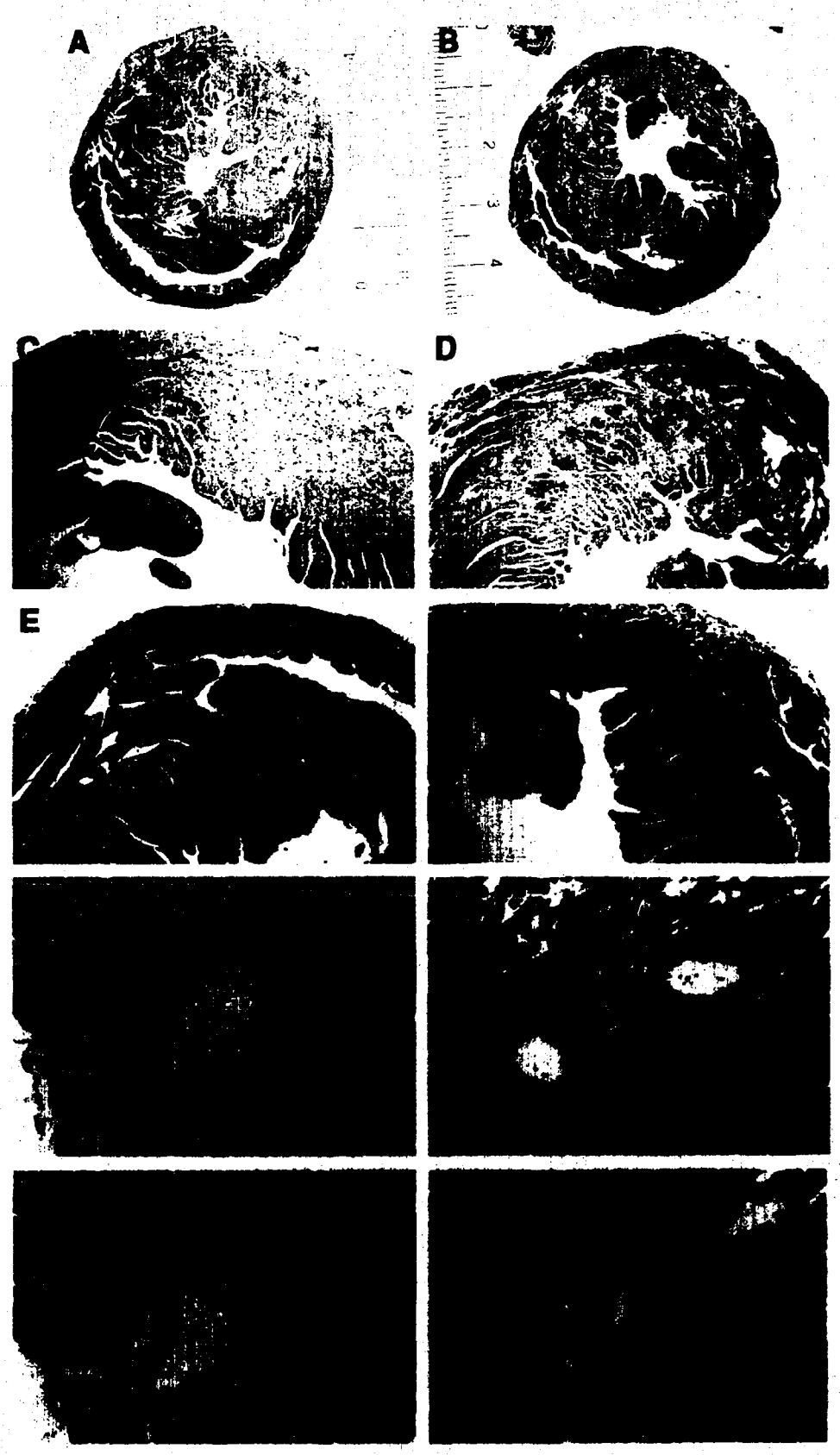

Figure 2. Histopathologic studies. In mice killed on day 35 (experiment II), severe cardiomyopathic lesions (i.e., dilated ventricles with remodeling, myocardial necrosis, calcification and interstitial fibrosis, were seen in the N^G-nitro-L-arginine methyl ester-treated group (**B, D, F**). These lesions were not marked in the infected untreated group (**A, C, E**). Perivascular fibrosis and medial thickening were more severe in N^G-nitro-L-arginine methyl ester-treated (**H, J**) than the infected untreated group (**G, I**). **A** and **B**: hematoxylin-eosin, ×15, reduced by 60% and 55%, respectively. **C** and **D**, Hematoxylin-eosin, ×100, reduced by 52%. **E** and **F**, Mallory-Azan, ×100, reduced by 52%. **G** and **H**, Mallory-Azan, ×1,000, reduced by 52%. **I** and **J**, Elastic van Gieson, ×1,000, reduced by 52%. **A** and **B**: scale = 0.1 mm.

Discussion

Coxsackievirus B3 myocarditis and nitric oxide. The present study clearly demonstrated that N^G-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor, increased myocardial virus titers, resulting in the aggravation of myocardial lesions in the early stage of coxsackievirus B3 myocarditis and induced more severe cardiomyopathic lesions in the later stage. In contrast, N^G-nitro-L-arginine methyl ester, the inactive enantiomer, was ineffective. On stimulation with INF-gamma and lipopolysaccharide, macrophages decreased coxsackievirus B3 titers. Restored coxsackievirus B3 titers in INF-gamma- and lipopolysaccharide-stimulated macrophages by treatment with N^G-nitro-L-arginine methyl ester, but not with N^G-nitro-D-arginine methyl ester, were observed in the in vitro study. Thus, nitric oxide played a protective role in the development of acute and chronic coxsackievirus B3 myocarditis.

Antiviral effects of nitric oxide. The transformed murine macrophage cell line RAW264.7 is highly nonpermissive for coxsackievirus B3 replication in the inactivated state. The virus yields were almost 10^4-fold less than those of VERO cells in the present study (data not shown). Treatment with N^G-nitro-L-arginine methyl ester, but not N^G-nitro-D-arginine methyl ester, reduced the ability of activated macrophages to suppress coxsackievirus B3 replication.

Nitric oxide in acute myocarditis. In the first in vivo experiment, a relation between myocardial virus titers and pathologic abnormalities was observed in the acute viremic experimental protocol, suggesting that nitric oxide plays a defensive role in the early stage of infection. Cardiac pathologic scores on days 4, 7 and 14 were higher, and myocardial virus titers on days 4 and 7 were higher, in the N^G-nitro-L-arginine methyl ester group than in the control group. In a previous in vitro study, Lowenstein et al. (19) reported that nitric oxide played a role in limiting coxsackievirus B3 replication when nitroprusside, a donor of nitric oxide, was added to HeLa cells. Nitric oxide may be cytostatic or cytotoxic for invading microorganisms. An antiviral effect of nitric oxide has been demonstrated (15) against herpes simplex virus type 1.

Nitric oxide in chronic myocarditis. In the second set of in vivo experiments, it was clearly demonstrated that nitric oxide synthase inhibition aggravated cardiac pathogenesis of chronic coxsackievirus B3 myocarditis. Indeed, there is increasing evidence that nitric oxide plays an important role in other chronic, nonviral inflammatory responses. For example, immune complex-induced vascular injury in rat lungs and dermal vasculature can be attenuated by nitric oxide synthase inhibitors (20). Furthermore, inhibitors of nitric oxide synthase ameliorate experimentally induced chronic ileitis (21). However, there have been reports suggesting that both inhibitors of nitric oxide synthase and nitric oxide donors protect against some forms of injury. This protection is probably due to the dual nature of nitric oxide, which is on the one hand cytotoxic, and on the other a vasodilative agent and is thus potentially protective (14).

Nitric oxide regulates vasomotor tone (22,23) and inhibits leukocyte and lymphocyte adherence and chemotaxis (24–26). Accordingly, reduced and diminished nitric oxide production may facilitate platelet aggregation, leukocyte adhesion and even smooth muscle proliferation. Although further studies are necessary, the enhancement of myocardial lesions by treatment with N^G-nitro-L-arginine methyl ester seen in the in vivo experiment may have been due to impaired regulation of vasomotor tone and aggregation of leukocytes or lymphocyte adherence and chemotaxis.

Nitric oxide in myocardial architectural remodeling. Treatment with N^G-nitro-L-arginine methyl ester (i.e., inhibition of nitric oxide synthesis) not only increased interstitial and perivascular fibrosis, but also induced more severe architectural vascular remodeling at the cardiomyopathic stage of this study. A mechanism by which nitric oxide may inhibit mitogenesis and proliferation of interstitial connective tissue as well as vascular smooth muscle has been proposed (27). Recently, it was demonstrated (28) that L-arginine treatment reduced myocardial fibrosis in another murine model of cardiomyopathy. Accordingly, we considered treatment with N^G-nitro-L-arginine methyl ester to be closely related to myocarditic and postmyocarditic ventricular and vascular remodeling in view of the observed effects on interstitial and perivascular fibrosis.

Implications and limitations of the present study. Although the key findings of this study showed small changes in viral titers, survival rates and pathologic grades, we consider that these were due to the oral administration protocol of the drugs. We did not directly measure nitric oxide production, and therefore no evidence of alteration in nitric oxide metabolism was obtained. Indeed, the inactive N^G-nitro-D-arginine methyl ester group demonstrated an intermediate effect between the N^G-nitro-L-arginine methyl ester and the control groups. We were unable to determine why there were no statistically significant differences in any of the variables of pathologic grades between the N^G-nitro-L-arginine methyl ester and N^G-nitro-D-arginine methyl ester groups. There is a common mechanism of action by both enantiomers on the effect of viral myocarditis (13,14), which may be due to mechanisms other than interference of nitric oxide synthase. Accordingly, the observed effects may have been due in part to nonspecific interactions of the drugs with nitric oxide synthase. Further studies will be needed to clarify these issues.

Conclusions. Treatment with the nitric oxide inhibitor N^G-nitro-L-arginine methyl ester aggravates both acute coxsackievirus B3 myocarditis by increased myocardial virus replication and chronic coxsackievirus B3 myocarditis by inducing more severe cardiomyopathic lesions. In vitro analysis of nitric oxide inhibition by treatment of the murine macrophage cell line RAW264.7 with N^G-nitro-L-arginine methyl ester may explain the possible in vivo anti-coxsackievirus B3 activity of nitric oxide in this experimental model. Thus, we demonstrated experimentally that nitric oxide plays a defensive role in the pathogenesis of coxsackievirus B3 myocarditis.

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