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*J Clin Invest.* 1994;**94**(3):1212-1217. <https://doi.org/10.1172/JCI117438>.

### Research Article

Controversy still exists concerning the therapy for viral myocarditis which manifests a wide variety of clinical symptoms. Vesnarinone, a quinolinone derivative that was developed as a positive inotropic agent with complex actions, including phosphodiesterase inhibition and cation channel modification, has recently been confirmed to improve the prognosis of patients with chronic heart failure. However, the precise mechanism of this beneficial effect is not yet clearly understood. In this study, using a murine model of acute viral myocarditis resulting from encephalomyocarditis virus infection, survival and myocardial damage were markedly improved by treatment with vesnarinone. In contrast, survival was not improved by treatment with amrinone, a phosphodiesterase inhibitor. Although vesnarinone did not inhibit viral replication or protect myocytes from viral direct cell injury, it did inhibit the increase in natural killer cell activity after viral infection. On the other hand, amrinone failed to inhibit natural killer cell activity. Both vesnarinone and amrinone suppressed the production of tumor necrosis factor-alpha. Therefore, we postulate that vesnarinone exerted its beneficial effects through an inhibition of natural killer cell activity, and that it serves as an immunomodulator providing new therapeutic possibilities for the treatment of viral myocarditis and/or immunological disorders.

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# Treatment of Virus-induced Myocardial Injury with a Novel Immunomodulating Agent, Vesnarinone

## Suppression of Natural Killer Cell Activity and Tumor Necrosis Factor- $\alpha$ Production

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### Abstract

Controversy still exists concerning the therapy for viral myocarditis which manifests a wide variety of clinical symptoms. Vesnarinone, a quinolinone derivative that was developed as a positive inotropic agent with complex actions, including phosphodiesterase inhibition and cation channel modification, has recently been confirmed to improve the prognosis of patients with chronic heart failure. However, the precise mechanism of this beneficial effect is not yet clearly understood. In this study, using a murine model of acute viral myocarditis resulting from encephalomyocarditis virus infection, survival and myocardial damage were markedly improved by treatment with vesnarinone. In contrast, survival was not improved by treatment with amrinone, a phosphodiesterase inhibitor. Although vesnarinone did not inhibit viral replication or protect myocytes from viral direct cell injury, it did inhibit the increase in natural killer cell activity after viral infection. On the other hand, amrinone failed to inhibit natural killer cell activity. Both vesnarinone and amrinone suppressed the production of tumor necrosis factor- $\alpha$ . Therefore, we postulate that vesnarinone exerted its beneficial effects through an inhibition of natural killer cell activity, and that it serves as an immunomodulator providing new therapeutic possibilities for the treatment of viral myocarditis and/or immunological disorders. (*J. Clin. Invest.* 1994, 94:1212–1217.) **Key words:** positive inotropic agent • encephalomyocarditis virus • congestive heart failure • cytokines • immunosuppression

### Introduction

Viral myocarditis exhibits a variety of manifestations, ranging from a total lack of clinical symptoms to congestive heart failure. There is increasing evidence that dilated cardiomyopathy is a late sequel of acute viral myocarditis (1–3), and appropriate treatment of viral myocarditis during the early stages of the disease process substantially modifies the development of chronic congestive heart failure. However, the management of

myocarditis is still a controversial subject (4). Vesnarinone, a quinolinone derivative, is a recently synthesized positive inotropic agent shown to have positive inotropic effects on congestive heart failure (5, 6). In contrast to the recent pessimistic views on inotropic agents for the treatment of congestive heart failure (7, 8), administration of this agent for six months improved the survival rate of patients with congestive heart failure (9). Although it has been suggested that the mechanisms of action of vesnarinone might be related to a slight inhibition of phosphodiesterase III, increased inward calcium current, and reduced potassium current (10, 11), the true mechanism by which the agent reduces mortality has not been clarified (12). One possible alternative explanation for its favorable clinical efficacy is a recent report that this agent can inhibit the production of some cytokines (13, 14). In this study, we investigated the effects of vesnarinone in an animal model of congestive heart failure due to acute viral myocarditis induced by encephalomyocarditis virus (EMCV),<sup>1</sup> a picornavirus (15, 16), and focused on novel immunomodulating effects.

### Methods

**Animals and drugs.** 4-wk-old male DBA/2 mice and pregnant DDY mice were purchased from Japan SLC Inc. (Kyoto, Japan). Vesnarinone was obtained from Otsuka Pharmaceutical Co. (Osaka, Japan), and was dissolved in 0.5% methylcellulose at concentrations of 1 mg/ml and 5 mg/ml for in vivo experiments and stored at 4°C. Vesnarinone was dissolved in 1 N HCl, and after filtration, diluted with heat inactivated FCS (Gibco, Auckland, New Zealand) at a concentration of 1 mg/ml for in vitro experiments and stocked at -20°C. Before use, the stock solution was thawed and diluted with medium and the pH was adjusted using 1 N NaOH. Amrinone was obtained from Yamanouchi Pharmaceutical Co. (Tokyo, Japan), and dissolved in 0.5 N lactic acid at a concentration of 50 mg/ml and diluted with distilled water (for in vivo experiments) or medium (for in vitro experiments) at various concentrations.

**Virus and virus titration.** EMCV m-variant has been maintained at our laboratory and the concentration of the virus was determined by the FL-plaque-assay described below and expressed as plaque-forming units (pfu) per ml or mg. FL cells (human amnion cells) were grown to subconfluent monolayer on 6-well plates (Corning Inc., Corning, NY) in 4 ml of Eagle's modified essential medium (EMEM) (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FCS at 37°C in 5% CO<sub>2</sub>. The wells were washed with PBS three times (FL cell monolayer). The samples were diluted 1:10 in EMEM, and 0.1 ml of the diluted samples was spread on the FL cell monolayer and incubated for 1 h with occasional shaking. The wells were then overlaid by 4 ml

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Received for publication 13 August 1993 and in revised form 18 May 1994.

*J. Clin. Invest.*

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0021-9738/94/09/1212/06 \$2.00

Volume 94, September 1994, 1212–1217

1. **Abbreviations used in this paper:** DME-ITS, DME plus 5  $\mu$ g/ml of insulin, 5  $\mu$ g/ml transferrin, and 5 ng/ml of selenium; EMCV, encephalomyocarditis virus; EMEM, Eagle's modified essential medium; NK cell, natural killer cell; pfu, plaque-forming unit.

of EMEM supplemented with 2% FCS and 1% methylcellulose, and incubated at 37°C in 5% CO<sub>2</sub> for 30 h. At the end of incubation, the wells were fixed by acidified ethyl alcohol and stained by crystal-violet, and the plaques were counted. Data values represent the means of duplicates (FL-plaque-assay).

**Survival experiment.** 4-wk-old male DBA/2 mice were inoculated with 10 pfu of EMCV i.p. Vesnarinone was orally administered at doses of 10 and 50 mg/kg and amrinone was administered at doses of 5 and 25 mg/kg daily, starting on the same day as EMCV inoculation and continuing for 14 d. Infected control mice were given vehicles. Each group consisted of 20 mice.

**Histopathologic examinations.** Mice were killed 5 d after EMCV inoculation by cervical dislocation after being anesthetized with ether. After measuring the weights of the body and heart, the heart was divided into two parts at the midportion of the left ventricle along the short axis. One part was fixed by formaline and stained by Masson's trichrome stain, and then observed under a magnitude 50 light microscope. Using a 0.1-mm square microgrid, the necrosis area was measured blindly as the ratio of the number of microgrid crosspoints on the necrotic area to the number of crosspoints on the left ventricle (17): this value ranged from ~300–500. The other part of the heart was immediately frozen and stored at –20°C until virus titration was performed. Frozen samples were thawed at room temperature, homogenized with 1 ml of EMEM, and centrifuged at 400 g for 5 min. The virus titer of the supernatants was determined by FL-plaque-assay.

**Preparation of murine myocytes.** Hearts obtained from neonates of DDY mice were minced and stirred for 5 min in 7 ml of trypsin-EDTA (Gibco, Grand Island, NY) in a 25-cm<sup>2</sup> small culture flask, then as much of the medium as possible was removed into a 50-ml conical tube containing DME supplemented with 20% FCS. This extraction procedure was repeated 7 times and the collected medium was centrifuged at 200 g for 10 min. Pellets were resuspended in 20 ml of culture medium (DME supplemented with 7% FCS and 0.6 mg/ml thymidine) and incubated on a 10-cm culture plate for 1 h at 37°C in 5% CO<sub>2</sub>. The nonadherent cells were collected and suspended to a suitable concentration. The suspension was cultured in 24- or 96-well flat-bottomed plates for 3 d and contraction of the myocytes was confirmed before the experiments were performed.

**Virus replication reduction assay.** Murine myocytes were cultured in 24-well plates and then infected with EMCV at an moi of 1. The plates were incubated for 24 h at 37°C in 5% CO<sub>2</sub> with 2 ml of DME plus 5 µg/ml of insulin, 5 µg/ml transferrin, and 5 ng/ml of selenium, (DME-ITS) with or without various concentrations of vesnarinone. Some wells were preincubated with vesnarinone for 8 h before EMCV infection, and some wells contained only the virus and 2 ml of the medium. After 24 h of incubation, the plates were frozen and thawed three times and centrifuged at 400 g for 5 min. Virus titration of the supernatants was determined by FL-plaque-assay. Data values represent the means of duplicated wells.

**Assay of virus-induced myocyte injury.** Murine myocytes were cultured on 96-well plates, labeled with <sup>51</sup>Cr for 8 h, washed three times with DME, infected with EMCV at an moi of 10, and then cultured for 30 h in up to 0.2 ml of DME-ITS with or without 10 µg/ml of vesnarinone at 37°C in 5% CO<sub>2</sub>. Total release was measured in three wells in which 1% Triton-x was added, and spontaneous release was measured in another three wells containing DME-ITS without the virus. Finally, 0.1 ml of the supernatant was sampled from each well and radioactivity was measured with an auto-gamma counter (Aloka Co. Ltd., Tokyo, Japan). The rate of <sup>51</sup>Cr release was calculated according to the following formula: 100 × (sample cpm – spontaneous cpm)/(total cpm – spontaneous cpm).

**Assay of NK cell activity.** 4-wk-old male DBA-2 mice infected with EMCV were killed on days 1, 3, and 5. Spleen cells were obtained by low density centrifugation using Lympholite-M (Cedarlane, Ontario, Canada) at 1500 g for 20 min. Finally, spleen cells were suspended in RPMI-1640 (Gibco) supplemented with 10% FCS (RPMI-FCS). YAC-1 cells (target cells) were labeled with <sup>51</sup>Cr for 2 h, washed three times and suspended at a concentration of 5 × 10<sup>4</sup> cells/ml in RPMI-FCS.

The target cells (5 × 10<sup>3</sup>/well) were incubated with spleen cells at E/T ratios of 25:1, 50:1, and 100:1 in up to 0.2 ml of RPMI-FCS in a round bottomed 96-well microplate. Incubation was carried out for 4 h at 37°C in 5% CO<sub>2</sub>, after which 0.1 ml of the supernatant from each well was sampled and radioactivity measured with an auto-gamma counter. All determinations were done in triplicate. The rate of cytotoxicity was then calculated as described above. In an additional experiment, mice were pretreated with 100 µg of rabbit polyclonal anti-asialo GM1 antibody i.p. (kindly donated by Dr. S. Habu, Tokai University, Japan) 1 and 3 d before EMCV infection.

Lytic units were calculated to represent the number of cells per 10<sup>7</sup> effector cells capable of lysing 20% of the target cell population.

**Assay of TNF-α production.** Spleen cells obtained from 4-wk-old DBA/2 mice were cultured in RPMI-FCS on round-bottomed microplates. Each well, which contained 1 × 10<sup>5</sup> spleen cells, was incubated at 37°C in 5% CO<sub>2</sub> with LPS (Difco Laboratories Inc., Detroit, MI), to which was added 2.6, 26, or 78 µM of vesnarinone or amrinone. After incubation, 0.1 ml of supernatant from each well was sampled and murine TNF-α was assayed using a commercially available ELISA kit (Otsuka Pharmaceutical Co., Osaka, Japan). The ELISA method was described in the instructions for the kit. The sensitivity of the kit was 50 pg/ml.

**Statistical analysis.** For statistical analysis, the log rank test was used for determining survival, analysis of variance with a multiple comparison test for histopathologic examinations and lytic units of NK cell activity, and Mann-Whitney U test for TNF-α production.

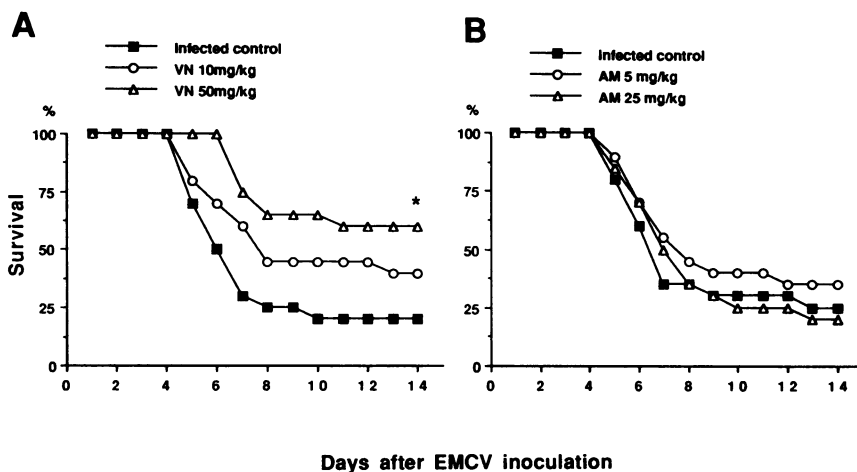
## Results

**Survival of the animals.** Fig. 1 shows that the infected control mice receiving no treatment began to die on day 5 after EMCV inoculation and that the majority of deaths occurred from day 5 to day 8. Mice treated with vesnarinone at 10 mg/kg also started to die on day 5, but the mortality rate was substantially reduced compared with the control. On the other hand, all animals treated with a higher dose of vesnarinone survived the first 6 d. The mortality rate was relatively low after the first 7 d in all groups. The cumulative survival rate was significantly increased in a dose-related manner, being 20, 40, and 60% for the no-treatment group and those treated with vesnarinone at 10 and 50 mg/kg, respectively (Fig. 1 A). Treatment with equivalent molar doses of amrinone did not improve the survival rate (Fig. 1 B). Thus, vesnarinone might exert its beneficial effects at a very early stage after viral infection.

**Histopathology of the heart on day 5 (Table I).** The heart weight to body weight ratio correlates closely with the severity of congestive heart failure, but there were no significant differences between the infected control group and the vesnarinone-treated group. The virus titer in the heart of mice treated with vesnarinone was similar to that of the infected control mice. Nevertheless, myocardial necrosis of the mice treated with 50 mg/kg of vesnarinone was significantly reduced compared with the infected control group.

**Effect of vesnarinone on virus replication in cultured murine myocytes.** The virus titer of the myocyte-free wells was 2.7 pfu/ml and the virus yield in infected control was 3.9 log pfu/ml. Treatment with various concentrations of vesnarinone, including preincubation for 8 h, did not affect virus yields (Fig. 2). Thus, vesnarinone did not directly inhibit virus replication.

**Effect of vesnarinone on direct virus-induced myocyte injury.** The rate of <sup>51</sup>Cr release for control wells without vesnarinone was 22.0 ± 3.4% (mean ± SE) and that for wells treated with 10 µg/ml vesnarinone was 21.1 ± 3.6% (Fig. 3). Thus, vesnarinone did not directly protect the myocytes from virus-induced cell injury.



**Figure 1.** Effect of vesnarinone and amrinone on survival rate after EMCV inoculation. 4-wk-old DBA/2 mice were inoculated intraperitoneally with 10 pfu of EMCV. (A) Two groups were treated with vesnarinone (VN) at doses of 10 and 50 mg/kg by mouth daily. Survival of the group treated with vesnarinone at 10 mg/kg was relatively improved compared with the control group, but there was no significant difference. Treatment with 50 mg/kg of vesnarinone significantly reduced mortality at a very early stage (\* $P < 0.01$  vs the control). (B) Mice were also treated with amrinone (AM) at doses of 5 and 25 mg/kg by mouth daily. Survival was not improved compared with the control. The vehicle alone was administered to the infected control mice. Each group started with 20 mice.

**Natural killer cell (NK cell) activity after EMCV infection.** The NK cell activity of the spleen cells obtained from infected mice, expressed as the rate of specific cytotoxicity (the rate of  $^{51}\text{Cr}$  release), began to increase from the second day after EMCV infection. Treatment with vesnarinone at 50 mg/kg inhibited increases in specific cytotoxicity caused by viral infection, but treatment with amrinone at 25 mg/kg (equal molar dose as vesnarinone) did not inhibit the increase in specific cytotoxicity on day 3 (Fig. 4). At the E/T ratio of 50:1, the rate of specific cytotoxicity obtained for infected control mice was  $6.5 \pm 1.1\%$  on day 1,  $22.4 \pm 2.6\%$  on day 3, and  $26.6 \pm 0.7\%$  on day 5, while the corresponding values for mice treated with vesnarinone were  $6.2 \pm 0.9\%$ ,  $13.1 \pm 1.7\%$ , and  $20.8 \pm 1.3\%$ . The value for mice treated with amrinone was  $20.3 \pm 3.0\%$  on day 3, and that for noninfected control mice was  $6.6 \pm 2.3\%$  (mean  $\pm$  SE). Lytic units were calculated, and the value for the infected control group was 48 on day 3 and 53 on day 5, that for the vesnarinone-treated group was 21 on day 3 and 42 on day 5, and for amrinone-treated group was 41 on day 3. Vesnarinone significantly reduced this value on days 3 and 5 ( $P < 0.05$  vs infected control), but the effect of amrinone was not significant. Spleen cells obtained from mice pretreated with anti-asialo GM1 antibody showed no response after viral infection (Fig. 5). Thus, the increase in specific cytotoxicity was due to the increase in the activity of asialo GM1-bearing NK cells, and vesnarinone inhibited this activity.

**Table I. Histopathologic Examinations on Day 5**

	n	Necrosis	Virus titer in heart	Heart/body
		%	log pfu/mg	wt. ratio $\times 10^{-3}$
Infected control	8	$16.7 \pm 5.6$	$7.6 \pm 0.7$	$6.5 \pm 0.5$
Infected + vesnarinone				
50 mg/kg	6	$9.7 \pm 5.0^*$	$7.4 \pm 0.5$	$6.3 \pm 0.8$
Noninfected control	10	0	ND	$4.8 \pm 0.3$

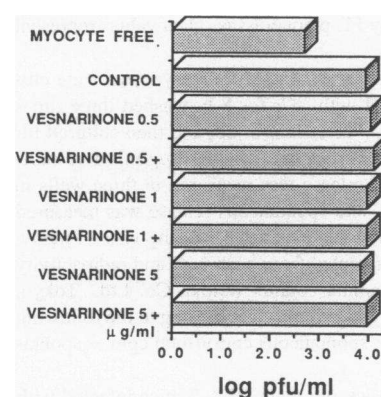
Treatment with vesnarinone 50 mg/kg daily improved myocardial necrosis after EMCV infection without affecting the virus titer in heart or the congestive state. Data values represent the mean  $\pm$  SD. (\* $P < 0.05$  vs infected control; ND, not done)

**TNF- $\alpha$  production.** The time course of TNF- $\alpha$  production from spleen cells by LPS stimulation showed that the TNF- $\alpha$  level reached almost the maximum after 9 h of culture (Fig. 6). Vesnarinone and amrinone significantly suppressed TNF- $\alpha$  production in a dose-dependent manner (Fig. 7).

## Discussion

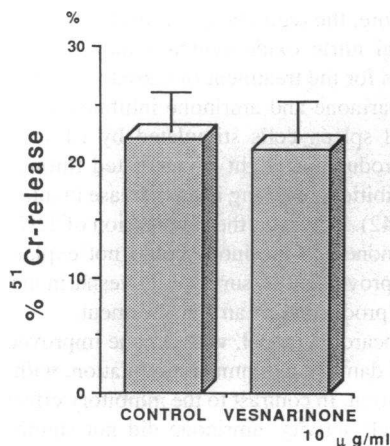
According to the current concept of heart failure management, the primary goals are to improve the quality of life and to prolong life. Newly developed inotropic agents which increase intracellular cyclic AMP either by stimulating  $\beta$ -adrenergic receptors or by inhibiting phosphodiesterase have produced dramatic short-term hemodynamic benefits in patients with advanced heart failure. However, long-term treatment with these agents results in an unfavorable outcome, with an acceleration of the disease process and an adverse effect on survival (7, 8).

In contrast to these agents, vesnarinone has been shown to improve both the quality of life and the prognosis of heart failure (18–20). Particularly, a recent report by Feldman and colleagues (9) demonstrated that in 238 patients assigned to



**Figure 2.** Effect of vesnarinone on viral replication in cultured murine myocytes. Murine myocytes from DDY neonates were infected with EMCV at an moi of 1, and incubated with various concentrations of vesnarinone (+ indicates 8 h of preincubation with vesnarinone preceding the infection) on 24-well plates. Myocyte-free wells contained only virus and culturing me-

di-um and control wells contained myocytes and virus. After 24 h of incubation, the plates were frozen and thawed three times and the virus titer of the supernatants was determined by FL-plaque-assay. Vesnarinone did not reduce viral replication. Data values represent the means of duplicates.

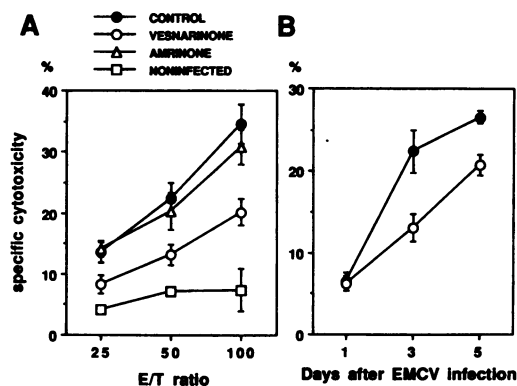


**Figure 3.** Effect of vesnarinone on myocyte protection against viral direct cytotoxicity. Murine myocytes cultured on a 96-well plate were labeled with <sup>51</sup>Cr, and infected with EMCV at an moi of 10. Vesnarinone was added at the beginning of the infection. After 30 h of incubation with up to 0.2 ml of medium with or without vesnarinone, 0.1 ml of the supernatant from each well was harvested and its radioactivity was

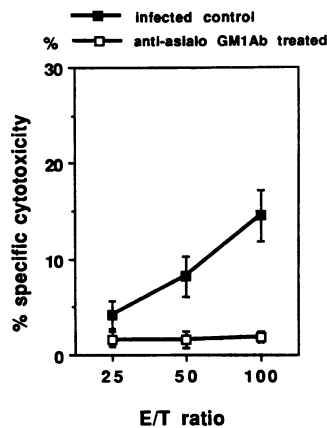
measured. The rate of <sup>51</sup>Cr release was calculated according to the formula described in Methods. Vesnarinone could not protect myocytes against direct viral cytolysis. Data values represent the means ± SE of 6 wells.

treatment with vesnarinone at 60 mg/d the combined endpoint of death or worsening heart failure was reduced by 50% during the 6-m study period compared with 239 patients receiving placebo. Vesnarinone also increases intracellular cyclic AMP by the inhibition of a specific isoform of phosphodiesterase, but unlike other phosphodiesterase inhibitors, vesnarinone prolongs action potential duration and slows heart rate by exerting an effect on ion channels.

A fall in contractility could be exacerbated by inotropic agents that potentially increase the heart rate in the failing heart, in which the force-frequency response is attenuated or even reversed (8). In this regard, in addition to its antiarrhythmic properties, the lack of an increase in heart rate is considered as the most likely cause of the beneficial effect of vesnarinone in long-term treatment.



**Figure 4.** Effect of vesnarinone and amrinone on NK cell activity after EMCV infection. Mice were treated as described in Fig. 1, and then killed on days 1, 3, and 5 after EMCV infection. (A) Percent specific cytotoxicity on day 3 at various E/T ratios. (B) Time course of percent specific cytotoxicity at a fixed E/T ratio of 50:1. Data values represent the means ± SE of noninfected mice ( $n = 4$ ), infected mice ( $n = 2$  on day 1 and  $n = 3$  on days 3 and 5), except for vesnarinone-treated mice and infected control mice on day 3 ( $n = 5$ ).

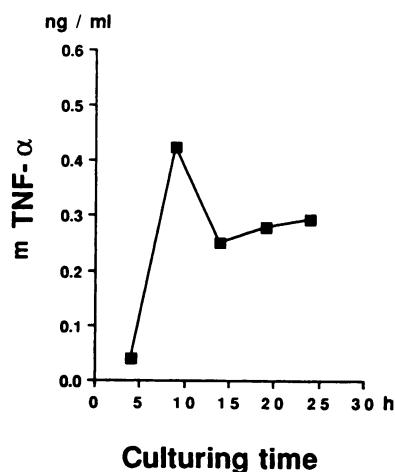


**Figure 5.** Effect of anti-asialo GM1 antibody on NK cell activity after EMCV infection. Spleen cells obtained from mice treated with rabbit anti-asialo GM1 antibody by intraperitoneal injection 1 and 3 d before EMCV infection showed no cytotoxicity on the target cells (YAC-1). Data values represent the means ± SE of infected control ( $n = 4$ ), and antibody-treated mice ( $n = 3$ ) on day 2 after EMCV infection.

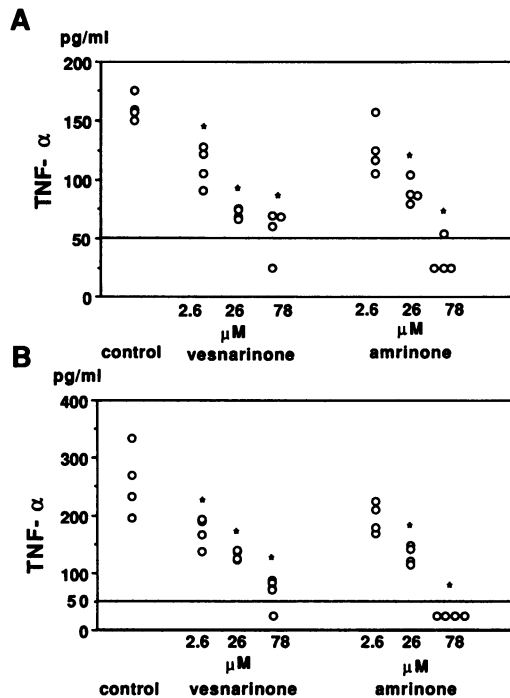
In this study, we employed an animal model of viral myocarditis to investigate the effects of vesnarinone from the immunological point of view.

Vesnarinone improved mortality in the acute stage of viral myocarditis. In this model, myocardial necrosis and cellular infiltration appeared about 4–5 d after viral infection, and some mice began to die on day 5, with others developing severe congestive heart failure after day 7 (15). Vesnarinone exerted its effect at the stage when the majority of deaths were caused by myocardial damage, before congestive heart failure developed. Histopathologic examination in the acute stage showed that vesnarinone reduced myocardial damage without significant effect on viral replication in the heart. Vesnarinone did not reduce viral replication in cultured myocytes or protect the myocytes from direct virus-mediated cell lysis. Therefore, the effect of vesnarinone could be due to altered host immune responses.

Anti-viral immune responses act to clear the virus and destroy the infected cells, and this is usually beneficial to the host. However, the injurious outcome can produce disastrous results when occurring in vital organs such as the brain, heart, etc. (21). Thus, immunosuppression may be reasonable and effective in certain circumstances. In a murine model of acute picornavirus myocarditis, the dominant population of infiltrating cells in the necrotic area at the very early phase are asialo GM1 positive cells without T cell markers, and cytotoxic T cells specific for viral antigen appear later (22, 23). We examined NK cell (nonspecific cytotoxic cell bearing asialo GM1 in mice) activity



**Figure 6.** Time course of TNF- $\alpha$  production after LPS stimulation. Spleen cells ( $1 \times 10^5$  cells/well) were stimulated with 1,000 ng/ml of LPS. After serial time incubation, the TNF- $\alpha$  concentration in the supernatant was assayed by ELISA. Data values represent the means of 2 to 4 samples.



**Figure 7.** Suppression of TNF- $\alpha$  production from spleen cells. Spleen cells ( $1 \times 10^5$  cells/well) were stimulated with LPS at doses of 100 (A) and 1,000 ng/ml (B). Vesnarinone and amrinone were added to the wells at doses of 2.6, 26, and 78  $\mu$ M. The control was the vesnarinone vehicle. After 9 h of incubation, the TNF- $\alpha$  concentration of the supernatant was assayed by ELISA. The sensitivity was 50 pg/ml. (\* $P < 0.05$  vs control)

after EMCV infection (24). The lack of an increase in cytotoxicity after treatment with anti-asialo GM1 antibody indicates that the assay we used was highly specific for NK cells (25) and the results lead us to conclude that vesnarinone substantially inhibited the increase in NK cell activity, while an equal molar dose of amrinone did not inhibit the increase in NK cell activity. In contrast to the mild effect observed in this study, excessive suppression or destruction of the immune system may lead to the expansion of the virus and severe tissue damage, as previously observed in the treatment of myocardial damage in viral myocarditis using cyclosporin A and/or corticosteroid (26–28).

Various cytokines are increased after viral infection (29) and TNF- $\alpha$  enhances anti-viral immune responses by activating lymphocytes, NK cells (30), or by producing other cytokines. TNF- $\alpha$  also inhibits virus replication and specifically kills virus-infected cells (31). But the action of TNF- $\alpha$  is not always protective in virus infection, because TNF- $\alpha$  has been shown to reactivate viral infection (32, 33). It also contributes to septic shock, resulting in multiple organ damage and a catabolic state (34, 35). We have already reported that TNF- $\alpha$  was increased after EMCV infection, and pretreatment with anti-TNF- $\alpha$  antibody improved myocardial damage in the acute stage in this model (36). In other reports, anti-TNF- $\alpha$  antibody suppressed cell-mediated immunity (37). Recently, the direct effects of pro-inflammatory cytokines on the contractility of the mammalian heart were studied (38–40). TNF- $\alpha$ , IL-2, and IL-6 inhibited myocardial contractility in a concentration-dependent, reversible manner, and this direct negative inotropic effect of the cytokines is largely mediated through a myocardial nitric oxide

synthase (39, 40). Therefore, the regulation of proinflammatory cytokines and myocardial nitric oxide synthase may provide new therapeutic strategies for the treatment of cardiac diseases. In our experiments, vesnarinone and amrinone inhibited TNF- $\alpha$  production in cultured spleen cells stimulated by LPS, so the effect on cytokine production might be mediated through phosphodiesterase III inhibition, resulting in an increase in intracellular cyclic AMP (41, 42). However, the suppression of TNF- $\alpha$  production by vesnarinone and amrinone could not explain the mechanism of the improvement of survival by vesnarinone, since amrinone failed to produce such an improvement.

In the acute viral myocarditis model, vesnarinone improved mortality and myocardial damage by immunomodulation, without affecting virus replication. In contrast to the inhibitory effect of vesnarinone on NK cell activity, amrinone did not significantly affect NK cell activity. The mechanism of this difference might be produced by differences in the pharmacological properties of these agents, including the inhibitory effect of vesnarinone on cation channels. Vesnarinone reduces outward potassium current, and some reports have suggested that voltage-gated potassium channel blockers are capable of inhibiting T cell activity (43). This additional action might be a major cause for the specific inhibition in NK cell activity, which was related to the improvement of viral myocarditis.

In our experiments, vesnarinone showed a protective effect at a dose of 50 mg/kg. This dose is about 7 times higher than the doses used in humans for the short-term therapy of congestive heart failure (5, 6), and about 50 times higher than the dose used for the treatment of chronic heart failure (9, 18, 19). It is difficult to compare doses in different animal species; however, on the basis of body surface area, a given dose in mice is comparable to a dose that is about 12 times lower in humans (44). Thus, a dose of 50 mg/kg in mice is equivalent to a dose of 4 mg/kg in humans. Even though this dose is still about four times higher than the dose used in the treatment of chronic heart failure, it does not exceed that in current clinical use. The serum concentration of vesnarinone in humans is  $\sim 5$ –10  $\mu$ g/ml (13 to 26  $\mu$ M) for a wide range of oral doses (single dose of 60–240 mg) (45) and the serum concentration may rise several fold in patients with chronic heart failure (13). Although we did not measure the serum concentration of vesnarinone in mice, a dose of 50 mg/kg should not exceed the reasonable dose for an experimental model.

Vesnarinone may therefore be of value in preventing the development of other diseases in which increased NK cell activity, as well as increased TNF- $\alpha$  levels, plays a major role in the pathogenesis. We believe that vesnarinone may provide a new therapeutic strategy for the management of viral infections and/or immunological disorders.

## Acknowledgments

We thank Mr. H. Toriyama and Mr. Y. Ohmoto for their assistance in the assay for TNF- $\alpha$ .

This work was supported, in part, by a Research Grant from the Ministry of Health and Welfare and by a Grant-in-Aid for Developmental Research and for General Scientific Research from the Ministry of Education, Science and Culture, and the Kanazawa Research Fund, Japan.

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