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EXPERIMENTAL STUDIES

Therapeutic Effects of FTY720, a New Immunosuppressive Agent, in a Murine Model of Acute Viral Myocarditis

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OBJECTIVES This study examines the efficacy of FTY720 (FTY), a new immunosuppressor, in the treatment of acute viral myocarditis in a murine model.

BACKGROUND Immunosuppressive agents have no proven therapeutic efficacy in experimental or clinical myocarditis.

METHODS Encephalomyocarditis virus was inoculated i.p. in DBA/2 mice on day 0. Postinoculation treatment consisted of FTY 10 mg/kg/day p.o. (FTY group), or cyclosporine A (CsA) 40 mg/kg/day p.o. (CsA group) or distilled water p.o. only (control group). Survival until day 14, as well as cardiac histopathology, virus concentrations, cytokines (interleukin [IL]-2, IL-12, interferon [IFN]-gamma and tumor necrosis factor [TNF]-alpha) and nitric oxide (NO) on day 5 were examined.

RESULTS In the control and CsA groups, all mice died within 10 and 7 days, respectively. However, in the FTY group, 27% of the animals survived up to day 14. Compared with the control group, 1) histological scores were significantly lower in the FTY group but unchanged in the CsA group; 2) virus concentration was significantly higher in the CsA group but not in the FTY group; 3) expressions of IL-2, IL-12 and IFN-gamma in the heart were suppressed in both the FTY and CsA groups, though suppression was weaker in the FTY group; 4) TNF-alpha and NO were significantly increased in the CsA group but not in the FTY group.

CONCLUSIONS FTY720 had a significant therapeutic effect in acute experimental myocarditis without inducing excessive virus replication. This report is the first to describe a beneficial effect by an immunosuppressive agent in the treatment of acute viral myocarditis. (J Am Coll Cardiol 2001;37:1713–8) © 2001 by the American College of Cardiology

Recent developments in the molecular analysis of autopsy and biopsy specimens have clarified the relationship between viral myocarditis and dilated cardiomyopathy (1,2). Therefore, treatments targeted toward viral infections are becoming important in the prevention of heart failure complicating myocarditis. However, despite intensive experimental and clinical research, specific therapies remain to be developed. Because both the direct cytopathic effects of the virus and the host immune response that it induces appear to be responsible for the manifestations of viral myocarditis (3), therapeutic effects have been expected from immunosuppression, a hypothesis supported by anecdotal, isolated case reports. However, treatment of myocarditis with immunosuppressors such as corticosteroids (4,5), cyclosporine A (CsA) (6–8) or FK506 (tacrolimus) (9) has been ineffective experimentally and in the recent clinical Myocarditis Treatment Trial (10).

The new synthetic immunosuppressor FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride, FTY), a derivative of ISP-I (myriocin) isolated from the fungus *Isaria sinclairii* (11,12), has unique properties, unlike those of CsA and FK506 or of corticosteroids (13). Its precise mechanisms of action remain unclear, though it has been shown to accelerate the sequestration of circulating mature lymphocytes into lymph nodes and Peyer's patches (14) and to decrease the number of peripheral blood lymphocytes and their infiltration into target tissues (15). In contrast to CsA and FK506, FTY does not suppress the proliferative response of T cells (16) or the production of IL-2 by lymphocytes in vitro (14). In experimental allogenic transplantation models of skin (14,17,18), liver (18), kidney (19) and heart (19,20), FTY has already been found as effective as, or more effective than, CsA in promoting the survival of allografts. Despite its importance in organ transplantation, the efficacy of FTY against infectious pathogens has been the object of only a few reports (21).

This study was performed to examine the efficacy of FTY in the treatment of experimental acute viral myocarditis induced by encephalomyocarditis virus (EMCV) in a murine model.

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MATERIALS AND METHODS

Pharmaceuticals. FTY, supplied as dry powder by Yoshitomi Pharmaceutical Industries, Ltd. (Osaka, Japan), was

Abbreviations and Acronyms

CsA	=	cyclosporine A
EMCV	=	encephalomyocarditis virus
FTY	=	FTY720
IFN	=	interferon
iNOS	=	inducible NO synthase
NO	=	nitric oxide
TNF	=	tumor necrosis factor

dissolved in sterile distilled water. Cyclosporine A was purchased from Sigma Pharmaceuticals (St. Louis, Missouri) and dissolved in olive oil (20).

Experimental viral myocarditis. Twenty-eight-day-old male DBA/2 mice were purchased from Shizuoka Agricultural Cooperation Association (Shizuoka, Japan) and housed in a special pathogen-free animal facility of the Kyoto University Hospital. The experiments were performed in a well-established murine model of EMCV-induced myocarditis (22). Briefly, the mice were inoculated intraperitoneally with 10 plaque-forming units (pfu) of the myocardiotrophic (M) variant of EMCV on day 0 and observed daily. Starting on day 0, FTY was daily administered orally in a dose of 10 mg/kg/day in one group of mice (FTY group), CsA was daily administered orally in a dose of 40 mg/kg/day in an other group (CsA group) and control mice received distilled water only (control group) (20). Each drug, or the distilled water, was administered via a gastric tube to guarantee reliable delivery. All experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoto University.

Survival study. In the survival experiments, the animals (nine in the CsA group, 21 in the FTY group, 22 in the control group) were observed daily between day 0 and day 14. In another series of experiments, the same doses of CsA or FTY were administered to uninfected mice (five in each group) in order to examine the effects of the drugs on survival in absence of infection.

Histopathologic examination. For histopathologic studies, hearts from surviving mice (six in the CsA group, eight in the FTY group, nine in the control group) were harvested on day 5, fixed in 10% formalin and embedded in paraffin. The ventricle was sliced transversally, stained with hematoxylin and eosin, and examined by light microscopy. Extent of cellular infiltration and myocardial necrosis were graded as follows: 0 = no lesion; 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; 4+ = lesions involving >75% of the myocardium. Extent of infiltration and necrosis was scored blindly by two independent trained observers, whose scores were averaged. The details of the method have been described previously (23). In another series of experiments, the same doses of CsA or FTY were administered to uninfected mice (five in each group) to examine the effects of the drugs on histopathology in absence of infection.

Intramyocardial virus concentration. For the measurements of intramyocardial virus concentration, the ventricles from surviving mice (five in the CsA group, seven in the FTY group, eight in the control group) were harvested aseptically on day 5, weighed, and homogenized in 1 ml of phosphate buffered saline. After centrifugation at 14,000 rpm for 20 min at 4°C, 0.1 ml of supernatant was inoculated into human amnion FL cell monolayers for 60 min at 37°C in 5% CO_2. The cells were overlaid with 3 ml of medium containing 4% fetal calf serum and 1% methylcellulose. After 20 h of incubation at 37°C in a humidified atmosphere containing 5% CO_2, the cells were fixed with acetic acid and methanol (in a ratio of 1:2) and stained with 1% crystal violet, and the plaques were counted under an inverted microscope (23). If the plaques were too numerous to count, the assay was repeated after appropriate dilution of the supernatants with Dulbecco's modified Eagle's medium (GIBCO BRL, New York, New York). Each value represents the average of two experiments. Myocardial virus concentration is expressed as plaque forming units/g of heart.

Intracardiac cytokine assay. For assays of intracardiac cytokine, the ventricles from surviving mice (six in the CsA group, seven in the FTY group, eight in the control group) were harvested on day 5, homogenized in 1 ml PBS solution with an ultrasonic homogenizer (Astrason Ultrasonic Liquid Processor, model XL2020, MISONIX Inc., Farmingdale, New York) and centrifuged at 14,000 rpm for 20 min at 4°C, and the supernatant was used for the assay of IL-2, IL-12, IFN-gamma and TNF-alpha. Each cytokine protein level was measured by enzyme-linked immunosorbent assay (24) with commercially available kits according to each manufacturer's instructions. Enzyme-linked immunosorbent assay kits for mouse IL-2 and IFN-gamma were purchased from GENZYME Corporation (Cambridge, Massachusetts) and kits for mouse IL-12 and TNF-alpha from ENDOGEN Inc. (Cambridge, Massachusetts).

The total protein concentration in each supernatant was also measured by the bicinchoninate acid method, and the ratio of cytokine concentration/total protein concentration was calculated (25). Each cytokine protein level was expressed as pg or ng/mg total protein.

Intracardiac nitric oxide assay. Intracardiac nitric oxide (NO) content was measured by modified Griess assay in the same supernatants as the intracardiac cytokines (25,26). In brief, 50 μl of supernatant or standard nitrite was mixed with 10 μl of 10 μM beta-NADPH, and 40 μl premixed master mix (500 μM glucose-6-phosphate, 160 U/l glucose-6-phosphate dehydrogenase, 80 U/l nitrate reductase, 0.2 mM phosphate buffer) was added and incubated for 45 min at 20°C. Subsequently, 50 μl of 1% sulfanilamide in 5% H_3PO_4 and 50 μl of 0.1% naphthylethylene diamine dihydrochloride were also added and incubated for 10 min at 20°C. The optical density was measured at 540 nm with a microplate reader. Nitrite concentration in each sample was calculated from standards. Measurements of all samples

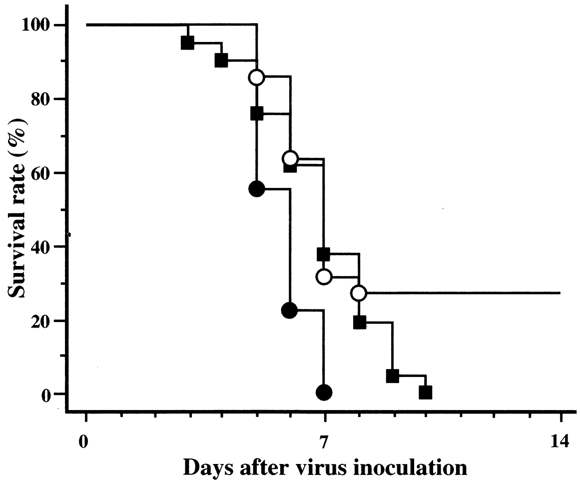

Figure 1. Fourteen-day cumulative survival after encephalomyocarditis virus inoculation in FTY720 treated mice **(open circles)**, versus cyclosporine A-treated mice **(filled circles)** versus control mice **(filled squares)**.

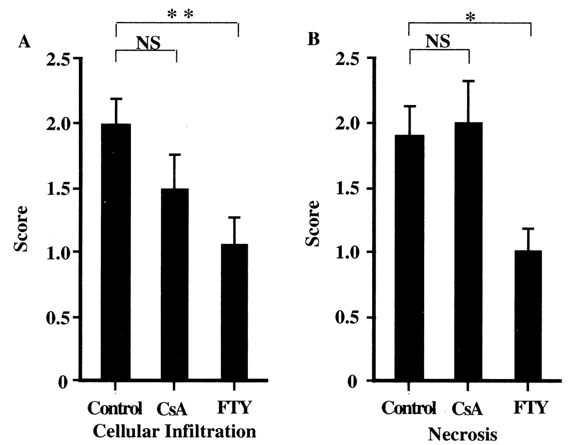

Figure 2. Myocardial histopathologic scores for cellular infiltration **(A)** and necrosis **(B)** on day 5 after encephalomyocarditis virus inoculation in surviving mice. *$p < 0.05$; **$p < 0.01$. CsA = cyclosporine A; FTY = FTY720; NS = not significant.

and standards were made in duplicate. The NO content in the heart was determined by dividing each nitrite concentration by the total protein concentration in each supernatant, expressed as μM/mg protein.

Statistical analysis. Statistical analysis of survival rates was performed by the Kaplan-Meier method, followed by Fisher's protected least significant difference. Histopathological scores; intracardiac virus concentrations; intracardiac IL-2, IL-12, IFN-gamma and TNF-alpha protein levels and NO content were compared by one-way ANOVA, followed by Fisher's protected least significant difference. A value of $p < 0.05$ was considered significant. Data are expressed as mean ± SEM.

RESULTS

Effects of FTY720 and CsA on survival in EMCV-infected mice. In the control group all mice died within 10 days. Cyclosporine A had a negative effect on survival rate, because none of the mice in that group survived past day 7 (Fig. 1), a significantly worse outcome than in the control group. In contrast, six of 22 mice (27%) treated with FTY were alive on day 14 ($p = 0.051$ vs. control, and $p = 0.0128$ vs. CsA). In the experiments performed in uninfected mice, all animals survived until day 14 after the administration of either drug (data not shown).

Histopathologic examinations. In the CsA group, the cellular infiltration score was lower, and the necrosis score slightly higher than in the control group (1.50 ± 0.26 vs. 2.00 ± 0.19 for infiltration, and 2.00 ± 0.32 vs. 1.89 ± 0.23 for necrosis), though these differences were not statistically significant. In contrast, the scores of both cellular infiltration and necrosis in the FTY group were significantly lower than in the control group (1.06 ± 0.19 vs. 2.00 ± 0.19 for infiltration and 1.00 ± 0.19 vs. 1.89 ± 0.23 for necrosis, Figs. 2 and 3). In the uninfected mice, neither drug had an apparent effect on the cardiac histology on day 5 (data not shown).

Intramyocardial virus concentration. Cyclosporine A increased virus replication in the heart 20-fold compared with control measurements ($9.94 \pm 6.30 \times 10^7$ vs. $5.18 \pm 2.73 \times 10^6$, Fig. 4). In contrast, FTY had no such effect on virus replication ($1.23 \pm 0.53 \times 10^7$ vs. $5.18 \pm 2.73 \times 10^6$, Fig. 4).

Measurements of intracardiac IL-2, IL-12, IFN-gamma and TNF-alpha. The effects of treatment with FTY and CsA on intramyocardial cytokines expression are summarized in Figure 5. Consistent with previous observations (27), protein levels of intracardiac IL-2, known to be associated with T-cell proliferation, were lower in both the CsA and the FTY groups than in the control group. However, the suppression of IL-2 in the FTY group was less prominent than in the CsA group. Protein levels of intracardiac IL-12, a Th1-specific cytokine (28), were

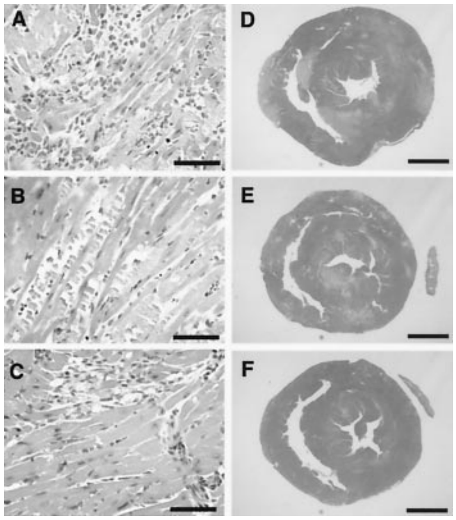

Figure 3. Representative photomicrograph of histopathologic sections of the heart. **A** and **D:** control mouse; **B** and **E:** cyclosporine A-treated mouse; **C** and **F:** FTY720-treated mouse. Hematoxylin and eosin stain, **A,B,C:** original magnification ×200, bar = 50 μm, **D,E,F:** original magnification ×2, bar = 1 mm.

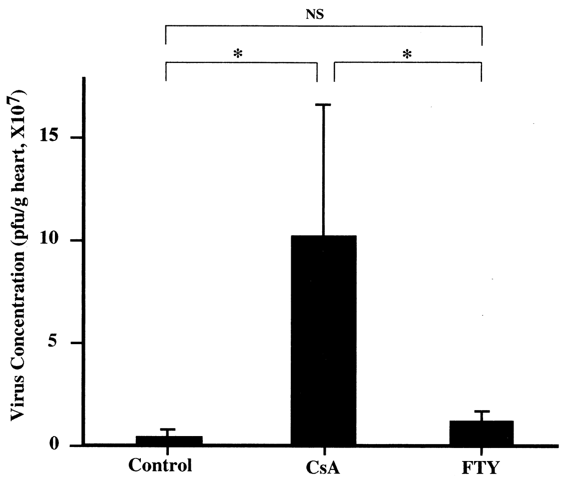

Figure 4. Effects of FTY (FTY720) and cyclosporine A (CsA) on intracardiac virus concentration on day 5 after encephalomyocarditis virus inoculation in surviving mice. $^{*}p < 0.05$. NS = not significant.

significantly lower in the CsA group than in both the FTY and the control groups. The difference between the FTY and control groups was also statistically significant. Similarly, intracardiac IFN-gamma, which inhibits virus replication (29), was markedly decreased in the CsA group and only mildly suppressed in the FTY compared with the control group. Conversely, concentrations of intracardiac

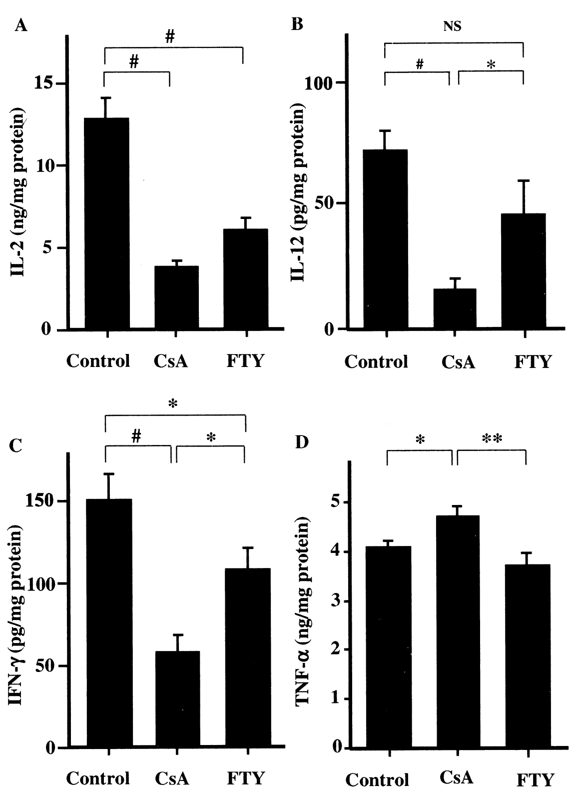

Figure 5. Intracardiac cytokine protein levels on day 5 after encephalomyocarditis virus inoculation in surviving mice. **A:** interleukin (IL)-2; **B:** IL-12; **C:** interferon (IFN)-gamma; **D:** tumor necrosis factor (TNF)-alpha. $^{*}p < 0.05$, $^{**}p < 0.01$, # $p < 0.001$. CsA = cyclosporine A; FTY = FTY720; NS = not significant.

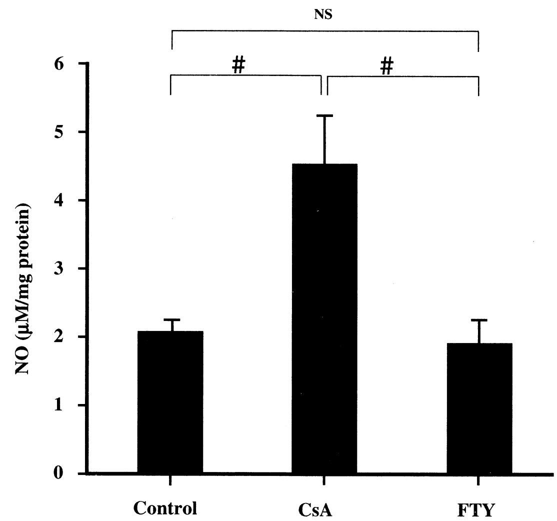

Figure 6. Intracardiac nitric oxide (NO) content on day 5 after encephalomyocarditis virus inoculation in surviving mice. #$p < 0.001$. CsA = cyclosporine A; FTY = FTY720; NS = not significant.

TNF-alpha, one of the proinflammatory cytokines, were increased in the CsA group, but not in the FTY group, compared with control.

Intracardiac NO content. In parallel with the changes observed in TNF-alpha, intracardiac NO content was significantly increased in the CsA group, but not in the FTY group, compared with control (Fig. 6).

DISCUSSION

Superiority of FTY720 over other immunosuppressors in viral myocarditis. This study showed that the new immunosuppressor FTY had notable therapeutic effects in this murine model of viral myocarditis, by a prolongation of survival and attenuation of histologic abnormalities. These effects were exerted by gentle cardiac immunosuppression, without the induction of excessive virus replication, despite the use of relatively high doses of FTY. The doses of CsA administered were also high because of its peculiar pharmacokinetics in mice (30,31). However, a detrimental effect of CsA in EMCV-induced myocarditis has been observed in other studies in which lower doses of the drug were used (6,7).

FTY is reported to be effective in a myosin-induced autoimmune myocarditis rat model (32). However, another immunosuppressor, FK506, which is ineffective in a viral myocarditis murine model (9), is also known to prevent the progression of myosin-induced autoimmune myocarditis in a rat model (33,34). A general complication associated with the use of immunosuppressors in the midst of a viral infection is uncontrolled virus replication (4,6,35,36), so the two models of myocarditis should be distinguished in terms of immunosuppressive therapy. In this study, virus concentrations in the heart were significantly increased by CsA, compared with control, as previously reported (4). This detrimental effect was not observed with FTY.

Characteristic effects of FTY on the expression of cardiac cytokines, on NO and on virus replication. To investigate the mechanism of this superior effect of FTY, we measured several cytokines associated with cell-mediated immunity, and one major proinflammatory cytokine. Interleukin-2 activates T lymphocyte proliferation, the suppression of which, at a transcriptional level, is known to be mediated by calcineurin-related immunosuppressant such as CsA and FK506 (13). In this study, as expected, CsA prominently suppressed the intracardiac expression of IL-2. In addition, both IL-12, which promotes differentiation to the Th1 cell subset (37), and IFN-gamma, a Th1 cytokine with antiviral activity, were markedly suppressed in the hearts of CsA-treated mice. This direct inhibition of T cell-mediated immunity may explain the observed accelerated virus replication. FTY also suppressed the expression of these three cytokines in the heart, though to a considerably lesser degree than CsA. The most likely proposed immunosuppressive mechanisms of FTY are an accelerated sequestration of circulating mature lymphocytes into lymph nodes and Peyer's patches (14), and a decrease in peripheral blood lymphocytes and infiltration into target tissues (15). In addition, FTY does not directly suppress the proliferative responses of T cells (16) or the production of IL-2 by lymphocytes in vitro (14). These mechanisms of action explain the absence of accelerated virus replication associated with the FTY treatment. Furthermore, intracardiac TNF-alpha and NO were proportionally elevated in the CsA group compared with the control group. Cyclosporine A is known to suppress TNF-alpha production from lymphocytes but not from macrophages in vitro (38). Immunohistochemical analyses have shown TNF-alpha staining in macrophages within the heart affected by EMCV-induced myocarditis (39). In addition, TNF-alpha mRNA was also induced in purified neonatal murine cardiac fibroblasts infected with EMCV in vitro (unpublished data), TNF-alpha concentrations in plasma and in the heart were increased in EMCV-induced myocarditis (23,25) and treatment with anti-TNF-alpha antibody had a therapeutic effect in this model (23). From these observations combined, one may hypothesize that excessive viral replication directly induced TNF-alpha in the hearts of CsA-treated mice. Nitric oxide is produced by inducible NO synthase (iNOS), which is induced by proinflammatory cytokines, including TNF-alpha. Nitric oxide not only has antiviral effects but also causes myocardial injury. Immunomodulating drugs, which suppress proinflammatory cytokines and iNOS (40–42), are therapeutic in EMCV-induced myocarditis (25). Increases in TNF-alpha and NO concentrations may also explain, at least in part, the worsening of heart failure associated with CsA treatment. In contrast, in our study, FTY did not increase the concentrations of TNF-alpha or NO.

Opportunistic infection is the most troublesome complication of immunosuppression in organ transplantation (35). In this respect, our results suggest that FTY may be superior to the other immunosuppressors, although its efficacy in the presence of common opportunistic infectious pathogens needs to be examined.

Study limitations. In a clinical situation, treatment of viral myocarditis cannot be initiated at the very onset of infection. Further experiments, designed with a delay between virus inoculation and onset of treatment, need to be performed. This study, nevertheless, remains noteworthy for having observed no increase in virus replication from treatment with FTY. In addition, further studies of the specific therapeutic effects of FTY are needed, as its precise pharmacologic properties remain incompletely understood.

In conclusion, this report is the first to describe a therapeutic effect by an immunosuppressive agent in the treatment of acute viral myocarditis.

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