TABLE 1. Hemodynamic Variables in Healthy Rats Treated With β-AR Agents

	Vehicle	Formoterol, 22.5 μg/kg	Salbutamol, 200 μg/kg	Metoprolol, 30 mg/kg	Propranolol, 10 mg/kg	*P*, ANOVA	*P*, Bonferroni 1 vs 2	1 vs 3	1 vs 4	1 vs 5	4 vs 5
No. of healthy rats	8	8	8	8	8						
HW/BW, %	0.48±0.02	0.52±0.03	0.50±0.02	0.50±0.02	0.49±0.03	0.88	...	...	...	...	...
Heart rate, bpm	438±6	448±4	446±6	328±8	342±6	<0.00001	0.88	0.95	<0.00001	<0.00001	0.20
SBP, mm Hg	133±2	139±4	142±4	111±3	113±4	<0.00001	0.18	0.06	<0.00001	<0.00001	2.26
DBP, mm Hg	88±3	82±3	90±2	73±2	74±2	<0.00001	0.09	0.89	<0.00001	<0.00001	2.11
Fractional shortening, %	76±3	79±3	79±3	76±3	75±4	0.68	...	...	...	...	...

Values are mean±SEM. 1 indicates vehicle group; 2, formoterol group; 3, salbutamol group; 4, metoprolol group; 5, propranolol group; SBP, systolic blood pressure; and DBP, diastolic blood pressure. One-way ANOVA was used to test for differences among groups, and, when appropriate, Bonferroni multiple comparison test was performed to test difference between 2 groups.

Results

Hemodynamics in Healthy Rats Treated With β-AR Agents

HR and BP were significantly decreased in the rats of the 2 β-AR antagonist groups compared with the vehicle group, with no significant difference between them. In contrast, no change in hemodynamic variables was seen in the formoterol or salbutamol groups (Table 1).

Administration of β-AR Agents Starting on Day 0

Mortality

Four diseased rats treated with vehicle or metoprolol (mortality: 4/18, 22%) died between days 19 and 21, and 8 diseased rats treated with propranolol (8/18, 44%) died between days 15 and 21. However, all diseased rats treated with the β2-AR agonists (0/18, 0%) survived until day 21.

Hemodynamics

Compared with the vehicle group, BP, HR, and fractional shortening were significantly decreased in the β-AR antagonist groups, whereas fractional shortening and BP were significantly increased with a decrease of HR in the β2-AR agonist groups. No significant differences in hemodynamic variables were seen between the β-AR antagonist groups (Table 2).

Severity of Disease

Macroscopic score, HW/BW, and area of cellular infiltration into the myocardium were significantly reduced in the 2 β2-AR agonist groups, indicating a significantly reduced severity of disease. In contrast, the propranolol but not the metoprolol group showed a significantly increased severity of disease compared with the vehicle group (Figure 1, Table 2).

Cytokine Profiles

Compared with the vehicle group, levels of IFN-γ and IL-10 mRNA in the myocardium were significantly increased in the propranolol but not in the metoprolol group. In contrast, levels were decreased in both the formoterol and salbutamol groups. However, IL-10/IFN-γ was significantly decreased in the propranolol group but increased in the 2 β2-AR agonist groups compared with the vehicle group (Table 2).

Administration of β2-AR Agonist From Day 14 After Immunization

Mortality

Three diseased rats treated with the vehicle (mortality: 3/12, 25%) died between days 19 and 21, whereas all diseased rats treated with the β2-AR agonists (0/12, 0%) survived until day 21.

TABLE 2. Histological and Hemodynamic Variables and Cytokine Profiles in EAM Rats Treated From Day 0

	Vehicle	Formoterol, 22.5 μg/kg	Salbutamol, 200 μg/kg	Metoprolol, 30 mg/kg	Propranolol, 10 mg/kg	*P*, ANOVA	*P*, Bonferroni 1 vs 2	1 vs 3	1 vs 4	1 vs 5	4 vs 5
No. of EAM rats	14	18	18	14	10	...	...	...	...	...	...
Macroscopic score	2.8±0.1	1.4±0.1	1.2±0.1	2.6±0.1	3.6±0.2	<0.00001	<0.00001	<0.00001	0.39	<0.00001	<0.00001
HW/BW, %	0.82±0.02	0.56±0.01	0.51±0.01	0.84±0.02	1.13±0.02	<0.00001	<0.00001	<0.00001	0.81	<0.00001	<0.00001
Cellular infiltration area, %	68.6±2.1	32.4±2.0	28.6±1.6	64.9±2.5	89.1±3.2	<0.00001	<0.00001	<0.00001	0.49	<0.00001	<0.00001
Heart rate, bpm	476±5	458±5	456±4	426±5	436±5	<0.00001	0.0031	0.00044	<0.00001	<0.00001	0.21
SBP, mm Hg	120±3	132±2	136±3	90±3	86±2	<0.00001	0.00001	<0.00001	<0.00001	<0.00001	0.47
DBP, mm Hg	82±2	80±2	88±2	60±2	58±3	<0.00001	0.89	0.17	<0.00001	<0.00001	0.94
Fractional shortening, %	46±3	66±2	70±3	31±2	26±3	<0.00001	<0.00001	<0.00001	<0.00001	<0.00001	0.25
IFN-γ, fold increase	0.99±0.02	0.46±0.02	0.42±0.02	0.93±0.02	3.82±0.21	<0.00001	<0.00001	<0.00001	0.19	<0.00001	<0.00001
IL-10, fold increase	0.98±0.01	0.88±0.02	0.83±0.02	0.95±0.01	1.63±0.02	<0.00001	<0.00001	<0.00001	0.28	<0.00001	<0.00001
IL-10/IFN-γ, fold increase	0.99±0.02	1.83±0.04	1.98±0.04	1.02±0.02	0.43±0.01	<0.00001	<0.00001	<0.00001	2.12	<0.00001	<0.00001

Values are mean±SEM. 1 indicates vehicle group; 2, formoterol group; 3, salbutamol group; 4, metoprolol group; 5, propranolol group; SBP, systolic blood pressure; and DBP, diastolic blood pressure. Level of IFN-γ, IL-10, or IL-10/IFN-γ in each group is represented as mean±SEM for fold increases over each average level in the control group.

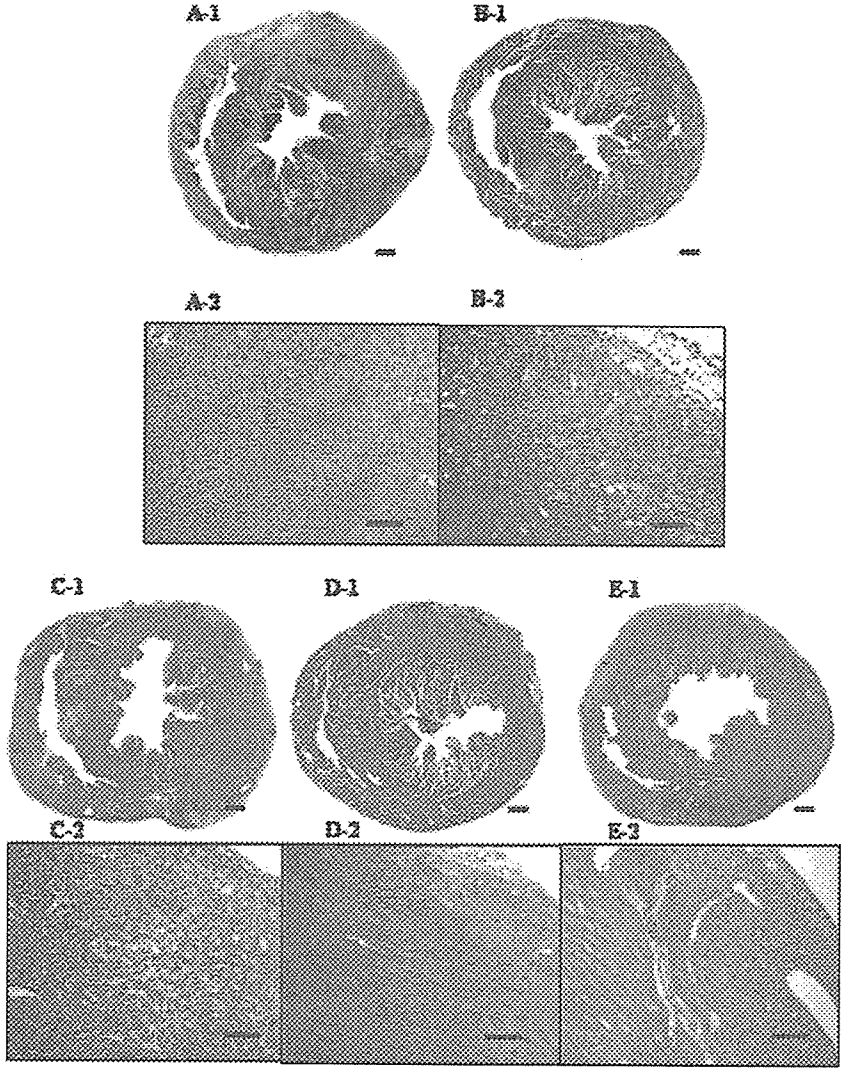

Figure 1. Histological findings in EAM hearts on day 21 of in vivo administration of β-AR agents. Administration of propranolol at 10 mg/kg (A) facilitated interstitial cellular infiltration compared with that of metoprolol at 30 mg/kg (B) or vehicle (C). Conversely, administration of either formoterol at 22.5 μg/kg (D) or salbutamol at 200 μg/kg ameliorated interstitial cellular infiltration (E). Bars=1 mm or 250 μm (thick, for ×1; thin, for ×40, respectively).

Hemodynamics

Among hemodynamic variables, significant increases in fractional shortening and systolic blood pressure were seen, with a significant decrease in heart rate in the formoterol and salbutamol groups compared with the vehicle group (Table 3).

Severity of Disease

Disease severity as indicated by macroscopic score, HW/BW, and area of cellular infiltration into the myocardium was significantly decreased in the formoterol and salbutamol groups compared with the vehicle group (Table 3). These macroscopic findings were reflected in microscopic findings, which included interstitial cellular infiltration and destruction of myocardial fibers (Figure 2).

Cytokine Profiles

Compared with levels in the vehicle group, myocardial mRNA levels of IFN-γ and IL-10 were significantly decreased in both the formoterol group and salbutamol group. In contrast, myocardial IL-10/IFN-γ mRNA levels were significantly increased in the β2-AR agonist groups compared with the vehicle group (Table 3).

Myocardiogenic T Lymphocytes and β-AR Stimulation

Formoterol as well as salbutamol strongly and dose-dependently suppressed cardiac myosin–specific T-lymphocyte proliferation and IL-12 production by antigen-presenting cells and IFN-γ production by T lymphocytes, whereas denopamine only slightly suppressed cardiac myosin–specific T-lymphocyte activity (Figure 3). ICI118,551 reversed these inhibitory effects of formoterol or salbutamol (Figure 4).

Influence of In Vivo Administration of β-AR Agents on Lymph Node Cell Activity

The proliferation of cardiac myosin–primed lymph node cells from treated EAM rats and their production of IL-12 and IFN-γ were significantly decreased in both the formoterol group (6399±297 cpm, 76±4.9 pg/mL, 3480±145 pg/mL, respectively; each $P<0.00001$ versus the vehicle group) and the salbutamol group (6140±295 cpm, 61±4.5 pg/mL, 3012±189 pg/mL, respectively; each $P<0.00001$ versus the vehicle group) compared with the vehicle group (13283±309 cpm, 173±12 pg/mL, 12913±429 pg/mL, respectively). Conversely, cell proliferation and cytokine production were

TABLE 3. Histological and Hemodynamic Variables and Cytokine Profiles in EAM Rats Treated From Day 14

	Vehicle	Formoterol, 22.5 μg/kg	Salbutamol, 200 μg/kg	P, ANOVA	P, Bonferroni 1 vs 2	P, Bonferroni 1 vs 3	P, Bonferroni 2 vs 3
No. of EAM rats	9	12	12				
Macroscopic score	2.9±0.1	2.2±0.1	2.0±0.1	0.00044	0.00005	<0.00001	0.62
HW/BW, %	0.82±0.03	0.72±0.02	0.68±0.02	<0.00001	<0.00001	<0.00001	0.06
Cellular infiltration area, %	70.6±2.7	48.4±1.3	42.4±2.0	<0.00001	<0.00001	<0.00001	0.0004
Heart rate, bpm	478±2	468±3	466±2	0.031	0.0079	0.0013	0.95
SBP, mm Hg	120±2	128±2	126±2	0.025	0.00078	0.029	0.32
Fractional shortening, %	44±2	56±2	58±2	<0.00001	<0.000001	<0.000001	0.70
IFN-γ, fold increase	1.01±0.03	0.44±0.02	0.41±0.01	<0.00001	<0.00001	<0.00001	0.24
IL-10, fold increase	1.02±0.03	0.89±0.02	0.84±0.02	0.00024	0.00002	<0.00001	0.08
IL-10/IFN-γ, fold increase	1.01±0.03	2.01±0.05	2.05±0.04	<0.00001	<0.00001	<0.00001	0.99

Values are mean±SEM. 1 indicates vehicle group; 2, formoterol group; 3, salbutamol group; and SBP, systolic blood pressure. Level of IFN-γ, IL-10, or IL-10/IFN-γ in each group is represented as mean±SEM for fold increases over each average level in the control group.

significantly increased in the propranolol group (28233±527 cpm, 402±24 pg/mL, 44022±1408 pg/mL, respectively; each $P<0.00001$ versus the vehicle group) but not in the metoprolol group (12388±253 cpm, $P=0.08$; 157±13 pg/mL, $P=0.96$; 13939±392 pg/mL, $P=0.58$; respectively) (Figure 5). Cell proliferation and cytokine expression were not observed in any culture without cardiac myosin (300±14 cpm).

Administration of formoterol and salbutamol increased intracellular cAMP levels in myosin-primed lymph node cells compared with vehicle (188±12, 179±9, versus 39±2 fmol, respectively; each $P<0.00001$), whereas that of propranolol decreased cAMP levels (11±0.8 fmol; $P=0.00007$ versus the vehicle group). Administration of metoprolol did not affect intracellular cAMP level (36±1 fmol; $P=2.39$ versus the vehicle group) (Figure 5).

Discussion

The role of β-adrenergic stimulation on myocarditis has been investigated. It has been shown that the β1-AR agonist denopamine prolongs survival in mice with viral myocarditis.[21] However, other recent reports demonstrated that metoprolol, a β1-selective AR antagonist, did not affect disease severity and mortality in rats with autoimmune myocarditis[22] and mice with viral myocarditis,[23] suggesting that β1-AR has only a weak modulatory effect on the development of myocarditis. Thus, the protective effect of denopamine on myocarditis may result from an improvement in hemodynamic deterioration via its positive inotropic effect rather than from any other effect. On the other hand, these reports also indicated the immunomodulatory potential on myocarditis of carvedilol, which has β-AR–blocking and antioxidative effects,[22,23] and indicated that this effect was explained mainly by its antioxidative effect. However, the role of β2-AR stimulation on myocarditis remains uncertain.

Wide use of β2-selective AR–stimulating agents such as formoterol and salbutamol has been restricted to bronchodilation in patients with asthma. Recently, however, interest in these agents was renewed for their potential immunomodulatory role in Th1 cytokine–induced autoimmune disease. Catecholamines and several adrenergic agonists have been shown to influence the production of Th1 cytokines, and β2-AR is involved in this mechanism.[12,13] Furthermore, intraperitoneal administration of salbutamol, a β2-selective AR agonist, suppressed Th1 cytokine–induced autoimmune arthritis via β2-AR in vivo.[14] In the present study, the 2 β2-selective AR agonists formoterol and salbutamol did not affect hemodynamics in healthy rats (Table 1) but ameliorated Th1 cytokine–induced EAM[10,11] on day 21 (Table 2) and reduced mortality. Furthermore, treatment with propranolol but not metoprolol exacerbated EAM on day 21 and increased mortality, despite showing equivalent hemodynamic effects (Table 2). The different effect of the 2 β-blockers according to β-AR selectivity supports the existence of an immunomodulatory role of β2-AR in the development of EAM. Together, the present results indicate that β2-AR–stimulating agents can ameliorate the development of EAM via β2-AR.

In myocarditis, the autoimmune process leads to myocardial inflammation and injury via the effect of activated Th1 T lymphocytes specific for cardiac myosin.[10,24] Th1 cytokines such as IL-12 and IFN-γ promote this process.[10] It has been demonstrated that β2-AR stimulation inhibits the production of IFN-γ and IL-12 in vitro.[12,13] Furthermore, inhibition of antigen-specific T-cell proliferation was reported to be a therapeutic strategy for retarding the development of myocarditis.[25] The protective effect on EAM of the 2 β2-selective AR agonists seen here can therefore be explained by the idea that β2-AR stimulation attenuates cardiac myosin–specific Th1 T-lymphocyte proliferation by suppressing the production of Th1 cytokines. However, the suppressive effect of β2-AR stimulation on cardiac myosin–specific Th1 T-lymphocyte activity has not been elucidated.

To clarify this point, we used in vitro experimental systems using the immunodominant myosin peptide–specific CD4-positive Th1 T-lymphocyte line, the transfer of which induces EAM,[18] and ex vivo experimental systems using cardiac myosin–primed lymph nodes from β-AR agent–treated rats. The myocarditogenic Th1 T-lymphocyte line was stimulated

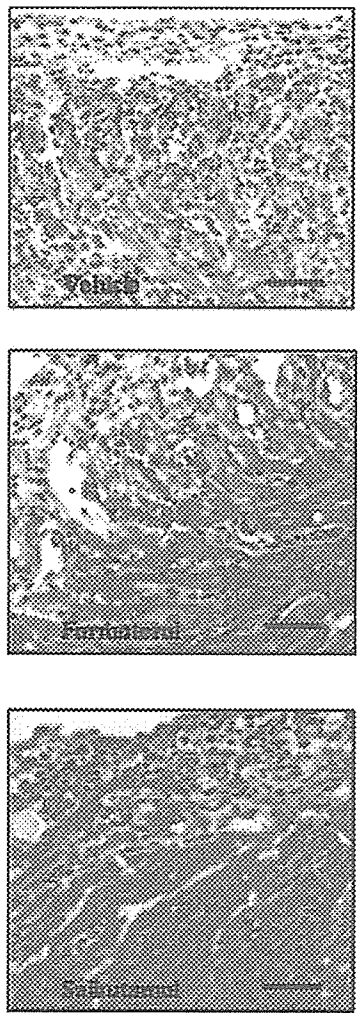

Figure 2. Microscopic findings in EAM rats treated with formoterol at 22.5 μg/kg, salbutamol at 200 μg/kg, or vehicle from days 14 to 21. β2-AR agonists reduced interstitial cellular infiltration and destruction of myocardial fibers compared with the vehicle. Bars=250 μm (thin, for ×100).

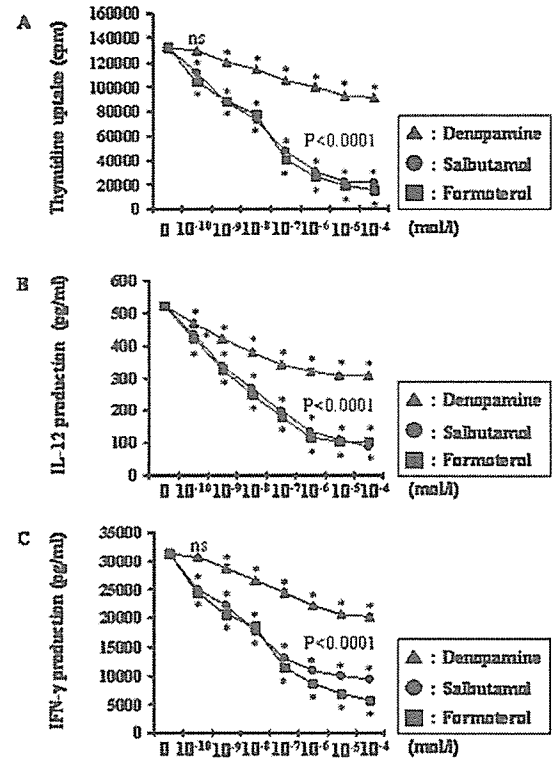

Figure 3. Effects of β-AR agonists on myocarditogenic T lymphocytes stimulated by specific antigen and antigen-presenting cells. Cell proliferation (A) and production of IL-12 (B) and IFN-γ (C) in the culture supernatant were determined by ^{3}H-thymidine uptake and an ELISA kit. Three series of experiments were performed for each investigation. Error bars represent SEM. β-AR agonists decreased cell proliferation and production of IL-12 and IFN-γ (*P<0.00001 vs culture with vehicle only), and inhibitory effects by formoterol and salbutamol were very much stronger compared with denopamine in each concentration (P<0.0001).

with antigen-presenting cells and the specific antigen to emulate the priming step of EAM in vivo. Both formoterol and salbutamol reduced cardiac myosin–specific T-lymphocyte proliferation by suppressing the production of IL-12 and IFN-γ (Figure 3). ICI118,551, a β2-selective AR antagonist, but not metoprolol (data not shown), completely reversed the inhibitory effects of formoterol and salbutamol (Figure 4). Along with the difference in myosin-primed lymph node cell proliferation among formoterol-treated, salbutamol-treated, propranolol-treated, metoprolol-treated, and vehicle-treated rats (Figure 5), these results suggested that β2-AR–stimulating agents ameliorated the induction of EAM by attenuating cardiac myosin–specific Th1 T-lymphocyte proliferation in the lymphoid organs associated with the suppression of Th1 cytokines via β2-AR stimulation. Previous reports have demonstrated that β2-AR is present on Th1 T lymphocytes and antigen-presenting cells and that its activation inhibits their production of Th1 cytokines by increasing intracellular cAMP levels.[12,13] In our ex vivo experiment, β2-AR stimulation inhibited myosin-primed lymph node cell activity adversely, in parallel with increasing intracellular cAMP levels (Figure 5). Thus, the inhibitory effect of β2-AR stimulation on Th1 T lymphocytes specific for the cardiac myosin–mediated immune response identified here may have contributed to the activation of the β2-AR–cAMP signaling pathway on Th1 T lymphocytes and antigen-presenting cells.

An imbalance in Th1 and Th2 cytokines modulates the pathogenesis of EAM,[11] and the Th2 cytokine IL-10 plays a protective role in the development of EAM.[26] Previous reports showed that shifting the immune response toward the Th2 pattern prevented the development of EAM.[10,27] Thus, we examined the production of the Th1 cytokine IFN-γ and Th2 cytokine IL-10 in the heart. Administration of either formoterol or salbutamol, but not of propranolol, starting on day 0 reduced myocardial IFN-γ expression on day 21 compared with the vehicle group. The influences of drugs on myocardial IL-10 expression showed a similar pattern, although to a lesser degree, which is in part associated with the interaction of IL-10 and IFN-γ during the course of their production. However, myocardial IL-10/IFN-γ mRNA expression was significantly increased in the 2 β2-AR agonist

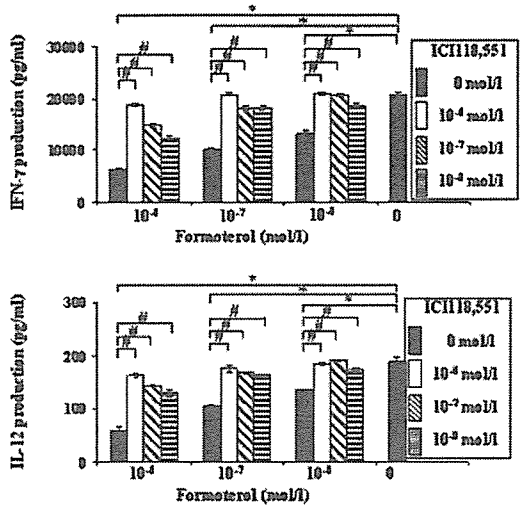

Figure 4. ICI118,551 reversed the inhibitory effect of β2-AR stimulation on the production of Th1-cytokines. Levels of IL-12 and IFN-γ in the culture supernatant were determined by an ELISA kit. Three series of experiments were performed for each investigation. Error bars represent SEM. *P<0.00001 vs culture with the vehicle only; #P<0.00001 vs each concentration of formoterol without ICI118,551.

groups, whereas it was significantly decreased in the propranolol group (Table 2). This finding suggests that β2-AR stimulation shifts the myocardial Th1/Th2 cytokine balance toward Th2 cytokines and that this effect in part contributes to modulating the development of EAM by β2-AR–stimulating agents.

Not only extremely suppressed myocardial IFN-γ expression by β2-AR stimulation but also in part enhanced IL-10 expression by it may contribute to alteration of the myocardial Th1/Th2 cytokine balance in vivo. Myocardial inflammation in EAM mainly involves macrophages and CD4-positive Th1 T lymphocytes, and the dominance of these cells is a constant finding at lesion sites and throughout the course of the disease.[28] The enhancement of IL-10 production in inflammatory cells such as monocytes, macrophages, and dendritic cells via β2-AR stimulation has been reported.[29,30]

Propranolol enhanced disease severity in the present study. However, recent studies in the BALB/c mouse model of viral myocarditis found that propranolol exerts a protective effect against myocarditis.[31] This difference may be explained in part by the promotion of a Th1 response in this infectious model, which clears the virus.[23,31]

In the present study, our intervention with β2-AR–stimulating agents was done in a later phase of EAM to examine their effect on established myocardial inflammation. Formoterol and salbutamol significantly reduced the severity and mortality of myocarditis (Table 3) and significantly increased myocardial IL-10/IFN-γ mRNA levels on day 21 compared with the vehicle (Table 3). β2-AR–stimulating agents also ameliorated established myocardial inflammation, and this beneficial effect was in part associated with an alteration in the myocardial Th1/Th2 cytokine balance. Given that human myocarditis is usually diagnosed after disease onset, this result has important implications for clinical treatment.

Chronic β-AR stimulation in heart failure induces myocardial apoptosis and thereby induces progressive myocardial remodeling.[32] However, recent reports have demonstrated opposing effects of β1- and β2-AR stimulation on cardiac myocytes. In rat cardiomyocytes, β1-AR stimulation induces apoptosis via a cAMP-dependent mechanism, whereas β2-AR stimulation inhibits this process.[33] A transgenic mice model overexpressing β1-AR developed dilated cardiomyop-

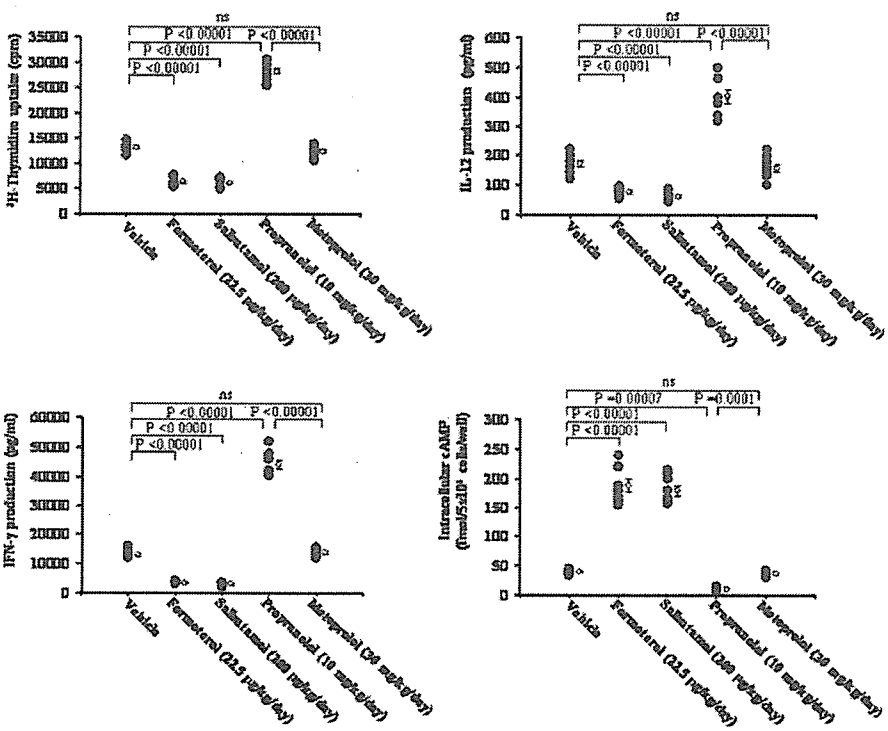

Figure 5. The proliferation of T lymphocytes and levels of IL-12, IFN-γ, and intracellular cAMP in cardiac myosin–primed lymph nodes from EAM rats treated with β-AR agent or vehicle. Cell proliferation, levels of Th1-cytokines, and levels of intracellular cAMP were determined by measuring radioactivity of incorporated ^{3}H-thymidine and an ELISA kit. Each group contained 9 rats. Closed circle indicates individual data; open circle and error bar, mean±SEM for each group. No significant difference was shown in comparisons of metoprolol and vehicle groups.

athy along with hemodynamic deterioration, whereas those overexpressing β2-AR did not.[34,35] In the present study, treatment with β2-AR agonists at doses that did not affect hemodynamic variables in healthy rats (Table 1) throughout the acute phase at least improved cardiac contractility in EAM rats (Table 2), and decreasing HR compared with the vehicle group (Table 2) may reflect an improvement of cardiac function. The new immunomodulatory effect of β2-AR stimulation identified here also contributed to this effect. β2-AR–stimulating agents thus may represent the preferred therapy for inflammatory myocardial conditions with hemodynamic deterioration derived from autoimmune processes.

Conclusions

To our knowledge, this study is the first to report that β2-AR–stimulating agents ameliorate the development of EAM by reducing cardiac myosin–specific T-cell activity in the lymphoid organs, which plays an important role in the initiation of myocarditis, and by altering the imbalance between Th1 and Th2 cytokines. These agents also ameliorate established myocardial inflammation. These findings indicate that β2-AR stimulation is a major immunomodulatory factor in the development of EAM. The potential of β2-AR–stimulating agents as new therapeutic drugs for the treatment of myocarditis requires further investigation.

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Disclosures

None.

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CLINICAL PERSPECTIVE

We present a compelling case for evaluating β2-adrenergic agonists in patients with myocarditic heart failure. Differences among in vivo therapies with β2-adrenergic agonists, propranolol as a nonselective β-adrenergic antagonist, and metoprolol as a β1-selective adrenergic antagonist in rat experimental autoimmune myocarditis (EAM) produced by immunization with cardiac myosin demonstrated that β2-adrenergic agonists modulate the development of EAM via β2-adrenergic stimulation. This effect was associated with modulating myocarditogenic Th1 T lymphocytes specific for cardiac myosin–mediated immune response in the lymphoid organs and shifting the imbalance of the Th1 cytokine and Th2 cytokine pattern toward the Th2 cytokine pattern in the myocardium, which was contributed to the suppression of Th1 cytokine production by β2-adrenergic stimulation. Therapy with β2-adrenergic agonists furthermore suppressed not only the induction of myocarditis but also established myocardial inflammation. It contributed to the improvement of hemodynamics and mortality in EAM. Taken together, these findings indicate the new role of the β2-adrenergic agonist as an immunomodulator in the development of EAM and may provide a novel approach for therapy in patients suffering from inflammatory myocardial conditions with hemodynamic deterioration, particularly those derived from autoimmune processes. Interest in β2-adrenergic agonists, which has been restricted to bronchodilators, should be renewed because of their potential as an immunomodulatory and therapeutic agent in myocardial inflammatory disease complicated by heart failure.

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Antigen-specific effects of autoantibodies against sarcolemmal Na–K-ATPase pump in immunized cardiomyopathic rabbits

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Abstract

Objectives: We examine antigen-specific actions of autoantibodies directed against sarcolemmal Na–K-ATPase.
Background: Autoantibodies against some receptors or pumps were detected in patients with dilated cardiomyopathy. Although immunoglobulin adsorption therapy improved cardiac function in such patients, direct pathogenic effects of autoantibodies remain to be proven.
Methods: Japanese white rabbits were immunized once a month with purified Na–K-ATPase (NKA rabbits, $n=10$) or a synthetic peptide corresponding to the second extracellular loop of beta1-adrenergic receptors (beta rabbits, $n=10$), respectively. Control rabbits ($n=10$) received vehicle in the same manner.
Results: At 6 months, cardiac hypertrophy along with increased left ventricular end-diastolic pressure was observed in both NKA and beta rabbits, and inhibitory G protein level increased in both NKA and beta rabbits. Histological findings showed similar myocyte hypertrophy and interstitial fibrosis in both rabbits. Enzymatic activities of Na–K-ATPase were lower in NKA rabbits than in other groups. Immunoblotting showed that alpha3-isoform of Na–K-ATPase was selectively reduced in myocardium from NKA rabbits.
Conclusions: Our present findings suggested that isoform-specific alterations of myocardial Na–K-ATPase activity were induced by immunizing rabbits. This was not secondary change due to cardiac hypertrophy. Thus, autoantibodies against sarcolemmal Na–K-ATPase have antigen-specific effect on the heart in vivo.

Keywords: Dilated cardiomyopathy; Autoantibodies; Na–K-ATPase

1. Introduction

Autoimmune mechanism is one of the causes of dilated cardiomyopathy (DCM) as well as genetic predisposition and viral infection. Production of anti-myocardial autoantibodies (Abs) is one of the outstanding autoimmune abnormalities in addition to persistent inflammation with alterations in cellular immunity and complement activation. Therefore, immunoadsorption (IA) therapy to remove various Abs was suggested as a new strategy to treat patients with congestive heart failure due to DCM [1]. Unsatisfactory hemodynamic improvement by IA therapy using protein-A column indicated the pathophysiological meaning of immunoglobulin G3, although the mechanism was not fully evaluated. In biopsy sample of the DCM patients, C5b-9 complex and the expression of TNF-alpha were observed along with the immunoglobulin deposits on the myocardial cell membrane [2]. This finding may provide a common mechanism leading to cardiac hypertrophy by any anti-myocardial Abs. However, we reported different clinical

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backgrounds of each Abs in patients with DCM [3]. So, we postulated antigen-specific effects of Abs and performed an experiment using immunizing rabbit model.

2. Materials and methods

2.1. Immunization

Experiments were performed in 30 male Japanese white rabbits, which were 10 weeks old (1.8 to 2.2 kg). The experimental protocol was approved by our Institutional Review Board. As antigens, porcine cerebral cortex Na–K-ATPase were obtained from Sigma Chemical Co. (St. Louis, Missouri) and a synthetic peptide corresponding to the second extracellular loop of rabbit beta1-adrenergic receptors (residues 197 to 222, HWWRAESDEARR-CYNDPKCCDFVTNR) was produced by Peptide Institute, Inc. (Osaka, Japan). Twenty rabbits were randomly divided into two groups and were immunized by subcutaneous injection of each antigens (1 mg) dissolved in 1 ml of saline conjugated with 0.5 ml of complete and incomplete Freund's adjuvant (NKA rabbits: $n=10$, beta rabbits: $n=10$). Ten control rabbits received saline containing adjuvant in the same manner. All rabbits were immunized once a month over 6 months.

2.2. Enzyme-linked immunoabsorbent assay (ELISA)

Each antigen (50 μl, 50 μg/ml in 0.1 mol/l Na_2CO_3) was used to coat individual wells of a 96-well microtiter plate. The wells were then saturated with phosphate buffer saline (PBS) supplemented with 3% skim milk, 0.1% Tween-20 and 0.01% merthiolate. Sera (50 μl) diluted 1:200 were added to the coated plates and incubated overnight at 4 °C. After three washes with PBS, an affinity-purified biotinylated goat anti-rabbit IgG antibody solution, diluted 1:1000 in merthiolate, was allowed to react for 1 h at room temperature. After three washes, the bound biotinylated antibodies were detected using streptavidin-peroxidase (1 μl/ml), H_2O_2 (2.5 mmol/l) and 2,2′-azino-di-(ethylbenzthiazoline) sulfonic acid (2 mmol/l). After 30 min, the optical densities (OD) at 492 nm were determined using an ELISA reader.

2.3. Ultrasonic echocardiography

Echocardiography was performed with the rabbit under anesthesia using 3 mg/kg thiopental sodium (Tanabe Seiyaku Co.). The parasternal long-axis views were obtained using a 7.5 MHz transducer connected to a Hewlett-Packard Sonos 500 ultrasonic echocardiographic system (model 77010CF), with the rabbit placed in the lateral decubitus position.

2.4. Hemodynamic and histological analysis

At the end of each observation period, hemodynamic measurements were performed in the open-chest condition under anesthesia with chloral hydrate as described previously [4]. Hearts were then rapidly removed. Excised hearts were fixed in 10% formalin and embedded in paraffin, and both transverse and cross sections (3 μm thickness) were obtained. Sections were stained with hematoxylin and eosin and were subjected to light microscopic examinations. Planimetry was performed macroscopically on cross sections of the hearts to evaluate left ventricular (LV) muscle mass and cavity area and microscopically on 100 cross-sectioned myocytes to determine cross-sectional area per myocyte, using NIH Image. Immunostaining was also performed in order to detect IgG on cardiac membrane. Endogenous peroxidase was inactivated by treatment with methanol+1% H_2O_2. After blocking with PBS supplemented with 3% BSA, each sections were incubated for 60 min with biotinylated goat anti-rabbit IgG. After three washes with PBS, the bound biotinylated antibodies were detected by the same method of ELISA.

2.5. Na–K-ATPase assay

The enzyme activity of myocardial Na–K-ATPase was determined by the colorimetric method. Membrane fractions were prepared as 50,000 g pellet as described previously [4]. Each membrane fragment was mixed with the reaction solution (100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA and 50 mM histidine, pH 7.2) at 4 °C. One hundred microliters of the membrane were mixed with 800 μl of the reaction solution and 100 μl of 30 mM ATP. For each sample, reactions with or without 2 mM ouabain were run in triplicate. We also determine the effects of purified IgG against myocardial Na–K-ATPase activity in each group. Myocardial membrane fragments were prepared from foreign healthy rabbits, and these Na–K-ATPase activities in the presence of 1 μg purified IgG were compared among the three groups. The IgG purification from rabbits serum was performed by protein A-agarose methods using the Affi-Gel Protein-A MAPS II Kit (Bio-Rad Laboratories, Hercules, California). The reactions were allowed to proceed for 2 h at 37 °C. Na–K-ATPase activity was expressed as inorganic phosphate produced 1 h/mg of membrane protein. The plasma and myocardial norepinephrine level was also determined by high-performance liquid chromatography.

2.6. Immunoblotting

Immunodetection of inhibitory G-protein (Gi) and Na–K-ATPase levels in the membrane fraction (20 μg) was performed using standard SDS-PAGE and immunoblotting techniques, as previously described [5]. Fixed samples were included on each gel as standard for quantification of the densities of each blot. Antisera against Gi (Santa Cruz Biotechnology) and Na–K-ATPase (Upstate Biotechnology, Inc.) were used as primary antibodies, and horseradish peroxidase-linked anti-rabbit IgG (Boehringer Mannheim) was used as a secondary antibody to detect individual protein

levels. The densities of each blot were quantified by densitometric scanning. Gi protein level was standardized by defining the mean density of membrane fraction from control rabbits as 1.0 densitometric unit. Na–K-ATPase levels were quantified by the relative intensity in comparison with 2 μg of porcine brain Na–K-ATPase. The specificities of antibody binding for each antigen were confirmed by neutralization assays using blocking antigens.

2.7. Statistics

Data are expressed as mean±S.E.M. Comparisons between three groups were performed by one-way ANOVA accompanied by a Bonferroni post hoc test when appropriate. Changes in OD on ELISA were analyzed using a repeated-measure ANOVA, whereas differences in mean values between the groups at a specific dose were determined by one-way ANOVA. Statistical significance was defined as $p<0.05$.

3. Results

OD values of ELISA in control group showed low throughout the observation period. In contrast, OD in beta and NKA group was high from the second through the sixth month, and there was no difference of OD between the two groups (Fig. 1).

Data on cardiac function are summarized in Table 1. Thickening of both the interventricular septum and the posterior wall was noted with a decrease in the LV end-diastolic dimension in beta and NKA group compared with control group on echocardiography (Fig. 2). Hemodynamic data demonstrated a relative bradycardia in only NKA group, and an elevation in LV end-diastolic pressure and a decrease in peak − d*P*/d*t* and cardiac output in both beta and NKA group. There was an increase in LV weight in beta and NKA group. Macroscopic examination of the hearts revealed LV hypertrophy, as evidenced by an increase in LV muscle mass and a decrease in the cavity area (Fig. 2). LV muscle mass corrected by the total cross-sectional area was increased in beta and NKA group. These macroscopic findings were endorsed by microscopic planimetry showing an increase in cross-sectional area per myocyte in both groups.

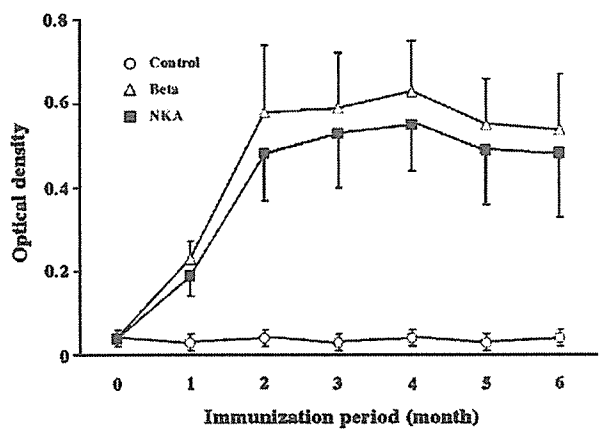

Fig. 1. ELISA of sera from three groups. Beta: a synthetic peptide corresponding to the second extracellular loop of beta1-adrenergic receptors, NKA: porcine cerebral cortex Na–K-ATPase, control: average of above both antigens. Data are expressed mean±S.D.

Table 1
Hemodynamic and morphometrical analyses

	Control	Beta	NKA
Ultrasonic echocardiography			
LV end-diastolic dimension (mm)	11.9±0.4	10.4±0.4*	10.5±0.4*
LV end-systolic dimension (mm)	7.2±0.4	6.9±0.3	7.0±0.3
Fractional shortening (%)	40±3	35±3	35±3
IVS wall thickness (mm)	2.6±0.1	3.3±0.2*	3.4±0.2*
LV posterior wall thickness (mm)	2.7±0.2	3.4±0.2*	3.4±±0.2*
Hemodynamics			
Heart rate (bpm)	253±9	254±8	213±7[a]
Aortic systolic pressure (mm Hg)	124±5	118±6	116±5
Aortic diastolic pressure (mm Hg)	90±6	92±5	91±4
LV end-diastolic pressure (mm Hg)	4.5±1.1	8.4±1.2*	9.4±1.1*
Peak + d*P*/d*t* (mm Hg/s)	4225±220	3975±175	4000±180
Peak − d*P*/d*t* (mm Hg/s)	3575±198	3060±159*	3025±162*
Cardiac output (ml/min/kg)	148±8	121±8*	120±7*
Weight			
Body weight (kg)	3.65±0.13	3.61±0.71	3.64±0.52
LV weight (g/kg)	1.42±0.02	1.54±0.04*	1.53±0.05*
RV weight (g/kg)	0.44±0.01	0.42±0.02	0.46±±0.02
Anatomic measurements			
LV wall area (mm^2)	117±7	138±7*	136±6*
LV cavity area (mm^2)	44±7	26±6*	25±7*
LV cross-sectional area (mm^2)	157±12	164±13	163±±11
LV wall area/cross-sectional area (%)	74±2	85±3*	84±2*
Myocyte cross-sectional area (mm^2)	250±15	338±25*	335±22*

IVS: interventricular septum, LV: left ventricle and RV: right ventricle.
[a] $p<0.03$ vs. control, beta.
* $p<0.03$ vs. control.

At 6 months, HE stain of organs except for heart had no significant pathological findings among the three groups. Histological findings of myocardium did not include any cellular infiltration in any group. However, myocardial hypertrophy with large nuclei, severe disorganization of the myofiber and interstitial fibrosis were present in the LV myocardium from beta and NKA group. Deposit of IgG around cardiac cell membrane was observed in beta and NKA group, although there was no stain in control group (Fig. 3).

Myocardial Na–K-ATPase activities were lower in the NKA group than other two groups, although plasma and myocardial norepinephrine concentrations had no difference

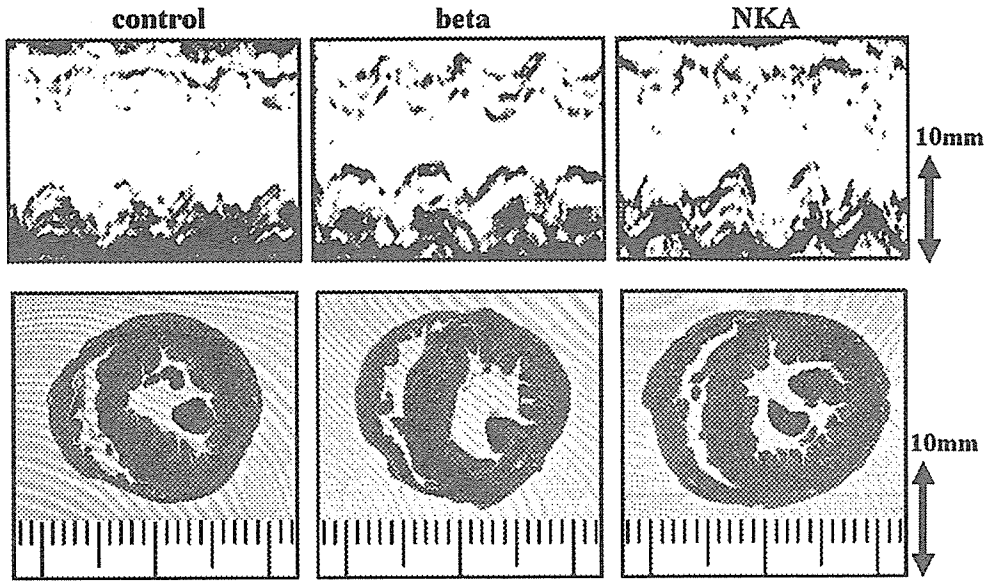

Fig. 2. Echocardiographic and macroscopic data from three groups. Beta, NKA and control; same as Fig. 1.

among the three groups. The effect of purified IgG against foreign healthy myocardium was compared among the three groups. Without adding IgG, Na–K-ATPase enzyme activity was 142 μM Pi/h/mg protein. The activity decreased by adding purified IgG from the NKA group, although there was no difference from control and beta group (Table 2).

Alpha3-isoform level of Na–K-ATPase was lower in the NKA group than control group, although there was no difference between beta and control groups. There was no difference in alpha1-isoform level of Na–K-ATPase in the three groups. Alpha2 level of Na–K-ATPase was also not different, either (data not shown). Gi protein level was higher in both beta and NKA groups than control group, without any difference between beta and NKA groups (Fig. 4).

4. Discussion

4.1. Cardiac hypertrophy induced by autoimmunity

Some previous observations have shown that repeated immunization using certain antigens corresponding to the second extracellular loop of beta1-adrenergic receptors and/

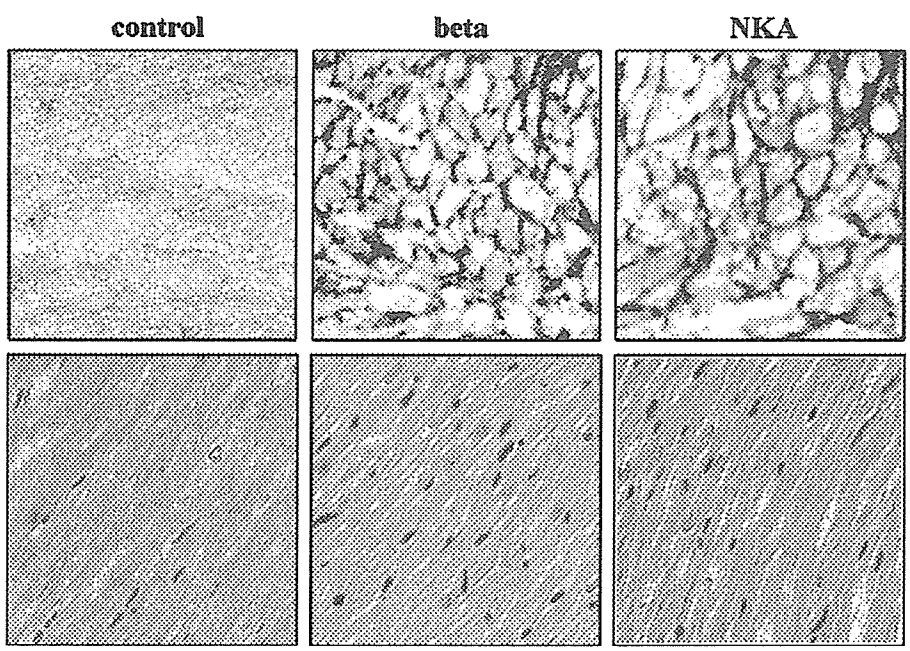

Fig. 3. Immunostaining of IgG and HE stain in myocardium from three groups. Beta, NKA and control; same as Fig. 1.

Table 2
Norepinephrine and Na–K-ATPase pump

	Control	Beta	NKA
Norepinephrine			
Plasma (pg/ml)	576±68	654±86	667±74
Left ventricle (ng/g tissue)	2092±193	2208±185	2147±215
Na–K-ATPase activity (μM Pi/h/mg protein)			
Each myocardium	143±20	133±25	94±16*
Foreign myocardium+purified IgG	145±12	138±15	98±12*

* $p<0.03$ vs. control, beta.

or M2-muscarinic receptors could induce cardiac hypertrophy [5,6]. Present study is the first report that repeated immunization using sarcolemmal Na–K-ATPase is able to induce cardiac hypertrophy mimicking DCM in rabbits. However, mechanisms underlying the development of cardiac hypertrophy have not been fully addressed. Inflammatory cell infiltration was observed at the early stage during immunization in previous studies. Our preliminary study also indicated that inflammatory cellular infiltration was observed in ventricular myocardium from rabbits immunized by Na–K-ATPase as well as those immunized by beta-adrenergic receptors 3 months after first immunization [5]. Whereas cellular infiltration in both groups subsided at 6 months, Abs against such antigens were persistently elevated. Our present study indicated antigen-specific production of Abs after myocarditis and that biochemical properties of hypertrophic myocardium were different by immunized antigens.

4.2. Alterations in Na–K-ATPase associated with cardiac hypertrophy

Sarcolemmal Na–K-ATPase activities declined in hypertrophic heart. Not alpha2- but alpha1- and alpha3-subunit of this pump decreased in human failing heart [7]. Protein level of the alpha3-subunit also decreased in high-frequency pacing model and in continuous norepinephrine infusion model [8]. Our present study showed that alpha1-subunit of Na–K-ATPase showed decrease tendency in beta and NKA group compared to the control group. The knockout mouse study indicated that less alpha1-subunit of Na–K-ATPase induced less contractility [9]. Short immunization period was thought as one of the reasons for no significant difference of this isoform in present study. In contrast, alpha3 protein level significantly decreased in NKA group than in other groups. Previous finding has shown that myocardial interstitial norepinephrine content was correlated with alpha3 protein levels [8]. However, myocardial as well as plasma norepinephrine levels were similar in the three groups in present study. As other humoral factor that could influence sarcolemmal Na–K-ATPase activity, there also exists ouabain that was reported to decrease alpha3-subunit mRNA level in vitro but to have no influence against alpha1-isoform [10]. Our preliminary study indicated that there were no differences of peripheral ouabain concentration among three groups.

4.3. Antigen-specific difference of hypertrophic myocardium

We have shown that Abs against Na–K-ATPase antibodies exhibits ouabain-like effects in vitro, inhibiting Na–K-ATPase catalytic activities [11]. And Abs against beta1-adrenergic receptors exhibit sustained sympathomimetic actions [5]. Complement activation is one of common mechanisms of cardiac hypertrophy induced by both Abs, but is not able to explain the difference of myocardial Na–K-ATPase activities in our present study. Our preliminary study using DCM patients' serum suggested that the epitope of anti Na–K-ATPase antibodies was a particular sequence with the alpha3-subunit of this pump. Therefore, we speculated that

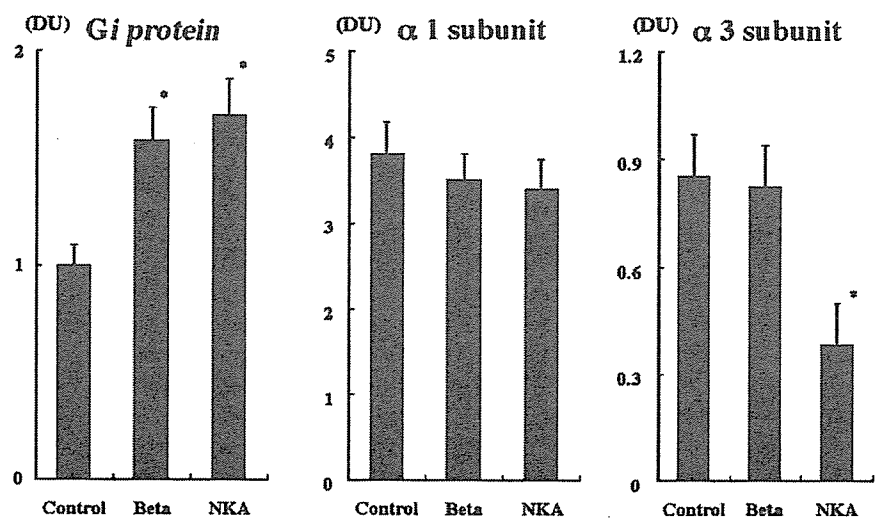

Fig. 4. Densitometric data of Gi protein, alpha1- and alpha3-subunit of Na–K-ATPase on immunoblotting. Beta, NKA and control; same as Fig. 1. DU: densitometric unit. Data are expressed mean±S.D. *$p<0.01$ vs. control.

this bioactive autoantibody would be one of the reasons for downregulation of alpha3-subunit of Na–K-ATPase.

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Case Report

A Case of Neonatal Lupus Erythematosus Presenting Delayed Dilated Cardiomyopathy With Circulating Autoantibody to Annexin A6

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SUMMARY

Patients with neonatal lupus erythematosus (NLE) often have congenital heart block with or without heart failure and are born from mothers who have anti-SS-A and/or -SS-B antibodies. NLE has been considered to result from the placental transmission of maternal autoantibodies into the fetal circulation causing myocardial damage. We report a case of NLE with congenital heart block who had pacemaker implantation at the age of 17, and then developed dilated cardiomyopathy (DCM) at the age of 19, extremely later than usual cases. The patient's mother was positive for anti-SS-A and -SS-B antibodies,whereas the patient was negative for both anti-SS-A and -SS-B antibodies. There were some autoantibodies against cell surface antigens of cardiac myocytes in the serum from the patient, and identified annexin A6 as one of the autoantigens. This is the first report demonstrating that annexin A6 was involved in the myocardial injury in patients with NLE. Our study showed a possibility that inhibition of annexin A6 function can prevent autoantibody-mediated myocardial injury in at least a part of DCM. (Int Heart J 2007; 48: -)

Key words: 2-D gel electrophoresis; anti-SS-A/-SS-B antibody; β-blocker; Ca^{2+} transient; congenital heart block; heart failure; mass spectrometry; pacemaker implantation; Western blot

PATIENTS with neonatal lupus erythematosus (NLE) are born from mothers who have anti-SS-A and/or -SS-B antibodies. They often have congenital heart block with or without heart failure, and cutaneous lesions, which subsequently disappear in about 6 months after birth.[1)] About 200 patients have been reported, and half of them have congenital heart block. NLE has been considered to result from the placental passage of maternal autoantibodies into

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the fetal circulation causing damage to otherwise normally developed heart. An inflammatory phase of cardiac injury is observed in the fetus heart. Because most patients with this syndrome die young, there have been few reports presenting the patients who lived until their maturity. About half of the patients with NLE have permanent pacemaker implantation before 3 months of age, and the most of the patients have pacemaker implantation before 10 years of age.[2)]

Here, we report a case of NLE with congenital heart block who had pacemaker implantation at the age of 17, extremely later than usual, and then developed dilated cardiomyopathy (DCM). The mother was positive for anti-SS-A and -SS-B antibodies, whereas the patient was negative for both anti-SS-A and -SS-B antibodies. We found that there were some autoantibodies against cell surface antigens of cardiac myocytes in the serum from the patient, and identified annexin A6 as one of the autoantigens.

CASE REPORT

The patient was a 37-year-old Japanese man in whom abnormal heart sound was found at 36 weeks' gestation. He was born at 43 weeks' gestation. At birth, cyanosis, poor sucking, and poor weight gain were observed. He suffered from pneumonia at 7 months of age. At 14 months of age, he came to our hospital because of cough and fever. Abnormal heart sounds, harsh holo-systolic murmur (Levine 3rd degree) and high-pitched diastolic murmur (Levine 2nd degree), were found. ECG showed complete AV block. At 19 months of age, angiography revealed patent ductus arteriosus (PDA), and closure of PDA was performed at 24 months of age. Then, he was medicated with oral digoxin. His heart rate was between 70 and 85 beats per minute, and cardiothoracic ratio (CTR) was about 52%. He could exercise normally except for running and swimming. At 17 years of age, pacemaker was implanted on DDD mode because of complete heart block. At 19 years of age, he suffered from cough and low grade fever. Then, general malaise, orthopnea, nausea, and vomiting occurred as the symptoms got worse. He was emergently hospitalized because of the first episode of heart failure. At that time, 3^{rd} and 4^{th} heart sounds were heard. Peripheral venous pressure (PVP) was 20 cmH_2O. C-reactive protein (CRP) and white blood cell (WBC) count were elevated (Table 1). Hypoxemia (PaO_2 67.3 mmHg) and hepatomegaly were observed. Chest X ray revealed pulmonary congestion and cardiac enlargement (CTR 60%). Echocardiography showed enlarged left ventricle (LV) and severely depressed LV contraction (ejection fraction [EF] 0.28). Right-sided cardiac catheterization revealed elevation of pulmonary capillary wedge pressure (26 mm Hg), and low cardiac index (1.8 $L/min/m^2$). LV biopsy sample showed interstitial fibrosis, thickening

of endocardium, and contraction necrosis. No obvious cell infiltration was found. These findings were compatible with those of DCM. He received O_2 inhalation, and was medicated with intravenous furosemide and digoxin. His CRP and WBC soon decreased to within normal range. The immunological data of the patient and his mother were summarized in Table 2. Although his speckled antinuclear antibody (ANA) test result was mildly elevated, other immunological data were within normal range. He had no symptoms suggestive of autoimmune diseases. As for his mother, she was positive for anti-SS-A and -SS-B antibodies as well as homogeneous and speckled ANA. Her serum levels of IgA and IgG were elevated. She had no symptoms suggestive of autoimmune diseases. After 2 months of hospitalization, he was discharged. However, he soon got exertional dyspnea, orthopnea, rapid gain of body weight, and he was rehospitalized because of worsening of congestive heart failure. He was medicated orally with furosemide (120 mg/day), spironolactone (50 mg/day), enalapril (5 mg/day), and digoxin (0.25 mg/day). Symptom-limited exercise test with expired gas analysis showed that his peak oxygen uptake was 18.2 ml/min/kg (51% of predicted normal values) and his anaerobic threshold was 13.9 ml/min/kg (71% of predicted normal values), suggesting that he had severe exercise intolerance and severe congestive heart failure. Oral administration of metoprolol, a β-blocker, was started and was increased to the dosage of 60 mg per day. His heart rate decreased from 90 to 65 beats per minute, and CTR also decreased from 54% to 49%. His symptoms of heart failure were dramatically improved. This was associated with a substantial improvement of peak oxygen uptake and anaerobic threshold. After the initiation of β-blocker therapy, the number of times of hospitalization decreased, and his exercise tolerance remained stable. He could work as an office worker for three years. Figure 1 shows the result of echocardiography in this period. However, his symptoms became gradually worse when he was 29 years old, and was hospitalized again because of worsening of heart failure. From that time, he had seven times hospitalization because of heart failure. At present, he is in the 3rd degree of New York Heart Association (NYHA) functional classification and has severe exercise intolerance under oral administration of carvedilol (a β-blocker, 15 mg/day), candesartan (an angiotensin II receptor blocker, 8 mg/day), furosemide (120 mg/day), spironolactone (25 mg/day).

METHODS

Because it is strongly suggested that some autoantibodies may play a role in myocardial injury in this patient, we investigated whether there were autoantibodies against some antigens of cardiac myocytes in the serum from the

patient and identified the antigens expressed on the surface of cardiac myocytes. This study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association, and was approved by the Ethics Committee of the University of Tokyo Hospital. The patient gave written informed consent after full explanation of the purpose, nature and risk of all procedures used. Primary culture of ventricular cardiac myocytes were prepared from neonatal rats as described elsewhere.[3] They were cultured for three days, washed in phosphate buffered saline (PBS), and the plasma membrane fraction of cardiac myocytes was prepared as described elsewhere.[4] Then, they were subjected to two dimensional (2-D) gel electrophoresis and then transferred onto polyvinylidene difluoride transfer membranes (NEN Research Products). The transfer membranes were blocked with 1% BSA and then incubated overnight at room temperature with the ten times diluted sera from the patient or an age and sex-matched healthy control. After incubated with biotinylated anti-human IgG (EY Laboratories, Inc., CA, USA), and then incubated with Vectasrain ABC-AP reagent (Vecter Laboratories, Inc., CA, USA), the blot was developed with a chemiluminescence detection kit (Phototope-Star Detection Kit, New England Biolabs, MA, USA). The same plasma membrane fraction was also subjected to 2-D gel electrophoresis followed by staining with Coomassie Brilliant Blue. The spots corresponding to the reaction products by Western blots were excised and in gel digested, then analyzed by mass spectrometry (MS). The procedures for 2-D gel electrophoresis, in gel digestion, and MS analysis were as described elsewhere.[5]

RESULTS

Figure 2 shows the results of Western blot of a healthy control subject (panel A) and the patient (panel B). There were almost no spots reacted with the serum from a healthy control subject (panel A), whereas there were several spots reacted with the patient's serum (panel B). LC-MS/MS analysis identified 2 of these protein spots indicated by arrows 1 and 2 as annexin A6 and vimentin, respectively (panel B). For other spots, we could not identify as known proteins.

DISCUSSION

Patients with NLE often have congenital heart diseases such as atrial septal defect and abnormality of great arteries, such as PDA, which was found in the patient. However, at least 10% of NLE patients with heart block develop heart failure due to myocardial injury by the disease process. [6] These patients generally develop heart failure at birth or very shortly after birth. Taylor-Albert,

et al.[7] reported two cases of NLE with complete heart block who had pacemaker implantation shortly after birth and developed heart failure due to DCM at 8 and 4 months after birth, respectively. The authors speculated that presence of high levels of maternal anti-SS-A antibody in the infant's sera caused delayed heart failure. The patient of our present study had pacemaker implantation and developed heart failure at the age of 17 and 19, respectively, extremely later than the usual course of NLE. Furthermore, although the mother of this patient was positive for anti-SS-A and -SS-B antibodies as well as homogenous and speckled ANA, the patient himself did not have both anti-SS-A and -SS-B antibodies at least when he developed heart failure.

It was reported that significantly higher rate of antibodies in the sera from mothers of children with congenital heart block capable of crossing the placenta reacted with fetal myocardial tissues than in the sera from control subjects.[8] In addition, there was deposition of immunoglobulin and complement components in all cardiac tissues of these infants, strongly suggesting the role of maternal antibodies in the development of fetal heart block.[8] In fact, Xiao et al.[9] demonstrated that the sera from mothers containing anti-SS-A and -SS-B antibodies whose children had congenital heart block significantly inhibited L-and T-type Ca^{2+} channels expressed in Xenopus oocytes. For roles of autoantibodies in DCM, sera from DCM patients significantly decreased isoproterenol-stimulated L-type Ca^{2+} currents in isolated rabbit cardiac myocytes.[10] Therefore, it is strongly suggested that autoantibody-mediated myocardial injury was involved in the development of complete heart block as well as DCM in the present patient and that autoantibodies other than anti-SS-A and -SS-B played a critical role in this process because the patient did not have both anti-SS-A and -SS-B antibodies and the onset of these conditions was extremely late. In the present study, we have showed that this patient had circulating autoantibodies against annexin A6 and vimentin. Vimentin is a member of the intermediate filament family proteins like desmin. They form the cytoskeletal structure along with microtubules and actin microfilaments, and are thought to play an important role in supporting and anchoring the organelles in the cytosol. Therefore, it seems unlikely that a circulating anti-vimentin antibody can significantly affect the myocardial contractile function. Annexins are ubiquitous proteins characterized by a conserved C-terminal domain with Ca^{2+} binding sites and a variable N-terminal domain. In general, C-terminal domain consists of 4 repeats containing α-helix structure except for Annexin A6 which has 8 repeats. Annexin A6 is the most abundant annexin in the heart and is constitutively expressed in cytosol and plasma membrane. Ca^{2+} binding leads

to conformational changes of the structure, high affinity for phospholipids, and relocation to plasma and nuclear membranes followed by membrane insertion. Annexin A6 has been suggested to act as a regulator of the Na^+/Ca^{2+} exchanger and /or SR Ca^{2+}-ATPase.[11),12)] Annexin A6 knockout has been found to induce faster changes in Ca^{2+} transient and increased contractility,[13)] whereas overexpression of annexin A6 has been shown to reduce contraction and relaxation of cardiac myocytes, leading to DCM.[14)] So, annexin A6 has been proposed to be a negative regulator of cardiac function. These data suggested that a circulating anti-annexin A6 antibody may bind annexin A6 on the surface of cardiac myocytes and stimulate its function resulting in the suppression of myocardial contractility. This is the first report demonstrating that annexin A6 was involved in the myocardial injury in patients with NLE. Our study showed a possibility that inhibition of annexin A6 function can prevent autoantibody-mediated myocardial injury in at least a part of DCM.

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