Identification of Autoantibodies With the Corresponding Antigen for Repetitive Coxsackievirus Infection-Induced Cardiomyopathy

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Background  The hypothesis that viral myocarditis causes an autoimmune response and subsequent dilated cardiomyopathy is controversial. To further investigate the autoimmune mechanism of cardiac dilatation and dysfunction after repeated episodes of viral myocarditis, the cardiac autoantigens induced by repetitive coxsackievirus B3 (CVB3) infection were examined.

Methods and Results  Male inbred A/J mice were inoculated intraperitoneally with CVB3 at 3 and 40 weeks of age. At 8 weeks after the second inoculation, the mortality of the repetitive CVB3 group was significantly increased compared with that of the control group, and was associated with a significant reduction in fractional shortening and marked left ventricular dilatation without inflammatory cell infiltration. The cardiac antigens in the repetitive CVB3 infection were identified by 2-dimensional electrophoresis and subsequent liquid chromatography/tandem mass spectrometry (LC-MS/MS) using the serum at 2 weeks after the second inoculation. LC-MS/MS and immunohistochemistry demonstrated α-cardiac actin and heat shock protein 60 (HSP60) as cardiac near-surface antigens induced by the repetitive CVB3 infection. Immunoelectron microscopy disclosed the selective localization of anti-IgM antibody on the membrane of the myocytes in the repetitive CVB3 group.

Conclusions  IgM antibodies against α-cardiac actin and HSP60, which were induced by repetitive CVB3 infection, may play an important role in the pathophysiology of the subsequent cardiac dysfunction and dilatation.

Key Words: Autoantigen; Autoimmunity; Coxsackievirus; HSP60; Viral Myocarditis

Dilated cardiomyopathy (DCM) is characterized by the dilatation and impaired contraction of the ventricles and clinically progressive heart failure. It may be idiopathic, familial/genetic, viral and/or immune, alcoholic/toxic, or associated with recognized cardiovascular disease in which the degree of myocardial dysfunction is not explained by the abnormal loading conditions or the extent of ischemic damage. Although the relationship between viral myocarditis and DCM remains controversial, a causal link has become more evident because of the tremendous developments in the molecular analyses of autopsies and endomyocardial biopsy specimens, new techniques of viral gene amplification, and advances in modern immunology.

It has already been reported that an autoimmune response plays a key role in the progression after viral myocarditis. This occurs in the context of a polyclonal stimulation of the immune system after the initial viral assault that may have been already cleared when the autoreactive B- and T-cell response occurs. Recently, we demonstrated that repetitive coxsackievirus B3 (CVB3) infection produced cardiac dilatation without inflammatory cell infiltration in the heart of mice with post-myocarditis, and autoimmunity mediated by the circulation of certain antibodies (eg, antibodies against the CVB3 genome or a CVB3-related protein) may be part of the pathogenic mechanism for this phenomenon. Moreover, only the autoreactive IgM antibody was apparent on the cell membrane of the myocytes and interstitial tissue in the repetitive infected mice, and may play a pivotal role in the early response to the virus in our repetitive viral myocarditic mice. To identify the autoantigen against the components of the myocardium in repetitive CVB3 myocarditic mice, particularly targeting the cell membrane, we examined whether IgM type-autoantibodies were present in the serum of these animals.

Methods

The experimental protocols used in this study were approved by the Ethics Committee for Animal Experimentation at Yamaguchi University School of Medicine, and carried out according to the Guidelines for Animal Experimentation at Yamaguchi University School of Medicine, and Law No. 105 and Notification No. 6 of the Japanese Government.
**Experimental Protocol**

Three-week old, inbred, certified, virus-free A/J (H-2a) male mice were purchased from Japan SLC (Shizuoka, Japan). Fourteen normal mice were also housed for 40 weeks as a control (3W–/40W–). The LV dimensions and wall thickness of the left ventricle were calculated according to the method of Yamaguchi et al.8 Briefly, the hearts at 2 weeks after inoculation were halved transversely and one portion was fixed with 10% formalin solution, then embedded in paraffin solution and sectioned into slices of 4-mm thickness stained with hematoxylin-eosin and Azan solutions.

**Morphometry and Histopathological Study**

The ventricles from the mice killed at 8 weeks after the second inoculation were halved transversely and one portion was fixated with 10% formalin solution, then embedded in paraffin solution and sectioned into slices of 4-mm thickness stained with hematoxylin-eosin and Azan solutions. The left ventricular (LV) dimensions and wall thickness were measured using the transverse section of the middle portion of the ventricle. The cavity dimensions and wall thickness of the left ventricle were calculated according to the method of Matsumori et al.9 Cardiac fibrosis was also evaluated quantitatively using a Fotovision FV-10 camera (Fuji Film Co, Japan) and a (Macintosh 8500/120) computer equipped with NIH Image version 1.62 software.

**Echocardiography**

Prior to death at 8 weeks after the second inoculation, the mice underwent light anesthesia with ether. The LV end-diastolic dimension (EDD), end-systolic dimension (ESD), and fractional shortening (%FS) were obtained by averaging the data from 3 cardiac cycles using an echocardiographic system (ALOKA 5500; Alokia, Japan) with a dynamically focused 10-MHz linear array transducer.

**Immunoelectron Microscopy**

Immunoelectron microscopy was performed by the method of Yamaguchi et al all Briefly, the hearts at 2 weeks after the second inoculation were fixed by perfusing with paraformaldehyde. For immunostaining, horseradish peroxidase (HRP) conjugated anti-mouse IgM was used. Sections 10–19 nm thick were examined with a JEOL 200 CX transmission electron microscope at 160 kV. Sarcomere lengths were measured in a blinded fashion at a magnification of 3,700. This process was repeated for 50 fields per animal.

**Two-Dimensional Western Blotting**

Fresh murine heart tissue at 2 weeks after the second inoculation was minced and resuspended in 5 volumes of STE buffer containing 320 mmol/L sucrose, 10 mmol/L Tris–HCl, pH 7.4, 1 mmol/L EGTA, 5 mmol/L NaN3, 10 mmol/L -mercaptoethanol, 20 mmol/L leupeptin, 0.15 mmol/L pepstatin A, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 50 mmol/L NaF, with a Polytron homogenizer (PT1200; Kinematica, Germany), at its maximum speed for 30 s, repeated 3 times. Homogenates were mixed with an equal volume of STE buffer and centrifuged at 1,000 G for 10 min, and the supernatant was centrifuged at 100,000 G for 60 min. The 1,000-G and 100,000-G pellets were designated as P1 and P2 fractions, respectively, and the 100,000-G supernatant as the S fraction. The P1 and P2 fractions were resuspended in STE buffer. The total protein concentration in each fraction was determined, using bovine serum albumin as a standard.

The extracted protein samples were diluted in a rehydration buffer (8 mol/L urea, 2% CHAPS, 2.8 mg/mL dithiothreitol (DTT), trace of bromphenol blue) containing 0.5% immobilized pH gradient (IPG) buffer (pH range 3–10; Amersham Pharmacia Biotech, Sweden), and loaded onto 7 cm ImmobilIBE Drystrips (Amersham Pharmacia Biotech) in the IPG reswelling tray (Amersham Pharmacia Biotech) at room temperature overnight. Up to 400 mg of the extracted proteins was applied onto the drystrip gels for western blotting, and up to 1,000 mg for the analysis by mass spectrometry. Isoelectric focusing (IEF) was performed in a horizontal electrofocusing apparatus (MultiPhor II; Pharmacia Biotech, Sweden) according to the manufacturer’s instructions. After the IEF, the IPG strips were equilibrated in 2 equilibration solutions. The first equilibration solution consisted of 10 mg DTT per 1 ml sodium dodecyl sulfate (SDS) equilibration buffer (1.5 mol/L Tris-Cl, pH8.8; 6 mol/L urea, 30% glycerol, 2% SDS), and the second equilibration solution consisted of 25 mg iodoacetamide per 1 ml SDS equilibration buffer. The equilibrated strips were placed on top of a 12.5% SDS polyacrylamide gel electrophoresis (PAGE) slab and sealed with 0.5% lower melt gel, and then the second electrophoresis was performed with a 40 mA constant current in the separating gel at 20°C.

After electrophoresis, the SDS-PAGE gels were stained with Coomassie Brilliant Blue (CBB) or used for protein transfer onto nitrocellulose membranes (Protran, Schleicher & Schuell, Germany). In the western blotting, the membranes were blocked in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% Tween 20 for 1 h, washed in PBS with 0.1% Tween 20 (PBST) for 30 min, and incubated with serum samples, which were collected from mice at 2 weeks after the second inoculation, diluted adequately in PBST containing 1% BSA for 1 h. After 5 washings in PBST, the bound antibodies were reacted with HRP-conjugated goat anti-mouse IgM (Sigma, St Louis, MO, USA) for 1 h. Finally, the bound antibodies were visualized by diaminobenzidine.

**Mass Determination and Mass Fingerprinting**

Liquid chromatography/tandem mass spectrometry (LC-MS/MS) was performed as follows to determine the molecular weight of the protein spots. Protein spots on the gel stained with CBB, which corresponded to the positive spots on the western blotting membranes, were recovered and then the recovered gel fragments were washed in double distilled water for 15 min, de-colored in 50 ml de-coloring solution (0.1 mol/L ammonium hydrogen carbonate, 50% methanol) at 40°C for 15 min, and cut into small pieces. The gel pieces were re-hydrated in 20 ml trypsin solution (0.1 pmol/ml trypsin in 50 mmol/L Tris-HCl; Wako Pure Chemical Industries, Ltd, Japan) and incubated at 37°C. The digested peptides were extracted from the gel pieces using trifluoroacetic acid (TFA) and acetonitrile.
Specifically, the digested products were added to 50 ml of 0.1% TFA/50% acetonitrile, vortexed, and sonicated for 10 min. After centrifugation, the supernatant was recovered. After 2 more cycles of this extraction, a similar extraction was performed with 50 ml of 0.1% TFA/80% acetonitrile. The collected supernatant was centrifuged again, filtered, and concentrated down to 50 ml in an evaporator, and was then desalted using a Zip-Tip desalting column (Millipore Corp, Milford, MA, USA). The peptide sample solution was stored at –20°C until mass spectrometry analysis.

The mass of the digested peptides in the samples was determined using a mass spectrometer with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF: Voyager DE-STR; PerSeptive Biosystems, USA). Alpha-cyano-4-hydroxycinnamic acid was used as an assisting matrix. A list of the determined peptide masses was made by a mass fingerprint search of the NCBI protein databases using the Mascot software program (Matrix Science, Ltd, UK), in which the NCBI protein databases were searched.

Immunohistochemistry

For immunoenzymatic staining, the remaining ventricular portion was quickly frozen with OCT compound (Miles, Inc, USA) in liquid nitrogen and stored at –80°C. The frozen specimens were sectioned at 4-mm thickness. Immunoenzymatic staining was performed with a DAKO LSAB kit (DAKO, USA) according to the manufacturer’s instructions. Antibody against murine heart shock protein 60 (HSP60) (Stressgen, Canada) was used as the primary antibody.

Statistical Analysis

Data were expressed as the mean±SD. Statistical analysis of the data was performed by an analysis of variance with multiple comparisons. The survival of all animals was assessed by Kaplan-Meier analysis. A level of p<0.05 was considered statistically significant.

Results

Survival Rates

Fig1 shows the survival curve at 8 weeks after the second inoculation at 40 weeks. Five of the 21 mice died after the second CVB3 inoculation (mortality rate: 23.8%, p<0.05). All of the dead mice had pleural effusion and ascites, indicating that they probably died from heart failure. No mice died after the second vehicle injection.

Physiological Analysis

The mean body weight in the 3W+/40W+ group was significantly reduced compared with that in the 3W+/40W– group (p<0.05). The heart weight and the ratio of heart weight/body weight in the 3W+/40W+ group was significantly increased compared with that in the 3W–/40W– group (p<0.05) (Table 1). There were no differences between the 3W–/40W– and 3W+/40W– groups in body weight or heart weight, or in the ratio of the heart weight/body weight. In addition, there were no significant differences in the weights of the lungs and livers among the 3 groups.

Table 1 Assessment of the Systemic Parameters of Viral Myocarditis

<table>
<thead>
<tr>
<th>Group</th>
<th>3W–/40W–</th>
<th>3W+/40W–</th>
<th>3W+/40W+</th>
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<tbody>
<tr>
<td>Physiological analysis</td>
<td></td>
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</tr>
<tr>
<td>No. of experiments</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<tr>
<td>Body weight (g)</td>
<td>29.2±2.8</td>
<td>30.2±2.5</td>
<td>26.3±2.0*</td>
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<tr>
<td>Heart weight (mg)</td>
<td>108±8.2</td>
<td>140±8.38</td>
<td>201±49.10*</td>
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<tr>
<td>Lung weight (mg)</td>
<td>212±6.6</td>
<td>199±92</td>
<td>221±93</td>
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<tr>
<td>Liver weight (mg)</td>
<td>1.17±0268</td>
<td>1.02±101</td>
<td>967±4</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>3.7±0.45</td>
<td>4.6±1.11</td>
<td>7.8±0.15*</td>
</tr>
<tr>
<td>LW/BW (mg/g)</td>
<td>7.2±0.90</td>
<td>6.3±2.75</td>
<td>8.5±4.38</td>
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<tr>
<td>LW/BW (mg/g)</td>
<td>40.2±3.31</td>
<td>34.2±2.44</td>
<td>36.2±1.99</td>
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<td>Morphometry</td>
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<tr>
<td>Wall thickness (mm)</td>
<td>0.8±01</td>
<td>0.7±01</td>
<td>0.7±01</td>
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<td>Fibrosis (%)</td>
<td>3.1±0.7</td>
<td>16.5±11.1*</td>
<td>26.9±6.0*</td>
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<td>Inflammatory grading</td>
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<td>0.2±0.4</td>
<td>0.2±0.4</td>
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<tr>
<td>Echocardiography</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>2.4±0.3</td>
<td>2.6±0.4</td>
<td>3.4±0.3*</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>0.9±0.1</td>
<td>1.3±0.3*</td>
<td>2.4±0.5*</td>
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<tr>
<td>%FS (%)</td>
<td>60.8±5.4</td>
<td>50.6±7.6*</td>
<td>29.6±7.9*</td>
</tr>
</tbody>
</table>

HW/BW, ratio of heart weight/body weight; LW/BW, ratio of lung weight/body weight; LV/BB, ratio of liver weight/body weight; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; %FS, fractional shortening.

Values are the mean±SD. *p<0.05 vs the 3W–/40W– group. †p<0.05 vs the 3W+/40W– group.
Histology
There was a significant increase in the LV dimension in the 3W+/40W+ group compared with the 3W+/40W– group (p<0.05). The ratio of cardiac fibrosis in the 3W+/40W– and 3W+/40W+ groups was significantly higher than in the 3W–/40W– group (p<0.05). The ratio of cardiac fibrosis in the 3W+/40W+ group was the highest among the 3 groups (Table 1). There was no significant difference in the mean wall thickness among the 3 groups. Inflammatory cell infiltration was less than 5% in all groups (Table 1).

Echocardiography
To evaluate cardiac function, we performed transthoracic echocardiography at 8 weeks after the second inoculation. Both the LVEDD and LVESD of the 3W+/40W+ group were significantly increased compared with those of the other 2 groups; %FS was significantly reduced in the 3W+/40W+ group compared with that in the other 2 groups (Table 1).

Immunoelectron Microscopy
The sarcomere length in the 3W+/40W+ group was 1.8±0.24 mm, which is comparable to the data previously obtained. Staining of bound IgM in the myocardium was recognized only in the 3W+/40W+ group. The cell membranes of the myocytes and interstitial tissue were positively stained with the anti-IgM antibody by immunoelectron microscopy (Fig 2).

Two-Dimensional Western Blotting and Subsequent LC-MS/MS
On 2-dimensional western blotting, 2 spots were detected as A3 (pI 5.2) and A5 (pI 5.9) in the membranous fraction that specifically cross-reacted with the serum in the 3W+/40W+ group (Fig 3). By subsequent LC-MS/MS, A3 (MW 42001 Da) and A5 (MW 60941 Da) were recognized as α-cardiac actin and HSP60, respectively (Fig 3).

Immunohistochemistry
Because α-cardiac actin is known to be ubiquitously distributed in myocytes, and only HSP60 is reported to be upregulated on the cell surface, as well as in the cytosol and mitochondria in response to many different stresses, we performed further immunohistochemical analysis for HSP60 at 2 weeks after the second inoculation. We showed that HSP60 was positively stained in the myocytes and interstitial tissue (yielding a brown color with a pale blue background) taken from the hearts in the 3W+/40W+ group (Fig 4A). No myocytes were positively stained with the HSP60 antibody in the 3W–/40W–, 3W+/40W– or 3W–/40W+ groups (Fig 4B, C and 4, respectively).

Discussion
There is some clinical evidence that DCM is a late sequel of acute or chronic viral myocarditis. Infectious and autoimmune myocarditis has also been extensively proven using murine and rat models. We previously demonstrated that repetitive CVB3 infection in mice could cause LV dilatation with dysfunction through autoantibodies,
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which were immunologically maximally activated at 2 weeks after the second CVB3 inoculation. Moreover, these pathophysiological changes were present even at 8 weeks after the second inoculation. Electron microscopy showed that these antibodies belong to the IgM subtype, and were distributed on the surface of the myocytes and interstitial tissue at 2 weeks after the second viral inoculation in mice. The results of our study are entirely different from the previously reported anti-heart antibodies in viral myocarditis. First, in the present study repetitive CVB3 infection produced cardiac dilatation and dysfunction without inflammatory cell infiltration in post-myocarditic mice. This poor T cell response may be related to the protective effect of neutralizing antibody against CVB3 induced by the first inoculation, as well as to CVB3 being less able to cause myocardial lesions related to senescence. Second, antibodies that react to the membranous fraction of normal myocytes were produced in the repetitive viral infection, but not in the post-myocarditis mice with simple viral infection. Third, the antibodies were classified as the IgM subtype, as distinct from the other IgG-type antibodies, such as ADP-ATP carrier protein and cardiac sarcoplasmic reticulum calcium ATPase, which were previously identified as cardiac autoantibodies. Fourth, the antibodies reacted to the membrane fraction of the myocytes, so that these antibodies identified in the serum of the repetitive myocarditic mice could easily bind the targeting antigen and cause immunological cytotoxicity accompanied by activation of the complementary system in vivo. It has also been reported that some anti-heart IgM autoantibodies can activate the complementary system and cause subsequent cardiac damage, especially in membranous proteins such as myolemmal. Fifth, we identified 2 autoantibodies (α-cardiac actin and HSP60) against the cardiac membrane protein. Finally, we have already reported that interleukin-1 and tumor necrosis factor-α were elevated in our model. They are able to potentiate the immune response and induce cell death, both of which appear to have a special importance in the pathogenesis of myocarditis. As a result, the IgM antibodies, which can activate the complementary system, and cytokines may cooperatively cause the cytotoxic effect on the target myocytes.

In patients with histologically proven myocarditis or familial DCM, autoreactive autoantibodies to components of the myocardium are often present, including intracellular targets such as the ADP/ATP translocator and other mitochondrial proteins. Dorffel et al demonstrated that the extraction of autoimmunoreactive antibodies by immunoadsorption results in a functional improvement in hemodynamics in DCM patients. Those authors have proven indirectly that autoantibodies against the ADP-ATP carrier, contractile proteins of cardiomyocytes, and the cardiac α-adrenergic receptor contribute to cardiac malfunction in DCM. They proposed that the immunoadsorption may be an additional therapeutic possibility for the acute hemodynamic stabilization of patients with severe DCM. Moreover, Kishimoto et al reported that antibody-mediated immune enhancement is involved in the pathogenesis of CVB3 myocarditis in mice. Nishimura et al also proved that PD-1, which binds to the 33-kD protein, may be an important factor contributing to autoimmune cardiomyopathy in mice. These results raise the possibility that some form of cardiomyopathy may have a CVB3-induced autoimmune basis, and that identifying possible autoantigen(s) may open up new diagnostic and therapeutic approaches for this disease. Regarding their results, molecular mimicry may be involved in these mechanisms. This occurs when an immune response mounted by the host against a specific determinant of an infecting viral or bacterial agent cross-reacts with a similar ‘mimicked’ host sequence, leading to autoimmune, and, in some cases, tissue injury and disease. However, one question that has been raised is how these antibodies recognize cytosolic antigen in intact myocytes, because these antigens are isolated and tolerated from circulating antibodies. Interestingly, Maish et al demonstrated that anti-membrane antibodies circulated not only in...
the peripheral blood, but were also bound to the sarcolemma and interstitial tissue in the endomyocardial biopsy specimens of patients. Their results are compatible with our electron microscopy findings, indicating that the antibodies belong to the IgM subtype, and respond to the surface of the myocytes in repetitive CVB3 infection. In this study, we compared the serum of repetitive CVB3 with other groups to identify autoantigens in the myocardium, and identified 2 cardiac antigens in the membranous fraction: α-cardiac actin and HSP60. Although α-cardiac actin is a well-known cytosolic component of myocytes, there is a technical limitation to purifying the membrane fraction in the process of extraction from a heart sample! As we observed in our study, α-cardiac actin has already been reported as a cytosolic autoantigen in CVB3 myocarditis.

In this study, we demonstrated that HSP60 may be a candidate for a membrane-bound autoantigen in repetitive CVB3 inoculation. The HSP family has been identified as a prominent target of ongoing immune responses during microbial infections. Cross-reactive immune responses between mammalian and microbial HSPs have been suggested as underlying several autoimmune and inflammatory disorders, including chronic arthritis, systemic lupus erythematosus, atherosclerosis, Crohn’s disease, and diabetes. In addition to constituting an endogenous stress response that protects cells from injury, members of the HSP family are also candidate molecules that potentially signal tissue damage or cellular stress to the immune system, the so-called ‘danger theory’. The expression of HSP is upregulated rapidly during several forms of cellular stress, and HSP can be released from damaged tissue. Porti et al reported that antibodies against HSP60 were found in the sera of patients with DCM, and may interfere with the functions of this stress protein plays in cell physiology (ie, protein transport, protein maturation, and protection of the cell under stress conditions). Latif et al also reported that not only was the anti-heart antibody against HSP60 present in the sera, but HSP60 was upregulated in the myocardium of patients with DCM. They confirmed that the cell surface expression of HSP60 after heat stress can be visualized using immunofluorescence. Taken together with our results, these findings suggest that IgM antibodies against α-cardiac actin and HSP60, which were induced by repetitive CVB3 infection, may play an important role in the pathophysiology of the subsequent cardiac dysfunction and dilatation.

Conclusions

In the present study, repetitive CVB3 infection caused cardiac dysfunction and dilatation with an induction of a variety of anti-heart antibodies. Exploring the nature of these autoantibodies found in the sera of our model will provide further immunological and virological insights into the mechanism of subsequent DCM after viral myocarditis.

Acknowledgments

We would like to thank Dr Akira Matsumori (Kyoto University, Kyoto, Japan) for his provision of CVB3. In addition, we would like to thank Rie Ishihara and Kazuko Iwamoto for their excellent technical assistance. This study was supported in part by funds from the Idiopathic Cardiomyopathy Research Group of the Ministry of Health and Welfare of Japan.

References


Circulation Journal Vol. 68, July 2004