Repetitive Coxsackievirus Infection Induces Cardiac Dilatation in Post-Myocarditic Mice

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The relation between myocarditis and dilated cardiomyopathy (DCM) is controversial. To clarify the pathogenic mechanism of these diseases, the present study examined the effect of repetitive inoculation with coxsackievirus B3 (CVB3) in post-myocarditic mice. Inbred 3-week-old A/J mice were inoculated intraperitoneally with CVB3 (Nancy strain; 2×10^4 plaque-forming units) and reinfected in the same manner with CVB3 at 40 weeks (3W+/40W+). All mice were killed at 42 weeks old. The weight of the hearts of the 3W+/40W+ group were significantly increased compared with those of the 3W–/40W– group, and both the heart weight/body weight and lung weight/body weight ratios of the 3W+/40W+ group were also significantly increased over those of the 3W–/40W– group, although the levels of serum neutralizing antibody titers were significantly increased in the 3W+/40W+ group over the level of the other groups. No increase in inflammatory cell infiltration or fibrosis progression was observed in the 3W+/40W+ group relative to the 3W–/40W– group, but the second inoculation resulted in a significant left ventricular dilatation and in left and right ventricular free wall thinning (3.31±0.20 mm vs 2.61±0.19 mm, p<0.05; 0.54±0.09 mm vs 0.72±0.16 mm, p<0.05, respectively). The sarcomere length was also significantly increased in the 3W+/40W+ group compared with that of the other groups, as determined by electron microscopy. Degenerative or necrotic areas in the infected hearts were not stained with anti-mouse IgG antibody, but were stained, only in 3W+/40W+ mice, with anti-mouse IgM antibody. The concentrations of TNF-α in the hearts of the 3W+/40W+ group were increased significantly over those of the 3W–/40W– group. Repetitive CVB3 infection produced cardiac dilatation without inflammatory cell infiltration in post-myocarditic mice. 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. Thus, repetitive virus infection might contribute to the pathogenesis of cardiac dilatation. (Jpn Circ J 1999; 63: 794–802)

Key Words: Dilated cardiomyopathy; Immunologic factors; Myocarditis; Virus

Dilated cardiomyopathy (DCM) is characterized by the dilation and impaired contraction of the ventricles. 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. Clinically, it is characterized by progressive heart failure.2 The need for the substantiation of a definite link between myocarditis and the development of DCM has been expressed in a number of clinical studies.3 Infectious and autoimmune myocarditis have been extensively investigated using murine and rat models. In addition, some models have demonstrated post-myocarditic DCM in the chronic phase of viral or self-antigen inoculation.4,5 However, it is quite difficult to definitively discriminate between the infectious effects and autoimmune mechanisms in these experimental models.6–8 Moreover, the inflammatory cell infiltration typically seen in these models is not a characteristic finding of DCM. Finally, acute myocarditis in mice does not always cause cardiac dilatation. However, one clinical report has demonstrated that repetitive viral myocarditis can result in severe cardiac dysfunction and cardiac death9 and another showed that successive infection with coxsackievirus B3 (CVB3) and encephalomyocarditis virus in mice induces additive myocardial lesions similar to chronic myocarditis or recurrent myocarditis,10 suggesting that repetitive infections with several virus types may lead to chronic myocarditis and DCM. Taken together, these results raise the possibility that repetitive viral infection is one of the causes of DCM.

In the present study, we performed histological, immunohistochemical, and serological examinations to clarify the cardiac effects of repetitive viral infection in mice.

Methods

Experimental Protocol

Thirty-six, 3-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 CVB3 (Nancy strain) was obtained from the American Type Culture Collection and stored at –80°C until use. Each mouse was initially injected by an intraperitoneal injection of 2×10^4
plaque-forming units of the CVB3 in 0.2 ml of saline. The infected mice were isolated, 5 per cage, in a special unit for 37 weeks (3W+/40W–). Seven mice first inoculated at 3 weeks were reinfected in the same manner with CVB3 at 40 weeks (3W+/40W+). In addition, 14 normal mice were halved transversely. One ventricular portion was fixed for an additional 2h in the same fixative at 4°C and washed in the same buffer containing 6% sucrose. The remaining area of the left ventricular wall from which the slices were taken was then cut into small pieces (1 mm³). The tissue was next postfixed for 2 h in 2% OsO₄ dissolved in 0.1 mol/L sodium cacodylate buffer (pH 7.4), at room temperature. Immediately after the perfusion, the heart was removed and transverse slices (1-mm thickness) were made at its mid-portion. The slices were fixed for an additional 2h in the same fixative at 4°C and washed in the same buffer containing 6% sucrose. The remaining area of the left ventricular wall from which the slices were taken was then cut into small pieces (1 mm³).

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**Morphometry and Histopathological Study**

The atria were removed from the hearts. The ventricles were halved transversely. One ventricular portion was fixed with 10% formalin solution, then embedded in paraffin solution and sectioned into slices of 4-μm thickness, which were stained with both hematoxylin-eosin and Azan solutions. Ventricular dimension 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 and right ventricles and of the interventricular septum were measured according to Matsumori et al. Cardiac fibrosis was also measured using a Fotovision FV-10 camera (Fuji Film Co, Japan) and a Macintosh 8500/120 computer equipped with NIH Image 1.60 software.

**Electron Microscopy**

The heart was fixed by retrograde perfusion through the thoracic aorta for 10 min with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4), at room temperature. Immediately after the perfusion, the heart was removed and transverse slices (1-mm thickness) were made at its mid-portion. The slices were fixed for an additional 2h in the same fixative at 4°C and washed in the same buffer containing 6% sucrose. The remaining area of the left ventricular wall from which the slices were taken was then cut into small pieces (1 mm³). The tissue was next postfixed for 2 h in 2% OsO₄ dissolved in 0.1 mol/L sodium cacodylate buffer (pH 7.4), at room temperature. Immediately after the perfusion, the heart was removed and transverse slices (1-mm thickness) were made at its mid-portion. The slices were fixed for an additional 2h in the same fixative at 4°C and washed in the same buffer containing 6% sucrose. The remaining area of the left ventricular wall from which the slices were taken was then cut into small pieces (1 mm³). The tissue was next postfixed for 2 h in 2% OsO₄ dissolved in 0.1 mol/L sodium cacodylate buffer (pH 7.4), at room temperature. Immediately after the perfusion, the heart was removed and transverse slices (1-mm thickness) were made at its mid-portion. The slices were fixed for an additional 2h in the same fixative at 4°C and washed in the same buffer containing 6% sucrose. The remaining area of the left ventricular wall from which the slices were taken was then cut into small pieces (1 mm³). The tissue was next postfixed for 2 h in 2% OsO₄ dissolved in 0.1 mol/L sodium cacodylate buffer (pH 7.4), at room temperature. Immediately after the perfusion, the heart was removed and transverse slices (1-mm thickness) were made at its mid-portion. The slices were fixed for an additional 2h in the same fixative at 4°C and washed in the same buffer containing 6% sucrose. The remaining area of the left ventricular wall from which the slices were taken was then cut into small pieces (1 mm³).

**Immunohistochemistry**

For immunoenzymatic staining, the remaining ventricular portion was quickly frozen with O.C.T. compound (Miles, Inc, USA) in liquid nitrogen and stored at –80°C. Frozen specimens were sectioned at 4-μm thickness. Immunoenzymatic staining was performed with the DAKO LSAB kit (Dako Co, USA) according to the manufacturer’s instructions. Briefly, the sections were preincubated with 0.3% hydrogen peroxide and normal goat serum (Dako Co) to block nonspecific reactions. Antibodies against mouse CD4 (1:10 dilution, Caltag Laboratories, Inc, USA), CD8 (1:10 dilution, Caltag Laboratories, Inc) and macrophages (1:10 dilution, Caltag Laboratories, Inc) were used as primary antibodies and incubated for 30 min at room temperature. Sections were then incubated with biotiny-
lated rabbit anti-rat immunoglobulins in phosphate-buffered saline (PBS) containing carrier protein and 15 mmol/L sodium azide for 20 min, followed by incubation with horseradish peroxidase-labeled streptavidin conjugated to alkaline phosphatase in Tris-HCl buffer, containing 15 mmol/L sodium azide, for 10 min. The slides were rinsed in cold Tris-buffered saline after each step of the incubation. Peroxidase activity was visualized with substrate-chromogen solution, containing New Fuchsin Chromogen (Dako Co). Finally, the slides were counterstained with hematoxylin solution. Negative controls were prepared with nonimmune serum instead of primary antibody.

For quantitation of the lymphocyte subsets, the sections were examined by 2 independent examiners in a blinded fashion. The extent of CD4+ and CD8+ T cells and macrophage infiltration in each group was assessed by determining the cell counts per high-power field (HPF). The number of lymphocytes per HPF in each section that was stained by each monoclonal antibody was recorded, and the number of stained lymphocytes was then calculated. This process was repeated for more than 20 fields.

Bound IgM and IgG in the heart were investigated by direct immunohistochemical staining using goat anti-mouse IgM FITC antibody (0.035 mg/ml, Caltag Laboratories) and rabbit anti-mouse IgG FITC antibody (0.025 mg/ml, Serotec, USA). After incubation with the antibody against IgM and IgG, sections were washed with PBS. The samples were then examined using a fluorescence microscope.

Fig 1. Photomicrograms showing cross-sections of normal and virus-infected mouse hearts. (a) 3W−/40W− group; (b) 3W−/40W+ group; (c) 3W+/40W− group; (d) 3W+/40W+ group. All samples in the 3W+/40W+ group revealed prominent left ventricular dilatation and remodeling. No further progressive fibrosis (blue staining) was observed in the 3W+/40W+ group compared with the 3W+/40W− group (Azan stain; original magnification, ×1.6).

Cytokine Assay
Mice from each of the infection groups (3W−/40W− group, n=6; 3W−/40W+ group, n=6; 3W+/40W− group, n=6; 3W+/40W+ group, n=6) were anesthetized, and the right atrium was then punctured and bled to obtain serum. The whole heart was then harvested for the cytokine assay and immediately immersed in liquid nitrogen. A volume of 500 μl of PBS was added to each heart, and this mixture was homogenized with an ultrasonic homogenizer. The concentration of interleukin (IL)-1 and tumor necrosis factor (TNF)-α in homogenates were determined in duplicate by using enzyme-linked immunosorbent assay kits for mouse IL-1α and TNF-α (Genzyme Co, USA). The minimum detectable concentration level was 15 pg/ml in each of the assays. All concentration levels were corrected to the corresponding heart weight.

Neutralizing Antibody Titer
Mice from each of the infection groups (3W−/40W− group, n=8; 3W−/40W+ group, n=5; 3W+/40W− group, n=7; 3W+/40W+ group, n=8) were anesthetized, and the right atrium was then punctured and bled to obtain serum for the assay for neutralizing antibody titers. The antibody titers were measured by a virus neutralization test. Briefly, the serum was inactivated at 56°C for 30 min. Each sample was titrated serially by determining the fourfold dilution in 5 ml EMEM that protected the CMKSI cell monolayer against a challenge of 100TCID50/25 μl of CVB3. After a 5-day incubation at 37°C, the cytopathic effect in each well was determined. The neutralizing antibody titer level for
Statistical Analysis

Data were expressed as mean±SD. A statistical analysis of the data was performed by an analysis of variance with multiple comparisons. A level of p<0.05 was considered statistically significant.

Results

Survival Rates

The A/J mice infected at 3 weeks after birth appeared ill and moved sluggishly 7–14 days after the first CVB3 inoculation. Some of the mice developed spastic paralysis, but some sick mice survived and appeared well after day 14. The survival rate of the 3-week infection group was 86%, whereas there were 4 deaths among 14 mice (survival rate 71%) in the 40-weeks inoculation group (Table 1).

Body, Heart, Lung and Liver Weights

The mean body weight of the infected group was significantly lower than that of the 3W–/40W– group (p<0.05), and there was no difference in body weight among the infected groups. The mean heart weight in the 3W+/40W+ group was significantly higher than that in the 3W–/40W+ group (p<0.05). In addition, both the heart weight/body weight and lung weight/body weight ratios in the 3W+/40W+ group were significantly higher than in the 3W–/40W– group (p<0.05). Comparing the 3W+/40W+ group with the 3W+/40W– group, there were no significant differences in the ratios of lung or liver weight to body weight.

Cardiac Morphology and Electron Microscopy

The left ventricular internal dimensions in 3W+/40W– and 3W+/40W+ groups were greater than those of both the 3W–/40W– and 3W–/40W+ groups. The mean left ventricular internal dimension in the 3W+/40W+ group was significantly greater than that of the other 3 groups (Fig 1, Table 2, p<0.05). The mean left ventricular internal dimension/body weight was greater in the 3 infected groups than in the 3W–/40W– group. In addition, the left ventricular internal dimension/body weight ratio of the 3W+/40W+ group was significantly larger than those of the other 3 groups (p<0.05). There was a tendency toward decreased wall thickness in the 3W+/40W+ group relative to the other 3 groups. Furthermore, there was a significant difference in the thicknesses of both the left and right ventricular free wall between the 3W+/40W– group and 3W+/40W+ group (p<0.05; Table 2). The sarcomere length in the 3W+/40W+ group was significantly greater than those of both the 3W–/40W– and 3W+/40W– groups (Fig 2).

Cardiac Fibrosis and Inflammatory Cell Infiltration

The ratio of cardiac fibrosis in the virus-infected (3W–/40W+, 3W+/40W+, and 3W+/40W+) groups was significantly higher than that of the 3W–/40W– group. The ratio of cardiac fibrosis in the 3W–/40W+ group was highest among the groups and was significantly higher than that of the 3W+/40W+ group (p<0.05). There was no statis-
tical difference between the ratio of cardiac fibrosis of the 3W+/40W– and 3W+/40W+ groups (Table 2).

Initial CVB3 inoculation at 40-weeks-of-age (3W−/40W+) resulted in marked inflammatory cell infiltration into the myocardium (Fig 3a). In contrast, little inflammatory cell infiltration was observed in either the 3W+/40W− or 3W+/40W+ groups (Fig 3b–c). At 40 weeks, mice inoculated with CVB3 at 3-weeks-of-age showed a significant cardiac dilatation compared with the vehicle mice, which showed only moderate fibrosis and calcification (Fig 3b). Furthermore, the immunohistological examination of the infected myocardium of both the 3W+/40W− and 3W+/40W+ mice demonstrated little inflammatory cell infiltration, which was positively stained with antibodies against macrophages, and CD4+ or CD8+ T cells (Fig 3d–f). The cell counts per HPF of CD4+T cells, CD8+ T cells, and macrophages in the myocardium of the 3W+/40W+ group were the same as those of the 3W+/40W− group (Table 2).

**Immunostaining of Immunoglobulin and Neutralizing Antibody Titer**

Staining of bound IgM and IgG in the myocardium was investigated in both the 3W+/40W− and 3W+/40W+ groups (Fig 4). The degenerative or necrotic areas in the myocardium caused by viral infection did not appear to be stained with anti-mouse IgG antibody in either the 3W+/40W− or 3W+/40W+ groups. In contrast, these lesions were stained with anti-mouse IgM antibody in the
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3W+/40W+ group, but not in the 3W+/40W– group. In addition, the antibody titers on day 14 after the second CVB3 inoculation in the chronic phase group (3W+/40W+) were significantly higher than in the other groups (p<0.05; Fig 5). There was no difference in antibody titers among the 3W–/40W–, 3W–/40W+, and 3W+/40W– groups.

Cytokine Assay

Although concentrations of both IL-1β and TNF-α in the sera of all 4 groups were under the cutoff level, the concentrations of TNF-α in the hearts of the 3W+/40W+ group were increased significantly over those of the 3W–/40W– and 3W+/40W– groups (p<0.05; Fig 6).

Discussion

Viral myocarditis is usually associated with a short clinical course, and little is known about whether repetitive virus infection causes similar cytotoxic effects on initially injured myocytes. In mice with chronic ongoing myocarditis lasting for at least 17 weeks after the first inoculation with CVB3, we previously reported histopathological changes and the continuous infiltration of macrophages, CD4+, and CD8+ cells as well as the expression of ICAM-1 and MHC class I and class II in the infected myocardium, with this inflammatory cell infiltration gradually disappearing after this phase of the ongoing myocarditis. Viral RNA genomes were not detected in mice with chronic ongoing myocarditis at 60 days after inoculation using the polymerase chain reaction.12,14 The present study has confirmed that, in the 3W+/40W– group, there was little inflammatory cell infiltration and little progression of fibrosis, suggesting that 40-week-old mice inoculated at 3 weeks after birth are
in a post-myocarditic state.

In the present study, we demonstrated that repetitive CVB3 infection produces significant heart weight gain and cardiac dilatation with an elongation of the sarcomeres. In addition, little inflammatory cell infiltration and no further progressive fibrosis occurred in the myocardium of the 3W+/40W+ group, as compared with the 3W+/40W– group. In comparison with the 3W+/40W– group, the 3W+/40W+ group showed no lung or liver congestion or body weight gain, indicating that the cardiac dilatation was not due to volume overload. Moreover, there was no difference in the level of fibrosis between the 3W+/40W– and 3W+/40W+ groups, which is interesting, considering that the 3W–/40W+ group had the highest level of fibrosis. This would suggest that there is some factor that is protective against fibrosis in the pre-infected group. Recently, Pauschinger et al demonstrated that the ratio of collagen subtype (Col III/I) mRNA abundance in the myocardium with chronic inflammatory processes greatly differs from that in histologically and immunologically confirmed idiopathic DCM.15 They have also proposed that this finding might be explained by the influence of cytokines and growth hormones that mediate inflammatory processes on the metabolism of collagens. In our present study, although the level of fibrosis in the repetitive inoculation group did not differ from that of the 3W+/40W– group, there might exist a differential ratio of Col III/I mRNA abundance in the repetitive inoculation group, according to the Pauschinger theory.

Although secondary infection by another virus can elicit secondary myocarditis,10,16 Okada et al have reported that the hearts of mice inoculated with CVB3 at both 4- and 8-weeks-of-age do not show additive myocardial lesions at 2 weeks after the second CVB3 inoculation. The absence of additive lesions may be due to the protective effect of neutralizing antibody against CVB3 induced by the first inoculation, as well as CVB3 being less able to cause myocardial lesions in 8-week-old mice than 4-week-old mice.10 The primary difference between the present study and the previously reported studies is the age of mice at the time of inoculation. We reinoculated with CVB3 in older post-myocarditic mice, suggesting a different susceptibility to the same virus during senescence. The poor T-cell response in 40-week-old mice observed in our study might indicate that aging contributes to the immunological mechanism causing cardiac dilatation in this model.17 Disturbances in both humoral and cellular immunity have been described in patients with DCM and have been implicated in the initiation and progression of this disease entity. Previous studies have shown that the sera of many patients with DCM are positive for several antibodies directed against cardiac antigens.18 The pathophysiological relevance of these autoantibodies, however, is far from clear. Recently, Dörffel et al demonstrated that the extraction of autoimmunoreactive antibodies by immunoabsorption results in a functional improvement in hemodynamics in DCM patients.19 These authors have proven indirectly that autoantibodies (eg, against the ADP–ATP carrier20 and contractile proteins of cardiomyocytes21 and cardiac ß1-adrenergic receptor22) contribute to cardiac malfunction in DCM. Certainly, it is difficult to clarify the potential role of a specific antibody directed against cardiac tissue because immunosorption is successfully used to effectively eliminate all immunoglobulins from plasma in a nonselective manner. The results of Dörffel et al, however, support the hypothesis that different autoantibodies may play a role in the hemodynamic deterioration in DCM patients. In the present study, we have demonstrated that the same viral challenge could induce repetitive myocarditis in the post-myocarditic phase, based on the results of significantly elevated neutralizing antibody titers and the myocardium staining positively with anti-IgM antibody in the 3W+/40W+ group. The distribution of this antibody was mainly on the surface of the myocardium, as observed by immune electron microscopy (data not shown). The antibody binding to the cell surface might indirectly influence carrier function (eg, ADP–ATP carrier) by activating a messenger system. Moreover, neutralizing antibody titers in the 3W+/40W+ group were significantly higher than those of the 3W+/40W– group, as well as those of the 3W–/40W– and 3W–/40W+ groups. These results suggest that the immunological response against the same viral antigen might be memorized and enhanced even in the healing stage of myocarditis. The latest viral inoculation
might have a booster effect, in which the T cells then assist the differentiation of B cells into plasma cells, which in turn produce antibodies directed against components of CVB3.

We have also demonstrated in the present study the activation of the cytokine system in the myocardium subjected to repetitive myocarditis. The concentration of TNF-α in the hearts of the 3W+/40W+ group was increased significantly over that of the 3W+40W− group. Over the past few years, there has been considerable interest in the role of cytokines in the pathogenesis of myocarditis and DCM. In particular, TNF-α has been studied in some detail, resulting in reports of elevated TNF-α levels in the serum of patients with chronic heart disease, including a subset of patients with myocarditis or DCM. TNF-α is able to potentiate the immune response and induce cell death, both of which appear to have a special importance in the pathogenesis of myocarditis. Kubota et al have described a transgenic mouse model of myocarditis in which TNF-α is expressed specifically in the myocardium, under the control of the α-myosin heavy-chain promoter. These animals develop gross interstitial edema, globular dilatation of the heart and cardiomegaly, suggesting that local TNF-α expression is sufficient to cause cardiac pathology and dysfunction, which supports the conclusion drawn from our data. In our present study, both the minimal inflammatory cell infiltration and elevated neutralizing antibody titers implied no further replication of CVB3 after the second viral inoculation. We also confirmed at the second inoculation that similar morphometric change and TNF-α expression occur even with inactivated CVB3 (Yamamoto et al, unpublished data). There is another possibility regarding the mechanisms causing cardiac dilatation in the present study, namely, that a complementary system activated by IgM might contribute to this host response.

**Study Limitations**

DCM is characterized by both cardiac dilatation and systolic dysfunction. Although we were able to demonstrate cardiac dilatation induced by repetitive viral infection in post-myocarditic mice, no hemodynamic studies were performed. Hemodynamic studies are necessary to show systolic dysfunction as a complete model for DCM. Moreover, to clarify the mechanism causing cardiac dilatation, it is also necessary to examine the morphological and immunological changes after the second inoculation at 40-weeks-of-age. In the present study, the repetitive group showed different histological features from those of DCM, including the absence of diffuse fibrous myocardial replacement at 2 weeks after the second inoculation. These studies will provide further insights into the mechanism of cardiac dilatation induced by repetitive viral infection in post-myocarditic mice.

**Conclusions**

In this experiment, following an initial inoculation with CVB3 at 3 weeks-of-age, a second CVB3 infection at 40 weeks failed to induce either inflammatory cell infiltration or further progressive cardiac fibrosis in post-myocarditic mice, but did cause a remarkable dilatation of the left ventricle only 2 weeks after the second inoculation. These findings suggest that both the initiation caused by virus infection in the infant and the reinfecion with the same virus in adulthood may result in left ventricular dilatation, suggesting that repetitive viral infection might contribute to the pathogenic mechanism of cardiac dilatation in this model. Further studies will be required to certify the autoimmune mechanism of repetitive myocarditis and the development of subsequent DCM.

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