A Pathogenic Mechanism of Chronic Ongoing Myocarditis

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To clarify the pathogenetic mechanism of chronic ongoing myocarditis, we produced Coxsackievirus B3-induced myocarditis in A/J mice and immunopathologically examined the microcirculation in the chronic phase of myocarditis. Forty-two 3-week-old A/J mice were inoculated intraperitoneally with Coxsackievirus B3 (Nancy strain) 2×10^4 PFU (plaque-forming units) and sacrificed 7, 14, 21, 50, 90, or 120 days later. To evaluate myocardial microcirculation, 18 of the hearts were perfused from the thoracic aorta with warm 2% gelatin/carbon solution. The remaining hearts were quickly frozen for immunologic analysis with an enzyme immunostaining assay using monoclonal antibodies against CD4, CD8, macrophages, intercellular adhesion molecule-1 (ICAM-1) and major histocompatibility complex class I or II. The presence of viral RNA genome in the myocardium at 40, 50, or 60 days after inoculation was evaluated using the polymerase chain reaction.

The lesions in chronic ongoing myocarditis consisted of myocardial damage, myocardial calcification, interstitial fibrosis, and infiltration of mononuclear cells. These infiltrated lymphocytes were predominantly CD4+ T cells. Furthermore, microvascular abnormalities, including dilatation, tortuosity, constriction, and abrupt termination, were observed around the lesions. There was marked infiltration by mononuclear cells around the microvessels. ICAM-1 was strongly expressed in the endothelial cells of the vessels. Coxsackie B3 viral genome was not detected in the myocardium of mice with chronic ongoing myocarditis in each stage examined. These results suggest that an autoimmune mechanism is involved in the persistent inflammation seen in chronic ongoing myocarditis.

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MYOCARDITIS is defined as an inflammatory cell infiltration in the myocardium accompanied by myocardial cell damage in a pattern that does not resemble ischemic heart disease¹. Most cases of acute myocarditis in developed countries are thought to be due to viral infection. Recently, with the advent of RNA hybridization

and the polymerase chain reaction, the presence of viral genome in myocardium with myocarditis or dilated cardiomyopathy (DCM) has been confirmed clinically². Although the pathogenesis of DCM is unclear, it has been suggested that an episode of subclinical viral myocarditis can initiate an autoimmune reaction that culminates in the development of DCM³.

The A/J mouse inoculated with Coxsackievirus B3 (CVB3) exhibits persistent myocarditis⁴. Therefore, this animal is a

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suitable model for studying the pathogenesis of ongoing myocarditis, with or without the virus genome. With the advance of immunology and molecular biology, various kinds of monoclonal antibodies to cell surface markers, adhesion molecules and cytokines are now available for studying the mechanism of inflammation. The characterization of infiltrated cells and the expression of adhesion molecules, especially intercellular adhesion molecule-1 (ICAM-1), which plays an important role in the infiltration of lymphocytes into lesions, can be determined.⁵ On the other hand, it has been suggested that disturbances of microcirculation in myocarditic lesions plays a role in the pathological changes in myocarditis⁶ and DCM.⁷ The vasculature itself may contribute to the migration of infiltrating inflammatory cells.

To clarify the pathogenetic mechanism of chronic ongoing myocarditis, we produced CVB3-induced myocarditis in A/J mice and analyzed the findings immunopathologically. Our results suggest that a virus-induced autoimmune process may play an important role in the progression of chronic ongoing myocarditis.

METHODS

Animals and infection

Forty-two 3-week-old, inbred, certified virus-free A/J (H-2a) male mice were purchased from Japan SLC, Inc, Shizuoka. CVB3 (Nancy strain) was obtained from the American Type Culture Collection. The virus was stored at -80°C until use. Each mouse was infected by an intraperitoneal injection of 0.2 ml of phosphate-buffered saline that contained approximately 2×10^4 PFU. The Coxsackievirus B3-infected mice were housed in a separate infectious cabinet. The room temperature and humidity were kept at $22 \pm 2^{\circ}\text{C}$, $45 \pm 5\%$, respectively. Mice were housed 5 per cage and given water and rodent chow ad libitum.

Histology

Mice were sacrificed 7, 14, 21, 50, 90, or 120 days after inoculation. In each phase, the heart was cut transversely into 2 slices. One part was fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin or

Azan solution. The other part was quickly frozen for immunostaining assay. Frozen slices were embedded in O.C.T. compound (Miles Inc, USA) and sectioned on a cryostat into slices $4 \mu\text{m}$ thick. Immunoenzymatic staining was performed with the DAKO LSAB kit (DAKO, USA). Briefly, the sections were pre-incubated with 0.3% hydrogen peroxide and normal goat serum to block nonspecific reactions. Antibodies against mouse CD4 (Caltag Laboratories Inc, USA), CD8 (Caltag Laboratories Inc, USA), macrophages (Caltag Laboratories Inc., USA), ICAM-1 (Serotec Inc, UK), and major histocompatibility complex class I (Cedarlane Inc, Canada) or class II (Serotec Inc, UK) were applied and incubated for 30 min at room temperature, respectively. Sections were incubated with biotinylated rabbit anti-rat immunoglobulins for 20 min, and then incubated with horseradish peroxidase-labeled streptavidin solution for 10 min. Slides were rinsed in cold Tris-buffered saline after each incubation. Peroxidase activity was visualized using a substrate-chromogen solution. Slides were counterstained with hematoxylin. The intensity of immunostaining was graded semiquantitatively on a 4-point scale from $-$ to $3+$. Grade $-$ indicated the complete absence of staining; grade $+$, immunostaining positivity associated with $<10\%$ of the cells; grade $2+$, positivity of 10 to 30% of the cells; and grade $3+$, positivity of $>30\%$ of the cells. Grading was performed independently by 2 investigators who had no knowledge of the data. Grades independently assigned by the 2 observers were agreed to within 1 grade; any differences were resolved by consensus.

Microcirculation

Three control mice and 18 mice with viral myocarditis were sacrificed 7, 14, 21, 50, 90, or 120 days after inoculation to study myocardial microcirculation.⁸ Just before sacrifice, the heart of each animal was prepared as follows. Under ether anesthesia, a polyethylene cannula (0.76 or 0.86 mm o.d.) was inserted into the thoracic aorta. The outflow was provided via a cut in the inferior vena cava. The heart was briefly perfused with saline to wash out blood, and then filled with warm 2% gelatin/carbon solution

Fig 1. Transverse section of 90-day myocarditis. Hematoxylin and eosin staining. (a)–(c), 90 days after inoculation, (d), normal control.

TABLE I DISTRIBUTIONS OF CD4, CD8, MACROPHAGES, ICAM-1, MHC CLASS I, AND MHC CLASS II WERE INVESTIGATED BY IMMUNOHISTOCHEMICAL STAINING IN MICE WITH CHRONIC ONGOING MYOCADITIS

	control	7 days	14 days	21 days	50 days	90 days	120 days
CD4	—	+	‡	‡	‡	‡	‡
CD8	—	+	‡	‡	‡	+	+
Macrophage	—	+	‡	‡	‡	‡	‡
MHC class I	—	+	‡	‡	+	‡	+
MHC class II	—	+	‡	‡	+	‡	+
ICAM-1	—	+	‡	‡	+	‡	‡

※RT-PCR

Intensity of immunostaining was graded as:

—, the absence of any staining

+, immunostaining positivity associated with <10% of the cells

‡, positivity of 10% to 30% of the cells

‡‡, positivity of >30% of the cells

Fig 2. Photomicrograms showing cross sections of chronic ongoing myocarditis (120 days). The distribution of CD4-positive cells (a), CD8-positive cells (b), macrophages (c), ICAM-1 (d), MHC class I (e), and MHC class II (f) were investigated by immunohistochemical staining. Intensity of immunostaining was graded semiquantitatively from $-$ to $3+$. (see Table I)

introduced via a hand-held syringe. Perfusion was continued until the entire epicardium turned black. The heart was then cooled with crushed ice until the gelatin/carbon mixture had gelled. The excised heart was fixed in cold 10% formalin, embedded in paraffin, serially sectioned at $4\ \mu\text{m}$, stained with hematoxylin-eosin, and examined by light microscopy.

Virologic Study

The presence of viral genome in the myocardium was investigated by the polymerase chain reaction (PCR) method using a primer of the 5' end of the CVB3 genome (CVB1 446–640) at 40, 50, or 60 days after inoculation, as previously reported^{9–11}. Briefly, nucleic acid extracted from the heart was isolated by the acid guanidinium thiocyanate-phenol-chloroform

Fig 3. Photomicrogramas showing microvessels with a gelatin/carbon fixation in the myocardium of acute (7 and 21 days) and chronic ongoing myocarditis (120 days) to study their microcirculation. On 7 days (a), small vessel dilatation was seen around the edematous lesion in the myocardium. At 21 days (b), microvascular abnormalities including dilatation, tortuosity, constriction, and abrupt termination, were observed around the lesions. At 120 days (c), lymphocytic infiltration were prominent around the abnormal microvessels (Hematoxylin-eosin staining).

method. Alpha-tubulin oligonucleotides were used to confirm nucleic acid extraction as a positive control. cDNA was then syn-

thesized using Moloney murine leukaemia virus (MMLV) reverse transcriptase (BRL, USA) and amplified by PCR. DNA was amplified in a DNA thermal cycler (Perkin Elmer Cetus), as described by Chapman et al¹⁰; denaturation for 1 min at 94°C, 10 cycles (1 cycle=94°C for 1s; 50°C for 1s; and 72°C for 1s) followed by 40 cycles (one cycle=94°C for 1s; 50°C for 1s). A total of 10 µl of a PCR mixture was electrophoresed at 80 V into 1.5% agarose gels in 0.2×TAE (1×TAE; 40 mmol/L Tris-acetate and 1 mmol/L EDTA (pH 8.0)). Gels were stained in ethidium bromide (1 µl/ml) to detect DNA by illumination with UV light, and photographed.

RESULTS

Histology

In the early stage (2 to 3 days after inoculation) of the infection, we observed several small degenerated myocytes. Between 7 and 14 days after inoculation, massive myocardial necrosis and marked infiltration by mononuclear cells were seen. After 21 days, we observed fibrosis and calcified lesions with persistent cellular infiltration and damage to myocardial cells. This latter stage was designated for chronic ongoing myocarditis. Histopathological changes at 90 days are shown in Fig 1. The distribution and time course of the severity of the infiltration by CD4- and CD8-positive cells, macrophages, and expression of ICAM-1, MHC class I, and MHC class II are summarized in Table I. In chronic ongoing myocarditis, infiltration consisted predominantly of CD4-positive cells around the lesion, accompanied by a few CD8-positive cells. ICAM-1 was expressed diffusely in myocardium in the acute phase, but it was expressed mainly in the endothelium of the small vessels around the chronic ongoing lesions. MHC class I and class II were also expressed on the surface of myocytes around the lesions (Fig. 2).

Microcirculation

At 2 to 3 days after viral inoculation, small vessel-dilatation was seen around the lesions in the myocardium (Fig 3a). In the acute or subacute phase (7 to 21 days after inoculation), severe cellular infiltration,

Fig 4. Expression of Coxsackievirus B3 genomic RNA and α -tubulin mRNA amplified by reverse transcriptase polymerase chain reaction. The A/J strain mice as well as the C3H/He strain of mice, were examined. Both strains were negative of 40, 50 and 60 days after inoculation. C3H/He: strain of C3H/He mouse, A/J: strain of A/J mouse. Virus (+) indicates samples extracted from the mice inoculated with Coxsackievirus B3. P, positive control; N, negative control.

microvascular abnormalities, including dilatation, tortuosity, constriction, and abrupt termination were observed in and around the lesions (Fig 3b). Similar microvascular abnormalities were also seen in the lesions in the chronic phase. In addition, numerous lymphocytes had infiltrated prominently around the dilated microvessels (Fig 3c).

Virologic Study

The products of all samples amplified with primers showed detectable bands at about 288 bp, which is the size of the products of α -tubulin mRNA, and which confirms that RNA extraction, reverse transcription, and enzymatic amplification all proceeded properly. Although a 195-bp band, which is the size of the product of Coxsackievirus B3 genomic RNA amplified with primers, was detected in positive controls, samples from

the infected mice at 40, 50, and 60 days after the inoculation and samples from non-infected controls were all negative for the 195-bp band (Fig 4).

DISCUSSION

Chronic myocarditis remains a controversial diagnosis histologically, although the clinical features of chronicity are well established.¹² Lerner proposed a biphasic model of pathogenesis based upon a mouse model, with an early infectious phase associated with active virus replication and a second immune-mediated phase.¹³ In this study, we also demonstrated that A/J mice inoculated with CVB3 developed acute myocarditis and subsequent myocarditis. The latter development may be due to an autoimmune reaction, since we did not find

viral genome in the myocardium. We defined this stage as chronic ongoing myocarditis.

Our histological findings suggest the following 3 mechanisms for the myocarditis in our model: (i) injury due to direct viral infection and replication in myocardial cells 2 to 3 days after inoculation, (ii) injury due to an immune response to viral infection on days 7 to 14, and (iii) injury due to an autoimmune response during chronic ongoing myocarditis. The first and second pathogenic mechanisms have been reported previously. Woodruff demonstrated the virus-mediated and infiltrating cell-mediated destruction of myofibers during the acute phase of myocarditis.¹⁴ Gauntt et al showed that some anti-CVB3 neutralizing monoclonal antibodies could induce cardiopathologic changes in vivo, and suggested that CVB3-induced inflammation can be sustained in the absence of continued virus replication.¹⁵ In particular, Woodruff first established that T lymphocytes were primarily responsible for myocyte necrosis in their model of CVB3-induced murine myocarditis. Interestingly, during chronic ongoing myocarditis, CD4-positive cells were the predominant infiltrate and viral genome was not detected by PCR. Recent experimental data suggest that CD4-positive T cells play a key regulatory role in autoimmune diseases.¹⁶ T helper 1 (Th1) cells may play a pathogenetic role and Th2 cells may have a protective role in T cell-mediated, organ-specific autoimmune diseases such as experimental autoimmune encephalomyelitis and insulin-dependent diabetes mellitus. Huber and Lodge isolated cytolytic T-lymphocyte of lymph node cells at 7 days after inoculation, and showed that the cytolytic activity to uninfected myocyte targets is mediated by a population of autoimmune T cells. They also provided evidence for autoimmunity to myocyte antigens.¹⁷ Furthermore, they showed that the Th1 cell phenotypic response contributed to the difference in susceptibility to CVB3-induced myocarditis between male and female BALB/c mice.¹⁸ If the CD4-positive cells detected in the chronic ongoing myocarditis in our models are Th1 cells, these findings suggest that a cell-mediated autoimmune mechanism is involved in the pathogenesis of chronic ongoing myocarditis.

Abnormalities of myocardial microcirculation, such as irregularity, dilatation, and focal narrowing of the microvessels, were observed in and around the lesions in the present study. The importance of abnormalities in microvessels was noted previously in the case of myocarditis and cardiomyopathies.^{6,19} Sonnenblick et al suggested that microvascular spasm and reperfusion injury caused a focal loss of cells with the subsequent development of focal fibrosis and reactive hypertrophy in experimental models of congestive cardiomyopathy.²⁰

The mechanisms for the accumulation of leukocytes at sites of inflammation are now clear. One of the most important mechanisms of this accumulation is adhesion between ICAM-1 on the endothelium and leukocytes. In the lesion of chronic ongoing myocarditis examined in the present study, expression of ICAM-1 was mainly seen on endothelial cells around the lesion, and on lymphocytes that infiltrated around the vessels. Therefore, the expression of ICAM-1 might reflect the activity of the lesions, and autoreactive T cells may have infiltrated through the microvessels around the lesion. The development of chronic ongoing lesion does not take a diffuse or random course, but seems to involve abnormal microvessels. It seems to spread around the initial lesions, which were constructed in the acute phase of myocarditis, as a ripple spreads after a stone is thrown into a pond.

Clinical and experimental studies have demonstrated a dramatic up-regulation of MHC antigen expression on various cardiac cells in viral infections such as myocarditis.^{21,22} Endogenous self-peptides have been obtained from both MHC class I and class II molecules. Smith and Allen demonstrated that myosin-class II major histocompatibility complexes are present on resident antigen-presenting cells (APC) in the normal mouse.²³ If the expression of these self-MHC class II complexes were enhanced, activated self-reactive T cells might cause chronic inflammation of the myocardium.²²

The pathogenesis of chronic ongoing myocarditis is still controversial. Lane et al showed that the development of autoimmune myocarditis was associated with the infiltration of inflammatory cells that secreted cytokines, interleukin-1 (IL-1) and tumor

necrosis factor α (TNF- α), and suggested that their local production may alter the consequence of cardiostrophic viral infection to induce a postinfectious autoimmune disease²⁴. These cytokines may further stimulate inflammatory cells, thus increasing mononuclear cell adhesion and the recruitment of these cells into the heart. The cytokine network may induce inflammatory cell infiltration from impaired microvessels, which occurs through the endothelium on which adhesion molecules are expressed. The intriguing possibility exists that ongoing tissue injury releases self-antigen, thus contributing to the persistence of autoimmunity.

Conclusions

Myocarditis in A/J mice infected with CVB3 may be a suitable model of chronic ongoing myocarditis. The progression of inflammation in the chronic stage of myocarditis in A/J mice may not be due to the presence of viral genome, but rather to an autoimmune response of cardiac tissue. The lesion progresses in a characteristic course in the chronic stage. In particular, ICAM-1 expression on microvessels and inflammation of CD4-positive cells around the vessels may play a major role in prolonging the inflammation of chronic ongoing myocarditis. Further experiments are required to certify the autoimmune mechanism of chronic ongoing myocarditis and the development of subsequent DCM.

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