Significance of Pericardial Cytokines in Giant Cell Myocarditis in Rats

— Pathological Comparison to Viral Myocarditis in Mice —

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To investigate the precise disease progression in myocarditis, Lewis rats were injected with porcine cardiac myosin, and C3H/He mice were inoculated with coxsackievirus B3. Both were killed serially, and the hearts were stained with hematoxylin-eosin to compare their pathological characteristics. In viral myocarditis, viral replication in the myocardium resulted in myocardial necrosis with inflammation, and the lesions were distributed transmurally, as previously reported. On the other hand, in giant cell myocarditis, inflammatory lesions appeared at first around the capillaries in the epicardium, and thereafter spread transmurally. Pericardial effusion was noticed in all the rats with myocarditis in the fulminant stage. Levels of interleukin (IL) -1β and IL-6 in the pericardial effusion were elevated compared with the serum cytokines at the peak of inflammation. However, interferon- γ in both the pericardial effusion and serum was not elevated. The cause of the myocardial lesions that developed in rats with giant cell myocarditis may be some active inflammatory process via the pericardial effusion. (*Jpn Circ J* 2000; 64: 977–981)

Key Words: Cytokines; Giant cell myocarditis; Pericardial effusion; Viral myocarditis

Infiltration of the myocardium with inflammatory cells occurs during infection with a variety of viruses. Usually, the infiltrate comprises mononuclear cells that are focally or diffusely scattered throughout the myocardium. Myofiber necrosis is an important feature of myocarditis. In addition, myocarditis is considered to be a cause of dilated cardiomyopathy. Immune or autoimmune mechanisms may be involved in the pathogenesis of this condition.^{1–3}

In viral myocarditis, the precise time course of the inflammatory lesions has already been reported,^{1,2} but there is another animal model of myocarditis available for analysis of the pathogenetic mechanisms responsible for immune or autoimmune reactions; immunization of cardiac myosin in rodents.^{4,5} Experimental giant cell myocarditis has been shown to be CD4⁺ T cell-mediated and strictly dependent on major histocompatibility complex (MHC) class II expression⁶ and this model is particularly suitable for studying the immunopathology of inflammatory heart disease.

In this study, we examined the time course of inflammatory lesions in viral, as well as in giant cell, myocarditis and discuss the pathological difference between these models. The studies were approved by the institution's Animal Care and Use Committee.

Methods

Viral Myocarditis

Virus The Nancy strain of coxsackievirus B3 (CB3) was used; the virus stock was prepared in cultures of VERO (kidney of African green monkey) cells in Eagle's minimum essential medium. Virus suspensions were centrifuged after the cytopathic effect had developed. Virus stock had a titer of more than 10^9 plaque-forming units (PFU)/ml determined in tissue cultures of VERO cells. Virus fluid was stored at -80°C until use.

Mice Inbred C3H/He mice had been maintained continuously by brother-sister matings. At 4–6 weeks of age, they were inoculated intraperitoneally with 0.1 ml of virus suspension containing 10^6 PFU/0.1 ml. The mice were observed daily, and were killed periodically on days 4 ($n=5$), 8 ($n=7$), 14 ($n=7$), 30 ($n=5$), and 90 ($n=3$). After gross inspection of the heart for alterations of myocardial appearance (ie, myocardial calcification, ascites or pleural effusion) the hearts and other organs were processed for histological or virological studies.

Fifteen mice were inoculated intraperitoneally with 0.1 ml of saline, and 3 mice each were killed on days 4, 8, 14, 30, and 90 (uninfected controls). The hearts were examined in the same manner as those of the infected mice.

Histopathology Hearts were fixed in 10% formalin solution, sectioned, embedded in paraffin, and stained with hematoxylin-eosin (H&E). The lungs, liver and other organs (thymus, spleen, pancreas, kidney and muscle) were also sectioned and stained with H&E. Myocardial lesions, including cellular infiltration, myocardial necrosis and fibrosis, were evaluated. The microscopic findings were graded: 0 (normal), 1 (mild myocarditis: only a few small

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Table 1 Serial Microscopic Gradings in Viral and Autoimmune Myocarditis

	Day				
	4 (n=5)	8 (n=7)	14 (n=7)	30 (n=5)	90 (n=3)
<i>Viral myocarditis in mice</i>					
<i>Infiltration</i>	2.2±0.21	2.4±0.24	1.9±0.34	1.2±0.20	0±0
<i>Necrosis</i>	1.6±0.25	2.6±0.24	2.6±0.20	1.8±0.38	0.3±0.36
<i>Fibrosis</i>	0±0	0±0	1.1±0.14	2.8±0.29	3.0±0
	Day				
	15 (n=4)	18 (n=4)	21 (n=4)	29 (n=3)	56 (n=3)
<i>Autoimmune myocarditis in rats</i>					
<i>Infiltration</i>	1.0±0	2.5±0.29	2.5±0.29	2.7±0.34	1.0±0
<i>Necrosis</i>	0±0	2.5±0.29	2.3±0.25	2.7±0.34	1.0±0
<i>Fibrosis</i>	0±0	0±0	0±0	1.3±0.34	2.7±0.34

Values are expressed as mean±SEM.

lesions per section), 2 (moderate myocarditis: multiple small lesions or a few moderate lesions), 3 (severe myocarditis: multiple moderate or large lesions). Because of the recognized propensity of C3H/He mice for spontaneous dystrophic calcification of the right ventricle, this condition was not included in the analysis. To avoid postmortem changes, the pathological study was performed only upon the killed mice.

Virological Study For infectivity assay, portions of hearts were removed aseptically, weighed, and homogenized in 2 ml phosphate-buffered saline. After centrifugation at 1,500rpm for 15 min, virus titers in the supernatants were determined by the plaque assay method, as previously described³

Giant Cell Myocarditis

Rats Six- to 7- week-old Lewis rats were purchased from Shimizu Laboratory Supplies Co, Ltd, Japan and maintained in our animal facilities.

Antigen Preparation and Immunization Autoimmune myocarditis was induced as previously described⁷ Porcine heart myosin (Sigma, M0531) was dissolved in phosphate-buffered saline at a concentration of 2 mg/ml. Rats were injected in their foot pads with 0.1 ml of myosin (1 mg/ml) mixed with an equal volume of Freund's complete adjuvant (FCA) supplemented with *Mycobacterium tuberculosis* H37Ra (Difco, 3113-60) on days 1 and 7. Littermate controls were injected with FCA alone.

Histopathology Rats were killed serially on days 15 (n=4), 18 (n=4), 21 (n=4), 29 (n=3), and 56 (n=3) under ether anesthesia. Control rats were killed on days 21 (n=1), 29 (n=1), and 56 (n=1). At death, macroscopic findings were obtained and pericardial effusion was examined. After macroscopic examination, the heart was removed from the pericardium, the ventricles were transversely sliced and stained with H&E and the same microscopic grading was performed as described before.

Inflammatory Cytokine Assay

Interleukin-1 β (IL-1 β , Cosmo Bio. KRC0010-SBO), IL-6 (R&D System RS-0476-00) and interferon- γ (IFN- γ , Cosmo Bio. KRC4020-SBO) in serum and pericardial effusion were assayed using commercially available kits.

Results

Viral Myocarditis in Mice (Table 1, Fig 1)

The precise disease progression has already been re-

ported;³ in brief, 4 days after CB3 inoculation, the mice appeared ill. Some developed coat ruffling, weakness and irritability. Grossly, the myocardium showed pale yellow patches that correlated with the inflammation, necrosis, and calcification seen microscopically. On day 4, necrotic foci with cellular infiltration and interstitial edema appeared in the myocardium. Thereafter, myocardial necrosis became more extensive, and cellular infiltration was most prominent on day 14. After day 8, dilatation of the ventricles with thrombi developed and fine endothelium appeared on the surface of the thrombi. On day 14, myocardial lesions were prominent in the subendocardial regions. On days 30 and 90, cellular infiltration was diminished, but myocardial calcification persisted. Myocardial fibrosis was evident in these periods. The semi-quantitative analysis showed that the degree of active myocardial lesions was maximal on days 8 and 14 (Table 1).

Viruses were isolated from the mice on days 4 and 8, but not from those on day 14.

None of the uninfected control mice developed myocardial lesions, except for spontaneous dystrophic calcification of the right ventricular wall.

Giant Cell Myocarditis in Rats (Table 1, Figs 2 and 3)

Macroscopic findings of immunized rats showed that there were no discolored areas on day 15; microscopically, there were cellular infiltrations around capillaries in the epicardium. On days 18–56, the hearts were enlarged and diffusely discolored. Microscopically, distinct inflammatory lesions around the epicardium of ventricular wall were noticed on day 18. The inflammatory lesions corresponded with macroscopic discolored areas, but not with the coronary perfusion areas. The inflammation consisted of numerous neutrophils, lymphocytes, macrophages, and fragments of degenerated myocardial fibers. On day 21, interstitial cellular infiltration and myocardial necrosis spread toward the endocardial ventricular wall. Multinucleated giant cells appeared in the center of the inflammatory lesions at this time. On day 29, inflammatory lesions were more extensive, and finally spread transmurally. However, interstitial cellular infiltration decreased, and disarrangement of myocardial fiber became apparent. On day 56, the inflammatory lesions almost disappeared. Pericardial effusion was observed in all the rats killed on days 18 and 21. The microscopic gradings for active inflammatory lesions were maximal on days 18 and 21.

None of the control rats showed myocarditis.

Fig 1. Typical histopathology of viral myocarditis in mice on day 14. (A) Extensive myocardial necrosis and cellular infiltration can be seen transmurally. Myocardial lesions are prominent in the subendocardial lesions. An arrowhead indicates the endocardial site of the left ventricle. (B) Thrombi developed in the left ventricular cavity. (C) Thrombi attached to the damaged endocardial site of the left ventricle. H&E; original magnification, $\times 58$ (A); $\times 10$ (B); $\times 200$ (C).

Fig 2. Typical histopathology of experimental giant cell myocarditis in rats on day 21. (A) The heart immunized with FCA. The myocardium is intact. (B) The heart immunized with myosin. Marked cellular infiltrations in the inflammatory focus are observed. Moreover, multinucleated giant cells are observed (arrows). H&E; original magnification, $\times 400$ (A, B).

Serum IL- 1β and IL-6 levels were constant throughout the course. IL- 1β and IL-6 in pericardial effusion were, however, elevated on day 21. IFN- γ was constant in the pericardial effusion and serum (Table 2).

Discussion

In the present study, we examined the time course of inflammatory lesions in both CH3/He mice with CB3 myocarditis and in Lewis rats with autoimmune myocarditis. As a result, a striking pathological difference was observed in

the fashion of the disease progression between the 2 animal models; that is, in viral myocarditis, viral replication in the myocardium resulted in myocardial necrosis with inflammation, and the distribution of lesions was mainly transmurally at any time. On the other hand, in giant cell myocarditis, the inflammatory response began with cellular infiltration around the capillaries in the epicardium on day 15. On days 18 and 21, inflammatory lesions spread transmurally. In addition, pericardial effusion appeared during this time. On days 29 and 56, active inflammation and pericardial effusion decreased. IL- 1β and IL-6 levels were elevated in the peri-

Fig3. Serial histopathology of giant cell myocarditis. Myocardial lesions began at the site of epicardium on day 15 (an arrow) and spread towards the endocardium with time on days 18 and 21. On days 29 and 56, transmural lesions developed. On day 56, myocardial fibrosis was noted. H&E stain. Original magnification $\times 18$.

Table 2 Cytokines in Autoimmune Myocarditis on Day 21

	Control rats*	Myocarditis rats	
	Blood (n=4)	Blood (n=4)	Pericardial effusion (n=4)
IL-1 β (pg/ml)	36.5 $\pm$ 4.5	35.3 $\pm$ 2.0	50.0 $\pm$ 5.9 [#]
IFN- γ (pg/ml)	1.0 $\pm$ 1.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
IL-6 (pg/ml)	135 $\pm$ 25	134 $\pm$ 25	3229 $\pm$ 165 [#]

Values are expressed as mean $\pm$ SEM. *The data were obtained from another set of experiments. [#] $p < 0.05$ vs values of blood in rats with myocarditis (by unpaired t test). [#] $p < 0.01$ vs values of blood in rats with myocarditis (by unpaired t test).

cardial effusion in parallel with the inflammatory process, but IFN- γ was not increased.

With respect to the anatomical aspects, a distinct pericardium is lacking in mice, so mice with viral myocarditis do not have pericardial effusion. With regard to the mechanism of the expansion of the inflammatory lesions in rats with giant cell myocarditis, we suppose that some inflammatory cytokines in the pericardial effusion may play a role in the development of the condition. This mode of expansion of inflammatory lesions might differ from that of viral myocarditis.¹⁻³

It has already been reported by Kodama et al that myocarditis is induced in Lewis rats by immunization with the cardiac myosin of other species, and that this model may mimic human giant cell myocarditis.⁵ Human giant cell myocarditis is often associated with various immunological disorders, such as myasthenia gravis, pernicious anemia, ulcerative colitis, malignant lymphoma, systemic lupus erythematosus, dermatomyositis, Sjögren syndrome, or thyroid disease. Therefore, it is presumed that autoimmune mechanisms are involved in the pathogenesis of giant cell myocarditis.

As far as disease susceptibility and resistance to myocarditis is concerned, several MHC genes are thought to be involved.⁷ It has been reported that B cell-deficient mice immunized with cardiac myosin developed myocarditis similarly to wild-type mice, and in that model the autoreactive T cells that caused myocarditis seemed to be activated by macrophages and dendritic cells.⁸ In murine models of coxsackievirus B3 myocarditis, the survival rate of TCR β^- knock out mice was higher compared with that of wild-type mice. TCR $\alpha\beta^+$ CD4⁻ CD8⁻ T cells, which provide MHC class II restricted help for CD8⁺ T cells and B cells, can participate in the immune response.⁹ It has recently been reported that human MHC class II molecules (HLA DQ6) and human α -myosin heavy chain derived peptides can trigger autoimmune heart disease.¹⁰

Apart from the immunological mechanisms, the role of inflammatory cytokines is thought to be that of active candidates for the disease pathogenesis.^{11,12} From this study, we consider that in rat model the inflammatory lesions began at the site of epicardium and spread gradually towards the endocardium, and that some cytokines in pericardial effusion played an important role in the development of the disease. Indeed, IL-1 β and IL-6 tended to be elevated in the pericardial effusion, while serum cytokines were almost constant.

In the clinical setting, it now appears reasonable from the results of present study that a negative endomyocardial biopsy can be obtained from some patients in whom active myocarditis was supposed to be diagnosed.

In conclusion, a different disease progression related to myocardial characteristics was demonstrated in different models of myocarditis.

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