Pathogenesis of Myocardial Injury in Myocarditis and Cardiomyopathy

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The pathogenesis of myocardial cell injury in myocarditis and cardiomyopathy was investigated. The presence of viral genomes in the murine heart in experimental coxsackievirus B3 myocarditis was studied by Northern blotting analysis using a $^{32}$P-labeled cDNA probe from the 5' end sequence. The strongest signal of positive autoradiograms was always at about 7.4 kilobases, corresponding to the size of the complete genome of the virus. Successive infections with coxsackievirus and encephalomyocarditis (EMC) virus infection showed simultaneous acute myocarditis and healed myocarditis, and the results suggest that successive virus infections cause additional myocardial damage, and develop lesions similar to chronic myocarditis or dilated cardiomyopathy. Anti-heart auto-antibody, induced during EMC virus infection, reacted predominantly with myosin. Indium-111 antimonyosin scintigraphy showed positive in some of the patients with cardiomyopathy, and the uptake was inversely correlated with left ventricular function. When mice were injected with antimonyosin antibody, mouse immunoglobulin G was detected in hearts in the chronic stage of EMC virus myocarditis, in myocytes surrounding fibrosis and calcification, suggesting deposition of antimonyosin antibody. Although further study is necessary to clarify the mechanism of uptake of antimonyosin in cardiomyopathy, antimonyosin antibody may accumulate in viable myocytes with ongoing degeneration as well as in necrosis.

I. A Northern Blotting Analysis of the Viral Genome in Murine Coxsackievirus B3 Myocarditis

Enteroviruses are thought to be etiologic agents in some cases of human myocarditis and dilated cardiomyopathy. Murine models of acute coxsackievirus B3 myocarditis implicate coxsackie B viruses as possible causes of human myocarditis. Indirect evidence implicating enteroviruses as causative agents in human heart disease is derived from serologic studies! More recently, direct evidence for enteroviral presence in diseased human heart tissues has been obtained by nucleic acid hybridization analyses.

Although the data suggest that enteroviral infections may be associated with 18% to 50% of cases of myocarditis or dilated cardiomyopathy, or both, causality has not been established. Recently, to evaluate the etiologic significance of enteroviruses in myocarditis and dilated cardiomyopathies, several investigators have assayed for viral genomes in hearts using nucleic acid probes. Bowles et al. claimed positive hybridization signals in 9 of 17 biopsy samples from patients with his-
Fig. 1. Autoradiogram of Northern blot of total RNA from the hearts of mice inoculated with coxsackievirus B3. Lane 1, day 3; lane 2, day 4; lane 3, day 5; Lane 4, day 6; lanes 5 and 6, day 7; lane 7, day 10.

tologic evidence of active or healing myocarditis or dilated cardiomyopathy with inflammatory changes, and in 6 of 17 explanted hearts from patients with end-stage dilated cardiomyopathy in the absence of a continuing cell-mediated or humoral immune response. Using the persistent infection of coxsackievirus B3 in myocardium of athymic mice as a model, another group showed the presence of the viral genome in the myocardium of immunodeficient mice until 56 days after inoculation of virus, and suggested that the presence of the viral genome might possibly be related to the pathogenesis of chronic dilated cardiomyopathy. In situ hybridization studies showed persistence of enteroviral RNA in human postmortem cardiac tissues and human biopsy samples. In situ hybridization has also been investigated on the natural course of encephalomyocarditis virus infection in mice. We have used Northern blot hybridization to detect coxsackievirus B3 in the experimentally infected murine heart, by both nucleic acid identity with the molecular probe and size of the viral infection. Four-week-old C3H/He mice were inoculated intraperitoneally with coxsackievirus B3 (Nancy strain). Four weeks after the first infection, mice were secondarily inoculated with encephalomyocarditis (EMC) virus. All surviving mice were sacrificed two weeks after the second infection, at the age of 10 weeks. Histopathology of the hearts of mice inoculated with coxsackievirus B3 alone showed myocardial calcification and fibrosis, but little cellular infiltration. There were cellular infiltration and necrosis, but little fibrosis, in the hearts of mice inoculated with EMC virus alone. In mice infected with both coxsackievirus B3 and EMC virus, acute myocarditis, with cellular infiltration and heart RNA were positive for viral RNA until day 7, and negative after day 10 and in control hearts. Positive autoradiograms always had the strongest signal at about 7.4 kilobases, corresponding to the size of the complete genome of the virus. The viral genomes were detected earlier than the appearance of the histopathologic changes in the murine heart (Fig. 1). We followed the course of coxsackievirus B3 infection in mice to correlate the presence of infectious virus in the heart with histopathologic changes induced by viral infection. Although inflammatory changes in the heart were present until 31 days after virus infection, neither infectious virus nor viral genomes were detectable by plaque assay or Northern blot analysis. This type of analysis of the viral genome in murine myocardium may be useful in evaluating the effects of specific drugs on experimental viral myocarditis at the level of the viral genome. Application of molecular approaches such as enzymatic amplification will help provide satisfactory answers to critical questions regarding enteroviral persistence in the heart, enteroviral identity, and the interactions of enteroviruses and immunosuppressive agents with the host.

II. An Animal Model of Chronic Myocarditis: Successive Infections with Coxsackievirus and Encephalomyocarditis Virus in Mice

Successive viral infections were investigated to see whether they may cause additional myocardial damage and develop lesions similar to chronic myocarditis or dilated cardiomyopathy. Four-week-old C3H/He mice were inoculated intraperitoneally with coxsackievirus B3 (Nancy strain). Four weeks after the first infection, mice were secondarily inoculated with encephalomyocarditis (EMC) virus. All surviving mice were sacrificed two weeks after the second infection, at the age of 10 weeks. Histopathology of the hearts of mice inoculated with coxsackievirus B3 alone showed myocardial calcification and fibrosis, but little cellular infiltration. There were cellular infiltration and necrosis, but little fibrosis, in the hearts of mice inoculated with EMC virus alone. In mice infected with both coxsackievirus B3 and EMC virus, acute myocarditis, with cellular infiltration and
Fig. 2. C3H/He mouse. Four weeks after the first infection with coxsackievirus B3, mice were inoculated with EMC virus and sacrificed two weeks later. Acute myocarditis, with cellular infiltration and myocardial necrosis, and healed myocarditis, with fibrosis and calcification, were seen simultaneously. Hematoxylin-eosin stain. X240

Fig. 3. Western blot of murine heart homogenate. Antimyosin monoclonal antibody (R11D10) and sera of mice with myocarditis (MC day 21) react with the same band.

Myocardial necrosis, and healed myocarditis, with myocardial fibrosis and calcification, were seen simultaneously (Fig. 2). The results suggest that successive virus infections cause additional myocardial damage and develop lesions similar to chronic myocarditis or dilated cardiomyopathy.

Fig. 4. Western blot of purified rabbit myosin. Anti-myosin monoclonal antibody (R11D10) and sera of mice with myocarditis (MC day 24) react with the same band. Only IgG was positive.

Fig. 5. The heart of a mouse with myocarditis a month after EMC virus inoculation. Twenty-four hours after intravenous injection of anti-myosin antibody, IgG was seen in myocytes surrounding fibrosis and calcification suggesting deposition of anti-myosin antibody. X 400.

III. Anti-myosin Autoantibody in Viral Myocarditis

Anti-heart autoantibodies were found in the sera from mice infected with EMC virus. Frozen sections of the hearts of uninfected mice were incubated with mouse sera and stained with anti-mouse IgG. Positive granular staining was first seen on day 4 and persisted to day 90; the titer was highest on day 21. The demonstration of anti-heart antibody in this model of viral myocarditis suggests a pathogenetic role in the previously demonstrated later cardiomyopathy. These sera were used to identify the target myocardial autoantigens. Sera were tested first against solubilized extracts from whole heart. Results from Western immunoblotting analyses demonstrated that antibodies to...
myosin were a pronounced feature in the sera of mice with EMC virus myocarditis (Fig. 3). The immunopathic sera were subsequently analyzed by Western blotting using purified myosin and were confirmed to react with purified myosin (Fig. 4). These studies suggest that myosin is one of the major auto-

antigens in EMC virus myocarditis.

IV. Prognostic Significance of Indium-111 Antimyosin Antibody Uptake in Patients with Myocarditis and Cardiomyopathy

Indium-111 antimyosin specifically binds to exposed cardiac myosin and has been successfully used to precisely delineate myocardial damage in patients with myocardial infarct. The usefulness of such an antibody to detect myocardial cell damage in other pathologic entities such as myocarditis and cardiac rejection has been suggested. Recently, a high prevalence of antimyosin uptake has been reported in patients with diluted cardiomyopathy, but correlation between positive scans and clinical features has not been studied. We investigated the prognostic significance of myocardial uptake of indium-111 antimyosin in patients with diluted cardiomyopathy and hypertrophic cardiomyopathy. The prognostic significance of myocardial uptake of indium-111 antimyosin was evaluated in 17 patients with idiopathic cardiomyopathy: 10 patients with dilated cardiomyopathy and 7 patients with hypertrophic cardiomyopathy. Seven of 10 patients with dilated cardiomyopathy showed positive images. Three of these 7 patients with strongly positive scans died within 3 months after scintigraphic examination. Six of 7 patients with hypertrophic cardiomyopathy showed positive images. Three of the patients with dilated left ventricle had pronounced positive scans and higher heart-to-lung ratios. The heart-to-lung ratio of antimyosin uptake was correlated in all patients with left ventricular end-diastolic dimension and ejection fraction measured by echocardiography. All three patients with myocarditis showed positive scintigrams within 4 weeks after the onset of the disease, and 1 of 6 patients was positive thereafter, and had a dilated ventricle and decreased cardiac function. Thus, indium-111 antimyosin antibody imaging may be useful to evaluate the prognosis of patients with cardiomyopathy and myocarditis.

V. Immunohistochemical Detection of Antimyosin Antibody in Viral Myocarditis in Mice

Immunoperoxidase staining was performed using a biotin-avidin peroxidase technique. DBA/2 mice were injected intravenously with 6 µg of non-labeled antimyosin Fab a month after EMC virus inoculation, and were sacrificed 24 h after the injection. Sections 4.0—6.0 µm thick from formalin-fixed paraffin embedded tissue blocks were cut and mounted on slides coated with 0.1% albumin. Slides were depara-
fined, and endogenous peroxidase activity was blocked by immersion of slides in a methanol-0.3% hydrogen peroxide bath for 30 min. Goat anti-mouse γ chain-specific antiserum labeled with biotin (1:40) was added and incubated for 30 min at room temperature in a humid atmosphere. The slides were washed with PBS and then avidin-peroxidase was added for 30 min. The slides were then incubated with diamino-
benzidine (DBA)/0.1% H₂O₂ in the dark. After washing with distilled water, the sec-
tions were counter-stained with methyl-green and rinsed in tap water. Slides were dehy-
drated, coverslipped, and examined by light microscopy. Mouse immunoglobulin G was detected in hearts in the chronic stage of EMC virus myocarditis, in myocytes sur-
rrounding fibrosis and calcification, suggest-
ing deposition of antimyosin antibody (Fig. 5).

Although further study is necessary to clarify the mechanism of uptake of anti-
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body might accumulate in viable myocytes with ongoing degeneration as well as in ne-
crosis.

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