Postcoxsackievirus B_3 Myocardiopathy in Mice[1] (37588)

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Group B coxsackieviruses have been implicated as the etiologic agents of acute carditis in man. Until recently, the disease in adults was thought to involve primarily the pericardium and to be self limited; a somewhat different picture has now emerged. Myocarditis as well as pericarditis is frequently encountered (1), and Burch and his colleagues have demonstrated endocardial involvement in both man (2, 3) and experimental animals (4, 5). Recurrent bouts of pericarditis may follow the acute episode, and chronic, even constrictive pericarditis has been reported (6, 7). There is little evidence at present to associate coxsackievirus infection with chronic idiopathic myocardiopathy, but the possibility is intriguing. For example, Burch *et al.* have recently reported the case of a middle aged man who had acute then chronic, progressive carditis resulting in his death about 1 yr after the onset of symptoms (3). The patient had serologic evidence of coxsackievirus B_4 infection and at autopsy, coxsackievirus B_4 antigens were demonstrated in his myocardium by immunofluorescence techniques.

A mouse model has been used by several investigators to study coxsackievirus heart disease, and the development of acute myocarditis is well documented in this species. Rytel and Kilbourne showed that even though coxsackievirus B_3 titers declined or became undetectable in the hearts of infected mice, the areas of inflammation persisted (and even progressed) during the 168 hr of observation (8). Wilson *et al.*, interested specifically in the possibility that chronic carditis may follow the acute infection with coxsackievirus B_3 in mice, reported that even though virus could not be detected beyond 9 days, cardiac inflammation continued for 6 mo (9). They concluded that at least some forms of idiopathic myocardiopathy in man may represent a sequel to clinical or subclinical coxsackievirus carditis. They further speculated that the continuance of inflammation long after the termination of acute infection may be the result of an immune response initiated by the persistance of virus antigen or by the development of cardiac neoantigens.

In the present study we have sought to confirm the findings of Wilson *et al.*, and to explore some of the possible pathogenetic mechanisms involved.

Materials and Methods. Animals and viruses. Random bred male swiss mice (Hormone Assay, Chicago, IL) were employed. They were 17 days old (weanling) at the time of infection. Coxsackievirus B_3 (Nancy strain) was passaged once in mouse heart. Each mouse was infected ip with 3×10^4 $TCID_{50}$ of the virus. The titer was determined in HEp-2 cells employing techniques described previously (8, 10). Uninfected cohorts were maintained in isolated cages as controls. There were 30 mice in the infected and 15 in the control groups.

Cardiac and viral antigens. Whole hearts were obtained from normal mice and mice with acute coxsackie myocarditis. Crude cardiac antigen was prepared from pooled hearts from several animals in each group. The hearts were first trimmed of fat and

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excess connective tissue, then minced with sterile iris scissors and homogenized with Teflon tissue grinders. The homogenate was then lyophilized and pulverized with mortar and pestle. The powder was suspended in phosphate buffered saline at pH 7.4, aggitated for 4 hr and left to sediment at 4° overnight. The supernate was decanted and used as the antigen. The protein concentration was determined by the micro-Kjeldahl method. The antigen preparation was used at a concentration of 300 μg/ml.

Inactivated viral antigens were prepared by exposing 10^4 $TCID_{50}$ of coxsackievirus B_3 prepared in HEp-2 cells to ultraviolet irradiation with a Germicidal lamp (GE G85) for 15 min at a distance of 6 in., with constant stirring.

Virology. Individual mouse hearts were screened for virus at a 10^{-1} dilution of a 10% w/v suspension of homogenized washed whole myocardium. When positive, viral titers were determined by serial 10-fold dilutions of myocardial suspensions in HEp-2 cells.

Serology. Viral neutralization was performed using HEp-2 tube cultures inoculated with 3×10^2 $TCID_{50}$ of coxsackievirus B_3, and serial 2-fold dilutions of mouse sera pooled for each experimental group.

Macrophage migration inhibition test. The macrophage migration inhibition test of George and Vaughan (11) was adapted to the mouse as previously described by us (12). Briefly, peritoneal macrophages were stimulated by injecting 3 ml of sterile mineral oil (Marcol 52, Esso) into the peritoneal cavity of each mouse. Two days later the exudate was harvested by washing the peritoneal cavity with 10 ml of cold Hanks' balanced salt solution (BSS) containing 10 units/ml heparin. The fluid was removed by exposing the peritoneum and aspirating its contents under direct vision with a syringe and 18 gauge needle. The cells were washed 3 times in cold Hanks' BSS without heparin, and finally suspended in MEM containing 15% fetal calf serum (FCS) (both from Microbiological Associates, Bethesda, MD). Small nonheparinized capillary tubes (Drummond Microcaps) (0.7 mm i.d.) were filled with suspension, sealed at one end with modeling clay and centrifuged lightly for 5 min. The tubes were broken off at the cell–fluid interface and mounted in Mackenass-type chambers in a dab of silicone grease. MEM + 15% FCS containing the antigen being tested was used to fill the chambers. Three chambers each containing five tubes were employed per experimental group for each antigen. The chambers were incubated at 37° for 24 hr at which time the migration from each capillary tube was measured. The image of the migration was projected through a microscope and traced on paper. The outline was measured by a planimeter. Results are expressed as migration percentage calculated as follows:

$$\text{migration } \% = \frac{\text{av area of migration of cells with antigen} \times 100}{\text{av area of migration of cells without antigen}}$$

Immunofluorescence methods. All hearts were stored at —80° until ready for study. Four micrometer frozen sections were cut on a cryostat. Sections were examined with a Zeiss phase-fluorescence microscope using an HBO 200 mercury bulb. For detection of coxsackievirus B_3 antigen, unlabeled rabbit derived specific antiserum (Microbiological Associates, Bethesda, MD), was used with 1 hr incubation. This was followed by staining with fluorescein labeled goat anti-rabbit serum (Hyland Laboratories, Costa Mesa, CA). Control sections were processed according to the second step only. For detection of mouse gamma globulin the direct (one-step) method was used with 30 min incubation. Labeled goat anti-mouse gamma globulin was supplied by Hyland Laboratories. Control sections were not used in the latter determinations since it was early recognized that specific fluorescence was confined to lesions recognizable by phase contrast. No technical difficulties were experienced except in efforts to demonstrate coxsackievirus B_3 antigen in chronically infected hearts. Here granular intracellular autofluorescence was seen in some cardiac fibers, but it was easily distinguished from specific fluorescence by its yellowish-gold color.

Results. Induction of carditis in mice. Mice were infected as described under Materials

and Methods. Virus was isolated from the hearts of infected mice killed 5 days after inoculation with an average titer of $10^{5.5}$ $TCID_{50}/g$ of cardiac tissue. Hearts obtained at 30 days or 6 mo after infection were uniformly negative for virus by isolation. Serum neutralizing antibody was less than 1:4 at 5 days but rose to 1:16 at 30 days and again was less than 1:4 at 6 mo after infection. These relatively low titers of neutralizing antibodies are in agreement with our previous observations (8). Neither virus nor antibody was detected in control mice at any time.

The hearts of infected and control mice were examined in the acute (5 days), convalescent (30 days) and chronic (6 mo) stages. The lesions during the acute stage, were similar to those reported previously (8, 13). They included gross epicardial lesions, microscopically extensive necrosis of the myofibrils, infiltration of acute inflammatory cells, and early mineralization. At 30 days most of the lesions showed healing by fibrosis as well as progressive calcification. Finally, 6 mo after infection (Fig. 1) there was still seen extensive myofibril necrosis, fibrosis and calcification. Furthermore, foci of chronic inflammation could be easily discerned (Fig. 2). Such inflammatory foci were scattered throughout the myocardium of all the mice studied in this chronic stage, with a frequency of about one per section under low power of magnification.

Immunologic studies. Immunofluorescent microscopy techniques were used to look for coxsackievirus B_3 antigens in cardiac tissues. Five days after infection specific viral antigen was easily detected by indirect immunofluorescence (Fig. 3). Heart sections from control animals and sections stained without previous exposure to coxsackievirus B_3 antibody were consistently negative. At 6 mo after infection, no coxsackievirus antigen could be detected. Deposits of IgG were found associated with abnormal areas in the hearts from chronically infected mice. However, speck-like deposits of IgG could be detected in the hearts of control mice as well.

To further delineate possible pathogenetic mechanisms experiments were performed using the macrophage migration inhibition

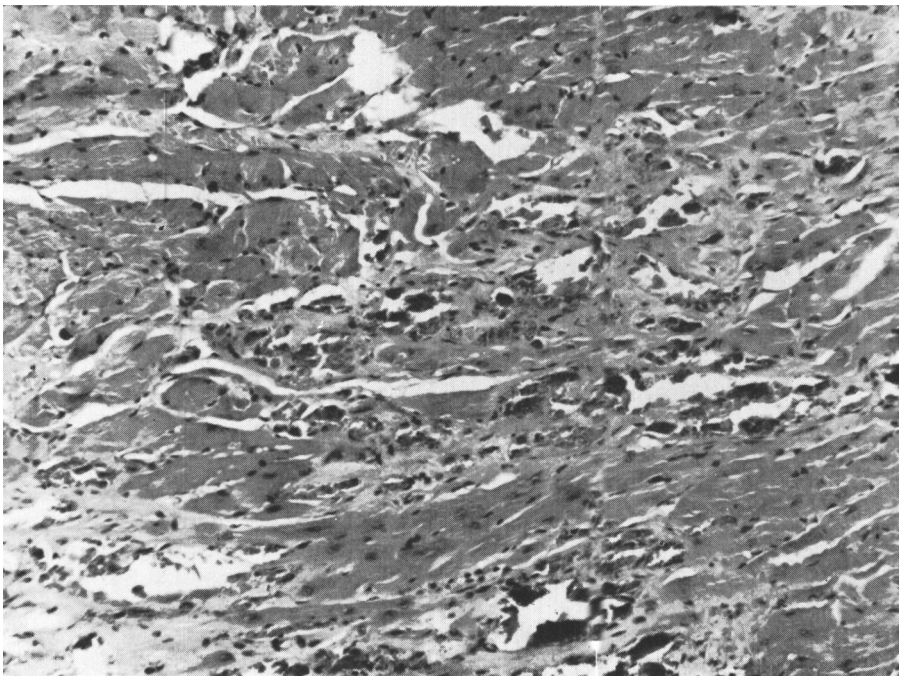

FIG. 1. Myocardium of coxsackievirus B_3 infected mouse at 6 mo showing severe calcification and fibrosis (H and E stain, ×100).

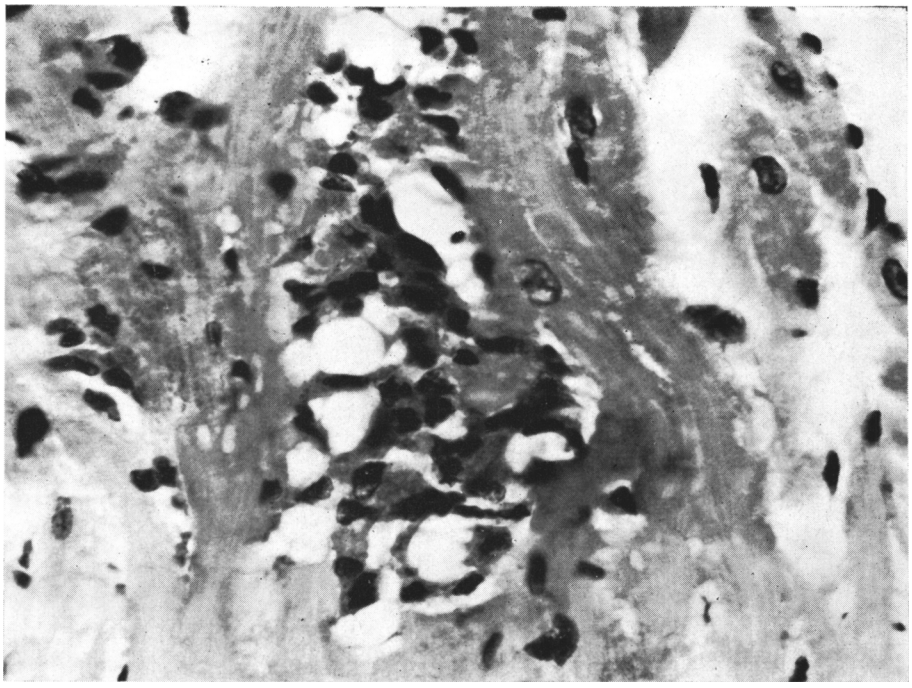

FIG. 2. Myocardium 6 mo after infection. Central area shows focal chronic inflammation with mononuclear cells filling space left by fiber fall out. Fibers to left show fine, granular calcification within cells (H and E stain, ×400).

method to study the delayed immune response of mice to coxsackievirus and to crude cardiac antigens. Peritoneal exudate cells were obtained from mice infected 30 days previously and in whom myocardial inflammation was demonstrated by histologic examination. The control group was comprised of uninfected mice. The peritoneal cells were exposed to cardiac antigens prepared from uninfected hearts, infected hearts and inactivated coxsackievirus B_3 antigens (see Materials and Methods). The results are summarized in Table I. There was no specific inhibition of macrophage migration indicating absence of delayed hypersensitivity to the antigens employed, at least as measured by this method. Comparison of the difference of the mean areas of migration between different categories using an extension of Student's t test showed no significant change ($p > 0.50$ in each case).

Discussion. We have confirmed the finding of Wilson *et al.* that chronic myocardiopathy follows acute coxsackievirus carditis in mice (9). The histologic findings characterizing this chronic stage consisting of myofibril necrosis, fibrosis, calcification and inflammation are similar to the changes seen in some forms of idiopathic myocardiopathy in man (3, 14). The mechanisms leading to this condition remain unknown. We could find no evidence that delayed hypersensitivity to viral or cardiac antigens as measured by macrophage migration inhibition method plays a pathogenetic role at least in the mouse. This possibility is not entirely ruled out, however. The antigens employed by us were crude saline extracts of homogenized infected and uninfected hearts. This was done because at the time these experiments were performed, cardiac antigens had been poorly defined, and there was no information as to which ones (if any) played a role in induction of autoimmunity. Espinosa and Kaplan have reported since that it is possible to separate cardiac tissue into many different antigens by acid extraction (15). Conceivably had we used more purified antigens in the macrophage migration inhibition studies, a measure of autoimmunity to some of them could have been demonstrated.

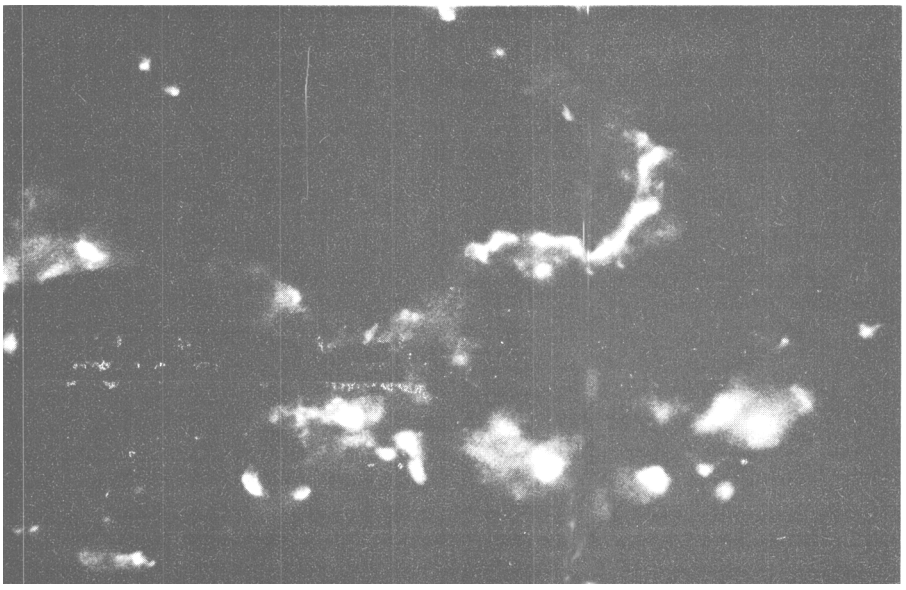

FIG. 3. Myocardium on fifth day of infection showing specific fluorescence in a lesion stained with rabbit anti-coxsackievirus B_3 antibodies and fluorecein conjugated goat anti-rabbit serum (×400).

No persistence of coxsackievirus antigens could be demonstrated in the myocardial sections from mice with myocardiopathy weighing against a latent or "slow" viral infection, and also against this being an antigen–antibody complex type of disease. No attempt was made to look for circulating antiheart antibodies, because it has been shown by Kaplan (16) and by Davies and Gery (17) that it is impossible to produce myocardial disease in animals by experimentally induced antiheart antibodies. We conclude therefore that it is likely that myocardiopathy in the mouse represents a sequel to replacement fi-

TABLE I. Determination of Inhibition of Migration of Peritoneal Macrophages from Coxsackievirus B_3 Infected and Control Mice.

Experimental group	Challenge antigen			
	None	Normal heart[c]	Infected heart[c]	Inactivated Coxsackievirus B_3[d]
Control				
$\bar{M}$ area[a]	992 (SE = 58)	929 (SE = 39)	814 (SE = 48)	712 (SE = 39)
% Mig.[b]	100	94	79	72
Infected				
$\bar{M}$ area	1163 (SE = 69)	1136 (SE = 51)	1027 (SE = 63)	827 (SE = 46)
% Mig.	100	97	88	65

[a] Average area of migration; SE = standard error.
[b] Migration percent (see Materials and Methods).
[c] 300 μg of protein/ml.
[d] 10^4 $TCID_{50}$/ml, inactivated by uv irradiation.

brosis which accompanies the healing of the acute cytolytic phase of viral infection.

It is interesting that even though histologic examination revealed extensive myocardial destruction, there was no mortality in the infected mice during the 6 mo period of observation. Such a situation corresponds to the clinical syndrome in man where at least symptomatic recovery from the acute viral infection is the rule (1). Why some patients experience no long-range ill effects and others may go on to develop myocardiopathy (3) is unknown.

Summary. "Idiopathic myocardiopathy" in man may represent a sequel to an antecedent coxsackievirus infection. To test this hypothesis and to elucidate the possible pathogenetic mechanisms involved, the following studies were performed. Weanling mice were inoculated ip with 3×10^4 $TCID_{50}$ of coxsackievirus B_3 (Nancy). Five days later myocarditis could be demonstrated by viral isolation from the heart and by the presence of acute inflammation, necrosis and early mineralization. Thirty days after infection when the virus could no longer be recovered, the inflammatory response continued although there was evidence of simultaneous healing. Six months later there was extensive fibrosis and calcification, but scattered foci of chronic inflammation persisted. Delayed hypersensitivity to cardiac and viral antigens was studied in these mice by the macrophage migration inhibition method. Thirty days after infection no delayed hypersensitivity could be demonstrated to antigens prepared from a crude saline extract of normal mouse myocardium, from the myocardium of mice with acute coxsackievirus carditis, or to inactivated coxsackievirus B_3. Coxsackievirus B_3 antigens were found in the myocardium of acutely infected animals by the indirect fluorescent antibody technique. However, no such antigens were detectable 6 mo after infection in animals with chronic inflammation. Thus, there is no evidence that delayed hypersensitivity to either viral or cardiac antigens or that persistence of viral antigens in the myocardium play a role in the pathogenesis of the postviral chronic myocarditis of mice. It is more likely that this myocardiopathy in the mouse represents a sequel to replacement fibrosis which accompanies healing of the acute cytolytic phase of viral infection.

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