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An Experimental Model for Dilated Cardiomyopathy after Rabbit Coronavirus Infection

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A rabbit model for coronavirus-induced dilated cardiomyopathy is described. Acute rabbit coronavirus infection results in virus-induced myocarditis and congestive heart failure. Of the survivors of rabbit coronavirus infection, 41% had increased heart weight and heart weight-to-body weight ratios, biventricular dilation, myocyte hypertrophy, myocardial fibrosis, and myocarditis consistent with the development of dilated cardiomyopathy. These changes were also seen in the remaining 59% of the survivors, except that the degree of myocyte hypertrophy was reduced and only right ventricular dilation was present. In most survivors, myocarditis was usually mild (1–5 foci/transverse section), but in some cases it was severe (>20 foci/transverse section). Interstitial and replacement fibrosis was more pronounced in the papillary muscles. These data suggest that rabbit coronavirus infection may progress to dilated cardiomyopathy.

Dilated cardiomyopathy (DCM) is a debilitating chronic illness with an incidence of ~0.73–7.5 cases/100,000 population/year in developed countries [1]. In developing countries, this number is probably significantly higher [1]. DCM is characterized by a grossly enlarged heart, ventricular dilation, and low ejection fractions that can frequently result in the formation of ventricular thrombi. Histologic examination reveals myocyte hypertrophy, fibrosis, and occasionally myocarditis [2, 3]. The 2- to 3-year survival rate of a patient with DCM is ~50% [4], with death frequently occurring from chronic congestive heart failure (CHF) and to a lesser extent from ventricular arrhythmias or pulmonary or peripheral emboli [2, 3].

The etiologic basis for DCM is unclear, but it may represent the end product of a previous injury to the heart muscle. Common risk factors associated with DCM include alcohol abuse, pregnancy, hypertension, and malnutrition [3, 5]. Viral infections of the heart muscle also have been suggested as an important initiating event in the development of DCM. Most of the evidence linking viral myocarditis to DCM has been obtained from retrospective serologic studies in humans [6–8], the identification of enteroviral RNA sequences in patients with myocarditis and DCM [9–13], and animal studies [14–16].

The most direct evidence suggesting that viral myocarditis may lead to the development of DCM has been demon-

strated after coxsackievirus B or encephalomyocarditis virus infection in mice. In these models, viral infection results in myocarditis and CHF, with a significant percentage of survivors developing DCM at a later stage in life [14–16].

A rabbit model for coronavirus-induced heart disease has been described [17, 18]. Infection with rabbit coronavirus (RbCV) resulted in virus-induced myocarditis and CHF with a mortality rate of ~60%. Morphologic and pathologic evidence indicates that a significant percentage of these animals were dying from heart failure [17]. We determined whether survivors of RbCV infection would develop DCM.

Materials and Methods

Animals and virus. Male New Zealand White rabbits weighing 2.5–3.0 kg were purchased from commercial suppliers (Franklin Rabbitry, Wake Forest, NC, or Robinson Services, Winston-Salem, NC). The animals were housed individually at room temperature (21°C) and given rabbit diet (Agway; Grandville Milling, Creedmoor, NC) and water ad libitum. RbCV stocks were obtained from moribund animals when peak titers were present at 4 days after infection [17]. Virus stocks were diluted to 10^3 – 10^4 rabbit ID₅₀ per milliliter and stored at –140°C. Animals were inoculated either intravenously via the marginal ear vein or occasionally intramuscularly in the thigh muscle with 0.2 mL of the 10^3 – 10^4 RID₅₀ virus stock. No differences were observed in day of death, clinical signs of infection, or histologic findings between the routes of inoculation. Animals were observed daily for signs of infection, which included weight loss, dullness of the sclera, hyphema, and severe congestion of the conjunctivae and irides.

For virus isolation from the heart muscle, 7 survivors at 30–111 days after infection were sacrificed by intravenous injection of 50 mg/kg sodium pentobarbital. The hearts were removed and flushed extensively or perfused with PBS, pH 7.0. The apex of the ventricles was removed, snap frozen in liquid nitrogen, then stored at –140°C until assayed. One gram of tissue was minced and homogenized on ice in 4 mL of PBS using a manual

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tissue grinder. Large pieces of tissue were removed by centrifugation at 12,000 g for 10 min in an Eppendorf centrifuge (Fisher Scientific, Norcross, GA) at 4°C. Rabbits were inoculated with 0.5 mL of the undiluted heart homogenate supernatant, then observed daily for 14 days for signs of viral infection. After 21 days, rabbits were challenged with 0.2 mL of stock serum at 10^3 – 10^4 RID₅₀/mL and observed daily for 14 days for signs of infection.

Pathology. Survivors of RbCV infection and uninfected control animals were sacrificed as previously described. Body weights were obtained to the nearest 0.10 kg. The heart was removed, separated from the pericardial sac, and flushed with PBS. The heart was weighed to the nearest 0.1 g; then the chambers were filled and fixed with 10% phosphate-buffered formalin. The heart was sectioned transversely at the widest dimensions of the ventricles. Four paraffin-embedded 6- μ m sections were cut at 150- μ m intervals and stained with hematoxylin-eosin. Additional heart sections were also stained with von Kossa's method or Masson trichrome stain. Sections of lung, liver, and spleen were also removed, fixed in 10% phosphate-buffered formalin, and stained with hematoxylin-eosin.

Morphometric studies. To determine the degree of myocyte hypertrophy, myocytes in the right and left ventricles and the interventricular septum were measured by using a software morphometry system (Image Measure; Phoenix Technology, Federal Way, WA) with an image processor (FG-100-AT; Imaging Technology, Woburn, MA). The myocardial fiber diameter was measured through the nucleus of 30–40 transverse sectioned fibers in the right ventricle, left ventricle, and interventricular septum. Myocardial fibers were measured in two consecutive cardiac transverse sections, and the mean and SD were calculated for each animal. Each cardiac section was measured independently by two of us (L.K.A., R.S.B.), and no significant differences in measuring were found.

To determine the dimensions of the cardiac walls and cavities and to assess the area within each ventricular cavity, the same software morphometry system and image processor were used. The wall thickness of the right and left ventricles and the thickness of the interventricular septum were determined by taking 15–20 measurements of each at regular intervals across the ventricular walls and septum. All measurements were statistically analyzed and presented as mean \pm SD. A one-way analysis of variance was used to evaluate the statistical significance of cardiac measurements. To examine which specific differences were significant, a post hoc contrast was used to compare group means [19].

Results

Mortality and course of RbCV infection. Seventy-nine New Zealand White rabbits were inoculated with 0.2 mL of a 10^3 – 10^4 RID₅₀/mL stock of RbCV. Consistent with earlier studies [17, 18], animals died at 2–12 days, with an overall mortality of ~60%. Twenty-four (30%) died in the acute phase with enlarged hearts, right ventricular dilation, pleural effusion, and pulmonary edema. Twenty-four (30%) died in the subacute phase with various degrees of pleural effusion,

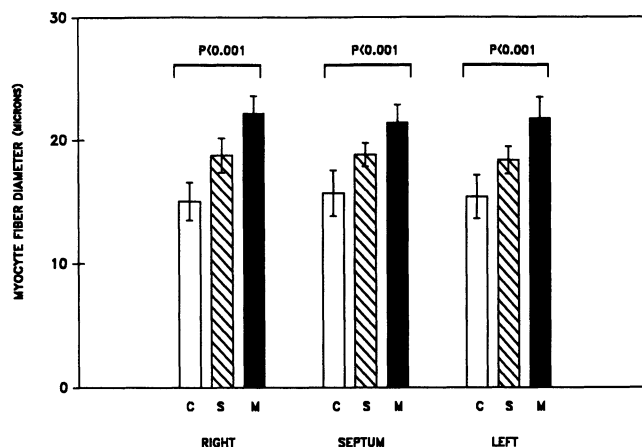


Figure 1. Myocardial fiber diameters in right ventricle, interventricular septum, and left ventricle of survivors of rabbit coronavirus infection compared with uninfected controls (C) sacrificed on similar days. Survivors had slight (S) or moderate (M) myocyte hypertrophy [20]. Measurements are mean \pm SD and were evaluated by analysis of variance.

pulmonary edema, enlarged hearts, gross biventricular dilation, and ascites, consistent with death due to CHF. Of the 79 animals, 40% survived and exhibited various degrees of ventricular dilation, myocarditis, interstitial and replacement myocardial fibrosis, and myocyte hypertrophy.

Myocardial fiber diameters. Myocardial hypertrophy is one of the hallmarks of DCM [2, 3, 15]. The cardiac muscle cells of most survivors exhibited signs of both nuclear and cellular hypertrophy. In humans with DCM, a classification system based on myocyte fiber diameter has been devised [20]. By this system, animals with mean myocyte diameters of 16–20 μ m would have slight myocardial hypertrophy, of 21–25 μ m would have moderate myocardial hypertrophy, and of >26 μ m would have severe myocardial hypertrophy. To document conclusively the presence of myocyte hypertrophy, the mean myocyte fiber diameter was determined in the hearts of 22 RbCV-infected survivors and 9 controls at 30–111 days after infection.

Thirteen animals (59% of the survivors) had evidence of slight myocardial fiber hypertrophy. The mean myocardial fiber diameters were 18.8 ± 1.4 , 18.8 ± 1.0 , and 18.4 ± 1.0 μ m in the right ventricle, interventricular septum, and left ventricle, respectively. Moderate myocardial fiber hypertrophy was present in 9 (41%) of the survivors. In these animals, the mean myocardial fiber diameters in the right ventricle, interventricular septum, and left ventricle were 22 ± 1.4 , 21.5 ± 1.4 , and 21.8 ± 1.7 μ m, respectively (figure 1). Statistically significant differences were present between the slight and the moderate groups ($P < .001$). No statistically significant differences were noted in the degree of myocyte hypertrophy measured in animals sacrificed early or late after infection ($P = .652$), and no animals had mean muscle cell diameters of >26 μ m in this study. From these studies, survi-

vors of RbCV infection were divided into two groups, those exhibiting zero to slight hypertrophy and those with moderate hypertrophy.

Control animals demonstrated significantly smaller mean myocyte diameters than either group of survivors ($P < .001$). The mean myocyte fiber diameters of the 9 control animals were 15.0 ± 1.5 , 15.7 ± 1.9 , and $15.4 \pm 1.8 \mu\text{m}$ in the right ventricle, interventricular septum, and left ventricle, respectively.

Body weight, heart weight, and heart weight-to-body weight ratios. Body weight, heart weight, and the heart weight-to-body weight (HW:BW) ratios were measured in RbCV-infected and control animals sacrificed on similar days. After infection, body weights initially decreased but then increased as animals recovered. There was no significant difference among the body weights of the 13 animals in the slight group ($3.23 \pm 0.355 \text{ kg}$), the 9 in the moderate group ($3.20 \pm 0.29 \text{ kg}$), or the 9 controls ($3.36 \pm 0.28 \text{ kg}$; $P = .52$).

The heart weights of the survivors (slight group, $8.35 \pm 1.21 \text{ g}$; moderate group, $8.21 \pm 0.48 \text{ g}$) were significantly greater than the heart weights of control animals sacrificed on similar days ($6.59 \pm 0.86 \text{ g}$; $P < .001$). There were no significant differences in the heart weights between animals in the slight and moderate groups ($P = .733$; figure 2A).

The HW:BW ratios of survivors were also significantly increased compared with the controls (slight group, $2.6 \pm 0.2 \times 10^{-3}$; moderate group, $2.5 \pm 0.2 \times 10^{-3}$; control group, $2.0 \pm 0.2 \times 10^{-3}$; $P < .001$). There were no significant differences between the HW:BW ratios in the slight and moderate groups ($P = .625$; figure 2B).

Dimensions of the cardiac walls and cavities. Changes in the size of the heart and dimensions of the ventricles were evident among the survivors (figure 3). These data suggested that biventricular dilation was clearly present within the moderate group, while survivors in the slight group probably had dilation of the right ventricle. To determine whether the degree of dilation correlated with the degree of myocyte hypertrophy, the thicknesses of the right and left ventricular cavity walls and the interventricular septum in both survivors and control animals were measured. No significant difference was evident in the thickness of the ventricular walls and the interventricular septum between survivors and controls (figure 4). For example, the mean thickness of the interventricular septum in control animals was $3364.46 \pm 583.21 \mu\text{m}$ compared with $3697.95 \pm 577.29 \mu\text{m}$ and $3367.13 \pm 520.63 \mu\text{m}$ in the slight and moderate groups, respectively ($P = .285$).

Significant changes in the size of the ventricular cavities were evident after infection. The right ventricular cavity in the slight group ($74.34 \pm 21.88 \times 10^6 \mu\text{m}^2$) was larger than in the control group ($44.77 \pm 13.83 \times 10^6 \mu\text{m}^2$; $P < .05$). In contrast, the left ventricular cavity area in the slight group was $63.61 \pm 21.45 \times 10^6 \mu\text{m}^2$. This was not significantly

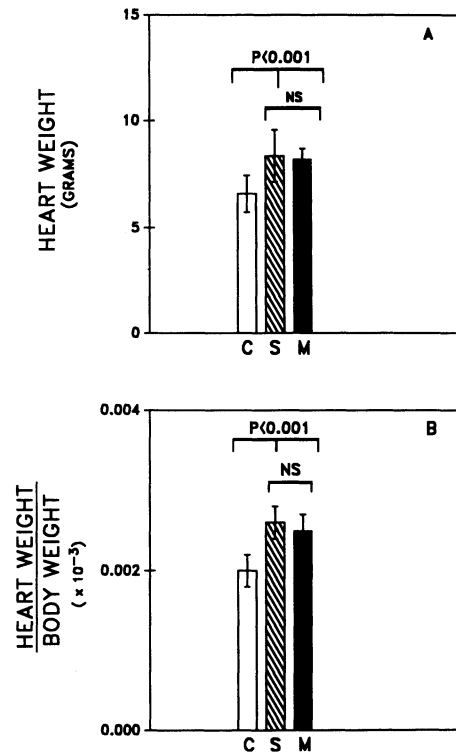


Figure 2. Heart weight (A) and heart weight-to-body weight ratios (B) of survivors of rabbit coronavirus infection compared with uninfected controls (C) sacrificed on similar days. Survivors had slight (S) or moderate (M) myocyte hypertrophy. Measurements are mean \pm SD and were evaluated by analysis of variance. NS, no significant difference.

different from that of the control group ($61.92 \pm 22.62 \times 10^6 \mu\text{m}^2$; $P = .89$). Dilation of the right ventricular cavity was more pronounced in the moderate group ($116.11 \pm 52.99 \times 10^6 \mu\text{m}^2$; $P < .01$). There was also a significant increase in the area of the left ventricular cavity in the moderate group compared with both the control and slight groups ($94.56 \pm 40.05 \times 10^6 \mu\text{m}^2$; $P < .05$; figure 5).

Pathology. Gross and microscopic lesions in animals dying between days 3 and 12 were as previously described [17, 18]. Hearts from survivors varied and either appeared normal or had dilation of the right or both ventricles. No pulmonary edema, pleural effusion, or ascites was observed, nor were lesions observed in other organs.

Microscopic analysis revealed that myocardial lesions commonly seen early in the acute and subacute stages of infection had resolved by 30 days after infection. Myocytes in both the slight and moderate groups exhibited various degrees of hypertrophy and demonstrated enlarged nuclei (figure 6A). Interstitial and replacement fibrosis was present but was usually not extensive (figure 6B). Fibrosis was most evident in the papillary muscles of both ventricles (figure 6C). Calcification was seen in only 1 of the survivors.

Myocarditis characterized by clusters of lymphocytes was

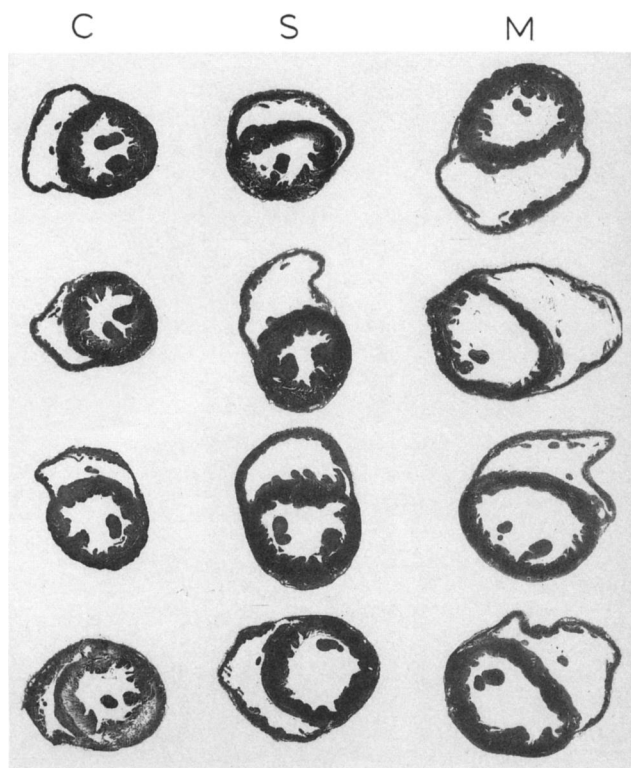


Figure 3. Cardiac dilation in survivors of rabbit coronavirus infection: representative sections from slight (S) and moderate (M) groups at 30–111 days after infection. Uninfected control (C) animals were sacrificed on similar days (36–106).

present in all survivors sacrificed 30–111 days after infection (figure 6D, E). Most rabbits had small scattered foci (1–5/section) of inflammatory infiltrate associated with degenerative or necrotic myocardial cells. In a few survivors, the de-

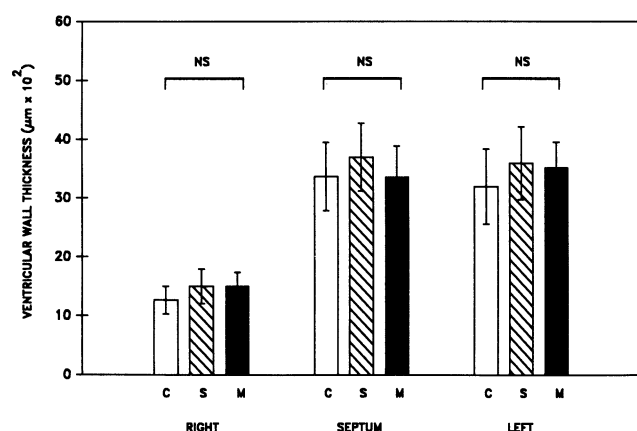


Figure 4. Dimensions of right ventricular walls, interventricular septum, and left ventricular walls in survivors of rabbit coronavirus infection compared with uninfected controls (C) sacrificed on similar days. Survivors had slight (S) or moderate (M) myocyte hypertrophy. Measurements are mean \pm SD and were evaluated by analysis of variance. NS, no significant difference.

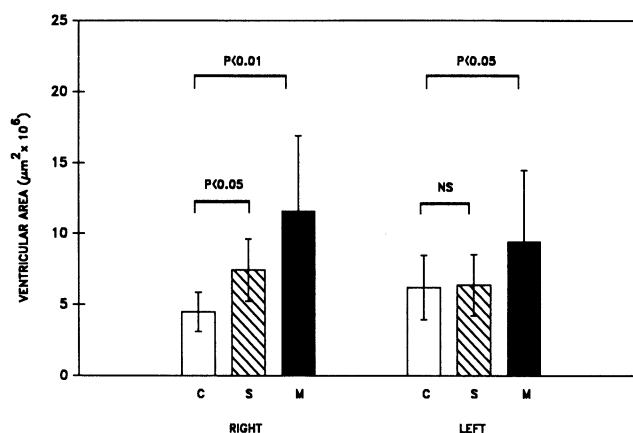


Figure 5. Area within right and left ventricles from survivors of rabbit coronavirus infection compared with uninfected controls (C) sacrificed on similar days. Survivors had slight (S) or moderate (M) myocyte hypertrophy. Measurements are mean \pm SD and were evaluated by analysis of variance. NS, no significant difference.

gree of myocarditis was quite severe (>20 foci/cross section). Other than the degree of myocyte hypertrophy, pathologic findings were similar in the slight and moderate groups.

The lungs were generally normal. In a few animals, a small residue of intraalveolar fluid was evidenced by pale pink-staining material in some of the alveoli. The compression of hepatic cords and necrosis of hepatocytes around central veins, seen early in infection, were not evident 30 days after infection.

Isolation of infectious virus. Between 30 and 111 days after infection, the heart muscle of 7 animals was examined by *in vivo* infectivity assay for the presence of virus. Infectious virus was isolated from the hearts of 4 of the 7 animals. Clinical signs of RbCV infection were not observed in animals inoculated with the heart homogenate. However, previous exposure to RbCV was demonstrated by protection from subsequent challenge with an RbCV stock of 10^3 – 10^4 RID₅₀/mL.

Discussion

A large number of RNA and DNA viruses are associated with heart disease in humans and experimental animals [21]. Viruses commonly linked to heart disease include enteroviruses, togaviruses, paramyxoviruses, orthomyxoviruses, coronaviruses, and others [21–23]. Infection may result in degeneration and necrosis of myocytes, myocarditis, arrhythmias, and CHF [21, 23]. Viral infection has long been suspected as an important initiating event in the development of DCM [24–26]. Direct viral involvement in both myocarditis and DCM, however, has been difficult to prove, because recovery of infectious virus has rarely been successful [26]. In addition, the mechanisms by which viruses induce myocarditis

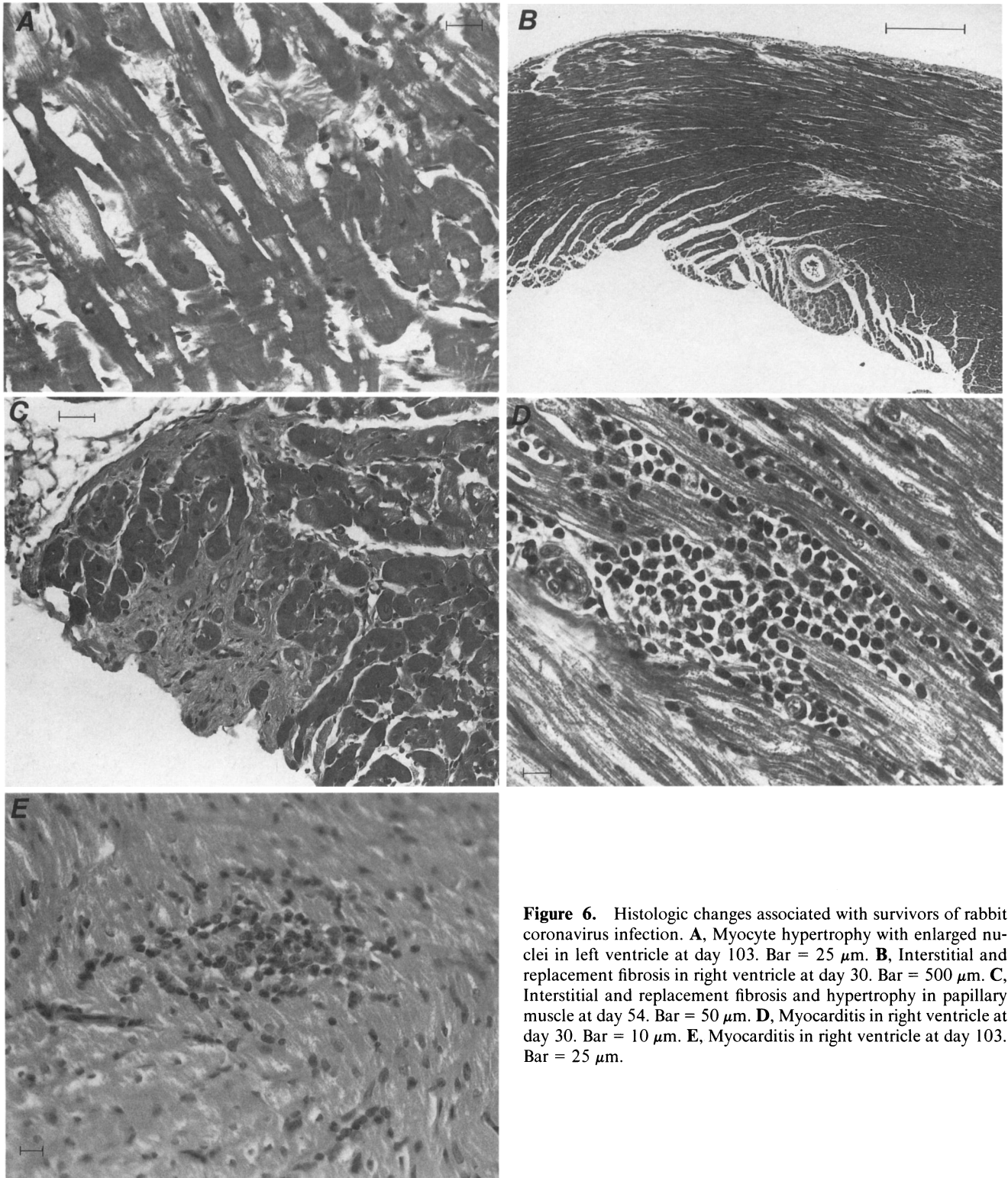


Figure 6. Histologic changes associated with survivors of rabbit coronavirus infection. **A**, Myocyte hypertrophy with enlarged nuclei in left ventricle at day 103. Bar = 25 μ m. **B**, Interstitial and replacement fibrosis in right ventricle at day 30. Bar = 500 μ m. **C**, Interstitial and replacement fibrosis and hypertrophy in papillary muscle at day 54. Bar = 50 μ m. **D**, Myocarditis in right ventricle at day 30. Bar = 10 μ m. **E**, Myocarditis in right ventricle at day 103. Bar = 25 μ m.

and DCM remain highly controversial, and more animal models are needed to provide insight into this phenomenon [27].

Previous studies in our laboratory have demonstrated that RbCV infection is divided on the basis of death and pathologic findings into acute and subacute phases. During the subacute phase, myocarditis is evident and animals are probably dying of heart failure [17, 18]. The etiologic agent for this disease is an enveloped RNA virus, originally designated the Stockholm agent. This virus is antigenically and morphologically related to group I human coronaviruses [18]. RbCV is also related to pleural effusion disease virus, which produces a similar disease in rabbits [18, 28–31].

In the present study, we describe a new animal model system for RbCV-induced DCM. In humans and in other experimental animal models, myocyte hypertrophy, fibrosis, increased heart weight, and ventricular dilation are the principal features of DCM [2, 3, 15, 32]. Survivors of RbCV infection were divided into two groups. On the basis of myocyte measurements, 59% of the rabbits were in the slight category while the rest had a moderate degree of myocyte hypertrophy. In addition to myocyte hypertrophy, the principal difference between the two groups was the presence of biventricular dilation in the moderate group. Heart weights, HW:BW ratios, pathologic findings, and day of death were not significantly different.

The slight group had a small but statistically significant dilation of the right ventricle, with the left ventricle remaining normal. In humans, right ventricular DCM in the absence of left ventricular dysfunction has been reported [33]. While our data indicate that viral infection may result in right ventricular dilation, there was no evidence of right heart failure in these animals.

Studies in humans suggest that hypertrophy and dilation represent an early stage of DCM, with CHF representing a later stage of the disease [20]. In the moderate group, biventricular dilation and hypertrophy were clearly evident in the absence of heart failure. These findings suggest that the rabbits in the moderate group may have had early DCM without clinical signs of CHF. Similar findings have been demonstrated in the coxsackievirus B murine models of DCM [14, 16]. In the encephalomyocarditis virus model of DCM, some animals had congestion of the lungs as early as 3 months after infection. The early development of CHF may be due to the severity of lesions in the chronic stages of encephalomyocarditis virus infection [15, 34].

DCM is thought to be a progressive disease after acute viral infection and myocarditis [25]. No progressive increase in myocyte hypertrophy and ventricular dilation was detected when animals were compared between early and late infection. It is therefore unclear whether some of these animals will later develop a more advanced form of DCM.

In humans with DCM, scattered foci of interstitial and replacement fibrosis are commonly seen throughout the

heart [2, 3, 35, 36], including the papillary muscles of the ventricular wall [5, 37, 38]. Histologic examination of the hearts of survivors of RbCV infection revealed lesions quite similar to those seen in human DCM [2, 3, 35–37]. Scattered foci of both interstitial and replacement fibrosis were present throughout the heart. Fibrosis usually was mild and most evident in the papillary muscles of both the right and left ventricles. Direct viral cytotoxicity of the myocytes and myocarditis has probably resulted in much of the fibrosis seen in the hearts. In addition, since papillary muscles are particularly sensitive to decreases in myocardial oxygenation, these data suggest that inadequate cardiac output due to impaired ventricular function has probably resulted in poor oxygenation of these tissues, resulting in fibrosis [39].

Calcification of myocytes was seen in only 1 rabbit examined >30 days after infection. This is also consistent with the findings in humans with DCM, in whom calcification is rare and usually limited to a few scattered myocytes [36]. In the encephalomyocarditis virus murine model, the extensive fibrosis and calcification seen after infection tends to be much more severe than that occurring in humans or in the RbCV or coxsackievirus B animal models. This may reflect the finding that encephalomyocarditis virus is more cardiotropic or that certain mouse strains are prone to calcification of soft tissues [14–16, 34, 36, 40, 41].

Histologically, DCM is often described as resembling “burned-out” myocarditis [42]. Active lymphocytic myocarditis is a common finding in patients with DCM [8, 43–46]. The inflammatory lesions seen are often small scattered foci of lymphocytic cells surrounded by necrotic tissue [2]. The frequency of myocarditis reported in DCM patients has varied greatly, ranging from 0 to 67% [44–48]. Small scattered foci of lymphocytic myocarditis, as defined by the Dallas classification system [49], were seen in all survivors of RbCV infection. The degree of myocarditis varied from slight to severe (1 to >20 foci/transverse section), which persisted through 111 days after infection. Most of the animals had scattered, small foci (1–5/transverse section) of lymphocytes that would have been difficult to detect by myocardial biopsy. In both the encephalomyocarditis virus and coxsackievirus B mouse models, mononuclear cellular infiltration of the heart was still evident 30 days after infection [14, 15]. At 90 days, inflammatory cells were no longer present in the hearts of survivors of either encephalomyocarditis virus or coxsackievirus B infection [15, 16]. As was seen in survivors of RbCV infection, myocarditis in mice with DCM infected with coxsackievirus B has been reported up to several months after infection [14].

Low levels of infectious virus ($<10^1$ RID₅₀) were detected in the heart tissue of 4 of 7 RbCV-infected rabbits sacrificed 30–111 days after infection. This finding is consistent with a previous study that showed RbCV and other pleural effusion disease virus isolates to persist at low levels ($<10^1$ – 10^2 RID₅₀/mL) in the serum of rabbits for almost 2 years after

infection. Survivors of pleural effusion disease virus infection also demonstrate fibrotic lesions in the heart muscle and focal myocarditis [31]. Although the presence of hypertrophy was not noted, these data suggest that some survivors of pleural effusion disease virus infection may also develop DCM.

Recent reports of enteroviral nucleic acid detected in the hearts of patients with DCM suggest that viral nucleic acid may persist in the heart muscle in the absence of detectable virus [9, 11–13]. Viral persistence and low-level expression of RbCV antigen in myocytes could result in chronic myocarditis through 111 days after infection. Alternatively, autoimmune mechanisms may explain the persistence of myocarditis. The difficulty in finding infectious virus in humans with DCM or in other animal models suggests that viral persistence or autoimmunity may play a significant role in chronic myocarditis in DCM [27]. At present, the mechanisms by which RbCV produce myocarditis are unclear and require additional study.

A model system for DCM after infection with RbCV has been described. Studies in this laboratory and others demonstrate that viral infection of the heart results in degeneration and necrosis of myocytes, myocarditis, and CHF [15, 17, 18, 34]. Acute infection of the heart may also lead to DCM at a later stage in life [14–16, 34]. Such findings support the theory that viral infection of the heart may lead to dilated cardiomyopathy in humans.

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