Persistence of the D Variant of Encephalomyocarditis Virus in the ICR-Swiss Mouse (43687)

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Abstract. **The D variant of encephalomyocarditis virus (EMCV-D) is used in the murine model to study virus-induced, acute-onset diabetes mellitus (IDDM) and myocarditis. In this model, viral replication and disease occur within seven days post infection (p.i.), and by Day 10 p.i., no infectious virus is detectable. The present study examined the possibility that EMCV-D persists in ICR-Swiss mice after the acute infection is resolved. The data show that viral antigen is detected at 28 days p.i. within the pancreatic islets of 8/10 males and 13/14 females, and within the heart valves of all animals tested. Histologic examination of the organs at 28 days p.i. suggests the development of chronic obstructive pancreatitis, and shows almost fully healed lesions in the myocardium. These observations indicate that the murine model for the study of EMCV-D induced IDDM may be extended to investigate chronic pancreatitis and heart-valve disease.** [P.S.E.B.M. 1994, Vol 205]

The D variant of the encephalomyocarditis virus (EMCV-D) is used in the murine model for studying virus-induced, insulin-dependent diabetes mellitus (IDDM). The disease produced by this virus has an acute onset, with IDDM apparent by Day 7 post infection (p.i.) (1). Myocarditis is also evident at this time (2). Studies show that following infection with EMCV-D, the onset of disease occurs concomitantly with viral titer peaks in the respective organ. No infectious virus is detectable by Day 10 p.i., and the virus is thought to be completely cleared from the animal (3).

Recent studies suggest that many picornaviruses including enteroviruses, Theilers murine encephalitis virus (TMEV), foot-and-mouth disease virus (FMDV), and Coxsackie viruses (CV) may persist past the acute phase of infection (4–9). The Coxsackie viruses are of particular importance considering their relation to human diabetes and heart disease (5, 6, 10–12, 14). While Coxsackie viruses have not been shown to persist in the human pancreas, studies demonstrate that, although no infectious virus can be isolated, Coxsackie virus RNA is detected for up to 10 years in the hearts of patients with dilated myocardiopathy (5, 10, 20). Coxsackie viruses have also been shown to establish persistent infections in cultured lymphoid cell lines (4, 6). Foulis *et al.* (11) have suggested that CVB3 may persist in defective form, since their genomes, but not viral proteins or infectious virus, have been demonstrated in human heart and pancreas biopsy samples (10). Further support for persistence of defective picornaviruses is a study showing cell cultures persistently infected with FMDV with deletions of up to 3 kb (7).

The purpose of the present study was to examine the possibility that EMCV-D persists in the murine host after the acute infection is resolved. This possibility was suggested by studies showing persistence of viral nucleic acid in the hearts of mice infected with either of two myotropic EMCV variants (15, 16) and in a cell line infected with EMCV-D (17). If found to persist, EMCV-D could be used as a model for the study of both acute-onset IDDM and persistent viral infections of the pancreas and possibly other organs.

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By using the natural murine host of this virus, the model would obviate the possible problems inherent when using an adapted human virus in a murine system, as is currently done with Coxsackie virus (13, 18).

Materials and Methods

Virus. EMCV-D was obtained from J. W. Yoon (University of Calgary, Calgary, Alberta, Canada). The virus was passed five times in L929 cells, once in BHK cells, and once again in L929 cells. Virus stock used for producing antisera was passed an additional time through HeLa cells to eliminate murine cellular antigens. Viral titers were determined by plaque assay on L929 cells. Virus was purified by the method described by Reuckert and Pallansch (21). Briefly, supernatant fluids from EMCV-D-infected L929 cell cultures were precipitated by adding 1/10 volume 2% protamine sulfate (Sigma), stirring for 30 min in the cold and centrifuging at 10,000*g* for 20 min (4°C). The resulting supernatant fluid was then precipitated by adding 30% Carbowax 8000 (Fisher Scientific) to 7% final concentration, followed by stirring and pelleting as above. The pellet was then resuspended in 1 ml 50 m*M* Tris pH 7.6, 4 ml 50% CsCl added, and the solution centrifuged for 18 hr at 200,000*g* in an AH-641 swinging bucket rotor (Sorvall). The viral band was withdrawn with a syringe, diluted to 10 ml with 50 m*M* Tris pH 7.6/1.0 *M* NaCl, layered over a cushion of 30% sucrose/20 m*M* Tris pH 7.6/1.0 *M* NaCl, and centrifuged for 3 hr at 100,000*g* in an AH-641 rotor. The viral pellet was then resuspended and stored in PBS. Test animals received 1600 pfu EMCV-D in 0.2 ml Hank's Balanced Salt Solution (HBSS) via the intraperitoneal route of inoculation (ip), while controls received an identical volume of HBSS.

Animals. Adult (9-week-old) male and female ICR-Swiss mice (Harlan Laboratories, Indianapolis, IN) were housed individually, given chow and water *ad libitum,* and rested for one week prior to the beginning of the study.

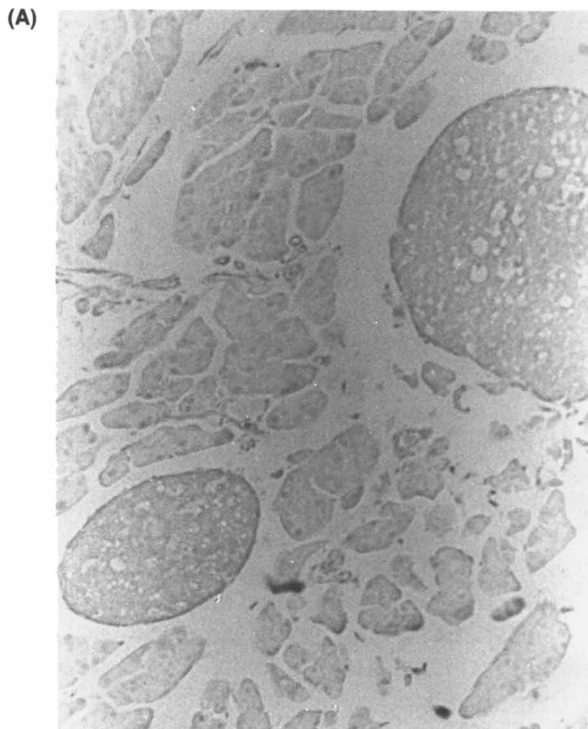
Antibodies. Antisera against EMCV-D (prepared and purified as described above) was produced by injecting UV-inactivated virus ip into five ICR-Swiss male mice. The animals received two more injections of inactivated virus subcutaneously at 10-day intervals. The serum was collected seven days after the last injection and heat inactivated (56°C, 15 min). The preparation had a titer of 10,000 PR_{50} neutralizing units per μl. Guinea pig antipig insulin was purchased from Sigma Chemical Co. (St. Louis, MO). Protein A (Sigma) was biotinylated with NHS-biotin (Vector Labs) following manufacturers directions.

Histology and Immunostaining. Immediately after sacrificing the animals, the organs were removed and fixed for 24 hr at 4°C in 3% paraformaldehyde (Sigma Chemical Co.) in PBS. The tissues were then dehydrated, cleared, and paraffin embedded using a Histomatic model 166A tissue processor (Fisher Scientific). Sections were cut at 7 μm, except the heart which was cut at 10 μm, and mounted on gelatin subbed slides. The sections were stained with hemotoxylin and eosin (H&E) for histological examination. After dewaxing and rehydrating, slides to be immunostained were washed in 50 m*M* Tris pH 7.6, blocked in 3% BSA (fraction V, Sigma)/50 m*M* Tris pH 7.6/0.02% NaN_3, and incubated overnight with primary antibody diluted in blocking buffer. The slides were then washed as above, incubated 30 min with biotinylated protein A, washed again, and incubated 30 min with avidin-HRP (elite Vectastain kit, Vector Labs). The slides were then washed and the color developed in 200 ml 50 m*M* Tris pH 7.6/150 mg DAB (Sigma)/7 μl 30% H_2O_2 for 10 min. The slides were then washed in H_2O, dehydrated, and coverslipped. Two milliliters of 1% CoCl was added to the color development buffer for all slides, except those containing heart tissues, to increase the staining intensity (18).

Results

Persistence of Viral Antigen. To determine persistence of EMCV-D after the acute phase of infection, 10 male and 14 female mice were infected ip with the virus. At 28 days p.i., the animals were sacrificed and their hearts, pancreases, and livers paraffin-embedded and immunostained for the presence of viral proteins. Twenty-eight days p.i. was chosen to be certain that any pathology seen was due to chronic infection and not to the final stages of acute infection which is resolved by Day 10 p.i. (3). Organs were also collected at 10 and 15 days p.i. and assayed for viral pfu content (mature virus). The results showed a positive signal in the pancreas and heart (Fig. 1 and 2), but not in the liver (data not shown). As expected, no mature virus was detected at 10 and 15 days p.i. (data not shown).

To demonstrate that the immune-signal was due to the presence of viral protein, the antisera was preincubated with CsCl purified EMCV-D. Figure 3 shows that the immunoreactivity in both the heart and pancreas was completely eliminated by the purified virus, showing that the tissues are displaying epitopes identical to those found on EMCV-D. Almost all of the animals (8/10 males and 13/14 females) demonstrated immunopositive cells in their pancreatic islets. Figure 1B shows a typical positive islet from a female. In all instances, the majority of the immunopositive cells were in the islet periphery, with only a few islets showing centrally located staining. The female pancreases stained more intensely and had a larger number of positive islets than the males. No antigen was detected in the acinar portion of the pancreas of either males or females. These results contrast with those obtained during the acute phase of infection, where viral pro-



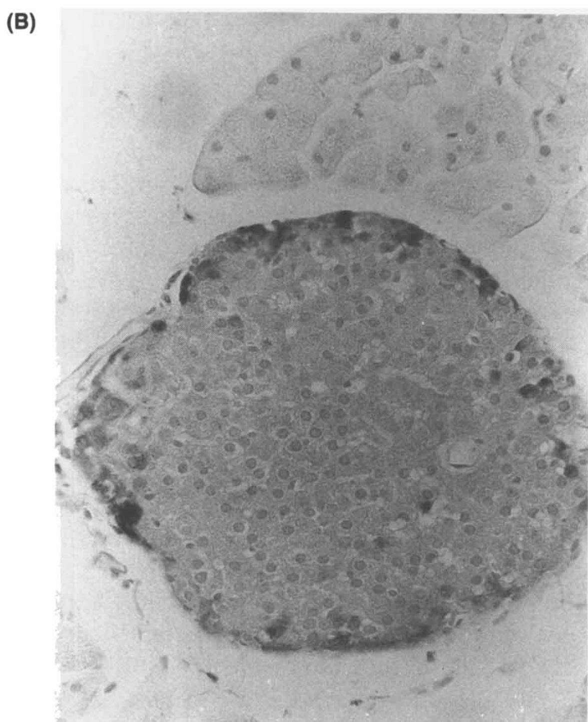


Figure 1. Pancreatic islets immunostained for EMC viral antigen. (A) Islet from an uninfected female showing no immunoreactivity. (B) Islet from female 28 days p.i. Notice immunoreactivity in periphery of islet, but not within the acinar pancreas.

teins were detected in both the acinar and islet portions of the pancreas (data not shown). Viral antigen was detected in the hearts of all infected mice (Fig. 2). Immunopositive cells were evident in the myocardium during the acute stage of infection, but were confined to the valves at 28 days p.i. Immunostaining of serial

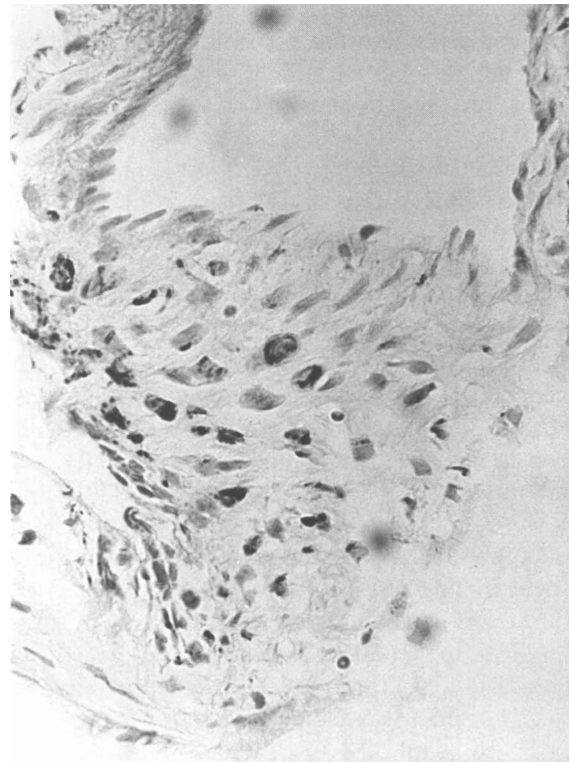


Figure 2. Heart immunostained for EMC viral antigen 28 days p.i. Area shown is sagittal section through valves.

sections of the heart showed that the viral lesion was focal, as much of the valve structure is immunonegative.

Effect of Viral Proteins on Insulin Production. To determine if the presence of viral antigen at 28 days p.i. affected insulin production, sections serial to those immunostained for viral protein were immunostained for the presence of insulin. As seen in Figure 4, the majority of the cells in islets from infected female mice, showed strong staining for insulin. The level of insulin-staining was similar to that of uninfected con trols (data not shown). This was true even in islet positive for the presence of viral proteins. In contrast insulin-staining in islets from infected male mice wa diffuse and much weaker than that seen in females an uninfected controls (Fig. 4B). Comparison of seri sections stained for viral antigen with those assayed for the presence of insulin showed that viral antigen was found only within the beta cells (data not shown).

Histology. The histopathology of various organs at 28 days p.i. was studied by examining H&E-stained sections. All the female pancreases showed some degree of pancreatitis (Fig. 5A), while only four showed a low level of insulitis (data not shown). The pancreatitis consisted primarily of scattered foci of mononuclear cells with a few macrophage in the acinar portion. Some perivascular cuffing was evident in a few pancreases. In animals with insulitis, only a few islets within the pancreas appeared to be involved. Eleven of the 14 animals also showed early signs of fat infil-

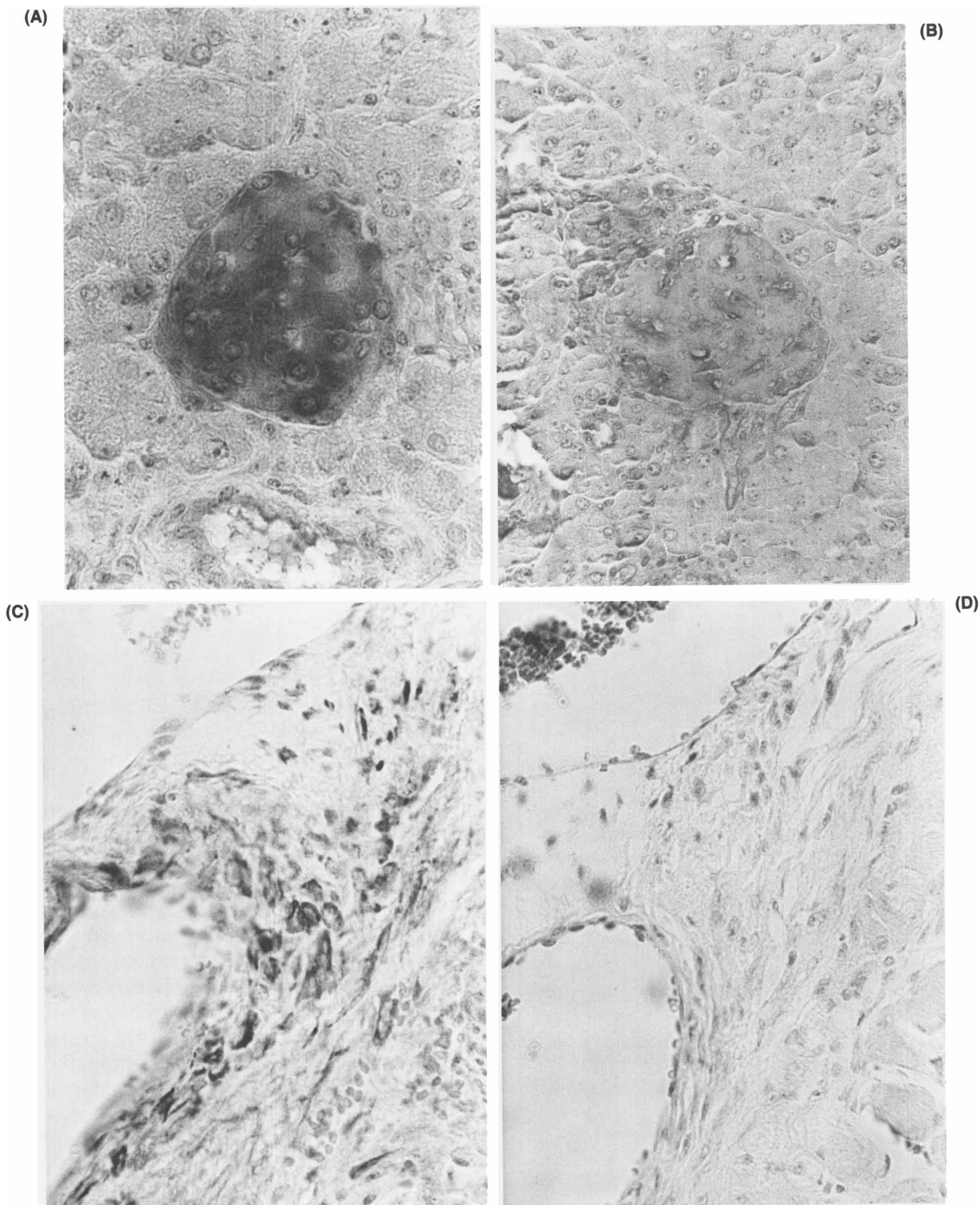


Figure 3. Preincubation of EMCV-D antisera with purified virus. (A) Pancreatic islet from infected animal immunostained with EMCV-D antisera. (B) Serial pancreatic section immunostained with EMCV-D antisera preincubated with purified virus. (C) Heart valve from infected animal immunostained with EMCV-D antisera. (D) Serial heart section immunostained with EMCV-D antisera preincubated with purified virus. Notice the complete absence of immunostaining by the preincubated antisera.

tration of the pancreas, where the acinar portion was apparently being destroyed by a mixed infiltrate of macrophage, polymorphonuclear, and mononuclear cells (Fig. 5C). One female showed complete replacement of the acinar tissue with adipose tissue, characteristic of chronic obstructive pancreatitis (Fig. 5D). Six of the males showed signs of pancreatitis; again, this was scattered and focal in nature with some peri-

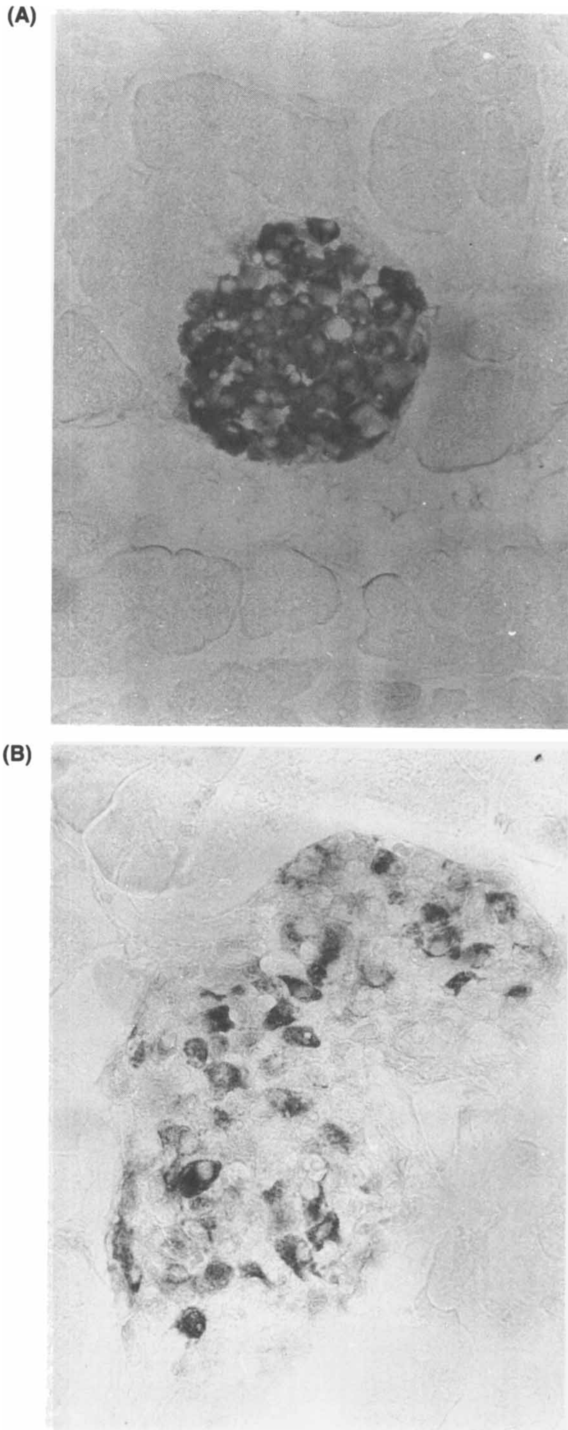


Figure 4. Pancreatic islets immunostained for the presence of insulin. (A) Female islet 28 days p.i. The islet contains over 90% insulin-positive cells; identical to that seen in uninfected control animals. (B) Male islet 28 days p.i. Male islets typically contained 20% or fewer insulin-producing cells.

vascular cuffing (Fig. 5B). Three of the males showed low levels of insulitis, with few islets involved, while four had signs of fat infiltration. No immune cells were observed near cells which were immunopositive for EMCV antigen.

Figure 6 is representative of scar present in the myocardium of both males (7/10) and females (8/14). The scars were limited to the myocardium and ranged in size from small discrete lesions to areas of large, diffuse involvement. The myocardial lesions are probably the result of acute myocarditis as very few lymphocytes or macrophage could be detected within them.

The spleen and liver were also examined but showed no unusual histology, except for an increase in circulating lymphocytes seen in the hepatic sinusoids in a few animals of both sexes (data not shown).

Discussion

The present study shows that EMCV-D-specific proteins are detected in ICR-Swiss male and female mice long after the acute infection has been resolved and mature virions can no longer be recovered. Viral antigens were present in the pancreatic islets and in the valves of the heart at 28 days p.i. Detection of viral antigen in females is particularly interesting in that, even though they are known to be resistent to the diabetogenic effects of EMCV-D, the immunostaining within their islets was more intense than that seen in susceptible males.

Day 28 p.i. was chosen to allow for the resolution of the acute infection. Previous studies have shown that viral replication in the heart and pancreas is complete by the end of the first week p.i. (3). Therefore, any remaining pathology must be due to the chronic/persistent aspects of EMCV-D infection.

The development of severe pancreatitis and insulitis within the first few days of infection indicates that EMCV-D replicates within both the acinar and islet portions of the pancreas during this period of infection. By Day 28 p.i., most of the cellular infiltration seen in the pancreas during the acute stage of infection was resolved, and viral antigens were restricted to the islets. Immunostaining of serial sections revealed that the antigen within the islets was located in beta cells. Low levels of pancreatitis and isolated cases of insulitis were also noted. The affect of viral infection on long-term insulin production, as shown by immunostaining (Fig. 4), was different in males compared to that in females. The intensity of the stain in islets of infected males was much lower, and more diffuse, than that seen in uninfected controls, indicating that insulin production was reduced by the virus infection. In contrast, the ability of females to produce insulin was apparently not affected by the virus, as the pattern and intensity of immunostaining in the beta cells was essentially identical in both infected and uninfected animals. Persistence of viral antigen did not appear to correlate with reduced insulin production in that a beta cell could be immunopositive for both viral antigen and insulin.

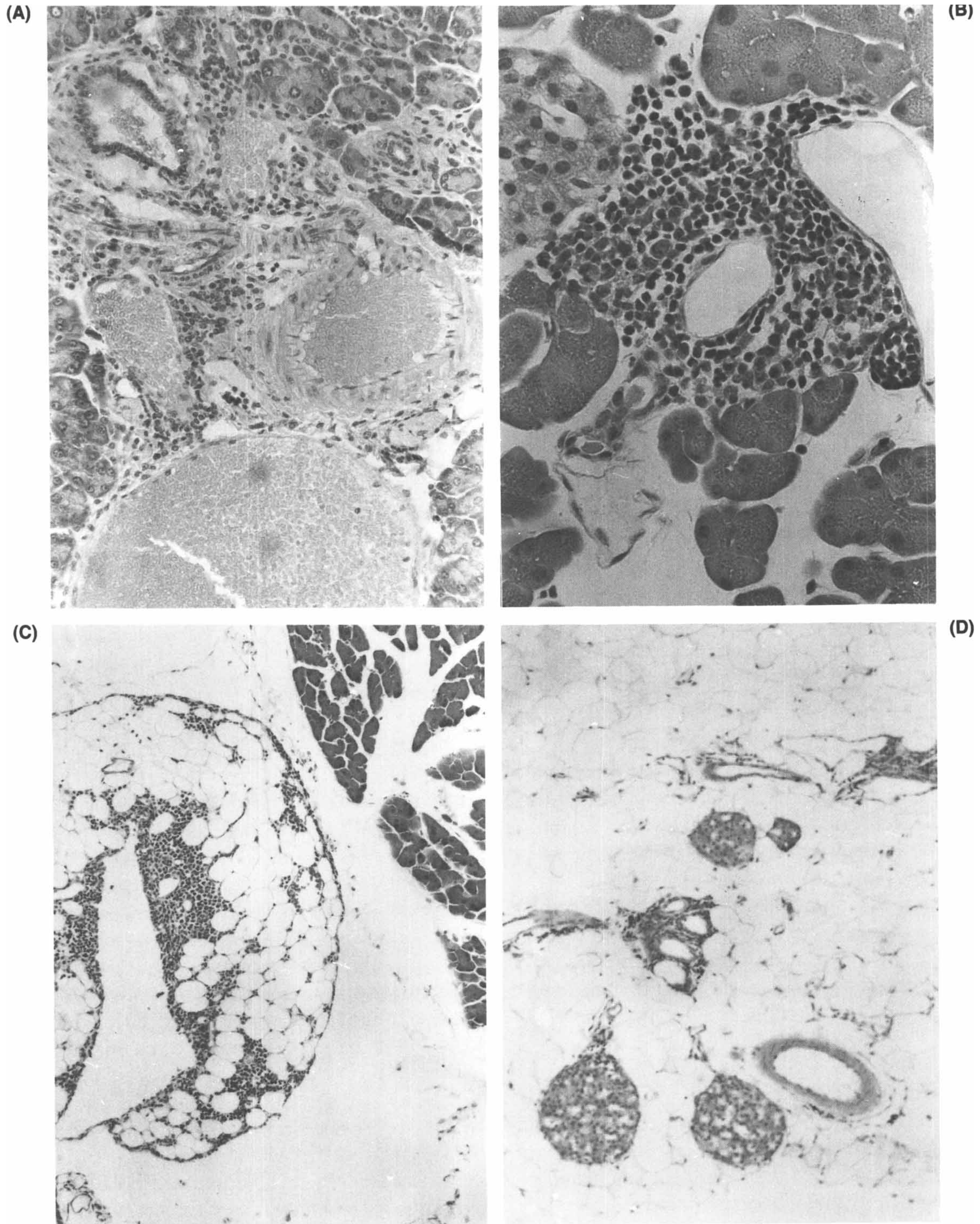


Figure 5. H&E-stained sections of pancreases 28 days p.i. (A) Example of diffuse pancreatic immune infiltration. These lesions did not usually involve more than one lobule and usually did not infiltrate islets. (B) Perivascular cuffing was the most common infiltrate pattern seen. Notice the lack of invasion into the neighboring islet. (C) Fatty infiltration with associated immune cell infiltrate bordering healthy acinar tissue. (D) Atrophied pancreas, where the entire acinar pancreas has been replaced by adipose.

The major histological change seen in the pancreas was infiltration of the acinar tissue by fat cells (Fig. 5C and D), indicative of obstructive chronic pancreatitis. This infiltration is most likely an indirect result of the acute stage of infection. Cell debris and inflammation during early infection could block the pancreatic ducts and lead to necrosis of the upstream acinar cells and their replacement with adipose tissue. The necrotic cells would then be removed by the immune cells accompanying the infiltrating fat. Similar histological lesions, occurring within the same time frame, have been reported in response to CVB3 infection, which,

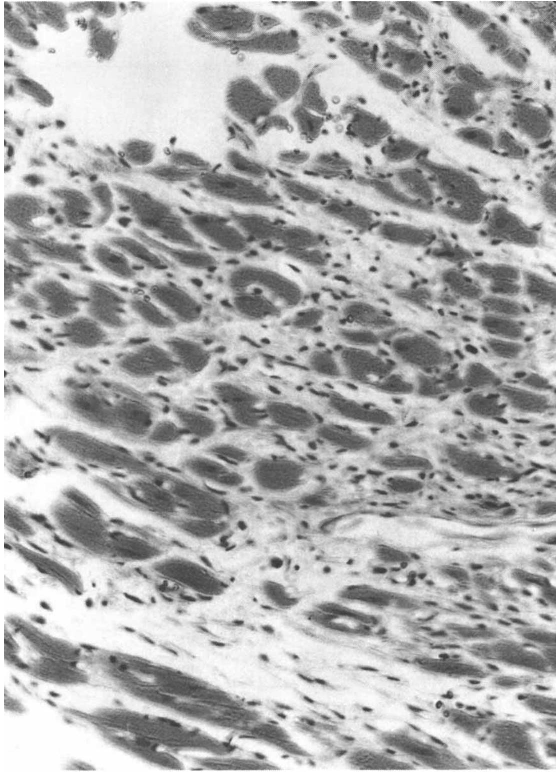


Figure 6. H&E-stained section of myocardium 28 days p.i. showing diffuse fibrotic scar.

on occasion, led to total replacement of the acinar tissue by adipose tissue (9, 20). Such an "atrophied pancreas" was seen in one animal in our study (Fig. 5D).

The low level pancreatitis seen at 28 days p.i. most likely reflects either final clearing of necrotic cells from the acute stage of infection, or early signs of ductal blockage effecting only a few acini. The apparent absence of virus-specific proteins in the foci might also suggest that the action of the infiltrating cells is directed against cells harboring viral proteins below the detection limits of immunostaining, or cells which contain inactive or partially active viral genomes.

During acute infection, replication of EMCV-D in the heart is limited to the myocardium and results in myocarditis (2). Immune cells, during acute infection, are restricted to the lesions in the myocardium. By Day 28 p.i., the lesions were essentially healed and the damaged myocytes replaced by connective tissue (Fig. 6). Viral antigen was evident in the heart valves of all infected animals at this time (Fig. 2). Apparently more viral antigen was present in the heart valves than in pancreatic islets. This conclusion is based on the fact that the immunostaining sensitivity had to be increased by the addition of CoCl to the color development step to detect the viral antigen in the pancreas, while the heart showed intense staining without the addition of CoCl. As in the pancreas, no immune response was detected against the antigen bearing cells. These observations contrast with the Coxsackie virus model where progressive myocardial damage occurs after the acute infection, apparently by an autoimmune mechanism (22–24). Coxsackie viruses have also never been detected within the heart valves.

Arguing for the specificity of the antisera to viral epitopes, are the results showing no immunostaining in tissues from uninfected animals, and the fact that the immunostaining was eliminated when the antisera was preincubated with purified EMCV-D (Fig. 3). Also, no staining was seen in infected tissues in the absence of the primary antibody. The possibility remains that the antisera recognizes a domain on the virus shared with a cellular protein whose expression was induced by the infection. This possibility, however, is unlikely in view of the different staining patterns seen in the early and late phases of infection. Specifically, all islets are immunopositive by Day 4 p.i., while only a few retain this immunoreactivity on Day 28 p.i. Within these islets only a few cells are positive even though many beta cells remain. The late phase also lacks any staining in the acinar tissue, which is seen during the acute infection. Even if the signal detected is due to an induced cellular protein, the presence of a protein so highly crossreactive with EMCV-D would most likely be pathogenic, perhaps by eventually triggering autoimmune responses.

The results reported in the present study differ significantly from previous studies showing persistence of EMCV in mice. Other investigators have shown persistence of low levels of viral nucleic acid in heart tissue (15, 16). In one study, the viral nucleic acid was present in such low levels that two rounds of PCR with nested promers was required for its detection (16). The presence of such low levels of viral nucleic acid raises the question of its significance in initiating pathologic responses. The low levels of viral nucleic acid found in these persistent infections may be due to the fact that myotropic EMC viruses, such as EMCV-M, have been shown to be mixtures of several different virus variants, some of which induce the production of interferon (1). It has been shown that interferon (IFN) plays a very important role in regulating the replication of EMC viruses (1, 25, 26). It is possible that low levels of IFN, induced by one of the EMC variants in the myotropic virus preparation, could prevent the expression of the persistent virus to any appreciable extent. The EMCV-D used in the present study was plaque-purified and may have circumvented this problem. This conclusion is based on the fact that we have for the first time demonstrated the persistence of easily detectable levels of virus-specific proteins. The presence of viral proteins strongly suggests the establishment of an active persistent infection. Such an infection may be biologically important.

The D variant of EMCV has proved to be very useful for studying virus-induced, acute-onset, IDDM.

This study demonstrates for the first time the ability of EMCV-D to persist within the murine pancreas and heart. We have shown that this virus induces a chronic pancreatitis which begins to develop at about four weeks p.i. The detection of viral protein within the heart valves is very intriguing, as no picornavirus has ever been shown to affect this structure. Experiments are currently in progress to determine if viral RNA can be detected in the organs that show the presence of viral proteins. We are also investigating the long-term effect of persistence on pancreatic and heart valve function.

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1. Yoon JW, McClintock PR, Onodera T, Notkins AL. Virus-induced diabetes mellitus XVIII. Inhibition by a nondiabetogenic variant of encephalomyocarditis virus. J Exp Med **152:**878–892, 1980.
2. Cerutis DR, Bruner RH, Thomas DC, Giron DJ. Tropism and histopathology of the D, B, K, and MM variants of encephalomyocarditis virus. J Med Virol **29:**63–69, 1989.
3. Gould CL, Trombley M, Bigley NJ, McMannama KG, Giron DJ. Replication of diabetogenic and nondiabetogenic variants of encephalomyocarditis (EMC) virus in ICR-Swiss mice. Proc Soc Exp Biol Med **175:**449–453, 1984.
4. Bendinelli M, Matteucci D, Conaldi PG, Giangregorio AM, Capobianchi MR, Dianzani F. Mechanisms of group B Coxsackie virus persistence in human cells. Eur Heart J **8**(Suppl J):441–444, 1987.
5. Bowles NE, Rose ML, Taylor PP, Banner NR, Morgan-Capner P, Cunningham L, Archard LC, Yacoub MH. End-Stage dilated cardiomyopathy persistence of enterovirus RNA in myocardium at cardiac transplantation and lock of immune response. Circulation **80**(5):1128–1136, 1989.
6. Cao Y, Schnurr DP. Persistent infection of YAC-1 cells by Coxsackie virus B3. J Gen Virol **69:**59–65, 1988.
7. De La Torre JC, Davila M, Sobrino F, Ortin J, Domingo E. Establishment of cell lines persistently infected with foot-and-mouth disease virus. Virology **145:**24–35, 1985.
8. Gebauer F, De La Torre JC, Gomez I, Mateu MG, Barahona H, Tiraboschi B, Bergmann I, DeMello PA, Domingo E. Rapid selection of genetic and antigenic variants of foot-and-mouth disease virus during persistence in cattle. J Virol **62**(6):2041–2049, 1988.
9. Vella C, Easton AJ, Eglin RP, Brown CL, Perry L. Coxsackie virus B4 infection of the mouse pancreas: I. Detection of virus-specific RNA in the pancreas by *in situ* hybridization. J Med Virol **35:**46–49, 1991.
10. Archard LC, Bowles NE, Olsen EGJ, Richardson PJ. Detection of persistent Coxsackie B virus RNA in dilated cardiomyopathy and myocarditis. Eur Heart J **8**(Suppl J):437–440, 1987.
11. Foulis AK, Farhuharson MA, Cameron SO, McGill M, Schonke H, Kandolf R. A search for the presence of the enteroviral capsid protein VP1 in pancreases of patients with Type I (insulin dependent) diabetes, and pancreases and hearts of infants who died of Coxsackie myocarditis. Diabetologia **33:**290–298, 1990.
12. Szopa TM, Ward T, Drondfield DM, Portwood ND, Taylor KW. Coxsackie B4 viruses with the potential to damage beta cells of the islets are present in clinical isolates. Diabetologia **33:**325–328, 1990.
13. Yoon JW, Onodera T, Notkins AL. Virus-induced diabetes mellitus XV. Beta cell damage and insulin-dependent hyperglycemia in mice infected with Coxsackie virus B4. J Exp Med **148:**1062–1080, 1978.
14. Bowles NE, Olsen EGJ, Richardson PJ, Archard LC. Detection of Coxsackie-B-virus-specific RNA sequences in myocardial biopsy samples from patients with myocarditis and dilated cardiomyopathy. Lancet **1086:**1120–1123, 1986.
15. Cronin ME, Love LA, Miller FW, McClintock PR, Plotz PH. The natural history of encephalomyocarditis virus–induced myositis and myocarditis in mice. Viral persistence demonstrated by *in situ* hybridization. J Exp Med **168:**1639–1648, 1988.
16. Wee L, Liu P, Penn L, Butany JW, McLaughlin PR, Sole MJ, Liew C. Persistence of viral genome into late stages of murine myocarditis detected by polymerase chain reaction. Circulation **86**(5):1605–1614, 1992.
17. Pardoe IU, Grewal KK, Baldeh MP, Hamid J, Burness ATH. Persistent infection of K562 cells by encephalomyocarditis virus. J Virol **64**(12):6040–6044, 1990.
18. Daldorf G, Gifford R. Adaptation of group B Coxsackie virus to adult mouse pancreas. J Exp Med **96:**491–497, 1952.
19. Hsu SM, Soban E. Color modification of diaminobenzidine (DAB) precipitation by metallic ions and its application for double immunochemistry. J Histochem Cytochem **30**(10):1079–1082, 1982.
20. Gomez RM, Lascano EF, Berria ML. Muring acinar pancreatitis preceeding necrotizing myocarditis after Coxsackie virus B3 inoculation. J Med Virol **35:**71–75, 1991.
21. Reuckert RR, Pallansch MA. Preparation and characterization of encephalomyocarditis (EMC) virus. Methods in Enzymology **78:**315–328, 1981.
22. Godney EK, Gauntt CJ. *In situ* autoradiographic identification of cells in heart tissues of mice with Coxsackie virus B3–induced myocarditis. Am J Path **129**(2):267–276, 1987.
23. Kishimoto C, Abelmann WH. *In vivo* significance of T cells in the development of Coxsackie virus B3 myocarditis. Circ Res **67:**589–598, 1990.
24. Gauntt CJ, Arizpe HM, Higdon AL, Rozek MM, Crawley R, Cunningham MW. Anti–Coxsackie virus B3 neutralizing antibodies with pathological potential. Eur Hrt J **12**(Suppl D):124–129, 1991.
25. Kaptur PE, Thomas DC, Giron DJ. The role of interferon in the protection of ICR-Swiss male mice by the nondiabetogenic variant of encephalomyocarditis virus against virus-induced diabetes mellitus. J Interferon Res **9:**671–678, 1989.
26. Giron DJ. Role of interferon in the propagation of MM virus in L cells. Appl Microbiol **18:**584–596, 1969.