Persistence of Viral Genome Into Late Stages of Murine Myocarditis Detected by Polymerase Chain Reaction

Lily Wee, MD; Peter Liu, MD; Linda Penn, PhD; Jagdish W. Butany, MD; Peter R. McLaughlin, MD; Michael J. Sole, MD; and Choong-Chin Liew, PhD

Background. Enteroviruses have been considered as the most common etiologic agents in clinical myocarditis and dilated cardiomyopathy; however, their pathogenetic role remains unknown. Hence, the relation of viral replication and development of cardiomyopathy has been determined in a murine model of myocarditis by evaluating the persistence of viral genome during acute and chronic stages of myocarditis by means of Northern blot hybridization and polymerase chain reaction (PCR).

Methods and Results. DBA/2 mice (n=146) were injected peritoneally with 10 plaque-forming units of encephalomyocarditis (EMC) virus, and the control mice (n=33) were injected with normal saline. Animals were randomly killed at 4, 7, 10, 14, 21, 28, 35, and 42 days after infection. Histology revealed acute myocardial necrosis with massive inflammatory cell infiltrate peaking on day 14 followed by increasing fibrosis and declining chronic inflammation features compatible with dilated cardiomyopathy between days 21 and 42. Northern blot analysis of control and infected hearts showed detectable viral RNA in the infected hearts initially at day 4, peaking by day 7, diminishing between day 7 and day 14, and absent at day 21 and day 28. However, potential viral remnants present in low quantities and undetectable by Northern blot were further detected by PCR followed by confirmation with an internal oligonucleotide probe after day 14 up to day 42.

Conclusions. Viral RNA signals on Northern blot showed a strong correlation with massive myocyte necrosis on day 14, but the viral RNA fragment was consistently detectable into late stages of cardiomyopathy on days 21, 28, 35, and 42 by PCR. This indicated that the mature virions are fully developed early in infection and are capable of persisting in the myocardium after virus-mediated myocytolysis stage. Therefore, PCR is an extremely sensitive method for detecting residual viral genome and viral persistence in the myocardium and may offer insights into the pathogenesis of chronic myocarditis leading to dilated cardiomyopathy. (Circulation 1992;86:1605-1614)

Key Words • encephalomyocarditis (EMC) virus • viral myocarditis • polymerase chain reaction (PCR) • dilated cardiomyopathy

Myocarditis is characterized by inflammatory infiltrates in the myocardium leading to necrosis and degeneration of the myocytes.1 The most common cause is considered to be viral, in which an infecting agent such as the enteroviruses initially causes virus-mediated cytolysis followed by autoimmune perpetuation of myocardial damage.2,3 However, the presence and role of the virus in late stages of the disease remain unknown. The clinical significance of viral myocarditis lies beyond its impact as an acute disease but extends to its contribution to chronic dilated cardiomyopathy resulting in congestive heart failure and its associated morbidity and mortality.4

To determine the pathogenetic role of the virus and to confirm the viral etiology in individual cases of myocarditis, various diagnostic techniques outlined below have been applied to the myocarditic tissue.4,5 However, the isolation of enterovirus from the heart muscle of patients with a history of myocarditis is generally unsuccessful.6,7 Similarly, immunofluorescent detection of enterovirus-specific antigen in myocardium is also unfruitful.8 The continuing autoimmune reaction mediates the progression of the disease from acute myocarditis to chronic myocarditis and implicates potential viral persistence in the myocardium.2,3,6,10 Several investigators have reported the detection of enteroviral RNA by slot blot, Northern blot, and in situ hybridization using nucleic acid probes complementary to viral genome in experimental models or in human myocardial biopsy or autopsy samples.11-14 Recently, we have introduced the polymerase chain reaction (PCR) gene amplification with slot blot hybridization techniques to demonstrate enteroviral persistence in endomyocardial biopsy samples from patients suspected of myocarditis.15 From this study, it remains unclear whether the viruses can always persist into the myocardium in late stages of
dilated cardiomyopathy and whether it can be detected consistently throughout all stages of the disease after viral infection.

Encephalomyocarditis (EMC) virus–induced myocarditis in murine models has demonstrated striking similarities to the clinical pattern of human myocarditis and dilated cardiomyopathy.3,5,6–18 The virus isolated from the mouse heart can only be cultured up to 14 days after infection,16 and the viral genome is detectable by in situ hybridization only up to 21 days of infection but is undetectable in the chronic phase.16

In this report, we used a DBA/2 murine myocarditis model with Northern blot hybridization and PCR to evaluate the persistence of EMC viral genome into the late stages of cardiomyopathy.

Methods
Propagation of Encephalomyocarditis Virus
Hamster kidney cells (BHK 21/C13), kindly provided by Dr. James Campbell, Department of Virology, University of Toronto, at a frozen stock concentration of \(10^6\) cells/ml were propagated into each of 25-cm² flasks containing alpha minimal essential medium (MEM, GIBCO BRL), antibiotics (units/ml penicillin 100 and 100 µg/ml streptomycin), 40 mM HEPES, and 10% fetal bovine serum (FBS, GIBCO BRL) at 37°C for 24 hours. The medium was then refreshed, and cell propagation was continued at 37°C until a confluent monolayer of cells was obtained. The BHK 21/C13 monolayers were infected with 2 ml of \(10^8\) plaque-forming units (pfu) of myotropic variant of EMC virus per flask at 37°C for 1 hour. Excess EMC virus was then removed, and 10 ml of medium containing alpha MEM, antibiotics, and 10% FBS was added. After the next 1, 3, 5, 7, 11, 23, and 47 hours, the virus-infected cells in each of the flasks were examined for cytopathic effects and used for extraction of viral RNA as positive controls.

Establishment of EMC Murine Myocarditis Model
Eight-week-old male DBA/2 mice (n=146, Harlan Sprague Dawley, Inc., Charles River) were inoculated intraperitoneally with 0.1 ml of 10 pfu of myotropic variant of EMC virus. Another group of DBA/2 mice (n=33) was injected with normal saline as controls. All mice were preweighed, fed a normal diet, and monitored daily for activity, fur characteristics, neurological deficits, and mortality before they were killed. Subgroups of these inoculated mice were randomly killed via cervical dislocation at day 4 (n=14), 7 (n=25), 10 (n=13), 14 (n=24), 21 (n=16), 28 (n=22), 35 (n=5), and 42 (n=5). Heart tissues of each subgroup were excised under aseptic conditions and quickly frozen in liquid nitrogen. From each subgroup, one to two heart specimens were randomly chosen and submitted for histopathological confirmation of myocarditis and associated damage. The remainder were used for RNA isolation. The heart weight of each animal was also measured.

Histopathology
The heart tissues from each subgroup representing different stages of myocarditis were cut transversely at the midventricle, fixed in 1% glutaraldehyde and 4% formaldehyde solution, dehydrated in graded alcohol,
The probe consisted of a 2.75-kb cDNA of EMC virus that corresponds to the 2A-POL(3D) portion of the viral genome. The probe was prepared by Neo I digestion of the recombinant plasmid pEM3, kindly provided by Dr. Ann Palmenberg, University of Wisconsin, Madison. The 2.75-kb fragment was further purified with glass milk (Bio/Can) and labeled with [α-3²P]dATP (specific activity, 3,000 Ci/mmol, ICN).

The total RNA of normal cells (40 μg), infected cells (0.3–10 μg), or tissues (40 μg) was denatured in formamide, added with ethidium bromide, run in 1.2% agarose–formaldehyde gel, and transferred to nitrocellulose filter (Schleicher and Schuell). The filters were incubated in prehybridization buffer overnight at 42°C. The filters were then hybridized in fresh prehybridization buffer containing 5% dextran sulfate and the denatured 3²P-labeled 2.75-kb probe (specific activity, 1.5×10⁹ cpm/μg) overnight at 42°C. The filter was sequentially washed for 30 minutes with 1×SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and 0.1% SDS at room temperature, then 1×SSC and 0.1% SDS at 42°C followed by 0.1×SSC and 0.1% SDS at 42°C and finally at 52°C until the radioactive background was negligible. The filter was autoradiographed at −70°C with intensifying screens.

To ensure equal loading of total RNA after the isolation procedure from heart tissues, plasmid pA50, a noncontractile protein housekeeping cDNA clone, was used. After washing off the previous hybridization with 0.1×SSC and 0.1% SDS solution for 10 minutes at boiling temperature, pA50 was introduced to probe for tissue β-actin messenger RNA (mRNA) found on the same nitrocellulose filter.

**Synthetic Oligonucleotide Primers and Probe**

With the documented sequencing data of the EMC viral genome, the location of the oligonucleotide EMC virus-specific primers A (nucleotide [nt] 5196–5216), B (nt4853–nt4873), C (nt4938–nt4958), D (nt5112–nt5132) that flanked near the viral protein genome (VPg or 3AB) area and probe (nt5017–nt5046) that positioned internally in between these primers or within VPg area (Figure 1A) were carefully screened, selected, and synthesized in computerized Gene Assembler Plus equipment (Pharmacia). The primers selected were based on the following criteria: a balance between G/C and A/T concentration with no palindromes at both 5’ and 3’ ends; a size between 20 and 27 mers with a melting temperature range from 55°C to 65°C; a sequence frame between 180 and 500 bp with a diagnostic restriction site. This area [between g(2C) and PRO(3C)] used for gene amplification overlapped a portion of the Northern probe. The 30-mer probe (16 pmol) was labeled at the 5’ end with 50 μCi [γ-3²P]ATP (specific activity, 3,000 Ci/mmol, Du Pont) and incubated in 1× kinase buffer I with 10 units polynucleotide kinase in a total volume of 20 μl for 45 minutes at 37°C. The labeled oligonucleotide was separated from unincorporated [γ-3²P]ATP by passing through a spin column of Sephadex G-25 (Boehringer Mannheim) according to manufacturer’s recommended protocol. The specific activity of the probe averaged 1.3×10⁹ cpm/μg.

**RNA Extraction and Northern Blot Hybridization**

Isolation of total RNA from frozen heart tissues and EMC virus–infected BHK-21/C13 cells was carried out by the acid guanidinium thiocyanate–phenol–chloroform extraction method as described by Chomczynski and Sacchi.
First-Strand cDNA Synthesis

The synthesis of the first-strand cDNA from total RNA samples was carried out by using virus-specific primer B (Figure 1A) and reverse transcriptase to form a stable RNA-DNA hybrid (Figure 1B). Approximately 2 μg of tissue RNA or 3 ng of EMC virus-infected cellular RNA was denatured at 70°C for 5 minutes, cooled, and added to the reaction mixture containing 1× avian murine virus (AMV) reverse transcription buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, and 10 μg/ml bovine serum albumin), 0.01 μg primer B, 1 mM deoxyribonucleotide triphosphate (dNTP) mixture, 0.5 mM spermidine-HCl, 40 mM sodium pyrophosphate, 50 units RNase inhibitor, and 10 units AMV reverse transcriptase (Pharmacia) in a total volume of 40 μl. These reactions were incubated at 42°C for 45 minutes and then at 65°C for 10 minutes to inactivate the AMV reverse transcriptase. The mixtures were kept at 4°C and used directly for PCR gene amplification.

PCR Gene Amplification

Four microliters of the first-strand cDNA of both tissues and cells was used as a template for virus-specific primers A and B and added to the 96 μl of master mix consisting of sterile distilled water, 1× reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% [wt/vol] gelatin), 0.2 mM each of dNTP, and 1 μM primer A and primer B. After denaturing the mixture at 94°C for 5 minutes, 2.5 units of Taq polymerase and 90 μl light mineral oil were added. The mixture was amplified in a DNA Thermal Cycler (Perkin Elmer Cetus) for 39 cycles by annealing at 55°C for 2 minutes, extension at 72°C for 3 minutes, and then denaturation at 94°C for 1 minute per cycle. After 39 cycles, 2 μl of each of the PCR products was reamplified for 30 cycles with nested primers C and D (Figure 1A) under the condition described above except extension was for 2 minutes.

Southern Blot Hybridization

After PCR, 20 μl of the product was analyzed by gel electrophoresis on a 2% agarose gel using 1× TAE (0.04
M Tris acetate and 0.001 M EDTA, pH 8.0) as running buffer. DNA bands were visualized after staining with ethidium bromide and exposure to ultraviolet light. The DNA bands were simultaneously denatured and transferred for 2.5 hours onto a positively charged nylon membrane (Hybond-N+, Amersham) via an adsorption process using 0.4 M of NaOH as both denaturant and transferring buffer. Membranes were then dried overnight at room temperature or 20 minutes at 37°C and prehybridized in 6×SSC, 1% SDS, and 0.5% skim milk powder for at least 10 minutes at 42°C. The prehybridization solution was removed completely, and membranes were hybridized in 6×SSC, 1% SDS, and 0.5% skim milk powder with 20 gl of 5'-end-labeled 30-mer probe overnight at 42°C. Membranes were washed in 6×SSC and 1% SDS for 10 minutes at 37°C and autoradiographed at −70°C with intensifying screens.

Tests for Specificity of EMC Primers and Probe

Four-week-old male DBA/2 mice (n=10) were inoculated intraperitoneally with 0.1 ml of 10 pfu of Coxsackie B3 (CVB3, Nancy strain) generously supplied by the Department of Microbiology, Hospital for Sick Children. At 5 and 7 days after infection, each subgroup of mice (n=5) was killed. The heart specimens were studied pathologically and molecularly similar to that of the EMC murine myocarditis model using EMC primers and probe (Figure 1A). In addition, the selected 5' end region of CVB3-specific primers TM1, TM2, TM3, TM4, and probe,11 generously supplied by Dr. Michael Sole, University of Toronto, were also used for the specific detection of CVB3 viral genome. The degenerate primer TM1 [nt1176(3')-CCNACCACCACCT-TQRANGG-(5')nt1195; N=A,G,T; Q=C,T; R=A,G], modified from Olive et al,32 was mixed with ~2 µg of the tissue RNA for the first-strand cDNA synthesis; then primers TM2 [nt448(5')-TCCGGCCCCCT-GAATG-(3t)nt462]33 and TM3 [nt625(3')-ACCTAAC-CGGTAGGCCAC-(5')nt642]33 and nested primers TM2 and modified TM4 [nt580(3')-ACCGAC-GAATACCCTGT-(5')nt597]15,34 were added to the first- and second-round PCR reaction mixture, respectively, at the same PCR parameters, temperatures, and number of cycles with that of EMC model. The PCR products were then run on a 2% agarose gel and confirmed by Southern blot hybridization using EMC probe and CVB3-specific probe [nt561(5')- ACAAGG-ACACCCAAAAGTAGTCGTTCC-(3')nt536].33

Statistical Analysis

All results were expressed as mean±SD. Comparisons between normal and infected subgroups of mice and among infected mice at different stages were performed by ANOVA with subsequent comparison between two subgroups done by Neuman-Keuls multiple range method.35
Results

Mortality and Incidence of Myocarditis

Four days after viral inoculation, the infected mice manifested systemic illness with sluggish movement, ruffling of the fur, and anorexia. Some of the mice developed unilateral or bilateral hind limb paresis, but most of the mice recovered fully and appeared well after day 14. In this study, 22 of 146 infected mice died during infection (mortality of 15%), primarily during the second week (17 of 22 dead mice, or 77.3%). All deaths were due to congestive heart failure as indicated by the presence of ascites and congestion of the lungs and liver during postmortem dissection. Gross myocardial lesions involving the biventricular surface of the heart were seen in 111 of 146 infected mice (incidence of 76%). Aside from the dead mice, these myocardial lesions were also observed in 89 of the 124 surviving mice (71.8%). No mice died after day 19. The lesions were continuously found in 43 of the 48 infected mice (89.6%) between day 21 and day 42. The control mice remained active and grew normally, and none died during the course of the experiment.

Body Weight, Heart Weight, and Heart Weight to Body Weight Ratio

Body weight, heart weight, and the heart/body weight ratio were measured in infected DBA/2 mice and in the controls in the acute and chronic stages of myocarditis. The results are presented in Figure 2. After day 4, the body weight of infected mice was significantly lower than that of control mice. The heart weight was also significantly greater than that of control mice in all groups throughout the infective stages. The heart weight/body weight ratio was significantly increased in infected mice when compared with control mice after day 4. These data are compatible with systemic illness in these animals with significant heart failure.

Histopathological Findings

The hearts of noninfected mice showed no cellular infiltration or myocardial cell necrosis. The infected hearts disclosed the typical lesions of acute myocarditis followed by chronic myocarditis and subsequently the nonspecific features of dilated cardiomyopathy (Figure 3). On day 4, myocardial cells appeared almost normal with only occasional tiny foci of myocytolysis surrounded by a minimal cellular infiltrate in the myocardium. On day 7, necrotic foci appeared in the myocardium with interstitial edema and the presence of an interstitial mononuclear cell infiltrate. By day 10, myocardial necrosis and extensive mononuclear cell infiltration were seen. Myocardial necrosis was extensive, and confluent areas of mononuclear cell infiltration were seen by day 14. On day 21, myocardial necrosis persisted but was associated with fibrosis and a decrease in cellular infiltration. On day 28, cellular infiltration decreased further with prominent myocardial fibrosis and calcification. On day 42, the myocardium showed areas of dense collagen, with virtually absent interstitial mononuclear cells. Islands of calcification of variable size were seen in the areas of inflammation from day 7 onward. Tissue sections were scored for cellular infiltrate and myocardial necrosis. Myocardial lesions were not readily apparent on day 4 but became increasingly severe from day 10 to 14 and then gradually diminished thereafter with replacement of myocardial fibrosis.
Northern Blot Analysis

Northern hybridizations of total RNA extracted from frozen tissues (noninfected mice as negative controls) and infected BHK 21/C13 cells (as positive controls) were probed with 2.75 kb cDNA of the EMC virus (Figure 4A). A band of ~7.84 kb representing the whole virus was seen 6 and 8 hours after viral inoculation on infected cells but was not present in noninfected cell and control tissue samples. This band was readily detectable in the infected myocardium on day 4, became very intense on day 7, gradually diminishing between day 7 and day 14, turned almost undetectable on day 14, and was absent on day 21 and day 28.

The same nitrocellulose filter after hybridizing with 2.75 kb EMC virus–specific probe was completely washed off and probed again with pA50 for β-actin mRNA in tissue RNA. The actin probe revealed an intense β-actin mRNA band of ~1.73 kb in size in all tissue RNA samples (Figure 4B).

PCR and Southern Blot Analysis

To determine whether viral persistence plays a role in chronic myocarditis/dilated cardiomyopathy, the PCR gene amplification technique was used to sensitively and specifically detect residual viral genome in EMC virus–infected murine hearts. The first-cycle PCR amplification using primers A and B showed that the 364 bp product was undetectable in most samples after agarose gel electrophoresis (data not shown). The product of the first reaction was reamplified for an additional 30 cycles using the nested primers C and D (Figure 1A). The 2% agarose gel stained with ethidium bromide revealed an amplified PCR band of 195 bp for infected cells and tissues from day 4 to day 28 that further persisted on day 35 and day 42 (Figure 5A). This was not present in normal tissues and water samples acting as negative controls. With the use of limited amount of tissue RNA and infected cellular RNA as starting materials, the same procedures were repeated more than 10 times for each group of the samples, and the consistency of the results ranged from 75% to 90%.

The identity of the PCR products was further confirmed by Southern blot using a synthetic radiolabeled internal 30-mer probe (Figure 5B). These amplified viral fragments supported not only the sensitivity and specificity of primers and probe being used for detection of EMC viral genome but also the persistence of EMC virus throughout 6 weeks of infective period.

Specificity of EMC Primers and Probe

The histological lesions of CVB3-infected murine hearts for both subgroups revealed focal myocytolysis with lymphocytic infiltrate found in the subepicardium of the left ventricle with more lesions found in the right ventricle. Because both subgroups of infected mice showed typical lesions of acute pericarditis, only the first subgroup of mice was chosen for study in this model.

The first PCR amplification using EMC primers A and B and CVB3 primers TM2 and TM3 showed no product and 195 bp, respectively, after agarose gel electrophoresis. Likewise, subsequent reamplification of the product of the first reaction with EMC nested primers C and D and CVB3 nested primers TM2 and TM4 for another 30 cycles revealed undetectable and 150 bp bands (Figure 5A, lanes 15 and 16), respectively. Further confirmation of PCR products by Southern blot hybridization exhibit-
ited negative viral signals with the EMC-specific probe (Figure 5B, lanes 15 and 16) but positive CVB3 viral signals with the CVB3-specific probe only in the amplified 150 bp fragment (Figure 5C, lane 16). The above results demonstrated the specificity of both the EMC and CVB3 primers and probes in the non-EMC myocarditis model.

Discussion

Acute myocarditis leads to dilated cardiomyopathy and frequently occurs as a complication of viral infection of the heart, particularly notable among the enteroviral group.5,36-38 Clinically, the definitive diagnosis of myocarditis and identification of its etiological agent remain difficult. This can be attributed to the frequently asymtomatic early phase of the disease, insensitivity of the traditional virological techniques,5-7 and relatively strict pathological criteria for diagnosis of myocarditis from myocardial biopsy samples.1 To date, direct evidence of viral infection by virus isolation from the heart muscle or pericardial fluid is generally unsuccessful. Recent viral hybridization techniques have identified the presence of enterovirus in myocardial biopsy samples with variable frequency.10,12,13,15 However, it is not clear at what stages of myocarditis these hybridization techniques identify, whether they are reliably present in all stages of myocarditis, and what pathogenetic implications this finding will produce. Because of the ongoing inability to arrive at a gold standard for the etiologic diagnosis of human myocarditis, we have resorted to a well-established animal model in which the etiologic agent is known. In this model, we have demonstrated by the use of PCR technique that viral residues persist well from the time of infection up into the late stages of dilated cardiomyopathy. This finding contrasts the results from traditional hybridization techniques, which fail to detect the viral residues in late myopathic stages of the disease.14,15,16

Our model of EMC virus-induced murine myocarditis conforms to previous descriptions of stages of infection and resembles human myocarditis in its chronology and natural history.16-18 After EMC viral inoculation, the heart weight of the infected mice increases significantly compared with normal control mice. There is a significant associated acute mortality (15%) caused by congestive heart failure. Almost all surviving mice manifest enlarged/dilated hearts with prominent inflammation, myocardial calcification, and fibrosis compatible with a diagnosis of dilated cardiomyopathy.

In our study, the EMC virus is detectable by Northern blot analysis in the myocardium on day 4 of infection before the histopathological changes are apparent. By day 7, viral signals reach peak level, paralleled by histological lesions exhibiting extensive cellular infiltration and myocardial cell necrosis. Between day 7 and day 14, the viral signals gradually diminish. By day 14, although the viral RNA signals are almost undetectable by Northern hybridization, histological changes become more severe with massive mononuclear cell infiltration and profound myocardial necrosis. This delay in pathological development of myocarditis is compatible with previous culture and molecular studies and suggests the possibility that the viral genome can be detected before the histopathological changes become apparent in my-ocarditis.5,14,16 After day 14, viral RNA is not detectable by Northern blot analysis.

In the later stages of the disease, despite the diminished detection of EMC virus, the inflammation flourishes for at least 4 more weeks, showing gradual diminution of mononuclear cell infiltration and replacement with increasing fibrosis after day 14, followed by chronic dilated cardiomyopathy after day 28. This dissociation of viral presence and inflammatory damage probably occurs through activation of autoimmune mechanisms.2,3 This immune response is usually activated early during the viral infectious process and continues despite a decrease in the number of viral particles.

In our model, the PCR-amplified viral fragments are consistently detected throughout 6 weeks after inoculation, well beyond the ability of detection by either traditional viral culture (10 days) or Northern blot analysis (14 days). It is likely that the virus may continue to persist indefinitely beyond this time point, because there are reports showing evidence of residual inflammation up to 12 weeks of infective period16 and continued dilatation and hypertrophy of the heart in infected mice until day 90 after infection.17,18 The pathology of our murine myocarditis model on day 42 shows only very few isolated foci of cellular infiltration but is dominated by fibrosis and dystrophic calcification.

The affirmative signals of viral genomic fragments in the myocardium from PCR raise the issue of whether the viral persistence may potentially play a role in perpetuating the inflammation and the myocardial damage and leading to the progression from myocarditis to dilated cardiomyopathy. It is well recognized that viral infections involve interruption in the synthesis of vital cellular components and may lead to alterations in myocyte characteristics that result in dilated cardiomyopathy.39,40 Although PCR may detect residual target viral signals when viral replications have ceased, further findings of viral persistence alone do not necessarily implicate pathogenesis. Moreover, it is possible that once the original viral infection triggers an autoimmune response through molecular mimicry,34,41 further presence of the viral genomic materials may not contribute significantly to the development of pathology. Positive Southern hybridization using the virus-specific internal oligonucleotide probe further confirms that the virus is probably existing integrally at a very low level per cell. It also confirms the ability of PCR to amplify the viral RNA with extremely high sensitivity and specificity beyond the capabilities of any previous diagnostic techniques.

In this study, the PCR products represented in Figure 5 can only be interpreted as positive or negative for the EMC viral fragment. Its relative quantities based on the intensity of the bands cannot be gauged accurately with this PCR protocol. The quantitation of the PCR results would be possible only once the synthetic internal standard RNA that consists of site-directed mutagenesis is present and coamplified with the specific target viral RNA at a lower number of PCR cycles without reaching the plateau stage.32 Nevertheless, the findings do represent interesting possibilities that definitely deserve further study.

Our laboratory has previously demonstrated that enteroviral RNA genome can also persist in some patients suspected of myocarditis by using the PCR technique.15 However, we were able to positively dem-
onstrate this in only 10–15% of the samples studied. It is therefore unclear whether this is attributed to the heterogeneity of the samples, generation of incomplete or defective forms of viral particles that may conceal from immunologic surveillance and perpetuate the disease process, or that human myocarditis is due to etiologic agents other than Coxsackie or enteroviruses. Our study has shown that virus probably will persist in the myocardium in the late or chronic stage of the disease, albeit in very low quantities detectable only by PCR. Successful infection by enterovirus in the myocardial cells is presumably based on continued synthesis of the viral precursor polyprotein and correct processing of viral-encoded polymerase and other gene products such as the coat proteins. However, the lack of correct processing of virus gene products may result in defective replication with a limited production of infectious progeny virus\textsuperscript{13,39,40,43} and viral RNA persistence without active infection. Furthermore, this study also suggests that a significant portion of suspected myocarditis may be due to viral agents other than enteroviruses, thus prompting the development of other viral probes for diagnostic application.

Our data therefore support an important continuing relation between the viral genome and myocarditis/dilated cardiomyopathy in this murine model. The data further support the need to use PCR for the clinical diagnosis of viral myocarditis from preclinical stage to the late stages of dilated cardiomyopathy. In view of the potential differences between human and murine models of the disease, independent confirmation in clinical settings will still be necessary.

The potential ability to diagnose definitely a viral etiology in the early and late stages of myocarditis/dilated cardiomyopathy has profound clinical implications. For instance, this approach may enable the epidemiology and risk profiles of the population to be defined. Furthermore, the ability to establish the viral diagnosis early in the course of the disease by molecular techniques opens up avenues of therapy such as specific antiviral agents\textsuperscript{44} that may terminate the impact of the disease early in its course and decrease the opportunities of viral persistence.

Summary
We have demonstrated that in a well-established murine myocarditis, the EMC virus persists well into the chronic stage of the disease. This suggests that PCR is very sensitive and specific for the residual viral RNA and will be important in the clinical diagnosis of myocarditis and dilated cardiomyopathy. The persisting viral genomic material found does not imply pathogenesis at this time. However, the potential relation between the molecular mechanism of virus persistence and the perturbed myocyte functions in the pathogenesis of dilated cardiomyopathy after myocarditis may represent a promising area for future investigation.

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