Cardiac Persistence of Cardioviral RNA Detected by Polymerase Chain Reaction in a Murine Model of Dilated Cardiomyopathy

Bun-sho Kyu, MD; Akira Matsumori, MD; Yukihito Sato, MD; Ikutaro Okada, MD; Nora M. Chapman, PhD; and Steven Tracy, PhD

**Background.** In our model of dilated cardiomyopathy (DCM), cardiac dilatation and hypertrophy developed after inoculation of encephalomyocarditis virus (EMCV), but the infectious virus was isolated only early after infection. In this study, we investigated whether viral RNA could be detected at later times using the polymerase chain reaction (PCR).

**Methods and Results.** In the in vitro study, FL (human amnion) cells infected with EMCV were harvested for RNA extraction, and viral cDNA was synthesized by reverse transcription with random hexamers. Using oligonucleotide primers with homology to the 5' noncoding region of EMCV, we enzymatically amplified a 121-base pair band, which was homologous to a probe specific for EMCV as demonstrated by Southern blot hybridization. The sensitivity of this PCR technique was at the level of about 10^2-10^3 copies of viral RNA genome. In the in vivo study, four-week-old DBA/2 mice were inoculated with EMCV intraperitoneally (10 pfu/mouse) and killed on days 1, 2, 3, 5, 7, 10, 14, 18, 28, 60, and 90. The hearts were divided into three parts for purification of total RNA, histopathological examination, and to culture for infectious virus. The infectious virus was isolated from the heart after the second day but never after the 14th day. The viral genome was detectable by PCR on the second day, when very little mononuclear cell infiltration around the blood vessels was histologically visible. Positive PCR signals were observed in all hearts through day 14. Viral RNA was also detected in four of six 28-day samples, four of six 60-day samples, and two of seven 90-day samples when diffuse myocardial fibrosis was prominent, but myocardial necrosis or cellular infiltration had disappeared.

**Conclusions.** The persistence of EMCV RNA was shown by PCR in the chronic stage of EMCV-induced myocarditis, a time when the inflammatory reaction had largely subsided. The PCR is a potentially useful method to test possible viral etiologies in idiopathic heart muscle disease or DCM. (Circulation 1992;86:522–530)

**Key Words** • polymerase chain reaction • cardiomyopathy, dilated • virus, encephalomyocarditis • myocarditis, viral

Dilated cardiomyopathy (DCM) has been defined as a heart muscle disease of unknown cause with ventricular dilation. It is considered to be the most likely end stage of myocardial damage from various factors such as alcohol, malnutrition, puerperium, toxins, genetic defects, and infection. Among these, the most important has been thought to be viral infection, especially with coxsackievirus B (CVB), because traces of humoral and cellular immune responses to viral agents as well as the detection of enteroviral RNA in myocardium have been found in some DCM patients. In addition, patients with viral myocarditis have progressed to DCM in long-term follow-up studies.

Unfortunately, conventional methods of viral detection, such as viral culture and serologic evaluation, are laborious and relatively insensitive; thus, definite clinical diagnosis is difficult even in acute myocarditis. Developments in molecular biological techniques, such as Southern and Northern blotting and in situ hybridization, have provided new tools for probing this field. Using an enterovirus group-specific cDNA probe in slot-blot hybridization studies, a group reported positive signals in six of 21 explanted hearts of end-stage DCM patients. The development of the polymerase chain reaction (PCR) can amplify few copies of a target sequence to a concentration for conventional analysis. This method enhances the diagnostic capability by rendering single copies of a gene detectable amid a background of 10^6 cells.

We have previously reported an animal model of DCM in inbred strains of DBA/2 mice infected with encephalomyocarditis virus (EMCV). After acute my-
ocarditis in this model, we demonstrated prominent myocardial fibrosis, hypertrophy of myocardial fibers, and very little cell infiltration in the chronic stage of viral myocarditis, which is similar to those seen in human DCM. Infectious virus was isolated from the heart only during 2–10 days after inoculation.

We have extended our study to the chronic stage of this model of EMCV-induced myocarditis to ask whether viral RNA is detectable longer after EMCV inoculation using the PCR.

Methods

Viruses

EMCV (M variant) and CVB3 (Nancy strain) were used in this study. Virus stocks were prepared by propagating viruses in FL (human amnion) cell monolayer cultures. FL cell monolayers were infected with 10 plaque-forming units (pfu) EMCV per 75-cm² culture flask and were harvested when cytopathic effects were complete. After three cycles of freezing and thawing, the viral suspensions were centrifuged, and the supernatant was divided into aliquots that were stored at −80°C until diluted for use. Virus titers were determined by plaque formation on FL cell monolayers.13

Viral Infection of Mice

Inbred DBA/2 mice were obtained from the Shizuoka Agricultural Cooperation Association (Shizuoka, Japan). At 4 weeks of age, mice were inoculated intraperitoneally with 0.1 ml of EMC virus diluted in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH₂PO₄, 7.2 mM KH₂PO₄, pH 7.2) to a concentration of 1×10⁶ pfu/ml. Mice (n=56) were killed by cervical dislocation on days 1, 2, 3, 5, 7, 10, 14, 18, 28, 60, and 90. Ten noninfected mice were used as controls. Immediately after the mice were killed, the hearts were removed aseptically and divided into three parts longitudinally. One portion was rapidly frozen in dry ice for RNA extraction. The second was immediately homogenized in Eagle’s minimal essential medium (EMEM) for viral culture, and the third was fixed in 10% formalin for histopathological analysis.

Nucleic Acids

EMCV-infected cells were collected 24 hours after infection by centrifugation, washed with ice-cold saline, and counted. Serial dilutions of cell samples were made and then lysed in a solution containing 8 M NaSCN, 50 mM EDTA, 5 mM EGTA, 50 mM Tris-HCl, pH 7.5, 142 mM 2-mercaptoethanol, 1% N-lauroyl sarcosine, proteinase K (500 μg/ml) for 10 minutes at 50°C and 5 minutes at 98°C. The mixtures were cooled (made 2.5 M in ammonium acetate), and nucleic acids were precipitated with ethanol.7 DNA was removed with ribonuclease-free DNase 1 (RQ1 DNase, Promega Corp., Madison, Wis.). Samples were then deproteinized with repeated phenol/chloroform extractions, ethanol precipitated, and suspended in 10 μl of sterile, distilled water. Preparations were stored at −70°C.

Samples (5–10 mg) of murine hearts were homogenized in ice-cold tissue culture medium. Homogenates were mixed with two volumes of 8 M NaSCN, 50 mM EDTA, 5 mM EGTA, 50 mM Tris-HCl, pH 7.5, 142 mM 2-mercaptoethanol, 1% N-lauroyl sarcosine, diluted to 1 M NaSCN with 50 mM EDTA, 50 mM Tris-HCl, pH 7.5, and nucleic acids were extracted twice with phenol/chloroform, once with chloroform and precipitated as above. Nucleic acid pellets were suspended in 100 μl of sterile, distilled water and stored at −20°C. All reusable glassware was thoroughly washed with detergent, followed by careful rinsing with deionized water and steam sterilization.

Oligonucleotides

Oligonucleotide primers were suspended in water and stored at −20°C. The EMCV oligonucleotides, EMC-1 through EMC-3, were derived from sequences in the 5′-nontranslated region of the EMC virus genome (Figure 1). Enzymatic amplification of EMC cDNA with the EMC-1 and EMC-2 primers results in the synthesis of a 121-base pair (bp) fragment. The middle 40-bp sequence (EMC-3) has no homology to EMC-1 and EMC-2 and was chosen as a hybridization probe. The primers used for the α-tubulin gene PCR amplification, AT-1 and AT-2, were derived from exon 4 of the human α-tubulin gene nucleotide sequences that contain conserved homologous genes of different species, including mouse (N=nucleotide, N2999-3286, GENBANK LOCUS HUMTUBAG; N535-822, GENBANK LOCUS MUSTUBALM). The amplified fragment that results is 288-bp.

Synthesis of cDNA and Enzymatic Amplification

cDNA was synthesized in 20-μl volumes containing 2 μl of total infected FL cell RNA or heart RNA sample, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (wt/vol) gelatin (Sigma Chemical Co., St. Louis, Mo.); 0.2 mM each deoxy (dT)ATP, dGTP, dTTP, and dCTP (Perkin Elmer Cetus, Norwalk, Conn.); 0.25 unit/ml at an optical density of 260 nm of random hexamers (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.); and 10 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Md.). Reactions proceeded at 40°C for 30 minutes.
For PCR amplification, the following reagents were added to 2 μl of the reverse transcriptase mixture: 11.2 μl of ddH2O; 1.8 μl of ×10 reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM MgCl2, 0.01% [wt/vol] gelatin); 2 μl of diluted mix of deoxynucleotides (2 mM each dATP, dCTP, dGTP, and dTTP); 1 μl each of EMC-1 and EMC-2 primers (optical density of 2.5/ml at 260 nm); and 1 μl of 0.5 units of Thermus aquaticus (Taq) DNA polymerase (GeneAmp kit, Perkin Elmer Cetus) per milliliter. Amplifications were performed in a DNA thermal cycler (Perkin Elmer Cetus) programmed as follows: after heat denaturation for 1 minute at 94°C, 40 cycles of denaturation at 94°C for 1 second and annealing at 55°C for 1 second. In this investigation, for all those PCRs that were negative after 40 cycles of amplification, 2 μl of PCR product was added to the same reagents substituting reverse transcriptase mixture, and 40 more cycles of PCR were performed as described above.

Agarose Gel Electrophoresis and Southern Blots

PCR reaction products (10 μl) were electrophoresed at 100 V in 1.5% agarose gels in 0.2×TAE (Tris acetate) (1×TAE: 40 mM Tris acetate [pH 7.8], 1 mM EDTA). Gels were stained in ethidium bromide, and DNA was detected by illumination with ultraviolet light (302 nm). Gels were immersed in 0.5 M NaOH/1 M NaCl for 1 hour at room temperature, and the DNA was transferred onto membranes (Magna Nylon; Micron Separations Inc., Westboro, Mass.) by Southern blotting in 0.05 M NaOH/1 M NaCl. Membranes were baked at 80°C for 2 hours.

Oligonucleotide probes were labeled with 32P using T4 polynucleotide kinase and [γ-32P]ATP (3,000 Ci/mmol; Amersham International plc, Buck's, UK) to a specific activity of 1×10⁸ cpm/pmml. Labeled probes were purified by Sephadex G-50 (Pharmacia) chromatography. Blots were prehybridized and hybridized in 500 mM NaCl, 0.1% (wt/vol) dry milk, 5 mM EDTA, 10 mM sodium phosphate buffer (pH 6.8), and 0.1% sodium dodecyl sulfate (SDS) at 37°C for 2–4 hours. Blots were washed in a covered container in 3 M tetramethylammonium chloride.19 Blots were exposed for various periods to x-ray film at −70°C with intensifying screens.20

Virus Isolation and Histopathology

For virus isolation, excised heart portions (n=16; four each from days 2, 3, 10, and 14) were ground with autoclaved sea sand in 2.0 ml of EMEM. After centrifugation at 1,500g for 15 minutes at 4°C, 0.1 ml of supernatant was inoculated onto FL cells containing 1.0 ml of MEM supplemented with 2.0% fetal calf serum. The tubes were observed daily for 7 days for the appearance of characteristic cytopathic effect.

Other heart sections (n=13; two from normal controls, three from day 90, and four each from days 2, 14, and 28) were fixed in 10% formalin solution, embedded in paraffin, and stained with hematoxylin–eosin for histopathological examination.

Results

Fidelity, Sensitivity, and Specificity of the Technique

Viral positive-strand RNA extracted from FL cells and murine hearts were used as templates to synthesize cDNA with random hexamers and reverse transcriptase. Thereafter, cDNAs were amplified by 40 cycles of PCR as described in “Methods.” PCR products were analyzed by agarose gel electrophoresis and Southern blot hybridization with the radiolabeled oligonucleotide probes.

The sequences of the EMC-1 and EMC-2 primers and their locations in the EMCV genome are shown in Figure 1. The EMC-3 oligonucleotide is located between the EMC-1 and EMC-2 primers and has no homology to EMC-1 and EMC-2 primers. A computer comparison of CVB1, CVB3, and CVB4 genomic sequences as well as those of picornavirus 1-3 detected only partial identity (data not shown).

EMCV-infected cell RNA was used to demonstrate the use of the EMC-1 and EMC-2 primers. The RNA was reverse transcribed and amplified as described (Figure 2, lane 1). As expected, a band of approximately 121 bp was observed. No bands were observed when uninfected cell RNA or RNA from CVB3-infected cells were used as templates (Figure 2, lanes 3 and 5, respectively). In each case, however, RNA capable of acting as a template was present, as the α-tubulin primers AT-1 and AT-2 generated the expected fragment of 288 bp in parallel amplifications (Figure 2, lanes 2, 4, and 6). In this work, parallel amplifications with AT-1 and AT-2 primers were used as positive controls for the presence of RNA in each sample analyzed for viral RNA by PCR.

The sensitivity of detection of this PCR protocol was assessed in the following manner. When all infected cells demonstrated advanced cytopathic effects (cell rounding), cells were harvested, counted, and serially diluted. RNA was purified from samples containing from 10³ through the equivalent of what would be 10⁵ cells. The EMCV-specific, 121-bp fragment generated

![Figure 2. Photograph of enzymatic amplification of RNA from encephalomyocarditis virus–infected, uninfected, and coxsackievirus B3–infected cells. Polymerase chain reaction (PCR) products were analyzed by agarose gel electrophoresis as described in “Methods.” Primers used for the PCRs and the expected sizes of the amplifiers are shown. Lanes 1 and 2, encephalomyocarditis virus–infected cells; lanes 3 and 4, uninfected cells; lanes 5 and 6, coxsackievirus B3–infected cells. E, EMC primers (121 base pairs [bp]); A, α-tubulin primers (288 bp).](http://circ.ahajournals.org/Download?uri=10.1161/01.CIR.86.2.524)
by amplification using EMC-1 and EMC-2 primers was observed at all times in as few as 10 cells (Figure 3). Occasionally, one infected cell was detectable (Figure 3). However, in general, the equivalent of 1–0.01 infected cells was not detectable in this manner. As it seemed likely that the inability to regularly detect one infected cell may be due to losses incurred during RNA purification, we diluted the RNA from one infected cell serially and always in the presence of the equivalent of 10^6 uninfected cells' RNA. These dilutions were then used as template for reverse transcription and PCR with the EMCV primers. Results from this experiment (Figure 4) demonstrated that the diagnostic EMCV-specific, 121-bp fragment could be detected in a 1 x 10^-2 dilution of infected-cell RNA in the presence of RNA from 10^6 uninfected cells. Although the band is very faint, reamplification of the PCR products showed a definite 121-bp band (Figure 4).

Detection of Viral RNA in Infected Murine Hearts

The viral genome was detectable in all five heart specimens on the second day after virus inoculation; Figure 5 shows representative results of these experiments. Viral RNA was detectable in every specimen from day 2 until day 14, in five of six on day 28, three of six on day 60, and two of seven on day 90 after 40 cycles of amplification, and one more on day 60 was detected after 40 more cycles of amplification (Table 1). Viral RNA was not detected in samples from 10 uninfected murine hearts. Amplification of α-tubulin RNA revealed that mRNA was present in all nucleic acid samples from uninfected and infected murine hearts.

Analysis of all amplification products by Southern blotting hybridization showed that only samples that demonstrated correct size (121 bp) hybridized with the EMCV-specific oligonucleotide probe (EMC-3) (data not shown), thus confirming the presence of EMCV...
RNA in the hearts. No hybridization of the EMC-3 probe was observed in PCR products without a visible band, even in the chronic stage of the disease.

**Pathological Findings**

No significant pathological changes were observed among day-2 heart section samples; a few mononuclear cells were apparent around some blood vessels (Figure 6B; compared with uninfected normal murine heart, Figure 6A). Extensive myocardial necrosis with calcification and prominent mononuclear cell infiltration was evident on day 14, however (Figure 6C). Heart sections from day-28 hearts displayed a decrease in cellular infiltration, and myocardial fibrosis was evident (Figure 6D). On day 90 after infection, myocardial fibrosis was prominent, and hypertrophy of myocardial cells was evident. Myocardial calcification persisted through 90 days after inoculation, but there was virtually no mononuclear cell infiltration (Figure 6E). There were two samples (one each from days 60 and 90) that revealed only pericardial calcification without myocardial injury: This was thought to be spontaneous calcification. PCR analysis did not detect the viral genome in these two samples (data not shown).

**Virus Isolation**

Infectious virus was isolated in all heart samples of infected mice on days 2 and 3 and in two of four samples from day 10 (Table 1). No infectious virus was detected in the hearts of mice after day 14.

**Discussion**

Although much evidence favors the concept that DCM can be a postviral disease, the actual prevalence and pathogenesis of viral heart disease in DCM has not been well explored because definite diagnosis of viral infection is still difficult. The current methods of viral detection include viral culture, immunological assay, and nucleic acid hybridization. Virus isolation procedures are time-consuming, relatively insensitive, and only possible during the first few days of acute illness, when symptoms are not generally obvious. Serological approaches, such as quantification of virus-neutralizing antibodies and determination of complement fixation

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**Table 1. Results of Polymerase Chain Reaction Analysis, Virus Isolation, and Histopathological Examination in Murine Hearts**

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>10</th>
<th>14</th>
<th>18</th>
<th>28</th>
<th>60</th>
<th>90</th>
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<tr>
<td>Number of hearts positive by PCR for viral RNA/total number of hearts</td>
<td>0/7</td>
<td>5/5</td>
<td>7/7</td>
<td>2/2</td>
<td>2/2</td>
<td>4/4</td>
<td>4/4</td>
<td>5/6</td>
<td>4/6</td>
<td>4/6</td>
<td>2/7</td>
<td>0/10</td>
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<tr>
<td>Virus isolation (positive/number examined)</td>
<td>ND</td>
<td>4/4</td>
<td>4/4</td>
<td>ND</td>
<td>ND</td>
<td>2/4</td>
<td>0/4</td>
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<td>Cell infiltration</td>
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<td>Myocardial necrosis</td>
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PCR, polymerase chain reaction; ND, not done.
titers, lack specificity because of the large number of serologically distinct viruses. Demonstration of at least a fourfold rise in specific antibody in paired acute (less than a week) and convalescent (2 weeks or longer) serum specimens is limited in use because of the lack of diagnosis in the early stage. Although nucleic acid hybridization is a potential tool for virus identification, the copy number of target nucleic acids to some extent is critical. Rotbart and colleagues reported that the sensitivity limits of detection of enteroviruses by single-strand RNA hybridization probes is approximately 10 pg of RNA, which translates to about 10^6 enterovirus RNA genomes.

In this study, we investigated the use of PCR to selectively increase the copy number of target cardiovirus sequences for ease of detection. The specificity of the primers EMC-1 and EMC-2 were demonstrated by the ability to prime the appropriately sized amplimer only from RNA derived from EMCV-infected cells or mice (Figures 2 and 5). In no case was the EMCV-specific, 121-bp fragment detected when using cDNA derived from reverse transcription of uninfected cells, normal murine heart tissues, or CVB3-infected cells. Computer searches of picornaviral genomes show no significant identity with human enteroviral sequences (data not shown), which is consistent with our results in which coxsackievirus RNA was not detected. All three EMC primers used in this study (Figure 1) should also be useful for the detection of Mengo virus, a related primate cardiovirus, as each primer is highly conserved in the sequences of this virus’ 5’NTR (nontranslated region) as well (personal

**Figure 6.** Photomicrographs of histopathological findings in murine heart sections. Panel A: Heart from an uninfected 4-week-old DBA/2 mouse; panel B: 2 days after inoculation; panel C: 14 days after inoculation; panel D: 28 days after inoculation; panel E: 90 days after inoculation. Hematoxylin–eosin stain; original magnification, ×350.
communication, A. Palmenberg to S. Tracy). However, comparing the primers with the sequence of the other well-characterized cardiovirus, Theiler's murine encephalomyocarditis virus (TMEV), suggests that TMEV will not be detected. That the primers EMC-1 through EMC-3 will detect two known cardioviruses suggests that these primers might be a useful reagent in any search of human tissue for a putative human cardiovirus.

The sensitivity of our PCR approach is at or below the level of a single EMCV-infected cell (Figures 3 and 4). When infected cells were aliquoted, extracted for nucleic acids, and probed by PCR to test how few cells were detectable by PCR using the EMC-1 and EMC-2 primers, 10 cells were reproducibly detectable. The detection of a single infected cell was variably successful and probably due to both the statistical uncertainties of adding a single cell to a tube and loss of nucleic acids during the extraction process (Figure 3). However, when purified RNA from infected cells was used in reconstruction experiments, the equivalent of 1 × 10⁻² of an infected cell’s viral RNA content was detectable (Figure 4). In addition, this was in the presence of RNA from 100,000 uninfected cells. Calculations of the copy number of picornaviral RNA molecules in infected cells in culture are on the order of 1 × 10⁹ to 10¹⁰ genomes per cell.¹²,²² Thus, the absolute sensitivity of our PCR procedure from reverse transcription through detection of the EMCV-specific amplimer on a gel is on the order of about 100 to 1,000 EMCV genomes, or fewer viral RNA molecules than would be present in a productively infected cell. Transcription of RNA into cDNA is less than 100% efficient; thus, the detection of single molecules of RNA in this fashion cannot be achieved. Reamplification of any PCRs that are negative in the first series of amplification should be considered in order to maximize the signal.

There are some problems of obtaining RNA from the small transcutaneous endomyocardial biopsy samples. The size of the specimen may be the essential one. However, the site (like endocardium), the ingredients, and RNAse should be considered. We found that at least 1 mg of heart tissue is necessary for adequate RNA extraction (unpublished observation), and samples (5–10 mg) used in this investigation were sufficient for study.

Animal models of viral myocarditis were extensively studied for elucidating the pathogenetic mechanisms of the relation between human viral myocarditis and DCM. We found that severe myocarditis was induced in BALB/c, C3H/He, and DBA/2 mice inoculated with EMC virus but not in A/J and C57BL/6 mice,²³,²⁴ which indicates that genetic factors play a role in susceptibility to infection and severity of the disease. Furthermore, lesions that resemble those seen in DCM developed in inbred strains of DBA/2 mice in the chronic stage despite that EMCV could not be isolated from the hearts and EMCV antigen could not be detected in the myocardium after the second week of infection.²⁴,²⁵

Using this sensitive and specific technique, the natural course of the EMCV-induced model of murine DCM was analyzed. To evaluate the acute stage (days 1–5 after inoculation), we amplified RNA from the infected murine hearts from the first day after viral inoculation. Positive results were first noted in hearts 2 days after inoculation, when no significant changes were noted upon histological examination except for slight mononuclear cell infiltration around some blood vessels. Infectious virus was also detected in viral culture from these samples, as shown in Table 1. These results revealed that PCR and viral culture could identify the virus in the very acute stage, when the virus began to replicate in the heart tissue well before visibly damaging the myocytes. Myocardial necrosis became apparent on the fourth day, and cellular infiltration gradually increased, which is consistent with our previous results.¹⁷ The most evident tissue necrosis and prominent mononuclear cell infiltration were noted on the 14th day, however. PCR detected viral RNA in all samples from day 3, 5, 7, 10, and 14; in contrast, infectious virus in cell culture was observed until day 10 but never after day 14. In the chronic stage, the viral genome was detected by PCR in four of six 60-day samples and two of seven 90-day samples when diffuse fibrosis was prominent, but myocardial necrosis or cellular infiltration had disappeared.

Various strategies for detecting picornaviruses with PCR have been investigated.²⁰,²⁶,²⁷ Using primers derived from consensus sequences in the 5' NTR region, PCR can detect a variety of viruses. Two groups have reported detecting enterovirus RNA by PCR in myocardial biopsy samples from patients with myocarditis and DCM.²⁶,²⁹ Using primers that detect a wide variety of enterovirus, Jin and colleagues²⁸ reported the presence of the viral genome in two of four samples (50%) with histological evidence of myocarditis and three of 21 samples (14%) with histological evidence of DCM. Weiss and coworkers²⁹ detected CVB3 viral RNA in one of five myocarditis samples (20%) and in none of 11 DCM samples. These levels of positive detection of enteroviruses by PCR are on the same order of magnitude as those reported using either blot or in situ hybridization approaches.⁷,¹⁰,¹³,¹⁴,³⁰

This and other studies³¹–³³ describing persistence of picornaviral infections in cells in culture and in experimentally infected mice raise broader questions in the issue of picornavirus-induced heart disease. What is the mechanism of picornaviral persistence? Host cell function alterations, the generation of defective interfering particles, and neoantigen expression are likely candidates.³⁴ If the mechanism(s) were known, procedures to interrupt and possibly eradicate the viral infection might be envisaged. Pharmaceutical delivery of antisense viral RNA derived from a conserved genomic region, compounds designed to disrupt viral proteases, or drugs that irreversibly bind viral capsids are being researched. How does a persistent picornaviral infection affect the host’s immune response in the development of chronic disease? Chronically infected myocytes may lose function, thus interfering with overall myocardial function and proceeding to cardiomyopathy. Cell-mediated and humoral immune mechanisms have been proposed, based on murine models and clinical observations.³⁵–³⁷

Of the lymphocytic component, most cells in myocardium in the acute stage (days 7 and 14) are T cells in this model.³⁸ As the disease progresses to the chronic stage, T cells decrease markedly and B cells increase and exceed T cells (days 30 and 75),³⁹ which suggests little interaction between T lymphocytes and chronic changes. In addition, the immunosuppressive therapy...
with prednisolone, cyclosporine, and anti–IL-2 receptor monoclonal antibody in EMCV myocarditis, after the period of viral replication and neutralizing antibody titer reaching high, does not improve the disease course.30–32 Finally, which picornaviruses are most commonly linked in the viral etiology of inflammatory heart disease and its sequelae? Clearly, enteroviruses are etiologic in at least some cases.7,10,13,14,37 To date, there is no evidence for a potential etiologic role of EMCV and/or other cardioviruses in human myocarditis and DCM. Our studies demonstrate that it is possible for the RNA genome of a highly lytic virus to persist for up to 90 days after inoculation in the hearts of experimentally infected mice and in the absence of detectable infectious virus. Using a technique capable of detecting fewer viral genomes than would be present in a productively infected cell, cardioviral RNA was nevertheless detected only in two of seven of these mice (28%). These data demonstrate that the detection of EMCV RNA, even under excellent experimental conditions, is not consistent within inbred animals. Therefore, detection of enteroviral RNA in DCM samples from humans, clearly an outbred population, may be more problematic. However, as the primers used in this study will permit the detection of two of the known cardioviruses, these primers might be useful in any screen of human myocarditic and DCM cardiac tissues for the presence of heretofore unidentified cardioviruses.

In situ hybridization has been shown to be a powerful tool with high sensitivity and specificity for studying viral infection. Using cloned CVB3 cDNA as a diagnostic probe, as few as 20 viral copies per cell were estimated to be detected in CVB3-infected Vero cell culture.33 Klingel and colleagues33 used strand-specific in situ hybridization in a model of persistent heart muscle infection and found that the amount of viral plus-strand RNA was the same as that of minus-strand RNA in myocardial cells, which suggests that viral replication may be restricted at the level of RNA synthesis. In situ hybridization is the technique of choice if it is desired to correlate nucleic acid presence with histopathological features, whereas PCR offers equivalent sensitivity and much greater rapidity of assay.

Summary

By using a sensitive PCR approach to the detection of EMCV RNA, we have demonstrated that EMCV RNA can persist in the absence of infectious virus for as long as 3 months after inoculation in the hearts of experimentally infected mice. This permits further investigation into the interaction of the cardioviral genome with the host cell in the model of EMCV-induced chronic murine heart disease. This PCR approach suggests a useful method for searching for unidentified cardioviruses.

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Cardiac persistence of cardioviral RNA detected by polymerase chain reaction in a murine model of dilated cardiomyopathy.

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