

Coxsackievirus B3 genomes detected by polymerase chain reaction: evidence of latent persistency in the myocardium in experimental murine myocarditis

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Summary. We have investigated the time course after infection in coxsackievirus B3 murine myocarditis to determine the extent (if any) of persistent or latent infection that might be responsible for recurrence. We employed a polymerase chain reaction (PCR) method that can detect an extremely small amount of genome by amplification techniques in four-week-old BALB/c mice (n=140) infected with coxsackievirus B3 by a single intraperitoneal injection of 1×10^4 plaque-forming units (PFU)/mouse (Group 1) and 1×10^2 PFU/mouse (Group 2). Mice were sacrificed on days 3, 5, 7, 10, 14, 21 and 28, and their hearts were resected for RNA extraction. Single chain DNA was synthesized from 1 μ g of RNA and the viral genome was amplified by PCR. The virus genome was strongly amplified in Group 1 from days 3 to 10, and in Group 2 from days 5 to 7, but afterwards both amplifications rapidly diminished. However, a positive signal, though very faint, persisted in both groups until day 28, by which time all histological evidence of myocarditis had disappeared in Group 2.

Our results demonstrated that there was persistent or latent virus infection in the myocardium throughout the entire study period of 28 days. Such persistence might provide a pathomechanism for the exacerbation and recurrence of myocarditis.

Key words: Coxsackievirus B3, Virus RNA, Persistent infection, Chronic myocarditis, Polymerase chain reaction

Introduction

Coxsackie B viruses are thought to be a major etiological factor in human myocarditis (Abelmann, 1973; Lerner and Wilson, 1973; Woodruff, 1980). Viral infections involving the heart result first in degeneration and then in necrosis of myocytes with inflammatory cell

infiltration. Interstitial fibrosis subsequently develops (Leslie et al., 1990). Clinically, viral myocarditis is diagnosed by retrospective serology, by histopathology on biopsy samples and rarely by either isolation of the infectious virus or by immunofluorescent detection of virus-specific antigens (Morgan-Capner et al., 1984). Direct demonstration of the virus in the myocardium by reisolation of infectious virus has been difficult to achieve in myocarditis.

In the coxsackie B virus murine myocarditis models developed by Reyes and Lerner (1987), the virus, which was first isolated from the myocardium on day 2, reached peak titers on day 3 and persisted through days 5 to 7 after infection. It was thought that the virus then disappeared fairly soon afterwards from the myocardium. However, Bowles et al. (1986) detected coxsackie-B-virus-specific RNA sequences that persisted after the acute phase in myocardial biopsy samples from patients with myocarditis and dilated cardiomyopathy. Adequate data are not yet available to determine whether or not, or by what mechanism, virus can persist in the heart for long periods of time.

A recently developed polymerase chain reaction (PCR) method (Saiki et al., 1985; Erlich et al., 1988) can be used to detect the coxsackie B3 viruses, as their molecular genetics are well understood (Lindberg et al., 1987). The coxsackie B3 genome is a single-stranded RNA molecule of 7.4 kilobases (kb). PCR can effectively detect very low levels of this genome. Therefore, it can be very useful in providing a better understanding of the natural history of the infection and in its diagnosis (Kato et al., 1990; Lampertico et al., 1990; Paterlini et al., 1990).

Using PCR, we have investigated the time course and persistence of infection in coxsackie B3 virus murine myocarditis.

Materials and methods

Virus and cells

Coxsackie B3 virus (Nancy strain) (CB3) was

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obtained from Dr. Minoru Hara of the National Institute of Health, Japan. The virus was grown in cultures of VERO (African green monkey kidney) cells at a density of 5×10^5 cells/ml. The virus was purified by the method of Hamada and colleagues (Hamada et al., 1988). Briefly, the VERO cells seeded with viruses were incubated in MEM (Eagle's minimum essential medium, Nissui, Tokyo) at 37 °C until complete destruction of the cells. The harvest was then frozen and thawed once, and subjected to further procedures, including CsCl isopycnic centrifuging. The final supernatant was again subjected to CsCl centrifugation, and the fractions of the peak areas were collected. The virus preparations were titered by the previously described plaque-forming assay (Huber et al., 1981). The virus titer was 7.0×10^7 plaque-forming units (PFU)/ml.

Purification of viral RNA

The viral RNA was purified by the method of Stålhandske et al. (1984). After the desalting of CsCl centrifugation fractions by ultrafiltration (Centriflo CF25, Amicon), the supernatant was twice subjected to phenol-chloroform-octanol (25:24:1) extraction. The water phase was then reextracted twice before the RNA was precipitated with 2.5 volumes of ethanol. The RNA was subsequently dissolved in TE buffer (0.01M Tris-HCl, (pH 7.9), 1mM ethylenediaminetetraacetate solution (EDTA)).

Molecular cloning

cDNA synthesis was performed using a cDNA Synthesis System Plus (Amersham). At first, we synthesized the first-strand cDNA. Five micrograms of RNA were added to 90 µl of mixture solution containing 10 µl of 5x first-strand synthesis buffer, 2.5 µl of sodium pyrophosphate, 50 units of human placental ribonuclease, 5 µl of deoxynucleotide triphosphate mixture (1mM of dATP, 1mM of dGTP, 1mM of dTTP and 0.5mM of dCTP), and 4 µg of oligo(dT)₁₂₋₁₈ primer, which annealed to be polyadenylated 3' region of viral RNA. After gentle mixing, 100 units of reverse transcriptase were added. These reaction mixtures were incubated at 42 °C for 60 minutes and then at 65 °C for 10 minutes. In the next step, we synthesized the second-strand cDNA. Fifty microliters of the first-strand cDNA reaction mixture were then added to 197.5 ml of mixture solution containing 93.5 µl of second-strand synthesis buffer, 4.0 units of *E. coli* ribonuclease H, and 115 units of *E. coli* DNA polymerase I. These mixtures were incubated at 12 °C for 60 minutes, then at 22 °C for 60 minutes, and at 70 °C for 10 minutes. After gentle spinning of the mixture was incubated at 37 °C for 10 minutes, after which the reaction was stopped by adding 10 µl of 0.25M EDTA (pH 8.0). The double-strand cDNA was extracted with phenol/chloroform, precipitated by ethanol, and again dissolved and precipitated by 4M ammonium acetate/ethanol for

purification.

The cDNA, linked with EcoRI adapter, as ligated into the λ gt10 EcoRI arm, and was then packaged *in vitro* into bacteriophage packaging λ particles. *E. coli* L87 were infected with bacteriophages, and a bacteriophage having the cDNA insert was screened. Finally, a 2.0-Kb CB3 cDNA fragment was obtained and subcloned into the EcoRI cleavage site of the pUC119 plasmid. The cDNA inserts were checked by agarose gel electrophoresis, and a ca. 1.0-kb BamHI-BamHI fragment and two BamHI-HindIII fragments (ca. 450 bp and ca. 620 bp) were then cleaved from the recombinant cDNA. This result was compatible with the cleavage sites for the restriction enzymes of the CB3 genome sequence (Stålhandske et al., 1984; Lindberg et al., 1987). Thus, we concluded that we had cloned a portion of CB3 cDNA, located ca. 2.0-kb from the 3'-terminal end of the genome.

Animals and experimental infection

Two hundred and ten BALB/c mice were obtained from the Central Institute of Experimental Animals in Japan and 4-week-old adult males of the strain were used for the experiment. The animals were divided into 3 groups (70 mice, each). The first group was inoculated intraperitoneally with 1×10^4 PFU of CB3 in 0.1 ml phosphate-buffered saline (PBS; pH 7.4) (Group 1); the second group was inoculated with 1×10^2 PFU of CB3 in 0.1 ml PBS (Group 2); and the third group was injected only with 0.1 ml PBS (Group 2); and the third group was injected only with 0.1 ml PBS, serving as controls. The animals were anesthetized with sodium pentobarbital and sacrificed on the 3rd, 5th, 7th, 10th, 14th, 21st and 28th postinoculation day. Ten mice in each group were sacrificed each day. The heart was excised above the origin of the great vessels, and blood was collected for virus titer assay. The heart was hemisected transversely at the midportion of the ventricle; the apical part was used for morphological study and the basal part was washed 5 times with PBS to remove as much blood as possible, and was then processed for the extraction of the viral mRNA.

RNA Isolation from the heart

Total RNA was extracted by the guanidinium/cesium chloride method (Sambrook et al., 1989). Briefly, the minced heart was homogenized in 5.2M guanidine isothiocyanate solution with a polytron homogenizer. Total cellular RNA was isolated by ultracentrifuging the homogenate through 5.7M cesium chloride/0.1M EDTA, followed by phenol and chloroform extraction and ethanol precipitation, and then dissolution in diethylpyrocarbonate solution.

Polymerase chain reaction testing

First-strand cDNA synthesis. We synthesized the first-

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strand cDNA using a cDNA Synthesis System Plus (Amersham). The assay was started from 1 µg of RNA, and the resulting synthesized first-strand cDNA samples were ready for PCR gene amplification.

PCR gene amplification. The virus domain was amplified by PCR using Taq polymerase. The amplified area was thought to be the coding region of CB3 RNA polymerase and the yield size was 461 bp (Hind III - BamHI fragment) (Fig. 1). Twenty microliters of the above-mentioned samples were added to 79.5 µl of 10mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin, 200 µM of each deoxynucleotide triphosphate and 0.1 µM of each primer (Fig. 1). Then, 2.5 units (5 U/µl) of Taq polymerase (Perkin-Elmer-Cetus, Norwalk, CT) and 100 µl of mineral oil were added. The PCR reactions were carried out on a Program Temp Control System PC-700 ASTEC, Japan). After heating at 94 °C for 2 minutes to ensure DNA denaturation, each PCR cycle consisted of incubation at 94 °C for 1 minute, incubation at 54 °C for 2 minutes and incubation at 74 °C for 3 minutes. After 28 PCR cycles, the PCR products were concentrated by a Speed Vac Concentrator (SAVANT, Farmingdale, NY) and were subjected to electrophoresis on 1.5% agarose gel. The identities of the PCR products were confirmed by sequence analysis automatically using an ABI 373 DNA sequencer. Sequencing results showed that the sequence of the PCR products was identical to that of the CB3 genome.

Neutralization test

The blood was obtained directly from the heart, and for serum antibody titration, neutralization tests were done in primary cynomolgus monkey kidney cell cultures using Eagle's minimal essential medium

according to the method of Hashimoto and Komatsu (1978).

Histopathological examination

After sacrificing, the hearts were fixed in 10% neutral-buffered formalin, processed routinely for paraffin embedding, sectioned, and stained with haematoxylin and eosin.

Results

Virus neutralizing titer

The virus titer peaked on day 5 postinoculation in both groups, and the means of the titers in Groups 1 and 2 were 14.0x and 12.9x, respectively. Afterwards, their titers gradually decreased, returning to 6 to 7x by day 14 in both groups.

Histopathological findings of the myocardium in each group

Findings of myocarditis in BALB/c mice were graded from (-) with no evidence of myocarditis to (3+) for severe myocarditis, as revealed in Figure 2. The data is summarized in Table 1. In Group 1 (inoculation of CB3 virus, 1x10⁴ PFU), 4 mice showed no signs of myocarditis and 6 mice showed grade (+) on day 3 postinoculation. The number of mice with severe inflammation (3+) peaked on day 7; this then rapidly subsided, with some mice becoming free of pathological inflammation by day 14, and more by days 21 and 28. In Group 2 (inoculation of CB3 virus, 1x10² PFU), no myocarditis was observed in 7 out of 10 mice on day 3, one mouse on day 10, two mice on day 14, eight mice on day 21, and all mice on day 28. The inflammatory reaction was most severe

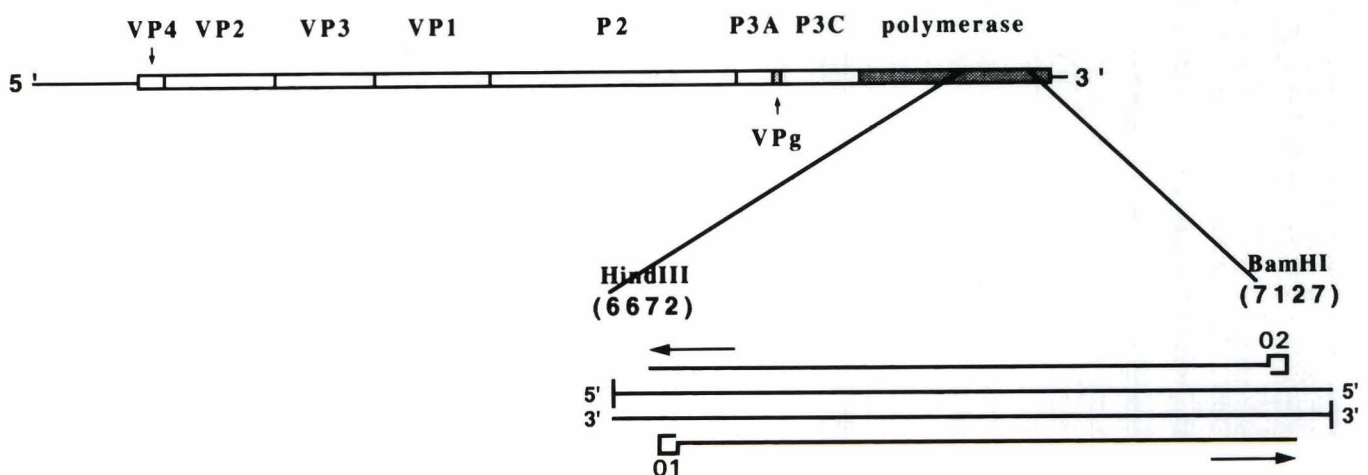


Fig. 1. Organization of the coxsackievirus B3 (CB3) genome (9), and a schematic representation showing the positions of the different oligonucleotides (each 20 bp long) used as primers for the specific amplification of a selected region of the CB3 genome. Oligonucleotide primers 01 (5'AAGCTTGGATACACGCACAA3') and 02 (5'GGATCCTTGGTCCATCTAAT3'), complementary to the CB3 coding sequences, respectively, were both added to the mixtures in the polymerase chain reaction.

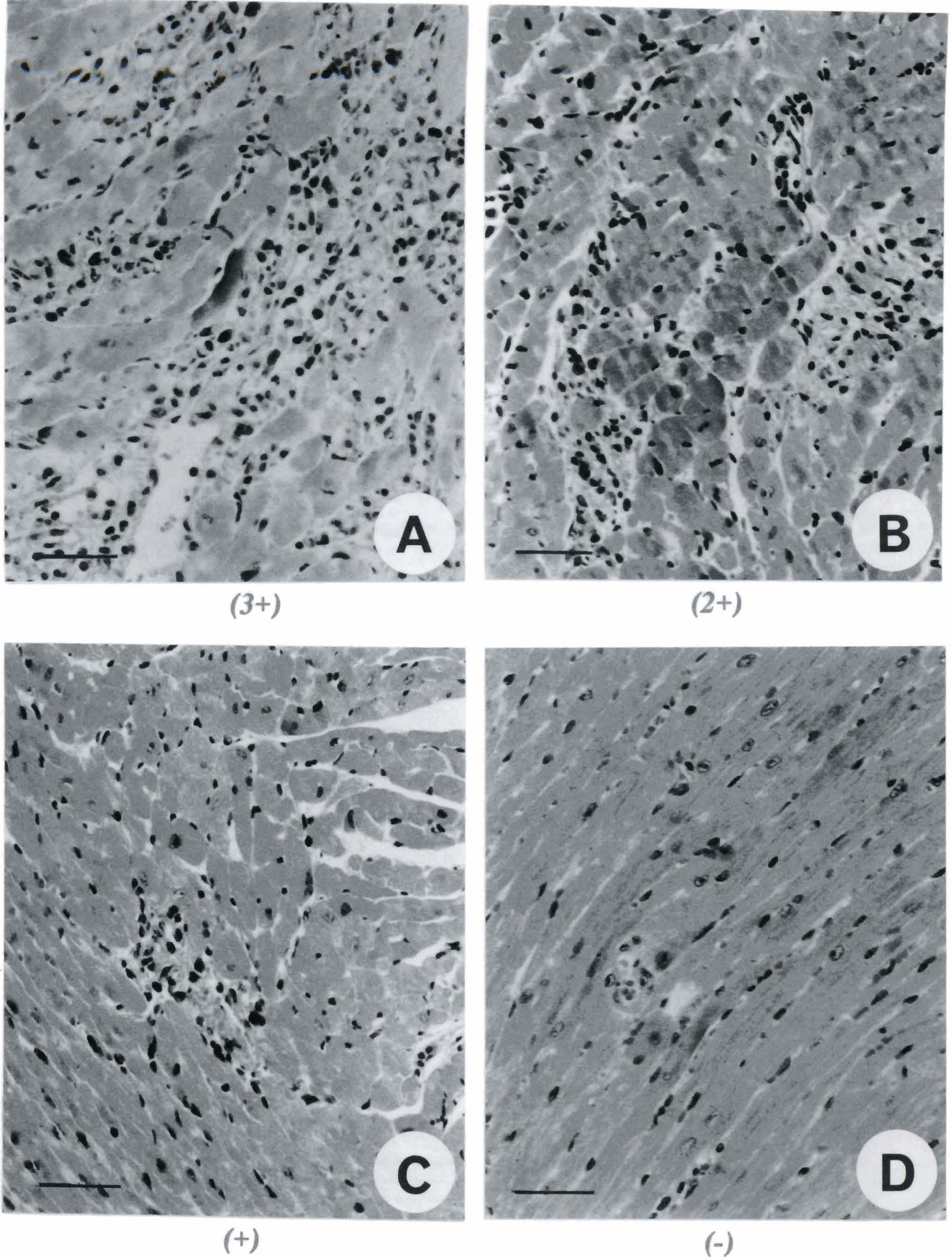


Fig. 2. Grading of the histopathological findings of myocarditis in BALB/c mice. 3+: severe myocarditis; 2+: moderate myocarditis; 1+: mild myocarditis; 0: no evidence of myocarditis; A: Day 7 postinoculation (Group 1); B: Day 7 postinoculation (Group 1); C: Day 10 postinoculation (Group 2); D: Day 28 postinoculation (Group 2). Bars: 50 μ m.

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on day 7, and this tendency was the same as that in Group 1.

Amplification of CB3 cDNA, inserted into pUC 119 plasmid, by polymerase chain reaction

CB3 cDNA inserted into pUC 119 plasmid was concentrated from 10^{-3} to 10^{-9} μg and was assayed by PCR. The amplified DNA could be observed in all lanes, and production of cDNA fragments was dependent on the dosage of the applied plasmid DNA

(Fig. 3).

Amplification of CB3 cDNA, synthesized from purified CB3 RNA, by polymerase chain reaction

CB3 cDNA synthesized from purified CB3 RNA was concentrated from 10^{-2} to 10^{-10} μg , and was assayed by PCR. The amplified DNA could be observed from 10^{-2} to 10^{-7} μg CB3 RNA, and the production of cDNA fragments was dependent on the dosage of the applied CB3 RNA (Fig. 4).

Table 1. The number of BALB/c mice showing inflammatory histopathological findings of the myocardium.

INFLAMMATORY REACTION (Grade)	DAYS POSTINOCULATION (n=10)						
	3	5	7	10	14	21	28
<i>Group 1</i> (1×10^4 PFU)							
3+	0	0	5	0	0	0	0
2+	0	3	3	3	2	0	0
1+	6	7	2	7	6	5	4
0	4	0	0	0	2	5	6
<i>Group 2</i> (1×10^2 PFU)							
3+	0	0	5	0	0	0	0
2+	0	2	4	1	1	0	0
1+	3	8	1	8	7	2	0
0	7	0	0	1	2	8	10
<i>Controls</i>							
0~3+	0	0	0	0	0	0	0

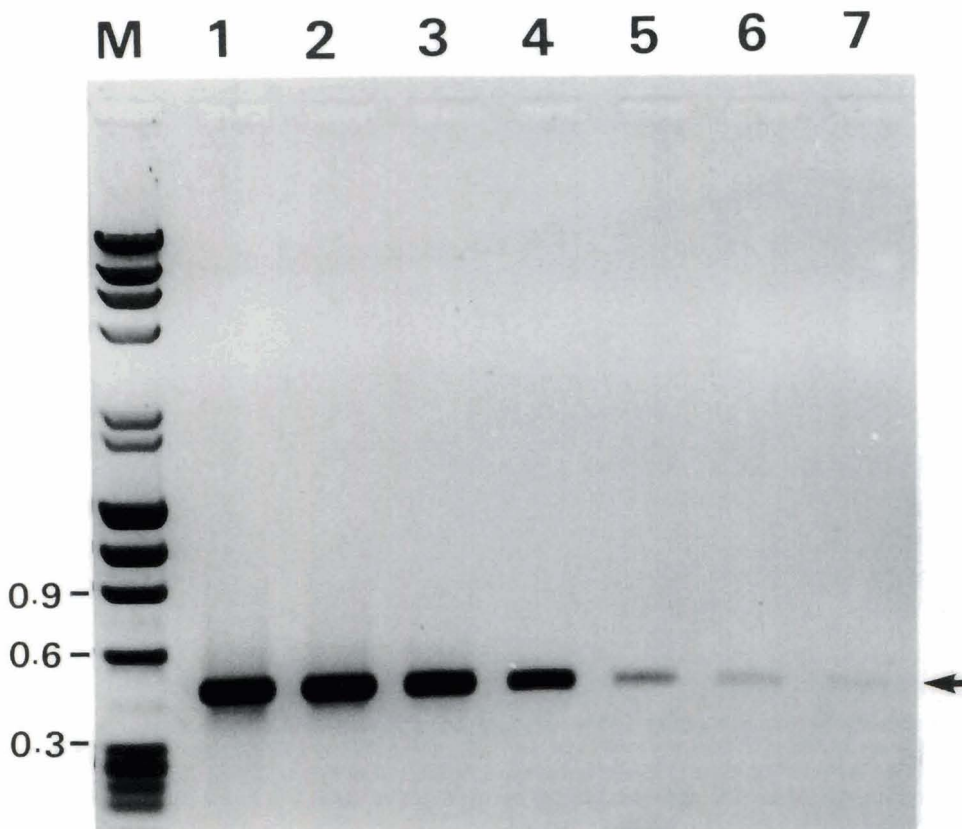


Fig. 3. Amplification of coxsackie B3 virus cDNA inserted into pUC 119 plasmids by polymerase chain reaction, agarose gel electrophoresis. Lanes 1-7, samples, assayed from 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} and 10^{-9} μg plasmid DNA, respectively. Lane M (molar calibration) contained molecular weight standards, relative molecular mass indicated on the left-hand side (Kb). Arrowhead, 461 bp. The figure represents the negative of ethidium bromide stained gel. The amplified DNA can be observed in all lanes, and production of cDNA fragments is dependent on the dosage of the applied plasmid DNA.

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Polymerase chain reaction of myocardial RNA from BALB/c mice in group 1, inoculated with CB3 (1×10^4 PFU)

Figure 5 gives examples of the PCR analysis of CB3 RNA from Group 1. The upper half of the figure shows that CB3 signals by PCR were observed on days 3, 5, 7, 10 and 28 postinoculation (lanes 1-4, lane 7). But on days 21 (lane 5) and 14 (lane 6), the signals were obscure, indicated here as negative amplification. The lower half of the figure shows that the amplified DNAs were observed on days 3, 5, 7, 10, 14 and 21 post-

inoculation (lanes 10-15). On day 28 (lane 16), it was negative. Usually, the amplification was most prominent on day 7 and abruptly diminished after day 14. Table 2 summarizes the data from Group 1. From days 3 to 10, CB3 signals by PCR were positive in all mice, while from days 14 to 28, 3 out of the 10 mice showed no CB3 signals. In comparison with pathological findings (Table 1), 4 out of 10 mice demonstrating positive CB3 signals on day 3 had no evidence of myocarditis. On day 21, 7 out of 10 mice were positive; 5 of them had pathologically demonstrated myocarditis, while the other 2 had no positive sign of myocarditis. On day 28, 7 mice

Table 2. The number of BALB/c mice in which virus genomes were detected in the myocardium by PCR.

DNA AMPLIFICATION	DAYS POSTINOCULATION (n=10)						
	3	5	7	10	14	21	28
<i>Group 1</i> (1×10^4 PFU)							
positive	10	10	10	10	7	7	7
negative	0	0	0	0	3	3	3
<i>Group 2</i> (1×10^2 PFU)							
positive	9	9	9	9	7	7	5
negative	1	1	1	1	3	3	5
<i>Controls</i>							
positive	0	0	0	0	0	0	0
negative	10	10	10	10	10	10	10

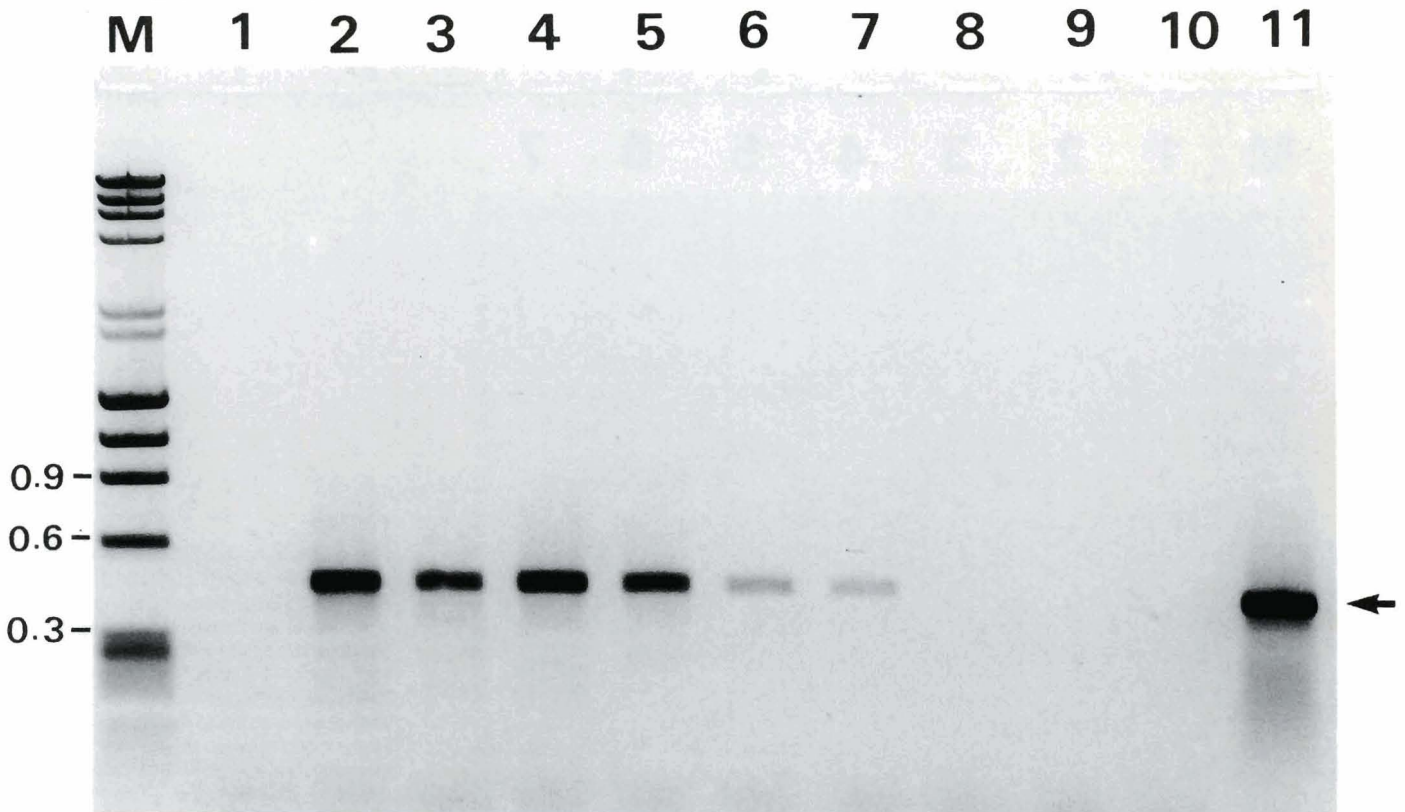


Fig. 4. Amplification of coxsackie B3 virus (CB3) cDNA synthesized from purified CB3 RNA by polymerase chain reaction, agarose gel electrophoresis. Lane 1: murine mRNA (10^{-2} μ g assay); lanes 2-10: samples assayed from 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} and 10^{-10} μ g purified CB3 RNA, respectively; lane 11: pUC119 plasmid inserting CB3 cDNA (10^{-4} μ g assay). Lane M contained molecular weight standards (Kb). Arrowhead: 461 bp. The figure represents the negative of ethidium bromide-stained gel. The amplified DNA can be observed from lanes 2 to 7, and production of cDNA fragments is dependent on the dosage of the applied CB3 RNA.

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had positive CB3 signals; 4 of them had myocarditis, while the other 3 had no myocarditis.

Polymerase chain reaction of myocardial RNA from BALB/c mice in group 2, inoculated with CB3 (1x10² PFU)

Figure 6 is a representative example of data from

Group 2. The amplified DNAs could be observed on all days. Usually, on day 7 it was strongest, and after day 14 it abruptly diminished, similar to that in Group 1. The amplification was not strong on day 3 in this group. Table 2 summarizes the data from Group 2. By day 10, the DNA amplification was positive in 9 out of 10 mice. On day 3, six out of 7 mice with no findings of myocarditis showed positive DNA amplification. On

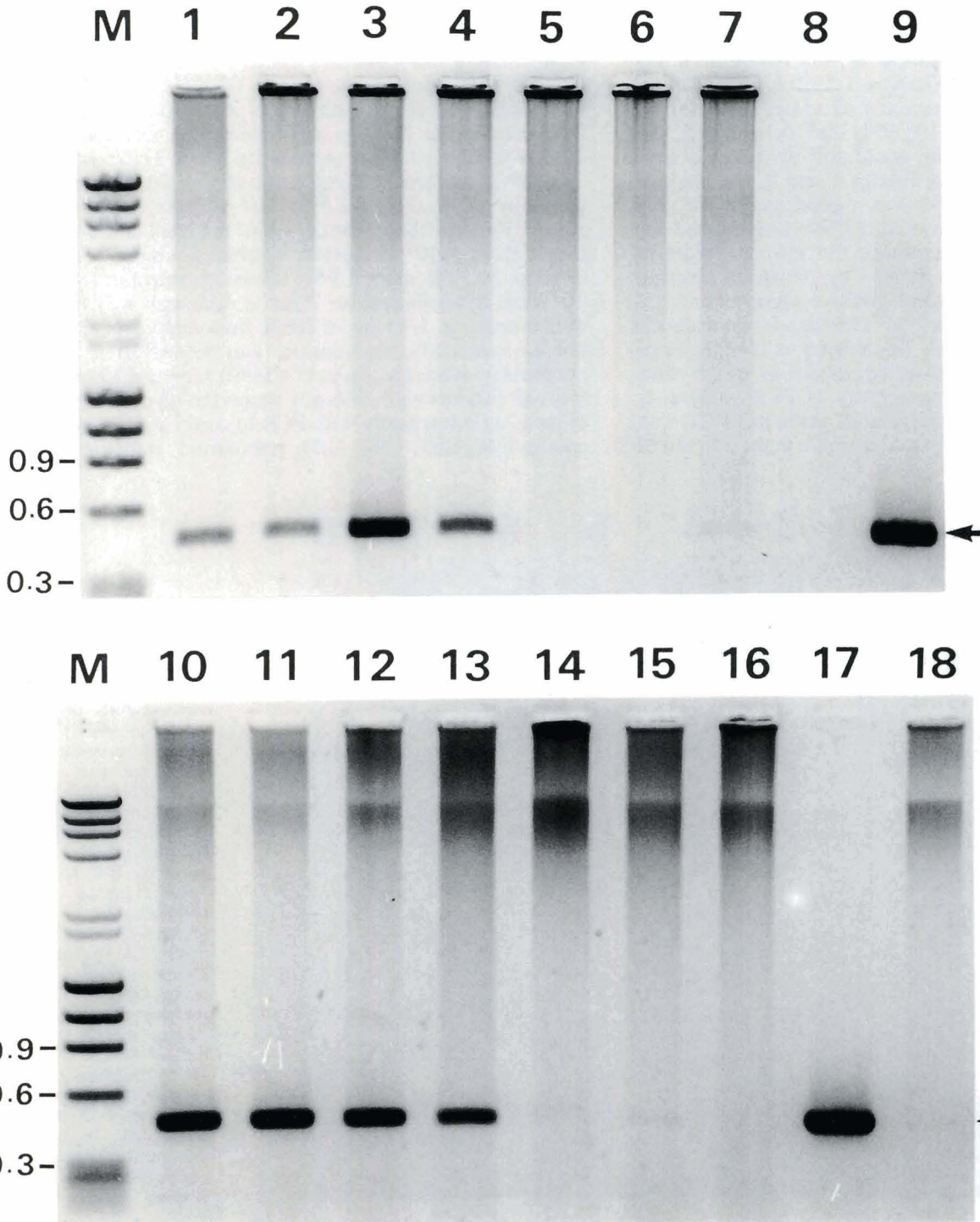


Fig. 5. Polymerase chain reaction of myocardial RNA of BALB/c mice in Group 1, inoculated with coxsackie B3 virus (CB3) (1x10⁴ PFU). Lanes 1-7: CB-3 infected murine myocardial RNA (1 µg) of days 3, 5, 7, 10, 14, 21 and 28 postinoculation, respectively; lane 8: poliovirus RNA (Mahoney strain) (10⁻³ µg); lanes 9 and 17: purified CB3 RNA as control (10⁻³ µg assay); lanes 10-16: CB3-infected murine myocardial RNA (1 µg) of days 3, 5, 7, 10, 14, 21 and 28 postinoculation, respectively; lane 18: non-infected murine myocardial RNA (1 µg). Lane M contained molecular weight standards (Kb). Arrowhead: 461 bp. The figure represents the negative of ethidium bromide-stained gel. The amplified DNA can be observed in lanes 1-4, 7, 9, 10-15 and 17.

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days 14 and 21, 7 mice were positive. On day 21, five out of mice with no myocarditis showed positive DNA amplification, and, on day 28, although all mice had almost normal hearts by pathology, half of them displayed positive DNA amplification.

Discussion

The virus genomes in murine myocarditis induced by CB3 were detected by the PCR, which was highly sensitive for detecting viral RNA sequences. Our results showed that positive signals were very strong until day 10 postinoculation in Groups 1 and 2. In addition, despite the different amount of virus inoculation (inoculation of CB3, 1×10^4 PFU and 1×10^2 PFU), the signals, though very faint, could still be detected until day 28 postinoculation in Groups 1 and 2, the latter of which at 1×10^2 PFU CB3 was a good model for very mild myocarditis. Okada et al. (1990) investigated the presence of viral genomes in the murine heart in experimental CB3 myocarditis by Northern blotting analysis. They could detect positive signals for viral RNA until day 7 but not signals after day 10 (inoculation of CB3, 4×10^4 PFU). This discrepancy is thought to be due mainly to differences in the sensitivity of the virus detection methods. The sensitivity of PCR analysis is extremely high. Generally, it is said that PCR can produce a greater than 10^5 -fold increase in the amount of

target sequences, enabling the analysis of as little as 1 ng of genomic DNA (Saiki et al., 1985; Erlich et al., 1988). Under our experimental conditions, the PCR assays from 10^{-9} μ g (1 fg) of CB3 cDNA inserted into pUC 119 plasmid and from 10^{-7} μ g (100 fg) of purified CB3 RNA yielded detectable CB3 signals near the detection limit of PCR. The sensitivity of the PCR assay starting from 1 μ g of purified CB3 RNA looked lower than that of 1 μ g CB3 cDNA. This may be explained by the fact that the assay for CB3 RNA must include the synthesis of single-strand DNA before PCR is applied. Such a two-step process may not generate complete conversion, in comparison with that for cDNA. Okada et al. (1990) mentioned that they could detect about 10-100 pg of RNA as a single band on a Northern blot, and that the 10-100 pg of viral RNA represented about 3×10^6 - 3×10^7 viral RNA molecules. Simple calculation demonstrates that our PCR analysis had 10^2 - 10^3 times higher sensitivity than their Northern blot analysis and could detect about 3×10^4 viral RNA molecules, which is equal to about 30 PFU, since 1 PFU is the equivalent of about 10^3 viral RNA molecules. That is, although we could obtain only ca. 1-15 μ g of RNA from endomyocardial biopsy material, this quantity can be sufficient for microassay. Recently, Jin et al. (1990) screened biopsies from 48 patients with clinically suspected myocarditis or dilated cardiomyopathy with PCR to detect signals of an enterovirus (CB1, CB3, CB4, poliovirus1, poliovirus2,

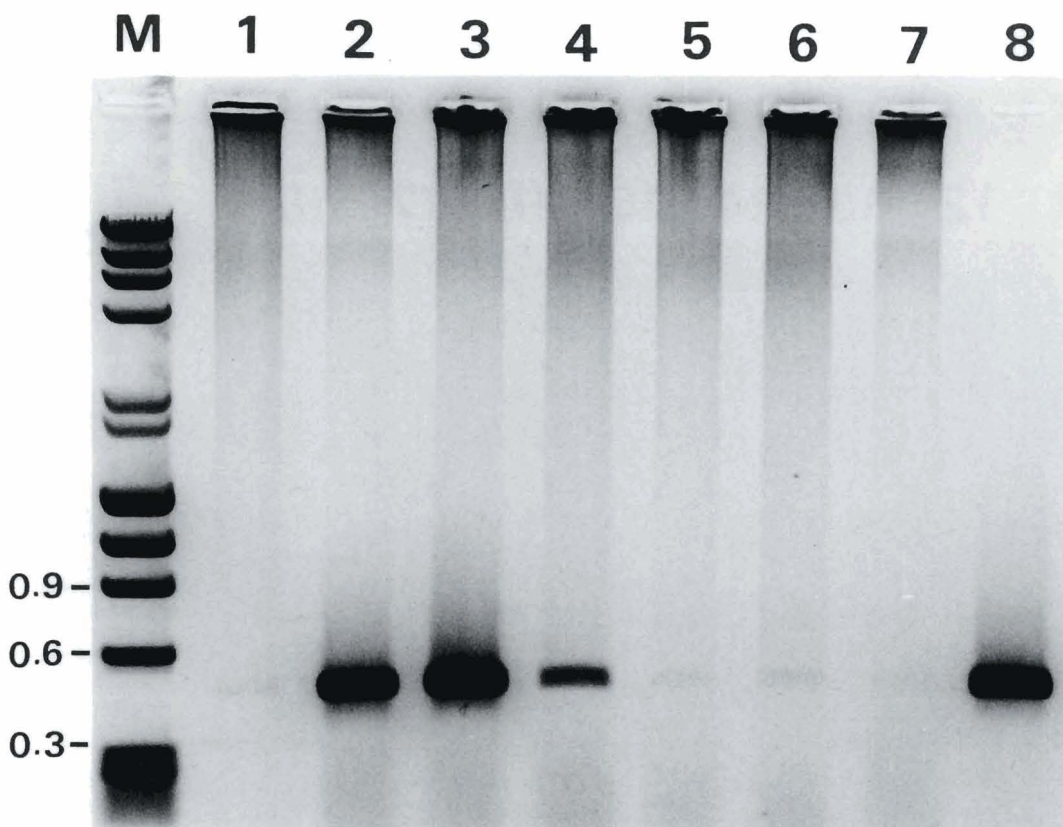


Fig. 6. Polymerase chain reaction of myocardial RNA of BALB/c mice in Group 2, inoculated with coxsackie B3 virus (CB3) (1×10^2 PFU). Lanes 1-7: CB3-infected murine myocardial RNA (1 μ g) of days 3, 5, 7, 10, 14, 21 and 28 postinoculation, respectively; lane 8: purified CB3 RNA as controls (10^{-3} μ g assay). Lane M contained molecular weight standards (Kb). Arrowhead: 461 bp. The figure represents the negative of ethidium bromide-stained gel. The amplified DNA can be observed in all lanes.

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and poliovirus3). Their results showed that only 5 patients were positive by PCR, but conventional slot-blot hybridization failed to exhibit a viral signal with coxsackie virus probes in 4 of these 5 patients. This may show the limitations of conventional detection although sampling heterogeneity may account for differences between PCR and standard hybridization techniques.

Our results show that mice inoculated with CB3 developed severe myocarditis on day 7 postinoculation, and the PCR amplification signals were strong by day 10 in both groups. Interestingly, the virus genomes were still detected in both groups, showing no histopathological evidence of myocarditis on day 28. In a case of human postpartal myocarditis, the pathology was negative for myocarditis 3 months after the biopsy-confirmed myocarditis, but the PCR findings were still positive (Jin et al., 1990). These results suggest that the viral genome may be present in the myocardium for a long period of time in both humans and mice, and that acute viral myocarditis may create a condition of persistent infection, predisposing the host to dilated cardiomyopathy. This could help to explain the role of coxsackie virus in the chronic stage of human diseases such as the exacerbation and recurrence of myocarditis.

Cellular immune mechanisms (Guthrie et al., 1984; Kishimoto et al., 1985; Godeny and Gauntt, 1986; Weller et al., 1989; Kishimoto and Abelmann, 1990; Seko et al., 1990), as one of the self-protective barriers in hosts, may strongly influence the course of viral infection. On the other hand, these mechanisms themselves may cause myocardial damage without persistent active viral infection. Our data suggest that the coxsackie B virus may be able to survive even under the stringent conditions of these immune mechanisms. The mechanism of maintaining such persistent infection is not yet clear. However, we speculate that the persistence of the viral RNA confers a resistance in infected cells against superinfection by homologous viruses and prevents the normal shutdown of protein synthesis during lytic virus infection, and that, under the above-mentioned circumstances, the virus survives and establishes the persistent infection.

Information is needed concerning the location of the residual viral genome in the persistently infected myocardium. By *in situ* hybridization, enterovirus RNA was not only detected in the early stage of myocarditis, but also in dilated cardiomyopathy, indicating the persistence of the virus in the human heart. In some cases of chronic dilated cardiomyopathy, the autoradiographic silver grains, indicating hybridization between viral RNA and the radiolabeled cloned cDNA, were clearly related to single myocytes in the myocardium (Kandolf et al., 1988). *In situ* experimental studies by Klingel et al. (1992) in mouse demonstrated a persistent presence of coxsackie viruses in the heart muscle. Interestingly, Schnurr and Schmidt (1984) showed that persistent coxsackie virus infection was maintained in mouse fibroblasts *in vitro*, and Kandolf et al. (1985) observed persistently infected human myocardial

fibroblasts *in vitro*. These results suggest that CB exists not only in myocytes, but also in interstitial cells, presumably fibroblasts. Other tissues, e.g., spleen and lymph nodes, may play a role in dissemination of virus or maintenance of a noncardiac viral reservoir. Further studies will be needed to clarify the mechanism of persistent infection in viral myocarditis.

Our results have precisely demonstrated the persistence of coxsackie viruses in the experimental murine myocarditis using PCR to follow minute trace quantities of the virus genome. These results confirm a work by Klingel et al. (1992) using *in situ* analysis that suggested the persistence of virus not only in the early stages but also in the chronic stages of myocarditis.

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