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Expression of Plus- and Minus-strand Viral RNA in Coxsackievirus B3-Infected A/J Mice

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In order to investigate the implication of viral replication in acute, subacute, and chronic infections of coxsackievirus B3 (CVB3), we examined the histopathological changes and plus- and minus-strand viral RNA dynamics in heart, pancreas, brain, and liver of CVB3-infected A/J mice. Mice were inoculated intraperitoneally with CVB3 and sacrificed on 1, 2, 3, 4, 7, 10, 14, 21, 30, 60, and 90 days post infection (p.i.). Plus- and minus-strand viral RNAs in the organs were quantitated and the organs were additionally evaluated histopathologically for inflammation. No inflammatory infiltrates were observed in the liver, brain, and heart. In contrast, massive lymphocyte infiltration and fat replacement were shown in the pancreas with loss of acinar cells. Both plus- and minus-strand viral RNA levels were detected by 21 days p.i. in heart, 90 days p.i. in pancreas, 4 days p.i. in liver, and 10 days p.i. in brain. The plus-strand RNA was found at least fifty fold higher than the minus-strand RNA by 4 days p.i. in heart and pancreas and by 3 days p.i. in liver. The plus- to minus-strand RNA ratio in brain was found less than 1:20. Our data indicate that viral replication was actively occurred in heart, pancreas, and liver during acute CVB3 infection, whereas viral replication was limited in brain. Furthermore, chronic persistent viral RNA was observed in pancreas. In conclusion, CVB3 at low dose of virus induces severe pancreatitis but marginal or no inflammatory changes in the heart, liver, and brain.

Key Words: Coxsackievirus B3, Pancreatitis, Viral RNA dynamics, A/J mice

INTRODUCTION

Enteroviruses (EVs) are members of the picornavirus family and include poliovirus (types 1-3), coxsackieviruses A (CVA; types A1-22, A24), coxsackieviruses B (CVB; type B1-6), echoviruses (type 1-9, 11-27, 29-33) and the newer numbered enteroviruses (types 68-71). CVB is associated with more severe clinical syndromes than CVA (18). CVB causes a wide spectrum of human diseases. CVB1 is known to be associated with fulminant hepatitis, insulin-dependent diabetes mellitus (IDDM), pleurodynia (14,23,28). CVB2 can cause IDDM, myocarditis, aseptic meningitis and hand-foot-mouth disease (11,23). CVB3 is the most common cause of human viral myocarditis and the subsequent development of dilated cardiomyopathy (2,26). CVB4 is associated with IDDM (29), and CVB5 is associated with several human diseases (5). Enteroviral infections have been known to be associated with a variety of acute and chronic phase of diseases (17). CVB3 is a non-enveloped virus, which has a single-stranded positive-sense RNA genome.

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During the viral replication period both plus- and minus-strand viral RNAs appear. The persistent enterovirus RNA has been found in patients with chronic fatigue syndrome, idiopathic inflammatory myopathy, sporadic motor neuron disease, dilated cardiomyopathy, and postpolio syndrome (24). The fact was further supported by experimental myocarditis and diabetes murine models (1,3,17,27). However, the association of persistent enterovirus RNA with chronic diseases such as dilated cardiomyopathy was not well understood. In the present study, we studied the histopathology of various organs including heart, pancreas, liver and brain for 90 days after CVB3 infection, and also examined the distribution of CVB3 after intraperitoneal inoculation. Furthermore, we performed quantitative analysis of plus- and minus-strand RNAs during acute, subacute and chronic phases of murine CVB3 infection by real-time reverse transcription-polymerase chain reaction (RT-PCR) using strand-specific primers.

MATERIALS AND METHODS

1. Viruses

CVB3 (Nancy strain, VR-30) purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) was grown in Vero cells. Virus supernatants were collected 3 days post infection (p.i.) and then centrifuged at 2,000 rpm for 20 min. Virus titers were determined by plaque assay on Vero cells and aliquots of virus preparations were then stored frozen at -80℃.

2. Animal experiment

Five-week-old male A/J mice, purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan), were inoculated intraperitoneally with 0.2 ml of DMEM containing 2×10^4 PFU of CVB3. Control mice were inoculated with uninfected Vero cell lysate in DMEM. Organs were removed from infected and uninfected mice on days 1, 2, 3, 4, 7, 10, 14, 21, 30, 60, and 90 p.i. Each group consists of five infected mice and one mock infected mouse. All experiments were performed following the regulations of the Animal Care Committee of the University of Ulsan (Seoul, Korea), in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

3. Histopathology

Tissues were fixed in 4% paraformaldehyde solution and embedded in paraffin. Three-μm-thick transverse sections were cut and stained with hematoxylin and eosin (H/E). Grading was performed in a blind manner by two independent investigators and any differences were resolved by consensus. Three adjacent sections of the heart were examined for the presence of myocarditic lesions. The severity of inflammatory lesions, necrosis, and fibrosis was graded semiquantitatively on a scale of 0 to 4, with 0 representing no necrosis, inflammation, or fibrosis; 1 representing between 1 and 10 foci per section; 2 representing between 11 and 20 foci; 3 representing between 21 and 40 foci; and 4 representing more than 40 foci (1). The grading of pancreatic damage was based on the percentage of damage in the examined area. The samples were graded as follows: absence of lesion=0; involvement of 1~10%=1; 11~25%=2; 26~50%=3; >50%=4.

4. Quantitation of plus- and minus-strand viral RNAs

Viral RNA was extracted with the Qiagen Viral RNA Kit (Qiagen, Crawley, UK) in according to the manufacturer's instructions. The enterovirus RNA standard was prepared using plasmid containing CVB3/20 genome, which was kindly provided by Dr. Tracy (University of Nebraska). An RNA standard representing on 5' NTR of enterovirus RNA was synthesized *in vitro* as follows. The plasmid was prepared in linear form and was used to perform an *in vitro* transcription reaction with T7 RNA polymerase (Roche, Indianapolis, IN, USA) at 37℃, for 2 hr. After digestion with RNase-free DNase, the resulting RNA transcripts were purified with RNeasy kit (Qiagen). The RNA transcripts were dissolved in RNase-free water and quantified using spectrophotometric analysis. Aliquots of the diluted RNA with 60 ng/μl were frozen immediately at -70℃ until used.

The primers and a FAM-labelled probe for enterovirus

detection were designed to target the 2A of the enterovirus genome. The design of primers and probes for the TaqMan assay was carried out using the PRIMER EXPRESS™ software (ABI, Foster City, CA, USA). Primers and the fluorescent producing probe used are as follows; forward primer, 5'-GCTTTGCAGACATCCGTGATC-'3, spanning nt 3692-3712 reverse primer, 5'-CAAGCTGTTCCACAT-AGTCCTTCA-'3, spanning nt 3772-3749 probe, 5' FAM-TGTGGCTGGAAGATGATGCAATGGA-TRAMRA 3, spanning nt 3716-3740. Rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified using *Taq-Man* GAPDH control reagent (PerkinElmer Biosystem, Waltham, MA, USA) as an internal control.

5. Plaque assay

To determine the infectious virus particles, plaque assay was performed as according to standard procedures (19). Briefly, dilutions of heart homogenates were incubated on confluent Vero cell monolayers for 30 min at 37℃ and 5% CO_2 to allow viral attachment, and then incubated for 3 days to allow plaque formation. Virus titers were expressed as the mean PFU/g tissue.

6. Statistical analysis

Viral RNA level and virus infectivity were calculated using Microsoft Excel program (Microsoft Corporation, Redmond, WA, USA). Chi-square test and Student's t-test in SPSS software (Statistical Package for Social Science, version 9.05, SPSS Inc., Chicago, IL, USA) were used for comparisons. A value of $p<0.05$ was considered statistically significant.

RESULTS

1. Histopathological changes in heart, pancreas, brain, and liver

To examine the time course of histopathological changes in heart, pancreas, liver, and brain, the organs were removed at various times after CVB3 inoculation (1 to 90 days) and stained with H/E using standard procedure. No inflammatory infiltrates were observed in heart during the 90-day observation period except 1 or 2 mice on 10 and 30 days p.i. (Fig. 1).

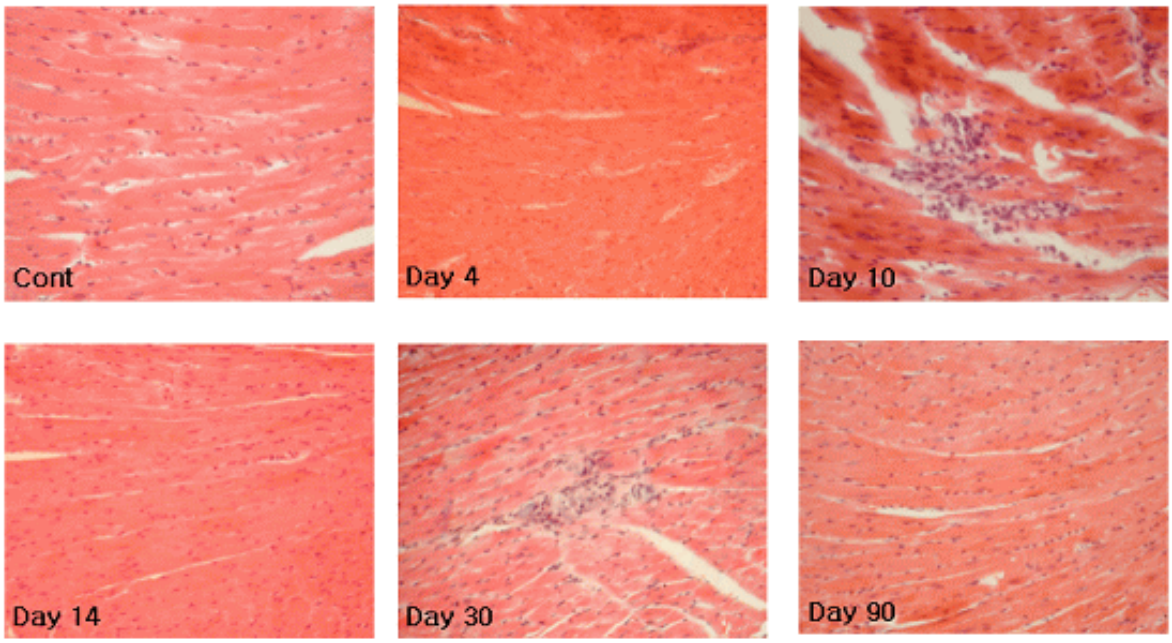

Figure 1. Hematoxylin and eosin (H&E)-stained paraffin-embedded murine heart tissue sections (3-μm) obtained on 1, 2, 3, 4, 7, 10, 14, 21, 30, 60, and 90 days p.i. of CVB3. No inflammatory infiltrates were observed during experiments except 1 or 2 mice at 10 and 30 days p.i. (×20).

A representative result of pathological changes in pancreas is shown in Fig. 2. By 4 days after infection, more than 75% of acinar cells were damaged and it was accompanied by massive inflammatory infiltration. By 10 days after infection, acinoductular metaplasia was shown. Although it is evident that fat replacement was shown by 10 days after infection, it was prominent from 21 to 90 days after infection. Particularly, 1 out of 5 mice after 30 days of inoculation and 2 out of 6 mice after 90 days of inoculation were recovered to normal except showing a little fat replacement.

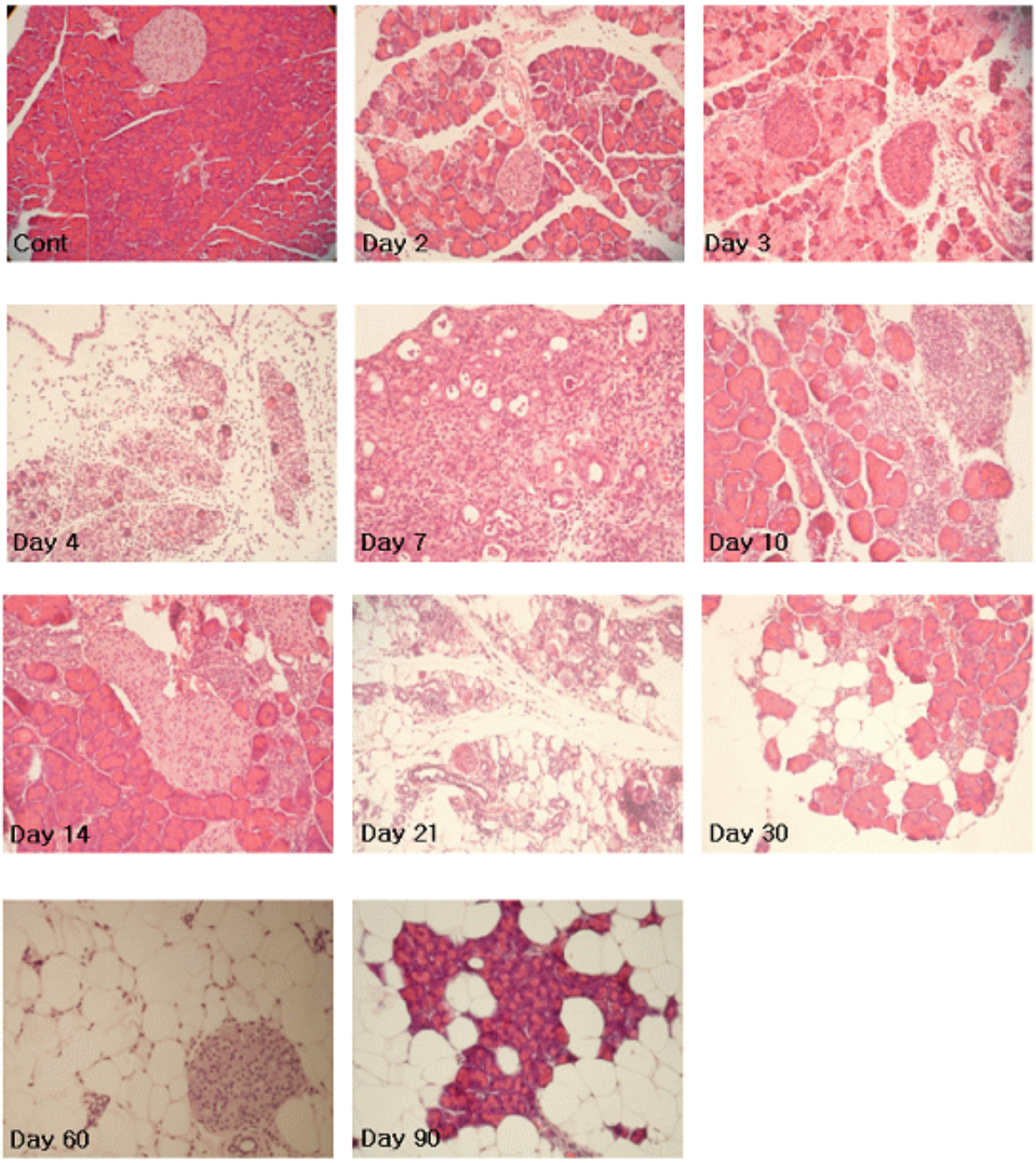

Figure 2. Hematoxylin and eosin (H&E)-stained paraffin-embedded murine pancreatic tissue sections (3-μm) obtained on 1, 2, 3, 4, 7, 10, 14, 21, 30, 60, and 90 days p.i. The affected pancreata displayed massive lymphocyte infiltration and loss of acinar cells, which peaked on 4 and 7 days p.i. of CVB3. Restoration of pancreatic tissues was observed between days 10 and 14 p.i., thereafter tissues were redestructed.

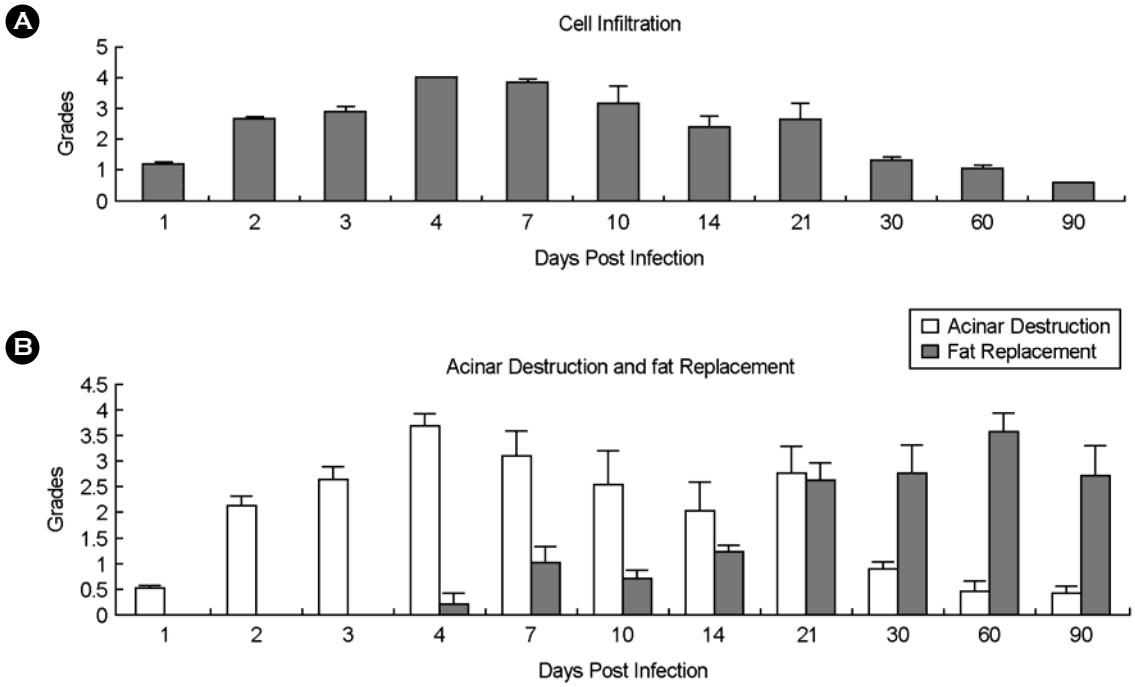

Figure 3. Increase of inflammatory cell infiltration and acinar cell destruction were found at acute phase in pancreatic tissue infected with CVB3. Fat replacement was prominent at chronic phase.

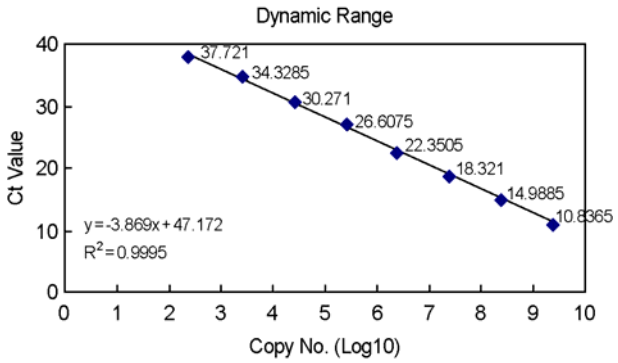

Figure 4. Standard curve used to determine CVB3 RNA concentration in tissue samples.

The islets of Langerhans were shown to be intact at the light microscopic level (Fig. 3). In summary, mice infected with CVB3 developed chronic pancreatitis, characterized by extensive acinar cell destruction and fat necrosis. The brain and liver showed no inflammatory cell infiltration in the experiment (data not shown).

2. Viral RNA distribution in CVB3-infected mice

The analytical sensitivity of TaqMan PCR was determined using serial dilutions *in vitro*-transcribed RNA transcripts (Fig. 4). The lowest dilution, containing 24 copies of the RNA transcripts, corresponded to a C_T of 39.10, whereas the highest dilution, containing 2.4×10^{10} copies of RNA transcripts, corresponded to a C_T of 7.81. The correlation coefficient r^2 was calculated to be 0.995. We subsequently used the standard curve to calculate the concentrations of viral RNA in tissue samples.

Viral RNA distribution during acute, subacute, and chronic phase of CVB3 infection was analyzed using real-time RT-PCR. Viral RNA in heart was detected from days 1 to 21 p.i, with maximum level at day 2 p.i. (Fig. 5A). No viral RNA was detected after 30 days p.i. in heart. On the other hand, viral RNA was peaked on 2 days p.i. and persistently retained until 90 days p.i. in pancreatic tissue. The viral RNA on 2 days p.i. was 2.35×10^8 and 5.28×10^5 on 90 days p.i. Plus-strand viral RNA was rapidly cleared in liver by 7 days and in brain by 14 days.

The minus-strand viral RNA in heart was peaked on 2 days p.i. and detected until 21 days p.i. (Fig. 5A). The minus-strand viral RNA in pancreas was peaked on 2 days p.i. and persistently presented through the end of experiment (Fig. 5B). The minus-strand viral RNA was peaked in

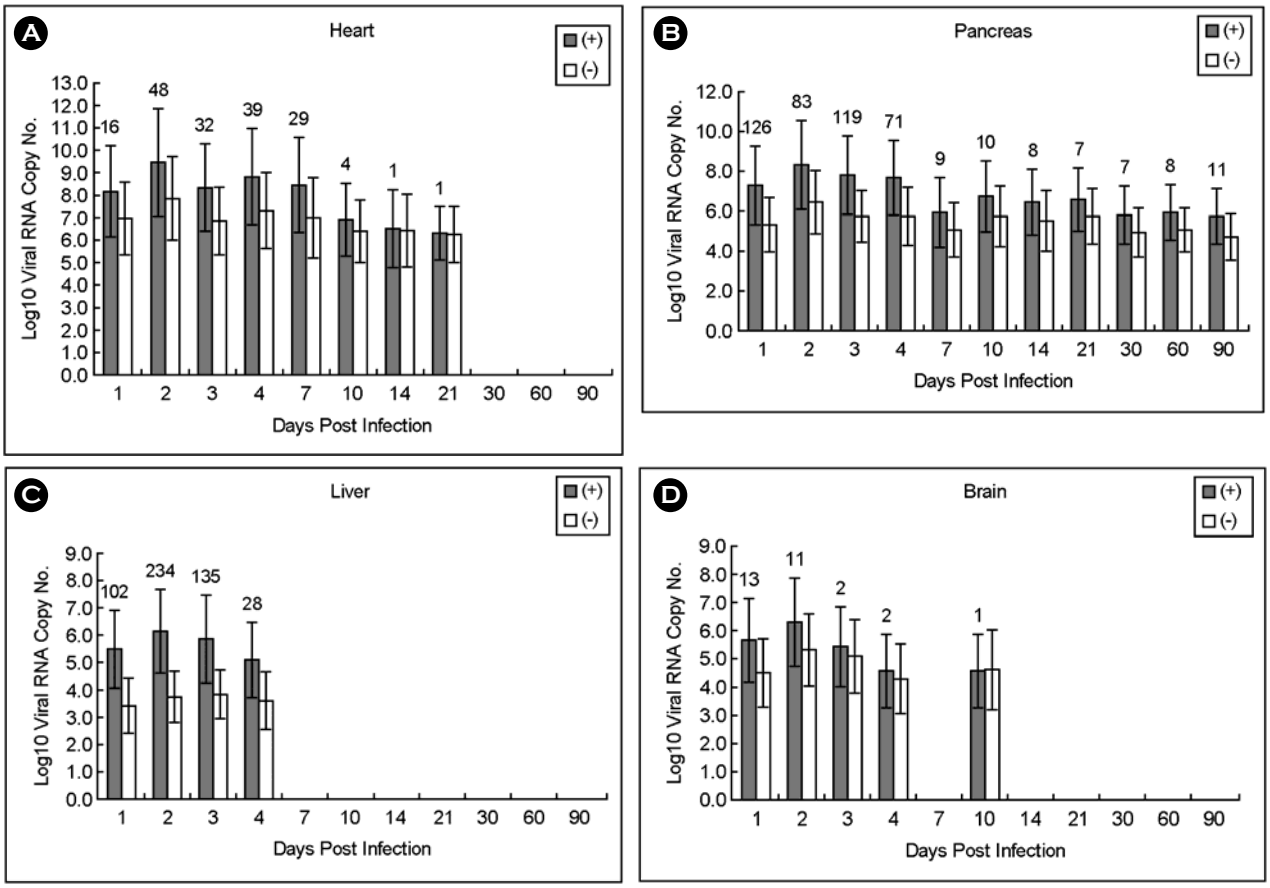

Figure 5. Copy number of plus- and minus-strand viral RNAs in heart, pancreas, liver and brain obtained from A/J mice infected with CVB3. Both plus- and minus-strand viral RNAs were peaked on 2 days p.i., then decreased in all tissue samples. **(A)** No viral RNA was detected after 30 days p.i. **(B)** Both plus- and minus-strand viral RNAs were detected until 90 days p.i. Both strand viral RNAs were detected until 4 days p.i. **(C)** and 10 days, respectively, except days 7 p.i. **(D)**. The numbers above histogram are the ratio of plus to minus strand viral RNA.

liver and detected until 4 days p.i. (Fig. 5C), and it was peaked in brain on 2 days p.i. and detected until 10 days (Fig. 5D).

3. Plus- and minus-strand viral RNA ratio

The ratio of plus to minus viral RNA in heart was > 20 between 2 day and 7 days p.i. (Fig. 6). The ratio was less than 5 between 10 days and 21 days p.i. In pancreas, the plus-strand RNA was at least fifty fold higher than the minus-strand RNA by 4 days p.i. The ratio of plus to minus was decreased to approximately 10 until the end of the experiment. The ratio of plus to minus viral RNA in liver was >20 between 1 day and 4 days p.i., but not detected after 7 days. In brain, relatively low ratio was observed.

DISCUSSION

Although histopathological changes of CVB3-induced pancreatitis have been reported, previous histopathological studies were focused on the early events in infection (20, 27). In the present study, we have analyzed chronic phase of CVB3-induced inflammatory diseases. CVB3 induced extensive inflammatory infiltration and acinar cell destruction of pancreas, followed by either repair of the acinar cell or fatty replacement and atrophy. Besides those findings, we have identified three phases of pancreatitis based on the histopathological changes. Phase I spanning 1 to 7 day p.i. was characterized by massive inflammatory infiltration and acinar cell destruction. Phase II spanning 10 to 21 day p.i.

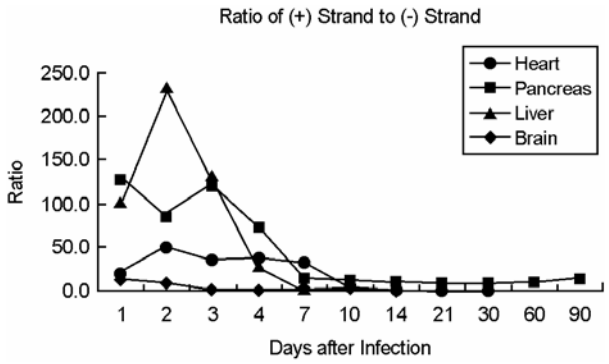

Figure 6. The ratio of plus- to minus-strand RNA in various organs obtained from A/J mice infected with CVB3. In heart, pancreas and liver in acute phase, high ratio (>20) of plus- to minus-viral RNA means the presence of active viral replication. Lower ratio was shown in brain.

was characterized by acinoductular metaplasia and fat replacement, although inflammatory infiltration and acinar cell destruction were still eminent. Phase III spanning 30 to 90 days p.i. was characterized by fat replacement and atrophy.

Meanwhile, no inflammatory infiltrates were observed in heart during the 90-day observation period except 1 or 2 mice on 10 and 30 days p.i. This is in contrast with the previous reports that CVB3 induced myocarditis in mice (1, 6,7,15,17,20). We also previously reported that CVB5 and CVB3 induced massive lymphocyte infiltration and loss of acinar cells of pancreas at early phase of infection (15,20). This disparity may be due, at least in part, to the differences in viral strains and in viral loading dose. In our study, CVB3 purchased from ATCC was serially passaged before experiments so that viral strain might be mutated and attenuated, losing cardiovirulence. Viral dose for the infection was also relatively low compared to other experiments. Thus, these factors might attribute in part to the no inflammatory change in liver and brain.

There are many molecular studies on pathogenesis of acute and chronic enteroviral diseases (1,9,10,16,17,24,25). For replication, minus-strand viral RNAs should be transcribed from the plus-strand genomic template, then multiple copies of viral plus-strand genome that is translated into enteroviral structural proteins are produced. Therefore, the presence of minus-strand enteroviral RNA may indicate active enteroviral RNA replication (13). Furthermore, *in vitro* study by Novak et al. (21) and Hellen et al. (12) suggested that higher ratio of plus- over minus-strand viral RNA may indicate active infection or virus RNA synthesis. Their findings support that the strand specific viral RNA kinetics should help differentiate between active enteroviral replication phase and persistent phase. In this study, both plus- and minus-strand types of CVB3 RNA were detected from acute phase of infection in heart, pancreas, and liver. In addition, higher ratio of plus-strand to minus-strand was observed in the heart, pancreas, and liver. On the other hand relatively low ratio was observed in the brain. It strongly suggests that viral replication was actively occurred in heart, pancreas, and liver during acute phase of infection, whereas viral replication was limited in brain. We also observed that viral RNA persists in heart on days 21 p.i., and no viral RNA was detected after days 30 p.i., which are similar to the previous reports (7,22).

Girard et al. reported the restriction of plus-strand RNA production is associated with decrease of virus replication and persistence of poliovirus in the central nervous system of paralyzed mice (8). It may be caused by reduction of RNA polymerase activity, leading to a corresponding lack of strand displacement and formation of the double-stranded replicative form (24). Our data also showed that the plus strand RNA is diminished rapidly and the ratio of plus- to minus-strand is decreased at chronic phase of CVB3 virus infection. Other studies have also reported the equivalent amount of plus- and minus-strands of enteroviral RNA in chronic phase (1,4).

In conclusion, at low dose of CVB3, the virus induces severe pancreatitis but marginal or no inflammatory changes in the heart, liver, and brain.

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