Variation in Serum Creatine Phosphokinase Activity as Indicated in Two-Phase EMC-D Virus-Induced Myocarditis

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Abstract: In this study, myocardial damage in the D-variant of encephalomyocarditis (EMC-D) virus-induced myocarditis has been investigated consecutively by measuring serum creatine phosphokinase (CPK) activity. CPK activity in 8 week-old male BALB/cA mice inoculated with EMC-D virus increased to a peak at 4 or 5 days postinoculation (DPI) and then gradually decreased. The CPK activity rose again after 7 DPI until it reached a second peak. In view of the kinetics of CPK activity, two-phase (early and late phase) myocardial damage in EMC virus infection were considered. In the late phase, an increase in cellular infiltration in the myocardium and a decrease in viral titer in the heart were observed. It was therefore suspected that the increase in CPK in the late phase may be caused by cellular infiltration, but not by viral replication. In our results, we suggested that a serial measurement of serum CPK activity might be a useful method for throwing more light on the myocardial damage caused by the autoimmune response. We also used a pathological (TUNEL) method to detect apoptotic cells and some apoptotic myocytes in the myocardium in late phase EMC virus-induced myocarditis.

Key words: CPK, EMC virus, histopathology, myocarditis

Introduction

Encephalomyocarditis (EMC) virus is one of the picornaviridae. Picornaviruses infect a number of different tissues and cause a variety of diseases in animals and man. The most notable human pathogens in picornaviridae are polio, coxsackie and echo viruses which have been implicated in insulin dependent diabetes, myocarditis, orchitis, meningitis and encephalitis [6, 9, 10, 11]. A number of picornavirus-induced diseases develop as a result of the autoimmune process [5]. A murine model is useful in the study of a number of human diseases associated with picornavirus infection. EMC virus infects the heart, pancreas and brain

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of mouse and causes myocarditis [2, 20, 29], diabetes [2, 4] and encephalitis [7, 24].

EMC virus induced myocarditis in several strains of mice [7, 14, 29]. In previous reports, EMC virus-induced myocarditis was evaluated by the mortality rate and histopathological observations [14, 20], but the kinetics of consecutive myocardial damage is necessary to throw more light on the myocarditic process. Tissue damage in individual surviving animals can be monitored by measuring the enzyme in serum. Creatine phosphokinase (CPK) is an enzyme which is found in myocytes. It is generally considered that the increase in the CPK level in serum is related to myocardial damage in man and animals [15, 21, 22], but no report has shown the developmental process of myocarditis by means of blood CPK levels in a murine pathologic model, such as EMC virus-infected mice.

In this study, consecutive myocardial damage in EMC virus-induced myocarditis has been investigated by measuring serum CPK activity. In addition, we conducted a pathological experiment to investigate whether or not apoptosis in myocytes was caused by EMC virus infection.

Materials and Methods

Animals: Eight-week-old male BALB/cAJcl mice were obtained from Clea Japan Inc., Tokyo. The mice were housed in an animal room at a temperature of 23 ± 2°C and relative humidity of 55 ± 5%. The mice were fed MF pellets (Oriental Yeast Co., Ltd., Tokyo) and watered. The above were in accordance with the principles outlined in the Guide for Animal Experiments, Faculty of Agriculture, University of Tokyo.

Virus inoculation: Fifty-seven mice were inoculated intraperitoneally with 10⁵ plaque forming units (PFU) of EMC-D virus (plaque-purified from heart-passaged M-variant) [27] diluted in 0.1 ml of phosphate buffered saline (PBS). Four mice were randomly chosen and sacrificed at 2, 4, 6, 8, 10 and 12 days postinoculation (DPI). The surviving mice were sacrificed at the end of the experiment (14 DPI). The heart and brain from each mouse were removed and cut aseptically. Histopathologic and virological studies of the heart were performed.

CPK level: A commercial CPK assay kit (Wako, Osaka) based on the principles of the NADPH (Rosalki) method [3] was used to measure serum CPK levels. Sera were obtained from the tail vein and used for CPK assay in this study. Sera were collected serially on the day before infection and throughout the 14 DPI. The CPK level was evaluated as abnormal if it was more than the mean ± 5 standard deviation for pre-inoculation serum.

Histopathology and Viral titration: For histopathological studies, one part of the heart was fixed with 10% buffered formalin and embedded in paraffin. Several sections were stained with hematoxylin and eosin (HE). The remaining part of the heart was homogenized in PBS, and stored at −80°C until viral titration. Titrations were performed by a standard plaque assay with mouse L929 cells [7] and virus titers were expressed as PFU/g of tissue. The brain was also sectioned and stained with HE.

In situ nick end labeling of heart: As described previously [8, 26], an Apop Taq peroxidase kit (Oncor, U.S.A.) based on the method of terminal deoxynucleotidyld transferase (TdT)-mediated dUTP nick end labeling (TUNEL) was used. Apoptotic cells had been identified microscopically by the characteristic formation of TUNEL-positive nuclei. The TUNEL method was used to detect the apoptotic cells of the heart obtained at 4, 6, 10 and 12 DPI. Sections of formalin-fixed and paraffin-embedded hearts were digested with proteinase K. Endogenous peroxidase was inactivated by covering the sections with 2.0% H₂O₂. The sections were then incubated with TdT and digoxigenin-dUTP at 37°C for 60 min. The samples were processed for immunocytochemical detection of digoxigenin-dUTP by peroxidase-labelled antidigoxigenin antibody, followed by an enzyme reaction with DAB as a substrate. The nucleus was counterstained with methyl green.

Results

CPK levels in EMC-D virus-infected mice: In normal mice, the CPK level in serum obtained from supraorbital veins ranged from 103.4 to 596.0 IU/l, while one in serum obtained from the tail vein ranged from 71.9 to 148.8 IU/l. Sera obtained from the tail vein were therefore used for the CPK assay in this study. Thirty-three mice were infected with EMC-D virus, and 19 mice survived the entire experimental period of 14 DPI.
Abnormal CPK levels were first observed at 3 DPI. The highest CPK levels in all mice were seen at 4 or 5 DPI (Fig. 1) and the CPK values were more than 300 IU/l in 17 of 19 mice (from 195 to 913 IU/l), and then these CPK levels decreased gradually, but CPK levels in all mice rose again after 7 DPI. The second maximum CPK values ranged from 242 to 577 IU/l. The second peak of CPK was seen in 17 of 19 mice from 9 to 13 DPI (2 mice on 7 DPI).

Histopathological observations: To determine whether abnormal CPK levels correlated with myocardial damage, sections of hearts of infected mice were examined microscopically. There were no significant alterations in the hearts at 2 DPI. The mice developed myocarditis at 4 DPI. Small foci of necrotic myofibers and minimal inflammation were observed (Fig. 2A). Inflammation was most prominent in the epicardium and endocardium. At 10 DPI, there were extensive inflammation, myocardial necrosis and mild fibrosis (Fig. 2B). In all sections of heart obtained in this late phase, myocardial cells in and around the residual foci often showed a variety of degenerative changes and were surrounded by numerous collagen fibers. Granulomatous lesions and infiltration of mononuclear cells were also observed.

In the brain, little focal inflammation was observed in mice with abnormal CPK levels at 7 DPI (data not shown).

Viral titers in heart: Viral titers in the heart rose to the maximum (more than $4.7 \times 10^7$ PFU/g) at 4 DPI and rapidly declined (Fig. 3). Viral titers were less than $1 \times 10^4$ PFU/g after 10 DPI.

In situ nick end labelling of heart: Examination of the paraffin sections by the TUNEL method showed that several cells in heart muscles underwent apoptosis at 10 and 12 DPI as judged by the presence of DNA fragmentation. The nuclei of apoptotic myocytes were darkly stained (Fig. 4). Several nuclei of infiltrated mononuclear cells were also stained. The TUNEL method has not been positive for myocytes at 4 and 6 DPI so far.

Discussion

Immunologic response may play an important role in the myocardial destruction in viral mediated myocarditis and the following progression to cardiomyopathy [1, 12, 29]. In our results, CPK activity increased to the peak at 4 or 5 DPI and then gradually decreased. The CPK activity rose again to form a second peak after 7 DPI. In view of the kinetics of CPK activity, two-phase (early and late phase) myocardial damage in EMC
Fig. 2. Histopathological observations in hearts obtained from mice inoculated with EMC-D virus at 4 and 10 DPI were done microscopically. A: Section of myocardium at 4 DPI showing focal necrosis and minimal inflammation. HE × 100. B: Histological alteration at 10 DPI showed severe necrosis, cellular inflammation and extensive fibrosis. HE × 100.

Fig. 3. Viral titer in heart from mice inoculated with EMC-D virus. Each point represents the individual viral titer in homogenate of heart at 2, 4, 6, 8, 10, 12 and 14 DPI.

virus infection was considered. In the early phase, the kinetics of CPK levels run parallel with that of the virus titer. In the late phase, an increase in cellular infiltration in the myocardium without an increase in viral titer in the heart was observed. This result that one of the causes of myocardial damage was viral multiplication in myocytes in the early phase and then infiltrating cells in the myocardium in the late phase. Kishimoto et al. had suggested the destruction of myocytes by T lymphocytes in the late phase of EMC-M virus infection. In their report, there were two peaks in the mortality rate in infected BALB/c-nt/nu mice, but only the first peak was seen in infected BALB/c-nt/nu mice [14]. Wong et al. have shown that cytotoxic T lymphocytes (especially autoreactive cytotoxic T lymphocytes) were involved in myocardial destruction induced by coxsackie virus B3 infection [28]. The increase in CPK in the late phase may therefore be caused by T lymphocytes, but not by viral replication, which resulted in the destruction of myocytes. Our report suggested that a serial measurement of serum CPK activity was useful in studying myocardial damage caused by the immunological response.

In a histopathological study at 14 DPI, the hearts of all mice with the second increase in CPK activity
showed signs of severe myocardial necrosis and cellular infiltration. The correlation of myocardial infarction size with CPK levels had been studied in man, dog and rabbit [15, 21–23], but in the present study the correlation between the size of the lesion and the CPK levels was unclear, since histopathological change was severe in all infected cases.

In the present study, CPK activity in serum obtained from the tail vein was consistent, but several serum samples obtained from supraorbital veins were inconsistent, even in normal mice. The inconsistency in CPK values of sera obtained from the supraorbital veins may be due to CPK from damaged tissues. This may be the reason why there are few reports about the duration of disease based on murine CPK tests.

Higher CPK activity was observed in EMC-D virus-infected mice than in non-infected mice. There was extensive inflammation in the heart, but little inflammation in the brain of abnormal CPK mouse (data not shown). Furthermore, hind limb paralysis which had been observed in EMC-D virus-infected mice with large brain lesions [25] was not observed in any of the mice during the period of our experiments. These results suggest that the appearance of abnormal CPK is caused by myocardial damage rather than brain damage. There is a need to have the three isoenzymes farther separated to ensure that the increase in CPK activity is derived from a heart injury, in addition to the total CPK assay.

In the late phase of EMC-D virus infection, TUNEL-positive myocytes were detected, when cellular infiltration spread in the myocardium, but there were no TUNEL-positive myocyte in the early phase (data not shown). This suggested that apoptosis may be one of the mechanisms causing myocyte damage in the late phase. We could not determine the cause of apoptosis of myocytes in this experiment, but it is unlikely that EMC-D virus infection is the direct cause of apoptosis. Apoptosis is induced by several factors, such as cytokines and cytotoxic T cells [16–19]. A number of apoptotic myocytes in human cases of chronic myocarditis have been observed [13]. Apoptosis observed in the late phase seems to be induced by infiltrating cells or secretory factors.

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**References**


