

## Original Article

## Mao-to Prolongs the Survival of and Reduces TNF- $\alpha$ Expression in Mice with Viral Myocarditis

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Goal of this study was to evaluate effects of Mao-to on development of myocarditis induced by encephalomyocarditis (EMC) virus in mice. Mice were randomly divided into five groups. Group N included uninfected controls ( $n = 18$ ), while group A, B and C underwent intraperitoneal injection of EMC virus. Group A was administered oral saline from day 0 to day 4. Group B was administered oral Mao-to ( $500 \text{ mg}^{-1} \text{ kg}^{-1} \text{ day}^{-1}$ ) from day 0 to day 4. Group C was administered Mao-to from day 2 to day 6. Group D was administered Mao-to from day 5 to day 10. Treated mice were followed for survival rates during 2 weeks after infection. Body weight (BW) and organ weights including heart (HW), lungs, thymus and spleen were examined on days 4, 6 and 14. Survival rate of group C (36.4%) was significantly improved compared with group A, B or D (0% of each,  $P < 0.05$ ). HW and HW/BW ratio in group C was significantly ( $P < 0.05$ ) lower than those in group A, B or D. Viral titers of hearts were significantly different among groups A, B and C. Cardiac expression in tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was significantly reduced in group C in comparison with group A, B or D on day 6 by immunohistochemical study. Administration of Mao-to starting on day 2 improves mortality resulting from viral myocarditis in mice with reduced expression of cardiac TNF- $\alpha$ . These findings suggest that timing of Mao-to is crucial for preventing cardiac damage in mice with viral myocarditis.

**Keywords:** Mao-to – viral myocarditis – tumor necrosis factor- $\alpha$

### Introduction

Myocarditis is an important cause of cardiomyopathy in young patients (1). Therapeutics for myocarditis have been restricted to supportive care including basic medications (2). Randomized trials of immunosuppressive

agents have failed to show a benefit (3). Immunomodulative agents have been tried with limited effects (4). In severe cases, heart transplantation presents the only therapeutic option (1). Anti-inflammatory and anti-viral agents are needed to improve outcomes in these patients.

Mao-to (Ma-Huang-tang in Chinese) is traditionally used in Japan and China for treatment of influenza-like illness (high fever, headache, pain and cough) since ancient times. Component herb names (botanical names) of Mao-to are as follows: *Ephedra Herba* (stem of *Ephedra Sinica* Stapf), *Cinnamomi Cortex* (bark of

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*Cinnamomum cassia* Blume), *Armenicae Semen* (semen of *Prunus Armeniaca* Linne) and *Glycyrrhizae Radix* (root of *Glycyrrhizae uralensis* Fisher) (5). *Ephedra Herba* was reported to show the *in vitro* anti-influenza viral effects and augments the production of inflammatory cytokines including interleukin-6 and interleukin-1 (6,7). Moreover, *Cinnamomi Cortex*, which contains Mao-to, suppress IL-1 $\alpha$  production in the influenza virus-infected mice (8). Moreover, Mao-to has been studied for its anti-viral and anti-autoimmune effects (9,10). Recently, anti-pyruvate effect of Mao-to was reported in patients with influenza infection (6). However, the effects of Mao-to in viral myocarditis have not been studied. Inflammatory cytokines are involved in the pathogenesis of myocardial injury in viral myocarditis.

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a proinflammatory cytokine (11) that plays a crucial role in the initiation and continuation of inflammation and immunity (12). This cytokine has been implicated in the pathogenesis of cardiovascular diseases, especially in viral myocarditis. Our previous work showed that cardiac expression of TNF- $\alpha$  mRNA is increased in a mouse model of viral myocarditis (13). Over production of TNF- $\alpha$  is generally considered to be harmful to the cardiovascular system, because systemic administration of TNF- $\alpha$  results in myocardial depression (14) and cardiomyopathy (15). Cardiac-specific over-expression of TNF- $\alpha$  has been reported to cause severe myocarditis in mice (16,17).

Therefore, we hypothesized that Mao-to would modify viral myocarditis through anti-viral and anti-inflammatory effects and that cardiac expression of TNF- $\alpha$  was studied as an indicator of myocardial damages.

## Methods

### Mice

Eight-week-old C3H female mice (Charles River, Japan) were used in the experiments.

### Virus

A myocarditic variant of encephalomyocarditis (EMC) virus was obtained from Y. Seto, Keio University, Tokyo, Japan. Encephalomyocarditis virus was cultured and purified followed by previous report (18). Animals were inoculated intraperitoneally with 500 plaque-forming units of EMC virus in 0.1 ml of saline.

### Chemicals

Mao-to which was supplied by Tsumura Co. (Tokyo, Japan) was dissolved in distilled water, and diluted with distilled water to the appropriate concentration. HPLC finger print pattern of Mao-to is shown in Fig. 1.

Mao-to solution at the dose of 500 mg<sup>-1</sup> kg<sup>-1</sup> was administered orally once daily to the mice. The dose of Mao-to was based on the findings in previous reports (19,20). The control mice were given saline.

### Treatment Protocol

A total of 135 mice were assigned randomly to five groups. Mice in groups B and C received Mao-to 10 mg per mouse in 0.1 ml saline (500 mg<sup>-1</sup> kg<sup>-1</sup> day<sup>-1</sup>) once daily for 5 days. Group A was administered with 0.1 ml saline from day 0 to day 4. Group B was administered with 0.1 ml Mao-to from day 0 to day 4. Group C was administered with 0.1 ml Mao-to from day 2 to day 6. Group D was administered 0.1 ml Mao-to from day 5 to day 10. Group N was the uninfected control group. The survival rate of each group was monitored during the observation period. Body weight and organ weight, and histopathologic changes in the heart were examined on day 4, 6 and 14 after infection.

### Pathologic Examination

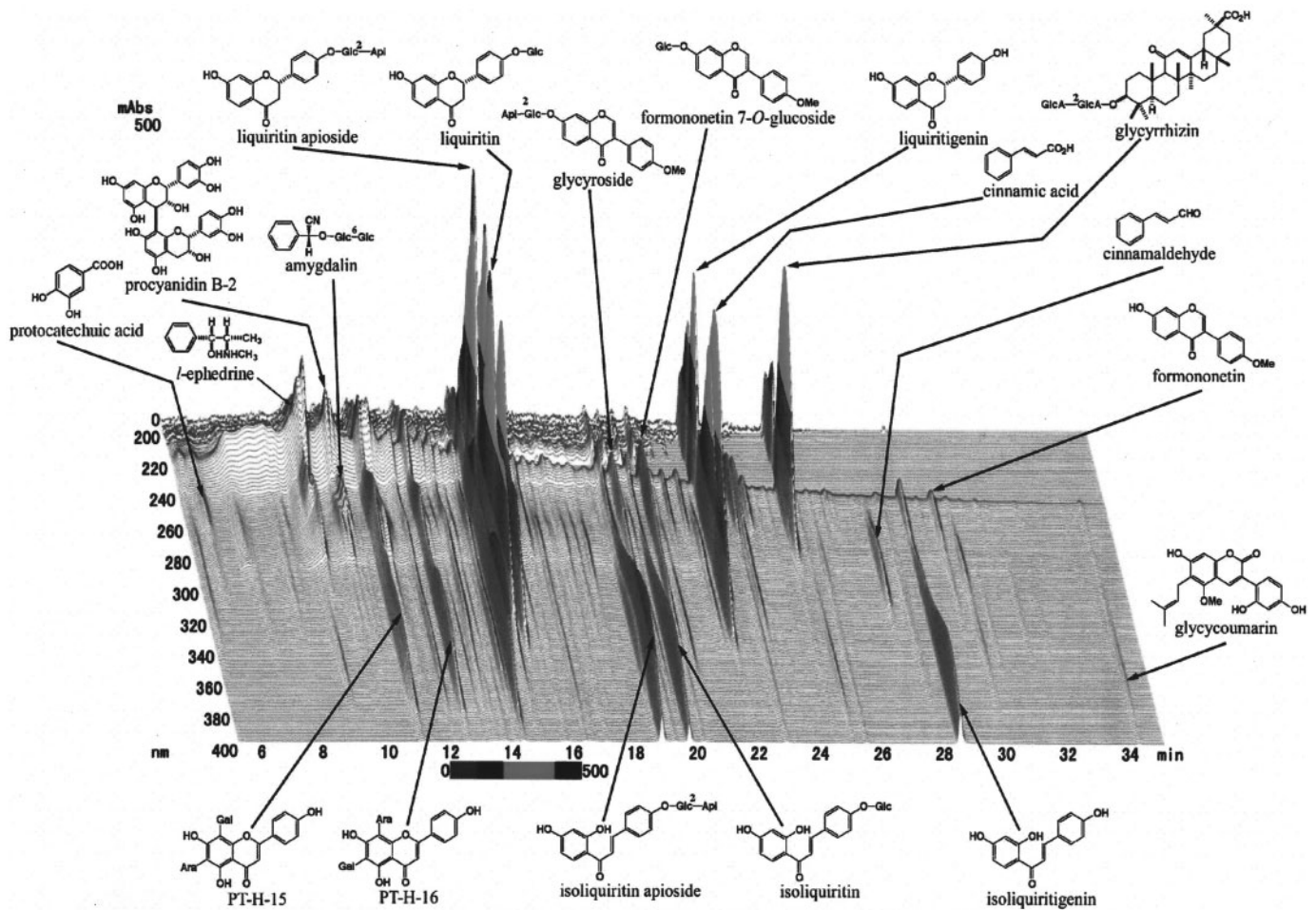
The heart and other organs were weighed. Body weight (BW) also was recorded. One half of each organ was fixed in 10% buffered formalin and stained with hematoxylin-eosin; the other half was frozen in embedding compound at -120°C for immunohistochemical studies. Transverse sections of ventricular myocardium were graded for severity of necrosis and mononuclear cell infiltration on a scale from 1 to 4 as follows: grade 1, lesions involving <25% of the ventricular myocardium; grade 2, lesions involving 25-50% of the myocardium; grade 3, lesions involving 50-75% of the myocardium and grade 4, lesions involving 75-100% of the myocardium. Tissues were evaluated blindly by an experienced pathologist who was familiar with grading murine viral myocarditis and had no knowledge of the study design.

### Measurements of Myofiber Diameter

In the lateral wall of the left ventricle, myocardial fiber diameter was determined by measuring the shortest diameter at the level of the nucleus of 50 myocardial fibers from each group with an ocular micrometer in the stained cross-sectional areas.

### Immunohistochemical Examination

To visualize the presence and anatomic localization of TNF- $\alpha$  within the myocardium, immunohistochemical studies were performed using an avidin biotin complex method (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) as previously described (21). To minimize the background staining, all sections were first blocked with normal goat serum for 20 min at room temperature.



**Figure 1.** HPLC finger print pattern of Mao-to. HPLC conditions: Pulp: LC-10AD vp (Shimadzu, Japan); column: TSK-GEL ODS-80TS column (250 × 4.6 mm; Tosoh, Japan); mobile phase: 0.05m AcONH<sub>4</sub> (pH 3.6) (5).

Next, the slides were incubated with an antibody directed against murine TNF- $\alpha$  (Alpha-Diagnostic International Inc., San Antonio, USA). Sections were counterstained with hematoxylin and eosin. TNF- $\alpha$  immunostaining was graded as follows: both nuclear and cytoplasmic staining 4; strong cytoplasm staining 3; moderate cytoplasmic staining 2 and slight cytoplasmic staining 1.

### Viral Titer in Heart

The EMC viral titer in the individual hearts was determined in terms of the viral cytopathic effects, and is expressed as the tissue culture mean infectious dose (TCID<sub>50</sub>). The hearts on day 4 after the inoculation ( $n = 3$  of each group) were homogenized in 2ml of MEM. After the centrifugation, the supernatants were added into 96-well microtiter plates containing human amnion cells in the MEM supplemented with 10% fetal calf serum as described previously (21). The microtiter plates were daily observed for 5 days to find the appearance of any cytopathic effects.

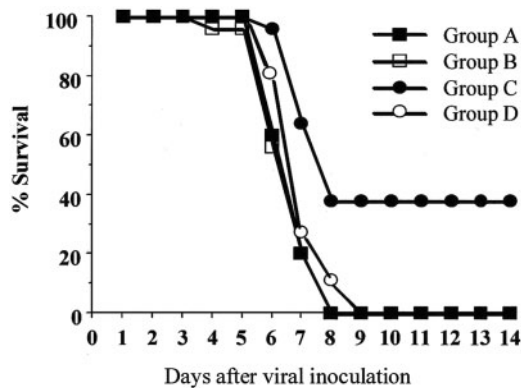
### Statistics

Data are reported as means  $\pm$  SD. The Kaplan–Meier curves were generated to analyse differences in survival. The differences in scores of myocardial damages were examined by two-way analysis of variance to reveal the combined effects of two different agents. Scheffes'  $F$ -test and Bonferroni/Dunn analysis were used for confirmation. A level of  $P < 0.05$  was considered statistically significant.

### Results

#### Prolonged Survival

Survival rates on day 7 were 18.2% in group A, 18.2% in group B, 63.6% in group C and 23.3% in group D. The survival rates on day 14 were 0% in group A, 0% in group B, 36.4% in group C and 0% in group D. The survival rate in group C was significantly higher than those in groups A, B or D ( $P < 0.05$ , Fig. 2). Thus, treatment with Mao-to starting on the day 2 after EMC



**Figure 2.** Survival in mice after viral inoculation. survival was significantly ( $P < 0.01$ ) improved in the group C as compared with mice in groups A, B or D. Group A: administered with 0.1 ml saline starting on day 0 to day 6, group B: administered with 0.1 ml Mao-to starting on day 0 to day 4, group C: administered with 0.1 ml Mao-to starting on day 2 to day 6, group D: administered 0.1 ml Mao-to from day 5 to day 10.

virus improved the survival, although Mao-to given earlier or later was not effective in this regard.

### Reduced Heart Weight

The results are shown in Fig. 3. The BW in group A and B on day 6 after virus inoculation was significantly ( $P < 0.05$ ) lower than that in group C. The HW/BW ratio in group A on day 4 was elevated compared with that of groups B and C ( $P < 0.05$ ). The HW and HW/BW ratio in group C on day 6 after virus inoculation was significantly ( $P < 0.01$ ) lower than that in group A. Additionally, the HW/BW in group C on days 4 and 6 were significantly ( $P < 0.01$ ) lower than those in group B. However, the HW and HW/BW ratio in group C on days 4, 6 and 14 did not differ significantly from those of the group N. In group D, HW and HW/BW ratio on day 6 were significantly higher than those in group N.

### Decrease of Lung Congestion

The lung weight (LuW)/BW ratio on days 4 and 6 was increased in groups A and B compared with group N. The LuW/BW ratio on days 4 and 6 were significantly reduced in group B and C vs. group A. In group C, the LuW/BW ratio on day 4 was significantly lower than that in group B, but that on day 6 was not significantly different than group B. In group D, LuW/BW ratio on day 6 was significantly higher than that in group N.

### Reduction of Thymus Weight and Enlarged Spleen

The thymus weight (ThW)/BW ratio in groups A, B, C and D on day 6 was significantly ( $P < 0.05$ ) lower than in

the group N and the spleen weight (SpW)/BW ratios in groups A, B and C on days 4 and 6 were significantly ( $P < 0.05$ ) higher than in group N (Fig. 4). Although the ThW/BW ratio on day 6 in group C did not differ from that in group A, the SpW in group C on day 6 was significantly ( $P < 0.05$ ) higher than in groups N and A. In group D, ThW/BW ratio on day 6 was significantly lower than that in group N and SpW/BW was significantly higher than that in group N.

### Decreased Myocardial Diameter and Myocardial Damage

The myocardial diameter was significantly smaller in group C than that in group A and B on day 6, as shown in Fig. 5. Myofiber diameter in group A was significantly larger than that in group N. The scores of myocardial necrosis and mononuclear cell infiltration were significantly reduced in group C compared with groups A, B and D on day 6 (Fig. 6A).

### Suppression of Expression of TNF- $\alpha$ in Heart

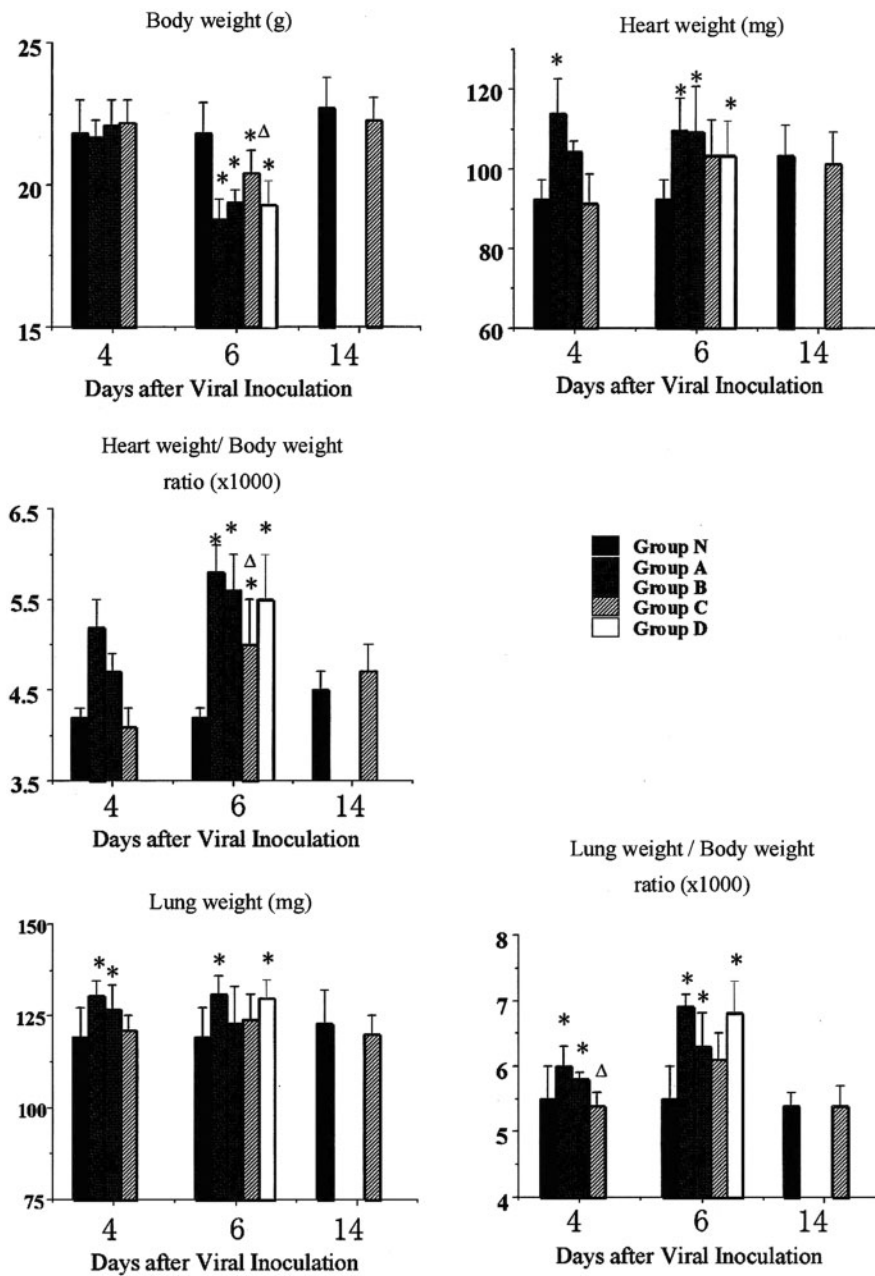
Localized expression of TNF- $\alpha$  in the heart is shown in Fig. 6B. Endothelial cells and myofiber were positive for TNF- $\alpha$  in groups A and B on days 4 and 6. Myofibers from group C were less positive than those from groups A and B on day 6. The grading of TNF- $\alpha$  immunostaining on day 6 was relatively lower in group C compared with those in group A, B or D ( $P < 0.05$ ,  $n = 3$  of each, Fig. 4). In addition, comparative expression of cardiac TNF- $\alpha$  mRNA in the group C were significantly less than in the groups A, B or D (Fig. 7).

### Viral Titer in Heart

On day 4, viral titer in the hearts was not significantly elevated in the Mao-to groups compared with that in the group A (group B;  $3.3 \pm 0.8$  TCID/mg, group C;  $3.0 \pm 0.7$  TCID/mg vs. group A;  $3.5 \pm 1.0$  TCID/mg), although the titer in group C was slightly lower than that in group A.

### Discussion

At present, we have shown that Mao-to, administered 2 days after viral inoculation in C3H/HeJ mice, improves survival rates, and reduces both myocardial necrosis and mononuclear cell infiltration in mice with viral myocarditis. Accordingly, SpW/BW ratios were elevated in these mice. However, the administration of Mao-to at the same time as virus inoculation did not influence survival or myocardial destruction. We conclude that Mao-to has an anti-viral effect on EMC viral myocarditis in this mouse model *in vivo* and involves the modulation of early immune responses with the reduction of cardiac TNF- $\alpha$



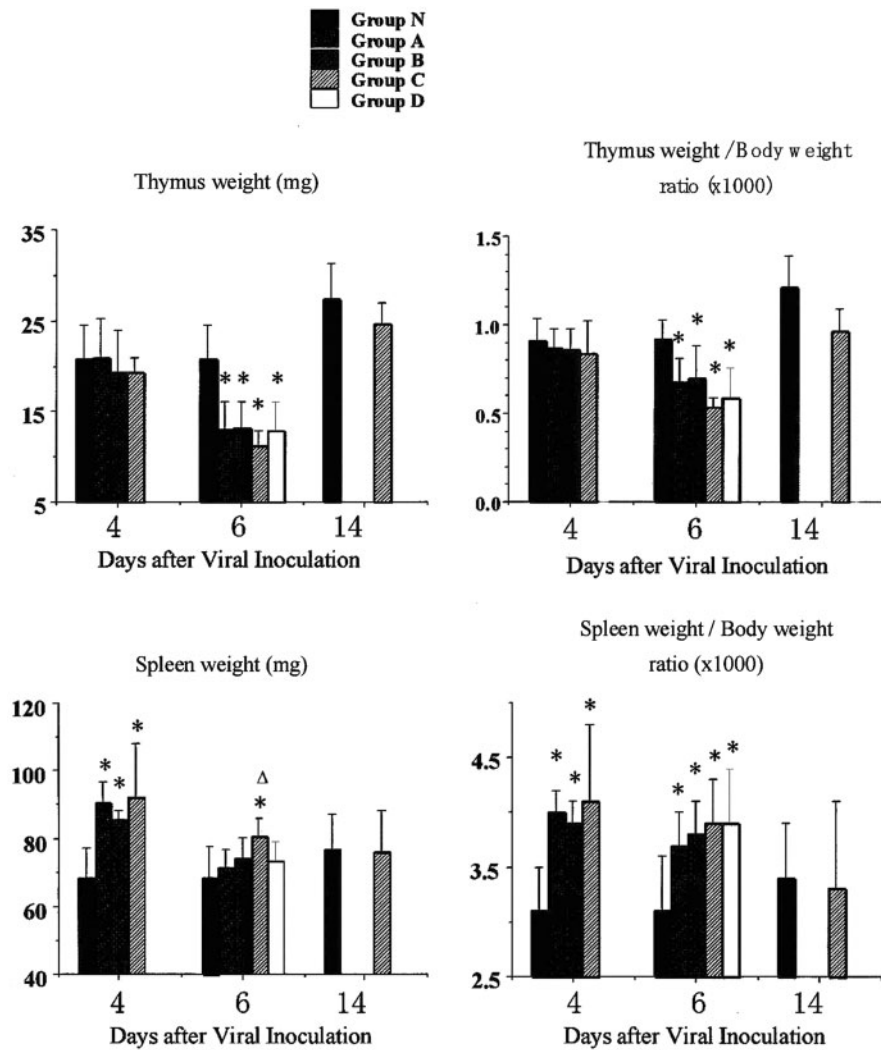
**Figure 3.** Heart and lung weight in murine viral myocarditis. Heart weight, heart weight/bodyweight ratio and lung weight/body weight ratio on day 6 was significantly reduced in group C compared with group N. Abbreviations; N: uninfected control mice, A: administered with 0.1 ml saline starting on day 0 to day 6, B: administered with 0.1 ml Mao-to starting on day 0 to day 4, C: administered with 0.1 ml Mao-to starting on day 2 to day 6. \* $P < 0.05$  vs. group N,  $\Delta P < 0.05$  vs. group A.

mRNA, although the viral titer of hearts were not significantly changed.

The LuW/BW ratio reflects the degree of lung congestion. The two days later administration of Mao-to and virus as group C leads to a significant reduction in LuW/BW on day 6. The reduction of LuW/BW ratios in group B are considered to reflect an improvement in congestive heart failure due to viral myocarditis. The effect of Mao-to on histopathological changes depends on the timing of administration. As noted, when

administered two days following viral inoculation, Mao-to led to significant reduction of myocardial necrosis and mononuclear cell infiltration.

The reasons why oral administration of Mao-to was started on 2 days after virus inoculation, should be discussed. Our previous data showed that the both peaks of serum TNF- $\alpha$  and interferon were identified on day 2 after viral inoculation in this murine model (21). Neutralizing antibody titer was confirmed on day 4. Oral administration of Mao-to was effective in patients



**Figure 4.** Thymus and spleen weight in murine viral myocarditis. Thymus weight in Mao-to-treated groups A, B, C and D were significantly reduced compared with group N on day 6. Spleen weight/body weight ratio were significantly increased in group C compared with group N. Groups as are described in Fig. 2. \* $P < 0.05$  vs. group N,  $\Delta P < 0.05$  vs. group A.

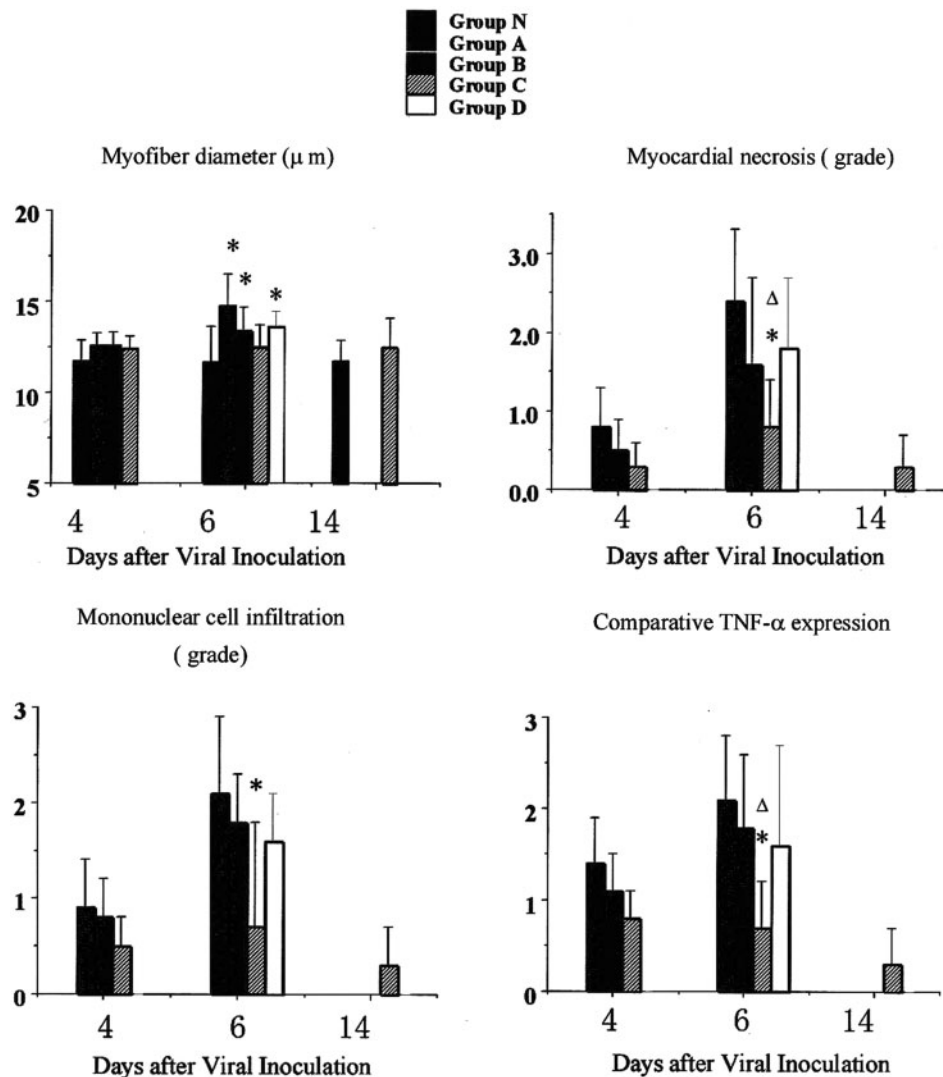
already infected with influenza virus (10). Moreover, Mao-to has been reported to show an *in vitro* anti-viral effect (6). These reports suggest that the starting time of administration, 2 days after virus inoculation, could suppress these reactions instead of other days after virus inoculation.

The reasons why Mao-to administration starting on day 0 (group B) did not prolong the survival should be discussed. It is known that Mao-to increase the blood pressure, heart rate and cardiac output and decrease the total peripheral resistance (22). From day 0 to 4, the main course of viral myocarditis is the infection of viral genome to myocytes, not the secondary immune cell infiltration in the heart. Mao-to administration on day 0 to 4 is possible to make myocyte damages worse by inducing excessive contraction of infected myocytes.

TNF- $\alpha$  is secreted primarily by myocytes and macrophages after injury (23). Elevation of TNF- $\alpha$  contributes

to the extent of ventricular dysfunction as shown in TNF- $\alpha$  knockout mice (24). Previous studies have demonstrated that cardiac-specific expression of TNF- $\alpha$  results in myocardial inflammation, cardiac hypertrophy, progressive dilatation and increased apoptosis, which leads to heart failure and death (25). TNF- $\alpha$  may play an important role in modulating left ventricular dysfunction (26).

The present study also suggests that Mao-to may play a role in cytokine regulation of host defense mechanisms against viral myocarditis in mice *in vivo*. Comparative studies on anti-immune effects of Mao-to were reported. Mao-to prevents passive cutaneous anaphylaxis and also inhibits histamine and leukotriene C4 release from the mast cells (27). These suppressive effects may be due to the interaction of its components such as *Ephedra Herba* and *Cassia Twig*. The former was showed to suppress interleukin-1 and 6 and the latter suppresses interleukin-1 $\alpha$



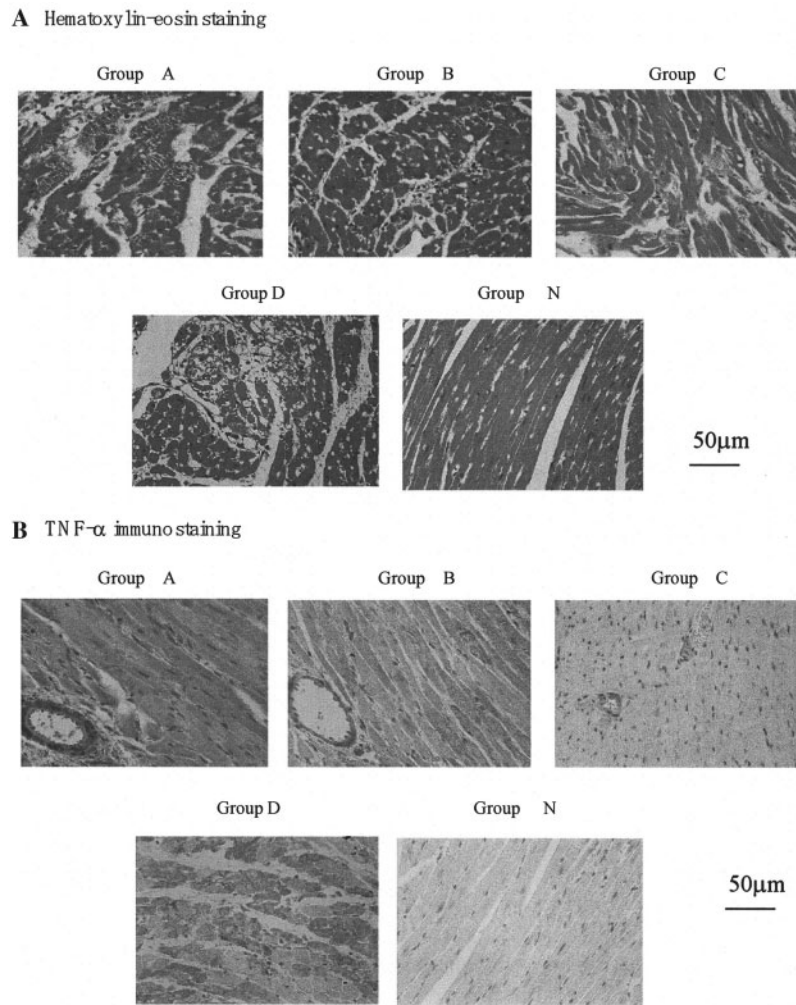
**Figure 5.** Myofiber diameter, cardiac histopathologic scores and grading of cardiac expression of TNF- $\alpha$  protein in murine viral myocarditis. Groups as are described in Fig. 2. Grades of severity of necrosis and mononuclear cell infiltration on a scale from 1 to 4 are as follows: grade 1, lesions involving <25% of the ventricular myocardium; grade 2, lesions involving 25-50% of the myocardium; grade 3, lesions involving 50-75% of the myocardium; and grade 4, lesions involving 75-100% of the myocardium. ND; not determined, \* $P < 0.05$  vs. group N,  $\Delta P < 0.05$  vs. group A.

(6–8). The importance of neutralization in early anti-viral host defense is well appreciated. The anti-viral mechanism of Mao-to in mice may be to elicit these immune responses and to enhance immune cell function rather than to directly interfere with viral replication (10). Indeed, Mao-to was reported to be effective in the suppression of hepatitis virus in combination with interferon beta (6). Recently, *Cinnamomi Cortex*, a component of Mao-to, was reported to inhibit nuclear factor-kappa B (NF- $\kappa$ B) activation in mice (28). NF- $\kappa$ B is the most regulator of the innate immune response (29). Therefore, Mao-to might contribute to the regulation of innate immunity *in vivo*.

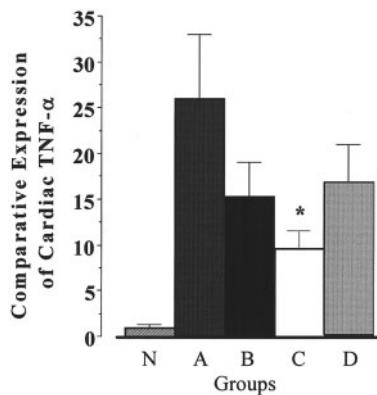
An additional reflection of immunomodulation by Mao-to was the enlargement of lymphoid organs, including spleen in group C. Although the precise mechanisms of Mao-to effect are not addressed by the examination of organ weights, the increased SpW/BW and ThW/BW

ratios may be related to the immunomodulatory effect of Mao-to (21,26).

In summary, oral administration of Mao-to two days after inoculation is beneficial to mice infected with the EMC virus and with subsequent active viral myocarditis. The present study supports the observations that Mao-to promotes temperately adaptive immune responses in spleen and heart muscle, and the consequent attenuation of viral replication when Mao-to is administered two days after the virus inoculation. However, Mao-to treatment must be concomitant with other treatment modalities. It would be better to further clarify the potential activities of Kampo medicine as well as Mao-to in basic investigations or clinical trials (30-32). Additional experiments are underway to explain specifically the mechanism by which Mao-to protects against viral myocarditis.



**Figure 6.** A. Pathologic findings of the heart in mice after viral inoculation. Myocardial necrosis and immune cell infiltration was observed on day 6. However, myocardial necrosis in group C on day 6 was reduced compared with the groups A, B and D. B. Immunohistochemical findings showed cardiac expression of TNF- $\alpha$  in the heart. Myofibers in the group C were less positive than in the groups A, B or D. Group A: administered with 0.1 ml saline starting on day 0 to day 6, group B: administered with 0.1 ml Mao-to starting on day 0 to day 4, group C: administered with 0.1 ml Mao-to starting on day 2 to day 6, group D: administered 0.1 ml Mao-to from day 5 to day 10.



**Figure 7.** Comparative expression of cardiac TNF- $\alpha$  mRNA. This expression in the group C were significantly less than in the groups A, B or D. Group A: administered with 0.1 ml saline starting on day 0 to day 6, group B: administered with 0.1 ml Mao-to starting on day 0 to day 4, group C: administered with 0.1 ml Mao-to starting on day 2 to day 6, group D: administered 0.1 ml Mao-to from day 5 to day 10.

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