THE EFFECT OF CYCLOSPORINE ON THE IMMUNOPATHOGENESIS OF VIRAL MYOCARDITIS IN MICE

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The effect of cyclosporine on the immunopathogenesis of viral myocarditis was studied using a murine viral myocarditis model. Mice in the treated group had a higher mortality rate compared with those of the infected control group before day 15 (47/67 vs. 14/31, p<0.05). On day 7, treated mice showed higher titers of anti-heart autoantibody than the control group (12±7 vs 4±2, p<0.05), but no significant difference was seen on day 14 (28±15 vs. 39±34). Histologic lesions, lymphocyte subsets in the peripheral blood and heart in situ, the neutralizing antibody, and virus concentrations in the heart showed no significant differences between these groups.

This study suggests that with the use of cyclosporine the production of anti-heart autoantibody was enhanced in the early stages of viral myocarditis in mice, and was associated with higher mortality rate.

Generally, viral myocarditis is thought to be benign; however, it can lead to congestive heart failure.1,2 A relationship between viral myocarditis and dilated cardiomyopathy has long been suggested3−5 and recently viral ribonucleic acid (RNA) was detected in endomyocardial biopsy samples taken from patients with myocarditis and dilated cardiomyopathy, using an in situ hybridization technique.6,7 Immunological mechanisms of the pathogenesis of viral myocarditis and its progression to dilated cardiomyopathy have been suggested3−5 however, the use of immunosuppressive agents for treating viral myocarditis is still controversial8−15 and a randomized trial is now in progress16.

We developed animal models of acute myocarditis17 and of subsequent dilated cardiomyopathy18 after infection with the encephalomyocarditis virus. Using these models, we found that ribavirin19 interferon20 and vaccination21 were effective in treating acute myocarditis; however, immunosuppressive therapy with prednisolone13 and cyclosporine15 were not effective and were associated with a higher mortality rate when administered early in the illness.

In this study, we investigated the effect of cyclosporine on the immunopathogenesis of viral myocarditis in mice.

Key words:
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Cyclosporine
Lymphocyte subsets
Antiheart antibody

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METHODS

Mice: BALB/c mice were originally purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Shizuoka, Japan.

Four-week-old mice were obtained from colonies of these mice, maintained at the Institute of Laboratory Animals, Faculty of Medicine, Kyoto University.

Virus: The myocardiac variant of the encephalomyocarditis virus was used. The virus stock was prepared in human amnion (FL) cell culture in Eagle's minimum essential medium (EMEM). Virus suspensions were centrifuged after the cytopathic effect had developed, and virus stock had a titer of \(10^{7.5}\) TCD\(_{50}\) (50% tissue culture infective dose) per 0.1 ml, determined in tissue cultures of FL cells.

Infection of mice: Mice were inoculated intraperitoneally with 0.1 ml of virus suspension containing \(10^{2.5}\) TCD\(_{50}\) per 0.1 ml.

Treatment with cyclosporine: Cyclosporine (kindly supplied by Sandoz Pharmaceuticals Ltd., Japan) was dissolved in 0.1 ml of a solution of 10% ethanol/90% olive oil, and administered subcutaneously at a dose of 25 mg/kg/day. This dosage was chosen because previous literature reported that it is well tolerated and effectively immunosuppressive in animals. Treatment with cyclosporine started on day 4 after infection. Control mice were given subcutaneous injections of 0.1 ml of the solution without cyclosporine. Mice were sacrificed on days 7 and 14. Blood was obtained from the retroorbital plexus, and the hearts were sectioned longitudinally through both atria and ventricles, and processed for later examination.

Histologic examination: The hearts were frozen immediately in OCT compound, and 4 \(\mu\)m-thick sections were stained with hematoxylin-eosin. The extent of cellular infiltration, myocardial necrosis, and calcification were scored blindly from 0 to 4. Grade 0 indicated no or questionable lesions, and grade 1 indicated those of less than 25%. Grade 2, 3, 4 indicated 25% increments, respectively.

Lymphocyte subsets in peripheral blood: The heparinized blood was diluted with phosphate-buffered saline (PBS), and layered gently onto 3 ml of Ficoll-Paque (Pharmacia), and centrifuged at 1500 rpm for 30 min. The lymphocytes obtained were washed twice in phosphate-buffered saline, and counted in a standard hemocytometer using trypan blue. They were suspended in RPMI-1640 medium with 2.5% fetal calf serum (FCS), and put into V-bottomed tubes at a concentration of \(1 \times 10^6\) cells/ml. B cells were determined by staining with fluorescein isothiocyanate (FITC) -labelled goat anti-mouse IgG (Cappel, Pennsylvania, U.S.A.). Biotin-conjugated rat anti-mouse Thy 1.2, Lyt 1, Lyt 2 (Beckton Dickinson, California, U.S.A.) were used as subsets of T lymphocytes. The tubes examining the subsets of T lymphocytes were centrifuged at 1500 rpm for 5 min, and the cell pellet was suspended in 50 \(\mu\)l of each antibody, diluted at 1:20. After incubation for 30 min at 4°C, the cells were washed twice and suspended in 50 \(\mu\)l of avidin-conjugated fluorescein (Vector, California, U.S.A.) diluted at 1:50, and incubated for 30 min at 4°C. The tubes examining the B lymphocytes were centrifuged at 1500 rpm for 5 min, and the cell pellet was suspended in 50 \(\mu\)l of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Cappel, Pennsylvania, U.S.A.) diluted at 1:50, and incubated for 30 min at 4°C. Fluorescence analysis was performed using flow cytometry (Ortho Spectrum III, Ortho Diagnostic System Inc., Massachusetts, U.S.A.).

Lymphocyte subsets in the heart in situ: Four \(\mu\)m-thick sections of the frozen hearts were obtained using a cryostat, placed on slide glass, air-dried for 1 h, and fixed in aceton for 10 min at 4°C. After washing 3 times in phosphate-buffered saline, the endogenous peroxidase was blocked with 0.3% H\(_2\)O\(_2\) in methanol for 10 min at room temperature. The sections were again washed 3 times in phosphate-buffered saline, and were then incubated with phosphate-buffered saline containing 0.5% bovine serum albumin (Sigma) for 30 min at room temperature. After washing 3 times in phosphate-buffered saline, they were incubated with biotin-conjugated rat anti-mouse Thy 1.2, Lyt 1, and Lyt 2, diluted at 1:100, for 2 h at room temperature. The sections were the washed 3 times in phosphate-buffered saline, and incubated with avidin.
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Fig.1. The survival rate of the cyclosporine group was significantly (p<0.05) lower than that of the control group on days 10 to 14.

TABLE I COMPARISON OF HISTOLOGIC LEGIONS ON DAY 7

<table>
<thead>
<tr>
<th></th>
<th>Cs (−)</th>
<th>Cs (+)</th>
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<tbody>
<tr>
<td>Infiltration</td>
<td>2.0±0.9</td>
<td>2.8±0.8</td>
</tr>
<tr>
<td>Necrosis</td>
<td>2.0±0.9</td>
<td>2.8±0.8</td>
</tr>
<tr>
<td>Calcification</td>
<td>0±0</td>
<td>0.2±0.4</td>
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Values are given as mean ± standard deviation. Cs = cyclosporine.

TABLE II COMPARISON OF LYMPHOCYTE SUBSETS IN THE PERIPHERAL BLOOD ON DAY 7 AND 14

<table>
<thead>
<tr>
<th></th>
<th>Day 7</th>
<th>Day 14</th>
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<tbody>
<tr>
<td></td>
<td>Cs (−)</td>
<td>Cs (+)</td>
</tr>
<tr>
<td>Thy 1.2 (%)</td>
<td>66±13</td>
<td>69±8</td>
</tr>
<tr>
<td>Lyt 1 (%)</td>
<td>39±11</td>
<td>37±6</td>
</tr>
<tr>
<td>Lyt 2 (%)</td>
<td>16±10</td>
<td>10±4</td>
</tr>
<tr>
<td>B cell (%)</td>
<td>5±2</td>
<td>5±2</td>
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Values are given as mean ± standard deviation. Cs = cyclosporine.

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TABLE III  COMPARISON OF LYMPHOCYTE SUBSETS IN SITU IN THE HEART ON DAY 7

<table>
<thead>
<tr>
<th></th>
<th>Cs (−)</th>
<th>Cs (+)</th>
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<tbody>
<tr>
<td>Thy 1.2 (%)</td>
<td>14±4</td>
<td>13±5</td>
</tr>
<tr>
<td>Lyt 1 (%)</td>
<td>6±1</td>
<td>4±1</td>
</tr>
<tr>
<td>Lyt 2 (%)</td>
<td>1±1</td>
<td>1±1</td>
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</table>

Values are given as mean ± standard deviation. Cs = cyclosporine.

were counted using an inverted microscope.

Neutralizing antibody titrations: Volumes (0.1 ml per well) of the FL cell suspensions described above, were laid in each well of 96-well plastic plates, and allowed to grow for 2 days at 37°C in the incubator. The sera, obtained under sterile conditions, were immobilized at 56°C for 30 min, and volumes (25 μl) of the sera which were serially diluted by two-fold increments were incubated for 30 min at 37°C with the same volume of suspensions containing 100 TCD₅₀ of the virus. The incubated sera were then adsorbed onto FL cell monolayers for 60 min at 37°C in the incubator, and the same volumes (50 μl) of Eagle’s minimum essential medium with 2% fetal calf serum were laid over them. Plates were observed daily for a week for signs of characteristic cytopathic effect. The highest dilution of the sera that inhibited these cytopathic effects in more than one of two wells containing the same dilution of serum was determined to be the titer of the neutralizing antibody.

Anti-heart autoantibody titrations: A heart was removed from an uninfected 4-week-old BALB/c mouse, frozen in OCT compound, and 4 μm thick sections were cut in a cryotome. The sections were overlaid with the sera of infected mice serially diluted by two-fold increments, and incubated overnight at 4°C. After washing 3 times in phosphate-buffered saline, the specimens were incubated for 6 h at 4°C with fluorescein isothiocyanate-conjugated rabbit antimouse IgG (Cappel, Pennsylvania, U.S.A.) diluted at 1:20. The sections were again washed 3 times in phosphate-buffered saline, and then overlaid with a solution of 50% phosphate-buffered saline, coverslipped, and examined using a fluorescent microscope (Nikon fluophoto). The highest dilution of the sera which showed a positive staining pattern was determined to be the titer of the anti-heart autoantibody.

Absorption of the sera was carried out by
incubating the sera at the highest dilution which showed a positive pattern with 10% (w/v) homogenates of the heart and liver of 4-week-old BALB/c mice. After incubation for 2 h at 37 °C and then overnight at 4 °C, the tubes were centrifuged at 500 rpm for 10 min, and the supernatants were examined using the method described above to determine whether the positive patterns still remained.

Statistical analysis: Survival data were analyzed using the Kaplan-Meier's method. Mice sacrificed on day 7 and 14 to obtain the peripheral blood and heart were excluded from this analysis. The other data were analyzed using a student's unpaired t test. Wilcoxon rank sum distribution test was also applied for the analysis of titers of neutralizing antibody and anti-heart autoantibody. All results were expressed as the mean ± SD (standard deviation).

RESULTS

Survival rate (Fig. 1): Mice appeared ill on day 4 after infection, and began to die on day 5. By day 15, 14 of the 31 mice in the control group had died, while in the cyclosporine-treated group, 47 of the 67 mice had died. About two-thirds (34 out of 47) of the mice in the cyclosporine-treated group died between days 7 and 10.

The survival rate of the cyclosporine-treated group was significantly lower than that of the control group after day 9.

Fig. 5. Fluorescence photographs demonstrating positive and negative anti-heart autoantibody using an indirect immunofluorescent technique.
a) Left, positive pattern (×185)
b) Right, negative pattern (×185)
The positive pattern shows striated stainings.
Histologic lesions (Table I): On day 7, mice in the cyclosporine-treated group showed a tendency towards more severe histologic changes; however, these differences were not found to be statistically significant.

Lymphocyte subsets in the peripheral blood (Table II): On day 7, the frequency of Lyt 2 decreased slightly in mice treated with cyclosporine compared with those of the control group. In both groups, Lyt 1 showed a tendency towards higher values on day 14 compared with day 7. Again, however, these changes were not statistically significant.

Lymphocyte subsets in the heart in situ (Table III): Subsets of T cells were examined on day 7; however, no significant differences were found.

Viral titers in the heart (Fig. 2): On day 7, a viral titer obtained from the heart of a cyclosporine-treated mouse showed a high value (870 plaque forming units (PFU)/mg heart weight); however, the histologic indexes of the same heart did not exhibit severe changes (the index of cellular infiltration was 3, that of myocardial necrosis was 3, and the index of calcification was 1). The average viral titer of the cyclosporine-treated group (444±245 plaque forming units/mg) did not show a significant change compared with that of the control group (275±161 plaque forming units/mg) on day 7, and no virus was found in either group on day 14.

Titers of neutralizing antibody (Fig. 3): On day 7, the titers of the cyclosporine-treated group were 1013±820, while the control group titers were 1387±629. On day 14, the titers were 2347±523 in the cyclosporine-treated group and 2027±850 in the control group. These differences were not significant.

Titers of anti-heart autoantibody (Fig. 4): Typical examples of both positive and negative anti-heart autoantibody patterns are shown in Fig. 5. The positive pattern (5a) was obtained using serum from a cyclosporine-treated mouse on day 14 diluted at 1:10, while the negative pattern (5b) was obtained using serum from a control mouse on day 7 diluted at 1:10. In the longitudinal sections of the myocardial fibers, the positive pattern showed striated stainings. On day 7, the sera from the cyclosporine-treated mice showed higher titers of anti-heart autoantibody compared with those from control mice (12±7 vs. 4±2, n=6 each, p<0.05); however, no significant difference was found in the tests performed on day 14 (28±15 vs. 39±34, n=6 each). After absorption by liver homogenates, the serum, described above, still exhibited a positive pattern. After absorption by homogenates of the heart, however, the serum showed a negative pattern.

DISCUSSION

Immunologic aspects of myocarditis: In viral myocarditis, the direct cytopathic effect of the virus may initiate myocardial injury, and alter the antigenicity in myocardial tissue. It has been postulated that the immunologic responses, stimulated by altered antigens, may play an important role in the successive myocardial injury of viral myocarditis and its progression to dilated cardiomyopathy. Some investigators reported that a marked decrease in the severity of cardiac inflammation and necrosis was observed in T-lymphocyte-depleted mice and in nude mice. Wong and Huber et al showed that cytotoxic T lymphocytes (especially autoreactive cytotoxic T lymphocytes) were involved in myocardial injury. If these hypotheses are correct, appropriate immunosuppressive therapy may lessen the degree of myocardial injury. Much conflict has arisen over the use of immunosuppressive agents in the treatment of viral myocarditis. Laboratory investigations revealed that steroids, given at an early stage, tended to aggravate the course of acute viral myocarditis; however, in clinical studies, clinical improvement associated with the resolution of inflammatory infiltrate in the cardiac biopsies was observed in some patients after immunosuppressive therapy with steroids. Cyclosporine is effective for many immunologically mediated diseases and transplant rejection but its clinical application for viral myocarditis is still under study. Some investigators demonstrated the deleterious effects of immunosuppression with cyclosporine in murine viral myocarditis. Monrad et al observed a higher mortality rate when cyclosporine was administered early in the illness, and a grea-
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ter heart failure rate when it was administered during the early recovery period. Our study yielded similar results with regard to mortality and pathologic changes. To elucidate this deleterious effect of cyclosporine, we examined lymphocyte subsets in peripheral blood as well as in the heart in situ, neutralizing antibodies, and anti-heart antibodies.

Lymphocyte subsets: Detailed evaluation of lymphocyte subsets has become possible because of the recent availability of monoclonal antibodies specific for cell surface markers and imbalance between OKT4-positive cells (helper/inducer cells) and OKT8-positive cells (suppressor/cytotoxic cells) has been reported in peripheral blood in patients with active myocarditis and dilated cardiomyopathy. Recently, serial changes of lymphocyte subsets in the peripheral blood and the heart in situ were studied in experimental murine myocarditis. Matsumori et al. reported that Lyt 1-positive cell concentrations decreased in peripheral blood, and increased in the heart in situ during the acute phase of viral myocarditis. Kishimoto et al. suggested that the investigation of the inflammatory site provided clues to the disease's pathogenesis which could not be found in studies of the peripheral blood. One possible explanation for the failure of cyclosporine to alter the lymphocyte subsets significantly, either in the peripheral blood or the heart in situ, is that cyclosporine has a different affinity for lymphocytes in different organs. Ryffel et al. reported that binding capacity of cyclosporine to lymphocytes in different organs was in the order of thymus > spleen > mesenteric lymph node (pure T cells) > mesenteric lymph node (pure B cells). Using the fluorescence activated cell sorting system, Hiramine et al. suggested that cyclosporine affected both thymus and spleen cells in vivo, preferentially impairing the L3T4+Lyt2- subset (helper T cells or their precursors) within the thymus, and the lymph node cells seemed to be relatively spared from the in vivo effect of cyclosporine compared with cells in the thymus and spleen.

Despite changes of lymphocyte subsets having been reported in myocarditis, the lack of further change with cyclosporine may indicate that the deleterious effect of cyclosporine is not associated with lymphocyte subsets.

Anti-heart antibody: Anti-heart antibody has been demonstrated in some heart diseases such as postmyocardial infarction syndrome, rheumatic valvular disease, Chagas' disease, and idiopathic cardiomyopathy. In dilated cardiomyopathy the prevalence of anti-heart antibody has varied widely in different studies. Yamakawa et al. found a higher frequency (81%) compared with other diseases, but Lowry et al. showed no consistent or specific abnormality between the diseases. Maisch et al. observed a higher incidence of anti-sarcolemmal antibody and anti-myolemmal antibody in patients who showed clinical or histologic evidence of previous perimyocarditis. They also studied anti-heart antibody in patients with acute myocarditis. This inconsistent frequency observed in dilated cardiomyopathy may reflect its multifactorial etiology and varied severity, and the fact that patients were studied in different stages of illness. Matsumori et al. showed that anti-heart antibody appeared before day 7, and was highest on day 21 in DBA/2 mice infected with encephalomyocarditis virus. It is still unclear as to whether anti-heart antibodies are a result of myocardial damage or whether they participate in a pathogenic process. In our study, the mice treated with cyclosporine had a higher mortality rate, higher titers of anti-heart antibody, and an increased frequency of myocardial injury in the early stage of viral myocarditis. Cyclosporine is thought to act as a suppressor of the T cell helper system. It is interesting to note that cyclosporine was associated with higher titers of anti-heart antibody.

One possible explanation for this is that cyclosporine accelerates the myocardial injury by immunologic for example, suppression of an appropriate immunologic response, inhibition of natural killer activity or nonimmunologic effects (for example, induction of renal insufficiency and altered renal handling of electrolytes), and this in turn enhances the production of anti-heart antibody. It was reported that the dose of cyclosporine which would not have a deleterious effect on the
kidney, would exacerbate renal function in case of renal ischemia. So, a dose of cyclosporine that would not produce any adverse effect in a normal heart may have deleterious effects in cases of myocardial dysfunction with myocarditis. Another possibility is that cyclosporine has a selective effect on the production of anti-heart antibody, which in turn induces myocardial injury. Using lymphocytic choriomeningitis virus, Huegin et al. reported that high doses (50-60 mg/kg) of cyclosporine are efficient in completely suppressing primary T cell responses; however, low doses (10-20 mg/kg) often enhance T cell responses. Cyclosporine also has a selective effect according to the antigenic stimulus used. Only one dosing regimen of cyclosporine was used in this study, and other regimens may yield different results.

Clinical implications: To the extent that a murine model of acute encephalomyocarditis infection may permit extrapolation to human myocarditis secondary to other virus infection, the results of this study suggest that great caution should be adopted in the use of cyclosporine in the treatment of acute viral myocarditis in man. However, immunosuppressive therapy in clinical myocarditis is targeted at patients in various stages of disease progression, and therefore the results of the present study limited to the acute phase may not be extrapolated to the later subacute and chronic stages of myocarditis.

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