Absence of Effects of Cyclosporine on Myocardial Lymphocyte Subsets in Coxsackievirus B3 Myocarditis in the Aviremic Stage

Chiharu Kishimoto and Walter H. Abelmann

To test the therapeutic efficacy of immunosuppression with cyclosporine upon the aviremic stage of coxsackievirus B3 (CB3) myocarditis, 2-week-old BALB/c mice were inoculated with $3 \times 10^2$ plaque-forming units of CB3, and the effects of cyclosporine on peripheral, splenic, and myocardial lymphocyte subsets were investigated. Cyclosporine, 25 mg/kg/day, was administered subcutaneously daily on days 10–31 (experiment 1) and days 30–51 (experiment 2). Treated groups were compared with infected controls for each experiment. In experiment 1, the survival rate of the cyclosporine-treated group was low (17/25 vs. 24/25, $p<0.05$). The severity of myocardial lesions and the distribution of lymphocyte subsets in myocardium and spleen on days 15–18 did not differ between treated and control groups; on the other hand, the percentages of peripheral Thy 1.2+ (pan T) and L3T4+ (activated helper T) cells on days 15–18 were decreased in the treated group, and those of B, Lyt 1+ (helper/inducer T), and Lyt 2+ (suppressor/cytotoxic T) subsets did not differ significantly. Notably, myocardial interleukin-2 receptor (IL-2R) positive cells, through which cyclosporine is considered to act, were scarce in both groups. In experiment 2, survival rates of two groups did not differ (treated, 32/34; untreated, 34/34; $p=NS$). The severity of myocardial lesions and the distribution of splenic lymphocyte subsets on days 35–38 also were not different between two groups. The percentages of peripheral lymphocyte subsets (Thy 1.2+ and L3T4+) were decreased in the treated group; those of B, Lyt 1+, and Lyt 2+ subsets did not differ significantly. In experiments 1 and 2, the thymus/body weight ratio in the treated groups was smaller than in the untreated group, but the spleen/body weight ratio in the treated group did not differ from the untreated group; histologically, medullary cellular depletion was evident in the thymus, not in the spleen, of the treated groups. We conclude that cyclosporine failed to change the distribution of lymphocyte subsets in the spleen as well as in the myocardium in CB3 myocarditis although it had effects on the peripheral blood and thymus, which may account for the higher mortality in the treated groups. The absence of beneficial effects of cyclosporine upon the CB3-infected myocardium may be related to the paucity of cyclosporine-sensitive cells (IL-2R, L3T4, and Lyt 2 positive cells) in the myocardium. (Circulation Research 1989;65:934–945)

There is increasing evidence that chronic dilated cardiomyopathy may result from prior viral myocarditis.1–4 Coxsackievirus B3 (CB3) is an enterovirus that can cause acute myocarditis in humans.1 We have previously shown that CB3 infection in various inbred strains of mice produces mild to severe myocarditis that is followed by chronic myocardial dysfunction and congestive heart failure.5 Evidence clearly indicates that tissue damage results not only from CB3-induced direct myocytolysis but also from T lymphocyte-dependent cytotoxic mechanisms.6 Furthermore, cells belonging to the Thy 1.2+ (pan T) and the Lyt 1+ and Lyt 2+ (immature T) subsets are primarily pathogenic in CB3 myocarditis although it had effects on the peripheral blood and thymus, which may account for the higher mortality in the treated groups. The absence of beneficial effects of cyclosporine upon the CB3-infected myocardium may be related to the paucity of cyclosporine-sensitive cells (IL-2R, L3T4, and Lyt 2 positive cells) in the myocardium. (Circulation Research 1989;65:934–945)

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Supported in part by a Research Fellowship from the American Heart Association, Massachusetts Affiliate (C.K.).

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Received April 2, 1988; accepted April 17, 1989.
autoimmune origin and in the suppression of allograft transplantation rejection. The in vivo and in vitro effects of cyclosporine on experimental viral myocarditis have already been reported\textsuperscript{11-14}; cyclosporine aggravated the course and the severity of the disease. However, to understand fully the in vivo actions of cyclosporine and the occasional associated immune abnormalities, one must have a clear understanding of the full spectrum of the immunopathologic effects. To address whether cyclosporine may suppress or aggravate the course of chronic cardiomyopathy, we examined the effects of immunosuppression with cyclosporine on the clinical course, cardiac pathology, and distribution of lymphocyte subsets in various organs in the aviremic stage of CB3 myocarditis.

**Materials and Methods**

**Infection Protocol**

The virus stock of CB3 (Nancy strain) (American Type Culture Collection) was prepared in cultures of VERO (kidney of African green monkey) cells in Eagle's minimum essential medium (EMEM). Virus suspensions were centrifuged after the cytopathic effect had developed and viral stock had a titer of more than 10\(^{9}\) plaque-forming units (PFU)/ml, determined in tissue cultures of VERO cells.

Two-week-old, male, inbred, certified virus-free BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) were studied under a protocol approved by the Beth Israel Hospital Animal Research Committee. They were supplied with mother mice until 4 weeks old and were maintained in laminar-flow isolation rooms throughout the study. They were inoculated intraperitoneally with 0.1 ml virus suspension containing 3x10\(^{5}\) PFU/ml.

**Treatment Protocol**

Cyclosporine was supplied by David Winter, MD (Sandoz, Nutley, New Jersey). Because of its strongly hydrophobic nature, the cyclosporine was dissolved in a 0.1-ml solution of 10% ethanol and 90% olive oil and administered subcutaneously daily at rotated sites at a dose of 25 mg/kg/day; the actual dose for each experiment was calculated from mouse weight at the beginning of the experiments. This dose was chosen because previous reports\textsuperscript{11-14} demonstrated this to be well tolerated and effectively immunosuppressive in animals.

**Experiment 1: Early protocol.** Sixty mice surviving 10 days after virus inoculation, at which time virus ordinarily can no longer be isolated from myocardium, were randomized either to no cyclosporine treatment \((n=30)\) or to treatment with cyclosporine \((n=30)\). Mice in the untreated group were sham-injected subcutaneously with 0.1 ml of 10% ethanol and 90% olive oil for the treatment period. Starting 10 days after inoculation, treatment was administered for 21 days. The mice were observed daily, and necropsy was performed immediately on those mice found dead. Mice surviving to the end of the treatment period were killed and necropsied. At necropsy, spleen, thymus, lung, liver, and heart were weighed. Body weight was also measured, and the ratios of organ to body weight were then calculated.

Additional control groups of uninfected mice that were treated for 21 days with cyclosporine (administered as above, \(n=5\)) or without cyclosporine (sham-injected subcutaneously with 0.1 ml of 10% ethanol and 90% olive oil, \(n=5\)) were also prepared. The treatment period for the control group (age-matched for the study) also began on day 10. For the analysis of lymphocyte subsets, five mice in each group (including uninfected groups) were killed on days 15–18; the spleen, heart, and peripheral blood were processed for immunologic studies. The infected hearts from mice killed on day 31 in both groups were also processed for immunologic study (each, \(n=4\)).

**Experiment 2: Late protocol.** Seventy-eight mice surviving 30 days after virus inoculation were randomized to either no treatment (sham-injected subcutaneously with 0.1 ml of 10% ethanol and 90% olive oil) \((n=39)\) or treatment with cyclosporine (administered as above) \((n=39)\). Treatment was administered for 21 days until 51 days after inoculation, at which time surviving mice were killed and necropsied. The mice were observed daily, and necropsy and organ weight measurements were performed on all mice that died during the course of the experiment.

Also, additional control groups of uninfected mice treated for 21 days with \((n=5)\) or without \((n=5)\) cyclosporine were prepared. The treatment period for the control group (age-matched for the study) also began on day 30. For the analysis of lymphocyte subsets, five mice in each group (including uninfected groups) were killed on days 35–38. The spleen and peripheral blood were processed for immunologic studies; myocardial lymphocyte subset study was not performed in experiment 2 because of paucity of infiltrating lymphocytes in the myocardium in this period.

**Immunologic Protocol**

**Immunofluorescence study.** The spleen and peripheral blood were subsampled (as described previously) and processed for the serial determination of lymphocyte subsets. Staining methods were similar to those described previously.\textsuperscript{6} The spleen was minced gently with a sterile stainless mesh. After mincing, the cell suspension was rapidly pipetted with a sterile Pasteur pipette into 20 ml Hanks' balanced salt solution (HBSS), filtered through nylon mesh to eliminate debris, and centrifuged at 1,500 rpm for 5 minutes. The cells were washed twice with HBSS. Peripheral blood \((0.8–1.3 \text{ ml})\) was collected in heparin and washed twice with HBSS. The red blood cells in the peripheral blood were lysed by hypotonic shock. The lymphocyte frac-
B cells were determined by staining the fluorescein isothiocyanate–labeled rabbit anti-mouse immunoglobulin (Miles Scientific, Naperville, Illinois). T cells were stained by use of monoclonal rat anti-Thy 1.2 (Becton Dickinson, Immunocytometry Systems, San Jose, California) and monoclonal rat anti-Lyt 1 (Becton Dickinson), rat anti-Lyt 2 (Becton Dickinson), and rat anti-L3T4 (Becton Dickinson) as the first layer of antibodies and fluorescein isothiocyanate–labeled rabbit anti-mouse immunoglobulin as the second.

Fluorescein isothiocyanate–labeled rabbit anti-mouse immunoglobulin was diluted 10-fold, and rat monoclonal antibodies were diluted 10-fold. The cells (1 × 10^6) were put into the V-bottomed tubes and centrifuged at 1,500 rpm for 3 minutes. After centrifugation, the cell pellet was suspended in 100 μl RPMI-1640 media with 2.5% fetal calf serum and 0.1% NaN₃ or the first layer of antibodies. After incubation for 30 minutes at 4°C, the cells were washed three times and suspended in 100 μl of the second layer of antibodies. The cells were washed twice with RPMI-1640 media with 2.5% fetal calf serum and 0.1% NaN₃ after 30 minutes of incubation at 4°C and suspended in 1.0 ml RPMI-1640 media with 2.5% fetal calf serum and 0.1% NaN₃.

**TABLE 1. Cardiac Histology and Organ Weights in Experiments 1 and 2**

<table>
<thead>
<tr>
<th>Cardiac histology</th>
<th>BW (g)</th>
<th>HW/BW (×10^-3)</th>
<th>SpW/BW (×10^-3)</th>
<th>ThW/BW (×10^-3)</th>
<th>LuW/BW (×10^-3)</th>
<th>LiW/BW (×10^-3)</th>
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</thead>
<tbody>
<tr>
<td>Infected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n=30)</td>
<td>0.7±0.7</td>
<td>1.0±1.2</td>
<td>1.1±1.1</td>
<td>0.5±0.9</td>
<td>19.5±4.2</td>
<td>6.6±1.8</td>
</tr>
<tr>
<td>Treated (n=30)</td>
<td>0.9±0.9</td>
<td>1.2±1.2</td>
<td>1.3±1.1</td>
<td>0.6±1.1</td>
<td>15.8±5.1*</td>
<td>6.8±2.3</td>
</tr>
<tr>
<td>Uninfected</td>
<td>NL</td>
<td>NL</td>
<td>NL</td>
<td>NL</td>
<td>20.5±1.5</td>
<td>5.9±0.4</td>
</tr>
<tr>
<td>Treated (n=5)</td>
<td>NL</td>
<td>NL</td>
<td>NL</td>
<td>NL</td>
<td>18.8±1.9</td>
<td>5.6±0.5</td>
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<tr>
<td>Infected</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (n=39)</td>
<td>0.8±0.6</td>
<td>0.9±0.9</td>
<td>1.1±0.7</td>
<td>0.6±0.7</td>
<td>21.5±2.2</td>
<td>5.9±0.8</td>
</tr>
<tr>
<td>Treated (n=39)</td>
<td>0.9±0.7</td>
<td>1.0±0.7</td>
<td>1.1±0.8</td>
<td>0.6±0.6</td>
<td>20.3±1.6</td>
<td>6.0±0.9</td>
</tr>
<tr>
<td>Uninfected</td>
<td>NL</td>
<td>NL</td>
<td>NL</td>
<td>NL</td>
<td>23.1±1.0</td>
<td>5.7±0.9</td>
</tr>
<tr>
<td>Treated (n=5)</td>
<td>NL</td>
<td>NL</td>
<td>NL</td>
<td>NL</td>
<td>22.4±0.4</td>
<td>5.6±0.3</td>
</tr>
</tbody>
</table>

Values are mean±SD. I, infiltration; N, necrosis; C, calcification; F, fibrosis; BW, body weight; HW/BW, heart weight/body weight ratio; SpW/BW, spleen weight/body weight ratio; ThW/BW, thymus weight/body weight ratio; LuW/BW, lung weight/body weight ratio; LiW/BW, liver weight/body weight ratio; NL, normal. Statistical significance of the observed differences in the infected or the uninfected protocol between the untreated and the treated groups is denoted by p value.

*p<0.01.

FIGURE 1. Graph showing the effects of cyclosporine on survival in murine (BALB/c mice) myocarditis with coxsackievirus B3. When the drug was administered early in the illness (experiment I), there was a significantly greater (p<0.05) mortality in the mice treated with cyclosporine (closed circles) than in control mice (open circles). Even when administered later in the illness (closed circles) (experiment II), no significant improvement (NS) compared with control mice (open circles) resulted. See text for details.

FIGURE 2. Photomicrographs showing the effects of cyclosporine on histopathology of thymus in coxsackievirus B3 myocarditis in experiment I. In a mouse treated with cyclosporine (top panel), medullary thymocyte atrophy was more severe compared with a control (infected) mouse (bottom panel). C, cortex; M, medulla. Stain, hematoxylin-eosin. Original magnification (both panels), ×500.
Table 2. Lymphocyte Subsets in the Peripheral Blood and Spleen on Days 15–18 in Experiment 1 and on Days 35–38 in Experiment 2

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Uninfected</th>
<th>Treated</th>
<th>Infected</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated (n=5)</td>
<td>Treated (n=5)</td>
<td>Untreated (n=5)</td>
<td>Treated (n=5)</td>
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<tr>
<td>Peripheral blood (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>25.9±7.0</td>
<td>24.2±10.9</td>
<td>29.5±7.9</td>
<td>19.2±11.1</td>
</tr>
<tr>
<td>Thy 1.2+</td>
<td>60.1±14.7</td>
<td>7.6±10.4+</td>
<td>28.5±16.1</td>
<td>4.3±4.7+</td>
</tr>
<tr>
<td>Lyt 1+</td>
<td>59.5±14.5</td>
<td>27.9±9.0t</td>
<td>22.6±12.6</td>
<td>19.5±11.6</td>
</tr>
<tr>
<td>Lyt 2+</td>
<td>39.1±15.3</td>
<td>13.0±13.5T</td>
<td>13.7±4.8</td>
<td>14.6±11.6</td>
</tr>
<tr>
<td>L3T4+</td>
<td>53.6±12.0</td>
<td>9.5±15.6</td>
<td>26.9±8.3</td>
<td>13.0±9.8+</td>
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<tr>
<td>TLC (x10⁶)</td>
<td>1.7±0.4</td>
<td>1.0±0.5</td>
<td>1.4±0.4</td>
<td>0.9±0.5</td>
</tr>
<tr>
<td>Spleen (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>41.8±21.5</td>
<td>41.9±6.5</td>
<td>40.7±11.0</td>
<td>30.4±16.3</td>
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<tr>
<td>Thy 1.2+</td>
<td>31.6±8.0</td>
<td>31.3±5.9</td>
<td>21.7±11.3</td>
<td>17.3±14.7</td>
</tr>
<tr>
<td>Lyt 1+</td>
<td>28.3±12.9</td>
<td>30.4±8.8</td>
<td>21.1±13.6</td>
<td>18.2±11.3</td>
</tr>
<tr>
<td>Lyt 2+</td>
<td>21.2±6.2</td>
<td>22.5±2.8</td>
<td>13.8±6.8</td>
<td>18.7±12.4</td>
</tr>
<tr>
<td>L3T4+</td>
<td>26.2±13.3</td>
<td>26.3±5.0</td>
<td>13.9±15.6</td>
<td>16.9±16.9</td>
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<tr>
<td>TLC (x10⁶)</td>
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<td>2.5±0.3</td>
<td>2.5±0.3</td>
<td>2.4±0.7</td>
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<tr>
<td>Experiment 2</td>
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<tr>
<td>Peripheral blood (%)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>B</td>
<td>27.8±8.9</td>
<td>26.9±4.5</td>
<td>27.4±6.9</td>
<td>22.7±5.4</td>
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<tr>
<td>Thy 1.2+</td>
<td>61.2±11.1</td>
<td>20.9±10.9+</td>
<td>45.9±10.9</td>
<td>11.1±7.0+</td>
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<tr>
<td>Lyt 1+</td>
<td>59.8±5.3</td>
<td>29.5±11.8+</td>
<td>31.9±10.8</td>
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<td>Lyt 2+</td>
<td>32.6±9.3</td>
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<td>L3T4+</td>
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<td>38.2±13.7</td>
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<td>TLC (x10⁶)</td>
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<td>1.4±0.4</td>
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<tr>
<td>Spleen (%)</td>
<td></td>
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</tr>
<tr>
<td>B</td>
<td>42.8±2.6</td>
<td>44.9±3.4</td>
<td>46.0±9.8</td>
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<tr>
<td>Thy 1.2+</td>
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<tr>
<td>Lyt 1+</td>
<td>30.4±10.0</td>
<td>30.5±10.1</td>
<td>22.9±13.5</td>
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<td>Lyt 2+</td>
<td>20.2±6.3</td>
<td>20.8±7.7</td>
<td>24.3±12.7</td>
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<td>L3T4+</td>
<td>21.2±6.9</td>
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<tr>
<td>TLC (x10⁶)</td>
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</table>

Values are mean±SD. Thy 1.2+, pan T cell; Lyt 1+ and Lyt 2+, immature T cells; L3T4+, activated helper T cell; TLC, total lymphocyte counts per one mouse. Statistical significance of the observed differences in the uninfected or the infected protocol between the untreated and the treated groups is denoted by p value.

* p<0.001.
† p<0.05.
‡ p<0.01.

Preparations of these cells were observed under a fluorescence microscope. The percentage of positive fluorescent cells in each specimen was determined by the examination of at least 100 cells. The percentages of T cells and subsets were obtained by subtracting the percentage of B cells from those obtained after staining with each monoclonal rat antibody and fluorescein isothiocyanate-labeled rabbit anti-mouse immunoglobulin.

Interpretation. As mentioned briefly earlier, Thy 1.2+ is a pan T cell; Lyt 1+ is a precursor of the other T cell subsets (immature T cell) and helper/inducer T cell; and Lyt 2+ is a precursor of the other T cell subsets (immature T cell) and suppressor/cytotoxic T cell. L3T4+ is an activated helper T cell.17

Immunopathologic study. Hearts were subsampled (as described previously) and sectioned into two equal cross sections in the short axis, and one half of the heart was processed for the immunohistologic study; hearts were embedded in OCT compound (Miles Laboratories), snap-frozen in a mixture of dry ice and acetone, and stored in a deep freezer (−120°C). The other half was processed for pathological study. Six-micrometer sections were cut from the frozen blocks on a cryostat at −20°C, placed on glass slides, air-dried for 1 hour, and fixed in 95% cold methanol. Cell surface markers were demonstrated in situ by 3,3'-diaminobenzidine tetrahydrochloride immunoperoxidase staining with the use of the same series of monoclonal rat anti-mouse alloantigen antibodies as in experiment 1 for the first layer of antibodies and peroxidase-conjugated rabbit anti-rat immunoglobulin (Dako, Santa Barbara, California) for the second. In addition, infiltrating B cells were stained by rat monoclonal antibody, Bet 1,18 which reacts specifically to...
Pathology Protocol

Tissues (heart, thymus, spleen, lung, liver, kidney, and pancreas) were processed by standard methods, embedded in paraffin, cut into 5-µm thick sections, and stained with hematoxylin-eosin. Myocardial sections were graded by the authors, who were blinded to the respective treatment groups, for severity of inflammation, necrosis, calcification, and fibrosis of the ventricles. The mean value was cited.

The pathological criteria for grading of the severity of myocardial inflammation, necrosis, calcification, or fibrosis were as follows: grade 1 (mild), one or two small foci; grade 2 (slight), several small foci; grade 3 (moderate), multiple small foci or several large foci; and grade 4 (severe), multiple large foci or diffuse inflammation, necrosis, calcification, or fibrosis.

The other organs were evaluated for evidence of viral or other pathological lesions.

Virological Study

For infectivity assays, hearts were removed aseptically, weighed, and homogenized in 2 ml EMEM after centrifugation at 1,500 rpm for 15 minutes at 4°C. Supernatants were inoculated into VERO cell monolayers, and plaque assays were performed. VERO cells were suspended at a concentration of 10⁶/ml in EMEM with 3% fetal calf serum plus 100 µg/ml penicillin and streptomycin in six-well plastic plates and were allowed to grow for 2 days at 37°C in 5% CO₂. After adsorption, the cells were overlaid with 3 ml medium containing 3% fetal calf serum and 1% methyl cellulose. After 2 days of incubation at 37°C in a humidified atmosphere containing 5% CO₂, cells were fixed with acetic acid and methanol (at a ratio of 1:3) and stained with 1% crystal violet, and plaques were counted with an inverted microscope.

Statistics

Differences in the percent of lymphocyte subsets, lymphocyte (lymphoid) cell numbers, organ weights, and pathological scores were evaluated by unpaired t test. Significant differences in the survival rate were determined by the χ² test with the Yates correction. A value of p<0.05 was considered statistically significant.

Results

Infection with CB3 produced a similar clinical and pathological picture to that reported previously. Three days after inoculation, the mice appeared ill; some developed coat ruffling, weakness, and irritability. Grossly, the myocardium had pale yellow patches that correlated with the inflammation, necrosis, and calcification seen microscopically. Plaque assay of myocardial viral titers showed peak viral titers at days 4-5 after inoculation. No virus could be isolated from the myocardium after day 8. The survival rate on day 10 was 92.7% (140/151).

Experiment 1

Mortality (Figure 1). One mouse in the control group and eight in the cyclosporine-treated group had died by day 31; the survival rate on day 31 was 96.0% (24/25) in the control group and 68% (15/25)
in the treated group. This difference was significant ($p<0.05$).

There were no deaths of uninfected mice throughout the entire period.

Cardiac pathology (Table 1). There were no significant differences in the score of cellular infiltration, myocardial necrosis, calcification, and fibrosis between the two infected groups.

No abnormalities were found in the heart of uninfected mice with or without cyclosporine treatment.

Pathology of other organs (Figure 2). A marked medullary thymocyte atrophy was noted in cyclosporine-treated mice (infected and uninfected). No marked atrophy was noted in the thymuses of untreated groups (infected and uninfected).

Pancreatitis, probably virus-induced, was noted in five control mice and in five treated mice. No viral lesions were noted in the lung, liver, kidney, spleen, or thymus. There were no significant changes of the incidence of congestion of lung and liver in either infected group.

No abnormal findings were observed in heart, lung, liver, kidney, or pancreas in uninfected groups.

Organ weights (Table 1). Body weight in the infected and treated group was significantly less than that in the infected and untreated group. The thymus weight/body weight ratio in treated groups (infected and uninfected) was significantly smaller than that in untreated groups (infected and uninfected). There were no significant differences in spleen weight/body weight, lung weight/body weight, liver weight/body weight, and heart weight/body weight ratios between treated and untreated groups.

Lymphocyte Subset Study.

Peripheral blood. As shown in Table 2, in uninfected mice, the percentages of B, Thy 1.2+, Lyt 1+, Lyt 2+, and L3T4+ cells in the cyclosporine-treated group markedly decreased on days 15–18 compared with the untreated group. In infected mice, the percentages of Thy 1.2+ and L3T4+ cells in the cyclosporine-treated group moderately decreased compared with the untreated group; those of B, Lyt 1+, and Lyt 2+ subsets did not differ significantly.

The numbers of lymphoid cells per mouse are also listed in Table 2.
**Spleen.** Surprisingly, the percentages of each lymphocyte subset did not differ between treated and untreated groups in either infected or uninfected animals.

**Myocardium.** As shown in Figure 3 and Table 3, in the diseased myocardium of the aviremic stage of myocarditis caused by CB3, most of the cells were positive for Thy 1.2 and Lyt 1; Lyt 2 and L3T4 positive cells occupied a very small part of the myocardium. B cells and IL-2R positive cells were sparse.

**Experiment 2**

**Mortality (Figure 1).** No mice in the control group had died by day 51; two mice in the cyclosporine group had died by day 51. The difference in the survival rate was not significant (untreated, 34/34; treated, 32/34; p=NS).

No mice in the uninfected groups died during the entire period.

**Cardiac pathology (Figure 4 and Table 1).** No significant differences in the score of cellular infiltration, myocardial necrosis, calcification, or fibrosis were noted between the two infected groups.

No abnormalities were found in the hearts of uninfected mice with or without cyclosporine treatment.

**Pathology of other organs.** The results were similar to those in experiment 1: marked medullary thymocyte atrophy was noted in cyclosporine-treated mice (infected and uninfected). No marked atrophy was noted in spleen of untreated groups (infected and uninfected).

Pancreatitis that was probably virus induced was noted in six control mice and in five treated mice. No viral lesions were noted in other organs. No significant changes were noted in the incidence of congestion of liver or lung in infected groups.

No abnormal findings were observed in heart, lung, liver, kidney, or pancreas of untreated groups.

**Organ weights (Table 1).** Thymus weight/body weight ratio in treated groups (infected and uninfected) was significantly smaller than that in untreated groups (infected and uninfected). There were no significant differences in spleen weight/body weight, lung weight/body weight, liver weight/body weight, and heart weight/body weight ratios between treated and untreated groups.

**Lymphocyte subset study (Table 2).**

**Peripheral blood.** Results are similar to those of experiment 1: in cyclosporine-treated groups (infected and uninfected), the percentages of Thy 1.2+ and L3T4+ subsets decreased on days 35–38 compared with untreated groups (infected and uninfected); however, those of Lyt 1+ and Lyt 2+ subsets did not differ significantly between infected mice with and without cyclosporine treatment.

**Spleen.** The percentages of each lymphocyte subset did not differ among the four groups.

**Discussion**

It is generally thought that a biphasic disease process results when mice are infected with CB3. In the acute phase, viral replication in the myocardium results in myocardial necrosis with inflammation during the first week. After the virus has been eliminated from the myocardium, a chronic inflammatory reaction results in progressive myocyte damage and hypertrophy, ventricular dilation, and heart failure. There is a suggestion that the chronic phase results from cell-mediated immune responses to a neoantigen that developed during the acute phase of the illness. Hence, immunosuppressive therapies for viral myocarditis have been attempted.

Recently, newer insights on the immunologic aspects of murine CB3 myocarditis have been gained. First of all, the susceptibility to CB3 infection appears to be primarily determined by the characteristics of the host (non-H-2 complex). Second, not B cells but T cells, especially the immature T cell subset, play a role in the development of myocarditis; this has been demonstrated by immunologic approaches revealing different kinetics of lymphocyte subsets in different organs (heart, spleen, and peripheral blood) in the acute stage. Third, we have to recognize that humoral immunity (for example, autoantibodies against heart) also may play a role in the pathogenesis of myocarditis, which may be dependent on the characteristics of the host (non-H-2 complex). Cyclosporine has been shown to inhibit preferentially T cell helper function, probably through inhibition of IL-2 production, while sparing T cell suppressor function, the primary deficiency of which may be responsible for the autoimmune process. Therefore, therapy with cyclosporine might restore a normal helper-suppressor balance.

IL-2 (T cell growth factor) is a T cell–derived lymphokine that stimulates the growth of T cells by interaction with a specific cell surface receptor. IL-2 is expressed on lectin- or antigen-stimulated T cells but not on resting T cells; IL-2 and IL-2R play a central role in T cell activation and possibly T cell neoplasia.

The present study demonstrates that cyclosporine aggravated the course of CB3 myocarditis even in the aviremic stage and that cyclosporine failed to change the distribution of lymphocyte subsets in the spleen or in the myocardium although an immunosuppressive effect on the peripheral blood and the thymus was demonstrated. This effect may have been responsible for the higher mortality observed in cyclosporine-treated groups. The paucity of...
cyclosporine-sensitive (IL-2, L3T4, and Lyt 2 positive) cells in the myocardium may have been responsible for the absence of beneficial effects of cyclosporine on cardiac histology in the subacute and chronic stages of CB3 myocarditis.

In this study, in cyclosporine-treated groups, an effective immunosuppressive state was demonstrated in the peripheral blood (by the immunofluorescence method) and in the thymus (by the pathologic study), but the incompleteness of this immunosuppressive state was shown in the spleen (by both methods). These findings are consistent with previous work; thymocytes have a higher binding capacity for cyclosporine than peripheral T lymphocytes, and in the peripheral blood, cyclosporine-sensitive cells have the receptor for pan T cells (OKT3-positive cells). Furthermore, cyclosporine caused medullary thymocyte depletion (maturation arrest) in the animal studies but induced incomplete depletion of T and B cell zones in the spleen.9,10

There were no significant changes in the distribution of myocardial lymphocyte subsets in untreated and treated groups in experiment 1. Notably, few IL-2R positive cells were present in the diseased myocardium in both groups. With regard to the precise assigned functions of each murine lymphocyte subset, the Lyt 1−-Lyt 2+ subset is the likely precursor of other T cell subsets and has been demonstrated to be an alloreactive and syngeneic cytotoxic lymphocyte subset in addition to the Lyt 1+ and Lyt 2+ subset. The Lyt 1+ and Lyt 2− subset is not only a helper/inducer in antibody and cytotoxic lymphocyte inductions but also an effector of delayed-type hypersensitivity, in which many lymphokines and monokines are involved.17 The L3T4+ subset is the activated helper T cell. So-called null (non-T and non-B) cells are also found. Godeny and Gauntt confirmed the protective role of natural killer cells during the initial period of CB3 myocarditis.

O'Connell et al reported an adverse effect of cyclosporine in the acute viremic stage of CB3 myocarditis, and Monrad et al reported similar results in another murine model. Estrin and coworkers confirmed in vitro resistance of the CB3 murine model to cyclosporine; in their study, two cytolytic T lymphocyte populations are recognized in CB3 infected BALB/c mice. One population belongs to the Lyt 2+ T lymphocyte subset and reacts specifically with uninfected heart cells (autoreactive cytolytic T); the other belongs to the L3T4+ T lymphocyte subset and reacts with infected targets. Although both immune T lymphocyte populations do not cause cardiac inflammation, the autoreactive cytolytic T lymphocyte subset predominantly causes tissue injury, and its generation is independent of IL-2 since neither anti-IL-2 antibody nor cyclosporine inhibits this response. Thus, we tried to demonstrate the effects of this agent on subacute and chronic (aviremic) stages of CB3 myocarditis in BALB/c mice (in which the cellular mechanisms were reported as the main etiologic factor in the development of myocarditis) to clarify the precise in vivo mechanisms by use of immunologic approaches.

The ability of cyclosporine to inhibit T cell activation strongly suggests that this drug would have the potential to function as a potent immunotherapeutic agent in autoimmune diseases; cyclosporine was useful for the prevention of delayed-type hypersensitivity responses, experimental allergic encephalomyelitis, autoimmune uveitis, or type-I diabetes mellitus. On the other hand, it aggravated the disease process of experimental thyroiditis and experimental collagen arthritis. From our data, we believe, one can classify viral myocarditis into the latter category, that is, disease aggravated by cyclosporine.

Several investigators reported that, during an acute stage of myocarditis, sensitized T cells migrate toward the target organ (i.e., heart), where pan T cells, helper/inducer T, and suppressor/cytotoxic T subsets may play a role in the development or prevention of myocarditis. Therefore, another possible explanation for the failure of cyclosporine to act favorably on the diseased myocardium is that because cyclosporine could not induce sufficient immunosuppression in the spleen, sensitized splenic T cells could migrate to the heart via the circulation; indeed, Lyt 1+ (helper/inducer) and Lyt 2+ (suppressor/cytotoxic) subsets in the peripheral blood in treated groups were still high in this study. Thus, even in cyclosporine-treated mice with CB3 myocarditis, cyclosporine-resistant lymphocyte subsets may remain within the host.

In conclusion, this study, by use of precise analysis of lymphocyte subsets, confirms the in vivo failure of cyclosporine to ameliorate the aviremic stage of CB3 myocarditis. Although the beneficial effects of cyclosporine on other inflammatory or autoimmune diseases have been demonstrated, the use of cyclosporine in clinical myocarditis of viral origin may be ineffective if not deleterious.

Acknowledgments

We thank Ms. Kathryn A. Thorp for her technical help and Ms. Carol Anne Centauro for her assistance in preparing this manuscript.

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Cyclosporine in Chronic Coxsackievirus B3 Myocarditis

Kishimoto and Abelmann


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KEY WORDS: myocarditis • coxsackievirus B3 • cyclosporine • lymphocyte subset • interleukin-2 receptor
Absence of effects of cyclosporine on myocardial lymphocyte subsets in Coxsackievirus B3 myocarditis in the aviremic stage.
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Circ Res. 1989;65:934-945
doi: 10.1161/01.RES.65.4.934

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/65/4/934

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