Immunosuppression With High Doses of Cyclophosphamide Reduces the Severity of Myocarditis but Increases the Mortality in Murine Coxsackievirus B3 Myocarditis

Chiharu Kishimoto, MD, PhD, Kathryn A. Thorp, BA, and Walter H. Abelmann, MD

To test the therapeutic efficacy of immunosuppression with cyclophosphamide (CYP) on coxsackievirus B3 (CB3) myocarditis, 2-week-old DBA/2 mice were inoculated with $3 \times 10^2$ plaque-forming units of CB3 virus. CYP (100 mg/kg/day s.c.) was administered daily on days 0–8 (experiment 1; group 2), days 8–21 (experiment 2; group 4), and days 21–34 (experiment 3; group 6). Groups 1, 3, and 5 were infected control groups for each experiment. Spleen, thymus, and body weights were measured. In experiment 1, survival rate in group 2 on day 8 was low compared with group 1 (nine of 51 versus eight of 28; $p=NS$), and myocardial virus titers in group 2 on day 8 were higher ($p<0.05$) compared with group 1; however, cellular infiltration and myocardial necrosis in group 2 were less severe ($p<0.05$), and serum neutralizing antibody titers were decreased ($p<0.01$). In experiment 2, survival rate in group 4 on day 21 was significantly lower (six of 24 versus 12 of 16; $p<0.01$), but cellular infiltration, myocardial necrosis, and calcification in group 4 were significantly less severe, and serum neutralizing antibody titers were decreased ($p<0.01$). In experiment 3, survival rate, cardiac histopathology, and serum neutralizing antibody titers did not differ between groups 5 and 6. In experiments 1, 2, and 3, the treated groups were characterized by lower spleen-to-body-weight and thymus-to-body-weight ratios and by marked cellular depletion in spleen and thymus. A similar reduction of cardiac histopathology, associated with lymphoid organ atrophy in the treated group, was demonstrated in the early study (day 4) in experiment 1. Thus, the administration of CYP (100 mg/kg/day) induced immunosuppression in CB3 myocarditis in mice. Notwithstanding an associated higher mortality rate, the severity of cardiac pathology was reduced in the acute stage. However, no beneficial effects were seen in the later stage. It is concluded that immune mechanisms play a role in the early stage of development of CB3 myocarditis. (Circulation 1990;82:982–989)

Infusion of the myocardium with inflammatory cells occurs during infection with a variety of viruses. Usually, the infiltrate comprises mononuclear cells that are focal or diffusely scattered throughout the myocardium. Myofiber necrosis is an important feature of this lesion.

Enteroviruses, especially coxsackieviruses B (CB3), are established as the predominant cause of viral myocarditis in humans. In addition, viral myocarditis is considered a cause of dilated cardiomyopathy; immune or autoimmune mechanisms may be involved in the pathogenesis of viral myocarditis and subsequent cardiomyopathy. Hence, attention has focused on the efficacy of immunosuppressive therapy for viral myocarditis. The use of immunosuppressive agents for the treatment of viral myocarditis, however, has been controversial, based on both clinical and experimental studies. To address unresolved questions, an experimental model of CB3 myocarditis in mice may be of value.

In the present study, we investigated the effects of total immunosuppression with high doses of cyclophosphamide (CYP) on different stages of experi-
mental murine CB3 myocarditis. To understand the in vivo action of CYP and the occasional associated immune abnormalities, one must have a clear understanding of the lymphoid organ alterations. The possible roles of the immune system and total immunosuppression with CYP in the development of virus-induced myocarditis are also discussed.

Methods

Virus and Cell

CB3 (Nancy strain, American Type Culture Collection) was stored at -70°C until it was diluted for use. Virus titers were determined by plaque formation on VERO (kidney of African green monkey) cell monolayers. Cells were suspended at a concentration of 1×10⁶/ml in Eagle’s minimal essential medium (EMEM) with 3% fetal calf serum (FCS) plus 100 μg/ml penicillin and streptomycin in six-well plastic plates and allowed to grow for 2 days at 37°C in 5% CO₂. Volumes (0.1 ml) of decimal dilutions of virus suspended in EMEM were adsorbed to VERO cell monolayers for 60 minutes at 37°C in 5% CO₂. After adsorption, the cells were overlaid with 3 ml of medium containing 3% FCS 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. Plaques were then counted with an inverted microscope.

Treatment of Mice

Two-week-old male DBA/2 mice (Jackson Laboratory, Bar Harbor, Me.) were inoculated intraperitoneally with 0.1 ml CB3 diluted in EMEM to a concentration of 3×10⁵ plaque-forming units (PFU)/ml. Mother mice were supplied for the maintenance of 2-week-old mice; when they became 4 weeks old, the mothers were removed. CYP (Sigma Chemical, St. Louis, Mo.) was administered subcutaneously daily at rotated sites beginning immediately after virus inoculation until day 8 (experiment 1, group 2; n=51), from the day 8 until day 21 (experiment 2, group 4; n=24), and from the day 21 until day 34 (experiment 3, group 6; n=6) at a dose of 100 mg/kg/day, the actual dose for each experiment being calculated from mouse weight at the beginning of the experiments. Control mice (group 1, n=28; group 3, n=16; group 5, n=6) were injected with an equal volume of saline. In experiment 1, five additional mice in each group were killed on day 4 for the evaluation of early cardiac pathology. Before starting experiment 2, an additional five mice in groups 3 and 4 were killed to confirm the homogeneity of both groups and processed for virologic and pathologic studies. Mice were observed daily; when found dead, complete necropsies were performed. Their spleens, thymus, lungs, livers, and hearts were weighed. Body weight was also measured, and the ratios of organ weight to body weight were then calculated. Surviving mice in experiments 1, 2, and 3 were killed on days 8, 21, and 34, respectively.

In parallel with experiment 1, additional control groups of noninfected mice (treated and untreated; n=5 each) that were treated or not treated with CYP (100 mg/kg/day for 8 days) were also studied.

Virus Titers of Hearts

For infectivity assays, hearts were removed aseptically, weighed, and homogenized in 2 ml EMEM. After centrifugation at 1,500g for 15 minutes at 4°C, supernatants were inoculated into VERO cell monolayers, and plaque assays were performed.

Neutralizing Antibody Titers

Blood was obtained under sterile conditions from the retro-orbital plexus, and the serum was inactivated at 56°C for 30 minutes. Each sample was titrated serially by determining the fourfold dilution in 10.0 ml EMEM, supplemented with 5% FCS that protected VERO cell monolayers in the tubes against a challenge of 100 PFU CB3. Tubes were observed daily for the appearance of characteristic cytopathic effects over a period of 5 days. Antibody titers were expressed as the highest serum dilution that neutralized 50% of the viral inoculum.

Pathologic Study

Gross pathology of heart. Gross abnormalities (i.e., white patches on the surface of the heart) were classified as 0 (no or questionable lesions), 1+ (lesions involving less than 25% of the myocardium), 2+ (lesions involving 25–50% of the myocardium), 3+ (lesions involving 50–75% of the myocardium), and 4+ (lesions involving 75–100% of the myocardium). After gross inspection, the hearts were sectioned into two equal cross sections processed for histologic and virologic studies.

To avoid postmortem artifacts, the ratio of the organ weight to body weight was compared only in killed mice in experiments 1 and 2.

Histology. The organs (heart, thymus, spleen, lungs, liver, and kidney) were fixed in 10% formalin solution, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. For myocardial lesions, cellular infiltration, myocardial cell necrosis, calcification, and fibrosis were scored blindly by two of the authors (C.K. and W.H.A.) on a scale of 1+ to 4+. A 0 score indicated no or questionable lesions. A 1+ score described a limited focal distribution of myocardial lesions. Scores of 2+ and 3+ described intermediate severity, whereas a 4+ score described the presence of multiple lesions over the entire heart. Sections of thymus and spleen were inspected primarily for cellularity and its depletion, and those of lung and liver were inspected for congestion or virus-induced lesions.

Again, to standardize the stage of myocarditis, histologic study in experiments 1 and 2 included only killed mice.
Statistics

The unpaired t test was used to evaluate differences in virus titers in the hearts, neutralizing antibody titers, organ weights, and pathologic scores. The \( \chi^2 \) test with Yates' correction was used to determine the significance of differences in survival rates. The average titers of neutralizing antibody (log 2) were given as mean±SD. A p value of less than 0.05 was considered statistically significant.

Results

Experiment I

Survival rate. Twenty mice in the control group (group 1) and 42 in the CYP group (group 2) died by day 8; the survival rate on day 8 was 28.6% (eight of 28) in group 1 and 17.6% (nine of 51) in group 2, respectively (Figure 1). The difference was not significant. On days 3–8, mice in both groups appeared ill; they showed coat ruffling, weakness, or irritability. However, running and irritability were more prominent in group 2 animals.

Myocardial virus titers. Myocardial virus titers of group 2 animals killed on day 8 (1.1±1.1×10^3 PFU/mg tissue; \( n=6 \)) were significantly higher (\( p<0.05 \)) compared with those of control animals (group 1) killed the same day (1.5±2.0×10^2 PFU/mg tissue; \( n=6 \)) (Table 1).

Neutralizing antibody titers. Neutralizing antibody titers (log 2) of group 2 animals on day 8 (0.4±0.9) were significantly lower (\( p<0.01 \)) compared with those of group 1 animals (2.8±0.8) (Table 1).

Cardiac pathology. Gross cardiac lesions were significantly less severe in group 2 animals compared with group 1 animals on days 4 and 8, respectively (Tables 1 and 2). As shown, cellular infiltration on days 4 and 8 was significantly less severe in group 2 animals compared with group 1 animals. Myocardial necrosis in group 2 animals was significantly less than in group 1 animals on day 8 but not on day 4. Myocardial calcification did not differ between group 1 and 2 animals on days 4 and 8. Myocardial fibrosis was not evaluated in experiment 1.

Other organs. A generalized cellular depletion in the thymus was more prominent in group 2 animals than in group 1 animals. Also, striking B zone and T zone lymphoid depletions in the spleen were evident in group 2 (Tables 1 and 2). Body weight in group 2 animals was less than in group 1 animals. The ratios of thymus and spleen weights to body weight in group 2 animals were significantly smaller than those in group 1. There were no significant differences in other organ-weight-to-body-weight ratios. Similar results were also demonstrated in the early study (day 4).

Uninfected groups. No mice died by day 8 in either group (treated or untreated). However, on days 3–8, only the drug-treated mice showed weakness, irritability, body weight loss, and running.

Pathologic examination revealed no abnormalities in myocardium, lung, or liver in either group. Marked cellular depletion and weight loss in spleen and thymus in the drug-treated group was evident; there was no cellular depletion in spleen or thymus in the drug-untreated group.

Figure 1. Plots of effects of cyclophosphamide (CYP, 100 mg/kg/day) on survival in DBA/2 mice with coxsackievirus B3 myocarditis. When drug was administered on day of virus inoculation (experiment 1), mortality rate in mice treated with CYP (GRP 1) was not significantly significant compared with untreated control group (GRP 2). When administered early (day 8) in illness (experiment 2), there was a significantly greater mortality in mice treated with CYP (GRP 3) than in control mice (GRP 4). When administered late (experiment 3), no significant change in treated mice (GRP 5) resulted, compared with that in control mice (GRP 6). GRP, group. (See text for details).
Table 1. Results of Experiment 1

<table>
<thead>
<tr>
<th>Group</th>
<th>CYP (mg/kg/day)</th>
<th>Myocardial virus titers (PFU/mg tissue)</th>
<th>Neutralizing antibody titers (log 2)</th>
<th>Cardiac pathology on day 8</th>
<th>Organ weight</th>
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In experiment 1, CYP was administered subcutaneously daily on days 0–8. Severity of myocardial lesions were scored blindly on a scale of 1+ to 4+ in terms of severity.

Values are given as mean±SD.

*p<0.05, †p<0.01, ‡p<0.001 versus group 1.

§Four values were not detected (less than 2 log 2).

Experiment 3

Survival rate. No mice in group 5 (n=6) died by day 34 (Figure 5). Two mice in group 6 (n=6) died by day 34 (Figure 5). The difference was not significant. Weakness was not evident in the course of this experiment. Neutralizing antibody titers did not differ between group 5 and 6 animals. Reduced cardiac weights in the degree of gross pathology in group 9 animals were significantly less than those of group 6 animals; the ratios of lung, thymus, and spleen weights to body weight were significantly less in group 9 animals than in group 6 animals. Other organs: Body weight of group 6 animals were significantly lower than those of group 5 animals. The ratios of lung, thymus, and spleen weights to body weight were significantly less than those of group 5 animals. (Table 4). No significant differences were noted in the livers, kidneys, or other organs of either group in experiments 1, 2, and 3.

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Table 2. Results of Experiment 1 on Day 4

<table>
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<th>Group</th>
<th>CYP (100 mg/kg/day)</th>
<th>Cardiac pathology</th>
<th>Organ weight</th>
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<tr>
<td></td>
<td>Gross</td>
<td>Histology</td>
<td>BW (g)</td>
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<tr>
<td>1 (n=5)</td>
<td>No</td>
<td>1.4±0.5</td>
<td>1.4±0.5</td>
</tr>
<tr>
<td>2 (n=5)</td>
<td>Yes</td>
<td>0.2±0.4*</td>
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</tr>
</tbody>
</table>

Five additional mice in each Group were killed on day 4 for the early evaluations in experiment 1. CYP, cyclophosphamide; PFU, plaque-forming unit; I, infiltration; N, necrosis; C, calcification; BW, body weight; HW/BW, heart weight/body weight; Th/BW, thymus weight/body weight; Sp/BW, spleen weight/body weight; Lu/W/BW, lung weight/body weight; Li/W/BW, liver weight/body weight.

In experiment 1, CYP was administered subcutaneously daily on days 0–8. Severity of myocardial lesions were scored blindly on a scale of 1+ to 4+ in terms of severity. Values are given as mean±SD. *p<0.01, †p<0.001 versus group 1.

Discussion

Previous studies have documented that immunosuppressive agents, such as steroids and cyclosporine, increase the severity of coxsackievirus heart disease.6-10 The present study clearly demonstrates that the administration of CYP (100 mg/kg/day) induced effective immunosuppression (severe cellular depletion of lymphoid organs and lower titers of serum neutralizing antibody) in mice in the early stage of CB3 myocarditis (experiments 1 and 2), associated with a higher mortality rate notwithstanding apparent reduction of cardiac pathology, and that no striking differences in cardiac effects were found between untreated and treated mice in the later stage (experiment 3). Thus, it would seem confirmed that immune mechanisms play a role in the development of CB3 myocarditis in the early stage.

A biphasic disease process results when mice are infected with CB3.1,2,7,11 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 dilatation, and heart failure in some strains of mice.12 It has been suggested that the chronic phase results from a cell-mediated immune response to a neoantigen that developed during the acute phase of the illness.1,2,11,13 Furthermore, besides the genetic contribution of the virus virulence phenotype (myocarditic or amycarditic), host genetics is a primary contributory factor to the development of the disease in addition to host, age, sex, and immune status.1,2,14

The immunosuppressive agents, corticosteroids, CYP, and cyclosporines have been tested in experimental models of myocarditis. Kilbourne et al.10 reported that a single injection of cortisone in an early stage of CB3 myocarditis increased both the severity of myocardial damage and the incidence of lethal disease in mice. Recently, cyclosporine has become a widely used primary immunosuppressive agent in allotransplantation, acting by suppressing helper T lymphocytes.15-17 O’Connell et al.17 reported an adverse effect of cyclosporine in the acute stage of CB3 myocarditis in mice. The results of their experimental studies led numerous investigators to denounce the use of these agents in treating acute viral myocarditis.

CYP is a well-known and powerful immunosuppressive agent with dose-specific effects.18 McCormick et al.18 reported that a low dose of CYP (30 mg/kg/day) acts mainly on B cell regions in lymphoid organs, but a high dose (300 mg/kg/day) affects both B and T cell regions. Thus, the mechanisms of immunosuppression of CYP may differ according to dosage. The effects of high doses of CYP were demonstrated in the present study, in which total cellular depletion in B cell as well as T cell regions in the lymphoid organ was evident. Indeed, there have been several reports18,19 that treatment with CYP (<50 mg/kg/day) aggravated the course and cardiac pathology of experimental viral disease.

The deleterious effect in the present study is based primarily on an increase in mortality in CYP-treated animals. It is difficult to attribute this increased mortality to cardiac changes; the cause of death remains obscure. However, the present study may have important implications with regard to the pathogenesis of viral myocarditis. The significance of T cells in the development of viral myocarditis has already been demonstrated in several animal models.20-25 Woodruff and Woodruff20 reported that the severity of myocardial necrosis and cellular infiltration in CB3-infected, antithymocyte serum-treated, and T cell-deprived mice was significantly less than that occurring in either intact mice or thymus-deprived irradiated mice that had been reconstituted with both thymus and bone marrow cells. Similarly, an animal model of mild myocarditis caused by encephalomyocarditis virus has been reported in BALB/c-nu/nu athymic mice.22 More recently, T cell-mediated immunity has been reported to play a role in the pathogenesis of murine CB3 myocarditis.11,22-25 Several investigators1,2 reported that during the acute stage of myocarditis, sensitized T cells migrate toward the target organ (heart), where T cells may play a role in the devel-
opment of myocarditis. Therefore, when animals with almost totally depressed T and B cell functions are infected with CB3, cardiac pathology alone would be expected to be less severe than in animals with normal T cell function, even if the former manifested higher myocardial virus titers and a higher mortality rate. The higher mortality rate in the CYP groups may be due to the total immunodeficiency state or the subsequent lower neutralizing antibody titers. The animals were kept in isolation, and there was no evidence of opportunistic infection at necropsy.
**Table 3. Results of Experiment 2**

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<th>Group</th>
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<th>Cardiac pathology on day 21</th>
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<td>4</td>
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In experiment 2, CYP was administered subcutaneously daily on days 8–21. Values are given as mean±SD.

*p<0.01, †p<0.05, ‡p<0.001 versus group 3.

**Table 4. Results of Experiment 3**

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<td>(n=5)</td>
<td></td>
<td>(n=6)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Yes</td>
<td>10.8±1.0</td>
<td>2.0±0.9</td>
<td>0.8±0.4</td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
<td></td>
<td>(n=6)‡</td>
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</tr>
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Values are given as mean±SD. CYP, cyclophosphamide; PFU, plaque-forming unit; I, infiltration; N, necrosis; C, calcification; F, fibrosis; BW, body weight; HW/BW, heart weight/body weight; ThW/BW, thymus weight/body weight; SpW/BW, spleen weight/body weight; LuW/BW, lung weight/body weight; LiW/BW, liver weight/body weight.

In Experiment III, CYP was administered (mean±SD) subcutaneously daily on days 21–34.

*p<0.05, †p<0.001 versus group 5. ‡Includes two hearts of mice died on days 32 and 33.
The lack of effects of immunosuppression by CYP on the chronic stage of myocarditis, when immunemediated myocardial injury is strongly suggested, remains unexplained. In this regard, it may be relevant that Gauntt and associates reported that after treatment with CYP, an amyocarditic strain of CB3 became myocarditic. In other studies of the immunopathogenesis of CB3 virus myocarditis, a role has also been attributed to humoral immunity; autoantibodies against heart were reported in the development of myocarditis, and their production was found to depend on the strain of the mouse.

We conclude that immune mechanisms play a role in the early stages of CB3 virus myocarditis.

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References

KEY WORDS • immunosuppression • coxsackievirus B3 • myocarditis • cyclophosphamide
Immunosuppression with high doses of cyclophosphamide reduces the severity of myocarditis but increases the mortality in murine Coxsackievirus B3 myocarditis.

C Kishimoto, K A Thorp and W H Abelmann

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