The effects of cyclosporine on acute murine Coxsackie B3 myocarditis

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ABSTRACT The effects of the immunosuppressant drug cyclosporine were studied in the murine model of Coxsackie B3 myocarditis. Ten BALB/c mice, given daily cyclosporine (15 mg/kg) intraperitoneally but not infected, were normal in all respects after 2 weeks. All 32 BALB/c mice infected, but given no cyclosporine, survived and had moderate myocardial mononuclear infiltrates and minimal necrosis at 7 and 14 days. In contrast, 24 mice concurrently infected and given cyclosporine had a high mortality rate (75%) and a significantly attenuated mononuclear infiltrate in the presence of enhanced necrosis when compared with control infected mice. Sixteen mice started on the drug 1 week after infection had a lower mortality rate (55%), but very similar histologic abnormalities. In contrast to negligible or no virus in the hearts of infected mice that were not given cyclosporine, drug treated, infected groups had easily detectable virus in their hearts 14 days after infection. An identical study in Swiss ICR mice yielded similar results. Cyclosporine, when given early during acute murine Coxsackie B3 myocarditis, causes a significant increase in myocardial necrosis and mortality, possibly secondary to enhanced viral survival. Circulation 73, No. 2, 353–359, 1986.

CYCLOSPORINE (CS), by virtue of suppressing T helper lymphocyte interleukin-2 production, is a quasiselective inhibitor of antigen-specific cytotoxic cell proliferation and has become a widely used primary immunosuppressive agent in allotransplantation. CS provides adequate control of rejection episodes and is associated with significant decreases compared with those achieved with other therapy in difficult to treat, life-threatening bacterial and fungal infections. The successful use of CS in renal, liver, and cardiac transplantation and the clarification of its mode of action has spurred a flurry of investigations designed to assess its efficacy in experimental and clinical diseases characterized by disturbed immunoregulation.

The postulate that immunopathologic mechanisms that culminate in a chronic dilated cardiomyopathy are set in motion during acute viral myocarditis serves as an attractive basis for unraveling the pathogenesis of primary myocardial disease in man. The source of this hypothesis is the documentation of a similar biphasic chain of events in the murine model; that is, an initial cardiotropic Coxsackie B3 (CVB3) viral infection leads to the development of cell-mediated cytotoxicity toward myocardial cells. The acute or initial phase is virally mediated and characterized by minimal myocyte necrosis and inflammation. The chronic or late phase develops after viral clearance has occurred, resulting in a smoldering inflammatory reaction, incremental myocyte necrosis, and ultimately, clinical emergence of congestive heart failure several months later.

It is apparent then that the development of therapeutic interventions attempting to modify the inflammatory response in the myocardium must be given at the appropriate stage of the disease. A miscalculation could be catastrophic, e.g., broad-spectrum immunosuppression during the acute phase of the illness may result in overwhelming viral dissemination and worsening of the early myocyte necrosis. Framed within this perspective, we studied the effects of CS when introduced at different times during the acute phase of infection.

Materials and methods

Animals. Three-week-old male BALB/c mice (Harlan Laboratories, Indianapolis) were held for 7 days before the experiment in a single, self-contained animal isolation unit to exclude pre-
diseased animals. They were maintained in disposable, filter-
topped cages and handled with gloves by gowned and masked
personnel. The intraperitoneal route was used for injection of
virus. All intraperitoneal injections were given in a 0.5 ml
volume by tuberculin syringe with a 27-gauge needle after pre-
paration of abdominal skin with iodine-alcohol. An identical
protocol was followed in outbred Swiss ICR mice (Harlan Lab-
oratories).

**Virus.** CVB3 (Nancy Strain) was obtained from the Ameri-
can Tissue Culture Collection, grown on either Hep-2 or VERO
cells, aliquotted, and maintained at −70 °C until use. At the
time of infection, seed virus was grown on either VERO or
LLC-MK-2 cells with Dulbecco’s modified Eagles medium,
12% fetal calf serum and gentamicin. Virus was harvested and
adjusted to an inoculum of 1.75 × 10⁴ plaque-forming units/0.5
ml RPM-1640. This infecting dose, one that resulted in myocar-
ditis in greater than 95% of animals so challenged in our labora-
tory, had been predetermined by serial dilution dosage studies in
comparably aged mice of an identical strain.

**CS dosage and assays**

*Drug. CS* (oral Sandimmune solution, 100 mg/ml) was dis-
solved in olive oil in a concentration so that a 0.15 ml final
volume contained 0.3 mg drug. This volume, equivalent to 15
mg/kg, was given subcutaneously with a tuberculin syringe
daily at rotated sites.

**Blood levels of CS.** Blood was obtained by cardiac puncture
before animals were killed and was allowed to clot and the
serum was held at −70 °C. On the day of analysis, serum
samples were diluted 1:50 with Trizma buffer, pH 8.5 (Sigma),
and 0.03% Tween 20. All reagents were prepared according to
the standard method described in the Cyclosporine RIA kit
(Sandoz). CS standards, radioactive 3H tracer, and CS antisera
were also obtained through Sandoz. Tests for serum levels were
run simultaneously with drug standards and actual serum level
was determined from a dose-response standard curve plotted on
log-log paper as percent relative binding vs standards.

**Tissue levels of CS.** Heart homogenates, assayed for CS,
were prepared similar to those for viral culture on tissue of
animals killed on day 14. Fifty microliters of homogenate was
diluted 1:50 with Trizma buffer, 8.5 pH, and assayed by the
standard radioimmunoassay. Quantitation of the tissue CS level
for a given homogenate was expressed as nanograms of drug per
milligram protein in the homogenate. Protein was quantitated
by the Bradford method. Severely necrotic hearts that were
likely to contain large amounts of collagen were coassayed by
the Lowry method.

**Treatment protocol.** Ten uninfected mice (group I) received
15 mg/kg CS only, and they served as the drug-treated, unin-
fected control group. Thirty-one mice were infected with CVB3
on day 0 and given no CS and they served as infected, nontreat-
et controls (group II). Nineteen of these animals were killed by
cervical dislocation 8 days after infection and the remaining 12
animals were killed on the fourteenth day after infection. Group
III consisted of 24 CVB3 infected animals that received 15
mg/kg CS daily, beginning on the day of infection. The four
survivors were killed on day 8. Group IV consisted of 16 CVB3
infected mice that were given a daily dose of CS beginning on
day 8 after CVB3 infection; survivors were killed on day 14.
Sham drug vehicle (olive oil) injections were not given to non-
drug-treated, infected animals. Multiple preliminary studies
have shown that sham olive oil injections have no deleterious or
beneficial effect on the natural course of CVB3 disease.

**Histologic analysis.** After cervical dislocation, the heart was
rapidly removed and divided in two equal cross sections. The
basal portion was snap frozen for isolation of virus and determi-
nation of CS tissue level. The apical portion was removed, im-
mersed in 10% formalin, and fixed overnight. The tissue was
then dehydrated, cleared, and embedded in paraffin in the stan-
dard semiautomated manner and 5 μm sections were mounted
and stained with hematoxylin-eosin and Masson’s trichrome
stains. The slides were examined by two observers blinded to
previous interpretation and slide code, and inflammation and
necrosis were quantitated as previously described.6 Necrosis
was given a score of 0 to 4, with 4 reflecting widespread necro-
sis. Inflammation was also scored on a 0 to 4 scale, with 4
representing 100 or greater mononuclear cells, usually confluent,
per high-power field.

**Detection of virus.** The bases of the individual hearts were
maintained at −70 °C until assayed. After thawing, they were
minced with a sterile scalpel, suspended in 1 ml RPMI-1640,
and homogenized in a glass tissue grinder. The suspension was
centrifuged at 8000 g for 10 min at 4 °C. Supernatants were
harvested and frozen at −70 °C until assay. Serial 10-fold
dilutions of heart homogenates in minimum essential medium
were layered on confluent, 72-hr-old VERO cells that had been
grown in 96-well microtiter plates (Falcon). Monolayers were
checked for cytopathic effects daily for 7 days for presence or
absence of virus and rate of cell destruction.

**Statistical analysis.** Survival was assessed daily and data
from CS-treated mice were compared with those from each
control population (virus only, drug only). Histopathologic se-
miquantitation of inflammation and necrosis after blind grading
was compared in treated vs control groups. Statistical analysis
was performed by means of a Student’s t test for unpaired
variables.

**Results (table 1)**

**Analysis of study groups at 7 days after infection**

*Group I BALB/c mice.* All uninfected but CS-treated mice
survived the initial 7 days of study with no apparent
clinical effects from the CS. There was no histologic
evidence of myocardial myocyte necrosis or inflammation
and no mortality in group I.

*Group II BALB/c mice.* All mice (infected, but no CS
treatment) survived the initial week of study (figure 1).
There was sporadic clinical evidence of viral infection,
mainly coat ruffling. There was mild-to-moderate
mononuclear infiltration and mild-to-moderate necrosis in all (figure 2). Examination of these hearts yielded an inflammation score of 1.4 ± 0.7 (figure 3) and a necrosis score of 0.5 ± 0.6 (figure 4).

**Group III BALB/c mice.** Of the 24 infected animals treated with CS beginning on day 0, 18 (75%) were dead by day 7 (figure 1); one died 24 hr after infection, but the majority of subsequent fatalities (14/17) occurred on days 5, 6, and 7 after infection. The remaining six animals were killed on day 8. All animals showed gross clinical signs of viral infection, including marked coat ruffling, irritability, and lethargy. Histologic analysis revealed an absent inflammatory response in the presence of significant necrosis (figure 5) in all hearts of group III animals (figure 4). Their inflammatory scores differed substantially from those of infected but untreated group II control mice (p < .001, figure 3). In contrast, their necrosis score was greater (1.1 ± 1.0) than that of the animals in group II (0.5 ± 0.6, p < .05; figure 4).

**Analysis at 2 weeks after infection.** Group I (drug only) animals were normal in all respects. The 12 infected, but untreated animals in group II killed on day 14 had an inflammation score of 1.1 ± 0.7 (figure 6), and

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**FIGURE 1.** Comparison of mortality rates in BALB/c mice after 1 week. Group I consists of 10 mice given cyclosporine only, group II of 32 mice given no cyclosporine, but infected with CVB3, and group III of 34 mice started on 15 mg/kg CS the same day they were infected with CVB3.

**FIGURE 2.** Mononuclear myocardial inflammatory infiltrate and myocyte necrosis in a BALB/c mouse 1 week after infection with CVB3. (Hematoxylin-eosin stain, original magnification × 200).
their necrosis score was 1.0 ± 1.8 (figure 7). The mortality rate in group IV was 0% at 7 days after infection, but rose to 55% by day 14. Fifty percent of these fatalities occurred within 72 hr after starting CS. The inflammation score in 16 mice infected on day 0 but treated with CS from day 7 was 0.6 ± 0.9, again not significantly different from that in the group II animals (figure 6). However, the necrosis score in group IV animals was 2.2 ± 1.4, significantly different from that in the control viral infected (group II) animals (p < .01), but there was wide scatter of necrosis scores in group IV (figure 7).

**Swiss ICR mice.** Identical experimental groups of Swiss ICR mice were also studied. No effect of CS or its vehicle was detectable in uninfected control mice. All group I and group II mice survived. Mortality rates, however, in groups III and IV were not as high as those in BALB/c mice. Group II mice had an inflammatory score of 2.0 ± 1.4 and a necrosis score of 1.5 ± 2.1. Group III mice (virus and CS from day 1), in contrast to the 75% death rate in BALB/c mice, had a 25% mortality rate. Group III mice, at 7 days, had a very low inflammatory score of 0.3 ± 0.5 and a necrosis score of 2.1 ± 1.2. When surviving group III mice were analyzed at 14 days, their inflammatory index was 1.7 ± 2, but the necrosis score was very high (3.7 ± 0.7). This was significantly different than the score in the group IV mice that had been treated since the eighth day of infection (t = 5.9, p < .01). Group IV mice had only a 5% (1/20) death rate, markedly lower than the 55% that occurred in BALB/c mice. Histologic analysis, however, revealed a similar lack of cellular infiltration but multiple foci of gaping necrosis comparable to that in the BALB/c mice (figure 6). This group had an inflammatory score of 0.2 ± 0.1 and a necrosis index of 1.2 ± 0.9.

**Viral cultures and serology.** All heart homogenates of group III or group IV BALB/c mice assayed at either 7 or 14 days after infection caused extensive (75% to 100%) cytopathogenic effects in Hep-2 cells within 72 hr, indicating the presence of virus. No cytopathogenic effect developed from homogenates from either group I or group II hearts taken from mice on day 14. Minimal amounts of cytopathic effect at 72 hr were present in three of 10 (30%) hearts taken from mice in group II killed on day 8.

**Cyclosporine levels.** The average CS serum levels in group I and III mice treated for a minimum of 7 days were 146 ± 34 and 225.7 ± 168 ng/ml. The mean serum level of CS in group IV was 243 ± 110 ng/ml after they were treated for 7 days. Tissue (heart) levels of CS averaged 157 ± 59 ng CS/mg protein, 435 ± 166 ng CS/mg protein, and 237 ± 174 ng CS/mg protein in groups I, III, and IV, respectively.

**Discussion**

Myocarditis is a common but usually clinically insignificant accompaniment to systemic enteric viral infections, especially Coxsackie B virus infections. However, recent epidemiologic, serologic, and clinical data suggest that viral myocarditis may initiate an immunopathologic process that clinically culminates in chronic dilated cardiomyopathy.2,7 Dilated cardiomyopathy is an important clinical syndrome that re-
results in severe morbidity and early mortality in a young patient population. The cause of dilated cardiomyopathy is usually obscure and therapeutic attempts to palliate congestive heart failure and ventricular arrhythmias have not improved a dismal natural history that is characterized by a 50% mortality within 2 years of diagnosis. Access to myocardium during life by way of endomyocardial biopsy has confirmed the presence of myocarditis in a significant number of patients with dilated cardiomyopathy. Detection of active myocardial inflammation offers promise that a "treatment window" in which modification of the natural course of the disease can be identified and acted upon.

The animal preparation of CVB3 myocarditis shows a progression from viral infection to myocardial hypertrophy and fibrosis. A biphasic disease process results when weanling mice are infected with CVB3. During the acute (viral-mediated) phase, viral replication in the myocardium results in variable necrosis with minimal inflammation during the first week. Immunosuppressive corticosteroid or cyclophosphamide therapy during this acute phase has resulted in overwhelming necrosis and death due to persistent viral replication. After the virus is cleared from the myocardium by myocyte and humoral responses, a chronic inflammatory reaction (immune-mediated phase) results in progressive myocyte damage and hypertrophy, ventricular dilatation, and heart failure within 1 to 2 years after infection. 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. At this phase of the disease, peri-infection thymectomy, thymocyte antiserum, or radiation attenuates the chronic inflammatory response without altering viral clearance.

There is burgeoning interest in the use of CS as a primary modulator in diseases characterized by abnormal immunoregulation. Favorable results have been seen in experimental preparations of autoimmune thyroiditis and also clinically in uveitis and diabetes mellitus. The latter two studies provide a precedent for evaluating CS effects in another viral-mediated disease with deleterious immunologic consequences.

When CS was given to uninfected mice in therapeutic doses and significant levels were achieved in target organ tissue, no drug-induced myocardial alterations...
FIGURE 6. Distribution of inflammation scores of group II BALB/c mice (CVB3 infected, but given no drug) and group IV BALB/c mice (CVB3 infected, but started on 15 mg/kg CS 1 week after infection) studied on day 14.

were found. Variations in CS levels may be explained by the fact that infected animals were ill, catabolic, of low weight, and somewhat volume depleted. Similar individual variations in dose and levels is seen in man due to idiosyncracy in drug metabolism. Random histologic examination of thymuses and spleens in this group of mice confirmed that CS was not being used in doses that could cause lymphoid atrophy and possibly secondary alteration of the response to CVB3. However, when CS was given in concert with CVB3 infection, there was a striking increase in mortality, with marked myocyte necrosis and attenuation of the expected inflammatory response. The cause of death was presumably cardiac due to the marked myocyte necrosis found histologically. The apparent inhibition of specific host cytotoxic responses to viral antigens by CS and high titers of viable virus that were present in myocardium may explain the disparity between myocardial necrosis and inflammation. However, the high mortality in mice that were not treated with CS until 1 week after CVB3 infection was a bit surprising, since virus should have been almost entirely cleared by monocyte, B cell, and natural killer responses by that time. These results in group IV further suggest that CS promoted persistence of or lack of antigen(s) clearance, thereby enhancing myocardial cell destruction. The mortality rate was especially high in BALB/c mice. The experiment was repeated in Swiss ICR mice to exclude a genetically based susceptibility of BALB/c mice to virus plus CS. For reasons that remain unexplained, these mice were more resistant in terms of fatality rate, but had almost identical, if even not more severe, necrosis and lack of inflammatory response.

The mechanism by which CS interferes with normal viral clearance is unknown. CS inhibits gamma interferon synthesis by lymphocytes and this may result in a delay in viral clearance with subsequent increased myocyte necrosis. Although CS has no direct effects on immunoglobulin synthesis, macrophages, neutrophils, or natural killer cells, it induces expansion of an antigen-specific T suppressor cell population by permitting or actually increasing suppressor growth factors. These may, in sequence, alter host defense mechanisms in vivo; T helper cell function, which is necessary for amplification of macrophage and B lymphocyte responses, is simultaneously directly attenuated. Infected animals that received CS beginning on day 1 may have been unable to amplify or activate normal monocytic clearance mechanisms. The demonstration that virus was detectable at 2 weeks after infection in the hearts of all animals who had received CS only from day 7 to 14 suggests that amounts of virus undetectable by our assay replicated to assayable levels after institution of CS therapy.

The effect of CS on CVB3-induced autoimmunity to myocyte or myocardial neoantigen production and/or display is also unknown. If a net result of CS is prolonged or altered neoantigen display as a result of decreased viral clearance, natural killer cell- and antibody-dependent cytotoxicity directed toward neoantigen would have an additive effect on increased myo-
cyte necrosis. Further studies are now necessary to determine whether initiation of CS therapy during the established chronic phase, after all virus has disappeared, will inhibit nonviral antigen(s), perpetuating myocardial destruction. Finally, CS may attenuate myocardial inflammation and enhance necrosis by its effects on prostacyclin metabolism.\(^7\) Inhibition of the latter may decrease blood flow and increase ischemic damage in areas of viral myocyte infection. We rarely, if ever, encountered thrombosis at any level of the myocardial circulation in any groups of infected BALB/c or Swiss ICR mice.

In summary, CS, when used as an immunosuppressive agent in acute viral myocarditis, may increase morbidity and mortality, presumably by altering viral clearance, blunting the inflammatory response, and permitting increased myocyte necrosis. The precise timing of CS intervention may be critical. CS may be hazardous if the drug is used for modification of myocardial inflammation during the acute viral phase. However, inhibition of cytotoxic responses by CS after viral disappearance may limit further necrosis. Studies are in progress to clarify these issues.

References

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