A Nonsteroid Anti-Inflammatory Drug Exacerbates Coxsackie B3 Murine Myocarditis

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Nonsteroid anti-inflammatory drugs are often used to treat myalgias and arthralgias in enteroviral infections, but their effects on acute viral myocarditis are unknown. The effect of the nonsteroidal anti-inflammatory drug, ibuprofen, on acute viral myocarditis was studied in 75 four week old male BALB/c mice infected with 1.75 × 10^7 plaque-forming units of Coxsackie virus B3 on day 0. Ibuprofen was given intraperitoneally at a dose of 15 mg/kg body weight daily. The mice were assigned to four groups—Group I, 18 uninfected mice given ibuprofen on days 1 to 14; Group II, 18 infected, untreated mice; Group III, 20 infected mice given ibuprofen on days 1 to 14; and Group IV, 17 infected mice given ibuprofen on days 7 to 14. Nine animals in Group I, eight in Group II and seven in Group III were killed on day 7; the remaining mice were killed on day 14. Heart viral cultures and histologic analysis were done. Cultures at days 7 and 14 were all negative. Inflammation and necrosis analyzed in each animal were graded 0 to 4, with grade 4 representing widespread inflammation and necrosis.

The heart was histologically normal in all 18 uninfected mice (Group I) given ibuprofen only. Inflammation and necrosis were not significantly different in Group II (infected, untreated) and Group III (infected, treated beginning day 1) mice killed at day 7. Inflammation scores of mice killed on day 14 were 2.1 ± 0.6 (Group II), 3.1 ± 0.7 (Group III) and 2.9 ± 1.0 (Group IV infected, treated days 7 to 14). Necrosis scores of mice killed on day 14 were 1.5 ± 0.8 (Group II), 3.0 ± 0.9 (Group III) and 2.7 ± 1.1 (Group IV). When compared with Group II (infected, but not treated), both inflammation and necrosis were significantly worse in Group III (p < 0.01) and Group IV (p < 0.05). These results indicate that ibuprofen worsens myocardial inflammation and necrosis during acute viral myocarditis. This effect, not attributable to viral persistence in the myocardium, may be related to immunomodulating properties of ibuprofen on prostaglandin-sensitive mononuclear cells.

The emerging hypothesis that viral myocarditis may lead to chronic idiopathic dilated cardiomyopathy underscores the importance of a more complete understanding of the role of the initial viral infection. The viral infection incites the complex immunologic responses that lead to persistent myocardial inflammation, fibrosis and, ultimately, cardiomyopathy. Moreover, because specific treatment for most primary myocardial diseases does not exist, a further understanding of the pathogenesis of viral-initiated myocardial inflammation may lead to the development of therapy strategies designed to treat the cause rather than ameliorate the congestive heart failure of dilated cardiomyopathy.

The murine Coxsackie B3 myocarditis model is ideally suited for studies attempting to modify immune responses in acute and chronic myocarditis. Multiple lines of investigation have documented the evolution of this myocarditis from an acute viral syndrome to a chronic state of fibrosis—a scenario that seems to parallel the pathophysiology of the human disease (1). After Coxsackie B3 infection, acute myocarditis ensues, but appropriate B cell and monocyte responses effect clearance of the virus. The myocardial histologic changes that occur early are patchy and usually consist of sporadic myonecrosis. However, a mononuclear inflammatory infiltrate persists in the heart of appropriate mouse strains for up to 6 months after the initial infection. Histologic analysis of these animals reveals fibrosis, dystrophic mineralization and myocardial hypertrophy. These progress to mural thrombi, myocardial disintegration and fibrotic scarring by 15 months (2).
The murine disease then appears to be two-phased. The initial phase is characterized by myocyte viral infection with reactive B cell, humoral antibody and monocyte responses, an appropriate rate of viral clearance by 10 days and a low fatality rate. The second, nonviral phase is characterized by absence of detectable virus in the face of mononuclear infiltrates that consist of monocytes, T lymphocytes and probably natural killer (NK) cells that perpetuate the disease to a terminal state of dilated cardiomyopathy (2,3). The dissociation of the acute from the chronic phase bears a strong similarity to the human disease and has prompted the use of this model for the evaluation of immunopharmacologic interventions that may be efficacious in myocarditis–cardiomyopathy.

The effects of nonsteroid anti-inflammatory drugs on inflammatory responses are presumably based on the common denominator of their ability to perturb prostaglandin and lipoxygenase synthetic pathways (4). These drugs are usually considered to be a primary focus of therapeutic interest in diseases with either an infectious or a noninfectious inflammatory basis. Thus, we believed that an initial step in seeking means to modify viral heart inflammation would be to evaluate the role of nonsteroid anti-inflammatory drugs in altering myocardial necrosis and the unfavorable outcome of myocardial destruction in the murine model.

**Methods**

**Animals.** Seventy-five 3 week old male BALB/c mice were held for 7 days before the experiment in a single, self-contained animal isolation unit to exclude animals with prior disease (Harlan Laboratory). The mice were maintained in disposable, filter-topped cages and handled with gloves by gowned and masked personnel. The intraperitoneal route was used for viral infection and all drug administration. All intraperitoneal injections were given in a 0.5 ml volume by tuberculin syringe with a 27 gauge needle after iodine-alcohol abdominal skin preparation.

**Drugs.** Ibuprofen (kindly provided by Upjohn Laboratories) was diluted, on the day of injection, in Hanks' balanced salt solution.

**Virus.** Coxsackie virus B3 (Nancy strain) obtained from the American Tissue Culture Collection was grown on either human epidermoid cell line 2 (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 monkey kidney-2 (LLC-MK-2) cells with Dulbecco’s modified Eagle’s medium, 12% fetal calf serum and gentamicin. Virus was harvested and adjusted to an inoculum of 1.75 × 10$^7$ plaque-forming units per 0.5 ml Roswell Park Memorial Institute 1640. This infecting dose had been predetermined by serial dilution studies in identical strain, comparably aged mice that led to myocarditis in greater than 95% of animals so challenged.

**Treatment protocol.** Ibuprofen or diluent only was given intraperitoneally daily at a dose of 15 mg/kg, the actual dose for each experiment being calculated from mouse weight at the time of infection. This drug amount is comparable with an average daily dose used in humans for synovial inflammation. The date of viral infection was termed day 0; the day of study completion was day 14. On day 0, 55 mice were infected; 73 mice were assigned to one of four groups. Group I consisted of 18 noninfected mice treated with ibuprofen on days 10 to 14, Group II consisted of 18 infected mice that were treated with diluent only for the duration of the experiment, Group III consisted of 20 infected mice given ibuprofen daily from days 1 through 14 and Group IV consisted of 17 mice infected at day 0 but receiving ibuprofen daily on days 7 through 14. On day 7, 24 mice were killed. The remaining survivors were killed on day 14.

**Histopathologic analysis.** After cervical dislocation, the hearts were immediately removed for histopathologic analysis. Each heart was divided into two coronal sections. The basal section was snap-frozen and held at −70°C for viral assay. After thawing, the sections were minced with a sterile scalpel, suspended in 1 ml Roswell Park Memorial Institute 1640 and homogenized in a glass tissue grinder. The suspension was centrifuged at 8,000 rpm for 10 minutes at 4°C; supernatants harvested and frozen at −70°C until assay. Serial 10-fold dilutions of heart homogenates in minimal essential medium were layered on confluent 72 hour old VERO cells that had been grown in 96 well microtiter plates (Falcon). Cytopathic effects were checked daily for 7 days and virus was determined by presence or absence of cell destruction. The remainder of the heart was immersed in buffered formalin and Bouin’s solution and processed by semiautomated dehydration and stained with hematoxylin-eosin and Masson’s trichrome stains.

**Data analysis.** The slides were coded and read independently by two of the investigators blinded to experimental group designations. Necrosis and inflammation were the two morphologic abnormalities analyzed, and each was graded according to the semiquantitative scoring system established by Woodruff and Woodruff (5) and modified by us (3). Basically, necrosis is scored 0 to 4, with a score of 4 reflecting widespread necrosis. Inflammation was graded 0 to 4, with a grade of 4 representing 100 or more mononuclear cells per low power field.

**Statistical analysis.** The unpaired Student’s t test and chi-square analysis were used to compare histologic abnormalities in experimental and control groups.

**Results**

**Histologic scores in mice killed on day 7.** One mouse died before the experiment and another on day 1 from a complication of the drug injection. There were no other complications secondary to drug injection during the study.
Table 1. Histologic Scores of 24 Mice Killed on Day 7

<table>
<thead>
<tr>
<th>Group</th>
<th>Inflammation</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (n = 9)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group II (n = 8)</td>
<td>1.6 ± 0.6;</td>
<td>1.2 ± 0.9;</td>
</tr>
<tr>
<td></td>
<td>(1–2.5)</td>
<td>(0–2.5)</td>
</tr>
<tr>
<td>Group III (n = 7)</td>
<td>1.8 ± 1.0;</td>
<td>1.4 ± 1.2;</td>
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<tr>
<td></td>
<td>(1–3.5)</td>
<td>(0–3)</td>
</tr>
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Results are expressed as mean ± SD; range of values shown in parentheses. Group I = noninfected mice given ibuprofen on days 1 to 14; Group II = infected mice given diluent only; Group III = infected mice given ibuprofen beginning on day 1.

One mouse in Group III was dead on day 14; three mice in Group IV died between days 12 and 14. These hearts could not be examined because of cannibalization. Nine animals in Group I, 8 in Group II and 7 in Group III were killed on day 7. Their histologic scores are shown in Table 1. None of the drug-treated, but uninfected, mice had evidence of any inflammation or necrosis. The histologic changes in infected mice were not significantly different from those in infected, drug-treated mice.

Histologic scores in mice killed on day 14. Nine animals in Group I, 10 in Group II, 12 in Group III and 14 in Group IV were killed on day 14. Inflammation and necrosis scores of the groups are depicted in Table 2.

A spectrum of inflammatory infiltrates and myofibrillar necrosis (Fig. 1) was present in the three experimental groups. Only 2 of 10 mice in Group II (virus only) had severe inflammation (score = 3), whereas 9 mice (75%) in Group III (virus plus ibuprofen from day 1) and 10 (71%) in Group IV (virus plus ibuprofen from day 7) had severe inflammation (score = 3) (*p < 0.05). Only one mouse in Group II had severe myofibrillar necrosis, whereas eight (66%) in Group III and nine (64%) in Group IV had severe necrosis (score = 3) (*p < 0.05). Three animals (25%) in Group III and four (28%) in Group IV had severe, widespread inflammation (score = 4); four animals (33%) in Group III and four (28%) in Group IV had severe, widespread necrosis (score = 4). No animal in Group I (drug only) had any inflammation or necrosis. Necrosis in the infected and drug-treated animals killed on day 14 was markedly different and characterized by extreme amounts of dystrophic calcification and gaping areas of necrosis surrounded by dense bands of mononuclear infiltrates (Fig. 2). This could be contrasted with the thin bands of mononuclear infiltrates and much less calcification and focal density of necrosis in infected mice that were not drug-treated (Fig. 3). Viral cultures of myocardium done at day 7 and day 14 were negative in all experimental animals.

Table 2. Histologic Scores of 45 Mice Killed on Day 14

<table>
<thead>
<tr>
<th>Group</th>
<th>Inflammation</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (n = 9)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group II (n = 10)</td>
<td>2.1 ± 0.6;</td>
<td>1.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>(1–3)</td>
<td>(0–3)</td>
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<tr>
<td>Group III (n = 12)</td>
<td>3.1 ± 0.7*;</td>
<td>3.0 ± 0.9*</td>
</tr>
<tr>
<td></td>
<td>(1.5–4)</td>
<td>(1–4)</td>
</tr>
<tr>
<td>Group IV (n = 14)</td>
<td>2.9 ± 1.0*;</td>
<td>2.7 ± 1.1*</td>
</tr>
<tr>
<td></td>
<td>(1–4)</td>
<td>(1–4)</td>
</tr>
</tbody>
</table>

* *p < 0.01 compared with Group II; † *p < 0.05 compared with Group II. Results are expressed as mean ± SD; range of values shown in parentheses. Groups I to III as in Table 1. Group IV = infected mice given ibuprofen on days 7 to 14.

Discussion

The experiment presented here demonstrates conclusively that ibuprofen, when given in the acute phase of murine myocarditis, exacerbates myocardial inflammation.
and necrosis. The effects of ibuprofen on chronic myocarditis have not been investigated. Although nonsteroid anti-inflammatory drugs are commonly used to treat the myalgias and arthralgias of viral infection, we chose to study ibuprofen for more basic than clinical reasons. The marked disparity between the drug-treated, virus-infected group and the virus-infected only group of animals should stimulate concern at the clinical level because many patients with viral infection are either purposely or coincidentally treated with nonsteroid anti-inflammatory drugs.

**Therapeutic interventions that alter Coxsackie virus B3 myocarditis.** Other drugs have already been identified that alter Coxsackie virus B3 heart disease. Administering corticosteroids before Coxsackie virus B3 infection or as late as the sixth day after infection results in extensive myocyte necrosis, viral persistence in the host and unusually high mortality (6). Presumably these agents inhibit the migration of inflammatory cells into the heart and also their ability to eliminate the virus. A more basic mechanism of...
effects during myocardial ischemia (10), should theoretically reduce viral-mediated inflammation and would not explain the acute phase of myocarditis, the highly necrotic myocardial lesions do not appear to be secondary to impaired viral clearance, a phenomenon shown to be dependent on relatively intact monocyte and humoral antibody function (9). Although we do not have serologic evidence of B cell responses, the lack of detectable virus at both 7 and 14 days after infection implies that clearance mechanisms were operative.

Ibuprofen, a phenylpropionate, blocks cyclooxygenase and lipooxygenase activity and subsequent formation of the inflammatory mediators prostaglandin E\(_2\), prostaglandin F\(_2\) and thromboxane A\(_2\). Nonsteroid anti-inflammatory drugs also have inhibitory effects on neutrophils that are not dependent on prostaglandin inhibition (4). These blocking mechanisms, recently shown to have cardioprotective effects during myocardial ischemia (10), should theoretically reduce viral-mediated inflammation and would not explain the marked disparity between the experimental groups in this study. Although the immunomodulating effects of ibuprofen on prostaglandin-sensitive mononuclear subpopulations have not been extensively studied, available evidence suggests that cyclooxygenase blockers may inhibit the actions of a population of prostaglandin-secreting, mononuclear suppressor cell populations (11,12). The prostaglandin E series can also inhibit the production or action of interleukin-2 (13). Thus, the inhibition of prostaglandin E mediators by nonsteroid anti-inflammatory drugs could enhance amplification of a cytotoxic population as they also inhibit the emergence of an immunoregulatory suppressor cell population. The end result would strongly favor a marked increase in both specific and autoimmune myocardial cell necrosis during Coxsackie virus B3 infection (14). Conversely, the mechanism of increased necrosis may be on a more mechanistic basis. Inhibition of prostaglandin synthesis may have negative effects on coronary flow or coronary vascular reactivity that enhance viral-induced cytotoxicity.

**Conclusions.** The superimposition of a perturber of prostaglandin synthesis (ibuprofen) during the acute and early chronic phase of Coxsackie virus B3 murine myocarditis leads to a marked exacerbation of both inflammatory and necrotic foci in the drug-treated animals. The Coxsackie virus B3 model appears useful for further study of both favorable and detrimental effects of immunomodulating drugs. The analysis of the morphologic outcomes after use of these immunomodulators may also lead to further understanding of inflammatory cell kinetics during viral infection of the heart and may identify important considerations in the use or nonuse of these drugs in appropriate clinical situations.

**References**


