Contractile depression and expression of proinflammatory cytokines and iNOS in viral myocarditis

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The purpose of the current study was to evaluate left ventricular performance in mice by means of echocardiography at different times up to 21 days after inoculation with 105 pfu of CVB3. CD-1 mice developed sackievirus B3 (CVB3) infection at 7, 14, and 21 days after inoculation with 10⁵ pfu of CVB3. CD-1 mouse developed inflammatory lesions at 7 days that nearly recovered by 21 days; C3H mice demonstrated persistence of infiltrates. Although there were differences in the baseline fractional shortening, it was further reduced at 7 and 14 days in both strains. It recovered in CD-1 mice but remained depressed at 21 days in C3H mice. Interleukin-6 and tumor necrosis factor-α transcripts were increased in both strains at 7 days. Levels dropped to near control in CD-1 mice at 21 days but remained elevated in C3H mice. Interleukin-1β was minimally elevated in CD-1 mice but increased progressively in C3H mice. mRNA for the inducible form of NO synthase (iNOS) was increased at 7 days in the CD-1 mice, returning to baseline by 14 days; it rose progressively in C3H mice, with a fivefold increase at 21 days. We conclude that mice infected with CVB3 show increased expression of proinflammatory cytokines as well as iNOS associated with reduced contractile performance. In more susceptible mice, contractile depression and cytokine and iNOS expression are more pronounced.

myocardial contraction; coxsackievirus; inducible nitric oxide synthase; mice

VIRAL MYOCARDITIS ACCOUNTS for significant morbidity due to congestive heart failure and has been the subject of intense study. Animal research has shown that the disease has two distinct phases: an early inflammatory state and a chronic phase in which there is progression to myocardial failure. Although the etiology of the latter is not fully defined, postulated mechanisms include an autoimmune process or persistent viral infection. In the early stage, inflammatory cells are present in the myocardium and elevated levels of circulating proinflammatory cytokines have been demonstrated (36). The known role of these cytokines in host defense against infection suggests that they may have a protective function. On the other hand, several recent studies have suggested that proinflammatory cytokines have a negative influence on cardiac function (11, 21, 33). In addition, exogenous administration of cytokines may promote myocarditis in mice that are ordinarily resistant to coxsackievirus B3 (24). Thus the precise nature of the participation of proinflammatory cytokines in the pathophysiology of viral myocarditis has not been fully defined.

Another mediator of inflammation that may participate in myocarditis is nitric oxide (NO). Although controversy exists regarding its precise role in myocardial function (10), Finkel et al. (11) showed reversible depression of isolated papillary muscle contractile function in the presence of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6, which is blocked when an L-arginine analog is added as a specific inhibitor of NO synthase (NOS). In addition, Brady and colleagues (5) showed that depressed contraction of isolated myocytes treated with endotoxin is blunted by pretreatment with inhibitors of NOS. In studies on hepatocytes, TNF-α and IL-1 led to strong induction of NOS activity as well as increases in mRNA for inducible NOS (iNOS) (12). Furthermore, TNF-α and, to a smaller extent, IL-1 led to an early increase in NO formation. Thus TNF-α and IL-1 are capable of stimulating NOS activity by various mechanisms. It is well established that cardiac tissue generates NO in response to soluble inflammatory mediators through the delayed expression of iNOS (32, 34). Although a recent study has shown the presence of iNOS mRNA in macrophages in hearts of mice infected with coxsackievirus (28), the time course of appearance of iNOS and its relation to myocardial performance have not been defined. Moreover, its relation to proinflammatory cytokines in the heart has not been fully explored.

In a recent study, Herzum and colleagues (19) evaluated the relationship between histological signs of myocardial inflammation and hemodynamic parameters measured using intracardiac pressures in an open-chest rat preparation. They showed that although there were overall correlations between scores of histological inflammation and rate of rise or fall of left ventricular pressure, the correlations were relatively weak and were present only when the data were assessed at day 10 postinoculation. On day 7 postinoculation, despite histological evidence of inflammation, the degree of inflammation was not correlated to the functional parameters. The reason for the lack of a clear relationship between histology and function is not clear. It is possible that assessment of myocardial function in open-chest animals was a confounding factor. In addition, whether histological scores accurately reflect the degree of tissue involvement in the inflammatory process is not clear. Myocardial tissue can produce proinflammatory cytokines, and it is possible that the level of these mediators may correlate more directly with contractile function than histological changes.
tion with coxsackievirus B3. In addition, expression of mRNA for the proinflammatory cytokines IL-1β, IL-6, and TNF-α, as well as for iNOS, was assessed by means of Northern blots. iNOS was assessed by Western blot and immunohistochemistry, and histological scores were semiquantified. Because there is a known strain-specific sensitivity to this infection, we compared results from two strains of mice. Results from CD-1 outbred mice, which are known to develop an acute myocarditis with resolution, were compared with results from inbred C3H.HeJ (C3H) mice, which are known to develop a chronic myocarditis.

**MATERIALS AND METHODS**

Experimental animals, diet, and inoculation with coxsackievirus B3. All animal protocols were carried out following the National Institutes of Health guidelines (29a). Four-week-old male mice of the CD-1 (Charles River Laboratories, Boston, MA) and C3H.HeJ (Jackson Laboratories, Bar Harbor, ME) strains were maintained in the Laboratory Animal Resources facilities of The University of Texas Health Science Center at San Antonio. Water and nutritionally adequate mouse chow were available ad libitum. Mice were maintained in plastic cages, and a 12:12-h light-dark cycle was followed. Mice were inoculated intraperitoneally with 10⁵ pfu of virus; the time of inoculation was defined as day 0. Control mice were inoculated with normal saline.

Physiological studies. After anesthesia, which was induced with a cocktail containing 44 mg/kg ketamine, 1 mg/kg acepromazine, and 8.5 mg/kg xylazine to a total volume of 0.2 ml and given intraperitoneally, the mice were positioned on their left sides. Echocardiograms were performed using an ultrasound machine and given intraperitoneally, the mice were positioned on acepromazine, and 8.5 mg/kg xylazine to a total volume of 0.2 ml and given intraperitoneally, the mice were positioned on their left sides. Echocardiograms were performed using an ultrasound machine.

**RESULTS**

Outbred mice, which are known to develop an acute myocarditis with resolution, were compared with results from CD-1 outbred mice, which are known to develop a chronic myocarditis.

**DISCUSSION**

It has been shown that myocardial cytokine expression.

**Tissue collection.** Mice were killed 7, 14, and 21 days postinoculation under anesthesia. The tissues were processed, embedded in paraffin, sectioned at 5 µm, and mounted on poly-L-lysine-coated glass slides, and used for hematoxylin-eosin staining and immunohistochemical localization.

Sections stained with hematoxylin-eosin were scored using a standard semiquantitative scale of 1–4, in which 1 represents <25%, 2 represents 26–50%, 3 represents 51–75%, and 4 represents >75% involvement of the myocardium with inflammatory lesions. Slides were read in a blinded fashion.

Northern blot analysis. Total RNA was extracted using the Northern blot analysis.

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Physiological studies. The results of the fractional shortening studies for the two strains of mice are seen in Fig. 1. There were baseline differences in fractional shortening between the two species of mice: baseline fractional shortening was 35.5 ± 4.1 and 45.8 ± 5.4% in the C3H and CD-1 mice, respectively. Serial determinations of fractional shortening in these groups showed a decrement in function in the CD-1 mice after 7 days of infection: percent fractional shortening was decreased from preinoculation levels by 22.9% (P < 0.01 vs. control). This decrement in function persisted to 14 days, with fractional shortening reduced by 31.7% (P < 0.01 vs. control). For this group, shortening at 21 days was not significantly different from control.

For the C3H mice, there was a significant reduction in function after 7 days of infection, although the change from baseline levels was somewhat less than the decrease at the same time for the CD-1 mice: fractional shortening was decreased by 13.0% at this time (P < 0.01 vs. control). Functional performance of the infected animals was further impaired at 14 days, with fractional shortening reduced by 31.7% (P < 0.01 vs. control); it was further impaired after 21 days, with a 36.7% decrease in fractional shortening compared with preinoculation levels (P < 0.006 vs. control). Thus, unlike the CD-1 mice, in which contractile abnormality appeared to be the most severe at 14 days, the C3H mice had reduced function at 14 days, which did not recover by the time the animals were killed at 21 days. Comparison of the strains showed significantly greater reduction in fractional shortening of the C3H mice than the CD-1 mice at 21 days (P < 0.02).

Histological studies. There were no signs of inflammation in the control mice of either strain. At each subsequent time, significant signs of inflammation were present in both strains (P < 0.05 at each time point vs. control for that strain). At 7 days, five of seven C3H mice had signs of inflammation (average score 1.1 ± 0.47), whereas signs were present in only three of seven CD-1 mice (overall average score 1.0 ± 0.41). At 14 days, signs were present in four of seven C3H mice (average score 1.2 ± 0.52) and in only one of seven CD-1 mice (average score 0.3 ± 0.12). The histological scores were consistently higher for the C3H mice, and the difference was statistically significant at 21 days (P < 0.05). Thus the C3H mice had more severe evidence of inflammation, and the signs of inflammation persisted longer in a higher percentage of these mice.

Proinflammatory cytokines. Results of the Northern blots for the three proinflammatory cytokines are shown in Fig. 2 for the CD-1 and C3H mice. In the CD-1 mice there was limited expression of IL-1β; signals for IL-6 and TNF-α were maximal after 7 days of infection. There was some interanimal variability in the expression. For the C3H mice the signals were substantially more intense. Again, interanimal variability was seen. In addition, the signal for IL-1β was present after 14 and 21 days, and signals for IL-6 and TNF-α were present at each of the times after infection, with the strongest signals after 7 days. Results of the autoradiographic analysis shown in Fig. 3 are the ratios of densitometric values of a specific gene to corresponding glyceraldehyde-3-phosphate dehydrogenase values. Comparison of baseline values of cytokine expression showed very similar values in each strain for all three cytokines before viral inoculation. In both strains of mice, IL-1β was not detected in control uninfected animals. Very low levels were detected in CD-1 mice, whereas levels increased in C3H mice, with maximal levels at 21 days postinoculation. In the case of IL-6, maximal levels were detected at 7 days postinoculation in CD-1 mice, with levels 4.1-fold higher than respec-
tive control, and declined thereafter (1.3-fold at 14 days and 1.4-fold at 21 days). On the other hand, in C3H mice the levels were 14-fold higher at 7 days and fell to 3.4-fold at 21 days. A similar trend was observed with TNF-α in both strains of mice, with maximum levels at 7 days postinoculation: 6.8-fold in CD-1 mice and 11.5-fold in C3H mice. By 14 days, TNF-α levels decreased to twofold in CD-1 mice but remained high in C3H mice (8.8-fold). TNF-α levels were similar in both strains of mice at 21 days postinoculation: 1.8-fold in CD-1 mice and 2.2-fold in C3H mice. These results demonstrate the higher signal level in the C3H mice as well as the persistence of the mRNA signal for the cytokines.

iNOS. Figure 4 shows the results of the Northern blots for iNOS mRNA and the values obtained from the autoradiographs. Figure 5 shows similar data for Western blots performed to quantify iNOS protein levels. Again, comparison of baseline results for mRNA and protein showed similar levels in each strain before viral inoculation. In CD-1 mice, iNOS mRNA levels were highest at 7 days postinoculation (2.2-fold vs. respective controls) and declined thereafter (0.8-fold at 14 and 21 days). In contrast, iNOS mRNA levels were more pronounced for the C3H mice, showing increases at 7 days (2.9-fold) that were progressively larger at 14 (3.4-fold) and 21 days (5.1-fold). In addition, Western blots demonstrate that protein levels correlate closely with the mRNA data in CD-1 (2-fold at 7 days and 1.1-fold at 14 and 21 days) and C3H mice (2.5-fold at 7 days, 3.0-fold at 14 days, and 4.1-fold at 21 days), indicating that transcription and translation occurred.

Detection of iNOS immunoreactivity. Immunostaining was not detected at any time point in uninfected mice from either strain. Because we used three controls (no primary antibody, rabbit preimmune sera in place of primary antibody, and addition of primary antibody after neutralization), the specificity of the primary
antibody and of the staining procedure is clearly confirmed. Very weak immunostaining was detected in the blood vessels in the hearts from uninfected CD-1 and C3H mice (score $0.13 \pm 0.14$ and $0.19 \pm 0.13$, respectively), with no staining of the cardiomyocytes (Fig. 6). iNOS protein was readily detectable in blood vessels and in cardiomyocytes at 7 days postinoculation in both strains of mice (score $2.33 \pm 0.58$ and $2.00 \pm 0.82$, $P = NS$). At 14 days postinoculation, the intensity of immunoreactivity in blood vessels as well as in cardiomyocytes was significantly increased in the C3H mice ($2.00 \pm 0.82$), whereas in CD-1 mice the vessels showed levels of iNOS similar to those seen at 7 days postinoculation, and fewer cardiomyocytes showed positive staining ($1.4 \pm 0.75$, $P < 0.05$ vs. C3H). For the C3H mice at 21 days postinoculation, scores remained high ($2.3 \pm 0.96$), whereas in CD-1 mice, levels in cardiomyocytes fell to that of controls, and levels in vessels remained high ($0.88 \pm 0.25$, $P < 0.25$ vs. C3H mice). In addition, iNOS immunoreactivity was detected in macrophages in both strains of mice at 7, 14, and 21 days postinoculation (data not shown). Results of immunohistochemical staining for a CD-1 and a C3H mouse are shown in Fig. 6.

Fig. 3. Autoradiographic semiquantitation of cytokine mRNA. Signals were higher for C3H mice, and signal for interleukin (IL)-1β showed a progressive rise. TNF-α, tumor necrosis factor-α. * $P < 0.05$; ** $P < 0.01$ vs. control of same strain. † $P < 0.05$; †† $P < 0.0001$ between strains.

Fig. 4. Northern blots (A) and autoradiographic densitometric values (B) for inducible NO synthase (iNOS) mRNA in CD-1 and C3H mice at each time point. In A, each lane represents 20 µg of total RNA from a single animal. mRNA for GAPDH was used to document loading equivalency. Densitometric values were higher for C3H mice, and a progressive rise in iNOS expression was seen. * $P < 0.05$, ** $P < 0.001$ vs. control of same strain. † $P < 0.01$; †† $P < 0.0001$ between strains.
DISCUSSION

Our results show for the first time that myocardial dysfunction, quantified by reduced fractional shortening in intact mice, parallels myocardial expression of proinflammatory cytokines and iNOS in coxsackievirus myocarditis. In addition, strains of mice with different sensitivities to the viral infection manifest unique temporal patterns of contractile dysfunction associated with different patterns of cytokine and iNOS expression. CD-1 mice show a reduction of contractile performance at 7 and 14 days, with recovery by 21 days. This pattern was associated with moderate increases of mRNA for proinflammatory cytokines, which peaked at 7 days and fell nearly to baseline levels at 21 days. C3H mice expressed a more marked cytokine message associated with sustained myocardial dysfunction. In addition, these mice showed progressive increases in IL-1β, which was only minimally expressed in the CD-1 mice.

Although the role of proinflammatory cytokines in viral myocarditis has been the subject of active investigation, their full impact has not been defined. It is well known that proinflammatory cytokines have a beneficial effect in viral clearance, but they may also serve to heighten the pathology of myocarditis. They are present in the myocardium during infection as part of the inflammatory cascade, and prior work has established that viral infection may induce production of proinflammatory cytokines (4, 13). Neumann et al. (30) suggested that they may be involved in pathological changes occurring in the interstitial compartment of the myocardium. In the intact animal, various cell types are capable of producing the cytokines, including myocytes and white blood cells. Henke et al. (17) specifically showed that coxsackievirus infection leads to production of IL-1, IL-6, and TNF-α by human monocytes.

The possible adverse impact of cytokines in myocarditis may manifest as altered susceptibility to disease. Lane and colleagues (24) showed that mice ordinarily resistant to coxsackievirus infection will develop inflammatory lesions when treated with TNF-α or IL-1. Similar results were found by Yamada et al. (36), who also showed that treatment of rats with monoclonal...
anti-TNF antibody before viral inoculation led to reduced mortality of infected mice. Thus proinflammatory cytokines may adversely affect the course of viral infection. In our study, levels of proinflammatory cytokines were persistently elevated in C3H mice for 21 days, a time well after viral clearance has been reported to occur in this model (31). In addition, despite the fact that inflammatory scores were returning to low values in these animals, biochemical aspects of the inflammatory condition appeared. Given the pleiotropic nature of the proinflammatory cytokines, it is likely that they may have other important local effects in the tissue.

A number of studies have emphasized the possible negative impact of proinflammatory cytokines on mechanical performance of the heart. In vitro studies using isolated perfused hearts and isolated cells from cats (37) as well as Syrian hamster papillary muscles (11) have shown that TNF-α exerts a transient myocardial depressant effect, which occurs within minutes. A recent study from this laboratory showed that exogenous administration of TNF-α to conscious dogs led to myocardial depression within 2 h that was sustained for 24 h (29). Other studies have shown that IL-1 (36) and IL-6 (11) also have independent negative inotropic effects. In view of the negative impact these cytokines are known to have on myocardial contractile function, it is plausible that the persistently depressed function we found in these mice was attributable, in part, to the presence of these inflammatory mediators in the local milieu. Further work is needed to define the degree to which myocardial cell loss and myodepressant influence of cytokines contribute to decreased contractile performance in myocarditis.

Although NO has also been linked to myocardial dysfunction, its role has not been definitively proven. Finkel et al. (11) showed that the reversible depression of isolated papillary muscle contractile function caused by TNF-α or IL-6 was blocked when an L-arginine analog was added as a specific inhibitor of NOS. Balligand and colleagues (3) showed that shortening of myocytes is impaired when they are exposed to media in which macrophages were stimulated with lipopolysaccharide and that this can be blocked by inhibition of NO synthesis. This implicates NO in the myodepression that follows cytokine exposure. The presence of iNOS in the myocardium of failing hearts is also suggestive of a possible role of this system in cardiac depression. In studies performed on biopsies from patients with heart failure, Haywood and colleagues (16) showed that in a
majority of cases iNOS mRNA can be found in the myocardium. They also showed the presence of iNOS protein by immunohistochemistry in the hearts in which iNOS mRNA was identified. In a similar study, Habib et al. (15) found iNOS immunoreactivity in tissue from patients with dilated cardiomyopathy. On the other hand, data from Crystal and Gurevicius (10) do not support a role for NO in acute cardiac depression, and studies by Yokoyama et al. (37) suggest that abnormalities of calcium handling, not NO, underlie the negative contractile impact of TNF-α. Although the possible negative inotropic impact of this factor has been emphasized, it is also possible that NO plays a role in infectivity of virus. Lowenstein and colleagues (27) recently showed that iNOS is markedly increased in hearts of mice infected with coxsackievirus B3 and that mortality was increased in infected mice by treatment with NOS inhibitors. These authors propose an important role for NO in defense against viral infection.

Our results are of interest because they confirm the notion that conditions for iNOS induction, including the presence of cytokines, are present after viral infection. In addition, more severe histological abnormalities and persistent contractile dysfunction were present in the strain of mice with the most pronounced cytokine and NOS expression. This strongly implicates these aspects of the inflammatory process in the pathogenesis of coxsackievirus myocarditis and in the attendant contractile depression. Our data do not allow us to separate effects of the individual cytokines or to differentiate between the impact of cytokines and iNOS. Because we show that these factors are concurrently present during inflammation in vivo, delineation of the role of each may be difficult. This may, however, offer insights into possible therapeutic interventions, especially if the activation of these cytokines occurs in a sequential fashion or if one plays a predominant role. Further studies are required to unravel these pathways.

A common underlying stimulant for the activation of cytokines and iNOS may be the presence of reactive oxygen intermediates in the tissue. Hiraoka et al. (20) showed a higher survival rate in mice pretreated with superoxide dismutase and catalase (bound to polyethylene glycol to prolong their half-lives) after inoculation with encephalomyocarditis virus than in mice not pretreated. This occurs despite no difference in viral titers in the groups, indicating a protective effect of free radical scavengers. In addition, we and others recently showed that, in hearts subjected to brief periods of ischemia followed by reperfusion, another condition known to be associated with free radical damage, cytokines are expressed (6, 18). In view of the occurrence of inflammatory cells in the heart, it is not unlikely that free radicals were present in the myocardium of our mice. Because free radicals are known to stimulate activation of the transcription regulator nuclear factor-κB (NF-κB) (25) and both proinflammatory cytokines and iNOS have NF-κB response elements (2, 23, 26, 35), there may be a unifying mechanism governing regulation of the cytokines in myocarditis. Further studies are necessary to directly address this issue.

As has been found by many prior investigators, our histological data showed myocarditis to be associated with patchy infiltrates throughout the myocardium. Although we have not defined precisely which tissue elements are the source of the cytokines or of iNOS, it seems likely that this was a more generalized process. First, echocardiographic assessment showed global decreases in myocardial function, not regional wall motion abnormalities. Second, expression of cytokines was found, particularly in the C3H mice, when most histological signs of inflammation had subsided. This suggests that even if the stimuli for cytokine generation arose in the regions of inflammatory infiltrate, they affected cells distant from the actual foci of inflammation. Finally, prior studies support this notion. Immunohistochemical data from the study of Lane et al. (24) showed that IL-1- and TNF-immunoreactive cells were located distant from major foci of inflammatory cells. Likewise, the immunohistochemical evidence of iNOS in patients with heart failure was not focal but was diffuse (16). This suggests that there are sequelae of the acute, focal inflammatory condition that affect the myocardium in a global fashion. This may, in part, account for sustained myocardial dysfunction seen in the C3H mice.

A limitation of this study is that different strains of mice were used to assess functional, cytokine, and iNOS response to viral infection. Clearly, differences in baseline contractile performance were present between the groups, such that the relative impact of the inflammatory process on function must be used for quantification. However, there were no differences in baseline cytokine mRNA, histological features, or iNOS mRNA or protein levels between the strains. Although the results of the functional studies correlate with the cytokine and iNOS results in each strain, further studies that do not use different strains will be valuable for corroboration of our results. Whether differences in genetic background may uniquely impact on how each strain responds to viral infection and the subsequent cytokine responses remains open to question. Despite this possibility, our data demonstrate the close correlation between inflammatory mediators and function in each strain.

In summary, we have shown increased expression of proinflammatory cytokines as well as iNOS in mice infected with coxsackievirus B3. This is associated with reduced contractile performance. In a strain of mice more susceptible to chronic myocarditis, the pattern of cytokine and iNOS expression is more severe. Further delineation of the precise mechanisms by which these factors are controlled will provide important insights into this important clinical entity.

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