Interleukin 1 or Tumor Necrosis Factor Can Promote Coxsackie B3-induced Myocarditis in Resistant B10.A Mice

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Summary

We have previously demonstrated that bacterial lipopolysaccharide (LPS) is capable of promoting Coxsackie B3 (CB3)-induced myocarditis in genetically resistant B10.A mice. Because LPS is known to increase production of various cytokines, we tested CB3-infected, LPS-treated mice for the presence of interleukin 1 (IL-1) and tumor necrosis factor (TNF). We found significantly increased amounts of both cytokines in the sera of CB3/LPS-treated mice compared with animals treated only with LPS. We also found immunohistochemical evidence for local production of these cytokines in the cardiac tissue of CB3/LPS-treated mice. Treatment with IL-1 or TNF alone promoted CB3-induced autoimmune myocarditis in resistant B10.A mice. Myocarditis was also observed when uninfected mice were immunized with syngeneic heart extract in the presence of IL-1 or TNF.

Infection of mice with Coxsackie virus B3 (CB3) results in myocyte necrosis and an inflammatory response within the myocardium consisting of focal infiltrates of polymorphonuclear cells, lymphocytes, and macrophages. The early pathological manifestations of viral injury gradually diminish so that by 14 d after infection, the acute phase of myocarditis is resolved. In some genetically predisposed mouse strains such as A/J (H-2^a), this acute phase is followed by a chronic, autoimmune myocarditis characterized by diffuse interstitial mononuclear cell infiltrates and by the presence of heart-specific autoantibodies (1). C57BL/10 H-2 congeneric mice (e.g., B10.A, H-2^b) are not susceptible to the chronic, autoimmune disease, and recover spontaneously from the initial myocarditis.

We have recently reported that LPS treatment of CB3-infected B10.A mice is capable of inducing autoimmune myocarditis in these genetically resistant animals (2). Because LPS is capable of stimulating increased production of various cytokines (3), it is possible that these immunomodulators, along with the CB3 viral infection of B10.A mice, contribute to the development of autoimmune myocarditis. To investigate this possibility we examined the sera and hearts from CB3-infected, LPS-treated B10.A mice for the presence of IL-1 and TNF, which are the major cytokines produced by macrophages when treated with LPS (4, 5). We found significantly increased amounts of both cytokines in the sera of CB3/LPS-treated mice when compared with animals treated only with LPS, or infected only with CB3, and immunohistochemical evidence for the local production of these cytokines in the cardiac tissue of CB3/LPS-treated mice. We also report the ability of treatment with IL-1 or TNF alone to promote CB3-induced autoimmune myocarditis in resistant B10.A mice. Additionally, when uninfected mice were cotreated with IL-1 or TNF, and immunized with syngeneic heart extract as a source of myosin, myocarditis was observed, but was absent in the mice injected only with the heart proteins. This finding indicates an ability of these immunomodulators to enhance immune reactivity against self-antigens.

Materials and Methods

Animals. The C57BL/10 (B10.A) mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME), and bred and maintained in our animal facilities.

Virus Preparation. Preparation and titration of CB3 (Nancy strain) using Vero monkey kidney cells has been previously described (6).

Preparation of Syngeneic Heart Extract. Hearts from untreated B10.A mice, 6–10 wk of age, were perfused with Tris buffered saline (TBS), pH 7.6, cut into small pieces, and then homogenized in TBS. Heart protein concentration was determined by the BCA protein assay (Pierce Chemical Co., Rockford, IL). Previous studies in our laboratory showed that myosin is the predominant autoantigen in heart extract (Foca et al., unpublished results).

Treatment Protocol. B10.A mice, 14–20 d of age, were inocu-
lated intraperitoneally with either CB3 and LPS (CB3/LPS), CB3 and IL-1 (CB3/IL-1), CB3 and TNF (CB3/TNF), CB3, LPS, IL-1, TNF, or saline (100 μl). The CB3/LPS group was inoculated on day 0 with a 1:3 dilution of 10^6 TCID_50 CB3 in 0.1 ml RPMI 1640, and with 25 μg LPS (from Salmonella minnesota Re 595; Sigma Chemical Co., St. Louis, MO) in 0.1 ml sterile saline. The CB3/IL-1 and CB3/TNF groups were similarly infected with virus, and treated with 100 ng of IL-1 synthetic peptide fragment 163-171 (Sigma Chemical Co.), and 250 ng TNF synthetic peptide fragment 114-130 (ICN Biochemicals, Costa Mesa, CA), respectively. Dosages of LPS, IL-1, and TNF were experimentally determined as the amounts necessary to induce the greatest amount of histological and serological evidence of myocarditis without causing >50% mortality in the mice. The CB3 group only received CB3 on day 0, the LPS, IL-1, and TNF groups received treatment diluted in sterile saline (no CB3). On day 4, the CB3/LPS and the LPS groups were treated again with LPS; the CB3/IL-1, and IL-1, and IL-1, and TNF groups were treated again with IL-1; the CB3/TNF and TNF groups of mice were treated again with TNF; and the CB3 and saline groups were treated with sterile saline (100 μl). Immunization of B10.A mice of similar ages was performed by subcutaneous injection into the inguinal region with 100 μg of B10.A heart extract (HE) emulsified in IFA on days 0 and 7, or cotreated with HE and individual immunomodulators. The immunomodulators were prepared and injected as above on days 0 and 4.

Histology. 2 wk after treatment, the mice were exsanguinated, perfused with heparinized saline, and the hearts removed. The hearts were cut sagitally. Half of each heart was frozen for immunohistochemistry, and the other half fixed in 10% buffered formalin for hematoxylin and eosin (H & E) staining.

Immunohistochemistry. The indirect immunohistochemistry procedure has been previously described (2) and was used to detect IL-1- and TNF-secreting cells in the heart tissue. IL-1- and TNF-secreting cells were identified by using a rabbit anti-murine IL-1 reagent (Genzyme Corp., Boston, MA), and rabbit anti-TNF (Endogen, Inc., Boston, MA), respectively.

ELISA for IL-1 and TNF Serum Levels. Goat anti–murine IL-1 (R & D Systems, Inc., Minneapolis, MN) or rat anti–murine TNF (Endogen, Inc.) (100 μl of 5 μg/ml solutions in PBS, pH 7.4) was added to the wells of ELISA plates (Costar Corp., Boston, MA), and incubated for 16–24 h at 4°C. The plates were washed with PBS containing 0.05% Tween-20 (PBS-Tween), and 200 μl/well of a solution of PBS containing 4% BSA was added and incubated for 1 h at 37°C. Serum samples were assayed neat and diluted 1:2 and 1:4 in PBS-1% BSA. Murine IL-1 (R & D Systems) or murine TNF (Endogen, Inc.) was diluted in PBS-Tween, 1% BSA (1,000, 500, 100, 25, 10, and 1 pg/ml). The plates were washed with PBS/Tween and 100 μl of rabbit anti–IL-1 (R & D Systems) or rabbit anti-TNF (Endogen, Inc.) were added to the wells of the appropriate assay plate and incubated for 2 h at 37°C. The plates were washed with PBS-Tween and 100 μl of peroxidase-conjugated goat anti–rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) were added to the wells, and the plates incubated for 2 h at 37°C. The plates were washed with PBS-Tween and 150 μl of ABTS substrate solution (Kirkegaard and Perry Laboratories; Gaithersburg, MD) were added to the wells for color development. A standard curve was obtained by plotting the resulting absorbance of the cytokine dilutions versus their concentrations. Cytokine concentration of the serum samples was determined by linear regression analysis.

Results and Discussion

We have previously demonstrated that LPS is capable of promoting CB3-induced autoimmune myocarditis in genetically resistant B10.A mice that is characterized by mononuclear cell infiltration of heart tissue, and by the presence of IgG autoantibodies to heart antigens (2). Gudvangen et al. (7) reported that treatment of CB3-infected, myocarditis-susceptible mice with another immunomodulator, levamisole, exacerbates the myocarditis that is observed compared with mice infected only with the virus. We have observed that levamisole treatment of CB3-infected B10.A mice, like LPS, induces an autoimmune myocarditis in these resistant animals (unpublished observations). Reports that LPS induces macrophage secretion of IL-1 and TNF (3–5), and that levamisole can enhance macrophage secretion of IL-1 (8), led us to examine what role these cytokines may have in our model of autoimmune myocarditis.

In this report, serum samples obtained 14 d after treatment were tested for concentrations of IL-1 (Fig. 1) and TNF (Fig. 2). IL-1 and TNF were detected in the sera of mice treated with CB3/LPS and LPS, but were not detected in mice infected with CB3 alone. Mice that were treated with saline did not have any detectable serum IL-1 or TNF (data not shown). Comparison of CB3/LPS- and LPS-treated mice revealed that both IL-1 and TNF serum levels were significantly greater in the CB3/LPS group (121.2 ± 10.6 pg/ml vs. 56...
± 13.2 pg/ml, p < 0.001 for IL-1, and 88.1 ± 8.0 pg/ml vs. 40.4 ± 6.2 pg/ml, p < 0.001 for TNF). The elevated serum levels of IL-1 and TNF observed for the LPS-treated mice were to be expected, based on the actions of this immunomodulator. Although both the CB3/LPS and LPS groups of mice were treated with equivalent amounts of LPS, the CB3/LPS group had significantly higher serum levels of the cytokines, which was evident 9 d after treatment (data not shown), than did mice treated only with LPS. The ongoing autoimmune process in the hearts of the CB3/LPS-treated mice may be attributed to this enhanced level of both cytokines. Increased levels of IL-1 and TNF have been observed in numerous chronic inflammatory conditions including autoimmune diseases. (9, 10).

The pathogenic relevance of the circulating serum levels of IL-1 and TNF was demonstrated by the observation of

Figure 3. Inflammatory cells in sequential sections of heart tissue of CB3/LPS-treated mice consisted of monocytes and CD3⁺ cells. These cells stained positive using the antibodies specific for IL-1 (A) and TNF (B). Note in areas away from the major focus of inflammatory cells (arrows), both IL-1- and TNF-staining cells were observed ×420.
inflammatory cells within the heart tissue appearing to secrete these cytokines locally. Heart tissue samples obtained from the CB3/LPS-treated mice 14 d after treatment were examined by immunohistochemistry for the presence of IL-1- and TNF-containing cells among the heart inflammatory cells. Within an inflammatory lesion, both IL-1- (Fig. 3 A) and TNF- (Fig. 3 B) staining cells were observed. No cytokine staining cells were detected in the heart tissue of control mice infected only with CB3 or treated only with LPS. Similar observations have been made in multiple sclerosis patients who have increased levels of TNF in the serum and spinal fluid (11), and who also have brain lesions containing TNF-secreting mononuclear inflammatory cells (12). In our mice, it is likely that, upon treatment with LPS, macrophages and monocytes are activated and secrete cytokines to cause the systemic increase of IL-1 and TNF that is observed in the serum. CB3 infection of heart tissue promotes the local production of these cytokines by activated monocytes within the heart as a response to viral infection. In B10.A mice not treated with LPS, these monocytes may cooperate with other lym-
phoid cells to remove virus for a resolution of the acute phase of myocarditis. In contrast, the highly activated monocytes in LPS-treated mice may result in the persistence of these cells within the heart tissue, even after virus is eliminated, and lead to the development of chronic myocarditis.

Supporting evidence for the involvement of IL-1 and TNF in promoting myocarditis was observed when CB3-infected B10.A mice were treated with IL-1 or TNF rather than LPS. Because LPS has many physiologic effects, in addition to cytokine production (13), peptide fragments of IL-1 and TNF were used. These peptides have been reported to have both in vitro and in vivo immunostimulatory activity (14–17), and with our mice similar results were obtained whether we used the peptide fragments or the whole IL-1 or TNF molecule (data not shown). 14 d after treatment, hearts from mice treated only with CB3 (Fig. 4 A), IL-1, or TNF appeared normal, while hearts from mice given combined CB3/IL-1 (Fig. 4 B) and CB3/TNF (Fig. 4 C) treatment were characterized by extensive mononuclear cell infiltration. These IL-1 and TNF peptides promoted disease similar to that seen with LPS, even though they represent the minimal structure necessary to be immunostimulatory without inducing LPS-like pyrogenic and inflammatory effects.

Our studies are the first to report the ability of cytokine treatment to convert a myocarditis-resistant strain of mice to become susceptible to myocarditis. There have been reports of cytokine modulation of other animal models of autoimmune disease. The effects of IL-1 on the development of collagen-induced arthritis was to accelerate the onset and progression of disease (18), and in the Biobreeding rat, in vivo treatment with IL-1 was found to modulate idiopathic autoimmune diabetes and thyroid disease in a dose-dependent manner (19).

It is interesting to note that treating infected mice with either IL-1 or TNF promoted similar disease. Regardless of which treatment the mice received, the other cytokine was detected in the serum and present within the cytokine-secreting cell population at equivalent levels (data not shown). This is most likely due to the ability of each cytokine to induce the production of the other (9, 20) and to act synergistically (21). Ongoing studies in our laboratory using anticytokine treatment are examining whether such synergy is operating in this model, or whether one of the cytokines is produced as a byproduct of the inflammatory process (10). We also are performing time course studies of these mice to identify the heart inflammatory cells, and to determine the local heart inflammatory cell production/serum levels of IL-1 and TNF (our manuscript in preparation). Preliminary evidence indicates that, regardless of whether infected mice are treated with LPS, IL-1, or TNF, an initial, limited, monocyte inflammatory population progresses by 9 d after treatment to an extensive monocyte/CD3+ cell population. This is at this time that serum cytokine levels are significantly increased compared with LPS-only- or cytokine-only-treated mice, and when cytokine-secreting cells can be detected within the heart tissue. Additionally, we find that the early, acute phase of myocarditis experienced by mice infected only with CB3 is not associated with detectable serum concentrations of either IL-1 or TNF, nor is there immunohistological evidence of cytokine secretion in the few inflammatory cells present in the heart tissue of these mice before resolution of the diseases.

Previous studies (22) in our laboratory have demonstrated that immunization of mice with mouse cardiac myosin results in chronic, autoimmune myocarditis, similar to that observed following acute CB3 infection. This disease is also genetically restricted, i.e., B10.A mice are resistant to the myosin-
induced autoimmune myocarditis. We found that immunizing B10.A mice with syngeneic heart extract, containing cardiac myosin in addition to other cardiac proteins but no CB3 virus, together with IL-1 or TNF resulted in myocarditis in all animals (n = 10 for both HE/IL-1 and HE/TNF), as seen in Fig. 5A. Treatment of B10.A mice with syngeneic heart extract without IL-1 or TNF (n = 5) produced no disease as seen in Fig. 5B. These observations and evidence presented in this report suggest that cytokine-mediated modulation of the immune response leads to the induction of chronic, autoimmune myocarditis.

It is possible that the extent of cytokine production during an organ-specific infectious process determines whether an autoimmune response will be directed towards that organ. B10.A mice may be resistant to the development of autoimmune myocarditis because the cytokine levels obtained during

Figure 5. H&E section of heart tissue obtained from B10.A mice cotreated either with HE and IL-1 (not shown), or HE and TNF (A) demonstrated mononuclear cell inflammation, whereas B10.A mice treated only with HE (B) experienced no inflammation ×420.
an immune response are properly balanced to ensure a controlled response to infected tissue. Manipulation of the cytokine levels upsets this balance, and may permit an uncontrolled immune response, or an inhibition of tolerance, to occur towards heart cell constituents, as observed in our whole heart extract experiments. If cytokines are found to play a significant role in the pathogenesis of this postinfectious autoimmune disease, then there would be potential therapeutic strategies for treating these diseases by reducing cytokine levels.

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