Antiviral and myocyte protective effects of murine interferon-β and -α2 in coxsackievirus B3-induced myocarditis and epicarditis in Balb/c mice

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Enteroviruses, especially coxsackievirus B3 (CVB3), are associated with human viral myocarditis, leading to cardiomyopathy, left ventricular dysfunction, and chronic heart failure. As yet, there is no effective therapy for this disease (22). Infection of CVB3 in Balb/c mice can also induce myocarditis with a pathological process resembling human disease: starting with an acute phase characterized by high viral replication, followed by a chronic phase characterized by much lower viral replication and an increase in cellular immune reaction, eventually leading to heart failure (23). Thus this experimental murine model has been widely used for studying both the acute infectious phase and chronic immune phase of human viral myocarditis and cardiomyopathy.

Type I interferons (IFN), including IFN-β and IFN-α, initially identified for their ability to protect cells from viral infections, are pleiotropic cytokines mediating a critical role in innate cellular defense against viral infection (25). Indeed, mice deficient in IFN-β or the IFN receptor chain IFN-β receptor showed impaired antiviral response and increased susceptibility of CVB3 infection (8, 28, 29). These studies mainly address the role of endogenous IFNs in the animal but do not define the potential for exogenously administered IFNs to inhibit viral replication. This is especially important in the setting of viral myocarditis, since there is evidence that endogenous IFNs may have a minor effect on viral replication within the cardiac myocyte (29, 30). Although IFN-β (Betaseron) is currently used for treatment of multiple sclerosis, a recent study also showed that IFN-β (Betaferon) eliminated viral genomes and improved left ventricular function in patients with left ventricular dysfunction and myocardial enteroviral or adenoviral persistence (14). However, the precise effect of IFNs on viral replication and myocardial pathology, as well as a proper comparison of these effects between IFN-β and IFN-α in the same study in intact animals, has not been thoroughly evaluated. To carefully evaluate the effect and efficacy of exogenously administered IFN on myocarditis and to determine whether there is a difference in the host response to IFN-β and IFN-α, we treated Balb/c mice with CVB3 and thoroughly evaluated viral replication, inflammation, and evidence of sarcolemmal disruption in CVB3-infected Balb/c mice. Indeed, the results demonstrated that both murine (m)IFN-β and mIFN-α2 had similar protection on CVB3-induced myocarditis. However, whereas mIFN-α2 only eliminated the CVB3 genome but not the infectivity, mIFN-β eliminated both. Further studies are needed to elucidate the potential mechanisms for the differential host responses to the two isoforms of IFN.

METHODS

General Procedures

Virus inoculation and animal handling postinoculation were performed in a biological safety cabinet according to biohazard level 2 regulations for infectious agents. The safety procedures were approved by the Environmental Health and Safety Department. The animal protocol was approved by the Institutional Animal Care and Use Committee.

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Six-week old male Balb/c mice (Charles River, Hollister, CA) were randomized for intraperitoneal injection with either vehicle or different doses of mIFN, followed by subcutaneous injection every other day until the day of being euthanized (day 4, or 14, n = 10–20 mice/group). One hour after the first dose, the mice were injected intraperitoneally with CVB3, a cardiotropic strain of Woodruff H3 variant derived from murine heart-passaged infectious cDNA copies of the wild-type viral genome (13, 31), at a dose of 30,000 plaque-forming units (PFU) in a volume of 100 μl using PBS as a vehicle (day 0). At the end of the experiment and 3 h before death, all mice were injected intraperitoneally with a 200 μl Evans blue solution (1% in PBS) to label early cardiomyocyte injury or death. The heart was then exposed and perfused with 5 ml PBS through the left ventricle, removed, weighed, and sectioned. Approximately 10 mg of tissue from the apex of the heart were excised and weighed for use in a plaque-forming assay and real-time RT-PCR. The remainder of the heart was fixed in 10% buffered formalin for histopathological evaluation.

Cloning, Expression, Purification, and Characterization of mIFN-β and mIFN-α2

The mIFN-α2 was PCR amplified from cDNA library created from vesicular stomatitis virus-infected L929 cells using the following primers: 5'-CCCCCGGCCGATGCTAGACTCTCTTCCTCC-3' and 5'-CCGGCTCTTATCTCCTCTCTCTCCT-3'. The resulting PCR product was DNA sequenced to confirm identity and then cloned into a modified pCEP4 mammalian expression vector using Clonase (vector PCEP4WPRI, Berlex Biosciences, Richmond, CA). The protein was then purified chromatographically on a GH-25 column. Protein determination was performed by UV 280 spectrum of the purified protein using the absorbance coefficient (mIFN-β = 1.65, and mIFN-α2 = 1.09). NH2-terminal protein sequences of both purified mIFN-β and mIFN-α2 matched the theoretical recovery for each, and no secondary sequence was observed. Analysis by analytical size exclusion chromatography showed a typical purity of 97.8–99.5%, depending on expression level. Endotoxin levels were acceptably low (≤5 endotoxin units/dose), determined by Limulus amoebocyte lysate assay before animal studies. Purified mIFN-β and mIFN-α2 remained stable when stored at −80°C at a concentration of 1 mg/ml in 20 mM Na-acetate with 100 mM NaCl.

The specific activities were 2 × 108 international units (IU)/mg for mIFN-β and 6.8 × 107 IU/mg for mIFN-α2, determined by a cytopathic-based antiviral assay (24). Purified mIFNs were stored in 50 mM Na-acetate with 150 mM NaCl. This buffer was also used as a vehicle to dilute mIFNs before administration to the concentrations of 2.5, 5, and 10 μg/ml for mIFN-β and 111 μg/ml for mIFN-α2. The total injection volume was 100 μl per mouse. The doses for the efficacy study were 2.5, 5, and 10 million (MIU)/kg for mIFN-β and 10 MIU/kg for mIFN-α2. An additional treatment group, the solution of mIFN-β was prepared in a plastic tube. Subsequent information suggests that the proteins adhere to the plastic walls of the tube. Indeed, the actual measured activity for this dose solution was below the detectable level of 0.1 MIU/kg. Thus this group was labeled with <0.1 MIU/kg.

Plaque-Forming Assay Determination of Cardiac Viral Load

The plaque-forming assay was performed as previously described (13). One piece of heart apex (~10 mg) was removed aseptically, weighed, and stored at −80°C. On the day of assay, the frozen tissue was homogenized in 200 μl of PBS and centrifuged at 12,000 rpm for 5 min. Viral titer (expressed in PFU) was determined in the supernatants by a plaque assay method on HeLa cell monolayers as previously described (12).

Real-time TaqMan RT-PCR Determination of Cardiac CVB3 Viral mRNA

Another piece of the heart apex was immediately placed into 500 μl of stabilization solution (nucleic acid purification lysis buffer, Applied Biosystems, Foster City, CA) and stored at −20°C until assayed by AppliedGeneX (Davis, CA). Real-time TaqMan RT-PCR primer sets were designed to recognize nucleotide sequences located in the CVB3 5' untranslated region (GenBank accession number, AY673831) using Primer Express software (Applied Biosystems). The fluorescent-labeled TaqMan probe (CVB3-516F) contains a 5' reporter dye FAM (6-carboxyfluorescein) and a 3' quencher dye TAMRA (6-carboxytetramethylrhodamine) with the sequence 5'-TCGTAACGGGCAACCTCTGGAGG-3'. Primer sequences were as follows: forward primer CVB3-457F, 5'-CCCTGAATGGCGGCTAATCC-3'; and reverse primer CVB3-566R, 5'-AACACGGACACCAGAATGTC-3'. A housekeeping gene, hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1), was used to correct for slight differences in the tissue mass collected for this assay.

Histopathology

The hearts were fixed in 10% neutral-buffered formalin for 24 h and then cut into 2-mm-thick systematic random cross sections (about 4 sections/heart) by IDEXX (West Sacramento, CA). All cross sections were embedded with the caudal face down. Serial sections (5 μm thick) were prepared. Slides stained with hematoxylin-eosin or trichrome were used to assess inflammatory infiltrations and myocyte injury or death. Unstained sections were analyzed using fluorescence microscopy to visualize intracellular uptake of Evans blue dye, an indicator of membrane permeability that reflects early injury in cardiomyocytes.

Slides were scored by a pathologist blinded with respect to treatment group. Inflammation, generalized myocardial fiber necrosis, and Evans blue dye fluorescence were all scored according to the following scale: 0 = absent, 0.5 = rare, 1.0 = 1–10% of myocardial area affected, 2.0 = 10–25% area affected, 3.0 = 25–50% area affected, and 4.0 = >50% area affected. Finally, all three scores (inflammation, myocyte injury, and fluorescence) were averaged to yield an integrated pathology (IP) score as an indicator of overall injury. Epidarditis was scored based on the surface area involved with 0.5 = focal, 1 = ~25%, 2 = ~50%, 3 = ~75%, and 4 = ~100%. Intraventricular thrombi were noted as present or absent.

Data Analysis and Statistics

Data are presented as means ± SE. Analysis of variance followed by the least significant difference for the repeated measurement was used for comparison among various groups. A P value of <0.05 was considered significant.

RESULTS

Therapeutic Effects of IFN-β and IFN-α2 in CVB3-Induced Myocarditis and Epidarditis in Balb/c Mice

General health and mortality. Naive Balb/c mice without inoculation of CVB3 appeared healthy. In contrast, CVB3-inoculated mice were ill with poor coat condition (ruffled and dirty fur), soft feces, lethargy, group huddling behavior, and progressive weight loss followed by death (Fig. 1).

Treatment with mIFN-β dose-dependently improved general appearance, reduced weight loss, and completely prevented death on day 7 (2.5–5 MIU/kg) and up to day 14 (10 MIU/kg).
The 7-day mortality in mice treated with <0.1 MIU/kg of 
miIFN-β was not significantly different from the vehicle group. Body weight gain was observed in the 10 MIU/kg of the 
miIFN-β-treated group, and at 14 days it was approaching 
toward that observed in the naïve group without inoculation of 
CVB3.

Mice treated with 10 MIU/kg miIFN-α2 showed little im-
provement in general clinical appearance with 1 of 10 mice 
dead on day 5. Although CVB3-induced weight loss was 
significantly reduced compared with the vehicle group, this 
reduction was only equivalent to that of the 2.5 MIU/kg dose 
of miIFN-β.

Cardiac viral load. Inoculation of CVB3 in Balb/c mice 
resulted in a large increase in cardiac viral load detected by 
both plaque-forming assay and CVB3 mRNA on day 4, which 
then spontaneously declined to 91 ± 30 PFU/mg heart tissue 
and 9.6 ± 5.5 copies of CVB3/HPRT mRNA on day 7 (Fig. 1). 
Treatment with miIFN-β dose-dependently reduced cardiac 
 viral load on day 4. Reduction in PFU, but not CVB3 mRNA, 
reached statistical significance, even at the lowest dose (<0.1 
MIU/kg). At 10 MIU/kg, cardiac viral load was reduced by 
99% as measured by plaque-forming assay and by 93% for 
CVB3 mRNA compared with the control mice treated with 
vehicle only. On day 7, there was a further reduction in cardiac 
 viral load in mice treated with miIFN-β compared with those in 
the vehicle group. On day 14, cardiac viral load was com-
pletely eliminated to nondetectable levels in mice treated with 
10 MIU/kg miIFN-β.

miIFN-α2 at 10 MIU/kg significantly reduced cardiac viral 
load to a level equivalent to that induced by 5 MIU/kg miIFN-β.

Fig. 1. The murine (m)IFN-β dose-depen-
dently and miIFN-α2 [10 million international 
units (MIU)] reduced weight loss (top), death 
(2nd panel), cardiac viral load determined by 
plaque-forming assay (3rd panel), and CVB3 
mRNA (bottom) in Balb/c mice infected with 
coxsackievirus (CVB3). Time course (left) 
and dose-response curve (right) are shown. 
PFU, plaque-forming units; HPRT, hypoxan-
thine guanine phosphoribosyl transferase.
as measured by the plaque-forming assay; however, mIFN-α2 had no effect on CVB3 mRNA.

**Myocarditis.** Histological examination of the hematoxylin and eosin- and trichrome-stained slides showed evidence of myocardial cell injury on day 4 after CVB3 administration (Fig. 2). Early injury was characterized by muscle fibers having a bright pink to red color associated with loss of myofibrillar cross striations. Inflammation was not a prominent feature at this time point, although some rare focal areas of muscle fiber phagocytosis were noted. Cases described as "inflammation 0.1" were characterized by some plump endothelial cells that could mask small monocytes in the perivascular space. The injured fibers noted on hematoxylin-eosin and trichrome were also strongly fluorescent for Evans blue dye in adjacent unstained sections. This observation served to validate the hematoxylin and eosin-based finding of acutely injured fibers in the absence of inflammatory infiltrates. The fluorescence method was able to detect early myocyte injury (e.g., on day 4) with higher sensitivity than the hematoxylin-eosin and trichrome stains. On day 7 following viral administration, myocardial fiber injury was more pronounced and an inflammatory response was present (Fig. 2). This response was characterized by some perivascular mononuclear cell infiltrates and by clusters of phagocytic cells ingesting injured fibers. Histological evaluation of the hearts showed that infection with CVB3 increased all three pathology scores (inflammation, myocyte injury, and fluorescence) from a low background of IP score of ~0.2 to 2–3 on day 7 (Fig. 3). The inflammation scores were always lower than the myofiber damage (fluorescence) scores because cell injury as assessed by Evans blue dye stain or hematoxylin and eosin stain was still ongoing on day 7.

Treatment with mIFN-β dose-dependently reduced all three pathology scores. A dose of 10 MIU/kg almost completely eliminated cardiac inflammatory infiltration and protected cardiac myocytes from damage with the average IP score on day 7 being reduced to 0.4 ± 0.1 (P < 0.001) compared with the vehicle group (Fig. 3). Histopathology scores from the low-dose mIFN-β (<0.1 MIU/kg) group were not statistically different from that of the vehicle-treated group. Treatment with 10 MIU/kg mIFN-α2 also reduced the IP score of myocarditis but at a level equivalent to that seen with 5 MIU/kg mIFN-β (Fig. 3).

**Epicarditis.** Hematoxylin and eosin- and trichrome-stained slides showed that naïve Balb/c mice without infection of CVB3 occasionally exhibited evidence of acute and/or chronic epicarditis. The incidence of epicarditis appeared to increase with CVB3 infection (Fig. 4). Treatment with mIFN-α2, but not mIFN-β, significantly reduced the epicarditis score on day 7.

**Cardiac intraventricular thrombosis.** Many of the mice had apparent intraventricular thrombi present, although an exact point of attachment to the endothelium could not be observed in most cases. The larger lesions were characterized by organized proteinaceous material with evidence of early neovascularization and collagen deposition. These thrombi appeared to be related to the severity of myocarditis, and their incidence was significantly reduced by both mIFN-β and mIFN-α2 (Fig. 4).

**DISCUSSION**

Consistent with the previous reports, the present data also demonstrated that, in the early stages of CVB3 infection, viral replication was a dominant pathological process, as evidenced by a dramatic elevation of CVB3 genome and plaque-forming...
capability with relatively low levels of inflammatory infiltration and tissue injury in the heart on day 4. This was followed by a spontaneous reduction of viral load and a further development of cardiac tissue inflammation and injury, which could compromise cardiac function as evidenced by deteriorated general health, weight loss, and death on day 7 to 8. The spontaneous decay of viral load could result from an activation of the innate cellular defense mechanism including the IFN system (8, 25, 28, 29). This is supported by the evidence that anti-IFN antibody caused a profound worsening of the pathology and an increase in the mortality rate of infected animals (4). On the other hand, cardiac tissue injury could result from the second phase of the innate immune response activation-induced inflammatory infiltration (23). Although the short time course of the acute phase in the current model differs from chronic viral cardiomyopathy observed in humans, the viral activity and replication in the heart, the virus-induced pathogenesis, and the subsequent cardiac tissue injury are similar. Thus this mouse model of CVB3-induced myocarditis adequately reflects the beneficial antiviral properties of IFNs in the target tissue, which are consistent with the suggested mechanism of action for IFNs in treating chronic human viral cardiomyopathy (14).

Treatment with mIFN-β dose-dependently reduced both viral genome and infectivity to a low level on day 4 and completely abolished them on day 7. This result is consistent with other studies which report that antiviral actions of IFN likely played an important role in the therapeutic efficacy in
CVB3-induced myocarditis (16). The central role of IFN in host defense against invasive viruses has been well established (25). Both in vivo (19) and in vitro (10) studies demonstrated the efficacy of type I IFNs in inhibiting CVB3 replication. Binding of IFN to specific cell surface receptors has been shown to result in the activation of multiple intracellular signaling cascades, such as the JAK-STAT pathway, leading to the synthesis of proteins that mediate antiviral responses (3). Although the current data demonstrate that an elimination of cardiac myocyte viral load by mIFN-α2 may contribute to the protection of mice from a lethal dose of virus infection, we cannot exclude the possibility that a general reduction of viral infection in other tissues, such as the liver and pancreas with a subsequent decrease in propagation of virus to the heart, could also contribute to the overall protective effects. This was supported by the observation that pancreatic expression of IFN-γ protected mice from lethal CVB3 infection and subsequent myocarditis, indicating that reducing the level of viremia early during infection may contribute to reducing the incidence of virus-mediated heart damage and autoimmunity (11). Thus whether systemic viremia or infections in other organs contribute to the effects of IFNs in the current experimental condition remains to be investigated.

In addition to its antiviral action, IFN also has immunomodulatory functions by modulating both B- and T-lymphocyte responses (3). For example, type I IFN influences T-cell development and function by regulating the expression of key cytokines, namely, IL-12, IL-15, and IFN-γ, thereby modulating lymphocyte trafficking and inflammatory responses (2) and by promoting Th1 differentiation (20). IFN-α directly promotes the survival of activated T cells by preventing their death, not by stimulating their division (17). IFN selectively inhibits IL-7 inducible growth of early T and B cells (15). In the clinic, Betaseron (IFN-β1b) is widely used for the treatment of multiple sclerosis, an autoimmune disease. The mIFN used in the present study has also been shown to be efficacious in a murine model of experimental allergic encephalomyelitis (26). Many have indicated that the second phase of reactive inflammatory infiltration involved in the extended cardiac tissue damage in the current murine model of CVB3-induced myocarditis could be associated with an autoimmune process. Thus, in addition to the antiviral action documented in the current study, modulation of immune reactive inflammation could also contribute to therapeutic effects of IFN, especially in the later stages of myocarditis by either increasing antiviral-mediated immune mechanisms or decreasing potentially detrimental au-
toimmune inflammation. This view is supported both experimentally, that type I IFN-β gene therapy suppresses cardiac CD8 during autoimmune myocarditis (1), and clinically, that IFN-β eliminated viral genomes and improved left ventricular function in patients with left ventricular dysfunction and low levels of myocardial enteroviral or adenoviral persistence (14). However, the rapidly progressive nature of CVB3 infection in this model would make it challenging to determine any effects of IFN treatment that begin after the animal shows signs of illness. It has to be pointed out that to maximize the therapeutic effect in this study, the first dose of IFNs was given before CVB3 inoculation. In a separate follow-up study, we further compared the pre- versus posttreatment of mIFN-β and, indeed, observed that the posttreatment reduced the therapeutic effects. Thus caution should be taken to translate the relevance of the pretreatment approach in the present study to clinical therapeutics.

Another interesting observation in the current murine model of CVB3-induced myocarditis is the thrombi attachment to the endothelium of the cardiac ventricular cavity. The presence of thrombi could be associated with inflammation-enhanced thrombogenicity, as well as impairment of the endocardial endothelial function. Other investigators also observed mural thrombus in viral myocarditis (27). In the present study, treatment with mIFN dose-dependently reduced the incidence of such intraventricular thrombosis. Although there is no direct evidence that mIFN affects coagulation and homeostasis, the indirect effects on reducing inflammation and protecting cardiac ventricular endothelial integrity may contribute to the reduction of thrombus formation.

In the present study, some naive Balb/c mice, without deliberate exposure to CVB3, exhibited histological evidence of acute and/or chronic epicarditis. Other investigators also observed such spontaneous epicarditis in naive Balb/c mice (5). Although the exact pathogenesis for the spontaneous epicarditis is not known, the present data clearly showed that CVB3 infection significantly increased the incidence and the degree of epicarditis. Interestingly, only mIFN-α2, but not mIFN-β, attenuated the CVB3-induced epicarditis to the background level, although both mIFNs had a similar efficacy on myocarditis. In addition, the present data showed that mIFN-α2 significantly reduced the formation of infectious virus but had little or no effect on the replication of the viral genome at the early stages of viral infection, whereas mIFN-β eliminated both. Matsumori et al. (18) also reported that IFN-α reduced viral infectivity and attenuated CVB3 myocarditis; however, they did not observe CVB3 RNA. Our observations suggest that there are differences in the mechanism of actions for the two IFN isoforms used in this study. Both mIFN-β and mIFN-α bind the same receptor and elicit similar biological responses; however, there are reported differences in their observed biological activities. For example, studies suggest that IFN-β engagement of the receptors and the degree of the receptor occupancies lead to a more stable interaction than that with IFN-α, likely due to the conformational differences that influence receptor-ligand interactions (6, 9). Indeed, in human vascular endothelial cells, the transcriptional activation of interferon-stimulated genes is IFN dose sensitive, with a subset of genes being activated at lower doses of IFN-β compared with IFN-α2 (7). It has been reported that distinct receptor interactions may be the basis for IFN-β selectively inducing the expression of the α-chemokine β-R1 gene (21). However, our understanding of the contributions of IFN to regulating immune responses by different immune effector cells is limited. In addition, questions remain as to the exact nature of IFN responses in the heart. For instance, it is not known whether the heart myocyte responds in a similar manner to IFN as do cells of hematopoietic origin. The challenge remains to continue to assign particular responses to specific IFN-inducible signaling cascades in the context of a coordinated biological outcome in the heart and in response to conditions leading to myocarditis.

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