Cardioselective Infection With Coxsackievirus B3 Requires Intact Type I Interferon Signaling

Implications for Mortality and Early Viral Replication

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Background—Interferons (IFNs) play an important role in antiviral defense and have therapeutic potential in coxsackievirus heart disease. However, little is known about the relative contributions of type I and type II IFN signaling in coxsackievirus B3 (CVB3) infection or their role in the cardioselective nature of CVB3 infection.

Methods and Results—Wild-type mice and mice deficient for either the type I or the type II IFN receptor (IFNR) were infected with CVB3. Infection of the type I IFNR–deficient mice with >10³ plaque-forming units (pfu) of CVB3 resulted in 100% mortality within 2 to 4 days after infection. Death was rare in wild-type and type II IFNR–deficient mice after inoculation with as much as 10⁶ pfu of CVB3. Surprisingly, the early mortality in the type I IFNR–deficient mice was not accompanied by higher virus titers in the heart. Unexpectedly, a dramatic increase of viral RNA in the liver was found to correlate with early mortality in type I IFNR–deficient mice.

Conclusions—Type I but not type II IFN signaling is essential for the prevention of early death due to CVB3 infection. Interestingly, neither type I or type II IFN signaling has a dramatic effect on early viral replication in the heart. However, lethal viral replication in the liver is controlled by type I IFNs. These results demonstrate that the IFN system is capable of modulating both viral pathogenicity and tissue tropism. (Circulation. 2001;103:756-761.)

Key Words: cardiomyopathy myocarditis immune system infection

Interferon (IFN) is the oldest known cytokine, discovered as a product of influenza virus–infected cells that conferred resistance to infection with the same or other viruses.¹ IFNs of the α/β subtype are referred to as type I IFNs and include IFN-α, IFN-β, IFN-α, and IFN-τ. IFN-γ is the only type II IFN. IFNs exert their effect by binding to specific receptors in the cell membrane that subsequently activate the intracellular signaling necessary for activation and expression of IFN-responsive genes. All type I IFNs bind to the type I IFN receptors (IFNRS), whereas the structurally unrelated IFN-γ binds to type II IFNRS. Genetic disruption of the type I or type II IFNRS completely and specifically abolishes the biological effect of type I or type II IFNs, respectively.²,³

Experiments in IFNR knockout mice have previously shown that the 2 IFNR subtypes exert their antiviral activities through distinct, partially nonredundant pathways.² For example, infection of mice that lacked the type I IFNR with vesicular stomatitis virus or the Semliki forest virus results in a markedly increased susceptibility to viral infection. However, disruption of type II IFN does not adversely affect the course of the infection by these viruses.² Furthermore, mice deficient in either type I or type II IFNR are more susceptible to infection with vaccinia virus and lymphocytic choriomeningitis virus.² Before the present study, little was known about how complete disruption of the IFN signaling pathways would affect coxsackievirus replication in the intact animal or whether there are tissue-selective antiviral effects of IFN.

Enteroviruses such as coxsackieviruses of group B (CVBs) induce myocarditis in mice and humans.⁴–⁶ Myocarditis variants of CVB3 cause a high virus load in the heart during acute infection compared with other tissues, and it is thought that this contributes to the capability of this virus to cause acute and chronic myocarditis.⁷ The mechanisms that mediate the cardiotropic nature of CVB3 infection are not known. Recently, it has been shown that CVBs bind to the coxsackievirus and adenovirus receptor that is expressed at high levels in the hearts, livers, lungs, and kidneys of mice.⁸ Although tissue-restricted expression of viral receptors is one of the mechanisms that may contribute to viral tropism, a variety of other virus-host interactions, including antiviral mechanisms of the IFN system, may play an important role in determining the efficiency of viral replication in a given tissue.

CVB is a member of the enterovirus genus of the picornavirus family. The 7.4-kb positive-strand RNA genome is encapsidated by 4 structural proteins.⁹,¹⁰ On entry into the
host cell, the single positive-strand RNA is released from the capsid, and viral protein synthesis is initiated by host translational mechanisms. One of the viral proteins is an RNA-dependent RNA polymerase (3Dpol) that allows replication of the viral RNA through a negative-strand RNA intermediate. Positive-strand RNAs are encapsidated, and progeny viruses are usually released by cell lysis during acute infection.

Previous experiments with cultured cells have suggested that both type I and type II IFNs can inhibit coxsackievirus replication.\textsuperscript{11,12} Administration of IFN-\(\alpha/\beta\) can ameliorate the effect of coxsackievirus myocarditis in mice\textsuperscript{13–15} and could also be shown that IFN-\(\gamma\) protects mice from lethal CVB4-induced pancreatitis.\textsuperscript{16} IFN therapy has also been administered for enteroviral infection in patients with cardiomyopathy in nonrandomized trials.\textsuperscript{17}

Because CVB3 normally replicates to high levels in the heart and because its replication can be inhibited by either type I or II IFNs, we hypothesized that complete disruption of either IFNR signaling mechanism would markedly affect coxsackievirus replication in the heart. In the present study, we demonstrate that only mice lacking the type I IFNR exhibit increased susceptibility to infection with CVB3. Furthermore, increased early mortality was found to be associated with a striking increase in viral RNA in the liver without significant changes in the amount or viral RNA in the heart.

\textbf{Methods}

\textbf{Virus}

The coxsackievirus used in the present study was derived exclusively from the infectious cDNA copy of the Woodruff variant of CVB3.\textsuperscript{18} Virus preparation and organ titers were quantified by plaque-forming assays as described previously.\textsuperscript{18,19} For animal infection, the virus was diluted in PBS to a final volume of 0.2 mL and injected intraperitoneally.

\textbf{Origin of IFNR Type I and Type II Knockout Mice and Confirmation of Gene Knockout}

Gene-targeted mice were obtained with the permission of Dr Michel Aquet from Genentech Inc (San Francisco, Calif) through B&K Universal Ltd, Aldbrough, Hull, UK.\textsuperscript{2,3} The murine genotype was confirmed by polymerase chain reaction with the appropriate primers on all gene-targeted breeder mice.

\textbf{In Situ Hybridization}

Tissues were fixed for 24 hours in phosphate-buffered 4\% paraformaldehyde and embedded in paraffin. Single-stranded \(^{35}\)S-labeled RNA probes for strand-specific detection of viral positive- or negative-strand RNA were synthesized from the dual-promoter plasmid pCVB3-R1 by using either T7 or SP6 RNA polymerase.\textsuperscript{20} Control RNA probes were obtained from the vector pSPT18. Pretreatment, hybridization, and washing conditions of dewaxed paraffin tissue sections (4 \(\mu\)m thickness) were performed as described previously.\textsuperscript{4,21} Slide preparations were subjected to autoradiography, exposed for 3 weeks at 4°C, and counterstained with hematoxylin/eosin.\textsuperscript{4}

\textbf{Quantification of In Situ Hybridization}

For quantitative evaluation of hybridized tissue sections, in situ autoradiographs (\(n=5\) mice per strain) were processed by an interactive image-analyzing system, with application of Optimas software (Stemmer). Slide preparations were analyzed by using a black and white video camera mounted on a microscope at a primary magnification of \(\times10\) and \(\times40\), respectively. Video signals were digitized, resulting in images of \(512\times512\) pixels with a gray value range of 0 to 255 for each pixel. By applying a chain-code algorithm, the autoradiographic signals were segmented from the background.\textsuperscript{4} Thereafter, areas of infected cells were automatically analyzed within 50 visual fields (each 100,000 \(\mu\)m\(^2\)) per tissue section; the visual fields were selected by systematic random sampling. Area fractions of infected tissues were expressed as percentages.

To compare the amounts of positive- and negative-strand CVB3 RNA in single infected cells, the optical densities of autoradiographic signals were measured within the same cells as visualized on consecutive tissue sections after strand-specific in situ hybridization. Thirty randomly selected infected cells from 5 hearts and livers of each mouse strain were quantified at a primary magnification of \(\times40\). Results were expressed as ratios of optical densities, representing negative- and positive-strand RNA, respectively.

\textbf{Results}

\textbf{Susceptibility to CVB3 Infection in Mice Lacking Type I or Type II IFNRs}

To determine whether the absence of IFN signaling changed the mortality associated with CVB3 infection, we infected wild-type 129SvJ mice and mice of the same genetic background that lacked either type I or type II IFNRs. 129SvJ mice, often used to generate genetic knockout mice, are from a BL6 background, in which mortality and inflammatory response after viral infection are generally mild.\textsuperscript{22} This was reflected in our experiments by a lack of death in CVB3-infected male and female wild-type mice, even after inoculation with as much as \(10^8\) plaque-forming units (pfu) of CVB3 (Figure 1). Similarly, in the type II IFNR knockout mice, there was minimal mortality up to day 14 after infection. However, there was very high mortality in mice that lack the type I IFNR. One hundred percent of type I IFNR null mice injected with \(\geq 10^4\) pfu CVB3 and 80\% of type I IFNR null mice injected with \(10^3\) pfu CVB3 died within 4 days after infection (Figure 1). The cause of death in a single type II IFNR–deficient mouse injected with \(10^4\) pfu is unknown.

\textbf{Increase of Viral RNA in Liver of Type I IFNR–Deficient Mice}

In spite of the low susceptibility of 129SvJ mice to CVB3 infection, we found a pattern of organ infection similar to that seen in susceptible mice of the BALB/c strain. At day 7 after infection, virus titer in the heart was 3 log units higher than that in the liver (Figure 2). Low levels of virus-infected cells were also detected in the spleen, kidney, and brain of these mice.\textsuperscript{23} To determine the level and pattern of CVB3 infection in different organs before the early mortality in the type I IFNR–deficient mice, we performed in situ hybridization experiments for the detection of both positive- and negative-strand coxsackievirus RNA at day 3 after infection. Area fractions of infection (expressed as percentages) were measured by quantifying autoradiographs after radioactive RNA/RNA in situ hybridization. As expected at day 3 after infection, only a minor part of the heart muscle cells was found to be infected in wild-type mice. As shown in Figures 3 and 4A, the area fractions of infection in the hearts (0.6\%) of these animals were found to exceed those in the livers.
slightly (0.2%). Surprisingly, compared with wild-type mice, type I IFNR–deficient mice revealed a dramatic increase in hybridization-positive areas in the livers of up to 75% without a significant change of the area fractions of infection in the heart muscles (0.6%) 3 days after infection (Figures 3 and 4A). This was associated with increased viral titers in the livers of type I IFNR–deficient compared with control mice (5.14 ± 6 2.38 ± 3 10^4 versus 3.9 ± 6 2.64 ± 3 10^2, respectively; data represent mean ± SE; n = 5 or 4; P = 0.052 by Wilcoxon analysis). There was a mild increase in the area fractions of infection in the hearts (1.6%) and livers (3.9%) in the type II IFNR–deficient mice compared with wild-type mice, but there was no detectable increase in viral titers. However, the changes in RNA levels were small compared with the increase of viral RNA in the livers of the type I IFNR–deficient mice (Figures 3 and 4A). Patterns of infection in other organs, such as the pancreas, spleen and lymph nodes, kidney, and lung, did not significantly differ in the 3 mouse strains investigated in the present study and were comparable to those observed in SWR/J mice.23

Acute enteroviral replication is characterized by asymmetric replication with >100 times more positive-strand RNA than negative-strand intermediate RNA.20 This observation was confirmed in both the heart (Figure 3A and 3D) and the liver (Figure 3G and 3J) of wild-type mice. However, as assessed by measurement of optical densities of autoradiographic signals representing negative- and positive-strand viral RNA in the livers of the type I IFNR–deficient mice, there was a reversal in the normal ratio of negative- to positive-strand viral RNA (Figures 3H, 3K, and 4B). However, the ratio of negative- to positive-strand RNA in the heart of type I IFNR–deficient mice did not change (Figures 3B, 3E, and 4B). The ratio of negative- to positive-strand RNA in the hearts and livers of the type II IFNR–deficient mice was not significantly different from that observed for the wild-type mice (Figures 3C, 3F, 3I, 3L, and 4B).

The large amount of viral RNA in the livers of type I IFNR–deficient mice was associated with a marked increase in alanine amino transferase, a serum marker for hepatic injury. There was not a statistically significant increase in total creatine kinase, a measure of muscle or brain injury, or lipase, a measure of pancreatic injury (Figure 5).

**Discussion**

The purpose of the present study was to elucidate the importance of type I and type II IFN signaling in acute coxsackievirus infection. Both type I and II IFNs can inhibit coxsackievirus replication in vitro.12 Although type II IFNs can have a direct effect on viral replication, it is thought that the major in vivo antiviral activities of the type II IFN, IFN-γ, are secondary to their role in the activation of various cell lineages of the immune system.24 Type I IFN signaling directly affects picornavirus replication.25 Type I IFNs are able to induce genes such as RNase L and 2-5(A) synthetase,
which mediate antiviral activity through paracrine and endocrine mechanisms.2,26,27

Our findings demonstrate that type I but not type II IFNR signaling is required to prevent early mortality from CVB3 infection in 129SvJ mice. In situ hybridization experiments and elevated levels of serum markers reflecting tissue injury reveal that early mortality in type I IFNR-deficient mice was associated with a marked increase in hepatic viral replication in 129SvJ mice. In situ hybridization experiments and elevated levels of serum markers reflecting tissue injury reveal that early mortality in type I IFNR-deficient mice was associated with a marked increase in hepatic viral replication.
with virus-mediated liver cell necrosis. Interestingly, there was no significant increase of viral RNA in the heart of these type I IFNR–deficient mice. These findings indicate that endogenous levels of the type I IFNs play an essential role in preventing early viral replication in the liver but that they have little effect on early viral replication in the heart. Furthermore, our results suggest that endogenous levels of type II IFNR signaling are not essential in the control of early viral replication but that type II IFNR signaling has a mild inhibitory effect on viral replication in the heart and liver. This hypothesis is further supported by our observations in IFN type II–deficient DBA/1 mice, in which we showed that mortality and the patterns of viral replication in the heart and liver are similar to those observed in the immunocompetent counterparts of DBA/1 mice at any time in the course of the disease (authors’ unpublished data, 2000). Interestingly, overexpression of IFN-γ in the pancreas has been shown to protect susceptible mice from lethal CVB3 infection.28 In addition, infection of IFN-γ–deficient mice with pancreatic CVB4, which is closely related to CVB3, results in the death of the animals within 3 to 4 days after infection.16 Although the mechanisms by which IFN-γ expression in pancreatic β cells prevents mortality after CVB3 or CVB4 infection are not yet defined, the phenotype is clearly distinct from that observed in the present study, in which the absence of IFN-γ signaling through the type II IFNR does not affect mortality after CVB3 infection.

There is considerable evidence that viral receptors are important determinants of viral tropism. The coxsackievirus and adenovirus receptor is expressed at high levels in the murine heart and liver.9 This permits infection of both myocytes and hepatocytes. However, in the normal mouse, viral replication is significantly greater in the heart than in the liver, suggesting that other mechanisms may contribute to the cardioselective nature of coxsackievirus infection. The results of the present study demonstrate that the lower viral titers in the liver of infected wild-type mice are not due to an inability of the virus to infect hepatocytes but rather to the inhibition of early viral replication via hepatic type I IFN signaling.

Conversely, coxsackievirus infects cardiac myocytes, but early viral replication is not significantly affected by type I IFN mechanisms. Because of the early mortality in the type I IFNR knockout mice, it is not possible to evaluate the effects of type I IFN signaling at later stages of viral infection. From a clinical perspective, the capacity of CVB3 to replicate in myocytes has also been demonstrated in patients in whom CVB3 has been identified as an important pathogenic agent of severe myocarditis5 and in early infancy, a stage at which it can cause fatal hepatitis.29

One of the surprising results from these experiments was the very high amount of the viral negative-strand RNA intermediates in the liver of mice that lacked type I IFNRs. A possible explanation for this observation is that type I IFNR stimulation induces the expression of 2-5(A) synthetase, which is activated in the presence of double-stranded RNA. This leads to activation of a latent ribonuclease, RNase L.9 There is considerable evidence that the 2-5(A) synthetase/RNase L pathway is the major pathway used by type I IFNs to inhibit the replication of picornaviruses.9 The large amount of both positive- and negative-strand RNA in hepatocytes of type I IFNR–deficient mice could be explained by a lack of activated RNase L in the infected cells, preventing degradation of viral RNA. Double-stranded RNA has been reported with low levels of viral replication, which can occur with viral persistence.30 It is less clear why the absence of type I IFN signaling leads to such a dramatic increase in negative-strand viral RNA, as assessed by in situ hybridization, without a significant change in the total amount of viral negative-strand RNA in the heart. These results were clearly distinct from those anticipated by our initial hypothesis, in which we expected a conserved pattern of viral positive- and negative-strand RNA in different tissues.

In summary, we have shown that the presence of type I IFN signaling is required to prevent high-level viral replication in the liver but that it has no significant effect on early viral replication in the heart. In contrast, the absence of type II IFN signaling does not have a significant effect on mortality and results in only a mild increase of the viral titers in heart and liver. These results demonstrate that in addition to viral receptor–mediated viral tropism, tissue-restricted antiviral mechanisms act as important determinants of the cardioselective nature of coxsackievirus infection. Whether type I or type II IFNs may be beneficial for the treatment of coxsackievirus disease in humans remains to be seen.

Acknowledgments

This study was supported in part by grant We-1811/1–1 from the Deutsche Forschungsgemeinschaft to Dr Wessely, by National Institutes of Health grant R01 HL-57365 to Dr Knowlton, and by grants from the Federal Ministry of Education, Science Research and Technology (Fö. 01KS9602) and the Interdisciplinary Center of Clinical Research (IZKF) Tübingen to Drs Klingel and Kandolf. We thank Martina Sauter and Carmen Ruoff for excellent technical assistance with the in situ hybridizations and David Rapaport (Department of Surgery, University of California, San Diego) for suggestions regarding microscopic images and use of his imaging facility. Dr Knowlton is an Established Investigator of the American Heart Association. This study represents equal contributions from the laboratories of Drs Kandolf and Knowlton.
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_Circulation_. 2001;103:756-761
doi: 10.1161/01.CIR.103.5.756
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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