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# The Gamma Interferon (IFN-γ) Mimetic Peptide IFN-γ(95-133) Prevents Encephalomyocarditis Virus Infection both in Tissue Culture and in Mice

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**We have demonstrated previously that the C-terminal gamma interferon (IFN-γ) mimetic peptide consisting of residues 95 to 133 [IFN-γ(95-133)], which contains the crucial IFN-γ nuclear localization sequence (NLS), has antiviral activity in tissue culture. Here we evaluate the efficacy of this peptide and its derivatives first in vitro and then in an animal model of lethal viral infection with the encephalomyocarditis (EMC) virus. Deletion of the NLS region from the IFN-γ mimetic peptide IFN-γ(95-133) resulted in loss of antiviral activity. However, the NLS region does not have antiviral activity in itself. Replacing the NLS region of IFN-γ(95-133) with the NLS region of the simian virus 40 large T antigen retains the antiviral activity in tissue culture. IFN-γ(95-133) prevented EMC virus-induced lethality in mice in a dose-dependent manner compared to controls. Mice treated with IFN-γ(95-133) had no or low EMC virus titers in their internal organs, whereas control mice had consistently high viral titers, especially in the heart tissues. Injection of B8R protein, which is encoded by poxviruses as a defense mechanism to neutralize host IFN-γ, did not inhibit IFN-γ(95-133) protection against a lethal dose of EMC virus, whereas mice treated with rat IFN-γ were not protected. The data presented here show that the IFN-γ mimetic peptide IFN-γ(95-133) prevents EMC virus infection in vivo and in vitro and may have potential against other lethal viruses, such as the smallpox virus, which encodes the B8R protein.**

Gamma interferon (IFN-γ) plays a critical role in normal immune functions. Its many biological effects include, but are not limited to, the induction of a number of antiviral proteins, up-regulation of major histocompatibility complex antigen expression, and involvement in B-cell maturation and immunoglobulin isotype switching (4, 6, 7). Among viral infections in which a protective role for IFN-γ is clearly demonstrated are infection with hepatitis B virus, herpes simplex virus, lymphocytic choriomeningitis virus, and some poxviruses (1, 2, 8). These activities are induced as the IFN-γ molecules interact with a single class of cell surface receptor consisting of a ligand-binding subunit α chain and an associated cofactor protein necessary for efficient signal transduction (15, 22).

We have shown previously that murine IFN-γ binds to a soluble form of its receptor via both the N terminus and the C terminus (21, 22). The IFN-γ N terminus binds to the extracellular domain of the receptor, while the C terminus of murine IFN-γ, consisting of residues 95 to 133 [IFN-γ(95-133)], binds instead to the membrane-proximal region of the cytoplasmic domain of the receptor (17, 19, 21, 22). Contained within this C-terminal region of IFN-γ is a required polycationic sequence, $^{126}$RKRKRSK$^{132}$, which is similar to the prototypical nuclear localization sequence (NLS) of simian virus 40 (SV40) T antigen (15). We have shown previously that the C-terminal IFN-γ peptide IFN-γ(95-133) possesses IFN-γ agonist activity, such as induction of major histocompatibility complex class II molecules (2, 21, 22) without toxicity, on macrophages. Furthermore, this mimetic peptide possesses antiviral activity similar to that of IFN-γ in tissue culture against vaccinia virus, vesicular stomatitis virus, and encephalomyocarditis (EMC) virus (1). Vaccinia virus, a member of the poxvirus family, encodes the B8R protein, which is an important virulence factor of poxviruses in that it neutralizes host innate antiviral defense mechanisms by binding to IFN-γ (13, 20). The therapeutic activity of IFN-γ(95-133) in the presence of B8R protein has not been determined in vivo in any viral infection models thus far. EMC virus is a rodent picornavirus belonging to the genus *Cardiovirus*, and it has an extremely wide range of hosts. It infects pigs, rodents, cattle, elephants, raccoons, marsupials, and primates such as baboons, monkeys, chimpanzees, and humans (5, 11, 25). Instances of human infection with EMC virus have manifested as generalized febrile illness, but the virus has also been isolated from patients with more-severe illnesses, such as encephalitis, meningitis, and cardiomyopathy (9, 11). In mice, EMC virus infection is lethal (10, 14, 25). In this study we characterized the antiviral effects of the IFN-γ mimetic peptide IFN-γ(95-133) with and without the NLS region, as well as a peptide with a substitution of the SV40 T antigen NLS [IFN-γ(95-133)SV40], and evaluated the therapeutic activity of the IFN-γ mimetic peptide in vivo in the presence and absence of the B8R protein in a model of lethal EMC virus infection in mice.

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## MATERIALS AND METHODS

**Cell culture, virus, B8R protein, and interferons.** Murine L929 fibroblasts were from the American Type Culture Collection (Manassas, VA) and maintained in Eagle's minimal essential medium (JHR Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium) at 37°C in a 5% $CO_2$ atmosphere incubator. EMC virus was obtained from the American Type Culture Collection and stored at −70°C until use. The B8R protein was a kind gift from Tilahun Yilma (University of California, Davis). Rat and mouse IFN-γ (both at $10^7$ U/mg) were purchased from PBL Biomedical Laboratories (Piscataway, NJ) and kept at −70°C until use.

**Peptide synthesis.** All peptides [IFN-γ(95-133), IFN-γ(95-125), IFNGR(253-287) (the IFN-γ receptor peptide consisting of residues 253 to 287), IFN-γ(126-133), and the SV40 T antigen NLS] were synthesized with an Applied Biosystems 9050 automated peptide synthesizer (Foster City, CA) using conventional fluorenylmethyloxycarbonyl chemistry as described previously (17, 18, 21). IFN-γ(95-133) and IFN-γ(126-133) peptides were synthesized without the cysteine residue at the C-terminal end (residue no. 133). The SV40 and the IFN-γ(95-133)SV40 peptides were synthesized with the addition of a cysteine at the N terminus of the sequence. The addition of a lipophilic group (palmitoyl-lysine) to the N termini of the synthetic peptides was performed as the last step by using a semiautomated protocol. Peptides were characterized by mass spectrometry and purified by high-performance liquid chromatography. Peptides were dissolved in deionized water and used for experimentation. All peptide solutions were negative for endotoxins as determined by a *Limulus* amebocyte lysate test (E-toxate kit; Sigma, St. Louis, MO).

**Antiviral assays.** Antiviral assays were performed by using a cytopathic effect reduction assay with EMC virus. Murine L929 cells ($6 \times 10^4$ cell/well) were plated in a 96-well plate and grown overnight to confluence for optimal growth. Various concentrations of IFN-γ, IFN-γ(95-133) and derivatives, and control peptides were incubated for various times (7 to 24 h) at 37°C. In other experiments, B8R protein (33 μg/ml) was preincubated for 2 h with rat IFN-γ and IFN-γ mimetic and control peptides. EMC virus (200 PFU/well) was added to the plate and incubated for 1 h, after which plates were washed and media added. Cells were incubated for at least 24 h and then stained with 0.1% crystal violet. Unbound crystal violet was aspirated, and the plates were thoroughly rinsed with deionized water, blotted, and allowed to air dry. Plates were then scanned using an Astra 2100U flatbed computer scanner (UMAX Technologies, Dallas, TX) and analyzed using Image J 1.29 software (National Institutes of Health, Bethesda, MD) to assess cell survival. Percentages of cell survival were determined by comparing survival for the experimental treatment groups with that for the virus-only control group.

**Virus yield reduction and plaque assay.** Murine L929 cells seeded to confluence in a 25-$cm^2$ flask were pretreated with 2,500 U/ml of mouse IFN-γ, 20 μM of IFN-γ(95-133), or 20 μM of the IFNGR(253-287) control peptide for 24 h, after which the cells were challenged with EMC virus at 100 PFU/ml for 1 h. The flasks were washed and incubated with Eagle's minimal essential medium containing 2% fetal bovine serum and incubated for 24 h (37°C, 5% $CO_2$). EMC virus produced was harvested via freezing and thawing three times in liquid nitrogen, and supernatants were titrated by a standard viral plaque assay using L929 cells, as previously described (21). Plaques for IFN-γ and IFN-γ(95-133) mimetic and IFNGR(253-287) control peptides were counted at a 1/200,000 dilution, whereas medium-only treatment was counted at a 1/2,000,000 dilution.

**In vivo studies of mice.** One-year-old mice (C57BL/6) were purchased from Jackson Laboratories and cared for at the Animal Resource Center of the University of Florida (Gainesville, FL). The Institutional Animal Care and Use Committee (IACUC) of the University of Florida approved all protocols prior to any study initiation. C57BL/6 mice were pretreated by intraperitoneal injection using a tuberculin syringe for 3 or 6 days with rat or mouse IFN-γ and IFN-γ(95-133) and control peptides at various concentrations (100 to 200 μg/mouse) every day. In some studies, B8R protein (250 μg/ml, diluted from crude stock preparation) was preincubated with the rat IFN-γ (200 U/mouse) and peptide (100 μg/mouse) injection cocktails prior to intraperitoneal administration. On the last day of treatment, mice were challenged with 50 PFU of EMC virus. In other studies, mice were injected with supernatants taken from murine L929 cells pretreated with IFN-γ and IFN-γ(95-133) mimetic and control peptides and infected with EMC virus. The numbers of surviving mice were recorded starting on the day of EMC virus challenge (day 0) and are presented as percent survival. Ten mice per treatment group were used in all mouse studies.

**Detection of EMC virus in mouse tissues.** For detection of virus in organs of infected C57BL/6 mice, heart, spleen, and liver tissues were extracted 6 days post-EMC virus injection from variously treated groups and homogenized into single-cell suspensions of 1-ml volumes. Cells were then lysed by freeze-thawing three times in liquid nitrogen. Samples were centrifuged at 2,000 rpm and supernatants collected. Supernatants were then diluted and incubated with murine L929 cells that were plated to confluence in a 96-well plate. After 24 to 48 h of incubation, plates were stained with 0.1% crystal violet and cell survival was assessed using Image J 1.29 software from NIH (Bethesda, MD) as described above in "Antiviral assays."

TABLE 1. Sequences of murine IFN-γ(95-133), derivatives, and control peptides[a]

| Peptide | Sequence |
|---|---|
| IFN-γ(95-133) | AKFEVNNPQVQRQAFNELIRVVHQLLPESSL**RKKRKRSR**C |
| IFN-γ(95-125) | AKFEVNNPQVQRQAFNELIRVVHQLLPESSL |
| IFN-γ(95-133)SV40 | AKFEVNNPQVQRQAFNELIRVVHQLLPESSL**PKKKRKV** |
| SV40 T antigen | PKKKRKV |
| IFN-γ(126-133) | RKRKRSRC |
| IFNGR(253-287) | TKKNSFKRKSIMLPKSLLSVVKSATLETKPESKYS |

[a] Peptides were synthesized as described in Materials and Methods. The NLS (RKRKRSR) of IFN-γ(95-133) and the NLS (PKKKRKV) of IFN-γ(95-133)SV40 are highlighted in boldface type.

## RESULTS

**The IFN-γ mimetic peptide IFN-γ(95-133) possesses antiviral activity against EMC virus infection in tissue culture.** The antiviral activity of IFN-γ(95-133) was determined on murine L929 cells by use of EMC virus. Table 1 lists the sequences of IFN-γ(95-133) and control peptides, including their derivatives, used in this study. Mouse IFN-γ (300 U/ml to 11 U/ml) and various lipophilic (palmitoyl-lysine) IFN-γ mimetic and control peptides (100 μM to 3.7 μM) were incubated with murine L929 cells grown to confluence. Addition of a lipophilic group (palmitoyl-lysine) to these peptides enabled cytoplasmic delivery into cells (23, 24). After 7 h of incubation, EMC virus (200 PFU/well) was added for 1 h of incubation, after which plates were washed with media and reincubated in media for at least 28 h for establishment of EMC virus cytopathic effect in EMC virus control wells. As shown in Fig. 1, mouse IFN-γ had antiviral activity at all concentrations. The IFN-γ(95-133) mimetic peptide had antiviral activity at the 100 μM and 33 μM concentrations. Deletion of the NLS region, as per peptide IFN-γ(95-125), resulted in a loss of antiviral activity, even at the highest concentration used (100 μM). Furthermore, the NLS region of IFN-γ, IFN-γ(126-133), did not have any antiviral activity. Substitution of the IFN-γ NLS with the SV40 large T antigen NLS, IFN-γ(95-133)SV40, resulted in antiviral activity at 100 μM and 33 μM concentrations similar to that of the IFN-γ(95-133) mimetic peptide. Thus, protection against EMC virus required an NLS as well as the IFN-γ C-terminal amino acids 95 to 125, whereas individually these peptides lacked antiviral activity.

In vitro toxicity studies using L929 cells grown to confluence on a 96-well plate and incubated with the mimetic and control peptides for 7 h demonstrated that all peptides, with the exception of SV40 large T antigen (at 100 μg), were nontoxic to

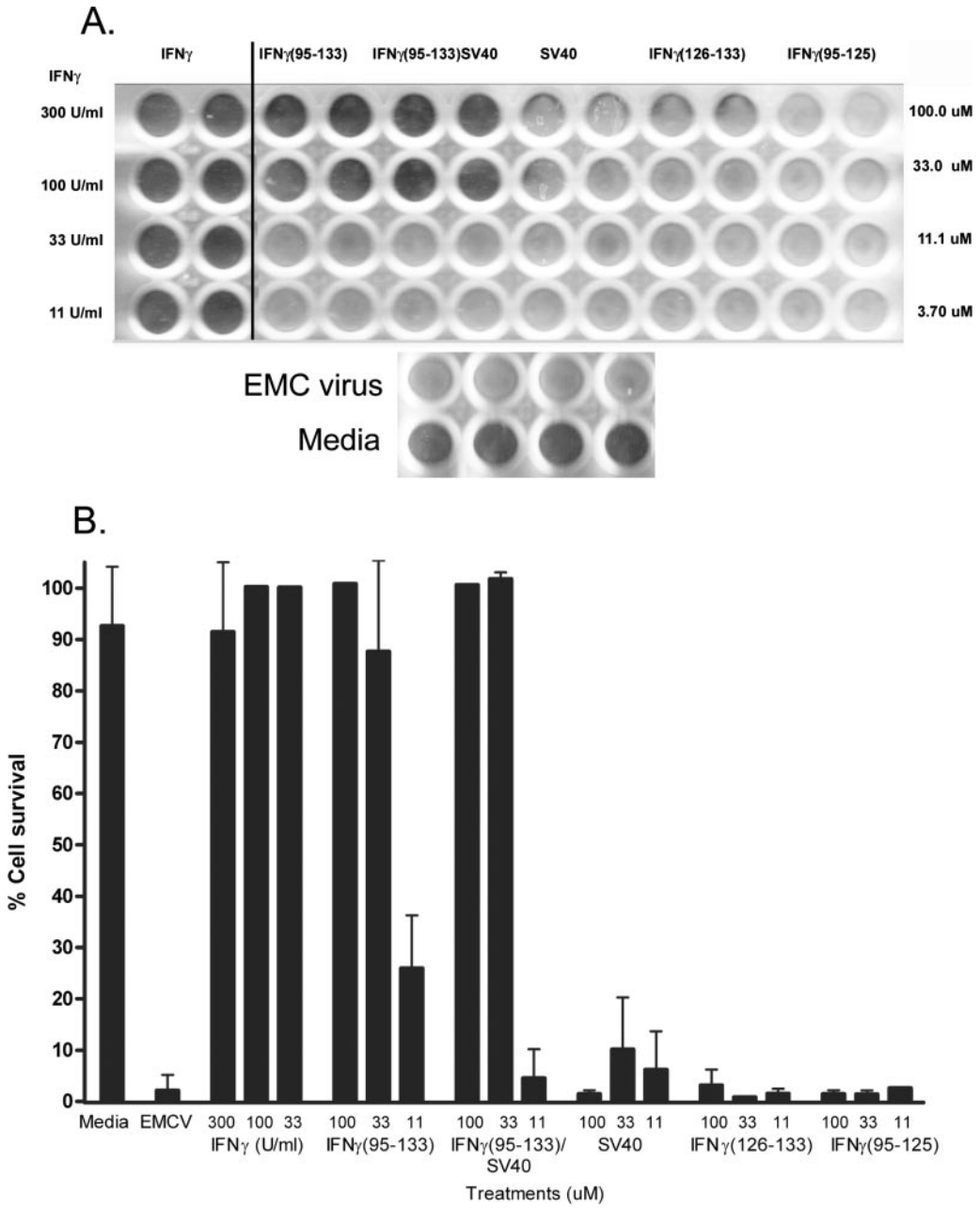


FIG. 1. IFN-γ mimetics possess antiviral activity against EMC virus in tissue culture. Mouse L929 cells were plated and grown to confluence on a 96-well plate. Various concentrations of mouse IFN-γ (300 U/ml to 11 U/ml) and various concentrations of IFN-γ(95-133), IFN-γ(95-133)SV40, SV40, IFN-γ(126-133), and IFN-γ(95-125) peptides, ranging from 100 μM to 3.7 μM, were incubated with L929 cells for 7 h, after which EMC virus (EMCV) (2,000 PFU/ml) was added. After 1 h, virus was removed and media added to all wells, followed by incubation for at least 28 h. Cells were stained with crystal violet solution, and plates were scanned and analyzed to assess cell survival. (A) Digital image of the plate. (B) Image J 1.29 software was used to analyze the image to obtain gray value to assess cell survival, presented here as percentages of the medium control value (100% cell survival). Representative data from one of three experiments are presented. Error bars indicate standard errors of the means.

cells at the concentrations used (100 μM to 3.7 μM), as shown in Fig. 2. Thus, the cytopathic effect shown in Fig. 1 was due to EMC virus infection rather than cellular toxicity caused by the IFN-γ mimetic and control peptides.

**IFN-γ and IFN-γ(95-133) have reduced EMC virus yield compared to control groups.** To determine if the mimetic peptide reduces virus yield after EMC virus infection in tissue culture, murine L929 cells seeded to confluence in a 25-cm$^2$ flask were pretreated with IFN-γ, IFN-γ(95-133), media, or the control peptide IFNGR(253-287) for 24 h, after which cells were incubated with EMC virus (100 PFU/ml) for 1 h. As shown in Table 2, IFN-γ and IFN-γ(95-133) reduced virus yield by 37.3- and 24.1-fold, respectively, compared to 0.7-fold for the control peptide IFNGR(253-287). Thus, similarly to IFN-γ, the IFN-γ(95-133) peptide reduced virus yield from EMC virus-infected cells.

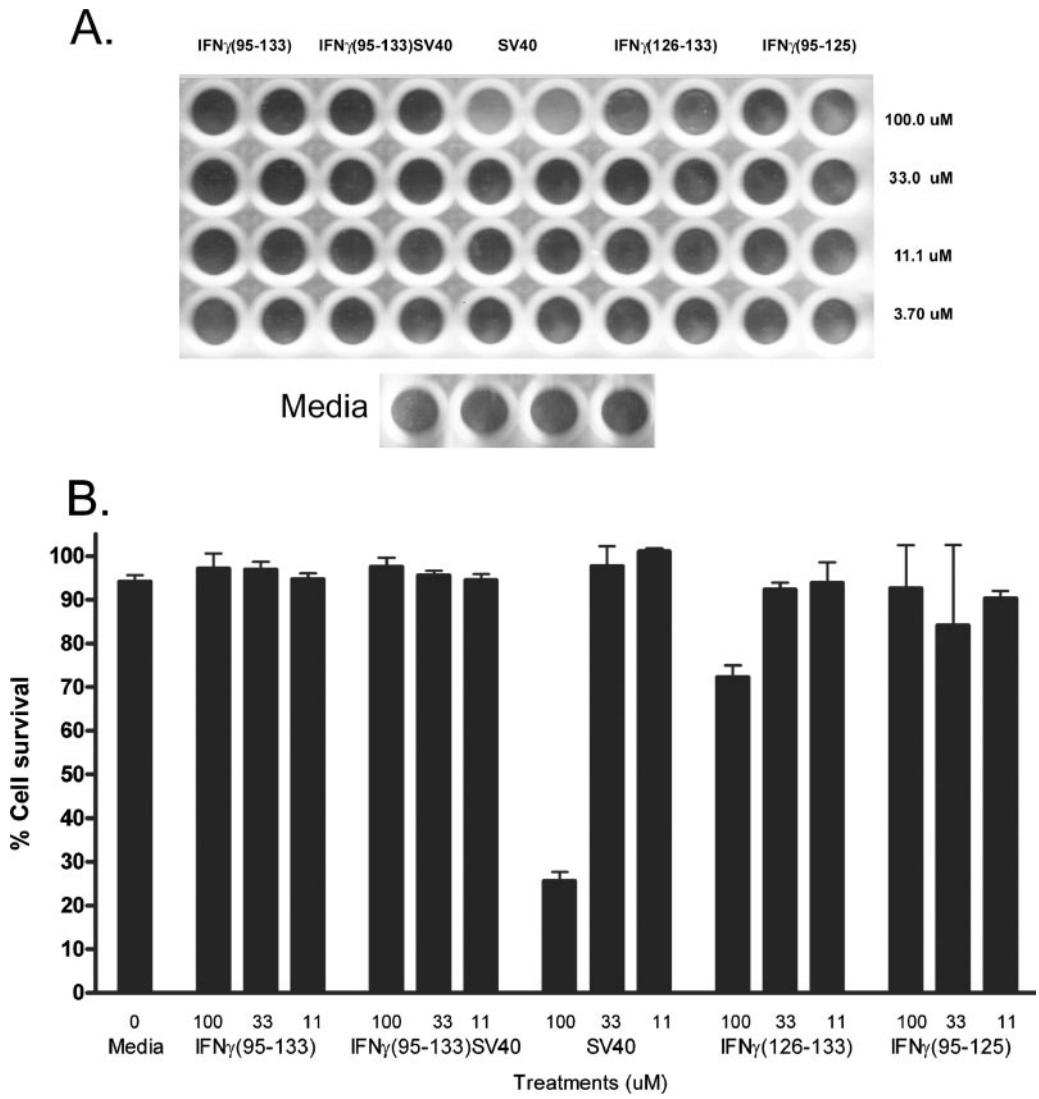


FIG. 2. Cellular toxicity of IFN-γ(95-133), derivatives, and control peptides. Mouse L929 cells were plated and grown to confluence on a 96-well plate. Various concentrations of IFN-γ(95-133), IFN-γ(95-133)SV40, SV40, IFN-γ(126-133), and IFN-γ(95-125) peptides, ranging from 100 μM to 3.7 μM, were incubated with L929 cells for 35 h. Cells were stained with crystal violet solution, and plates were scanned and analyzed to assess cell survival. (A) Digital image of the plate. (B) Image J 1.29 software was used to analyze the image to obtain gray value to assess cell survival, presented here as percentages of the medium control value (100% cell survival). Representative data from one of three experiments are presented. Error bars indicate standard errors of the means.

TABLE 2. IFN-γ and IFN-γ(95-133) reduction of EMC virus yield[a]

| Treatment | Concn | Virus yield (PFU/ml) | Fold reduction |
|---|---|---|---|
| Medium only | | $4.1 \times 10^8$ | |
| IFN-γ | 2,500 U/ml | $1.1 \times 10^7$ | 37.3 |
| IFN-γ(95-133) | 20 μM | $1.7 \times 10^7$ | 24.1 |
| IFNGR(253-287) | 20 μM | $6.3 \times 10^8$ | 0.7 |

[a] Mouse L929 fibroblasts seeded to confluence in a 25-cm$^2$ flask were pretreated with 2,500 U/ml of IFN-γ, 20 μM of IFN-γ(95-133), or 20 μM of IFNGR(253-287) control peptide for 24 h, after which cells were challenged with EMC virus at 100 PFU/ml for 1 h. Flasks were washed and incubated with media for another 24 h. Virus produced was harvested and titrated by a standard viral plaque assay (see Materials and Methods). The numbers of PFU/ml of the original samples were reported and reduction (*n*-fold) calculated via dividing the number of PFU/ml of the medium-only control by the number of PFU/ml of each experimental sample.

**Supernatants from EMC virus-infected cells treated with IFN-γ and IFN-γ(95-133) protect mice against lethal EMC virus infection.** Supernatants taken from cells from experiments shown in Table 2 were injected into mice for determination of infectivity as measured in a lethal model of EMC virus infection. In the lethal EMC virus infection, mice become lethargic and eventually die, starting 4 days after the EMC virus injection (50 PFU or greater/mouse) (14, 25). As shown in Table 3, supernatants taken from cells treated with media and injected intraperitoneally into mice resulted in 75% death (25% survival), while supernatants taken from IFN-γ-treated cells resulted in 50% death. In contrast, mice injected with supernatants taken from cells treated with IFN-γ(95-133) did not succumb to EMC virus lethality (100% survival). No change was seen in any group after the eighth day after the supernatant injection. Thus, IFN-γ(95-133)-treated cells

TABLE 3. Induction of EMC virus infection in C57BL/6 mice[a]

| Treatment | % Survival at the indicated day after EMC virus infection | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 4 | 5 | 6 | 7 | 8 |
| Medium | 100 | 100 | 50 | 50 | 25 | 25 |
| IFN-γ | 100 | 100 | 100 | 75 | 50 | 50 |
| IFN-γ(95-133) | 100 | 100 | 100 | 100 | 100 | 100 |

[a] Mouse L929 fibroblasts were treated with media, $2.5 \times 10^3$ U/ml of IFN-γ, or 20 μM of IFN-γ(95-133) for 24 h, after which cells were incubated with 100 PFU/ml of EMC virus for 1 h. Cells were then washed three times with media to wash away residual IFN-γ and peptides from the cells. Cells were resuspended with media for 24 h and supernatants collected. All supernatants were diluted with media (1:200,000) prior to intraperitoneal injection of mice on day 0. Mortality from EMC virus infection was recorded, and the percentage of surviving mice for each treatment group is presented. No change was seen in the mice after the eighth day postinjection with supernatants. A total of eight mice per group were used.

produced EMC virus amounts that were not lethal, as reflected by survival of mice.

**Protection of mice from EMC virus challenge by the IFN-γ mimetic peptide IFN-γ(95-133) is dose dependent.** Based on the tissue culture results showing antiviral activity of IFN-γ(95-133) and the in vivo results presented in Table 3, the IFN-γ mimetic peptide IFN-γ(95-133), along with a control peptide, IFN-γ(95-125), which does not have any antiviral activity in tissue culture due to the loss of the NLS region, were tested directly in vivo for their therapeutic effects in the mouse model of lethal EMC virus infection used as described above. C57BL/6 mice were prophylactically treated with phosphate-buffered saline (PBS), IFN-γ (3,000 U/day), IFN-γ(95-133) (100 μg/day), and the control peptide IFN-γ(95-125) for 6 days. On the last day of intraperitoneal injection, EMC virus (200 PFU/mouse) was also included in the injection cocktail. Mice were monitored every day and the numbers of surviving mice recorded. As shown in Fig. 3A, all PBS- and control peptide IFN-γ(95-125)-treated mice died by the seventh and eighth days after the EMC virus challenge, respectively. In contrast, mice injected with IFN-γ at 3,000 U/mouse per day showed 100% survival, while administration of the IFN-γ mimetic peptide at 100 μg/mouse per day resulted in approximately 30% of mice surviving EMC virus challenge ($P < 0.05$). Thus, administration of IFN-γ mimetic peptide at 100 μg/day for 6 days protected mice significantly compared to administration of PBS and control peptide.

To determine if an increased dosage of the IFN-γ mimetic peptide results in enhanced therapeutic activity against EMC virus challenge using the above-described mouse model of lethal EMC virus infection, IFN-γ(95-133) was administered at 200 μg/day intraperitoneally for 3 days. This treatment regimen differs from that described above for Fig. 3A in that a higher dose (200 μg/day) was administered in a shorter pretreatment time (3 days). On the third day of treatment, mice were challenged with EMC virus and the numbers of surviving mice recorded. As shown in Fig. 3B, PBS and control peptide IFN-γ(95-125) did not have any antiviral protection in mice (0% survival) whereas 100% of mice were protected from EMC virus challenge by IFN-γ(95-133) treatment. Thus, a higher dose of IFN-γ(95-133) in a reduced treatment time period enhances antiviral activity in mice infected with the EMC virus.

**Analysis of sera and tissues of EMC virus-infected mice treated with the IFN-γ mimetic peptide.** Infection with lethal EMC virus dosages results in certain organ failures and thus mortality (11, 14, 25). In cases where levels of EMC virus are below lethal doses, viral particles may still be found in the internal tissues of infected mice. Thus, viral titers of EMC virus in the blood and liver, spleen, and heart tissues of mice treated with IFN-γ, IFN-γ(95-133), PBS, and the control peptide IFN-γ(95-125) from the experiments performed as described above for Fig. 3B were determined. Treatment with the control peptide IFN-γ(95-125) showed detectable levels of EMC virus at dilutions of 1/270, 1/21,870, and 1/90 in the spleen, heart, and liver tissue samples, respectively, as shown in Fig. 4A to D.

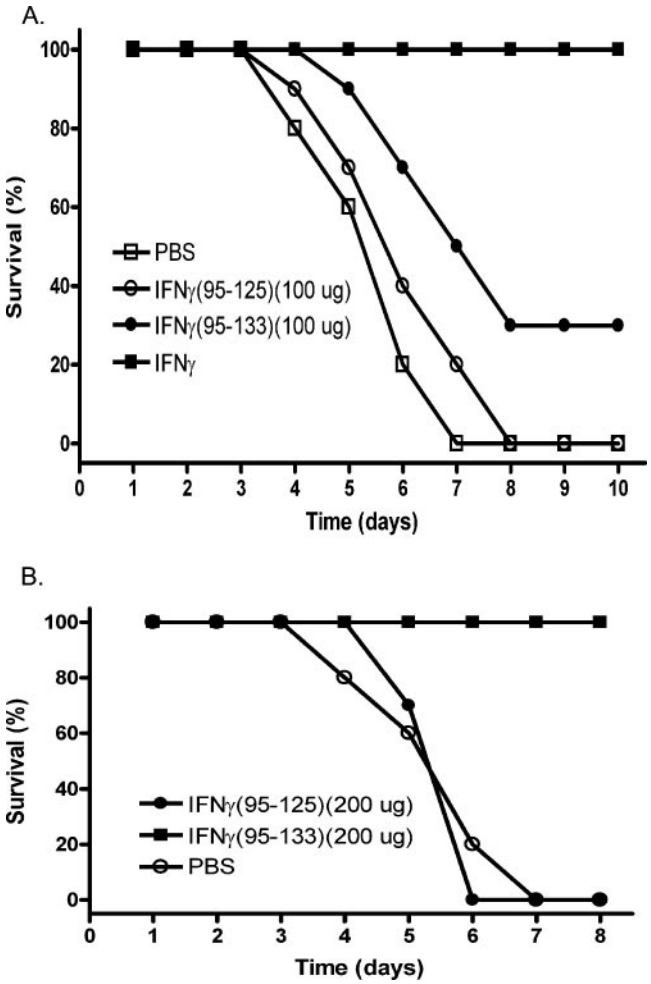


FIG. 3. Protection of mice from EMC virus infection by IFN-γ and the IFN-γ mimetic peptide IFN-γ(95-133) is dose dependent. (A) C57BL/6 mice (10 mice per treatment group) were pretreated by intraperitoneal injection for 6 days with PBS, IFN-γ(95-125) and IFN-γ(95-133) peptides (100 μg/day), or IFN-γ (3,000 U/day). (B) Mice (five mice per treatment group) were pretreated with 200 μg/day of either IFN-γ(95-133) or IFN-γ(95-125) peptide for 3 days. On the last day of treatment, mice were challenged by intraperitoneal injection with 50 PFU of EMC virus. The numbers of surviving mice were recorded starting on the day of EMC virus challenge (day 0) and are presented as percent survival. Representative data from one of two experiments are shown.

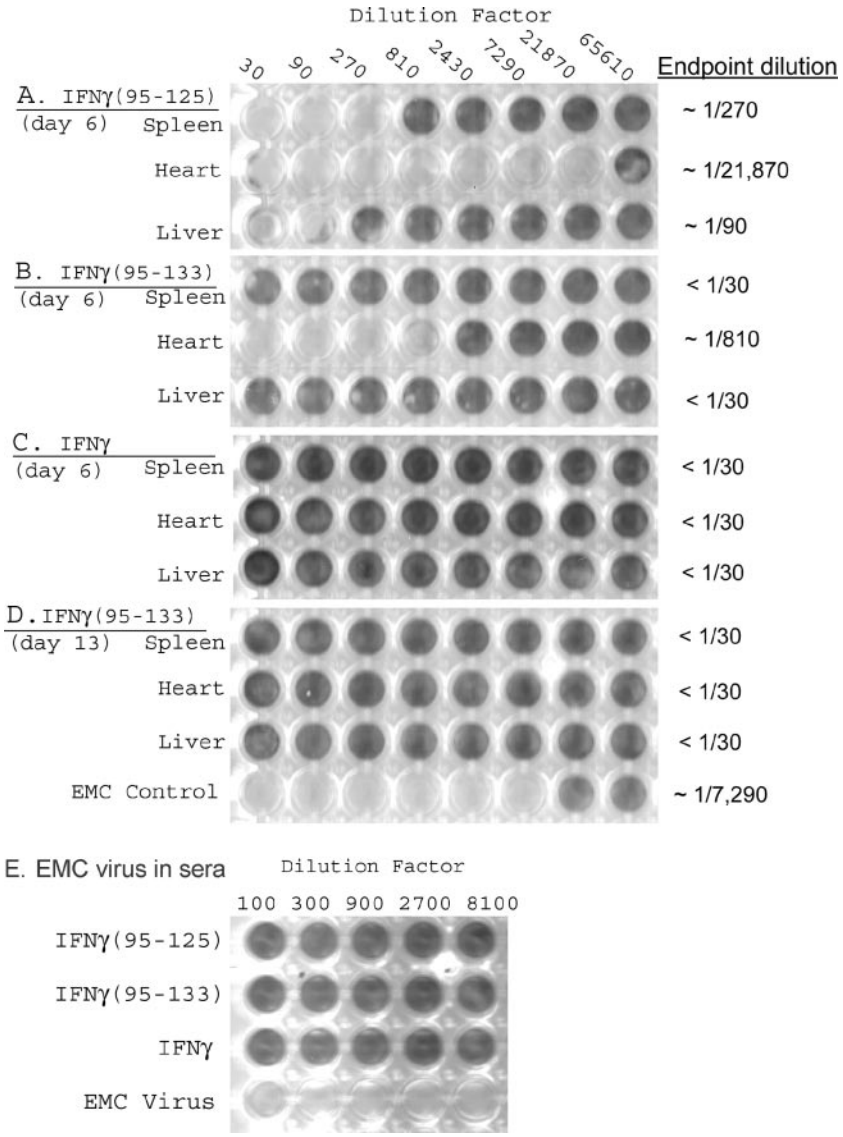


FIG. 4. Mice treated with IFN-γ(95-133) peptide have reduced or no EMC virus particles in sera and various tissues. C57BL/6 mice were pretreated for 3 days with IFN-γ (3,000 U/day), IFN-γ(95-133) (200 μg/day), or the control peptide IFN-γ(95-125) (200 μg/day). On the last day of treatment, mice were challenged with 50 PFU of EMC virus. On day 6 after EMC virus infection, mice were bled and sacrificed, after which equal amounts of heart, spleen, and liver tissues were extracted from each group, homogenized, and lysed by thawing/freezing. (A to D) Samples were centrifuged, and supernatants were diluted and incubated on murine L929 cells plated to confluence in a 96-well plate for detection of EMC virus cytopathic effect after being stained with crystal violet solution. (E) Sera from various treatment groups were diluted and incubated with L929 cells as described above for determination of cytopathic effect. Tissues and sera from three mice per treatment group were analyzed, and representative data from one of two experiments are shown.

Spleen and liver tissue samples from mice treated with IFN-γ(95-133) did not have any quantifiable levels of EMC virus. Although the heart tissue did have detectable levels of EMC virus up to a dilution of 1/810 for IFN-γ(95-133), none of these mice died as indicated previously for Fig. 3B. The viral levels in the heart tissues of IFN-γ(95-133)-treated mice were 27 times lower than those for the control peptide [IFN-γ(95-125)] group. This is consistent with results seen in virus reduction in tissue culture experiments. Furthermore, 7 days later (13 days after the EMC virus challenge), EMC virus was not present in any tissue from the IFN-γ(95-133)-treated mice. Tissue samples from mice that were treated with IFN-γ had no detectable levels of EMC virus. In addition, as shown in Fig. 4E, EMC virus was not present in any serum samples from mice, including the control groups. Thus, IFN-γ(95-133)-treated mice have no or low EMC virus levels, consistent with mimetic protection.

**Antiviral activity of IFN-γ(95-133) peptide in the presence of the B8R protein in tissue culture and in vivo.** Since we have shown previously that IFN-γ(95-133) binds to the cytoplasmic domain of the IFN-γ receptor and thus triggers signal transduction events associated with IFN-γ, this mimetic peptide was evaluated in the presence of the B8R protein, which is produced by poxviruses for neutralization of IFN-γ activity. The

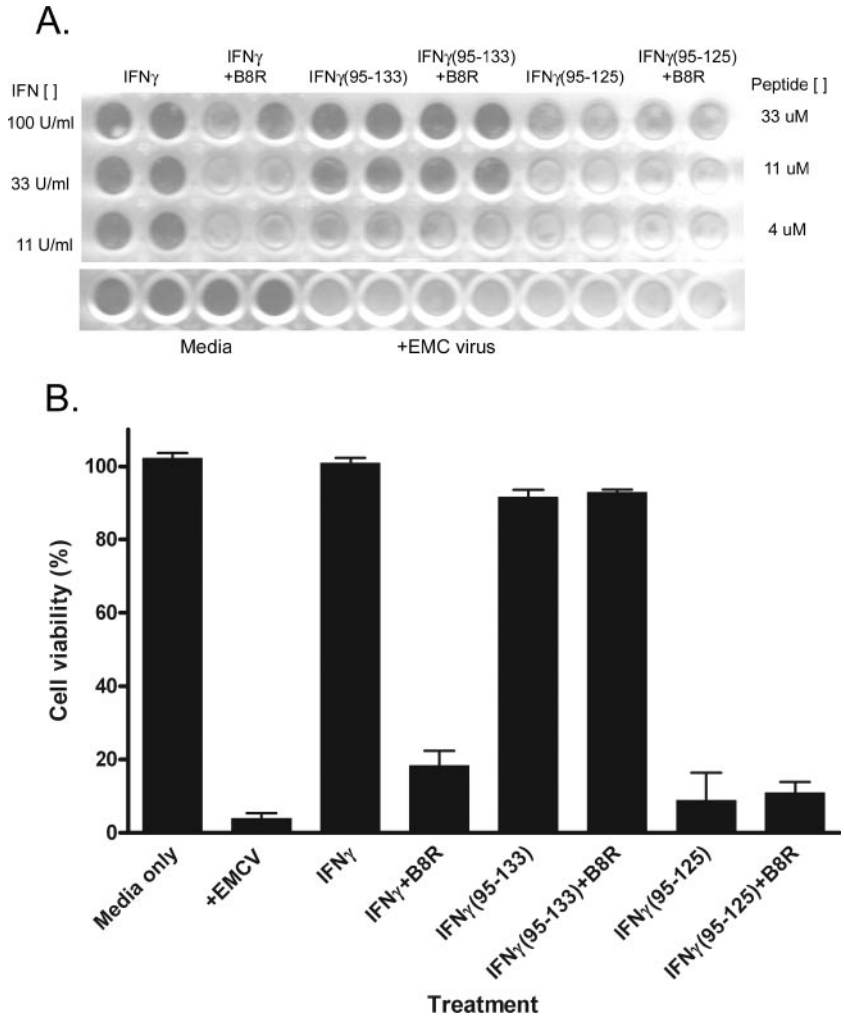


FIG. 5. B8R neutralizes IFN-γ but not IFN-γ(95-133) antiviral activity. Murine L929 cells were plated to confluence, after which media and various concentrations of IFN-γ, IFN-γ(95-133), and IFN-γ(95-125) that were preincubated for 2 h with or without B8R (33 μg/ml) were added to the plate. After 24 h of incubation, EMC virus (EMCV) (200 PFU/ml) was added for 1 h of incubation and washed with media. Cells were then incubated with media for 24 h, after which wells were stained with crystal violet and washed. (A) Digital image of the plate. (B) The plate was scanned for cell viability assessment using Image J software (NIH). Percent cell viability is presented for 33 U/ml of IFN-γ and 11 μM of IFN-γ(95-133). Error bars indicate standard errors of the means.

B8R protein is a homolog of the IFN-γ receptor extracellular domain; therefore, IFN-γ(95-133) should retain its antiviral activity in the presence of this virulence factor of poxviruses. To demonstrate this in tissue culture, murine L929 cells were plated to confluence on a 96-well plate, after which rat IFN-γ, IFN-γ(95-133), and IFN-γ(95-125), which were all preincubated for 2 h with B8R protein, were added to the plate. Rat IFN-γ was used here instead of mouse IFN-γ due to the fact that B8R does not bind to mouse IFN-γ but does bind to rat IFN-γ (3). Furthermore, rat IFN-γ has activity on mouse cells, as shown previously (3). After overnight incubation, EMC virus was added, and the cells were washed and reincubated in media for 24 h, followed by determination of EMC virus cytopathic effects. As shown in Fig. 5A, IFN-γ had antiviral activity against EMC virus at all concentrations (100 U/ml, 33 U/ml, and 11 U/ml) used, but in the presence of B8R, IFN-γ antiviral activity was lost at 33 U/ml and 11 U/ml. In contrast, the antiviral activities of IFN-γ(95-133) were similar in the presence or absence of B8R protein, as denoted by almost 100% cell viability at the 11 μM peptide concentration used (Fig. 5A and B). The control peptide IFN-γ(95-125) did not have any antiviral activity in either case. Thus, B8R protein neutralized IFN-γ antiviral activity but not IFN-γ(95-133) antiviral activity against EMC virus in tissue culture.

Based on the above-described tissue culture study, the antiviral activity of IFN-γ(95-133) was assessed in the presence of B8R protein in the mouse model of lethal EMC virus infection. C57BL/6 mice were pretreated for 3 days with PBS, IFN-γ(95-133) (100 μg/day), or rat IFN-γ (200 U/day). The 200-U/day dose of rat IFN-γ, which still had significant antiviral activity, was administered in order to detect the neutralizing effect of the B8R protein. On the last day of treatment, mice were challenged with 50 PFU of EMC virus. The numbers of surviving mice were recorded over time. As shown in Fig. 6, injection of rat IFN-γ resulted in 20% survival of mice in response to EMC virus challenge. In contrast, administration

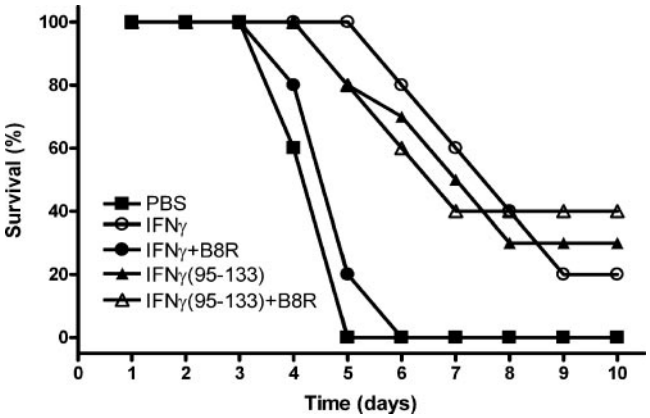


FIG. 6. Protection of mice from EMC virus challenge by the IFN-γ(95-133) peptide in the presence of B8R protein. C57BL/6 mice were pretreated for 3 days with PBS, IFN-γ(95-133) (100 μg/day), or rat IFN-γ (200 U/day) in the presence or absence of the B8R protein (25 μg). On the last day of treatment, mice were challenged with 50 PFU of EMC virus. The numbers of surviving mice were recorded starting on the day of EMC virus challenge (day 0) and are presented as percent survival. Ten mice per treatment group were used, and representative data from one of two experiments are shown.

of B8R with rat IFN-γ resulted in 0% of mice surviving (100% death), which was similar to results for PBS controls. Administration of IFN-γ(95-133) in the presence or absence of B8R resulted in 40% and 30% survival, respectively, 9 days after the EMC virus challenge. Furthermore, there was a delay in the onset of lethality in IFN-γ(95-133)- and rat IFN-γ-treated groups compared to results for PBS- and IFN-γ/B8R-treated groups. These percentages of survival are similar to those seen for Fig. 3A, where 100 μg/day of IFN-γ(95-133) peptide was used. Thus, the IFN-γ mimetic peptide IFN-γ(95-133) has antiviral activity in tissue culture as well as in vivo in the presence of B8R protein, which neutralizes IFN-γ and which is encoded by poxviruses.

## DISCUSSION

The data presented here demonstrate that the IFN-γ C-terminal peptide encompassing amino acids 95 to 133 has antiviral activity similar to that of IFN-γ itself both in tissue culture and in vivo in the murine model of lethal EMC virus infection. IFN-γ(95-133) had antiviral activity on L929 cells in a dose-dependent manner against EMC virus challenge. Reduction of EMC virus yield with IFN-γ(95-133) treatment was over 24-fold greater than that achieved with control treatments. Furthermore, both IFN-γ(126-133) (NLS region) and IFN-γ(95-125) (region without the NLS) were required for antiviral activity. The NLS peptide, IFN-γ(126-133), and IFN-γ peptide, IFN-γ(95-125), did not possess any antiviral protection against EMC virus in the L929 viral cytopathic assay, indicative that intact IFN-γ C-terminal amino acids 95 to 133 are critical for antiviral activity against EMC virus. The region of amino acids 95 to 133 contains an alpha helix that we have shown previously to be necessary for mimetic activity (21, 22). Replacement of the IFN-γ(95-133) NLS region with the NLS of the SV40 large T antigen resulted in a similar antiviral activity against EMC virus challenge in tissue culture. This suggests that the NLS in IFN-γ and IFN-γ(95-133) is classic and functions through the components of the Ran/importin pathway utilized by the SV40 T antigen NLS for transportation into the nucleus for signal transduction events triggered by receptor-ligand binding (15, 17, 19). Also shown here, the concentrations of IFN-γ(95-133) that had antiviral effects in tissue culture were not toxic to cells. Thus, IFN-γ(95-133) possessed antiviral activity similar to that of IFN-γ itself in tissue culture against EMC virus. Given that the mimetic and the other control peptides are small and stable (data not shown) relative to IFN-γ, it suggests the IFN-γ(95-133) peptide's potential as an IFN-γ drug.

Protection against EMC virus challenge in vitro correlated well with in vivo experiments utilizing the lethal model of EMC virus infection in mice. In our studies, intraperitoneal administration of 50 PFU of EMC virus resulted in 100% lethal effect in all control mice. Treatment of mice with 100 μg/day of IFN-γ(95-133) for 6 days prior to EMC virus challenge resulted in approximately 30% survival, compared to 0% survival for control groups. Increasing the dosage of IFN-γ(95-133) to 200 μg/day resulted in 100% survival of mice against EMC virus challenge, similar to the IFN-γ treatment survival rate. Internal tissues processed for viral titer 6 days after the EMC virus infection showed no or low EMC virus in the hearts, livers, and spleens of IFN-γ(95-133)-treated mice, whereas tissue samples from control peptide- and PBS-treated groups had high levels of EMC virus. All control peptide- and PBS-treated mice died 6 to 7 days after the EMC virus challenge in these studies. Tissue samples taken from IFN-γ(95-133)-treated mice showed no detectable EMC virus 13 days after the EMC virus challenge, suggesting that IFN-γ(95-133) had established an antiviral state in these mice, thus giving the immune system time to mount an appropriate immune response to clear the EMC virus. Previous studies have shown EMC virus-specific antibodies in sera from mice infected with a nonlethal dose of EMC virus (12). Thus, in addition to the antiviral activity of IFN-γ(95-133) shown in tissue culture, most importantly, the IFN-γ mimetic peptide has antiviral activity against EMC virus in vivo.

We have demonstrated previously that murine IFN-γ binds to the extracellular domain of the receptor α chain through its N terminus and subsequently to the intracellular domain of the receptor via its C terminus following receptor-mediated endocytosis via lipid microdomains (15, 17). The C terminus of murine IFN-γ, consisting of residues 95 to 133, binds to the membrane-proximal region of the cytoplasmic domain of the receptor and thus can independently activate IFN-γ signal transduction mechanisms while bypassing the need of binding the N terminus of the IFN-γ receptor, which is at the extracellular side of the membrane (16, 19, 21, 22). This is consistent with the data shown here that IFN-γ(95-133) peptide has antiviral activity in tissue culture and in mice in the presence of the poxvirus B8R protein. The B8R protein is a homolog of the extracellular domain of the IFN-γ receptor and can therefore bind to intact IFN-γ and prevent its interaction with the receptor (1, 20). The B8R protein is an important virulence factor of poxviruses, such as vaccinia virus and variola virus, enabling these viruses to propagate in the infected host (1, 20). Thus, our studies here show that while IFN-γ can be neutralized by the B8R protein in tissue culture and in vivo, the IFN-γ

mimetic peptide IFN-γ(95-133) is not neutralized due to its binding to the cytoplasmic domain of the IFN-γ receptor, therefore retaining its antiviral activity against EMC virus.

We have shown here that the IFN-γ mimetic peptide IFN-γ(95-133) has antiviral activity against lethal EMC virus challenge in tissue culture and in mice. Furthermore, and in contrast to IFN-γ, the IFN-γ mimetic peptide induces antiviral activity against EMC virus in the presence of B8R protein in both tissue culture and in vivo experiments. Thus, the data presented here and in our previous studies (1, 2, 21) demonstrate that IFN-γ(95-133) has antiviral activity and, therefore, could be a potential antiviral therapeutic against lethal viruses in humans, such as the smallpox virus, which encodes the B8R protein.

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