Bovine Type III Interferon Significantly Delays and Reduces the Severity of Foot-and-Mouth Disease in Cattle

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Interferons (IFNs) are the first line of defense against viral infections. Although type I and II IFNs have proven effective to inhibit foot-and-mouth disease virus (FMDV) replication in swine, a similar approach had only limited efficacy in cattle. Recently, a new family of IFNs, type III IFN or IFN-λ, has been identified in human, mouse, chicken, and swine. We have identified bovine IFN-λ3 (boIFN-λ3), also known as interleukin 28B (IL-28B), and demonstrated that expression of this molecule using a recombinant replication-defective human adenovirus type 5 (Ad5) vector, Ad5-boIFN-λ3, exhibited antiviral activity against FMDV in bovine cell culture. Furthermore, inoculation of cattle with Ad5-boIFN-λ3 induced systemic antiviral activity and upregulation of IFN-stimulated gene expression in the upper respiratory airways and skin. In the present study, we demonstrated that disease could be delayed for at least 6 days when cattle were inoculated with Ad5-boIFN-λ3 and challenged 24 h later by intradermal inoculation with FMDV. Furthermore, the delay in the appearance of disease was significantly prolonged when treated cattle were challenged by aerosolization of FMDV, using a method that resembles the natural route of infection. No clinical signs of FMD, viremia, or viral shedding in nasal swabs was found in the Ad5-boIFN-λ3-treated animals for at least 9 days postchallenge. Our results indicate that boIFN-λ3 plays a critical role in the innate immune response of cattle against FMDV. To this end, this work represents the most successful biotherapeutic strategy so far tested to control FMDV in cattle.

The synthesis of antiviral cytokines such as type I interferons (IFN-α/β) is the first cellular response to a virus infection. Recently, a new family of IFNs, type III (IFN-λ), has been described in several species, including humans, mice, swine, and chickens (27, 28, 44, 45, 46), and our group has identified, expressed, and characterized a member of the type III IFN family in bovines (boIFN-λ3) (15).

Similarly to IFN-α/β, IFN-λ is rapidly produced within a cell after infection with viruses or intracellular bacteria (25). Compared with type I IFN, type III IFN induces similar innate antiviral responses but signals through very different receptors. IFN-λs mediate their biological activity through a heterodimeric cellular receptor composed of two subunits, interleukin 28 receptor alpha (IL-28Rα) and IL-10Rβ, the last shared by the IL-10 family of cytokines (28, 45). The binding of IFN-λ to its receptor results in activation of the Janus kinase–signal transducer and activator of transcription (JAK-STAT), a signal transduction cascade that ultimately induces IFN-stimulated gene (ISG) expression (55). Most cell types express IFN-α/β receptors as well as the IL-10R subunit of the IFN-λ receptor; however, only a limited range of cells express the IL-28Rα receptor subunit, which is specific for signaling only by IFN-λ. In fact, IL-28Rα is significantly expressed in epithelial cells, and as a consequence, cells of epithelial origin in the skin and mucosae respond to treatment with this cytokine (2, 11, 46, 50, 51).

The main biological function of IFN-λ, as well as of the other IFNs, is to inhibit virus replication in virus-infected cells and to protect uninfected cells from virus infection. Such IFN-λ-induced antiviral activity has been demonstrated against many different viruses, most of them replicating primarily in epithelial cells from the respiratory tract, including influenza virus, respiratory syncytial virus, human metapneumovirus, and coronavirus (33). Other viruses, such as hepatitis B and C viruses, HIV, and herpes simplex virus 2, are also sensitive to the activity of IFN-λ (3, 23, 41). Foot-and-mouth disease (FMD) is an acute viral disease of domestic cloven-hoofed animals, including swine, cattle, goats, and sheep, and many wild animals. The etiological agent is the FMD virus (FMDV), a positive-sense single-stranded RNA virus belonging to the Aphthovirus genus of the Picornaviridae family. FMDV is characterized by a high replication rate, short incubation times, a high level of virus excretion via aerosol, and a high level of contagiousness within susceptible animals. Moreover, FMDV has a high antigenic variation, as displayed by the presence of 7 serotypes (A, O, C, Asia 1, and South African Territories 1, 2, and 3) and multiple subtypes (17, 20). The main natural route of FMDV infection is via aerosol through the upper respiratory tract (1). Experimental infection of cattle by aerosol has shown that the nasopharyngeal region is the primary site of viral replication, with subsequent spread to pneumocytes in the lungs (4, 37). Moreover, in ruminants, the virus can persist by unknown mechanisms in the pharyngeal region of the upper respiratory tract for many years after the resolution of the acute infection (carrier state), increasing the threat of new FMD outbreaks under certain conditions (47).

In areas where it is enzootic, FMD control is achieved by vaccination with chemically inactivated vaccines formulated with adjuvants (16, 42). Although they are effective, the use of these vaccines presents limitations that have led the World Organization of
Animal Health (OIE) to impose more severe trade restrictions on countries that opt to vaccinate instead of slaughtering animals (20). These restrictions make FMD-free countries reluctant to use the inactivated vaccine. An alternative vaccine that overcomes some of the limitations of the commercially available inactivated vaccine has been recently developed and is in the pipeline to be approved for commercial use (21). This vaccine uses a replication-defective human adenovirus type 5 (Ad5) to deliver empty FMDV capsids (21, 31). However, neither this live vectored nor the inactivated FMDV vaccine is able to confer complete protection prior to 7 days postvaccination (dpv). In an effort to address this limitation, we have previously shown that treatment of swine with Ad5s that express porcine type I IFN (Ad5-poIFN-α/β) resulted in complete protection against FMDV infection when challenge was performed at 1 day postinoculation, and protection lasted for 3 to 5 days (8, 12, 32). Unfortunately a similar approach in cattle only delayed disease by 1 to 2 days and resulted in the appearance of milder clinical signs (52). Our recent studies with boIFN-α3 demonstrated antiviral activity against FMDV and vesicular stomatitis virus (VSV) in bovine cell culture. Moreover, we showed that treatment of cattle with Ad5-boIFN-α3 induced systemic antiviral activity and upregulation of ISGs in multiple tissues, including those of the upper respiratory tract and skin (15).

In the current work, we evaluated the efficacy of treatment of bovines with Ad5-boIFN-α3 against FMDV challenge at 24 h after Ad5 inoculation. Our results demonstrated that treatment with Ad5-boIFN-α3 alone or in combination with Ad5-poIFN-α resulted in significant delay (6 days) and reduced severity of FMD when animals were challenged by intradermological (IDL) inoculation with FMDV. Interestingly, treatment with Ad5-boIFN-α3 alone resulted in milder and delayed disease for at least 9 days when the challenge was performed by aerosolization of FMDV. These results suggest that type III IFN is a potent biotherapeutic against FMDV in cattle and that its use in prevention and treatment of this feared disease deserves further consideration.

MATERIALS AND METHODS

Cell and viruses. Human 293 cells (ATCC CRL-1573) were used to generate and propagate recombinant Ad5s. Embryonic bovine kidney (EBK) and porcine kidney (IB-RS2) cells were obtained from the Foreign Animal Disease Diagnostic Laboratory (FADDL) at Plum Island Animal Disease Center (PIADC), Greenport, NY. Baby hamster kidney cells (BHK-21, clone 13, ATCC CL-10) were used for propagation and titration of FMDV serotype A24. Bovine kidney LF-BK cells (48) were used for titration of FMDV serotype O1 Manisa. MDBK-t2 cells (Madin-Darby bovine kidney cells transfected with plasmid expressing the human MxA promoter linked to a chimeraphenolic acetyltransferase [CAT] reporter and with resistance to blasticidin [18]) were kindly provided by B. Charleston (Institute for Animal Health, Pirbright, United Kingdom). All cells were maintained in Eagle’s minimal essential medium (EMEM) containing either 10% calf serum or 10% fetal bovine serum (FBS) and supplemented with antibiotics, glutamine, and nonessential amino acids. MDBG-t2 medium was also supplemented with 10 μg/ml blasticidin (Invitrogen, Carlsbad, CA). Replication-defective recombinant Ad5s containing type I IFN, bovine and porcine IFN-α, boIFN-α3, and vector control Ad5-Blue (empty vector, negative control) were produced as previously described (15, 32, 52). For cattle challenge, FMDV A24 (strain Cruzeiro, Brazil, 1995), a gift from A. Tanuri, Federal University of Rio de Janeiro, Brazil, was obtained from vesicular lesions of the coronary band of an FMDV A24-infected steer. The challenge virus FMDV O1 Manisa was obtained from macerated tongue epithelia of two experimentally infected cattle (37). The 50% bovine infectious doses (BID₅₀) of both FMDV strains were determined in bovines by inoculation of multiple dilutions intradermally in the tongue (22). FMDV titers were determined by standard plaque assay on BHK-21 cells for A24 and on LF-BK cells for O1 Manisa. Alternatively, FMDV RNA levels were determined by quantitative real-time PCR (6).

Determination of boIFN-α3 biological activity against FMDV serotype O1 Manisa in vitro. The biological activity of recombinant boIFN-α3 was measured in EBK cells as previously described (15). Briefly, EBK cells were treated with different dilutions of supernatants from IB-RS2 cells infected with Ad5-boIFN-α3 which had been centrifuged to remove virus particles. Twenty-four hours later, treated cells were washed with phosphate-buffered saline (PBS) and infected with FMDV O1 Manisa at a multiplicity of infection (MOI) of 1. One hour after the infection, cells were washed with 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6) for 5 min, followed by an incubation in cell culture medium for an additional 7 h. Supernatants were then collected, and virus yield was measured on LF-BK cells by standard plaque assay (9). Results were expressed as log₁₀ PFU/ml of supernatant.

Animal experiments. All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Plum Island Disease Center. Animals were held for at least 1 week for acclimatization before the start of experiments and were kept under strict controlled conditions at the PIADC biosafety level 3 animal facilities. Two different experiments using a total of 26 Holstein steer calves, of about 400 lb each, were performed to evaluate the efficacy of boIFN-α3 in vivo.

Experiment I: efficacy of Ad5-boIFN-α3 and Ad5-poIFN-α against FMDV intradermological challenge. Twelve animals divided in 4 groups of 3 animals each were intramuscularly (i.m.) immunized in the neck with 10¹ⁱ PFU/animal of Ad5-poIFN-α, one group was treated with 10¹³ PFU/animal of Ad5-boIFN-α3, and two groups were treated with a combination of Ad5-boIFN-α3 and Ad5-poIFN-α at a dose of 10¹³ PFU/animal (“high-dose combination”) or 5 × 10¹⁰ PFU/animal (“low-dose combination”) of each Ad5. Two other animals were inoculated with 2 × 10¹³ PFU Ad5-Blue as control group. One day after the Ad5 inoculation (1 day postinoculation [dpi]), all animals were challenged with 10⁶ BID₅₀, FMDV A24 Cruzeiro by IDL inoculation at 4 sites (100 μl/each). Rectal temperatures were monitored daily. Clinical signs of FMD were evaluated at 0, 2, 4, 6, and 8 days postchallenge (dpc), equivalent to 1, 3, 5, 7, and 9 dpi, respectively. A clinical score of 1 to 5 was assigned to describe the severity of the disease, with a maximal score of 5 (a score of 1 was assigned when one or more vesicular lesions were detected in one foot, a score of 2 was assigned if two feet had vesicular lesions, a score of 3 was assigned if three feet had vesicular lesions, etc., and an additional score of 1 was assigned if lesions were found in the mouth at other than the inoculation site). Animals were considered protected when no clinical signs of disease were detected during the course of the experiment. Animals werebled daily from the day of the Ad5 inoculation, –1 dpi, until 7 dpi and at 14 and 21 dpc for viremia, serology analysis, and antiviral activity determination. Nasal swabs were obtained daily from 0 until 7 dpc for measuring FMD viral loads and antiviral activity.

Experiment II: efficacy of Ad5-boIFN-α3 and Ad5-boIFN-α against FMDV aerosol challenge. Twelve animals divided in 4 groups of 3 animals each were subcutaneously (s.c.) inoculated with 4 ml of recombinant Ad5s at 2 sites in the neck as follows: two groups were inoculated with either 1.5 × 10¹¹ PFU/animal of Ad5-boIFN-α or Ad5-boIFN-α3, a third group was inoculated with a combination of 7.5 × 10¹⁰ PFU/animal each of Ad5-boIFN-α3 and Ad5-boIFN-α, and the fourth group, a control group, was inoculated with 4 ml of PBS/animal. The control group was inoculated with PBS based on previous experiments where this treatment gave the same results as treatment with the control vector Ad5-Blue or Ad5-Null (12, 13). Twenty-four hours later, all animals were challenged with 10⁶ BID₅₀ of FMDV O1 Manisa in a total volume of 2 ml by aerosol exposure for 12 min as previously described by Pacheco et al. (37). Ani-
mals were monitored for clinical signs at 0, 3, 5, 7, 9, and 12 dpi, and blood and nasal swabs were collected for analyses as described for experiment I. The clinical score was evaluated as in experiment I, but detection of one or more lesions in the mouth was always scored as 1. The complete blood count (CBC) was analyzed in a Hemavet 950 analyzer (Drew Scientific, Waterbury, CT). Animals were kept for 3 weeks postchallenge and humanely euthanized according to IACUC guidelines.

**Determination of antiviral activity in sera and nasal swabs.** MDBK-t2 cells were seeded into 24-well tissue culture plates at 2 × 10⁴ cells/well, and after 24 h of incubation at 37°C and 5% CO₂, the culture medium was replaced with 0.25 ml of medium containing 0.1 ml of either the serum samples or the nasal swab secretions. Known amounts (from 1.95 to 1,000 U/ml) of recombinant human IFN-α2A were used as a standard. Known amounts (from 0, 4, 7, 14, and 21 dpc) were heat inactivated (30 min, 56°C) and used in a microtiter neutralization assay on LF-BK cells. Serial dilutions of serum were incubated with 100 50% tissue culture infective doses (TCID₅₀) of FMDV O1 Manisa for 1 h at 37°C and 5% CO₂, followed by infection of monolayers of LF-BK cells in 96-well plates for 72 h. Titters were calculated as the reciprocal of the highest dilution of serum that neutralized the virus in 50% of the wells.

**Analysis of gene expression by qRT-PCR.** The expression of IFN and ISGs was determined in purified peripheral blood mononuclear cells (PBMCs). PBMCs from −1, 0, 1, and 2 dpi were purified by density gradient centrifugation on Ficoll-Hypaque (density, 1.007 g/liter) and lysed in RLT buffer (Qiagen, Valencia, CA). Total RNA was isolated using an RNeasy kit (Qiagen) following the manufacturer’s protocol, and gene expression was measured by quantitative real-time PCR (qRT-PCR) in an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA) using primers and probes previously described (15). Relative mRNA levels were determined by comparative threshold cycle (Cₚ) analysis (User Bulletin 2; Applied Biosystems), utilizing as a reference the samples at 0 dpi and GADPH (glyceraldehyde-3-phosphate dehydrogenase) mRNA expression as internal control.

**Statistical analysis.** Differences in antiviral activities, neutralizing antibodies, and percentages of lymphocytes in blood were analyzed by the Student t test using GraphPad Prism software version V5.04 (GraphPad Software, Inc., San Diego, CA).

**RESULTS**

**boIFN-α3 displays antiviral activity against FMDV O1 Manisa in vitro.** We had previously demonstrated that boIFN-α3 displays antiviral activity against VSV and FMDV A24 in cell culture. In both cases the antiviral activity of the IFN-α3 was dose dependent, and when it was combined with IFN-α, an additive effect was observed (15). In the current study, we tested the bioactivity of boIFN-α3 alone or in combination with bovine type I IFN, boIFN-α, in EBK cells but using FMDV O1 Manisa. We tested this serotype because we anticipated that we would perform an in vivo experiment using the aerosol inoculation model recently optimized at Plum Island Animal Disease Center, which had given very consistent results, particularly with FMDV O1 Manisa (37). Similarly to our previous studies, treatment of EBK cells with boIFN-α3 or boIFN-α alone (from 0 to 4 U) reduced the FMDV O1 Manisa titer in a dose-dependent manner from 5.5 × 10⁵ to 2.0 × 10⁴ PFU/ml, with statistically significant differences among the tested concentrations (P < 0.05) (Fig. 1). In contrast, the combination of both IFNs did not show a clear additive effect at the tested concentrations. No statistically significant differences were detected when the effects of the various combinations of IFNs were compared to that of each IFN alone. We concluded that as for FMDV A24, boIFN-α3 and boIFN-α display antiviral activity against FMDV O1 Manisa in bovine cell culture.

**Inoculation of cattle with Ad5-boIFN-α3 results in delayed disease after IDL challenge with FMDV A24.** In our previous studies, we demonstrated that inoculation of cattle with Ad5-boIFN-α3 results in systemic antiviral activity and induction of ISGs. We therefore examined the protective effect of Ad5-boIFN-α3 treatment, alone or in combination with Ad5-IFN-α, against FMDV infection in cattle. We decided to use Ad5-poiIFN-α because in earlier experiments, a slightly higher antiviral activity had been detected when steer calves were inoculated with Ad5-poiIFN-α than when inoculation was with Ad5-boIFN-α (52), despite the similar antiviral activities measured in bovine and porcine cultured cells (7). The experimental design is described in Materials and Methods. As shown in Fig. 2, FMD clinical signs appeared the earliest in control animals (Ad5-Blue group), by 2 dpi, and reached the maximum clinical score of 5 by 6 dpi. Elevated temperature (≥40°C) was observed in both animals by 1 dpi and continued intermittently until 4 to 5 dpi, while viremia and nasal shedding were detected between 1 and 4 dpi. All animals of the Ad5-poiIFN-α group showed a 2-day delay in the appearance of FMD clinical signs compared to the control group, but by 4 dpi they had high scores (4 or 5), with elevated temperatures after 2 dpi. High viremia was detected between 2 and 4 dpi. Viral shedding in nasal swabs was detected in 2 out of 3 animals between days 3 and 5, although one animal remained negative throughout the experiment. Similar results for viremia and nasal shedding were found in the “low-dose combination” group. However, 2 out of 3 animals showed a delay in the appearance of FMD compared to the control and the Ad5-poiIFN-α groups. Moreover, the severity of the disease in the “low-dose combination” group...
was reduced; 2 out of 3 animals had clinical scores of only 1 and 3 by 8 dpc. In the “high-dose combination” group, 2 out of 3 animals showed a very low clinical score (1) at 4 dpc, and one animal remained free of FMD until 8 dpc. By 8 dpc, all 3 animals had vesicles only on the tongue, at other than the challenge site. Remarkably, all animals inoculated with only Ad5-boIFN-α3 had a considerable delay in the appearance of FMD compared with the control animals and the other vaccinated groups. Similar to the “high-dose combination” and “low-dose combination” groups, one animal did not show clinical signs until day 8. In general, viremia was delayed until 2 to 3 dpc, and viral shedding was delayed until 3 to 4 dpc, continuing for only one additional day in the Ad5-boIFN-α3 and “high-dose combination” groups. One animal from the Ad5-boIFN-α3 group remained negative for viremia throughout the experiment. Overall, most animals in the Ad5-boIFN-α3 and “high-dose combination” groups developed less severe clinical signs, viremia, and nasal shedding than those in the other three groups. Elevated rectal temperatures were observed in all animals in the Ad5-poIFN-α and “high-dose combination” groups at a time when these animals presented FMD clinical signs (between 2 and 4 dpc) and lasted for approximately 2 to 3 days. However, only one animal in each of the Ad5-boIFN-α3 and
The presence of FMDV-neutralizing antibodies was detected by a microtiter neutralization assay on LF-BK cells in sera of animals treated as described for Fig. 2 at 0, 4, 7, 14, and 21 days after IDL challenge with FMDV A24 Cruzeiro. Titers are reported as the log_{10} of the reciprocal of the highest dilution of serum that neutralized the virus in 50% of the wells. Each data point represents the mean ± standard deviation (SD) for each group. *, statistically significant difference between groups (P < 0.05).

“high-dose combination” groups had elevated temperature for 1 day. All animals developed neutralizing antibodies against FMDV by 7 dpc, with values that increased by 14 dpc. The neutralizing-antibody titers at 21 dpc were statistically significantly lower (P < 0.05) in all the IFN-treated animals than in the Ad5-Blue-inoculated animals, consistent with reduced FMDV infection (Fig. 3).

With respect to antiviral activity, all IFN-treated animals showed measurable levels of antiviral activity in serum 24 h after the Ad5 inoculation (0 dpc) which declined substantially by 1 dpc (Fig. 4). Animals inoculated with Ad5-poIFN-α, alone or in combination, showed the highest levels of antiviral activity, in a dose-responsive manner. However, we could not detect any direct correlation between the levels of systemic antiviral activity and the lag in appearance of disease. Although low levels of systemic antiviral activity were detected in the animals treated with Ad5-boIFN-α,3, disease was delayed until 6 to 8 dpc. In contrast, Ad5-poIFN-α inoculated animals, which showed the highest levels of antiviral activity in serum by 1 dpc, developed disease by 4 dpc, with high clinical scores. The control group showed some levels of systemic antiviral activity at 2 and 3 dpc, concurrent with the peak of viremia. No measurable antiviral activity was detected in the animals treated with Ad5-boIFN-α,3 alone did not develop clinical signs until 9 dpc, and 1 out of the 3 animals in the group remained free of FMD throughout the experiment (12 dpc). Consistent with the appearance of clinical signs, animals of the control and Ad5-boIFN-α groups were positive for FMDV in blood and nasal swabs by virus isolation at 2 and 3 dpc, respectively, and most of them remained positive for additional days. Two of the three steer calves from the combination group were positive for viral shedding in nasal swabs, although at later times postchallenge (4 to 8 dpc). Two of these animals had no detectable viremia. Interestingly, none of the animals in the Ad5-boIFN-α group was positive for viremia or viral shedding in nasal swabs at any time point after the challenge as determined by virus isolation.

Inoculation of cattle with Ad5-boIFN-α3 alone or in combination with Ad5-poIFN-α caused delayed and reduced severity of disease after IDL challenge with FMDV. All animals from the control group showed the maximum clinical score of 5 by 3 dpc. Similarly, all animals inoculated with Ad5-boIFN-α developed vesicles at 3 dpc, reaching a maximum score at 5 dpc. In contrast, animals treated with Ad5-boIFN-α3, either alone or in combination with Ad5-boIFN-α, showed a clear delay in the appearance of FMD. Within the combination group, 2 out of 3 animals were negative for vesicular lesions at 5 dpc and 1 animal remained negative until 12 dpc. Most interestingly, animals inoculated with Ad5-boIFN-α3 alone did not develop clinical signs until 9 dpc, and 1 out of the 3 animals in the group remained free of FMD throughout the experiment (12 dpc). Consistent with the appearance of clinical signs, animals of the control and Ad5-boIFN-α groups were positive for FMDV in blood and nasal swabs by virus isolation at 2 and 3 dpc, respectively, and most of them remained positive for additional days. Two of the three steer calves from the combination group were positive for viral shedding in nasal swabs, although at later times postchallenge (4 to 8 dpc). Two of these animals had no detectable viremia. Interestingly, none of the animals in the Ad5-boIFN-α3 group was positive for viremia or viral shedding in nasal swabs at any time point after the challenge as determined by virus isolation. In order to confirm these results, we measured the levels of viral RNA in serum and nasal swabs by a more sensitive method, qRT-PCR (Fig. 6A). Threshold cycle (C_{T}) values of 40 or less were considered positive. Animals from the control and Ad5-boIFN-α groups were viral RNA positive as early as 2 dpc and continued to be positive for 3 to 4 consecutive days. Animals from the combination group were positive for FMDV in serum from 3 to 5 dpc.
Animals inoculated with Ad5-boIFN-A3 only had detectable viral RNA by 5 dpc, which disappeared by day 6 in 2 out of the 3 animals of the group.

The percentage of lymphocytes in total white blood cells was calculated. As shown in Fig. 6B, the percentage of lymphocytes decreased by 3 dpc in animals of the control and Ad5-boIFN-α groups. Despite the high individual variation, this decrease was statistically significant (P < 0.05) in the PBS group at 3 and 4 dpc relative to −1 dpc. The percentage of lymphocytes recovered by 6 dpc.

(ii) Neutralizing-antibody response. The levels of neutralizing antibodies were consistent with the delay in the appearance of clinical signs and viremia. As seen in Fig. 6C, the Ad5-boIFN-A3 group, which showed the longest delay in disease onset or no disease during the course of the experiment, had lower levels of anti-FMDV neutralizing antibodies than the control, Ad5-boIFN-α, and combination groups, with statistically significant differences by 21 dpc (P < 0.05). Consistently, the combination group, which showed clinical signs earlier than the Ad5-boIFN-A3 group, displayed intermediate levels of neutralizing antibodies by 7 dpc; however, by 14 and 21 dpc these animals reached levels similar to those for the control and Ad5-boIFN-α groups.

(iii) Antiviral activity in sera and nasal swabs. The levels of systemic antiviral activity in all animals were measured using an Mx1-CAT ELISA (18). All Ad5-IFN-treated animals showed the highest levels of antiviral bioactivity in sera before challenge (1 dpi...
or 0 dpc) or at 1 dpc (Fig. 6D). These levels declined with time and mostly disappeared by 6 dpc. However, different dynamics were observed in the group inoculated with PBS. Control animals developed antiviral bioactivity levels by 1 dpc with a peak between 2 and 4 dpc, correlating with the levels of viremia. By 6 dpc, the levels of antiviral activity returned to baseline values (\( \text{log}_{10} \)). The presence of FMDV-neutralizing antibodies was detected by a neutralization assay on LF-BK cells. Titers are reported as the group average relative fold induction for each gene at 0, 1, and 2 dpc with respect to the values obtained at 1 dpc, the inoculation day. Values of 2-fold induction or higher were arbitrarily considered upregulation. Table 1 shows that at 1 dpi (0 dpc), only the Ad5-IFN-\( \lambda \)-treated groups had upregulation of most of the analyzed ISGs. Overall, the Ad5-boIFN-\( \lambda \) group showed the highest values. Meanwhile, control animals showed upregulation of the same genes only after FMDV challenge (1 and 2 dpc). Control animals showed upregulation of IFN-\( \beta \) and its receptors

(iv) Expression of ISGs in PBMCs. It is known that both type I and type III IFNs induce the expression of ISGs, resulting in antiviral functions (3, 55). Some of these ISGs include those for chemokines (such as CCL2, CCL3, CCL20, and CXCL10), those for activators of the IFN pathway (IRF7, MDA5, and RIGI) and genes with known antiviral activity (ISG15, Mx1, OAS1, and PKR). We measured the levels of these ISGs along with IFN-\( \beta \) and IFN-\( \lambda \) by qRT-PCR using mRNA extracted from PBMCs at 0, 1, and 2 dpc. We also examined the expression of the type III IFN receptor subunits, IL-28Ra and IL-10R\( \beta \). Results were expressed as the group average relative fold induction for each gene at 0, 1, and 2 dpc with respect to the values obtained at 1 dpc, the inoculation day. Values of 2-fold induction or higher were arbitrarily considered upregulation. Table 1 shows that at 1 dpi (0 dpc), only the Ad5-IFN-\( \lambda \)-treated groups had upregulation of most of the analyzed ISGs. Overall, the Ad5-boIFN-\( \lambda \) group showed the highest values. Meanwhile, control animals showed upregulation of the same genes only after FMDV challenge (1 and 2 dpc). Control animals showed upregulation of IFN-\( \lambda \)3 and its receptors

FIG 6 Experiment II. (A) Viremia determined by qRT-PCR. FMDV RNA was detected by RT-PCR in all animals daily from 0 to 10 dpc. \( C_T \) values of 40 or lower were considered positive. (B) Lymphocyte count. The percentage of lymphocytes in whole blood was determined. Cells were counted in a Hemavet 950 analyzer and expressed as the percentage of lymphocytes with respect to the total number of white cells. *, statistical significance within a group relative to \( -1 \) dpc. (C) Neutralizing antibody titer. The presence of FMDV-neutralizing antibodies was detected by a neutralization assay on LF-BK cells. Titers are reported as the group average relative fold induction for each gene at 0, 1, and 2 dpc with respect to the values obtained at 1 dpc, the inoculation day. Values of 2-fold induction or higher were arbitrarily considered upregulation. Table 1 shows that at 1 dpi (0 dpc), only the Ad5-IFN-\( \lambda \)-treated groups had upregulation of most of the analyzed ISGs. Overall, the Ad5-boIFN-\( \lambda \) group showed the highest values. Meanwhile, control animals showed upregulation of the same genes only after FMDV challenge (1 and 2 dpc). Control animals showed upregulation of IFN-\( \lambda \)3 and its receptors

or 0 dpc) or at 1 dpc (Fig. 6D). These levels declined with time and mostly disappeared by 6 dpc. However, different dynamics were observed in the group inoculated with PBS. Control animals developed antiviral bioactivity levels by 1 dpc with a peak between 2 and 4 dpc, correlating with the levels of viremia. By 6 dpc, the levels of antiviral activity returned to baseline values (\( -1 \) dpc). As observed in experiment I, no correlation between the levels of systemic antiviral activity before challenge and the delay of disease observed thereafter was detected. In fact, animals treated with Ad5-boIFN-\( \lambda \), which displayed the longest delay of FMD onset, had the lowest levels of systemic antiviral activity at 0 dpc, 24 h after Ad5 inoculation In contrast, all animals in the Ad5-boIFN-\( \alpha \)-treated group, which had slightly higher levels of antiviral activity at 0 dpc, developed disease by 3 dpc, with the highest clinical scores (4 or 5). The combination group also showed higher levels of antiviral activity than the control group, but again no correlation with the delay or severity of disease was observed. We did not detect any antiviral activity in nasal swabs at any time point (data not shown).
peak at 1 dpc. In contrast, no or little induction of type III IFN or its receptors was observed in Ad5-IFN-treated animals either before or after the challenge. Altogether, these results indicated that the expression of similar ISGs is induced in animals treated with type I and/or type III IFN before FMDV challenge, while control animals showed similar patterns but only after challenge.

**DISCUSSION**

The role of type III IFN in conferring resistance to viral infections has been previously demonstrated in several species, including human, avian, porcine, and murine, mainly using *in vitro* models (3, 41, 44, 49). Moreover, the role of IFN-α in protection has been demonstrated *ex vivo* in primary cells of porcoid bats (54) and, more importantly, *in vivo* against human viruses, including influenza A virus, metapneumovirus, and herpesvirus (2, 33), but using animal models of viral infection. In a previous study we had shown that boIFN-α displays antiviral activity against FMDV (serotype A24) in bovine cell culture; furthermore, cattle treated with an Ad5-boIFN-α had upregulation of ISGs in multiple tissues susceptible to FMDV infection (15).

Here we demonstrate that type III IFN (boIFN-α3) significantly delays and reduces the severity of disease caused by two different FMDV serotypes (A and O) in cattle when the animals are exposed to the virus by either direct inoculation in the epithelia of the tongue, as recommended by OIE in efficacy testing (51a), or by controlled aerosolization, a viral exposure method that best resembles the natural route of infection (37). In general, there was a good correlation, within the groups of animals included in both experiments, between the delay in the appearance and the levels of viremia and viral shedding as well as in the titers of neutralizing antibodies after FMDV challenge. Upregulation of ISGs was detected in PBMCs. Interestingly animals treated with boIFN-α3 displayed the longest delay of disease in both experiments, and one animal was protected for the whole course of the experiment. Animals treated with poIFN-α alone, but not with boIFN-α, had a slight delay of the disease and in the appearance of viremia and nasal shedding compared to the control animals, which is in agreement with a previous study (52). However, porcine or bovine IFN-α treatment seemed to have little or no effect in protection against FMD when combined with boIFN-α3. In fact, at comparable doses, combination treatment resulted in a more severe clinical outcome than boIFN-α3 treatment alone (Fig. 5), results that were consistent with the data obtained with cultured EBK cells (Fig. 1). Previous studies utilizing other viruses, encephalomyocarditis virus and herpes simplex virus 2, have shown that complex interactions might occur when a combination of type I and type III IFN is used (3). A cooperative effect was seen in human HepG2 cells treated with IFN-α1 or -α2 and IFN-α only under a limited range of IFN dose combinations. However, a dose-dependent response was detected when either IFN was used alone (3). Further biochemical studies are required to understand cooperative, redundant, or synergistic effects in the antiviral properties of these cytokines. In our previous cattle study (15), we tested the activities induced by both IFNs by examining the expression of multiple ISGs in tissues by qRT-PCR. These results demonstrated that treatment with both IFNs induced enhanced expression of many ISGs compared to the levels observed after treatment with either IFN alone. In the present study, we observed that protection against or a significant delay in the appearance of FMD is clearly dependent on type III IFN. However, we could not detect a correlation between this delay and the levels of antiviral activity detected in serum on the day of challenge (0 dpc) and 1 day after (1dpc). In the first experiment, the groups of animals treated with Ad5-poiIFN-α alone or in combination with Ad5-boIFN-α3, displayed the highest levels of antiviral activity at 0 dpc, in a dose-dependent manner. The high levels detected in the animals inoculated with Ad5-poiIFN-α decreased rapidly by 1 dpc. These results suggested that detection of levels of antiviral activity in serum is not a good predictor of protection against or delay in the appearance of clinical signs of FMD in cattle. It is reasonable to hypothesize either that the method of detection of antiviral activity used in these studies is not sensitive or accurate enough to evaluate the systemic activity induced by type III IFN or that this family of IFNs preferentially induces an antiviral response locally.

### TABLE 1 Analysis of gene expression by qRT-PCR

<table>
<thead>
<tr>
<th>Combination</th>
<th>Ad5-boIFN-α</th>
<th>Ad5-boIFN-α3</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>IFN-β</td>
<td>1.5 ± 0.7</td>
<td>1.5 ± 0.6</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>IFN-α3</td>
<td>1.4 ± 0.8</td>
<td>1.4 ± 0.6</td>
<td>1.2 ± 0.7</td>
</tr>
<tr>
<td>IL-28RA</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.4</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>IL-10Rβ</td>
<td>1.6 ± 0.3</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>CCL2</td>
<td>5.3 ± 4.4</td>
<td>4.9 ± 5.2</td>
<td>1.3 ± 1.0</td>
</tr>
<tr>
<td>CCL3</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>CCL20</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>CXCL10</td>
<td>14.9 ± 10.7</td>
<td>9.3 ± 3.3</td>
<td>11.2 ± 12.1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>7.2 ± 4.3</td>
<td>2.6 ± 1.0</td>
<td>2.7 ± 1.3</td>
</tr>
<tr>
<td>ISG15</td>
<td>55.6 ± 31.6</td>
<td>12.3 ± 3.3</td>
<td>16.5 ± 2.0</td>
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<td>MDA5</td>
<td>6.6 ± 0.8</td>
<td>5.4 ± 2.1</td>
<td>4.6 ± 2.2</td>
</tr>
<tr>
<td>MX1</td>
<td>9.2 ± 1.9</td>
<td>5.4 ± 3.1</td>
<td>7.0 ± 4.5</td>
</tr>
<tr>
<td>OAS1</td>
<td>12.6 ± 3.1</td>
<td>10.0 ± 2.0</td>
<td>9.1 ± 2.9</td>
</tr>
<tr>
<td>PRK</td>
<td>6.4 ± 0.8</td>
<td>4.4 ± 1.9</td>
<td>3.8 ± 2.3</td>
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<tr>
<td>RIGI</td>
<td>17.0 ± 1.6</td>
<td>11.4 ± 4.7</td>
<td>9.6 ± 5.0</td>
</tr>
</tbody>
</table>

a RNA was extracted from PBMCs of the animals from experiment II at 0, 1, and 2 days postchallenge with FMDV by aerosol (equivalent to ~1, 0, and 1 days postinoculation with PBS or Ad5s). Gene expression was measured by RT-PCR and expressed as the group average relative fold induction compared to the values before treatment (~1 dpsi). Shading denotes >2-fold induction.
in epithelial tissues that are also susceptible to FMDV infection. We therefore tested the antiviral activities in serum by a classical bioassay, treating MDBK cells that were subsequently infected with vesicular stomatitis virus (32). Consistent with the results of the Mx-CAT ELISA, animals treated with Ad5-type I IFN showed the highest detectable levels of systemic antiviral activity at 0 dpc, in a dose-dependent manner, reaching values of 200 to 400 units/ml, while animals treated with Ad5-boIFN-λ3 alone had lower levels (50 units/ml). We had previously observed in swine studies that despite the systemic antiviral activity induced by inoculation with an Ad5-type I IFN, controlling FMD requires a local, tissue-specific response (13). Consistently, several studies have shown that IFN-λ plays a key role in the defense against pathogens that infect mucosal surfaces of the respiratory tract and intestine (3, 33, 36, 38, 49).

Indeed, in our second animal experiment we decided to study the effect of bovine type III IFN against FMD when the animals were exposed to aerosolized FMDV. This method of challenge with FMDV uses a nebulizer to administer a definite amount of virus in the upper respiratory tract, thus resembling FMDV transmission in nature or in an animal production facility (37). Furthermore, by using FMDV aerosol exposure, Arzt et al. (4) identified the nasopharynx as the initial site of virus replication. Induction of an antiviral state in this tissue should correlate with better protection against FMD. The results from our second animal experiment (aerosol challenge) supported this hypothesis and correlated with previous reports in the literature showing in other species that the antiviral activity of type III IFN protects the mucosa of the upper respiratory tract from viral infection (24, 33, 38, 50). The delay of disease in cattle challenged by aerosol with FMDV 01 Manisa lasted for at least 9 days, and one out of three animals was free of disease throughout the experiment. In contrast, treatment with boIFN-α had little or no effect on protection, delaying viremia and nasal shedding for only 1 day.

Treatment with boIFN-λ3 not only delayed clinical signs but inhibited viremia and viral shedding in nasal swabs as demonstrated by virus isolation. Utilizing a more sensitive method, qRT-PCR, we did detect viral RNA but at much lower levels than in the animals that developed clinical signs ($C_T$ values of 37 for the boIFN-λ3-treated groups versus 27 to 29 for the control or boIFN-α groups) and lasting for a shorter period of time (2 versus 4 to 5 days). Consistently, the titer of neutralizing antibodies developed in animals treated with Ad5-boIFN-λ3 was lower than that detected in the control or the Ad5-boIFN-α groups. We do not know whether the viremia detected in the animals treated with Ad5-boIFN-λ3 originated from the initial exposure to FMDV at day 0 or if the steer calves were reinfected with residual virus present in the environment after challenge. Nevertheless, it seemed that Ad5-boIFN-λ3 treatment prevented FMDV replication for several days, probably until the effect of the IFN weakened. As reported for type I IFNs (41), type III IFN may have a rapid but short antiviral activity. Therefore, treatment of cattle with a combination of type III IFN and FMD vaccine should be evaluated as a method to induce a rapid and complete protection against FMDV. Correlating with viremia and clinical disease, we observed a significant decrease in the percentage of lymphocytes in control animals by 3 and 4 dpc. Lymphopenia has also been reported in earlier studies in cattle (30) and swine (5, 14, 35) infected with FMDV. However, the mechanism involved in the induced lymphopenia in bovines and swine still remains unclear. It has been proposed that either infection of lymphocytes (14) or the presence of IFN-α in serum after FMDV infection (35) could account for the observed lymphopenia in swine. Recently, Reid et al. (40) have shown that bovine plasmacytoid dendritic cells are the major producers of IFN in response to FMDV infection and that despite the relative high levels of IFN, no lymphopenia is observed in cattle. Perhaps the lymphopenia observed in our experiment is FMDV serotype specific, as previously reported for swine (34). Alternatively, some other, unknown mechanisms related to the pathogenesis of FMDV could be involved.

Despite the lack of correlation between the delay in the appearance of disease and the systemic levels of antiviral activity, we analyzed the pattern of expression of several ISGs in PBMCs. A similar pattern of gene expression was detected in animals treated with Ad5-boIFN-α and/or Ad5-boIFN-λ3 as previously reported in bovines or other species (3, 13, 43, 53). Basically, no differences in gene expression that supported the differences in the clinical response were detected for the analyzed genes. These results suggest that type III IFN might be selectively inducing ISGs that were not measured in our experiments and that could be responsible for the observed delay of FMD. Alternatively, type III IFN could induce the localized expression of ISGs in tissues directly affected by FMDV. The expression of ISGs in PBMCs was similarly up-regulated in the control (PBS) group at 1 to 2 dpc compared to the IFN-treated groups at 0 dpc (1 dpi). Interestingly, in contrast to the case for the Ad5-boIFN-α/λ-treated animals, the mRNAs for IFN-λ3 and its receptor subunits (IL-28BRα and IL-10Rβ) were also upregulated in the control group. These results are consistent with previous reports showing that IFN-λ is the most predominant IFN induced by respiratory viruses in nasal epithelial cells (36), including in influenza A virus infection in vivo (26). Moreover, these results corroborate our previous results with primary bovine cell culture showing that FMDV infection induces the expression of IFN-λ3 (36).

Altogether, our results indicate that type III IFN is able to limit FMDV replication and spreading in cattle, one of the economically most important susceptible livestock species, especially when the virus infects using its natural route. No other biotherapeutic approach has shown such an effective activity against FMD in cattle. Although the mechanism that mediates the observed significant delay in disease onset is still unknown, type III IFN has a strong potential to be developed as a biotherapeutic strategy against FMD and possibly against other bovine viral respiratory infections.

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REFERENCES

10. Reference deleted.
19. Reference deleted.
29. Reference deleted.


