RESEARCH REPORT
SWINE HEALTH

Title: Development of an Antiviral and Vaccine Approach to Control Foot-and-Mouth Disease **- NPB #03-056**

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Abstract: We have tested the efficacy of a combined foot-and-mouth disease virus (FMDV) subunit vaccine and the antiviral molecule type I porcine interferon alpha/beta (pIFNα/β) as a method of enhancing the protection against FMD induced by a FMD subunit vaccine alone. Swine were inoculated with a low or high dose of replication-defective human adenovirus type 5 vector containing the capsid and 3C proteinase coding regions of FMDV (Ad5-A24 subunit vaccine) and/or an Ad5 vector containing pIFNα (Ad5-pIFNα) and challenged 42 days later with virulent FMDV. Inoculation of groups of swine with a high dose of Ad5-A24 protected 1 of 5 animals and the remaining animals had very limited disease. Four of 5 swine given both a high dose of Ad5-A24 and Ad5-pIFNα were protected from disease, while the fifth animal only had a lesion at the site of challenge. Swine given a 10-fold lower dose of Ad5-A24 developed severe disease, while the group given the low dose of Ad5-A24 and Ad5-pIFNα had delayed and less severe disease. Furthermore, the addition of pIFN-α to the low dose vaccine inoculated group reduced the level of viremia as compared to the group that received only low dose vaccine. These results indicate that IFN-α acts as an adjuvant to enhance the vaccine induced adaptive immune response. Further work to identify the mechanism of the IFN-induced enhancement of protection is essential and should aid in the development of improved disease control strategies for FMDV and potentially other acute infectious diseases.

We have also performed preliminary experiments to determine if pIFN-β can provide rapid protection against FMD. We showed that an Ad5-pIFNβ vector induces an antiviral response in swine, but have not yet tested the efficacy of this vector against challenge with FMDV. Furthermore, we are in the process of producing the reagents necessary to quantitatively detect the expression of pIFNβ.

Introduction: Foot-and-mouth disease (FMD), a highly infectious and rapidly spreading viral disease of cloven-hoofed animals, is economically the most important disease of domestic animals, and a serious threat to U.S. agriculture. The presence of FMD in a country can have severe economic consequences from the loss of animal productivity and imposition of trade embargoes as well as effects on other aspects of the economy. Although FMD has not occurred in the U.S. since 1929, it is still a major problem in many parts of the world including South America, Africa, and parts of Asia, and has recently spread to developed countries in Asia which have been free of FMD for many years and to western Europe. For example the outbreak of FMD in the Spring of 1997 in Taiwan, which had been FMD-free for 68 years, resulted in the eventual death or destruction of approximately four million swine, and a multibillion dollar loss due to cleanup costs and lost export revenue. Currently Taiwan has still not achieved FMD-free status. In the Spring of 2000 more limited outbreaks occurred in South Korea and Japan, countries which had been free of FMD for many decades. Most recently an outbreak of FMD occurred in the United Kingdom in February, 2001. During this outbreak the U.K. did not vaccinate, but instead slaughtered over 6.5 million animals. The total cost to the economy was over $15 billion U.S. This outbreak also spread to the European continent including the Netherlands which controlled the outbreak by vaccination and slaughter, although all vaccinated animals were subsequently slaughtered. The slaughter of large numbers of infected and susceptible in-contact animals in the U.K. and vaccinated animals in the Netherlands raised considerable public concern.

The U.S. FMD policy includes vaccination as a control strategy. Whole virus inactivated vaccines are used in FMD control programs, and it has been estimated that worldwide more than a billion doses are used annually. There are seven distinct serotypes of FMDV and numerous subtypes. Current FMD vaccines require production of large quantities of live virus in high containment facilities followed by imine inactivation. These vaccines have been successfully utilized in controlling the disease. However, there are a number of concerns with the use of the inactivated vaccine in disease-free countries, including, the inability to reliably differentiate vaccinated from infected animals with currently approved diagnostic tests, the inability to rapidly induce protection, and the need for expensive high-containment facilities to produce vaccine (the U.S. can not produce FMD vaccine since federal law prohibits the use of FMDV in this country except at the Plum Island Animal Disease Center (PIADC)). Other concerns with the vaccine include selection of antigenic variants upon passage of virus in cell culture for preparation of vaccine stocks. At the present time the PIADC houses the North American FMD Vaccine Bank that includes small amounts of antigen for some of the different serotypes. These reserves are available for an outbreak in the U.S., Canada, or Mexico, but would not be sufficient to control a wide-spread outbreak. To supply the North American FMD Bank, vaccine antigen has been purchased from companies in Western Europe. As a consequence of the above concerns, alternate vaccine approaches are being investigated.

Our approach towards development of a safe, effective and rapidly-acting FMD control strategy is to produce viral subunit vaccines and antivirals utilizing genetic engineering technology. This approach is based on the demonstration that empty viral capsids, virus particles lacking infectious nucleic acid, are naturally produced in infected cells and are indistinguishable from infectious virus in their ability to stimulate an immune response and the known sensitivity of FMDV replication to IFNα/β. The

advantages of this approach include, 1) no infectious FMDV required or produced, 2) no tissue culture passage of FMDV required, 3) no large scale biocontainment facilities needed for production, 4) the possibility of producing this type of vaccine on the U.S. mainland, 5) the ability to easily distinguish vaccinated from infected or convalescent animals using current technology, which detects a viral nonstructural protein that is not encoded in our subunit vaccine, and 6) the ability of IFNα/β to rapidly induce a protective response against all FMDV serotypes.

Objectives: The objective of this proposal is to develop a combination strategy including prophylactic antiviral treatment with IFN-α/β and co-administration of a FMD empty viral capsid subunit vaccine to induce rapid as well as long-lasting protection against FMDV. Specifically, replication-defective recombinant human Ad5 vectors containing pIFN-α or pIFN-β genes and an Ad5 vector containing the FMDV serotype A24 (Ad5-A24) capsid coding region and serotype A12 3C proteinase coding region will be used to inoculate swine. The ability of the IFN proteins to induce a protective antiviral response as well as their ability to enhance the protective response induced by the co-administered FMD vaccine will be examined.

Materials and Methods: In the animal experiment in Objective A, 5 groups, 5 animals per group, each group housed in a separate room, were inoculated IM with $5x10^9$ or $5x10^8$ pfu/animal of Ad5-A24 in the presence of $1x10^9$ pfu Ad5-pIFNα or $1x10^9$ pfu Ad5-Blue/animal, or a control group inoculated with $6x10^9$ pfu of Ad5-VSVG/animal (Table 1). The animals were bled prior to inoculation, daily for the first 7 days, and weekly until the day of challenge. Plasma samples were assayed for antiviral activity and pIFN-α expression by ELISA. Serum samples were assayed for an FMDV-specific neutralizing antibody response by a plaque reduction neutralization assay, antibody isotype by ELISA, and induction of antibodies against viral specific proteins by radioimmunoprecipitation (RIP) and ELISA. We also assayed for a number of IFN induced mRNAs including double-stranded RNA-dependent protein kinase (PKR), 2'-5' oligoadenylate synthetase(OAS)/RNase L, and Mx mRNA as well as IFN-α and IFN-β mRNAs by real-time RT-PCR using RNA extracted from peripheral blood mononuclear cells (PBMC's) obtained daily for 7 days after Ad5 vector inoculation At 42 dpv all animals were challenged by direct inoculation in the heel bulb of both rear feet with a total of 10^5 50% bovine infectious doses of animal-derived A24. The animals were monitored clinically and serologically for 21 days postchallenge (dpc). Blood samples and nasal swabs were obtained daily for the first 7 dpc to determine viremia and virus shed, respectively. Serum samples were obtained weekly to assay for neutralizing antibody response and virus specific antibodies by RIP.

In the animal experiment outlined in Objective B, swine, one animal per dose, were inoculated IM with $1x10^9$ or $5x10^9$ pfu Ad5-pIFNβ or $5x10^9$ pfu Ad5-VSVG. Antiviral activity was measured by a plaque-reduction neutralization assay. To further characterize the antiviral response induced by pIFN-β we have also initiated a program to produce reagents necessary to develop a pIFN-β specific ELISA including purified pIFN-β and pIFN-β-specific monoclonal antibodies. We have produced amino- and carboxy-terminal peptides of pIFN-β and affinity purified rabbit polyclonal antisera against each peptide. We have constructed a bacterial expression plasmid containing the pIFN-β gene and produced *E. coli* expressed pIFN-β. We have also expressed significant amounts of the protein in a mammalian expression system infected with Ad5-

pIFNβ. Using the above reagents we have developed an ELISA to screen for pIFN-β positive monoclonal antibodies.

Results: Objective A: Immunomodulatory role of pIFN-α.
All animals in the groups that received Ad5-pIFNα developed a significant antiviral response in plasma by 1 day postvaccination which lasted for an additional 1-2 days (Table 2). All these animals had between 6-25,000 pg/ml pIFN-α on the first day and the protein was still detectable for 2-3 additional days. We measured the level of induction of IFN-α and IFN-β mRNA as well as a number of IFN induced genes in PBMC's after inoculation with the different Ad5 vectors. The groups that received Ad5-pIFNα had 2.5-3.5-fold increased levels of PKR and OAS, 5-7-fold increased levels of Mx, and about 15-fold increased levels of IFN-β mRNAs in PBMC's by one day postvaccination as compared to non-Ad5- pIFNα vector inoculated animals. However, there was little induction of IFN-α mRNA. We have previously shown that IFN-induced proteins that inhibit FMDV replication include PKR and to a lesser extent OAS/RNase L (Chinsangaram et al., J. Virol. 5498-5503, 2001). Thus, the groups that received IFN-α had higher levels of induction of IFN-induced genes that are known to inhibit FMDV replication than the groups given the non-Ad5-pIFNα vectors.

The groups given the high dose of Ad5-A24 had significantly higher levels of FMDV-specific neutralizing antibodies than the groups given the low dose of vaccine (Table 2). However, addition of Ad5-pIFNα did not significantly increase the FMDV-specific neutralizing antibody response in either the high or low dose Ad5-A24 inoculated animals.

After challenge the Ad5-A24 high dose-inoculated animals were either completely protected (Table 3, #3868 no lesions) (Moraes et al., Vaccine 1631-1639, 2002) or the 4 remaining animals in this group had significantly reduced disease as compared to the control group (Table 3, compare Groups 4 and 1). Furthermore, the one lesion present on swine 3865 and 3866 and the 2 lesions on #3869 were at the site of challenge and thus did not spread systemically, while #3867 had one lesion at the site of inoculation and the second lesion on a front foot indicating limited systemic spread. The addition of Ad5-pIFNα improved the efficacy of the vaccine, ie., 4 of 5 animals were completely protected and the other animal had only 1 lesion at the site of inoculation (Table 3, Group 2). None of the animals in groups 2 or 4 had detectable viremia.

Likewise, addition of Ad5-pIFNα to the low dose Ad5-A24 group delayed the onset of disease and reduced disease severity, although all 5 animals had systemic disease spread (Table 3, compare Groups 3 and 5).
Addition of pIFN-α to the Ad5-A24 low dose inoculated group significantly reduced the level of viremia. In the low dose vaccine group, 3 animals had viremia for 2-3 days, while only 1 animal had detectable viremia for 1 day in the group given pIFN-α and low dose vaccine.

Objective B: Ability of pIFN-β to rapidly protect swine from FMDV infection.

Antiviral activity was only detected in the animal given the high dose of Ad5-pIFNβ, and not the low dose or control vector inoculated animals (Table 4). The level of antiviral activity induced by Ad5-pIFNβ is lower than that induced by Ad5-pIFNα (Chinsangaram et al., J. Virol. 1621-1625, 2003). To attempt to enhance the level of expression of

pIFNβ and other foreign genes in general, we have previously constructed second-generation Ad5 vectors containing improved promoters necessary for increased expression of foreign genes. We have constructed a second-generation Ad5-pIFNβ virus and are currently examining for expression in infected IBRS2 cells.

To obtain the antibodies required to detect pIFN-β in inoculated animals by ELISA we followed two tracts. To rapidly produce antisera we designed peptides to the amino and carboxy termini of the protein and produced rabbit polyclonal antibodies to these peptides. We obtained pIFN-β from the supernatants of IBRS2 cells infected with Ad5-pIFNβ and demonstrated that both antibodies reacted with the expressed protein and designed a capture ELISA that detected the protein in Ad5-pIFNβ inoculated animals. Concurrently we have collaborated with Dr. Antonio Garmendia, University of Connecticut at Storrs, to produce monoclonal antibodies against the pIFN-β. We provided Dr. Garmendia with a mammalian expression vector containing the pIFN-β gene, ie., pCI-pIFNβ. Dr. Garmendia inoculated a number of mice with the plasmid and demonstrated that after several inoculations the mice had high titer antibody against pIFN-β. He has performed several fusions and using the ELISA we developed with the pIFN-β polyclonal antibodies we detected a number of clones that are expressing positive sera. Dr. Garmendia is currently in the process of expanding and cloning these positive antibody-secreting cells.

We have also expressed pIFN-β as a soluble protein in an E. coli expression system and have partially purified the protein by His-Tag column chromatography. We are currently attempting to purify the protein by affinity chromatography and will use this protein with the monoclonal antibodies or the antipeptide sera to quantitate the amount of protein in Ad5-pIFNβ inoculated animals.

Discussion: We have previously demonstrated that one high dose of Ad5-A24 vaccine, ie., $5x10^9$ pfu/ml, completely protected swine from direct inoculation challenge as early as 7 days postchallenge and as long as 42 days later. We also demonstrated that inoculation of swine with Ad5-pIFNα can afford complete protection when the animals are challenged with virulent FMDV as early as one day postinoculation or as long as 3-5 days postinoculation. The combination of the Ad5-A24 subunit vaccine and the Ad5-pIFNα antiviral completely protects animals challenged with FMDV 5 days postinoculation. IFNα/β induces a number of genes whose protein products are able to block virus replication. In cell culture studies we have shown that two of these induced proteins, PKR and to a lesser extent OAS/RNase L, can block FMDV replication.

In the current study we demonstrated a direct correlation between inoculation of swine with Ad5-pIFNα, the presence of pIFN-α in the plasma and the induction of the mRNAs encoding the proteins that have FMDV antiviral activity. Although the swine in this experiment were not challenged directly after administration of the Ad5 vectors, the results strongly suggest that these proteins are involved in inhibition of FMDV replication in susceptible animals.

Studies indicate that IFN-α/β in addition to directly inducing an antiviral response has an immunomodulatory role and can enhance the humoral immune response to soluble antigens as well as act as an adjuvant when administered with a human influenza subunit vaccine. The aim of Objective A was to examine the potential adjuvant effect of pIFN-α in conjunction with our FMD subunit vaccine. The results indicate that pIFN-α enhanced the efficacy of the vaccine, although the mechanism of action is unclear. In this study we only examined the neutralizing antibody response to

our subunit vaccine and did not find a significant enhancement in the presence of pIFN-α. In future studies we plan to examine various parameters of the cell-mediated and innate immune responses to determine their possible role in IFN-α induced protective enhancement.

In Objective B, our ongoing studies with pIFN-β have yielded very promising results. We are in the process of generating the reagents needed to directly assess the role of pIFN-β as an antiviral reagent for FMDV as well as use these reagents along with the reagents we have generated to more completely understand FMDV-host interaction in swine. These studies will allow us to improve the novel FMD control strategies we have developed and potentially apply these methods to combat other acute viral diseases of swine.

Lay Interpretation: Foot-and-mouth disease virus (FMDV) causes a highly contagious disease of cloven-hoofed animals including swine, cattle, sheep and goats. The economic impact of an FMD outbreak can be devastating as demonstrated by the 1997 and 2001 outbreaks in Taiwan and the United Kingdom, respectively. Although an FMD vaccine is available, its use in the event of an outbreak in a previously FMD-free country such as the U.S. is uncertain because of a number of drawbacks. We have previously developed an subunit FMD vaccine that overcomes many of these limitations and recently combined this subunit vaccine with an antiviral approach, using interferon alpha, to induce very rapid protection. In this study we have demonstrated that delivery of the combination of our FMD subunit vaccine and interferon alpha can enhance the long-term protection afforded swine with the vaccine alone. Furthermore, we are developing additional antiviral reagents, ie., interferon beta, that may allow us to supplement our current approach as well as develop a more comprehensive understanding of the interactions between FMDV and its host.

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TABLE 1
Experiment Design to Examine the Potential Immunomodulatory Role of pIFN-α

Group	Animal #	Inoculum[a]	Dose (pfu)	Challenge[b]
1	3875-3879	Ad5-VSVG	$6x10^9$	42 dpv
2	3855-3859	Ad5-A24 + Ad5-pIFNα	$5x10^9$ $1x10^9$	42 dpv
3	3860-3864	Ad5-A24 + Ad5-pIFNα	$5x10^8$ $1x10^9$	42 dpv
4	3865-3869	Ad5-A24+ Ad5-Blue	$5x10^9$ $1x10^9$	42 dpv
5	3870-3874	Ad5-A24+ Ad5-Blue	$5x10^8$ $1x10^9$	42 dpv

[a] Animals were inoculated IM in the neck with 1 ml of the indicated dose of Ad5-vector.
[b] All animals were challenged by intradermal inoculation in the heel bulb of the rear feet with 10^5 bovine infectious $doses_{50}$ of animal-derived FMDV A24.

TABLE 2

Serological Response after Ad5 Vector Administration

Group	Animal #	PRN_{70}[a]	Antiviral Activity[b]	pg/ml[c]
1	3875-3879	<8,<8,<8,<8,<8	<25,<25,<25,<25,<25	246, 0, 0, 0, 63
2	3855-3859	512,256,128,64,256	800,200,200,200,200	22,875, 8725, 7175, 6300, 9575
3	3860-3864	16,16,16,32,32	800,200,400,400.400	22395, 9870, 8395, 11,395, 24320
4	3865-3869	256,256,64,256,128	<25,25,25,25,25	0, 0, 0, 0, 0
5	3870-3874	8,8,8,8,32	25,<25,<25,<25,<25	0, 0, 0, 0, 0

[a] Neutralizing antibody titer at day of challenge
b Highest dilution that reduced FMDV A12 plaque number by 50% in IBRS2 cells from 1 day postinoculation plasma samples
[c] pg/ml by ELISA 1 day postinoculation

TABLE 3
Serological and Clinical Response after FMDV Challenge

Group	Animal #	Viremia[a]	Lesion Score[b]
1	3875-3879	$2.2x10^4$/3, $6.8x10^4$/3, $1.6x10^5$/3, $4.8x10^3$/3, $1.7x10^4$/3	15,16,16,17,16
2	3855-3859	0,0,0,0,0	0,0,0,0,1
3	3860-3864	$2.5x10^2$/4,0,0,0,0	5,16,2,14,13
4	3865-3869	0,0,0,0,0	1,1,2,0,2
5	3870-3874	0, $7.3x10^2$/3, $7.3x10^4$/3, $9.5x10^3$/4, 0	13,17,17,17,8

[a] maximum pfu/ml/ day postchallenge
[b] The lesion score is the number of digits and snout on which the swine exhibited lesions when examined at 15 dpc.

TABLE 4

Dose-Response of Swine to Ad5-pIFNβ

Inoculum[a]	Dose (pfu)	Antiviral activity of plasma samples[b]					
		0 dpi	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi
Ad5-VSVG	$5x10^9$	<25	<25	<25	<25	<25	<25
Ad5-pIFNβ	$1x10^9$	<25	<25	<25	<25	<25	<25
Ad5-pIFNβ	$5x10^9$	<25	200	100	<25	25	<25

[a] Animals were inoculated IM in the neck with 1 ml of the indicated dose of Ad5-vector.
[b] Highest dilution that reduced FMDV A12 plaque number by 50% in IBRS2 cells.