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Atividade do interferon tipo I suíno na proteção contra o vírus da

febre aftosa (FMDV)

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Sônia de Avila Botton

PPGMV

Santa Maria, RS, Brasil

2005

ATIVIDADE DO INTERFERON TIPO I SUÍNO NA PROTEÇÃO CONTRA O VÍRUS DA FEBRE AFTOSA (FMDV)

por

Sônia de Avila Botton

Tese apresentada ao Curso de Doutorado do
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ATIVIDADE DO INTERFERON TIPO I SUÍNO NA PROTEÇÃO CONTRA O VÍRUS DA FEBRE AFTOSA (FMDV)

elaborada por
Sônia de Avila Botton

como requisito parcial para obtenção do grau de
Doutor em Medicina Veterinária

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Tese de Doutorado

Programa de Pós-graduação em Medicina Veterinária

Universidade Federal de Santa Maria, RS, Brasil

ATIVIDADE DO INTERFERON TIPO I SUÍNO NA PROTEÇÃO CONTRA O VÍRUS DA FEBRE AFTOSA (FMDV)

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Santa Maria, 8 de março de 2005.

Esse trabalho tem como objetivo avaliar o efeito adjuvante do interferon alfa suíno em animais imunizados com uma vacina recombinante de um adenovírus defectivo contendo as regiões codificadoras das proteínas do FMDV, bem como investigar alguns dos aspectos moleculares envolvidos na interação FMDV e a célula hospedeira em uma espécie susceptível. Na primeira fase do trabalho, o efeito adjuvante do interferon alfa suíno (pIFNα) foi avaliado em suínos imunizados com uma vacina recombinante, tendo como vetor o adenovirus humano tipo 5 (Ad5), contendo regiões do capsídeo do FMDV A24 e da proteinase 3C^{pro} do FMDV A12 (Ad5-A24). Os suínos foram separados em 5 grupos e inoculados com baixa (5×10^8 PFU) e alta (5×10^9 PFU) dosagem de Ad5-A24 na presença ou na ausência de pIFNα (Ad5pIFNα, 10^9 PFU). O grupo controle foi inoculado com 6×10^9 PFU da glicoproteína do vírus da estomatite vesicular (VSV) cepa *New Jersey* (Ad5VSNJV-G). Todos os suínos foram desafiados aos 42 dias pós-vacinação (dpv) com FMDV-A24. Após a inoculação, as amostras de sangue foram examinadas para a produção de IFN, a indução de genes induzidos pelo IFN e os anticorpos neutralizantes e resposta de imunoglobulinas específicos para o FMDV. Depois do desafio um número de parâmetros foram analisados incluindo a avaliação clínica, viremia, linfopenia; além dos anticorpos contra as proteínas estruturais e não estruturais do FMDV. Os resultados obtidos indicam que ambos os grupos que receberam Ad5-A24 em alta dosagem desenvolveram níveis de anticorpos neutralizantes pelos 14-21 dpv, que foram mantidos até o dia do desafio. Os níveis de IgG1 foram maiores que de IgG2 nesses dois grupos, sendo que a IgG1 é considerada a mais relevante para

conferir proteção ao FMDV. Dentre esses grupos, o que recebeu o IFN apresentou níveis significativamente mais altos desta imunoglobulina. Atividade antiviral e o IFNα foram detectados nos animais que receberam o IFN. A respeito da presença dos genes induzidos pelo IFN nos leucócitos dos suínos vacinados com Ad5-pIFNα, todos os três genes incluídos neste estudo, PKR, OAS e Mx1, foram detectados pelo real time RT-PCR. Após o desafio todos os animais do controle desenvolveram viremia, linfopenia, lesões vesiculares e os anticorpos contra as proteínas não estruturais do FMDV. Os animais que receberam baixa dose de Ad5-A24 sem IFN tiveram sinais clínicos similares, exceto que poucos animais desenvolveram viremia. Porém, os suínos inoculados com a mesma dose da vacina de Ad5-A24 com o IFN apresentaram as lesões vesiculares com início tardio e somente um animal teve detectável viremia. Os animais vacinados com a alta dose de Ad5-A24 sem IFN não tiveram nenhuma viremia e poucas lesões foram detectadas tardiamente após a inoculação do FMDV. Quatro dos cinco suínos, que receberam a alta dose da vacina com o IFN, foram protegidos da doença e somente um animal neste grupo teve uma lesão vesicular, restrita ao local do inoculação do vírus por ocasião do desafio. Esses resultados indicam que IFNα realça o nível da proteção induzido pela vacina do adenovírus-FMD contra o FMDV homólogo, suportando o uso do IFNα como um adjuvante potencial em estratégias de vacinação de FMD. Para avaliar os efeitos da infecção pelo vírus da FMD na indução da resposta de IFNα/β do hospedeiro, células de origem suína foram previamente infectadas em diferentes multiplicidades de infecção com o FMDV e com um vírus mutante que teve o gene que codifica a protease L ou L^{pro} deletado, o FMDLLV. A síntese de mRNA do IFNα e β bem como de três dos genes induzidos pelo IFN, PKR, OAS e Mx1 foram avaliados por real time RT-PCR. A infecção das células pelo FMDLLV induziu altos níveis de mRNA do IFNβ quando comparados com os do FMDV original. Contudo, não foi possível detectar os níveis de mRNA do IFNα na presença de ambos os vírus. O aumento nos níveis de mRNA do IFNβ foi relacionado ao aumento nos níveis de indução dos mRNAs de PKR, OAS e Mx1, assim como dos altos níveis de atividade antiviral. Pelo uso da tecnologia de interferência do RNA, usando siRNA (*silencing* RNA) para bloquear a expressão do mRNA da PKR, foi possível demonstrar que o título do FMDLLV aumentou cerca de 200 vezes. Desta forma foi possível confirmar o papel desta proteína como um inibidor da replicação do FMDV. Os resultados obtidos demonstram que a L^{pro} tem um importante papel na regulação da resposta imunológica inata do hospedeiro quando da infecção pelo FMDV em vários níveis. Estudos anteriormente realizados indicaram que o controle era efetuado ao nível da tradução pela clivagem do eIF4G. Os dados obtidos

neste trabalho indicam que a regulação também ocorre ao nível da transcrição e pela inibição da indução do IFNβ através de um mecanismo ainda não conhecido.

Palavras-chave: Febre aftosa; FMDV; Adenovírus; Interferon; Vacina; Proteína líder; L^{pro}.

ABSTRACT

Doctoral Thesis

Graduate Program in Veterinary Medicine

Federal University of Santa Maria, RS, Brazil

ACTIVITY OF SWINE TYPE I INTERFERON IN PROTECTION AGAINST FOOT-AND-MOUTH DISEASE VIRUS (FMDV)

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ADVISER: RUDI WEIBLEN

Santa Maria, March, 8th, 2005.

The objective of this study is to evaluate the adjuvant effect of interferon alpha (IFNα) in swine vaccinated with a recombinant replication-defective adenovirus containing foot-and-mouth disease virus (FMDV) protein coding regions, as well as to understand the molecular mechanisms involved in the interaction of FMDV with its host. In the first part of this thesis, the adjuvant effect of pIFNα was evaluated in swine vaccinated with a recombinant vaccine delivered by a human adenovirus type 5 (Ad5) vector containing FMDV capsid (P1-2A) and 3C^{pro} proteinase coding regions (Ad5-A24). Swine were separated into 5 groups and inoculated with low ($5x10^8$ PFU) or high ($5x10^9$ PFU) doses of Ad5-A24 in the presence or absence of pIFNα (Ad5-pIFN, 10^9 PFU). Control animals received $6x10^9$ PFU of an Ad5 vector containing the glycoprotein gene of vesicular stomatitis virus (Ad5-VSNJV-G). All swine were challenged at 42 days post vaccination (dpv) with FMDV-A24. Prior to challenge, blood samples were examined for IFN production, induction of IFN-induced genes (ISG's), FMDV-specific neutralizing antibodies and FMDV-specific antibody isotypes. After challenge, a number of parameters were analyzed including clinical score, viremia, lymphopenia and antibodies against FMDV structural (S) and non-structural (NS) proteins. The results indicate that both groups that received high-dose Ad5-A24 developed an FMDV-specific neutralizing antibody response by 14-21 dpv, which was maintained until the day of challenge. Both high-dose groups developed high levels of IgG1 and IgG2, however the IgG1 response was higher. The high-dose Ad5-A24 with IFN group developed higher levels of

IgG1 than the group administered only high-dose Ad5-A24 and this difference was statistically significant. Antiviral activity and IFNα were detected in the groups that received IFN. The three ISG's examined, PKR, OAS and Mx1, were detected by real time RT-PCR in leukocytes from Ad5-pIFNα-vaccinated swine. After challenge, all animals in the control group developed early viremia, vesicular lesions, considerable lymphopenia and antibodies to FMDV NS proteins. The animals that received low-dose Ad5-A24 without IFN had similar clinical signs, except that fewer animals had viremia. In contrast, pigs inoculated with the low-dose Ad5-A24 and IFNα had a delayed onset of vesicular lesions and only one animal had detectable viremia. Animals vaccinated with high-dose Ad5-A24 without IFNα had no viremia, showed fewer lesions, one animal had no lesions, and delayed onset of disease compared to the low-dose Ad5-A24 groups. Four of five pigs vaccinated with high-dose Ad5-A24 and IFNα were completely protected from disease and only one animal in this group had a vesicular lesion restricted to the site of challenge virus inoculation. The results indicate that IFNα enhances the level of protection induced by the Ad5-FMD vaccine against homologous FMDV, supporting the use of IFNα as a potential adjuvant in FMD vaccination strategies. To investigate the effect of FMDV infection on the induction of the host IFN-α/β response, swine cells were infected with wild-type (WT) FMDV and a mutant FMDV lacking the L proteinase (L^{pro}) coding region (A12-LLV) at different multiplicities of infection. The synthesis of IFN-α and IFN-β mRNAs and three well characterized ISG's, PKR, OAS, and Mx1 mRNA, were evaluated by real time RT-PCR. A12-LLV infection resulted in significantly higher levels of induction of IFN-β mRNA as compared to WT virus infected cells, while IFN-α mRNA was not induced after either infection. The increased levels of IFN-β mRNA in A12-LLV-infected cells correlated with higher levels of induction of PKR, OAS and Mx1 mRNAs and antiviral activity. By using RNA interference (RNAi) technology to knock-down PKR mRNA expression, it was possible to demonstrate that the yield of A12-LLV was increased up to 200-fold, supporting the role of PKR as an inhibitor of FMDV replication. These results confirm that L^{pro} down regulates the innate immune response to FMDV infection at multiple levels. Previous studies indicated that control was at the translation initiation level by L^{pro} cleavage of translation initiation factor eIF-4G. The present data demonstrates that regulation also occurs at the level of transcription by inhibition of IFN-β mRNA induction through an unknown mechanism.

Keywords: Foot-and-mouth disease; FMDV; Adenovirus; Interferon; Vaccine; Leader protein; L^{pro}.

INTRODUÇÃO

A febre aftosa (*Foot-and-mouth disease* ou FMD) é uma doença vesicular de caráter agudo e altamente contagioso que afeta bovinos, suínos, ovinos e outros animais domésticos e selvagens biungulados (CALLIS & MCKERCHER, 1986, HOUSE & HOUSE, 1999). Os surtos de FMD comumente produzem drásticos efeitos econômicos e sociais para países considerados livres da enfermidade, devido aos custos diretos e indiretos que na maioria das vezes estão relacionados aos embargos sofridos pelo comércio internacional de animais e produtos de origem animal (MATTHEWS, 2001).

A enfermidade é causada por um vírus RNA de fita simples e polaridade positiva (*Food-and-mouth disease virus* ou FMDV) pertencente ao gênero aftovírus da família *Picornaviridae* (FENNER et al., 1993, RODRIGO & DOPAZO, 1995, RUECKERT, 1996). O vírus consiste de sete sorotipos denominados O, A, C, SAT1, SAT2, SAT3 e ASIA1 (MURPHY, 1996) Existe uma considerável diversidade antigênica dentro de cada sorotipo, particularmente no tipo A (KITCHING et al., 1989, De CLERCQ, 2001, DOEL, 2003). O sorotipo A foi identificado como o agente causador da maioria dos surtos de FMD na Argentina, Uruguai e Sul do Brasil ocorridos em 2000 e 2001 (KNOWLES & SAMUEL, 2003, SUTTMOLLER et al., 2003). Esta cepa tem sido incluída nas vacinas em diversos países na América do Sul (DOEL, 2003, KNOWLES & SAMUEL, 2003).

O genoma do FMDV contém uma única fita de RNA que é circundado por um capsídeo icosaédrico composto por 60 cópias de quatro proteínas estruturais: VP1 (1D), VP2 (1B), VP3 (1C) e VP4 (1A) (GRUBMAN & BAXT, 2004, RUECKERT & WIMMER, 1984, RUECKERT, 1996). O RNA genômico contém uma longa ORF (*open reading frame* ou seqüência aberta de leitura) a qual é traduzida em uma poliproteína, que é processada em várias proteínas maduras (estruturais e não-estruturais) através das proteases codificadas pelo próprio vírus durante a tradução bem como após a mesma (CHINSANGARAM, et al., 1998, MASON et al., 2003a, RUECKERT, 1996). A protease L, ou proteína *leader*, é a primeira a ser traduzida, cliva ela mesma da poliproteína na sua porção carboxi-terminal e também cliva o fator de iniciação da tradução do hospedeiro (eIF4G *ou eucariotic initiator factor*), resultando na supressão da tradução dos RNA mensageiros (RNAm) do hospedeiro e tradução dos mRNAs virais via *internal ribossome entry site* (IRES) que não necessita do eIF4G intacto (CHINSANGARAM et al., 2001, DEVANEY et al., 1988, MASON et al., 1997, PICCONE et al., 1995).

Nos estudos realizados por CHINSANGARAM et al. (1999) e GRUBMAN & CHINSANGARAM (2000) foi proposto que a protease L é um fator de virulência do FMDV, uma vez que ela cliva o eIF4G e conseqüentemente suprime a síntese dos interferons tipo I (IFNα e IFNβ). A supressão da produção IFN permite a rápida replicação e disseminação do FMDV nas células do hospedeiro. No entanto, um mutante do FMDV que perdeu o gene que expressa a protease L, denominado de *Leaderless vírus* (FMDLLV) obteve uma replicação ineficiente em células capazes de sintetizar ambos IFNα e β (CHINSANGARAM et al., 1999, 2001, GRUBMAN & CHINSANGARAM, 2000).

Muitos experimentos têm demonstrado que os interferons constituem a primeira linha de defesa da célula hospedeira contra muitas das infecções virais (BIRON & SEN, 2001, HORISBERGER, 1995, MULLER et al., 1994, SAMUEL, 2001). Momentos após a infecção viral, as células infectadas são induzidas a expressar e secretar IFN α/β. O interferon secretado liga-se ao seu receptor específico na superfície celular, ativando os elementos que participam nos mecanismos moleculares da "cascata" do interferon. Desta forma, tanto as células infectadas quanto as células vizinhas tornam-se resistentes ao vírus devido a uma série de eventos que induzirão os genes estimulados pelo interferon (ISGs ou *IFN α/β-stimulated genes*) (BIRON, 1998, BIRON & SEN, 2001, HORISBERGER, 1995,VILCEK & SEN, 1996).

Os ISGs têm sido amplamente caracterizados e incluem principalmente os seguintes genes: proteína kinase dependente da dupla fita de RNA (PKR), 2'-5' oligoadenilato-sintetase (OAS) que por sua vez ativa uma nuclease latente, a RNAse L, e o gene da GTPase Mx (ou proteína Mx). Os produtos desses genes podem ser considerados os mediadores dos efeitos biológicos dos interferons. Eles têm a capacidade de afetar os vírus em diferentes estágios do seu ciclo de replicação, bem como os diferentes vírus são susceptíveis aos diferentes produtos dos ISGs (CHINSANGARAM et al., 2001, HORISBERGER, 1995, SAMUEL, 2001, VILCEK & SEN, 1996).

O efeito inibitório do IFN α/β sobre o FMDV pode ser presumido pela ligação ao receptor do IFN α/β iniciando uma série de eventos que levam à ativação dos ISGs dentro das células hospedeiras (CHINSANGARAM et al., 2001, SAMUEL, 2001, VILCEK & SEN, 1996). O bloqueio da replicação do FMDV *in vitro* ao nível da tradução protéica foi investigado por CHINSANGARAM et al. (2001), o qual sugeriu que a PKR desempenha um importante papel no bloqueio da replicação viral. O efeito inibitório da PKR foi constatado através do uso de um inibidor da PKR, a 2-aminopurina sobre células de cultivo secundárias,

havendo um aumento do título viral na presença dessa substância. O mecanismo celular antiviral induzido pelo IFN envolvido na inibição da replicação do FMDV foi investigado em células derivadas de fibroblastos de camundongos *knock-out* para o ISGs. Tais estudos sugerem um importante papel exercido pela PKR na resposta da célula hospedeira à infecção pelo FMDV, interferindo na replicação viral.

Os mecanismos moleculares que interferem na replicação do FMDV *in vivo* não estão bem estabelecidos até o presente momento, ressaltando-se a importância de se encontrar um sistema *in vitro* que mimetize o que aconteceria com o hospedeiro natural desse vírus. Dessa forma possibilitaria a pesquisa dos mecanismos moleculares envolvidos na replicação do FMDV. O suíno tem sido o alvo de importantes estudos sobre FMD, por ser um dos hospedeiros naturais do vírus e também um significante fator na disseminação da doença (HOUSE & HOUSE, 1999, SUTMOLLER et al., 2003). Constituindo-se de um excelente modelo experimental para a pesquisa de FMDV. As células oriundas dessa espécie têm sido bastante utilizadas na pesquisa em diferentes aspectos da infecção pelo FMDV.

As medidas para conter os surtos de FMD incluem o controle do movimento de animais, sacrifício dos animais infectados e dos animais em contato, a desinfecção e vacinação (MASON et al., 2003b).

Atualmente, vacinas convencionais inativadas por etilenamina binária (*binary ethyleneimine* ou BEI) e emulsificadas com adjuvantes têm sido amplamente usadas em programas de controle e erradicação da doença em muitos países na América do Sul, África e Ásia. Contudo, países considerados livres de FMD mantêm estas vacinas somente para uso em casos emergenciais (BROWNLIE, 2001, DOEL, 2003). As vacinas convencionais requerem que haja o crescimento e inativação de vírus vivo, pelos quais podem ser um risco em potencial para o escape de vírus vivo destes locais ou pela preparação vacinal inadequada. Esses fatores levaram os países livres da doença proibir a fabricação das vacinas anti-FMD, visto que para tal procedimento os laboratórios fabricantes devem possuir um altíssimo nível de biossegurança (DOEL, 2003).

Muitas tentativas de desenvolver vacinas alternativas anti-FMD têm sido feitas, e incluem a construção de vírus vivo modificado, proteínas biossintéticas, peptídeos sintéticos, vetores de DNA e vírus recombinantes (CHINSANGARAM et al., 1998, GRUBMAN & MASON, 2002, MAYR et al., 1999, MORAES et al., 2002, RODRIGUEZ et al., 2003, SANZ-PARRA et al., 1999a,b, WANG et al., 2002). O adenovírus humano tem sido usado como um vetor para vacinas de FMD, com a obtenção de resultados variados. SANZ-PARRA et al. (1999a) usaram o adenovírus humano tipo 5 (Ad5) contendo a seqüência codificadora

das proteínas estruturais (P1) do vírus da FMD e demonstraram que suínos inoculados com este vetor foram parcialmente protegidos, mas não desenvolveram uma resposta por anticorpos neutralizantes. MAYR et al. (1999) demonstraram que houve uma completa proteção de suínos após duas inoculações do Ad5 recombinante contendo regiões codificadoras para a P1 do vírus A24 e para a protease 3C^{pro} do vírus A12 (Ad5-A24). Com esse estudo foi comprovada a importância do processamento das proteínas precursoras do capsídeo pela 3C^{pro} do vírus. Tal processamento foi imprescindível à indução de anticorpos neutralizantes e, conseqüentemente, uma resposta imune protetora ao vírus.

No estudo realizado por MORAES et al. (2002) verificou-se que utilizando uma única dose da vacina recombinante de Ad5 (Ad5-A24), obteve-se uma boa indução de anticorpos e os suínos foram completamente protegidos após o desafio com o vírus homólogo. MORAES et al. (2003) utilizaram a vacina recombinante Ad5-A24 combinada com um tratamento antiviral profilático, baseado no uso de Ad5 expressando o interferon alfa suíno (Ad5-pIFNα).

O presente trabalho tem por objetivo: avaliar o efeito adjuvante do interferon alfa suíno em animais imunizados com uma vacina recombinante de adenovírus humano tipo 5 contendo as regiões codificadoras das proteínas do FMDV e investigar alguns dos aspectos moleculares envolvidos na interação FMDV e a célula hospedeira, em uma espécie susceptível.

CAPÍTULO 1

REVISÃO DE LITERATURA

A febre aftosa ou *foot-and-mouth disease* (FMD) é uma doença altamente contagiosa de todos os animais da ordem artiodactila, incluindo bovinos, caprinos, ovinos e suínos (CALLIS & MCKERCHER, 1986, HOUSE & HOUSE, 1999). A doença foi descrita como uma epidemia em 1514 e o agente responsável foi identificado pela primeira vez em 1897 por Loeffler e Frosh (BROWN, 2003, De CLERCQ, 2001). FMD é uma doença debilitante que causa grandes perdas de produtividade (PERRY et al., 1999). As perdas diretas são devido a morte de animais jovens, redução na produção de leite e carne, bem como diminuição na performance. Os custos devido a erradicação ou controle são elevados e as perdas indiretas estão relacionadas às restrições impostas ao comércio internacional de animais susceptíveis e dos seus produtos derivados (De CLERCQ, 2001, MATTHEWS, 2001).

1.1. Agente etiológico

O agente etiológico da FMD é um vírus do gênero aftovírus, membro da família *Picornaviridae* (FENNER et al., 1993, RODRIGO & DOPAZO, 1995, RUECKERT, 1996), o FMDV (*foot-and-mouth disease virus*). O genoma do FMDV contém uma fita simples de RNA de polaridade positiva, com aproximadamente 8500 nucleotídeos que servem como RNA mensageiros (mRNA) (HOUSE & HOUSE, 1999). O RNA viral é circundado por um capsídeo de simetria icosaédrica, medindo cerca de 22 a 30 nm de diâmetro e composto por 60 cópias de quatro proteínas estruturais: VP1 (1D), VP2 (1B), VP3 (1C) e VP4 (1A) (GRUBMAN & BAXT, 2004, RUECKERT & WIMMER, 1984, RUECKERT, 1996).

O FMDV é bastante heterogêneo antigenicamente. Na atualidade, encontram-se descritos sete sorotipos: O, A, C, ASIA1, SAT1, SAT2 e SAT3 (SAT ou *Southern African Territories*) (MURPHY, 1996). Há uma grande diversidade antigênica, originando múltiplos

subtipos dentro de cada sorotipo (KITCHING et al., 1989), particularmente dentro do grupo A (DOEL, 2003). A infecção com um sorotipo não confere proteção contra os outros seis sorotipos (DOEL, 2003).

1.2. Propriedades do vírus

O FMDV é sensível à temperatura e ao pH. Em pH abaixo de 6 e acima de 9 o vírus é rapidamente destruído. O vírus pode sobreviver por longos períodos de tempo ao abrigo da luz e em locais úmidos, mas é rapidamente inativado quando são combinados os fatores: dissecação, pH e temperatura (De CLERCQ, 2001).

1.3. O genoma e proteínas do FMDV

O RNA do FMDV possui uma pequena proteína, a VPg, covalentemente ligada na extremidade 5' e uma cauda de poli A na sua extremidade 3'. O RNA genômico contém uma única *open reading frame* (ORF), sendo traduzido como uma única poliproteína, a qual é processada em várias proteínas maduras (estruturais e não estruturais) através das proteases codificadas pelo próprio vírus (*Leader* ou L^{pro}, 2A e 3C ou $3C^{pro}$) durante a tradução e após a mesma (CHINSANGARAM et al., 1998, 2001, MASON et al., 2003a, RUECKERT, 1996). O genoma é dividido em três regiões principais: (i) a 5' UTR (região não traduzida ou *untranslated region*) que é uma região regulatória, (ii) a região codificadora da poliproteína ou ORF (subdividida em L/P1, em P2 e em P3); e (iii) a 3'UTR, que também é uma região regulatória (Figura 1).
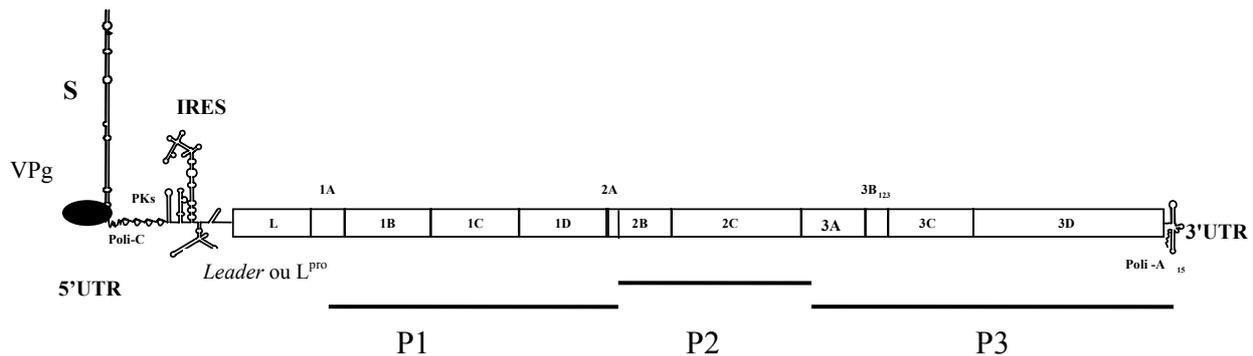
Figura 1. Esquema do genoma do FMDV. Regiões regulatórias (5' UTR e 3' UTR) estão indicadas nas extremidades do genoma. A região codificadora das proteínas (ORF) está demonstrando as proteínas no seu interior (de L a 3D). Encontra-se divida em 3 regiões funcionais principais (P1, P2 e P3). A VPg é a proteína covalentemente ligada à região 5'UTR do RNA e na porção 3'UTR há uma cauda de poli-A.

A região 5'UTR inclui a extremidade 5', o segmento S (cerca de 360 nt), uma região poli-C com comprimento variável (geralmente 100-400 nt). Após o IRES (local de reconhecimento pelo ribossoma), uma região denominada de *pseudoknot* (PKs) de aproximadamente 440 nt serve para a iniciação interna da síntese da proteína de uma forma CAP-independente e que possivelmente estão envolvidas na replicação do RNA (DOMINGO et al., 2002).

A síntese da proteína começa em dois códons funcionais AUG, separados por aproximadamente 80 nucleotídeos, e codificam uma poliproteína de aproximadamente 2330 amino-ácidos. As diferenças no comprimento de regiões codificadoras e não codificadoras são observadas entre isolados naturais de FMDV e entre vírus com diferentes passagens em cultivo celular (DOMINGO et al., 2002). A região P1 codifica as quatro proteínas estruturais do capsídeo: VP1 (1D), VP2 (1B), VP3 (1C) e VP4 (1A) (GRUBMAN & BAXT, 2004, RUECKERT & WIMMER, 1984, RUECKERT, 1996), e a P2 e a P3 codificam as proteínas não estruturais envolvidas na replicação do RNA e na maturação viral (DOMINGO et al., 2002).

O capsídeo do FMDV é composto por 60 cópias de cada uma das quatro proteínas estruturais. Essas proteínas são codificadas pela P1, sendo primeiramente processada em uma forma intermediária P1⁄2A, as quais ao serem processadas formarão os protômeros, que contém uma cópia de cada proteína: VP0 (é a forma precursora da VP2 e VP4), VP3 e VP1. Cada 5 protômeros reunidos originarão um pentâmero e 12 pentâmeros podem se reunir e no

seu interior receber um RNA viral, originando o provírion (GUTTMAN & BALTIMORE, 1977) ou o capsídeo viral vazio (GRUBMAN et al., 1985). Para que haja a formação do vírion é necessária a clivagem da VP0 para originar VP2 e VP4 (RUECKERT, 1996).

A função de diversas proteínas não estruturais ainda não está completamente elucidada embora algumas de suas proteínas sejam homólogas entre os demais picornavírus. Nesta revisão maior ênfase será dada às proteínas L^{pro} (proteína L, *leader,* proteína líder ou protease L) e 3C^{pro} [1].

A L^{pro} é a primeira a ser traduzida, além disso, é considerada um importante fator de virulência do FMDV. Esta proteína é considerada uma protease semelhante à papaína (PICCONE et al., 1995). No genoma de todos os sorotipos de FMDV existem dois códons de AUG que irão determinar qual será o tamanho da proteína a ser gerado; assim sendo dois produtos serão codificados, denominados de fragmento Lab e Lb. No estudo realizado por CAO et al. (1995) sugere que o produto Lb é o mais abundante gerado *in vivo*, é oriundo da leitura do segundo códon de AUG. A L^{pro} realiza a autoclivagem da poliproteína na sua porção carboxi-terminal, bem como, cliva um importante fator de iniciação da tradução do hospedeiro, o *eucariotic initiator factor* (eIF4G), resultando na supressão da tradução dos RNA mensageiros (RNAm) do hospedeiro e na tradução dos mRNAs virais de maneira CAP-independente, ou seja, via *internal ribossome entry site* (IRES) a qual não necessita do eIF4G intacto (CHINSANGARAM et al., 2001, DEVANEY et al., 1988, MASON et al., 1997, PICCONE et al., 1995).

PICCONE et al. (1995) utilizando a engenharia genética gerou um vírus mutante que foi deletado para a região codificadora da proteína L^{pro}, denominado de *leaderless* (FMDLLV) verificando que este vírus causou menor efeito citopático do que o vírus originário não deletado, e que este efeito seria devido à clivagem do eIF4G, resultando na interferência da síntese protéica celular. Porém, este vírus demonstrou crescimento similar ao vírus que continha a L^{pro} em dois tipos de células, BHK-21 e IBRS-2, sugerindo que a L^{pro} não é requerida para o crescimento viral. Posteriormente, quando FMDLLV foi inoculado em animais, evidenciaram-se sinais clínicos de FMD mais brandos e o vírus não foi transmitido aos animais não inoculados que estavam em contato (CHINSANGARAM et al., 1998, MASON et al., 1997). Indicando que a virulência do FMDV está relacionada à presença da L^{pro}, sendo também o gene da L^{pro} importante para causar a doença e permitir a transmissão entre os animais susceptíveis.

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[1]Para maiores detalhes sobre as proteínas estruturais do FMDV ver a revisão elaborada por MASON et al. (2003a).

Outra evidência que a L[pro] é um fator crítico para a patogênese originou-se do trabalho realizado por BROWN et al. (1996) em bovinos expostos ao aerossol que continha o FMDLLV ou o FMDV com a L[pro] presente no seu genoma Os animais que foram infectados com o FMDLLV não apresentaram doença detectável após 72 h de exposição. O FMDLLV permaneceu restrito em áreas localizadas dos pulmões e não foi disseminado a outros locais de predileção nas infecções pelo FMDV com a L[pro]. Posteriormente o FMDLLV foi utilizado para verificar as interações do vírus com a resposta à infecção gerada no hospedeiro[1].

A proteína 3C[pro] é uma protease que é responsável pela maioria das clivagens da poliproteína viral. Também tem sido associada com a clivagem de algumas proteínas celulares podendo estar relacionada com a inibição da transcrição da célula hospedeira, como por exemplo, o eIF4A, que é parte do complexo de ligação do CAP e funciona como uma RNA helicase (BELSHAM et al., 2000). A 3C[pro] também está envolvida no processamento do precursor P1⁄2A, ocasiona a formação de protômeros contendo uma cópia de cada proteína estrutural do capsídeo (VP0, VP3 e VP1) viral. MAYR et al. (1999) demonstraram que o processamento do capsídeo é essencial para o desenvolvimento de uma resposta de anticorpos neutralizantes anti-FMDV eficiente para proteger os animais.

As proteínas 2A e 2B têm sido implicadas na indução de efeito citopático dos picornavírus, 2B demonstrou habilidade de aumentar a permeabilidade da membrana e bloquear as vias secretórias de proteínas, 2C é envolvida na síntese do RNA. A proteína 3A parece estar ligada à adaptação das cepas de FMDV ao hospedeiro; assim como a 3A e o intermediário 3AB estão associadas a fatores de replicação do RNA ligados as membranas celulares. A 3B codifica três cópias da VPg, a proteína covalentemente ligada a extremidade 5' do RNA (Figura 1). A proteína 3D é a RNA polimerase dependente do RNA. A região 3'UTR provavelmente é um sítio de interação com as proteínas virais e está envolvida na replicação do RNA; a extremidade 3' contém uma seqüência de poliA com diferença no comprimento entre os diferentes picornavírus. Apesar da clássica distinção entre as regiões regulatórias e codificadora do vírus, há evidências sugerindo que a região codificadora pode estar envolvida na regulação viral (MASON et al., 2003a).

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[1]Ver "sensibilidade do FMDV ao interferon" na página 22.

1.4. Epidemiologia e distribuição geográfica

A FMD é considerada a mais contagiosa doença de rebanhos. Essencialmente todas as espécies de casco bipartido são susceptíveis ao FMDV, sendo que o suíno, bovinos, ovinos, caprinos, búfalos africanos as principalmente afetadas. A mortalidade em animais adultos é baixa, porém a morbidade é bastante alta (HOUSE & HOUSE, 1999).

Algumas cepas de FMDV parecem estar restritas a um hospedeiro sob condições de campo, como exemplificado pelo surto ocorrido em Taiwan 1997, onde o tipo O1 atingiu um grande número de suínos sem ter sido isolado de bovinos naquele país por ocasião do surto (HOUSE & HOUSE, 1999).

Os animais infectados são capazes de eliminar grandes quantidades de vírus por pelo menos sete dias. A exposição via aerossol é a principal forma de disseminação do vírus, particularmente em climas temperados, onde a alta umidade e moderadas temperaturas podem favorecer a disseminação do vírus a longas distâncias (SELLERS & PARKER, 1969). Grandes quantidades de vírus são geradas nos suínos e disseminadas via aerossol; sendo os suínos designados como os "amplificadores" do FMDV (SELLERS & PARKER, 1969, DONALDSON & FERRIS, 1980). Os suínos podem produzir cerca de 10^8 doses infectantes de vírus nos aerossóis por dia durante o pico de viremia (SELLERS et al., 1971), aproximadamente 1500 vezes mais vírus do que a quantidade produzida pelos bovinos (DONALDSON & FERRIS, 1980).

Em áreas quentes e áridas a doença é transmitida por contato, pois a disseminação dos aerossóis a longas distâncias se torna restrita devido à baixa umidade e altas temperaturas (HOUSE & HOUSE, 1999). Outras formas de transmissão podem incluir o sêmen, carne, leite e derivados (CALLIS, 1996), além dos fomitos (HOUSE & HOUSE, 1999).

Nos animais que o FMDV persiste por mais de 28 dias após a infecção são designados como portadores (SALT et al., 1996, SUTMOLLER & COTTRAL, 1967, SUTMOLLER et al., 2003). A condição de portador é independente do *status* imunológico do animal no período de exposição à infecção viral. Esta condição é caracterizada por uma infecção inaparente onde pode haver um isolamento intermitente do vírus da orofaringe, que é atualmente a única medida de detecção do indivíduo portador (DAVIES, 2002). Portadores têm sido descritos em búfalos africanos (HEDGER & CONDY, 1985), bovinos, ovinos, caprinos. Não tem sido reportado em suínos (DAVIES, 2002, SUTMOLLER et al., 2003).

DAWE et al. (1994) demonstraram a transmissão de FMDV de búfalos africanos para bovinos sob condições experimentais.

A prevalência de portadores imediatamente após a infecção é alta, declinando com o tempo, sendo que bovinos portadores podem excretar o vírus num período de 4-5 meses após a infecção. O período de portador parece variar de acordo com a espécie animal envolvida, aproximadamente 12 meses em bovinos, 9 meses em ovinos e caprinos e até 5 anos em búfalos africanos (DAVIES, 2002).

Embora a condição de portador em FMD exista, a evidência de transmissão da infecção a outros ainda é motivo de discussões. DAVIES (2002) cita que não há dados a campo que possam embasar o risco de um animal portador. Os dados obtidos de condições experimentais indicam que, se a transmissão da infecção pelos portadores acontece, sua ocorrência estaria em uma baixa freqüência ou sob um particular conjunto de circunstâncias ainda não identificados.

Surtos de FMD com repercussões econômicas e sociais bastante drásticas ocorreram em março de 1997 em Taiwan e em fevereiro de 2001 no Reino Unido. No surto de 1997 houve o envolvimento de suínos, resultando no abate de aproximadamente 4 milhões de animais e com a derrocada da indústria suinícola daquele país (YANG et al., 1999). O segundo evento resultou na morte de 4 milhões de animais, principalmente ovinos e as perdas econômicas foram estimadas entre 12,3 – 13, 8 bilhões de dólares, com aproximadamente 36% relacionadas à indústria do turismo (THOMPSON et al., 2002). Em ambos os surtos o sorotipo O foi identificado como o agente etiológico.

Atualmente como zona livre é considerada somente o continente Australiano e América do Norte (GRUBMAN & BAXT, 2004). Na América do Norte a última ocorrência de FMD foi no México em 1946-1954 e no Canadá em 1952. Nos EUA o último surto ocorreu em 1929 (HOUSE & HOUSE, 1999, SUTMOLLER et al., 2003). Na América do Sul é endêmica em muitos países. Os sorotipos A, O e C são de ocorrência na América do Sul. Na África são reportados 6 sorotipos (A, O, C, SAT1, SAT2 e SAT3). Na Ásia e no Oriente Médio estão os sorotipos A, O, C e Ásia 1.

As ocorrências de surtos de FMD relatados pela OIE podem ser encontrados no site: http://www.iah.bbsrc.ac.uk/virus/picornaviridae/Aphthovirus/index.html.

1.5. Patogenia

A principal rota de transmissão do FMDV é a aerossol (HOUSE & HOUSE, 1999), embora a infecção também possa ocorrer via abrasões na pele ou nas membranas mucosas (DONALDSON, 1987). Os suínos também podem se contaminar ingerindo comida infectada pelo FMDV, por contato direto com animais infectados ou por serem colocados em um local onde foi previamente habitado por animais infectados. Porém os suínos são menos susceptíveis à infecção por aerossol do que os bovinos; todavia excretam muito mais aerossóis que os bovinos e ovinos (ALEXANDERSEN & DONALDSON, 2002).

As partículas virais se aderem ao trato respiratório superior e inferior. O ácido nucléico viral é encontrado nos tecidos epiteliais de bovinos cerca de 6 horas após a exposição via aerossol (BROWN et al., 1992). E em suínos inoculados intradermicamente no focinho, o ácido nucléico viral foi encontrado disseminado após 24h da infecção (BROWN et al., 1995).

O vírus atinge os tecidos-alvo como: epitélio, mucosa e miocárdio, replica-se e a viremia persiste geralmente por 3-5 dias. Dentro de 2-3 dias há o desenvolvimento de vesículas e abrasões mecânicas podem ocorrer (HOUSE & HOUSE, 1999). As lesões normalmente estão associadas à banda coronária dos cascos, na mucosa da boca, língua e palato. Pode haver um aumento na temperatura corporal de 3- 4ºC (DAVIES, 2002).

A replicação do FMDV ocorre também no epitélio da glândula mamária, podendo o vírus ser encontrado no leite de bovinos até 10 dias após a infecção, correspondendo ao desenvolvimento de anticorpos neutralizantes (BLACKWELL et al., 1983). Em bovinos já foi mencionado que o vírus pode ser encontrado no leite até sete semanas, mesmo na presença de anticorpos neutralizantes no soro (BURROWS et al., 1971). O vírus pode ser excretado via sêmen, urina e fezes (DONALDSON, 1987)

O FMDV também pode ser detectado em secreções orais e respiratórias em grandes quantidades de dois a sete dias após a exposição viral. Em animais jovens pode haver uma severa necrose do miocárdio (HOUSE & HOUSE, 1999).

1.6. Diagnóstico

As técnicas de diagnóstico estão descritas no manual de teste de diagnóstico e vacinas (manual of standards for diagnostic tests and vaccines) da OIE (*Office International des Epizooties*) e revisado pela AVIS (*Advanced Veterinary Information System*) no endereço eletrônico http://www.aleffgroup.com/avisfmd/.

Uma vez que uma doença vesicular em animais da ordem artiodactila é detectada pode-se estar diante de FMD; desta maneira, precauções devem ser adotadas para garantir a seguridade das amostras provenientes de animais suspeitos, e os laboratórios de diagnósticos devem possuir um alto nível de biossegurança. As amostras preferenciais para detecção viral são epitélios e fluido vesicular. Fluido esofagofaringeal, bem como sangue com anticoagulante e leite também podem ser utilizados (De CLERCQ, 2001).

Para isolar e crescer o vírus utiliza-se a inoculação das amostras em cultivo celular sensível ao FMDV, tais como: células primárias de rim de cordeiro, células de linhagem da tireóide de bovinos (BTY), de rim de suíno (IBRS-2) ou células de rim de hamsters neonatos (BHK-21). Uma vez observado efeito citopático (CPE), o sobrenadante dos cultivos celulares devem ser testados para identificação viral através de um teste de ELISA (De CLERCQ, 2001).

A detecção do antígeno pode ser realizada por ELISA do tipo sanduíche indireto (ROEDER & Le BLANC SMITH, 1987) usando anticorpos policlonal e/ou monoclonal, o qual indicará o sorotipo de FMDV envolvido.

Técnicas para identificar o ácido nucléico viral têm sido empregadas. A reação de polimerase em cadeia em tempo real (real time RT-PCR) ou a RT-PCR podem ser empregadas para detectar e tipificar o genoma viral e para diferenciar o FMDV de outros agentes de enfermidades vesiculares. Quando há suspeita de infecção subclínica, coleta das amostras antes da constatação dos sinais clínicos ou após a resolução da doença clínica, ou quando processando amostras de saliva ou provenientes de *swabs*, essas técnicas têm sido consideradas mais sensíveis e mais rápidas do que múltiplas passagens em cultivo celular (De CLERCQ, 2001, SUTMOLLER et al., 2003).

As cepas de FMDV normalmente são identificadas e caracterizadas para a eleição de vacinas mais apropriadas e nos levantamentos epidemiológicos em um surto. Para a caracterização pode-se utilizar a sorotipagem com anticorpos monoclonais (MEYER et al.,

1994). Painéis de anticorpos monoclonais estão disponíveis contra diversos sorotipos de FMDV, desta forma se pode fazer uma rápida triagem dos vírus de campo e determinar o perfil antigênico dos mesmos comparando-se os perfis antigênicos das cepas já conhecidas. A caracterização genômica do vírus é feita através do seqüenciamento de nucleotídeos. Este método identifica a relação genômica das cepas coletadas na mesma ou em diferentes regiões em diferentes tempos. A seqüência de nucleotídeos da cepa de campo é comparada com as seqüências de outras cepas de campo ou de referência, as quais são mantidas em uma central de dados. Com esta comparação é possível compor um dendograma dos vírus isolados (KNOWLES et al., 2001).

Amostras de sangue de animais suspeitos ou em contato devem ser coletadas e uma análise sorológica dever ser efetuada. A detecção de anticorpos anti-FMDV indica uma infecção prévia ou vacinação. A vigilância sorológica pode ser realizada mesmo na ausência da doença ou para verificar os níveis de proteção e o *status* dos animais em contato em uma zona de vigilância após um surto. Amostras de soro pareado podem ser coletadas para verificar aumento de título de anticorpos específicos contra o FMDV (De CLERCQ, 2001). O teste de neutralização viral (GOLDING et al., 1976) é o teste de referência para detectar anticorpos contra o FMDV. Ele tem a desvantagem de levar 2-3 dias para a obtenção do resultado, além de requerer cultivo celular, precisa utilizar vírus vivo e ser efetuado em unidades com alto nível de biosseguridade. Anticorpos também podem ser detectados por ELISA, tais como: ELISA competitivo de fase sólida (MACKAY et al., 2001) ou ELISA de bloqueio de fase líquida (HAMBLIN et al., 1986).

Uma maneira de distinguir animais vacinados de animais infectados ou convalescentes seria medir os anticorpos contra as proteínas não estruturais do vírus através de um teste de ELISA específico para as mesmas (LUBROTH & BROWN, 1995, de DIEGO et al., 1997), pois a detecção de anticorpos contra essas proteínas indica infecção pelo vírus, devido as mesmas apresentarem-se somente durante a multiplicação viral. Tais proteínas também são produzidas nos cultivos celulares utilizados na produção de vacinas. Nas formulações mais modernas esses cultivos celulares são altamente purificados antes de ser incluídos nas vacinas e desta forma quase todas as proteínas não estruturais são eliminadas. Conseqüentemente, anticorpos contra essas proteínas não serão desenvolvidos após a vacinação, sendo possível a distinção entre os animais vacinados e infectados. Desta forma, o emprego de vacinas altamente purificadas deveria ser permitido nas campanhas de vacinação.

Teste para discriminar animais vacinados dos infectados e convalescentes seria de grande valor durante um monitoramento sorológico após uma vacinação emergente para

declarar um país ou região livre da infecção. Um teste de ELISA para a detecção de proteínas não estruturais do FMDV poderia ser bastante útil para a vigilância sorológica em regiões de alto risco de introdução da enfermidade. Na Europa o teste mais utilizado é o ELISA para detectar anticorpos contra as proteínas não estruturais 3ABC (BROCCHI et al., 1997). Em 1999, em Taiwan foi utilizado um teste de ELISA contra peptídeos não-estruturais para diferenciar animais convalescentes dos que foram vacinados (SHEN et al., 1999).

1.7. Diagnóstico diferencial

FMD é uma enfermidade clinicamente indistinguível de outras doenças vesiculares. As doenças vesiculares de causas virais que podem ser confundidas com FMD incluem: doença vesicular do suíno, estomatite vesicular, estomatite papular bovina, doença das mucosas, rinotraqueíte infecciosa bovina, febre catarral maligna, peste bovina, mamilite herpética bovina, ectima contagioso, língua azul, febre dos pequenos ruminantes, poxvírus caprino e ovino, encefalomiocardite dos suínos. Outras enfermidades ou achados de causas não virais também devem ser consideradas no diagnóstico diferencial de FMD, tais como: *footrot*, dermatite fototóxica, irritantes químicos e algumas lesões traumáticas (HOUSE & HOUSE, 1999, De CLERCQ, 2001).

1.8. Controle da enfermidade com o uso de vacinação

Nesta revisão a classificação das vacinas foi baseada na terminologia empregada por GRUBMAN & BAXT (2004).

1.8.1. Vacinas convencionais

As vacinas convencionais são as atualmente utilizadas e são preparadas com o vírus inteiro inativado, posteriormente são adicionados os adjuvantes, antes do seu uso a campo.

Alguns países têm mantido banco de vacina de FMD, onde o antígeno concentrado está estocado em nitrogênio líquido; sob tais condições o antígeno se mantém estável por um longo período de tempo. Os bancos contêm uma diversidade de sorotipos de FMDV para o suprimento de vacinas (DOEL, 2003).

Desde o desenvolvimento das vacinas inativadas os surtos de FMD foram drasticamente reduzidos nas áreas enzoóticas, porém muitos problemas com a produção e uso dessa vacina que tem limitado o seu emprego em programas de controle emergenciais, entre os quais estão incluídos: a preparação das vacinas convencionais requer laboratórios com alto nível de biossegurança, pois é necessário trabalhar com grandes quantidades de vírus vivo para crescimento em cultivos celulares. Os vírus incluídos nessas vacinas são inativados quimicamente (BARTELING & VREESWIJK, 1991). O uso da formalina foi associado com inativação insuficiente e contaminações com vírus vivo residual (BECK & STROHMAIER, 1987); no entanto com o emprego da etilenamina binária estes problemas foram em grande parte contornados. Para garantir o sucesso do processo de inativação é imprescindível que os vírus estejam bem purificados antes do mesmo. Muitas preparações de vírus usadas nas vacinas estavam contaminadas com proteínas não estruturais de FMDV, desta forma os animais vacinados desenvolviam anticorpos contra as proteínas contaminantes, tornando difícil distingüir os animais vacinados dos infectados ou convalescentes com os testes de diagnósticos atualmente aprovados (GRUBMAN & BAXT, 2004).

A eficiência das vacinas inativadas para FMD também depende da adição de bons adjuvantes. Os mais usados são o hidróxido de alumínio e saponina (uso em ruminates) ou as formulações oleosas (uso em suínos e bovinos) (DOEL, 2003).

O FMDV é um vírus com uma grande variabilidade antigênica (KITCHING et al., 1989) e a vacinação não protege os animais de uma infecção com cepas pertencentes ao outro subtipo de FMDV; por este motivo há a necessidade de formular uma vacina que contenha o sorotipo do vírus de campo por ocasião de um surto (DOEL, 2003).

A vacina inativada não induz uma proteção imediata após sua aplicação, deixando uma janela de susceptibilidade aberta antes da indução de uma resposta imunológica adaptativa eficaz. Os animais vacinados podem se tornar portadores após o contato com o FMDV (GRUBMAN & BAXT, 2004).

1.8.2. Vacinas alternativas

Devido aos problemas decorrentes do emprego das vacinas inativadas, muitas pesquisas têm sido feitas para tentar desenvolver vacinas alternativas para FMD que não empregam o vírus vivo. Tais como: proteínas e peptídeos; vacinas com o vírus vivo atenuado e as vacinas contendo o capsídeo viral vazio.

1.8.2.1. Proteínas e peptídeos

Baseadas nas informações a respeito da estrutura do capsídeo do FMDV, incluindo a superfície proeminente da VP1 e o *loop* G-H da superfície da VP1, que é um sítio importante imunologicamente, muitos estudos têm sido desenvolvidos. Inicialmente incluíram o uso da VP1 oriunda do vírus ou produzida por técnicas de recombinação do DNA (KLEID et al., 1981); o uso de peptídeos derivados da VP1 (STROHMAIER et al., 1982) ou de peptídeos da VP1 quimicamente sintetizados (BITTLE et al., 1982, DiMARCHI et al., 1986, FRANCIS et al., 1991); o uso de vetores expressando proteínas de fusão da VP1 (KIT et al., 1991, KITSON et al., 1990); a inoculação de DNA expressando somente os epítopos da VP1 (WONG et al., 2000) ou co-administrados com DNA codificando a interleucina 12 (IL-12) (WONG et al., 2002) e o uso de plantas transgênicas expressando genes que codificam as regiões do capsídeo viral (CARRILLO et al., 1998). Embora todas essas estratégias induzem altos títulos de anticorpos neutralizantes nem sempre elas conferem proteção do rebanho contra o vírus desafio (DIMARCHI et al., 1986, MULCAHY et al., 1992). A imunogenicidade dessas vacinas de subunidade parece ser devido à presença de epitopos imunologicamente importantes do *loop* G-H da superfície da VP1. Apesar desses epitopos parecerem imunodominantes em muitos sistemas testados eles não são os únicos epitopos neutralizantes no vírion (CROWTHER, et al., 1993), nem reconhecidos similarmente em todas as espécies hospedeiras (MATEU et al., 1995). Outro problema relacionado com o uso desses peptídeos sintéticos seria a seleção de variantes antigênicas. No trabalho realizado por TABOGA et al. (1997) detectaram limitada proteção no desafio e mutantes de escape viral nos animais vacinados.

1.8.2.2. Vacinas com o vírus vivo atenuado

Cepas atenuadas foram produzidas por passagem em espécies não susceptíveis tais como: camundongos, coelhos e ovos embrionados até o enfraquecimento da sua virulência em bovinos. Alguns estudos realizados com essas vacinas demonstraram capacidade de induzir um determinado grau de proteção. As cepas virais atenuadas em uma determinada espécie foram virulentas quando inoculadas em outras espécies susceptíveis. Tem sido difícil obter vírus que são atenuados e imunogênicos concomitantemente, além disso, existe a possibilidade de reversão à virulência (GRUBMAN & BAXT, 2004).

Diante disso, pesquisas têm sido realizadas no intuito de desenvolver vacinas atenuadas, utilizando a engenharia genética para modificar ou deletar regiões do genoma. Pelo uso da tecnologia de DNA recombinante foi gerado um vírus no qual a RGD (a região de ligação do FMDV ao receptor celular que está situada no *loop* G-H da VP1, composta pelos três aminoácidos: arginina, glicina e aspartato) foi deletada. Este vírus apresentou ineficácia em ligar-se às células e em produzir doença em camundongos e suínos. No momento que bovinos foram inoculados com esse vírus não desenvolveram doença clínica e os animais apresentaram anticorpos neutralizantes específicos similares aos induzidos pela vacina inativada, sendo que após o desafio foram protegidos da doença clínica (McKENNA et al., 1995).

A deleção da região não codificadora de uma proteína não estrutural que não é essencial para replicação viral em cultura celular é uma alternativa para gerar um vírus vivo atenuado. Contudo, para ser utilizado como uma vacina esse vírus deletado precisa ser capaz de replicar em animais susceptíveis. A vantagem disso comparado ao método clássico de atenuação é que o risco de reversão à virulência é bastante reduzido (GRUBMAN & BAXT, 2004).

A L^{pro} é uma proteína codificada pelo vírus e está envolvida na inibição da síntese protéica do hospedeiro, permitindo o vírus utilizar a maquinaria da célula para sintetizar as proteínas virais. Um vírus mutante foi gerado pela deleção da região codificadora a proteína L^{pro} do genoma do FMDV A12, o vírus foi denominado *leaderless*. O vírus replicou em células BHK21, mas não causou doenças em bovinos ou suínos (CHINSANGARAM et al., 1998, MASON et al., 1997, PICCONE et al., 1995). Utilizou-se, então, como um candidato à vacina de vírus vivo atenuado. A inoculação induziu uma resposta de anticorpos neutralizantes específicos para o FMDV em bovinos e suínos, não sendo suficiente para

induzir uma proteção total (MASON et al., 1997). Porém, quando a L^{pro} do sorotipo O1 Campos foi deletada, o vírus gerado foi avirulento em bovinos e causou uma doença branda em suínos (ALMEIDA et al., 1998). Esses resultados indicam um potencial uso dessas vacinas vivas atenuadas, porém, é necessário encontrar um vírus capaz de induzir uma resposta imunológica eficaz.

1.8.2.3. Vacinas contendo o capsídeo viral vazio

Vacinas contra a FMD contendo os sítios imunogênicos presentes no vírus intacto, mas que não contêm o ácido nucléico têm sido desenvolvidas e pesquisadas (BELSHAM et al., 1991, GRUBMAN et al., 1985, 1993, LEWIS et al., 1991). Tais vacinas necessitam conter as regiões do genoma viral necessárias à síntese, processamento e formação do capsídeo viral pelas proteínas estruturais do vírus, incluindo as regiões da P1-2A e da 3C^{pro}. Essas estruturas são normalmente produzidas em células de cultivo infectadas pelo FMDV, são também antigenicamente semelhantes às partículas virais e imunogênicas em animais (GRUBMAN et al., 1985, RWEYEMAMU et al., 1979). Os animais imunizados com essas vacinas poderiam ser diferenciados dos animais infectados ou convalescentes em testes para detectar a presença das proteínas não estruturais. Os capsídeos vazios do FMDV foram primeiramente expressos em *Escherichia coli* ou baculovírus recombinante e inoculados em animais. Embora os produtos obtidos por esses sistemas oferecessem proteção não atingiam a mesma eficácia oferecida pelas vacinas inativadas, as quais contêm o vírus inteiro, pois somente poucas quantidades do antígeno eram produzidas (BELSHAM et al., 1991, GRUBMAN et al. 1993, LEWIS et al., 1991).

Alternativas para aumentar a expressão do antígeno e entregar o capsídeo viral vêm sendo testadas, incluindo o uso de vetores, para expressar as estruturas do capsídeo e induzir ambas as respostas humoral e celular. Estudos em camundongos inoculados com DNA intradermicamente resultaram na indução de resposta neutralizante de anticorpos contra FMDV expressando o capsídeo vazio e também a protease (3C^{pro}) (BEARD et al., 1999, CHINSANGARAM et al., 1998). Quando inoculados em suínos, foram necessárias grandes quantidades de DNA e pelo menos de 2 a 3 inoculações para induzir uma branda resposta de anticorpos neutralizantes e com níveis variáveis de proteção (BEARD et al., 1999, CEDILLO-BARRON et al., 2001).

Outro sistema para a entrega de antígeno tem sido estudado e envolve um vetor com vírus vivo. O adenovírus e poxvírus humano têm sido utilizados para a expressão de diferentes genes oriundos de outros patógenos (ELOIT et al., 1990, IMLER, 1995, PANICALI & PAOLETTI, 1982) e para entregar as proteínas do capsídeo do FMDV (ABRAMS et al., 1995, BERINSTEIN et al., 2000, MAYR et al., 1999, 2001, MORAES et al., 2003, SANZ-PARRA, 1999a). O adenovírus humano sorotipo tipo 5 possui baixa patogenicidade tanto em humanos como em animais. Uma forma de replicação defectiva do desse adenovírus (Ad5), apresentando uma deleção no seu genoma tem sido utilizada como vetor. Esses vetores crescem somente em células específicas (células 293) que possuem fatores de replicação que não estão presentes nos vírus defectivos (GRAHAM et al., 1977, GRAHAM & PREVEC, 1992). O Ad5 usado como vetor apresentou a capacidade de ligar-se e internalizar-se efetivamente em células animais, como bovinos e suínos (PREVEC et al., 1989), garantindo rápida internalização e expressão dos genes desejados nessas espécies.

Um Ad5 contendo as regiões do capsídeo do FMDV sorotipo C1 Oberbayern, mas sem a região codificadora da 3C^{pro} (Ad5-C1) foi gerado, mas quando inoculado em suínos e bovinos não conferiram resposta de anticorpos contra o FMDV e nem proteção por ocasião do desafio (SANZ-PARRA et al., 1999 a, b). O mesmo fato foi evidenciado em suínos inoculados com o vírus mutante do sorotipo A12 sem a protease (MAYR et al., 2001). Demonstrando que é necessário haver o processamento da poliproteína precursora do capsídeo viral (P1-2A) pela protease (3C^{pro}) para que a vetor, contendo o capsídeo vazio viral, seja efetivo.

O Ad5 expressando as regiões do capsídeo do FMDV sorotipos A12 e A24 Cruzeiro, com o gene da 3C^{pro}, também foram gerados e utilizados como vacina em animais. Suínos que receberam duas inoculações do de tais vacinas desenvolveram anticorpos neutralizantes específicos contra o FMDV e foram protegidos por ocasião do desafio, tanto pelo contato ou por inoculação direta (MAYR et al., 1999, 2001, MORAES et al., 2002).

Nos estudos realizados por MAYR et al. (1999, 2001) também foi demonstrada a segurança do vírus recombinante Ad5 empregado como vacina, pois não houve a disseminação do vírus para os animais não inoculados os quais foram mantidos no mesmo local onde estavam os suínos vacinados.

Numa situação emergencial, diante de um surto, uma vacina contra a FMD deve ser empregada em uma única dose e ser capaz de induzir uma rápida e eficaz resposta de anticorpos para conferir proteção aos animais. Para examinar esse critério, MORAES et al. (2002) construíram a vacina recombinante de Ad5-A24 (Figura 2), expressando a região do

capsídeo do sorotipo A24 Cruzeiro, o qual é utilizado em muitas vacinas na América do Sul, e o gene da 3C^{pro} do sorotipo A12.
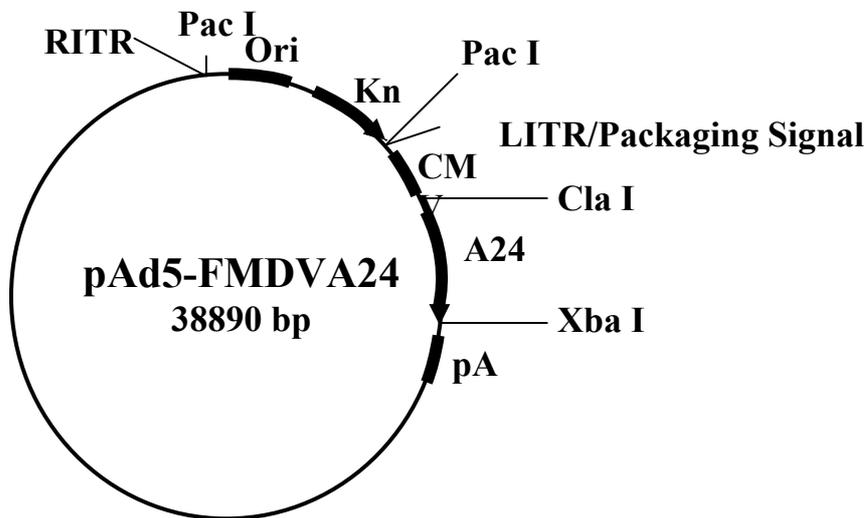
Figura 2. Esquema do plasmídeo contendo o Adenovírus humano tipo 5 recombinante expressando a região codificadora do capsídeo do FMDV A24 e da 3C^{pro} do FMDV A12 (pAd5-FMDVA24)[1].

A potência e eficácia dessa vacina foram avaliadas em suínos em diferentes regimes de vacinação e também foram comparados com a vacina comercial inativada. Os animais vacinados com Ad5-A24 e com a vacina comercial apresentaram uma resposta de anticorpos neutralizantes anti-FMDV anteriormente ao desafio e após o mesmo foram completamente protegidos. Nos animais vacinados com Ad5-A24 não foi detectada viremia.

Uma vez que o regime de única dose de vacina foi suficiente para proteção dos animais, um segundo trabalho foi realizado para verificar quando o Ad5-A24 seria capaz de gerar uma resposta imunológica eficaz contra o FMDV. Os animais depois de vacinados com uma única dose de 5×10^9 *pfu* (unidades formadoras de placas) da vacina de Ad5-A24 foram capazes de produzir anticorpos neutralizantes contra o FMDV; também foi evidenciada uma proteção dos animais depois do desafio diretamente com o FMDV aos 7 e aos 14 dias após a vacinação (MORAES et al., 2002). Os animais não demonstraram viremia ou vírus nos fluidos nasais ou sinais de replicação viral.

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[1] Para ver detalhes do plasmídeo de Ad5 e das enzimas de restrição verificar o trabalho de MORAES et al. (2001).

Vacinas empregando o capsídeo viral como imunógeno em bovinos ainda estão em testes. No estudo preliminar, bovinos foram inoculados com duas doses de Ad5-A24 e desenvolveram uma boa resposta de anticorpos neutralizantes específicas contra o FMDV, além de não apresentarem sinais clínicos da doença ou sorologia contra o FMDV após o desafio direto na língua e do contato com um animal infectado (GRUBMAN & MASON, 2002).

Os experimentos utilizando o Ad5 como vetor expressando o capsídeo vazio do FMDV, sugerem que esta vacina auxiliaria a abrandar os problemas existentes com a vacina inativada atualmente empregadas contra esta enfermidade, pois não requer FMDV infeccioso, o vírus defectivo recombinante não é eliminado para os demais animais em contato com os vacinados. Também o Ad5 recombinante não possui muitos dos genes que codificam as proteínas não estruturais do FMDV, desta forma os animais vacinados podem ser diferenciados dos infectados e convalescentes. Além do mais esta vacina confere uma boa eficácia e induz rapidamente uma imunidade protetora equivalentemente observada com a vacina atualmente usada (GRUBMAN & MASON, 2002).

1.9. Sensibilidade do FMDV ao interferon

Uma das respostas iniciais do hospedeiro à infecção viral é a indução do interferon (IFN) tipo I, principalmente o IFNα/β (BIRON & SEN, 2001). Seguidamente à infecção viral, as células infectadas iniciam a expressar e secretar IFN α/β. O interferon secretado liga-se ao seu receptor específico na superfície celular, ativando os elementos que participam nos mecanismos moleculares da "cascata" do interferon. Desta forma, tanto as células infectadas quanto as células vizinhas tornam-se resistentes ao vírus devido a uma série de eventos que induzirão os genes estimulados pelo interferon (ISGs ou *IFN α/β-stimulated genes*) (BIRON, 1998, BIRON & SEN, 2001, HORISBERGER, 1995,VILCEK & SEN, 1996).

Atualmente os ISGs que têm sido bem caracterizados são os genes da: proteína kinase dependente da dupla fita de RNA (PKR), 2'-5' oligoadenilato-sintetase (OAS), que por sua vez ativa uma nuclease latente, a RNAse L, e o gene da GTPase Mx (ou proteína Mx). As proteínas oriundas da tradução destes genes são consideradas como os mediadores dos efeitos biológicos dos interferons. Sua ação baseia-se na habilidade de afetar os vírus em diferentes

estágios do seu ciclo de replicação. Da mesma forma que os diferentes vírus possuem susceptibilidade diferente para cada produto dos ISGs (CHINSANGARAM, et al., 2001, HORISBERGER, 1995, SAMUEL, 2001, VILCEK & SEN, 1996).

A L^{pro} é um fator de virulência do FMDV, uma vez que ela cliva o eIF4G e conseqüentemente suprime a síntese dos interferons tipo I. A supressão da produção IFN permite a rápida replicação e disseminação do FMDV nas células do hospedeiro. No entanto, um mutante do FMDV que não possui o gene que expressa a L^{pro}, o FMDLLV obteve uma replicação ineficiente em células capazes de sintetizar ambos IFNα e β (CHINSANGARAM, et al., 1999, CHINSANGARAM et al., 2001, GRUBMAN & CHINSANGARAM, 2000). Células previamente tratadas com o IFNα/β tornam-se resistentes ao FMDV (CHINSANGARAM et al., 1999, 2001).

O bloqueio da replicação do FMDV *in vitro* ao nível da tradução protéica foi investigado por CHINSANGARAM et al.(2001) usando o FMDLLV o qual sugeriu que o efeito inibitório do IFN α/β sobre o FMDV poderia ser devido a secreção e a ligação ao receptor do IFN α/β iniciando uma série de eventos, tais como a ativação dos ISGs dentro das células hospedeiras.

Primeiramente foi verificado se a PKR desempenharia um papel importante no bloqueio da replicação do FMDV. O efeito inibitório da PKR foi constatado através do uso de um inibidor da PKR, a 2-aminopurina sobre células de cultivo secundárias, havendo um aumento do título viral na presença desta substância. O mecanismo celular antiviral induzido pelo IFN envolvido na inibição da replicação do FMDV foi investigado em células derivadas de fibroblastos de camundongos *knock-out* para o ISGs. Tais estudos sugerem que a PKR exerce um importante papel na resposta da célula hospedeira à infecção pelo FMDV, interferindo na replicação viral (CHINSANGARAM et al., 2001).

1.10. Combinação de um agente antiviral com vacinas de subunidade

Os experimentos realizados com as vacinas que utilizam o Ad5 como vetor e expressando o capsídeo vazio do FMDV têm demonstrado que tais vacinas constituem um método seguro para a indução de uma resposta imunológica rápida e segura para suínos e os estudos preliminares em bovinos sugerem sua utilização em bovinos. Contudo, existe um

período após a vacinação que antecede o desenvolvimento de uma resposta imunológica específica contra o FMDV. Geralmente, ambas as vacinas (inativada e recombinante de Ad5) induzem proteção ao redor do sétimo dia após a vacinação, durante esse período o animal fica susceptível à infecção pelo vírus. No controle de um surto é imprescindível levar em consideração esse período. Para contornar esta situação, a possibilidade de empregar um agente antiviral em estratégias de vacinação tem sido analisada. Na seleção de um antiviral leva-se em consideração que o mesmo deveria: providenciar uma proteção antiviral rápida, ser eficaz contra os sete sorotipos e induzir de uma reposta imunológica adaptativa contra a vacina co-administrada. Esta combinação deveria propiciar uma reposta imediata e por um longo período (GRUBMAN & MASON, 2002).

Muitos pesquisadores têm demonstrado que as células que foram tratadas previamente com o interferon (IFN) do tipo I (IFNα/β), a replicação do FMDV foi inibida pelos produtos dos genes induzidos pelo IFN, a proteína quinase dependente da dupla fita de RNA (PKR) e pela 2'- 5' oligoadenilato sintetase (OAS)/RNase L (CHINSANGARAM et al., 1999, 2001, SELLERS, 1963). O IFN é uma das primeiras linhas de defesa da célula contra as infecções virais (VILCEK & SEN, 1996) e pode rapidamente induzir uma resposta protetora não específica contra todos os sorotipos de FMDV testados até o momento (CHINSANGARAM et al., 1999, 2001). Para uma efetiva proteção é necessário que a vacina contenha a cepa circulante no campo no momento de um surto e a mesma deve induzir uma resposta imunológica bastante rápida. Com base nesses dados, a terapia antiviral poderia resolver esses problemas inerentes às vacinas atuais de FMDV. O IFNα/β poderia ser empregado como um agente anti-FMDV para um tratamento profilático em animais susceptíveis (GRUBMAN & BAXT, 2004).

Estudos efetuados com IFNα/β em humanos têm demonstrado que a proteína é rapidamente eliminada do organismo, sendo necessário para o tratamento o emprego de múltiplas inoculações e em alta dosagem (LUKASZEWSKI & BROOKS, 2000, SANTODONATO et al., 2001). Para que o IFN seja efetivo em um surto de FMD ele deve ser entregue em uma única inoculação e em combinação com a vacina para que haja a indução de uma rápida proteção antes do início da resposta imunológica adaptativa. No trabalho de CHINSANGARAM et al. (2003) foi desenvolvido um Ad5 defectivo contendo o gene do IFNα suíno (Ad5-pIFNα). Suínos inoculados com uma dose desse vírus recombinante foram completamente protegidos quando desafiados com o FMDV um dia após a inoculação. Os animais não apresentaram sinais clínicos, nem viremia ou anticorpos contra as proteínas virais

não estruturais. Os níveis de proteção foram correlacionados com a dose do vírus e com o nível plasmático do INFα. Estudos posteriores realizados por MORAES et al. (2003) indicaram que somente o Ad5-pIFNα poderia ser usado para proteção de suínos 3 a 5 dias após inoculação. Animais que receberam o IFNα e foram desafiados um dia após, demonstraram reduzida viremia, excreção viral e severidade da doença clínica. Quando o pIFNα foi combinado com a vacina de Ad5-A24 induziu proteção imediata e, também, a longo prazo. Nos estudos com bovinos, a administração de IFNα expresso pelo Ad5 recombinante não induziu proteção completa dos animais após o desafio com o FMDV, porém a doença teve o início retardado e foi mais branda quando comparada ao grupo controle (WU et al., 2003). Esses resultados sugerem que a baixa proteção em bovinos, quando comparada aos suínos, pode estar relacionada aos reduzidos níveis plasmáticos de IFN.

Outro problema relacionado com o uso das vacinas convencionais é a sua inépcia em bloquear a infecção viral e o animal vacinado pode vir a se tornar portador. Tem sido sugerido que os indivíduos portadores poderiam ser prevenidos ou tratados pelo uso de um agente antiviral ou por componentes dirigidos a um sítio de privilégio da replicação viral (GRUBMAN & MASON, 2002).

O desenvolvimento e os testes de vacinas recombinantes de Ad5 expressando as proteínas do capsídeo do FMDV têm demonstrado que essa vacina é segura e eficaz, podendo ainda sobrepujar muitas das limitações das vacinas atuais para FMD. A combinação da vacina com um antiviral possibilitaria o desencadeamento de uma resposta imunológica protetora imediata e em longo prazo, além de ser uma alternativa para atuar na prevenção e/ou tratamento portadores. Entretanto, estudos são necessários para verificar a viabilidade dessas vacinas e do uso dos antivirais, incluindo outros sorotipos de FMDV (GRUBMAN & MASON, 2002). Nos surtos da enfermidade no Reino Unido em 2001, com o abate de milhares de animais e com prejuízos econômicos bastante onerosos, evidenciou-se a necessidade de encontrar novas alternativas e estratégias de controle para a FMD.

CAPÍTULO 2

IMMUNOPOTENTIATION OF A RECOMBINANT ADENOVIRUS-FMD VACCINE BY IFNα IN SWINE

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Abstract

The adjuvant effect of porcine interferon alpha (pIFNα) was examined in swine vaccinated with a recombinant replication-defective adenovirus containing foot-and-mouth disease virus (FMDV) capsid (P1-2A) and 3C proteinase coding regions (Ad5-A24). Swine were divided into 5 groups, each containing five animals, and inoculated with high ($5x10^9$ pfu) or low ($5x10^8$ pfu) doses of Ad5-A24 in the presence or absence of pIFNα (Ad5-pIFNα, 10^9 pfu). Control animals received $6x10^9$ pfu of Ad5-VSNJV-G containing the glycoprotein gene from vesicular stomatitis virus New Jersey. All swine were challenged at 42 days post vaccination (dpv) with the homologous strain of FMDV. Both high-dose Ad5-A24 inoculated groups developed a significant FMDV-specific neutralizing antibody response (PRN_{70}) by 14-21 dpv which was maintained until the day of challenge. While both low-dose inoculated groups had lower PRN_{70}, the IFN group had an enhanced response. After challenge all animals in the control group developed viremia, vesicular lesions, lymphopenia, antibodies to FMDV nonstructural proteins, and high PRN_{70}. Animals receiving low dose Ad5-A24 with no IFN had similar clinical disease, except that fewer animals developed viremia. In contrast, pigs inoculated with the low dose vaccine plus IFN had a delayed onset of vesicular lesions and only one animal had detectable viremia. Animals vaccinated with high dose Ad5-A24 without IFN had no viremia, significantly fewer lesions and delayed onset of disease compared to the group given a low dose vaccine and no IFN. Four of five pigs vaccinated with high dose Ad5-A24 plus IFN were completely protected from disease and only one animal in this group had a vesicular lesion which was restricted to the site of challenge virus inoculation. Furthermore, this latter group had a statistically significant higher IgG1 response than the high vaccine dose only group. Our results indicate that pIFN-α enhances the level of protection induced by the adenovirus-FMD vaccine against homologous FMDV, supporting the use of IFN-α as a potential adjuvant in FMD vaccination strategies.

Keywords: Foot-and-mouth disease; Adenovirus; Interferon; Vaccine.

1. Introduction

Foot-and-mouth disease virus (FMDV), the type species of the Aphthovirus genus of the family *Picornaviridae,* is the causative agent of foot-and-mouth disease (FMD) the most important disease of livestock worldwide [Grubman, Baxt, 2004]. FMD is a highly contagious acute vesicular disease affecting cattle, pigs, sheep and other domestic and wild cloven-hoofed animals [Callis, McKercher, 1986]. Although FMD results in low mortality, it can cause a significant decline in production of meat and dairy products and in young animals, myocarditis may cause death [Grubman, Baxt, 2004]. The virus spreads rapidly via aerosol, by contact with infected animals, or by movement of contaminated farm equipment, humans, and other non-susceptible animals [Bachrach, 1978]. FMD-free countries maintain rigid quarantine and import restrictions on animal and animal products from infected countries to prevent introduction of the disease, since an outbreak can result in drastic economic losses as a result of decrease in livestock production and international constraints on exports of animals and animal products [Bachrach, 1978]. Currently, the most effective method of control of the spread of FMD through livestock populations consists of the inhibition of movement of susceptible animals and their products, slaughter of infected and exposed animals, disinfection and vaccination.

Conventional vaccines based on chemically inactivated viruses are used to control and eradicate FMD in many countries. However, FMD-free countries are often hesitant to vaccinate during an outbreak because of the difficulty in distinguishing vaccinated from infected animals and the possibility of vaccinated animals becoming disease carriers following contact with live virus [Grubman and Baxt, 2004]. To overcome some of the problems associated with conventional FMD vaccines various FMD subunit vaccines delivered as peptides, by DNA vaccination, or by live virus vectors have been developed, but they have not successfully protected naturally susceptible animals [Taboga et al; Rodriguez et al., 2003; Wang et al., 2002; Beard et al., 1999; Cedillo-Barron et al., 2001; Benvenisti et al., 2001; Sanz-Parra et al., 1999a, 1999b]. We have recently developed a replication-defective human adenovirus type 5 (Ad5) vector containing the FMDV capsid and 3C proteinase coding regions, but lacking the coding regions for a number of FMDV nonstructural (NS) proteins [Mayr et al., 1999, 2001]. Infection of cells with this vector results in synthesis of the FMDV capsid precursor protein, its processing by the viral 3C proteinase, and assembly of the processed structural proteins into capsid structures. Animals administered this vaccine develop a FMDV-specific neutralizing antibody response, but can be readily distinguished from infected animals using diagnostic assays based on the NS proteins not present in this

subunit vaccine. We have demonstrated that one inoculation of this vaccine can successfully protect swine and cattle from virulent FMDV challenge [Moraes, et al., 2002; Pacheco et al., 2005]. However, both the conventional vaccine and the Ad5 vectored vaccine require 7 days to induce full protection. In FMD outbreaks in disease-free countries the induction of rapid protection is necessary to inhibit or limit disease spread so as to reduce the number of animals that need to be slaughtered.

Type I IFN (IFNα/β) is the first line of host cell defense against virus infection [Biron and Sen, 2001]. Virus-infected cells are induced to express and secrete IFNα/β, generating a virus-resistant state in cells via a series of events leading to activation of IFNα/β-stimulated genes (ISG's). Previous studies have demonstrated that FMDV is highly sensitive to IFNα/β and its replication is inhibited by the presence of two ISG products: double-stranded RNA-dependent protein kinase (PKR) and 2'-5' oligoadenylate synthetase (OAS)/RNase L [Chinsangaram et al., 1999, 2001]. These results indicated that IFNα/β may be helpful in vivo as an anti-FMDV agent. We have recently demonstrated that Ad5 expressing porcine IFN-α or -β (Ad5-pIFNα or -β) can deliver biologically active IFNα/β to animals [Chinsangaram et al., 2003, Wu et al., 2003a]. Inoculated swine are totally protected from FMDV infection when the animals are challenged 1 day post administration and protection can last for 3-5 days [Moraes et al., 2003]. In additional studies, we demonstrated the efficacy of a combination of Ad5-pIFNα and a FMD subunit vaccine (Ad5-A24) for rapid protection [Moraes et al., 2003].

Numerous studies indicate that in addition to antiviral activity IFNα/β can act as an adjuvant and enhance the immune response to antigens [Le Bon et al., 2001; Proietti et al., 2002; Cull et al., 2002; Brassard et al., 2002]. In the present study, we examined the adjuvant effect of pIFN-α in swine when combined with an Ad5-A24 vaccine. To determine whether pIFNα enhanced long-lasting immunity, different regimens of vaccination were administered. Swine were inoculated with different doses of Ad5-A24 in the presence or absence of Ad5-pIFNα and challenged at 42 days post vaccination (dpv) with FMDV, and protection was evaluated by clinical and serological parameters. The results indicate that pIFN-α enhanced the efficacy of the Ad5-A24 vaccine.

2. Materials and methods

2.1. Cells and viruses

Human 293 cells (ATTC CRL-1573) were used to generate and propagate recombinant human adenovirus serotype 5 viruses (Ad5) and determine virus titer [Graham et al., 1977, Moraes, et al., 2002]. The recombinant Ad5 viruses used in this study included: Ad5-A24 which contains the capsid coding region of FMDV-A24 Cruzeiro plus the 3C proteinase coding region of FMDV-A12 [Moraes et al., 2002], Ad5-pIFNα containing the porcine IFNα gene [Chinsangaram et al., 2003, Moraes et al., 2003], Ad5-Blue containing the β-galactosidase gene [Moraes et al., 2001], Ad5-VSNJV-G containing the glycoprotein gene from vesicular stomatitis virus New Jersey (VSNJV) [Moraes et al., 2001; Moraes et al., 2002]. All viruses were purified by CsCl gradient centrifugation and viral titer was determined by the method of tissue culture infectious dose ($TCID_{50}$) and converted to plaque-forming units (pfu/ml) [Wu et al., 2003b]. Baby hamster kidney cells (BHK-21, clone 13, ATCC CCL-10) were used to measure FMDV titers in plaque assays. Swine kidney cells (IBRS2, Foreign Animal Disease Diagnostic Laboratory [FADDL] at the Plum Island Disease Center) were used in a plaque reduction assay to evaluate antiviral activity of plasma from inoculated swine [Chinsangaram et al., 2001, Moraes et al., 2003]. FMDV serotype A24 (Cruzeiro) was obtained from vesicular fluid of a FMDV A24 infected swine and used as challenge virus. FMDV A12 was generated from full-length infectious clone pRMC35 [Rieder et al., 1993] and used for the biological assay of IFN.

2.2. Animal experiment

All animal experiments were performed in the disease secure isolation facilities at the Plum Island Animal Disease Center following a protocol approved by the Institutional Animal Use and Care Committee. Twenty-five Yorkshire swine (5-weeks old and weighing about 35-40 lbs each) were divided into five groups, each containing 5 animals. All groups were housed in separate rooms. Groups were inoculated with a high ($5x10^9$ pfu) or low ($5x10^8$ pfu) dose of Ad5-A24 per animal and Ad5-pIFNα ($1x10^9$ pfu) or Ad5-Blue ($1x10^9$ pfu). One group was inoculated with Ad5-VSNJV-G ($6x10^9$ pfu) as a control. All animals were inoculated intramuscularly (im) in the neck with the different doses of recombinant Ad5 and challenged at 42 dpv by intradermal inoculation in the heel bulb of each rear foot with a total of 100 PHID (pig heel infectious doses) FMDV A24 in 100 μl (50μl in each rear foot).

Prior to challenge, blood samples were examined for IFN production, induction of IFN-induced genes, FMDV-specific neutralizing antibodies (PRN_{70}), and antibody isotype. After challenge a number of disease parameters were analyzed including clinical score [Moraes et al., 2002], viremia, antibodies against viral structural and NS proteins, PRN_{70}, and antibody isotype. Nasal swabs were collected for 7 days post challenge (dpc). Temperatures were also monitored on a daily basis. Animals with a high temperature were given Banamine®. The experiment was ended at 63 dpv and all animals were humanely euthanized.

2.3. IFN assay

2.3.1. Antiviral activity in plasma samples

Antiviral activity was evaluated in plasma samples as previously described (Moraes et al., 2003). In brief, the samples were obtained at 0-7 dpv, diluted and applied in duplicate wells of six-well plates containing IBRS2 cells, and incubated overnight at 37°C. The inoculum was aspirated and the cells were infected with approximately 100 pfu FMDV A12. After adsorption of the virus for 1 h at 37°C, the inoculum was aspirated and the cells were overlaid with 2 ml of gum tragacanth and incubated 24 h at 37°C before staining with crystal violet to visualize the plaques [Chinsangaram, 2001]. Antiviral activity of IFN was reported as the reciprocal of the highest sample dilution that resulted in a 50% reduction in the number of plaques relative to the untreated cells.

2.3.2. Quantification of pIFNα in plasma samples

A porcine IFNα ELISA double capture assay was previously developed in our lab and was used as described [Moraes et al., 2003]. Briefly, ELISA plates (Immunolon 2HB, Thermo Labsystems, Franklin, MA) were coated overnight at 4°C with one recombinant monoclonal antibody (MAb) against pIFNα (K9, R&D Systems, Minneapolis, MN) diluted in carbonate-bicarbonate buffer (Sigma, St Louis, MO). The plates were blocked with PBS containing 0.05% Tween 20 and 5% dry non-fat milk (blocking buffer) for 2 h at room temperature (RT) and then washed 4 times with PBS containing 0.05% Tween 20 (washing buffer) in a Skan Washer 300 (Skatron Instruments, Lier, Norway). Plasma samples obtained from swine inoculated with different recombinant adenoviruses were used directly or serially 10-fold diluted in minimal essential media plus 5% fetal calf serum and added to the plates in duplicate for 1 h at 37°C. The plates were washed as above and a second anti-pIFNα MAb (F17, R&D Systems) previously biotinylated was diluted and added to the plates for 1 h at

37°C. The plates were washed as above and peroxidase-conjugated streptavidin (Streptavidin-HRP, Kirkegaard & Perry Laboratory [KPL], Gaithersburg, MD) was diluted and added to the plates for 30 min at 37°C. The amount of bound peroxidase was determined by incubation with 3,3,'5,'5-tetramethyl benzidine (TMB) substrate (KPL) for 15 min at RT. The reaction was stopped by adding 1M H_2SO_4, and the plates were read at 450-570 nm on a Bio-Kinetics Reader EL-312e (Bio-tek Instruments, Winooski, VT). The pIFNα concentrations were determined from a standard curve with recombinant pIFNα using Microsoft Excel.

2.4. IFN induced genes

A quantitative real time RT-PCR assay was used to evaluate the mRNA levels of three IFN induced genes: PKR, OAS and Mx1. RNA was extracted from the white blood cells (WBC's) prepared from 1mL heparinized blood collected at 0-4 and 7 dpv by using a QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA). 5μl of RNA total samples were treated with deoxyribonuclease I (Sigma) and cDNA was synthesized with M-MLV reverse transcriptase and random hexamers (Invitrogen, Carlsbad, CA). The relative levels of PKR, OAS, and Mx1 mRNAs were measured using Ampli Taq gold (Applied Biosystems, Foster City, CA) in a ABI PRISM 7700 sequence detection system. Primers (Invitrogen) and probe were designed using Primer express® software (Applied Biosystems) and their sequences are listed in Table 1. The TaqMan MGB probes (Applied Biosystems) were labeled with a fluorescent dye (FAM™) and used in this assay. 18s rRNA was used as an internal standard control and was amplified for each sample. Primers and probe used for 18s rRNA were from the TaqMan® Ribosomal RNA Control Reagents (Applied Biosystems). This kit provides rRNA probe (VIC™ probe), and rRNA forward and reverse primers. The assay was standardized and mRNA from 0 dpv was used as normalizer gene. The Ct values of rRNA obtained from each sample were analysed and were between 9-11. The delta Ct, delta-delta Ct and fold increase for each gene with each sample was determined.

2.5. Antibody analysis

2.5.1. Plaque reduction neutralization (PRN$_{70}$) assay

FMDV-specific neutralizing antibody titers were measured by PRN$_{70}$. Briefly sera was collected at 0 and 5 dpv and weekly thereafter and 2-fold serial dilutions were mixed with an equal volume of approximately 100 pfu of FMDV A24 and incubated at RT for 1 h. This mixture was applied, in duplicate, to a six-well plate of BHK-21 cells. After adsorption of the

virus for 1 h at 37°C, the inoculum was aspirated and the cells were overlaid with 2 ml of gum tragacanth and incubated 24 h at 37°C before staining with crystal violet to visualize the plaques [Mason et al., 1997, Mayr et al., 1999]. Neutralizing titers were reported as the serum dilution yielding a 70% reduction in the number of plaques (PRN$_{70}$).

2.5.2. Radioimmunoprecipitation (RIP) of [^{35}S]-methionine labeled FMDV A24 infected cell lysates

Swine sera were examined by RIP for the presence of antibodies against FMDV structural and NS polypeptides as a sensitive measure of challenge virus replication. In brief, [^{35}S]-methionine-labeled FMDV A24-infected BHK-21 cells lysates (approximately 200,000 - 250,000 cpm per sample) were immunoprecipated with individual swine sera from 0 and 63 dpv. After 1 h incubation at RT, the immune complexes were precipitated with *Staphylococcus aureus* protein A and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel and visualized by autoradiography [Moraes, et al., 2002].

2.5.3. 3ABC ELISA assay

Swine sera from 0, 42, 49, 56 and 63 dpv were examined for the presence of antibodies against FMDV NS proteins using an ELISA developed by the FADDL at the Plum Island Animal Disease Center. Briefly, ELISA plates were coated overnight with a preparation of baculovirus-expressed FMDV 3ABC protein and baculovirus-expressed negative control antigen in 8M urea [Meyer et al., 1997]. Control, standard and sera samples to be analyzed were added to both the 3ABC and negative control antigen microplate wells. The samples were diluted 1:100 and added in duplicate and incubated 30 min at RT. The plates were washed and horseradish peroxidase conjugated recombinant protein A/G (Pierce, Rockford, IL) diluted at 1:800 was added and incubated 30 min at RT. The plates were washed and TMB substrate (Sigma) was added for 4 min at RT. The reaction was stopped by addition of 1M H$_2$SO$_4$, and the plates were read at 450-650 nm on a microplate reader. Values obtained from negative control antigen wells are subtracted from values obtained from the 3ABC wells to give corrected absorbance values. The corrected absorbance values are normalized to the standard sera. All calculations were performed using Microsoft Excel.

2.5.4. Antibody isotype assay

ELISA plates (Immulon 2HB) were coated overnight at 4°C with a 1:2000 dilution of rabbit anti-FMDV A5/22/24 (Institute for Animal Health Laboratory, Pirbright, Surrey, UK)

in carbonate-bicarbonate buffer, pH 9.6 (Sigma). The plates were blocked with blocking buffer for 2 h at 37°C in constant agitation and washed 3 times with washing buffer in a Skan Washer 300 (Skatron Instruments). Pairs of wells were incubated with blocking buffer containing a 1:20 dilution of binary ethylenimine inactivated (BEI) FMDV A24 infected BHK-21 infected cell culture supernatant for 1 h at 37°C on a shaker and then washed as above. Swine sera from the control and vaccine groups were diluted 1:50 in blocking buffer and added to antigen-containing and antigen-negative wells (1:20 dilution of BEI treated cell culture supernatants from uninfected BHK-21 cells), and incubated for 1 h at 37°C. The plates were washed and a 1:500 dilution of anti-swine IgM, IgG1, or IgG2 isotype monoclonal antibodies (Serotec, Raleigh, NC) in blocking buffer were added and the plates incubated for an additional hour at 37°C with shaking. The plates were washed and a 1:1000 dilution of goat anti-mouse peroxidase conjugate (KPL) in blocking buffer was added and the plates incubated for 1 h at 37°C. The amount of peroxidase bound was determined by incubating with 2,2'-azino-di- (3-ethylbenzthiazoline-6-sulfonate) in 0.01% hydrogen peroxide (ABTS$^{®}$/H$_2$O$_2$) solution (KPL) for 10 min at RT, addition of stop solution (1% SDS), and detection at 405 nm in a Bio-tek microplate reader. To minimize the difference of results within and among assays, each plate contained a positive and a negative control serum. The positive control sera for IgM was obtained from a swine at 7 days postinfection with virulent FMDV A24, and for IgG1 and IgG2 was from a swine at 35 days postimmunization with a commercial BEI-inactivated FMD A24 vaccine (Vecol, Colombia). Positive control sera were chosen for their ability to generate a definitive signal in their respective isotype-specific assays. Negative control sera for each assay were pre-immune sera from the same animals. Results are expressed as percentage of the positive reference serum reaction in the same plate [Wright, 1993]. Statistical analysis of the data was performed by Tukey's test, ie., P<0.05.

3.   Results

3.1. Antiviral response in swine inoculated with pIFNα

All swine in the groups that were inoculated with Ad5-pIFNα (Groups 1 and 2) developed a significant antiviral response in plasma samples by 1 dpv which lasted for 1-2 additional days (Table 2; data not shown). The levels of IFN-α detectable by ELISA correlated with the antiviral activity and were in the order of 6,000 – 24,000 pg/ml by 1 dpv.

Animals in the groups that did not receive IFN-α had essentially no detectable antiviral activity or IFN-α in their plasma (Table 2).

Previously we showed that PKR and OAS/RNAse L were involved in the inhibition of FMDV replication in cell culture [Chinsangaram *et al*., 2001]. The induction of these two IFN-induced genes as well as Mx1, another ISG, were measured by real time PCR amplification of RNA extracted from white blood cells collected following the inoculation of the different recombinant Ad5 viruses. An increase of all 3 ISG mRNAs was detectable by 1 dpv in the groups that received Ad5-pIFNα (Groups 1 and 2) (Table 3). This induction decreased on day 2, but significantly increased on day 3, reached a peak on day 4, and was still detectable on day 7, the last day of sampling. Although the standard deviation for all 3 ISG mRNAs was large, especially for Group 1, this was because one animal in this group, 3858, had a much higher fold increase of all 3 ISG's on all the days examined. Nevertheless, all 4 remaining animals in this group also had a significant induction of the ISG's. Groups 3 and 4, which received Ad5-A24 vaccine but no pIFN-α, either had no induction or only a 2-4 fold induction of all ISG's. The control group, 5, which was administered Ad5-VSNJV-G had essentially no response on days 1 and 2 postvaccination, but showed a significant response of all 3 ISG's by day 3 which was sustained until day 7.

3.2. FMDV-specific antibody response in Ad5-inoculated swine

The FMDV-specific neutralizing antibody titer was evident around 5-7 dpv in the groups that received Ad5-A24 and was maintained until the day of challenge at 42 dpv (Figure 1). The groups that were inoculated with the high dose of Ad5-A24, 1 and 3, developed significantly higher levels of FMDV-specific neutralizing antibodies as compared to the groups that received the 10-fold lower dose of the vaccine, 2 and 4, through the challenge day. Groups 1 and 3 developed approximately 13-fold higher levels of FMDV-specific neutralizing antibodies than Groups 2 and 4. (Table 2, compare Groups 1 and 3 with 2 and 4). The presence of Ad5-pIFNα did not augment the levels of FMDV-specific neutralizing antibodies in the animals inoculated either with high or low doses of the vaccine. The control group given Ad5-VSNJV-G did not develop an FMDV-specific neutralizing antibody response.

ELISA assays were used to characterize the isotype response in vaccinated animals throughout the course of the experiment. The data presented in Fig. 2 shows the mean of the response in each group. The animals vaccinated with the high dose of vaccine, Groups 1 and 3, developed an FMDV-specific IgM response by 14 dpv, while the groups given the low dose

of vaccine had a lower IgM response, and the control group, Group 5, had no detectable response. Only the high dose vaccinated groups had a switch to IgG1 at 21 dpv with a peak at 28-35 dpv and a switch to IgG2 by 28 dpv with a peak at 28-35 dpv. The low dose vaccinated groups had little or no detectable IgG1 or IgG2 response prior to challenge. The high dose vaccinated group inoculated with Ad5-pIFNα developed both higher IgG1 and IgG2 responses compared to the high dose vaccine group inoculated in the absence of IFN-α (compare Fig. 2B and 2C, Groups 1 and 3). Statistical analysis, performed by Tukey`s test (P< 0.05), revealed that the increased IgG1 response of Group 1 compared to Group 3, at 35 dpv, was statistically significant.

3.3. Protection of swine challenged with homologous FMDV: Clinical response and viral excretion

All swine were challenged at 42 dpv with FMDV A24. All animals in the control group, Group 5, developed lesions at 2 or 3 dpc as well as viremia and virus in the nasal fluid. All the animals in this group had a significant clinical score and presented detectable lymphopenia at 3-5 dpc during the acute phase of FMD infection as previously demonstrated [Bautista et al., 2003] (Fig. 3). The animals inoculated with low-dose vaccine in the absence of IFN-α, Group 4, had similar clinical signs as the control group and lesions were evident by 2-3 dpc in all animals. Lymphopenia was reduced as compared to the control group and only detectable for two days and viremia was detectable in only 3 of 5 animals, but virus was present in the nasal fluid of all swine. However, 2 of 5 animals in the group receiving low-dose vaccine with IFN-α, Group 2, had lower clinical scores and 3 of 5 animals in this group had delayed onset of disease as compared with the control and the low-dose Ad5-A24 without IFN-α groups (Table 4, compare group 2 with groups 4 and 5). Although the animals in this group showed virus in nasal fluid, lymphopenia was not as pronounced and only detectable for two days, and only one animal had a low level of viremia at 4 dpc that lasted for one day. In the group that was vaccinated with a high dose of Ad5-A24 without IFN-α, one animal (Table 4, swine no. 3868) was completely protected with no viremia, lymphopenia or virus in the nasal fluid and the four remaining animals showed remarkably reduced clinical scores when compared with the control group. Two of these animals (3865 and 3869) only had lesions at the site of challenge and 3865 only developed a lesion 10 dpc. None of the animals in this group had viremia or lymphopenia and only a low virus titer was detectable in the nasal fluid of 4 of 5 animals. In the group inoculated with high dose Ad5-A24 with IFN-α, Group

1, four swine had no lesions, no viremia, no lymphopenia and no virus evident in the nasal fluid. Only one animal in this group had a lesion and it was restricted at the site of challenge.

3.4. Protection of swine challenged with homologous FMDV: Antibody response against the nonstructural proteins

After challenge the control group, Group 5, and the two groups given low dose vaccine, Groups 2 and 4, all developed a strong IgM response, which subsequently switched to IgG1 and 2. In contrast, the two groups given high dose vaccine, Groups 1 and 3, developed an immediate boost in IgG1 and IgG2, but not IgM.

Swine sera collected at 0, 42 and 63 dpv were examined by RIP for the presence of antibodies against FMDV NS proteins as a sensitive measure of challenge virus replication. At 42 dpv (day of challenge) only the animals in the groups given a high dose of Ad5-A24, Groups 1 and 3, had antibodies against the viral structural proteins and no swine in these groups had antibodies against FMDV NS proteins, ie., $3D^{pol}$ or 2C (data not shown).

At 63 dpv (21 dpc) all animals in the control group had a strong antibody response against FMDV structural and NS proteins (Table 4). The animals inoculated with low-dose vaccine without IFN-α, had antibodies against the structural proteins and $3D^{pol}$. In this group three of five animals showed a weak reaction against $3D^{pol}$. In the group receiving low-dose vaccine with IFN-α, three animals showed positive reaction against the NS proteins, while two animals had no NS antibodies. In the group inoculated with high-dose vaccine in the absence of IFN-α, two animals had no NS antibody response, while the remaining animals had low, but detectable levels of antibodies against $3D^{pol}$. The group vaccinated with high-dose Ad5-A24 with IFN-α only had antibodies against the viral structural proteins and had no detectable antibodies against NS proteins by RIP.

We also used a 3ABC ELISA as an additional serological method to measure challenge virus replication. This assay is currently used to differentiate FMDV-vaccinated animals from infected or convalescent animals. The results were similar to the RIP data for Groups 4 and 5. However, in Groups 1-3 there were some differences. In Group 3 inoculated with high-dose vaccine and no IFN-α, animal 3868 which was protected from clinical disease and had no viremia or virus in the nasal fluid was weakly positive by RIP but negative by ELISA and animal 3869, which developed two lesions and virus in the nasal fluid, was negative in RIP but positive by ELISA. In Group 2 animal 3863, which developed significant clinical disease, was strongly positive by ELISA, but only suspicious by RIP. In Group 1,

vaccinated with high-dose vaccine with IFN-α, which had no viremia, virus in the nasal fluid and only one animal showed a lesion in the site fo inoculation, only two animals were positive by ELISA (one weakly, animal 3858 and 3859 same animal with a lesion in the site of inoculation), but were negative by RIP (Table 4).

4. Discussion

Type I interferons have multiple biological activities [Biron and Sen]. They were initially identified because of their antiviral function [Isaacs and Lindemann], but subsequently they have been shown to effect many aspects of the host immune system [Biron and Sen]. In particular, recent studies have demonstrated that when co-administered with an antigen including soluble protein [Le Bon], a killed vaccine [Proietti], or DNA encoding a transgene [Cull] IFNα/β can function as an adjuvant. Administration of IFN-α together with an influenza virus subunit vaccine resulted in protection of mice against virus challenge and the induction of a Th1 type of response [Proietti et al., 2002]. Likewise co-administration of DNA expressing IFN-α9 or IFN-β with DNA encoding the glycoprotein B gene (gB) of cytomegalovirus (CMV) provided better protective immunity in mice against CMV-induced myocarditis than gB DNA alone [Cull et al., 2002].

We have been examining novel approaches to develop protection against FMD and have proposed that a new FMD control strategy should combine an antiviral to induce rapid non-specific protection and a subunit vaccine to induce long-lasting specific protection against the vaccine antigen. Recently we have shown that pIFN-α can induce an almost immediate antiviral response in swine and within one day protect inoculated animals from challenge with FMDV [Chinsangaram et al., 2003; Moraes et al., 2003]. The basis of the rapid protection by IFN-α correlated with the synthesis of IFN and the induction of an antiviral response. In the current study, we confirmed that the Ad5-pIFNα inoculated animals rapidly produced high levels of pIFN-α that was biologically active since these animals developed an antiviral response, and were induced to produce increased levels of ISG's, two of which have been shown to directly inhibit FMDV replication [Chinsangaram et al., 2001]. We further demonstrated that the biologically active pIFN-α enhanced the protective adaptive immune response of an FMDV subunit vaccine. Swine inoculated with both a high dose of Ad5-A24 and Ad5-pIFNα were completely protected from systemic disease after challenge with FMDV 42 days later, ie., only one animal had a single lesion at the site of challenge. In contrast,

although animals administered only a high dose of Ad5-A24 had significantly reduced disease as compared to controls, two of five animals in this group had a lesion at a site other than the inoculation site. Likewise, the group that received low dose Ad5-A24 and Ad5-pIFNα had reduced viremia and delayed and reduced clinical disease as compared to the low dose Ad5-A24 vaccinated group.

We examined two in vitro serological parameters as a basis of understanding the potential adjuvant effect of pIFN-α. The in vitro neutralizing antibody titers did not allow discrimination between the groups that received either pIFN-α and Ad5-A24 or only vaccine. However, examination of the antibody isotype composition enabled us to correlate a serological parameter with enhanced protection. A role for induction of a specific antibody isotype by the inactivated vaccine in promoting protection against FMD has been suggested [Mulcahy et al., 1990; Capozzo et al., 1997]. These researchers showed that cattle vaccinated with conventional inactivated FMD whole virus vaccine develop a virus-specific antibody response in which the IgG1 titers predominate over those of IgG2. Furthermore, there was an almost linear correlation between the IgG1 response and the animals' capacity to be protected from challenge [Capozzo et al, 1997]. In contrast, FMD synthetic peptide vaccines are not as effective in inducing protection against disease as inactivated virus vaccines and in peptide-vaccinated cattle the FMD-specific IgG1:IgG2 ratios were significantly lower than in animals inoculated with inactivated virus vaccine [Mulcahy, 1990]. These results imply that the induction of protection is related to the antibody isotype produced and that the IgG1 isotype has particular relevance to protection against FMDV. It has been suggested that in vivo the major protective immune response against FMDV requires antibody-dependent opsonization-enhanced phagocytosis [McCullough et al., 1992] and in cattle there is evidence that IgG1 interacts with the Fc receptor on bovine macrophages [Mulcahy et al., 1990]. At this time our knowledge of the swine immune system is relatively rudimentary and the identification of IgG subtypes, the cytokines involved in their induction, and their role in biological processes needs more studies. Nevertheless, our results show that the group that received both the high dose Ad5-A24 and Ad5-pIFNα had increased levels of IgG1 and IgG2 postvaccination as compared to the group that received only high dose vaccine. Furthermore, the increase in the IgG1 response in the dual-inoculated group as compared to the group that only received high dose vaccine was statistically significant on day 35 postvaccination. In addition, the dual-inoculated group had an increased IgG1:IgG2 ratio as compared to the vaccine only inoculated group. Clearly additional work is necessary to determine the role enhanced IgG1

and IgG2 levels have in protection against FMD in swine. Likewise a better understanding of additional factors including possible cytokine induction and cell-mediated immune response is essential to gain a more comprehensive understanding of the mechanism of the adjuvant effect of IFN-α.

An unexpected finding in this study was the enhanced expression of the 3 ISG mRNAs in the control Ad5-VSNJV-G inoculated animals (in the absence of detectable antiviral activity). The induction of the ISG's can be attributed to the expression of the VSV G protein, since the 2 groups that received only high or low dose Ad5-A24 did not show this response. There is evidence that viral glycoproteins can induce an innate IFN-α synthesis. Transmissible gastroenteritis virus is a potent inducer of IFN-α and it has been shown that the viral M glycoprotein plays a role in this process [Laude et al., 1992]. Furthermore, glycosylation of this protein has an important role in efficient induction of IFN-α. More recently it has been shown that toll-like receptors (TLRs) are involved in initiating antiviral responses. Various viral proteins including the hemaglutinin protein of measles virus and the fusion protein of respiratory synctial virus have been implicated in activation of TLRs [Bowie and Haga, 2005].

It is well documented that some strains of VSV are efficient inducers of IFN [Marcus], but we are unaware of any evidence implicating a role for G protein in this process. We have previously demonstrated that infection of cells with Ad5-VSNJV-G results in the expression of G on the cell surface (Moraes et al., unpublished observations). Therefore, the G protein travels through a number of cellular organelles including the ER, golgi, and plasma membrane during its life cycle. It is known that TLRs are present in these organelles and it is possible that an interaction of G with a TLR may occur and induce expression of ISG's. Likewise the presence of G on the cell surface of infected cells could also serve to activate expression of ISG's in cells that interact with these infected cells including antigen presenting cells or other immune cells.

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Figure legends

Fig. 1. Neutralizing antibody response in swine inoculated with Ad5-A24. Serum samples were collected at 0, 5 dpv and weekly following the vaccination. Neutralizing titers were reported as serum dilution yielding a 70% reduction in the number of plaques (PRN_{70}) and represent the mean of each group. Group 1 [high dose Ad5-A24 (5×10^9) + Ad5-pIFNα (10^9)], Group 2 [low dose Ad5-A24 (5×10^8PFU) + Ad5-pIFNα (10^9PFU)], Group 3 [high dose Ad5-A24 (5×10^9PFU) + Ad5-blue (10^9PFU)], Group 4 [low dose Ad5-A24 (5×10^8PFU) + Ad5-

blue (10^9PFU)], and Group 5 [Control group, Ad5-VSNJV 6x10^9PFU)]. Error bars shown refer to the S.D. for each mean data point, n= 5 samples.

Fig. 2. Isotype of immunoglobulins response in swine vaccinated with Ad5-A24. A. IgM response, B. IgG1 response and C. IgG2 response. The data represent the mean of each group. Group 1 [high dose Ad5-A24 (5x10^9) + Ad5-pIFNα (10^9)], Group 2 [low dose Ad5-A24 (5x10^8PFU) + Ad5-pIFNα (10^9PFU)], Group 3 [high dose Ad5-A24 (5x10^9PFU) + Ad5-blue (10^9PFU)], Group 4 [low dose Ad5-A24 (5x10^8PFU) + Ad5-blue (10^9PFU)], and Group 5 [Control group, Ad5-VSNJV 6x10^9PFU)]. Error bars shown refer to the S.D. for each mean data point, n= 5 samples.

Fig. 3. Percentage of lymphocytes in swine inoculated with Ad5-A24. Heparinized blood was collected one day before challenge and at 1-6 and 10 days post-challenge. The data represent the mean of each group. Group 1 [high dose Ad5-A24 (5x10^9) + Ad5-pIFNα (10^9)], Group 2 [low dose Ad5-A24 (5x10^8PFU) + Ad5-pIFNα (10^9PFU)], Group 3 [high dose Ad5-A24 (5x10^9PFU) + Ad5-blue (10^9PFU)], Group 4 [low dose Ad5-A24 (5x10^8PFU) + Ad5-blue (10^9PFU)], and Group 5 [Control group, Ad5-VSNJV 6x10^9PFU)]. Values lower than 40% were considered as lymphopenia. Error bars shown refer to the S.D. for each mean data point, n= 5 samples.
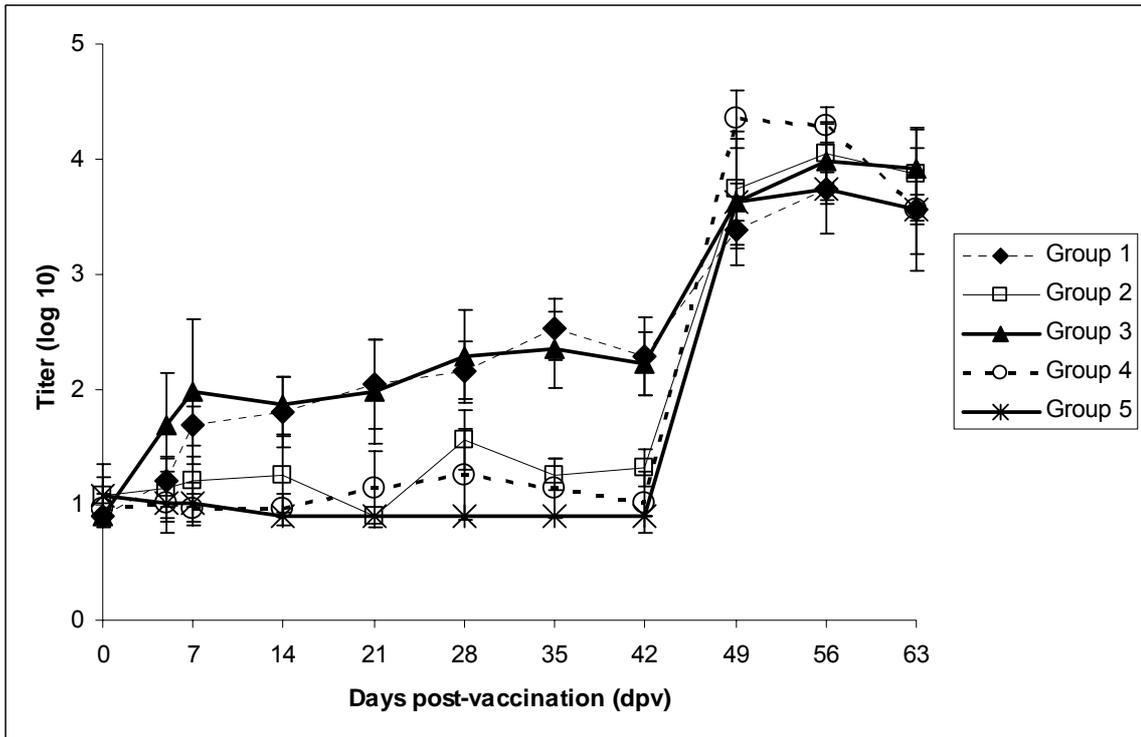
Fig. 1. Neutralizing antibody response in swine inoculated with Ad5-A24.

A. IgM

B. IgG1
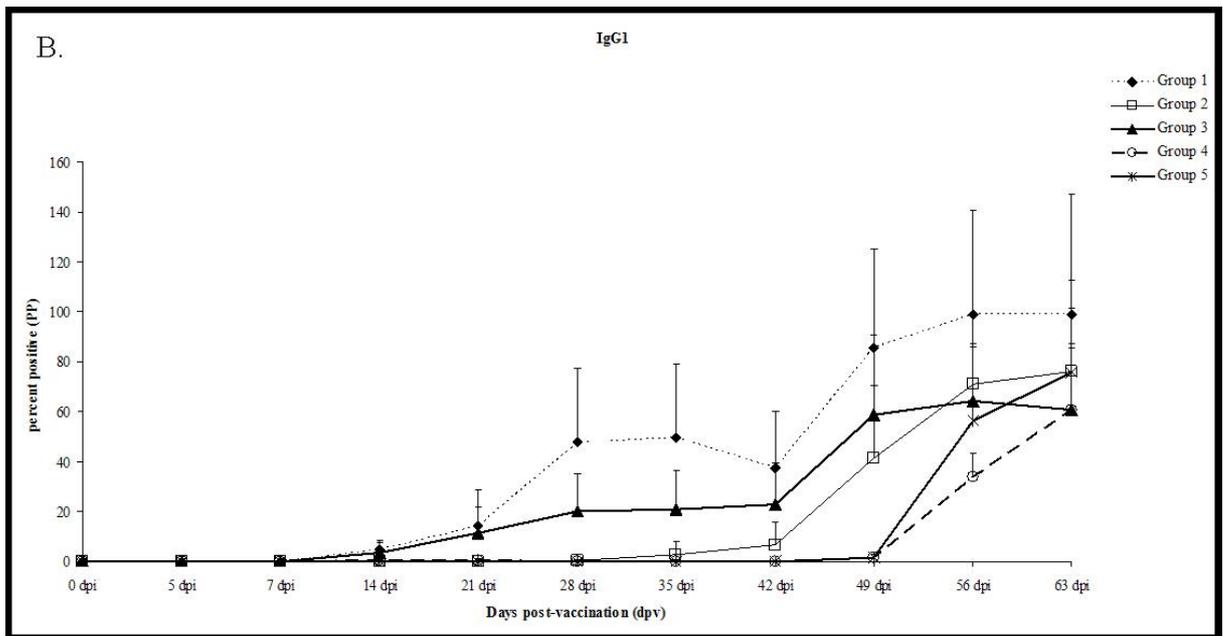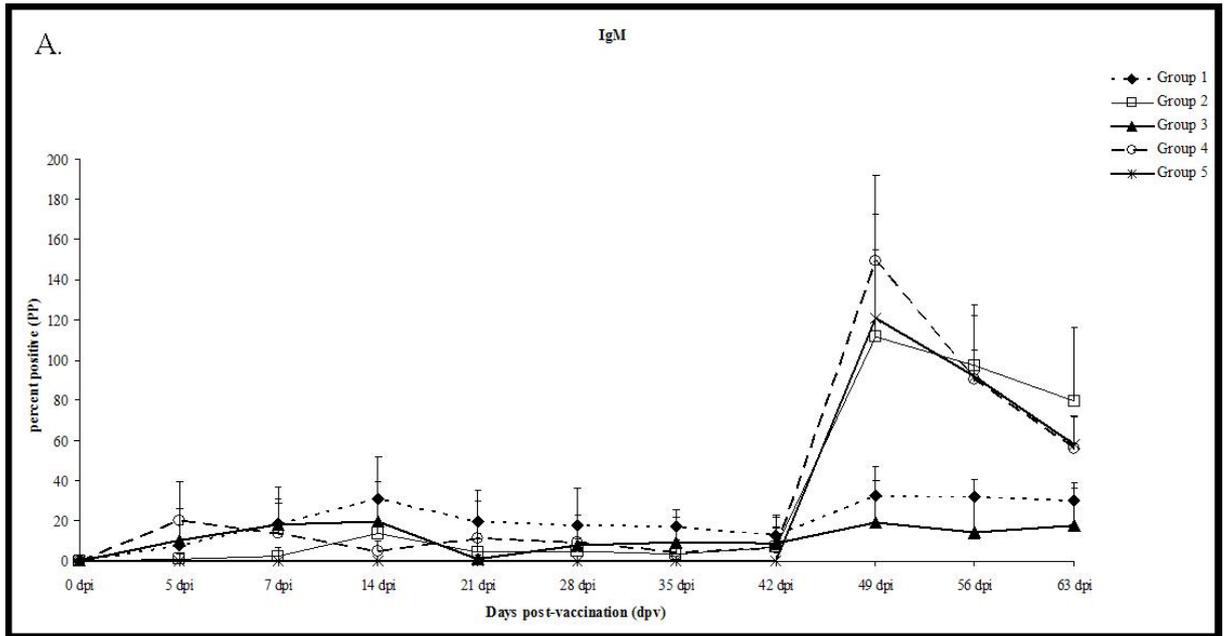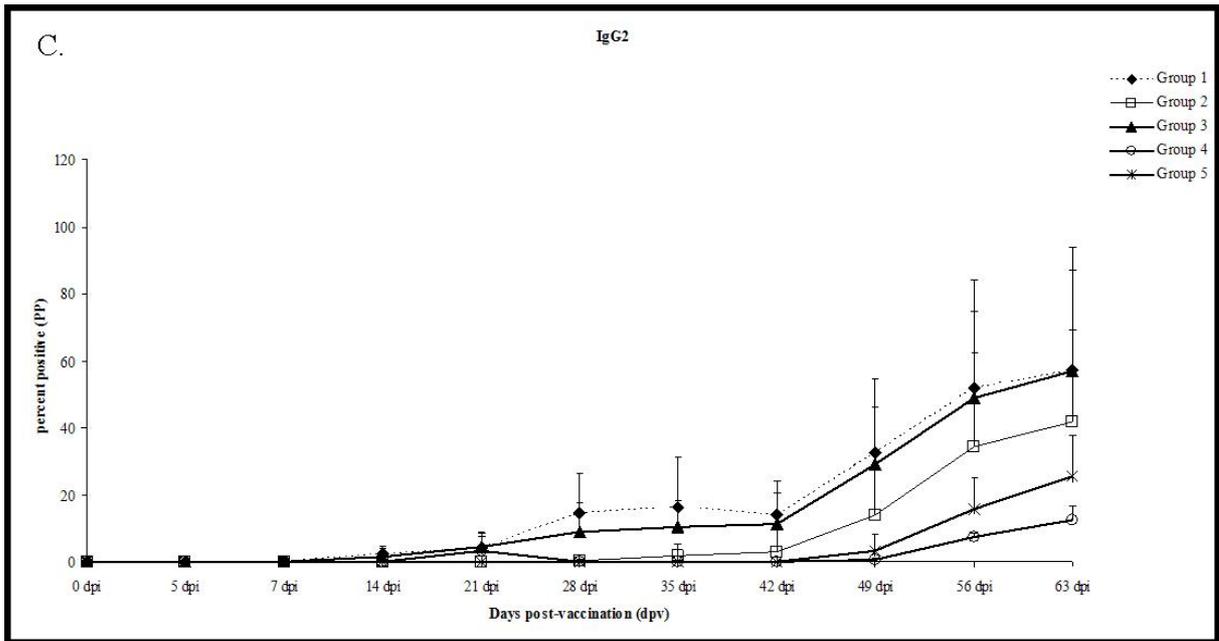
Fig. 2. Isotype of immunoglobulins response in swine vaccinated with Ad5-A24.
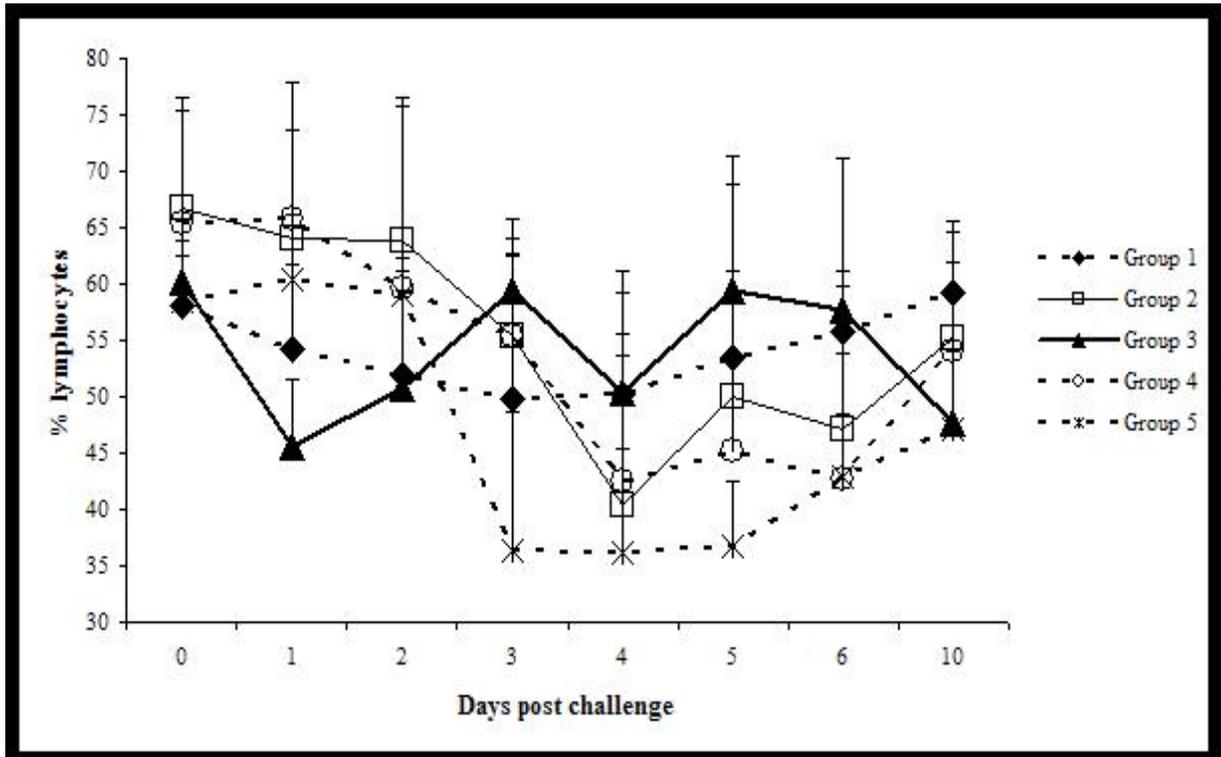
Fig. 3. Percentage of lymphocytes in swine inoculated with Ad5-A24.

Table1

Oligonucleotide primers and probes sequences for amplification of porcine IFN and IFN-induced genes used in real time RT-PCR

Gene	Primer and probe sets	Sequence	GenBank [1]
Mx1	porcine Mx1-803F[2]	5'GAGGTGGACCCCGAAGGA3'	M65087
	porcine Mx1-859R[3]	5'CACCAGATCCGGCTTCGT3'	
	porcine Mx1-824T[4]	5'AGGACCATCGGGATC3'	
OAS	porcine OAS-889F[2]	5'CTGTCGTTGGACGATGTATGCT3'	AJ225090
	porcine OAS-954R[3]	5'CAGCCGGGTCCAGAATCA3'	
	porcine OAS-919T[4]	5'TCAAGAAACCCAGGCCT3'	
PKR	porcine PKR-968F[2]	5'GGAAGAAAACAAACACAGCTTGAA3'	AB104654
	porcine PKR-1048R[3]	5'CCAAATCCACCTGAGCCAATT3'	
	porcine PKR-994T[4]	5'CCAGGTT TGTCGAAGAT3'	

[1] NCBI GenBank accession code. [2] Forward primer. [3] Reverse primer. [4] TaqMan MGB Probe.

Table 2

Antiviral and serological response of swine inoculated with Ad5 virus

Group	Inoculum	Dose (PFU/ml)[a]	Animal #	Antiviral activity[b]	pIFNα (pg/ml)[c]	PRN$_{70}$[d]
1	Ad5-A24[e] Ad5-pIFNα[f]	5×10^9 1×10^9	3855	800	22875	512
			3856	200	8725	256
			3857	200	7175	128
			3858	200	6300	64
			3859	200	9575	256
2	Ad5-A24 Ad5-pIFNα	5×10^8 1×10^9	3860	800	22395	16
			3861	200	9870	16
			3862	400	8395	16
			3863	400	11395	32
			3864	400	24320	32
3	Ad5-A24 Ad5-Blue[g]	5×10^9 1×10^9	3865	‹25	0	256
			3866	25	0	256
			3867	25	0	64
			3868	25	0	256
			3869	25	0	128
4	Ad5-A24 Ad5-Blue	5×10^8 1×10^9	3870	25	0	8
			3871	‹25	0	8
			3872	‹25	0	8
			3873	‹25	0	8
			3874	‹25	0	32
5	Ad5-VSNJV-G[h]	6×10^9	3875	‹25	246	‹8
			3876	‹25	0	‹8
			3877	‹25	0	‹8
			3878	‹25	0	‹8
			3879	‹25	63	‹8

[a] Individual dose of inoculum expressed as plaque forming units per ml.
[b] Highest dilution that reduced FMDV A12 plaque number by 50% in IBRS2 cells in plasma samples 1 dpv.
[c] Amount of pIFNα determined by ELISA capture assay in plasma samples 1 day post inoculation.
[d] Neutralizing antibody response reported as serum dilution yielding a 70% reduction in the number of plaques (PRN$_{70}$) at the day of challenge (42dpv).
[e] Recombinant human adenovirus serotype 5 virus (Ad5) containing the capsid coding region of FMDV-A24 Cruzeiro plus the 3C proteinase coding region of FMDV-A12.
[f] Ad5 containing the porcine IFNα gene.
[g] Ad5 containing the β-galactosidase gene.
[h] Ad5 containing the glycoprotein gene from vesicular stomatitis virus New Jersey (VSNJV).

Table 3

RNA- fold induction of three interferon-induced genes: PKR, OAS and Mx1 in swine white blood cells

Group	Inoculum	Dose (PFU/ml)[b]	DPV[c]	PKR	OAS	Mx1
				RNA-fold induction (Mean ± SD[a])		
1	Ad5-A24[d] Ad5-pIFNα[e]	5 x 10⁹ 1 x 10⁹	1	3.60 ± 1.62	3.54 ± 1.59	14.12 ± 9.12
			2	1.55 ± 1.17	1.16 ± 0.50	4.38 ± 3.06
			3	23.25 ± 25.90	27.88 ± 30.58	73.56 ± 89.93
			4	34.78 ± 27.14	33.72 ± 25.56	78.36 ± 70.33
			7	25.07 ± 25.01	24.35 ± 28.98	58.09 ± 82.06
2	Ad5-A24 Ad5-pIFNα	5 x 10⁸ 1 x 10⁹	1	2.54 ± 1.25	2.08 ± 1.70	8.38 ± 6.16
			2	2.01 ± 0.74	1.28 ± 0.90	4.60 ± 1.99
			3	14.15 ± 11.26	27.54 ± 22.11	36.74 ± 23.84
			4	27.86 ± 11.49	60.57 ± 45.82	51.60 ± 24.70
			7	12.72 ± 2.11	16.42 ± 12.73	23.32 ± 3.35
3	Ad5-A24 Ad5-Blue[f]	5 x 10⁹ 1 x 10⁹	1	0.87 ± 0.17	1.18 ± 0.49	1.42 ± 0.24
			2	0.90 ± 0.89	0.57 ± 0.53	0.76 ± 0.45
			3	0.37 ± 0.13	0.24 ± 0.17	0.62 ± 0.32
			4	1.54 ± 0.53	1.32 ± 0.80	2.72 ± 1.63
			7	1.35 ± 0.25	0.80 ± 0.52	2.00 ± 0.67
4	Ad5-A24 Ad5-Blue	5 x 10⁸ 1 x 10⁹	1	1.29 ± 1.02	2.02 ± 1.94	2.16 ± 1.59
			2	0.55 ± 0.45	0.49 ± 0.48	0.81 ± 0.84
			3	0.48 ± 0.24	0.54 ± 0.23	0.96 ± 0.36
			4	1.91 ± 1.42	2.41 ± 1.85	4.40 ± 3.23
			7	1.63 ± 0.82	2.68 ± 1.89	3.84 ± 2.00
5	Ad5-VSNJV-G[g]	6 x 10⁹	1	1.00 ± 0.49	2.18 ± 1.38	2.44 ± 1.49
			2	1.95 ± 4.35	0.60 ± 0.20	1.29 ± 0.62
			3	10.66 ± 6.71	13.21 ± 5.74	20.38 ± 19.78
			4	9.48 ± 7.67	12.47 ± 7.53	15.08 ± 11.47
			7	11.45 ± 8.18	16.98 ± 10.04	33.73 ± 32.63

[a]Data represent mean ± SD (standard deviation) for $n = 5$ samples.
[b]Individual dose of inoculum expressed as plaque forming units per ml.
[c]Days post-vaccination.
[d]Recombinant human adenovirus serotype 5 virus (Ad5) containing the capsid coding region of FMDV-A24 Cruzeiro plus the 3C proteinase coding region of FMDV-A12.
[e]Ad5 containing the porcine IFNα gene.
[f]Ad5 containing the β-galactosidase gene.
[g]Ad5 containing the glycoprotein gene from vesicular stomatitis virus New Jersey (VSNJV).

Table 4

Clinical score, virus isolation and antibody response against NS proteins in swine challenged with FMDV

			Peak Viral Titer		NS Proteins	
Group	Animal #	Clinical Score[a]	Blood	Nasal Fluid	3ABC ELISA[b]	RIP[b,c]
1	3855	0	0	0	-	-
High Ad5-A24	3856	0	0	0	-	-
+	3857	0	0	0	-	-
Ad5-pIFNα	3858	0	0	0	+	-
	3859	1^d (3^e)	0	0	++	-
2	3860	5 (5)	0	20 (3^g)	++	++
Low Ad5-A24	3861	16 (2)	250(4^f)	2000 (5)	+++	++
+	3862	3 (7)	0	100 (3)	+/-	-
Ad5-pIFNα	3863	14 (3)	0	350 (3)	+++	+/-
	3864	13 (5)	0	225 (3)	+++	++
3	3865	1^d (10)	0	30 (5)	-	+/-
High Ad5-A24	3866	1 (4)	0	35 (4)	+	+
+	3867	2^d (5)	0	10 (5)	+	+
Ad5-Blue	3868	0	0	0	-	+
	3869	2^d (6)	0	50 (2)	++	-
4	3870	13 (3)	0	1000 (3)	+++	+
Low Ad5-A24	3871	17 (2)	725 (3)	6000 (4)	++	+
+	3872	16 (2)	72500 (3)	550 (4)	++	+++
Ad5-Blue	3873	17 (3)	9500 (4)	500 (2)	+++	++
	3874	8 (2)	0	500 (3)	++	+
5	3875	15 (3)	22250 (3)	350 (3)	+++	+++
	3876	16 (2)	67500 (3)	14000 (3)	+++	+++
Ad5-VSNJV-G	3877	16 (2)	157500 (3)	600 (3)	++	+++
	3878	17 (2)	4750 (3)	65 (4)	+++	++
	3879	16 (2)	16500 (3)	1450 (3)	+++	+++

[a] The clinical score was determined by the number of toes with lesions and the presence of lesions on the snout or tongue. The maximum score is 17. [b]Data from serum collected at 63 dpc, [c] Radioimmunoprecipitation, [d]Lesion at site of inoculation, [e] Date in parenthesis indicates the dpc when the vesicular lesions were first observed, [f, g] Date in parenthesis indicates the dpc with highest viral titer and are expressed in plaque forming units (PFU/ml),- Negative, +/- Suspicious, + Weak positive, ++ Positive, +++ High positive.

CAPÍTULO 3

THE LEADER PROTEINASE OF FOOT-AND-MOUTH DISEASE VIRUS INHIBITS THE INDUCTION OF INTERFERON BETA MRNA AND BLOCKS THE HOST INNATE IMMUNE RESPONSE

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ABSTRACT

Foot-and-mouth disease virus (FMDV) is a positive-stranded-RNA virus in the family *Picornaviridae*. We have previously shown that a FMDV mutant lacking the leader proteinase (L^{pro}), LLV2-FMDV, is attenuated in cell culture, swine and cattle. Apparently this attenuated phenotype results from the inability of LLV2-FMDV to block the production of type I interferons (IFNs) which have a strong inhibitory effect on FMDV replication. The L^{pro} cleaves itself from the viral polypeptide and inhibits host translation by targeting the translation initiation factor eIF4G. Here we show that in addition to prevent IFNα/β protein synthesis L^{pro} blocks the early induction of IFNβ and interferon-stimulated genes (ISG's) such as: double-stranded RNA-dependent protein kinase (PKR), 2'-5' A synthetase (OAS) and protein GTPase Mx (Mx1) mRNA. Down-regulation of cellular PKR by RNA interference (RNAi) allowed LLV2 infected cells to grow to significant higher titers indicating a direct role of this gene in controlling FMDV replication. The observation that L^{pro} also controls the transcription of genes involved in innate immunity reveals a novel role of this protein in antagonizing the cellular response to viral infection.

1. INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious viral disease of wild and domestic cloven-hoofed animals, including pigs and cattle, that is characterized by temporary and debilitating oral and pedal vesicles. Countries where the disease is enzootic can suffer severe economic losses as a result of a decline in livestock production and international restrictions on exports of animals and animal products making FMD the most economically important disease of livestock world-wide (Callis and McKercher, 1986; Grubman and Baxt, 2004).

The causative agent, FMD virus (FMDV), belongs to the *Aphthovirus* genus, of the *Picornaviridae* family and contains a single-stranded, positive-sense RNA genome of approximately 8500 nucleotides surrounded by an icosahedral capsid composed of 60 copies each of four structural proteins [VP1 (1D), VP2 (1B), VP3 (1C) and VP4 (1A)] (Grubman and Baxt, 2004, Rueckert and Wimmer, 1984, Rueckert, 1996). Upon infection the viral RNA is translated as a single, long open reading-frame into a polyprotein that is co-translationally processed by three viral-encoded proteinases, leader (L^{pro}), 2A and $3C^{pro}$, into the four structural proteins and a number of non-structural proteins, which function in various aspects of the replication cycle (Mason et al., 2003, Rueckert, 1996). L^{pro}, the first protein to be translated, is a papain-like proteinase (Kleina and Grubman, 1992; Piccone et al., 1995; Roberts and Belsham, 1995; Skern et al., 1998) that cleaves itself from the polyprotein precursor and also cleaves host translation initiation factor eIF4G resulting in the shut-off of host cap-dependent mRNA translation (Strebel and Beck, 1986, Devaney et al., 1988, Medina et al., 1993, Kirchweger et al., 1994). FMDV mRNA, in contrast, is translated by a cap-independent mechanism via an internal ribosome entry site (IRES), and does not require intact eIF4G for viral protein production (Kuhn et al., 1990; Belsham and Brangywyn, 1990). Thus, as a result of FMDV infection host cell protein synthesis is rapidly shut-off without affecting translation of viral mRNA thereby diverting the cell protein synthesis machinery to the rapid production of large amounts of virus.

To examine the role of L^{pro} in pathogenesis we constructed a virus lacking this coding region (A12-LLV2, leaderless virus) (Piccone et al., 1995). Surprisingly leaderless virus grew almost as well as wild-type (WT) virus in some cell lines including BHK-21 and swine IBRS-2 cells suggesting that L^{pro} is not required for growth in cell culture. However, in contrast to WT virus, leaderless virus is highly attenuated in both cattle and swine (Mason et al., 1997; Chinsangaram et al., 1998) and after aerosol infection of cattle it does not spread systemically

beyond the initial site of infection in the lungs (Brown et al., 1996). Based on this information we proposed that L^{pro} is an important virulence factor in livestock hosts.

To understand the molecular basis for the difference in virulence of leaderless virus between cell culture and susceptible animals, we screened a number of primary/secondary cells for their ability to differentially support WT virus but not leaderless virus plaque formation. We identified swine, bovine, and lamb cells in which leaderless virus infection does not result in plaque formation, causes only limited cytopathic effects (CPE), and produces significantly lower virus yields than WT virus infection (Chinsangaram et al., 1999, 2001). We found that these cells have an active type I interferon (IFN-α/β) system, while BHK-21 and IBRS-2 cells do not (Chinsangaram et al., 1999, 2001). Supernatants from leaderless virus infected primary cells contained higher levels of antiviral activity than supernatants from WT virus infected cells and this activity is IFN-α/β specific (Chinsangaram et al., 1999). Utilizing mouse embryonic fibroblasts derived from knock-out mice, we showed that two IFN-α/β-stimulated genes (ISGs), double-stranded RNA-dependent protein kinase (PKR) and RNase L, are involved in the inhibition of FMDV replication (Chinsangaram et al., 2001). These results suggest that in WT virus infected primary cells and in susceptible animals, L^{pro} inhibits the translation of capped host mRNAs, including IFN-α/β mRNAs, thereby blocking or reducing the innate immune response to virus infection (Biron and Sen, 2001, Vilcek and Sen, 1996). As a result FMDV rapidly replicates and spreads. In contrast, in leaderless virus infected cells the absence of L^{pro} allows the translation of IFN-α/β mRNA and IFN protein secretion thereby resulting in the induction of an antiviral state in neighboring cells via a series of events leading to activation of ISG's some of which, including PKR and RNase L, inhibit FMDV replication (Biron and Sen, 2001, Vilcek and Sen, 1996). We further demonstrated that WT FMDV replication is inhibited by pretreatment of cells with IFN-α/β suggesting that FMDV can not overcome the antiviral effects of already expressed ISG products (Chinsangaram et al., 2001). Based on this information we recently showed that administration of IFN-α to swine, by a replication defective human adenovirus, can completely protect these animals from virulent FMDV challenge 1-5 days later (Chinsangaram et al., 2003; Moraes et. al., 2003).

To more closely examine the effect of WT and leaderless virus infection on the induction of the host IFN-α/β response, we have followed the synthesis of IFN-α and IFN-β mRNAs and three well characterized ISG's, PKR, OAS, and Mx1 (Vilcek and Sen, 1996, Biron and Sen, 2001, Samuel, 2001), by real-time RT-PCR. Leaderless virus infection

resulted in significantly higher levels of induction of IFN-β mRNA as compared to WT virus infected cells, while IFN-α mRNA was not induced after either infection. The increased levels of IFN-β mRNA in leaderless virus infected cells correlated with higher levels of induction of PKR, OAS and Mx1 mRNAs and higher levels of antiviral activity. Utilizing RNA interference to knock-down PKR mRNA expression, we demonstrated that the yield of leaderless virus was increased up to 200-fold confirming the role of this gene product as an inhibitor of FMDV replication. These results show that L^{pro} down regulates the innate immune response to FMDV infection at multiple levels. Previous information indicated that control was at the level of translation initiation by cleavage of eIF4G. The present data demonstrates that regulation also occurs at the level of transcription by inhibition of IFN-β induction via an unknown mechanism.

2. MATERIALS AND METHODS

2.1. Cells and viruses. Porcine kidney cell lines, IBRS-2 and SK6, were provided by the Foreign Animal Disease Diagnostic Laboratory, Plum Island Animal Disease Center, Greenport, NY. Primary porcine kidney cells were provided by Animal Plant and Health Inspection Service, National Veterinary Service Laboratory, Ames, Iowa. All cells were maintained in minimal essential medium (MEM, GIBCO BRL, Invitrogen, Carlsbad, CA) containing either 10% heat inactivated fetal bovine serum (FBS) and supplemented with 1% antibiotics and non-essential amino acids. These cells were used to perform all experiments with FMDV. IBRS-2 cells were used to test the biological activity of IFN produced in porcine cells. BHK-21 cells (b*aby hamster kidney cells* strain 21, clone 13, ATCC CL10), obtained from the American Type Culture Collection (ATCC, Rockville, MD) were used to propagate virus stocks and measure virus titer by plaque assay. These cells were maintained in MEM containing 10% calf serum and tryptose phosphate broth supplemented with 1% antibiotics and non-essential amino acids. All cultures were incubated at 37ºC in 5% CO_2.

FMDV A12-IC was generated from the full-length serotype A12 infectious clone, pRMC$_{35}$ (Rieder et al., 1993) and A12-LLV2 (leaderless virus) was derived from the infectious clone lacking the Lb coding region, pRM-LLV2 (Piccone et al., 1995). The viruses were concentrated by polyethylene glycol, aliquoted and maintained at -70ºC. The viruses

were used to perform all cell experiments and the multiplicity of infection (MOI) used was based on titration in BHK-21 cells.

2.2. Single-step growth assay. To verify differences in growth by both viruses in SK6 cells a single-step growth experiment was performed. SK6 and BHK-21 were infected with FMDV A12-IC and A12-LLV2 at an MOI of 10 at 37ºC. After 1 h of adsorption at 37ºC cells were rinsed with 150 mM NaCl, 20 mM morpholineethanesulfonic acid (MES pH 6) to inactivate unadsorbed input virus, and incubated with MEM at 37ºC. Supernatants were collected at 1 and 24 h post-infection (hpi) and titrated in BHK-21 cells (Chinsangaram et al., 1999).

2.3. FMDV infection. Porcine cell monolayers in 6-well plates were infected, in duplicate, with FMDV A12-IC and A12-LLV2 at different MOI's (1 and 10) for 1 h at 37ºC. After adsorption, cells were rinsed and incubated with MEM at 37ºC. Supernatants and cell lysates were collected at different times post-infection (0, 1, 3, 6, 11, and 24 hpi). Mock-infected cells were prepared at each time point for all cells used in this experiment.

2.4. IFN assays. To characterize the IFN response in FMDV-infected cells biological and ELISA assays for IFNα and β were performed by using the supernatants from FMDV-infected cells.

2.4.1. Biological activity. Biological activity was evaluated in supernatants from cells infected with A12-IC or A12-LLV2 at MOI's of 1 and 10 and collected at 0, 1, 3, 6, 11, and 24 hours post-infection (hpi). The samples were diluted and applied in duplicate wells of six-well plates containing IBRS-2 cells, and incubated overnight at 37°C. The inoculum was aspirated and the cells were infected with approximately 100 pfu of FMDV A12-IC. After adsorption of the virus for 1 h at 37°C, the inoculum was removed and the cells were overlaid with 2 ml of gum tragacanth and incubated for 24 h at 37°C before staining with crystal violet to visualize the plaques (Mayr et al., 1999, Moraes et al., 2003). Antiviral activity of IFN was reported as the reciprocal of the highest sample dilution that resulted in a 50% reduction in the number of plaques relative to the untreated cells.

2.4.2. Porcine IFNα ELISA. A porcine IFNα ELISA double capture assay previously developed in our lab (Moraes et al., 2003) was used to quantitate IFNα protein. Briefly, ELISA plates (Immulon II, Dynatech Ltd, Chantilly, VA) were coated overnight at 4°C with a recombinant monoclonal antibody (MAb) against pIFNα (K9, R&D Systems, Minneapolis, MN) diluted in carbonate-bicarbonate buffer (Sigma, St Louis, MO). The plates were blocked with PBS containing 0.05% Tween 20 and 5% non-fat milk (blocking buffer) for 2 h at room

temperature (RT) and then washed 4 times with PBS containing 0.05% Tween 20 (washing buffer) in a Skan Washer 300 (Skatron Instruments, Lier, Norway). The supernatants obtained from infected cells were added directly to the plates, in duplicate, and incubated for 1 h at 37°C. The plates were washed and a second anti-pIFNα MAb (F17, R&D Systems) previously biotinylated was added to the plates for 1 h at 37°C. The plates were washed and peroxidase-conjugated streptavidin (Streptavidin-HRP, KPL, Gaithersburg, MD) was added for 30 min at 37°C. The amount of bound peroxidase was determined by incubation with 3,3,'5,'5-tetramethyl benzidine (TMB) substrate (KPL) for 15 min at RT. The reaction was stopped by adding 1M H_2SO_4, and the OD at 450-570 nm was measured on a Bio-Kinetics Reader EL-312e (Bio-tek Instruments, Winooski, VT). pIFNα concentrations were determined by extrapolation on a standard curve prepared with recombinant pIFNα using Microsoft Excel.

2.4.3. Porcine IFNβ ELISA. ELISA plates were coated overnight at 4°C with a polyclonal antibody produced in rabbits against the N-terminal fragment of pIFNβ protein diluted in carbonate-bicarbonate buffer (Sigma). The plates were blocked with blocking buffer for 2 h at RT and then washed 4 times with washing buffer in a Skan Washer 300 (Skatron Instruments). The supernatants obtained from FMDV-infected and mock-infected cells were added directly to the plates, in duplicate, and incubated for 1 h at 37°C. The plates were washed and a biotinylated rabbit polyclonal antibody against the C-terminal fragment of pIFNβ was added for 1 h at 37°C. The plates were washed and 6 μg/ml of peroxidase-conjugated streptavidin (Streptavidin-HRP, KPL) was added for 30 min at 37°C. The amount of bound peroxidase was determined by incubation with TMB substrate (KPL) for 15 min at RT. The reaction was stopped with 1M H_2SO_4, and the OD at 450-570 nm was measured on a Bio-Kinetics Reader EL-312e (Bio-tek Instruments). The amounts of pIFNβ, in U/ml, were determined by extrapolation on a standard curve prepared with known amounts of biologically active supernatants from IBRS-2 cells infected with Ad5-pIFNβ by using Microsoft Excel

2.5. IFN-induced genes. A quantitative real-time RT-PCR assay was used to evaluate the mRNA levels of porcine IFNα and IFNβ genes and three IFN induced genes: PKR, OAS and Mx1. RNA was extracted from monolayers of porcine cells (FMDV-infected or mock-infected) collected at different time points by using an RNeasy Mini Kit (Qiagen, Valencia, CA). Primers and probe were designed using Primer express® software (Applied Biosystems, Foster City, CA). The Applied Biosystems TaqMan probe labeled with a fluorescent dye was

used in this assay. 18s rRNA was used as an internal standard control and was amplified for each sample. Primers and probe used for 18s rRNA were from the 18s rRNA control reagent kit (Applied Biosystems). In addition, a second internal control, the porcine GAPDH enzyme (glyceraldehyde 3-phosphate dehydrogenase) was used. The sequence for the primers and probes are listed in Table 1. The assay was standardized and mRNA from mock-infected cells was used as normalizer gene. The Ct values of rRNA obtained from each sample were analysed and were between 9-11. The delta Ct, delta-delta Ct and fold increase for each gene with each sample was determined.

2.6. RNA interference (RNAi) for porcine PKR. Specific small interference RNAs (siRNAs) were designed by Dharmacon (Dharmacon, Chicago, IL) using the sequence of porcine PKR (NCBI GenBank accession code NM_214319). A pool containing four siRNAs targeting PKR coding sequences: 5'-GCACATAACTTGAGGTTTA-3', 5'-GGAAGAACGTCACAGAGAA-3', 5'-GAGCAAAGCATGAATACTT-3', 5'-CCTGAAGGCTGGCGTCTTA-3' were synthesized by Dharmacon. siControl Risc-free (Dharmacon) was used as a negative control.

2.7. siRNA transfection and FMDV challenge. Twelve-well plates were seeded with SK6 cells in MEM containing 10% FBS and supplements. When the cells were about 80-90% confluent, the media was replaced by OptiMEM I (Invitrogen) and after 4 h the pool of siRNAs (200mM) was transfected using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's instructions. FBS (0.5%) was added 4 h post-transfection. After 24 h a second transfection under identical conditions was performed. Twenty-four hours later the cells were infected with FMDV A12-IC and A12-LLV2 at MOIs of 0.1, 0.01, 0.001 and 0.0001. Supernatants for virus titration and cell lysates for RNA extraction were collected 24 hpi.

3. RESULTS

3.1. Replication of A12-IC and A12-LLV2 in porcine kidney cells. We examined the swine kidney cell line, SK6 cells and primary swine kidney cells, PK cells, for their susceptibility to FMDV and their ability to inhibit leaderless virus spread. Cytopathic effects were observed in A12-IC infected SK6 cells beginning at 3-4 hpi, and the cell monolayer was destroyed between 9-24 hpi at MOI 1. Virus yields, however, as determined in a single-step growth experiment, are approximately 10-fold lower than in BHK-21 cells. In contrast CPE in A12-LLV2 infected SK6 cells started approximately 9-10 hpi (CPE around 20%) and at 24

hpi around 80-90% of the monolayer was still intact when compared to SK6-mock infected cells. No plaques were visualized after infection of SK6 cells with A12-LLV2. Similar results were obtained with PK cells, ie., A12-IC was able to grow and form plaques but no plaques were visualized after A12-LLV2 infection (data not shown).

To determine if A12-LLV2 could replicate in SK6 cells, a single-step growth experiment was performed. Supernatants from SK6 and BHK-21 cells infected with either virus were collected at 1 and 24 hpi and titrated on BHK-21 cells. A12-LLV2 grew much more slowly than A12-IC. Also the virus yield was significantly lower in A12-LLV2 infected SK6 cells as compared to A12-IC infected cells (Fig.1). In contrast, A12-IC grew to only slightly higher yields than A12-LLV2 in BHK-21 cells (Piccone et al., 1995). As we have previously shown BHK-21 cells do not have an active antiviral and thus A12-LLV2 can grow to high titers and spread in these cells.

3.2. Host cell antiviral response. To evaluate the factors involved in the inability of A12-LLV2 to spread in some porcine cells, we analyzed supernatants from infected SK6 and PK cells for antiviral activity. Cells were infected with each virus at different MOI's and supernatants collected at different times post-infection. The supernatants from the A12-LLV2-infected porcine cells showed antiviral activity, while A12-IC and mock-infected cell supernatants had no antiviral activity (data not shown). The maximum inhibitory effect achieved was 16 U with supernatants from A12-LLV2-infected SK6 cells at MOI 1 at 30 hpi, and A12-LLV2- infected PK cells at MOI 1 at 24 hpi.

3.3. Induction of type I IFN following A12-IC and A12-LLV2 infection in porcine cells. The synthesis of IFN-α and-β mRNA were followed in A12-IC and A12-LLV2 infected SK6 and PK cells, by real-time RT-PCR. The results are shown for IFNβ mRNA and represent the mean of three experiments in SK6 cells and two experiments in PK cells (Figs. 2- and 3-A).

Both A12-IC and A12-LLV2 are poor inducers of IFN-α mRNA. Likewise treatment of cells with poly IC, a known inducer of IFNα/β, did not result in the induction of IFN-α mRNA in SK6 or PK cells. Since the sequence of only a limited number of porcine IFN-α genes have been identified it is conceivable that the primers and probe we used are not able to detect other IFN-α mRNAs. Based on a conserved region in the sequence of the known mouse and human IFN-α mRNAs, we designed an additional primer/probe set to assay for IFN-α mRNA induction. No IFN-α mRNA induction was detected in A12-IC, A12-LLV2 or poly IC treated cells (data not shown).

A12-IC is a relatively poor inducer of IFN-β in SK6 and PK cells, while in A12-LLV2 infected porcine cells the levels of IFN-β mRNA were 15-20 fold higher (Fig. 2A, 3A).

3.4. Induction of ISGs following A12-IC and A12-LLV2 infection in porcine cells. Among the best characterized ISG's are dsRNA-dependent protein kinase (PKR), 2'-5' A synthetase (OAS) and Mx1 induced by the IFN after virus infection. The synthesis of PKR, OAS and Mx1 mRNA were followed after the infection with A12-IC and A12-LLV2 in SK6 and PK cells by real-time RT-PCR. The results shown represent the mean of three experiments in SK6 cells and two experiments in PK cells (Figs. 2- and 3-B, C and D).

A12-IC was also poor inducer of all three ISG's mRNA analyzed as compared with leaderless virus. The presence of A12-LLV2 in both porcine cells induced an increase in the mRNA levels of PKR 4-fold (Figs. 2- and 3-B). Surprisingly, the higher mRNA levels were achieved for OAS, 18-265-fold and Mx1, 10-20 fold (Figs. 2- and 3-C and D).

3.5. IFNα/β response in porcine cells. The production of pIFN-α or −β in supernatants of porcine cells was measured by ELISA. pIFN-β could not be detected in supernatants from SK6 or PK cells infected with either A12-IC or A12-LLV2, while pIFN-α was detected in both SK6 and PK cells. The highest levels of pIFN-α were detected in leaderless-infected cells at the latest times (Fig. 4). Notably the levels of pIFN-α was approximately 3 times higher in A12-LLV2-infected PK cells than in A12-LLV2-infected SK6 cells (651,83 pg/ml in PK cells and 221,52 pg/ml in SK6 cells). Two experiments with SK6 cells infected with A12-IC and A12-LLV2 were performed and the data shown represents the mean of both experiments (Fig. 4).

3.6. Silencing of PKR in SK6 cells. We have previously shown in mouse embryonic fibroblasts derived from knock-out mice that PKR has a major role in the inhibition of FMDV replication (Chinsangaram et al., 2001). To confirm the role of PKR in the IFN-induced inhibition of A12-LLV2 replication in porcine cells we used RNA interference to knock-down the level of PKR expression. SK6 cells were transfected with a mixture of PKR specific siRNAs and subsequently infected with A12-LLV2 or A12-IC at different MOIs. The yield of A12-IC in PKR-specific siRNA transfected cells was the same as in control transfected, SiGLO, or untransfected cells at all MOIs examined. However, the yield of A12-LLV2 in cells transfected with PKR-specific siRNA was significantly increased as compared to SiGLO or untransfected cells. At an MOI of 0.001 there was an approximately 200-fold increase in virus yield (Fig. 5).

4. DISCUSSION

IFNs play an essential role in innate immunity by inhibiting the replication and spread of viral pathogens (Sen, 2001, Stark et al., 1998, Vilcek and Sen, 1996). IFNα/β bind to specific receptors on cells priming them to a virus-resistant state by induction and expression of several gene products that effectively inhibit different stages of viral replication (Biron and Sen, 2001, Horisberger, 1995, Vilcek and Sen, 1996).

To overcome the host antiviral response, FMDV has developed a mechanism to counteract the IFN actions. FMDV encodes a papain-like proteinase, L^{pro}, which cleaves of eIF4G that is an initiation translation factor of most eukaryotic mRNAs. This cleavage results in shut-off of host cell protein synthesis, which is cap-dependent; but allowing the virus to use the host cell protein synthesis machinery by cap-independent mechanism, via IRES (Chinsangaram et al., 1999, Devaney et al., 1988, Medina et al., 1993, Piccone et al., 1995). As a consequence of the shut-off of cellular protein synthesis, the virus prevents the host IFNα/β synthesis, and allowing a sudden onset of viral replication and dissemination of virus in cells (Chinsangaram et al., 1999) and in the animals infected (Brown et al., 2000, Mason et al., 1997).

Wild-type FMDV, A12-IC, spreads rapidly in infected cells (Chinsangaram et al., 1999) and animals (Brown et al., 1996). In contrast, a genetic mutant lacking the coding region for the non-structural L^{pro} protein, A12-LLV2, is attenuated in animals (Brown et al., 1996, Mason et al., 1997) and grows and forms plaques in BHK-21 cells, which is deficient in some aspect of the IFN signal transduction pathway and∕or IFN-regulated cellular protein. As a result, A12-LLV2 can grow in high titers in BHK-21 (Chinsangaram et al., 1999, Piccone et al., 1995). However in secondary bovine cells that are susceptible to A12-IC infection, A12-LLV2 can not form plaques but grows in considerable lower titer (Chinsangaram et al., 1999). It was demonstrated that supernatants of A12-LLV2–infected secondary bovine cells display type I IFN antiviral activity. In contrast supernatants of A12-IC-infected secondary bovine cells do not show antiviral activity.

To understand the molecular basis of the difference in virulence between these both viruses, we examined the effect of A12-IC and A12-LLV2 infection in the induction of the host IFNα/β response. In this study, we selected a porcine kidney cell line, SK6 and primary kidney cells, PK; both cells are highly susceptible to A12-IC infection and A12-LLV2 can not form plaques but grow in lower titers.

We demonstrated antiviral response in both porcine cells upon A12-LLV2 infection. This antiviral response was higher than that induced by A12-IC. Similar to results previous obtained with bovine cells (Chinsangaram et al., 1999). To evaluate this antiviral response, we used IFN-α and-β ELISA assay. The production of porcine IFN-α or –β was measured in supernatants of SK6 and PK cells. We were able to detect porcine IFNα in higher levels in both A12-LLV2-infected porcine cells. However we could not detect any porcine IFNβ in supernatants of both cells infected with each virus.

The well-characterized IFNα/β- induced products are: dsRNA-dependent protein kinase (PKR), its action is associated to inhibit protein expression; 2'-5' A synthetase (OAS) which activates a latent ribonuclease, RNaseL, to cleave cellular and viral RNAs; and Mx1 protein which blocks transport of viral ribonucleoproteins to the nucleus (Biron and Sen, 2001, Horisberger, 1995, Vilcek and Sen, 1996). Here, we followed the kinetics of expression of these three ISG's and the IFN-α and –β mRNAs in both A12-IC and A12-LLV2- infected porcine cells. Deletion of L^pro of FMDV has resulted in the inability of A12-LLV2 to spread from the site of infection. Two factors have been linked to this characteristic, the slow replication of A12-LLV2 and the expression of IFNα/β in A12-LLV2-infected cells (Chinsangaram et al., 1999). In this work, we primarily examined the mRNA levels of IFN-α and –β in SK6 and PK cells. We were able to detect porcine IFNα in supernatants by ELISA, but we could not detect any increase in the IFNα mRNA levels when the cells were infected with both viruses. We treated the porcine cells with poly-IC (poly-inosinic and poly-cytidylic acid, which is a potent IFN inducer) and still had no effect on IFNα mRNA levels from these cells. We also designed new set of primers and probe based on alignments with other possible conserved regions among the IFNα genes in mammalian species available in GenBank and we could not reach any increase of mRNA expression of this cytokine.

Conversely, when we examined the IFNβ mRNA levels we could detect high level, in order to 15-20-fold in A12-LLV2 than A12-IC-infected porcine cells. We also demonstrated that the three ISG's had higher levels of mRNA induction in A12-LLV2 than A12-IC– infected SK6 and PK cells. Surprisingly, the mRNA levels for OAS and Mx1 were much higher than PKR. We previously demonstrated that in cell culture PKR and RNase L are ISG's that play role in inhibition of FMDV replication, but it was not possible demonstrate the effect of the Mx1 on FMDV infection (Chinsangaram et al., 2001). It would be interesting to directly demonstrate that these mRNAs would be translated in IFN-induced products and their potential antiviral activity in FMDV replication. In this study, the increased levels of

IFNβ mRNA in A12-LLV2-infected porcine cells were associated to higher levels of induction of PKR, OAS and Mx1 mRNAs and higher levels of antiviral activity.

The cellular IFN-inducible antiviral mechanism involved in the FMDV replication was investigated by using ISG knockout mouse embryonic fibroblast cells (EF) for both genes PKR and RNase L. It was evidenced that PKR has a major role in the inhibition of FMDV replication (Chinsangaram et al., 2001). To confirm the role of PKR in the IFN-induced inhibition of A12-LLV2 replication in porcine cells we used RNA interference technology to knock-down the PKR expression by using small interfering RNAs (siRNAs). When the PKR-siRNA transfected cells infected with A12-IC no difference in the virus yield related to non-siRNA treated cells. However, the yield of A12-LLV2 was greatly increased in SK6 cells transfected with PKR-specific siRNA. We results corroborate with previous studies (Chinsangaram et al., 2001) to demonstrate that PKR is one mechanism of type I IFN induces inhibition of FMDV replication.

Our results demonstrate that L^{pro} limits induction of IFNβ mRNA. Lpro blocks translation of capped mRNAs including type I IFN by degradation of eIF4G. However, FMDV is susceptible to pretreatment with type I IFN and does not appear to inhibit antiviral activity of type I IFN-induced gene products. This suggests that besides the inhibitory effect on the translation host machinery other unknown mechanisms can be affecting the cell in FMDV infection.

The innate immune response is important in containing FMDV replication if we understand molecular mechanisms involved in interactions virus and host could allow us to rationally develop an effective disease control strategy for FMD.

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TABLE 1. Oligonucleotide primer and probe sequences for amplification of porcine genes used in real time RT-PCR

Gene	Primer and probe sets	Sequence	GenBank [a]
GAPDH	porcine GAPDH-327F[b]	5'-CGTCCCTGAGACACGATGGT-3'	AF017079
	porcine GAPDH-380R[c]	5'-CCCGATGCGGCCAAAT-3'	
	porcine GAPDH-348T[d]	5'-AAGGTCGGAGTGAACG-3'	
Mx1	porcine Mx1-803F[b]	5'-GAGGTGGACCCCGAAGGA-3'	M65087
	porcine Mx1-859R[c]	5'-CACCAGATCCGGCTTCGT-3'	
	porcine Mx1-824T[d]	5'-AGGACCATCGGGATC-3'	
OAS	porcine OAS-889F[b]	5'-CTGTCGTTGGACGATGTATGCT-3'	AJ225090
	porcine OAS-954R[c]	5'-CAGCCGGGTCCAGAATCA-3'	
	porcine OAS-919T[d]	5'-TCAAGAAACCCAGGCCT-3'	
PKR	porcine PKR-968F[b]	5'-GGAAGAAAACAAACACAGCTTGAA-3'	AB104654
	porcine PKR-1048R[c]	5'-CCAAATCCACCTGAGCCAATT-3'	
	porcine PKR-994T[d]	5'-CCAGGTT TGTCGAAGAT-3'	
pIFNα	porcine IFNα-236F[b]	5'-TGGTGCATGAGATGCTCCA-3'	M28623
	porcine IFNα-290R[c]	5'-GCCGAGCCCTCTGTGCT-3'	
	porcine IFNα-256T[d]	5'-CAGACCTTCCAGCTCT-3'	
pIFNβ	porcine IFNβ-11F[b]	5'-AGTGCATCCTCCAAATCGCT-3'	M86762
	porcine IFNβ-69R[c]	5'-GCTCATGGAAAGAGCTGTGGT-3'	
	porcine IFNβ-32T[d]	5'-TCCTGATGTGTTTCTC-3'	

[a] NCBI GenBank accession code. [b] Forward primer. [c] Reverse primer. [d] TaqMan MGB Probe.
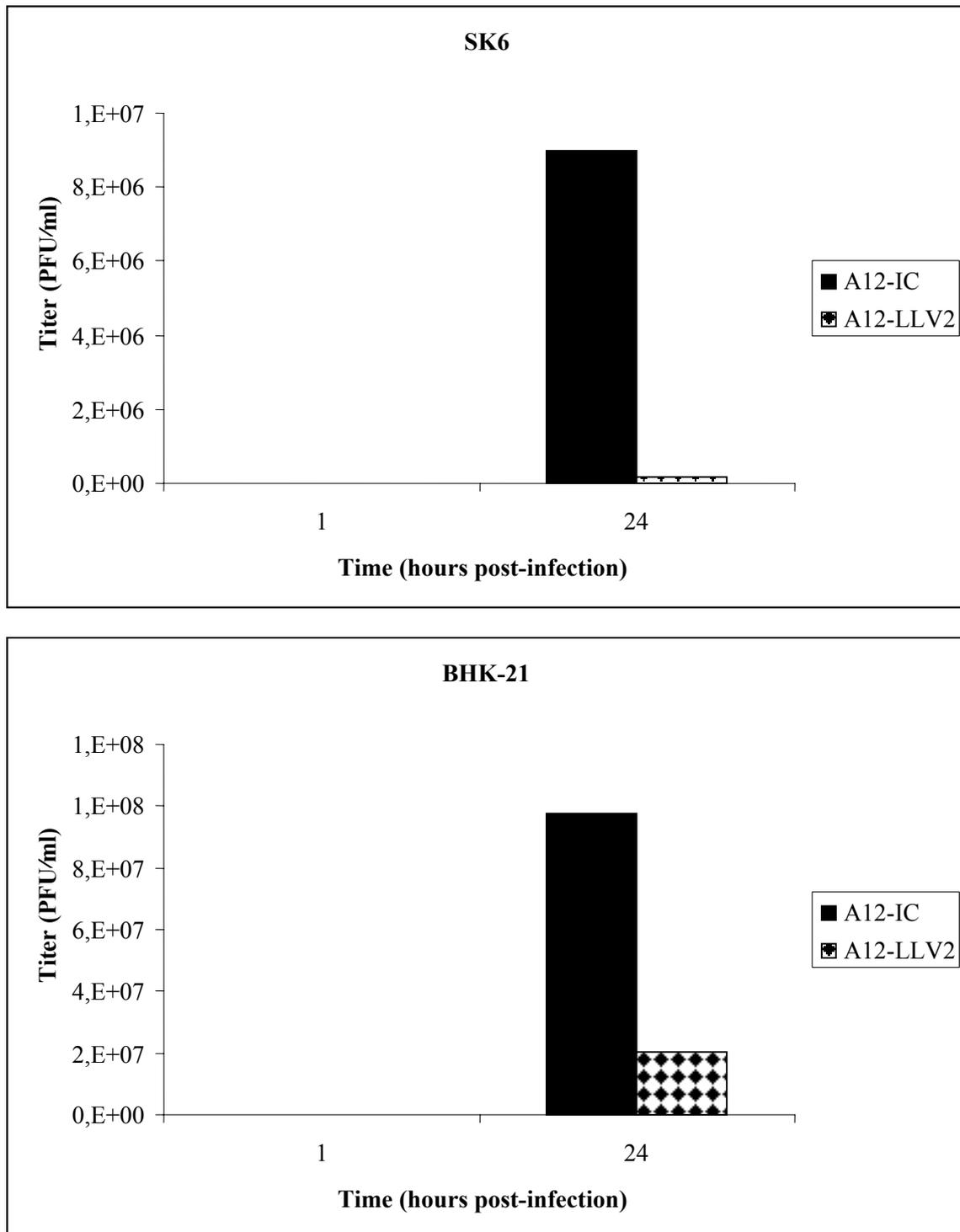
FIG. 1. Single-step growth curve. SK6 cells were infected with A12-IC or A12-LLV2 at an MOI of 10 at 37ºC. After 1h, cells were rinsed with 150 mM NaCl, 20 mM MES (pH 6.0) and incubated with MEM at 37ºC. Supernatants were collected at 1 and 24 hpi and titrated in BHK-21 cell. In parallel BHK-21 were also infected with both viruses as a control.

A. IFNβ mRNA in SK6 cells
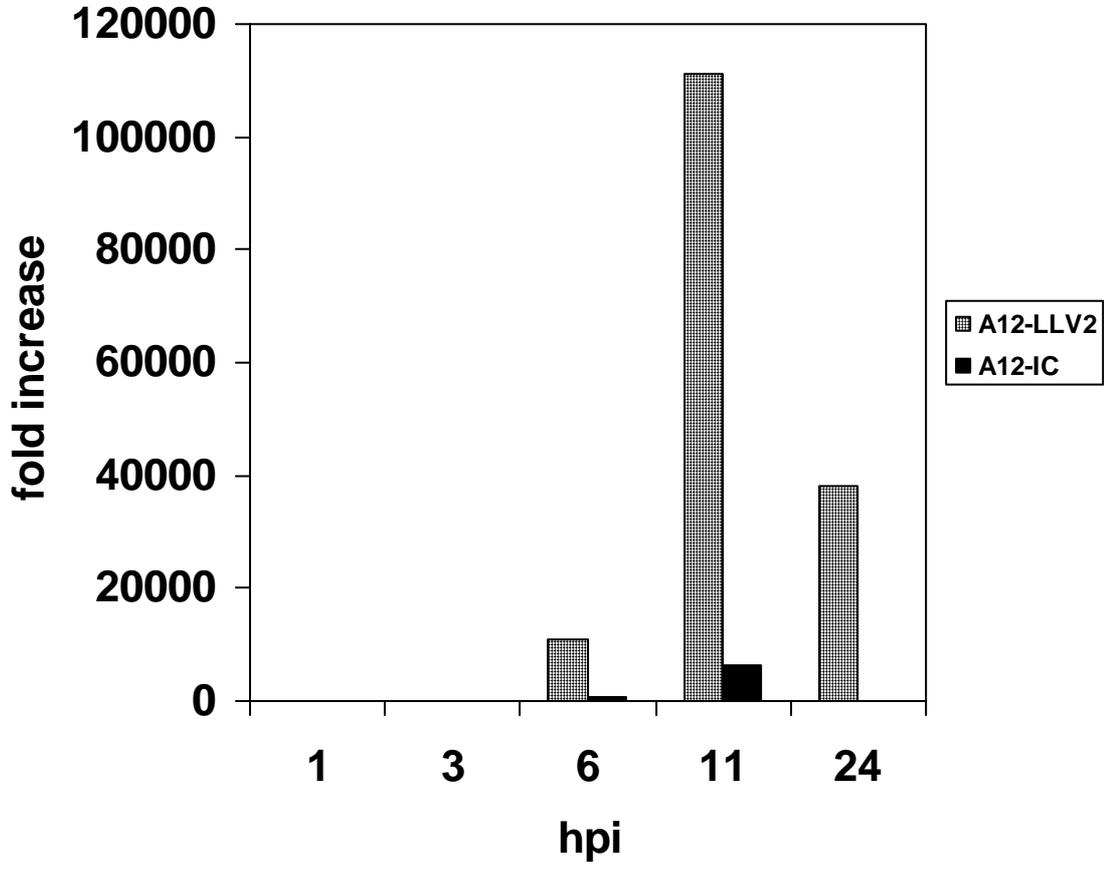
B. PKR mRNA in SK6 cells
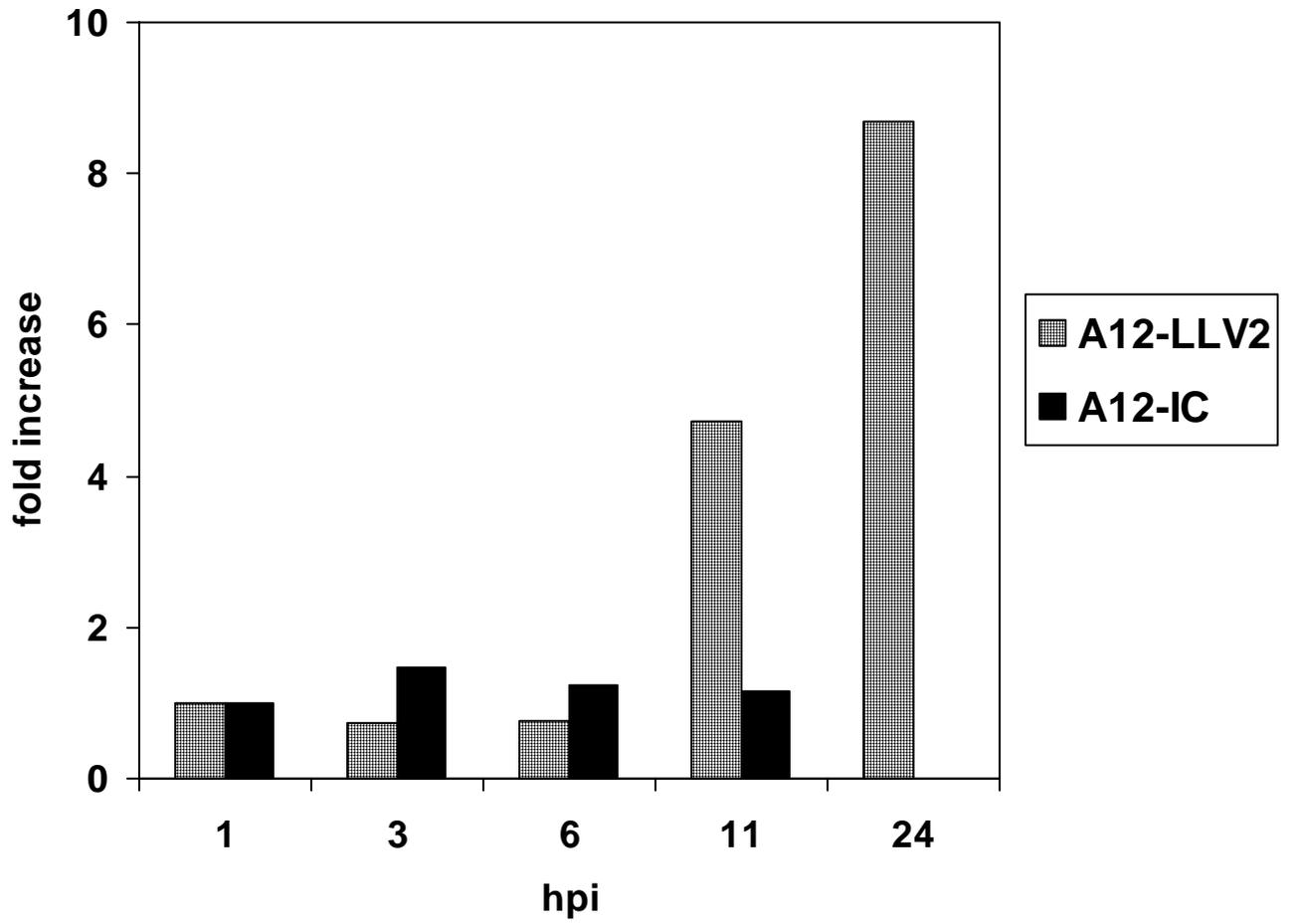
C. OAS mRNA in SK6 cells
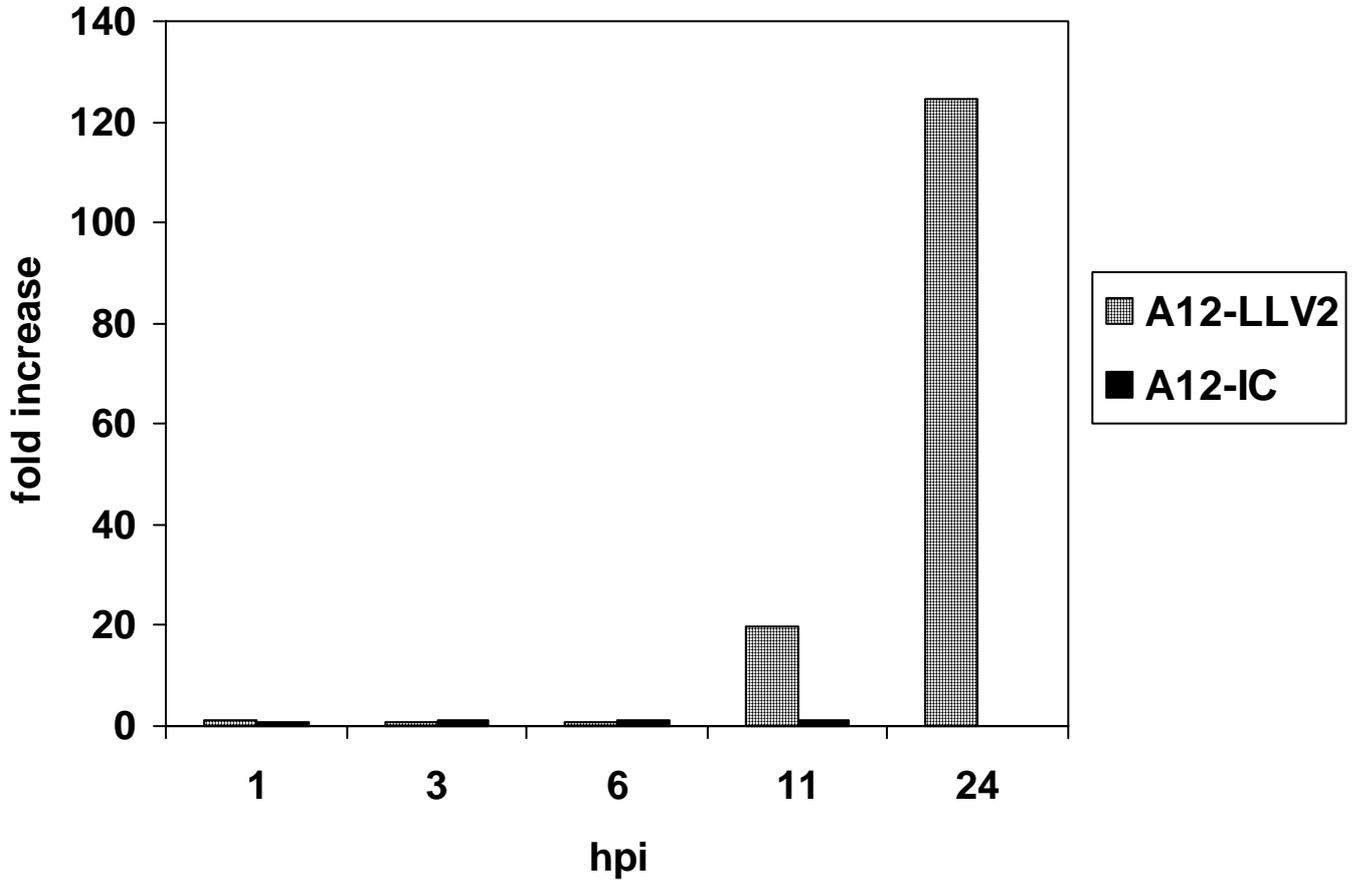
D. Mx1 mRNA in SK6 cells
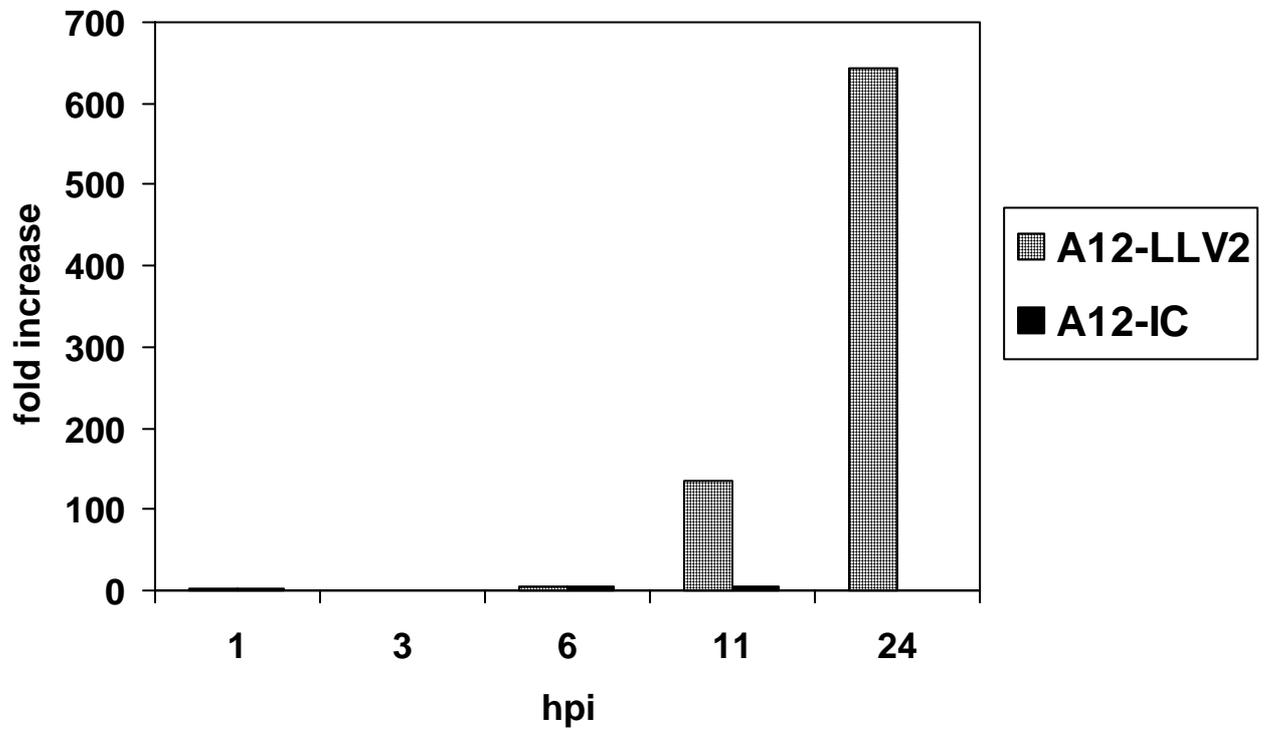
FIG. 2. Induction of type I IFN and IFN-induced genes mRNA following A12-IC and A12-LLV2 infection in SK6 cells. A. IFNβ mRNA, B. PKR mRNA, C. OAS mRNA, and D. Mx1 mRNA.

A. IFNβ mRNA in PK cells
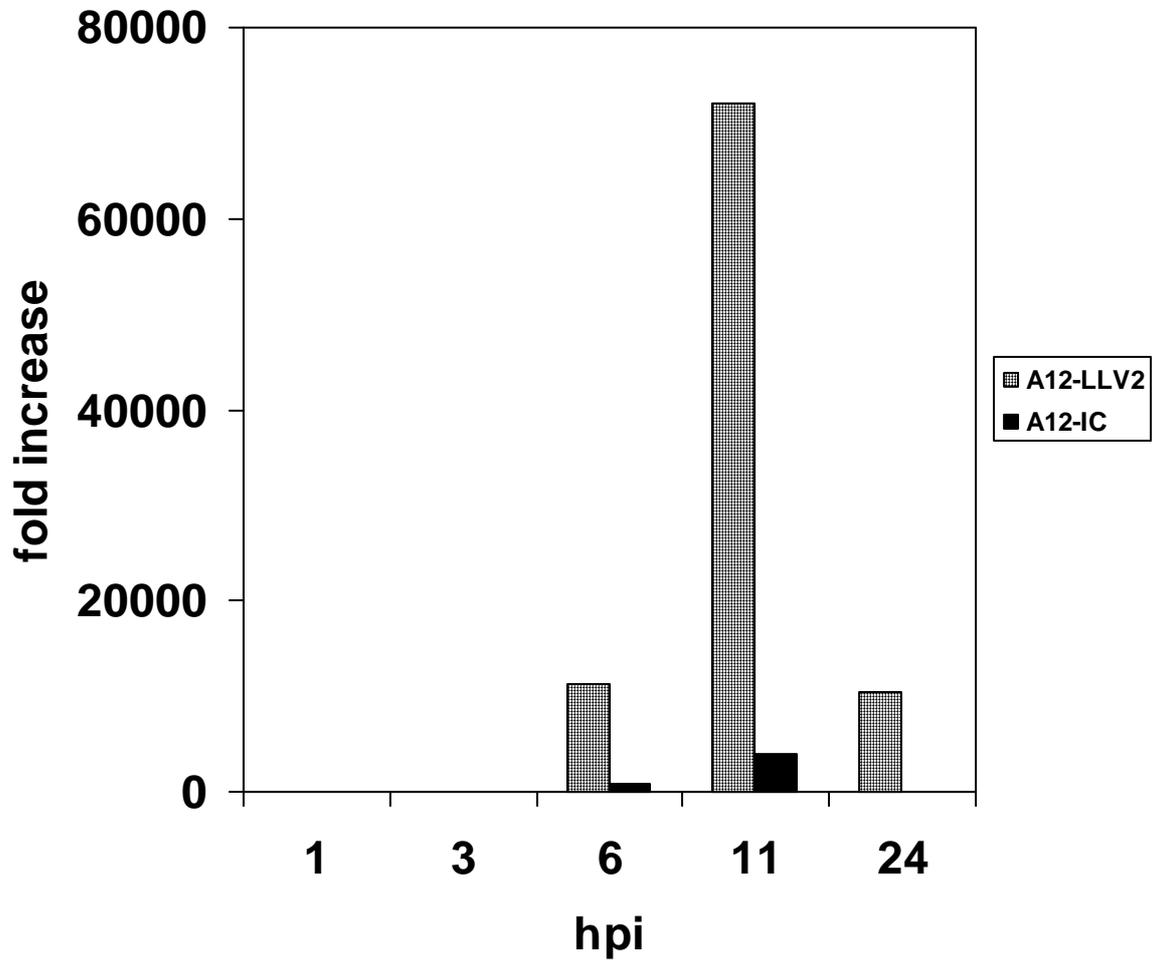
B. PKR mRNA in PK cells
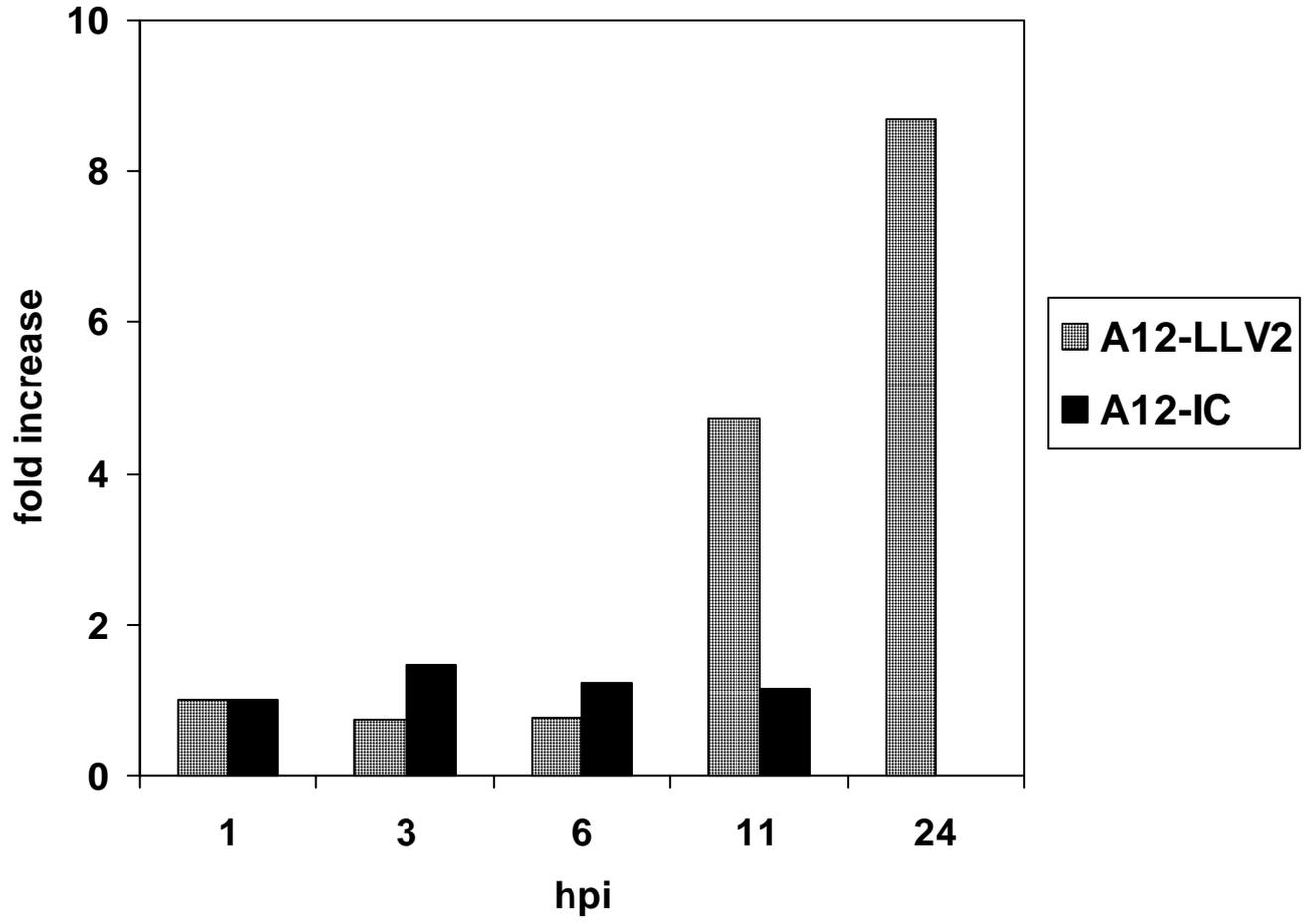
C. OAS mRNA in PK cells
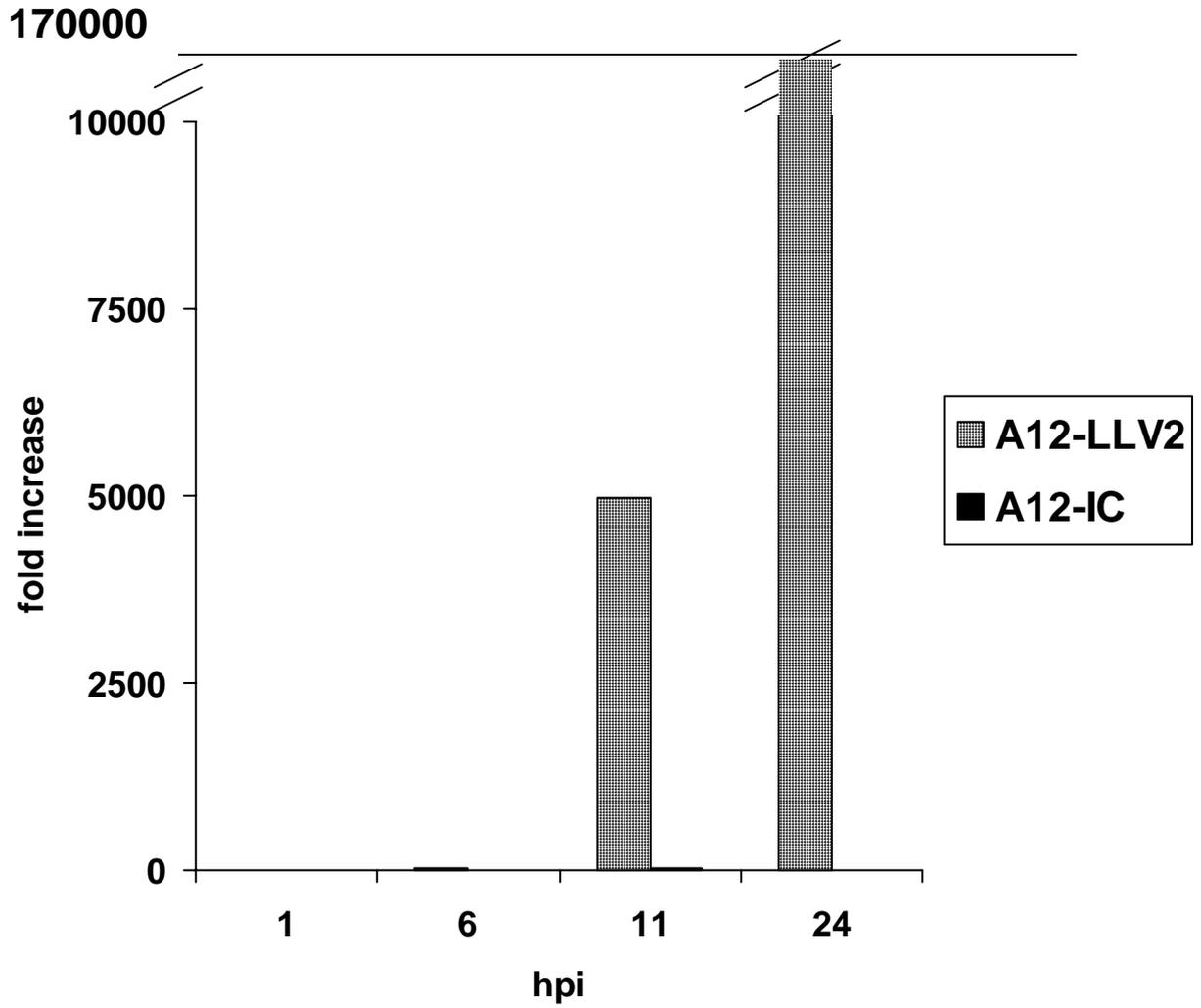
D. Mx1 mRNA in PK cells
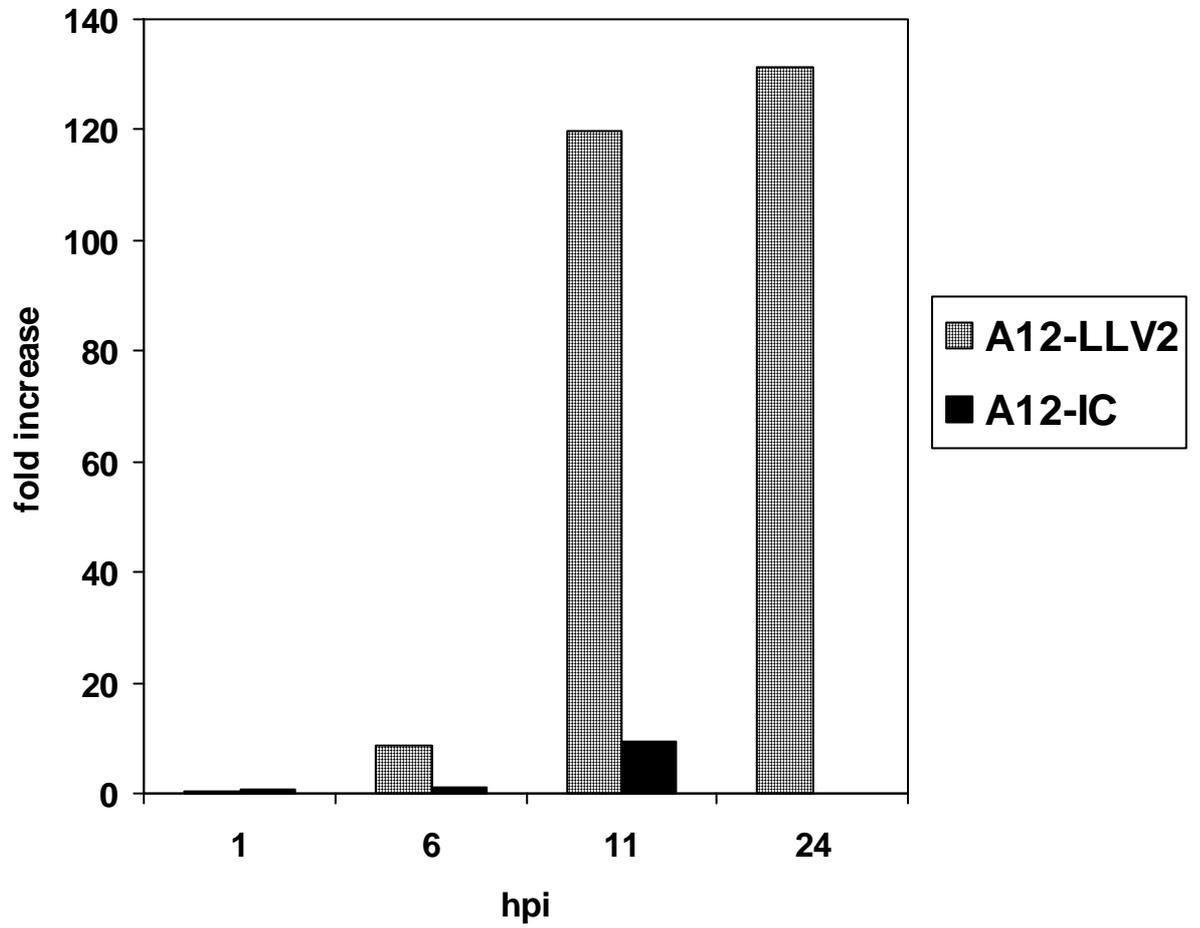
FIG.3. Induction of type I IFN and IFN-induced genes mRNA following A12-IC and A12-LLV2 infection in PK cells. A. IFNβ mRNA, B. PKR mRNA, C. OAS mRNA, and D. Mx1 mRNA.
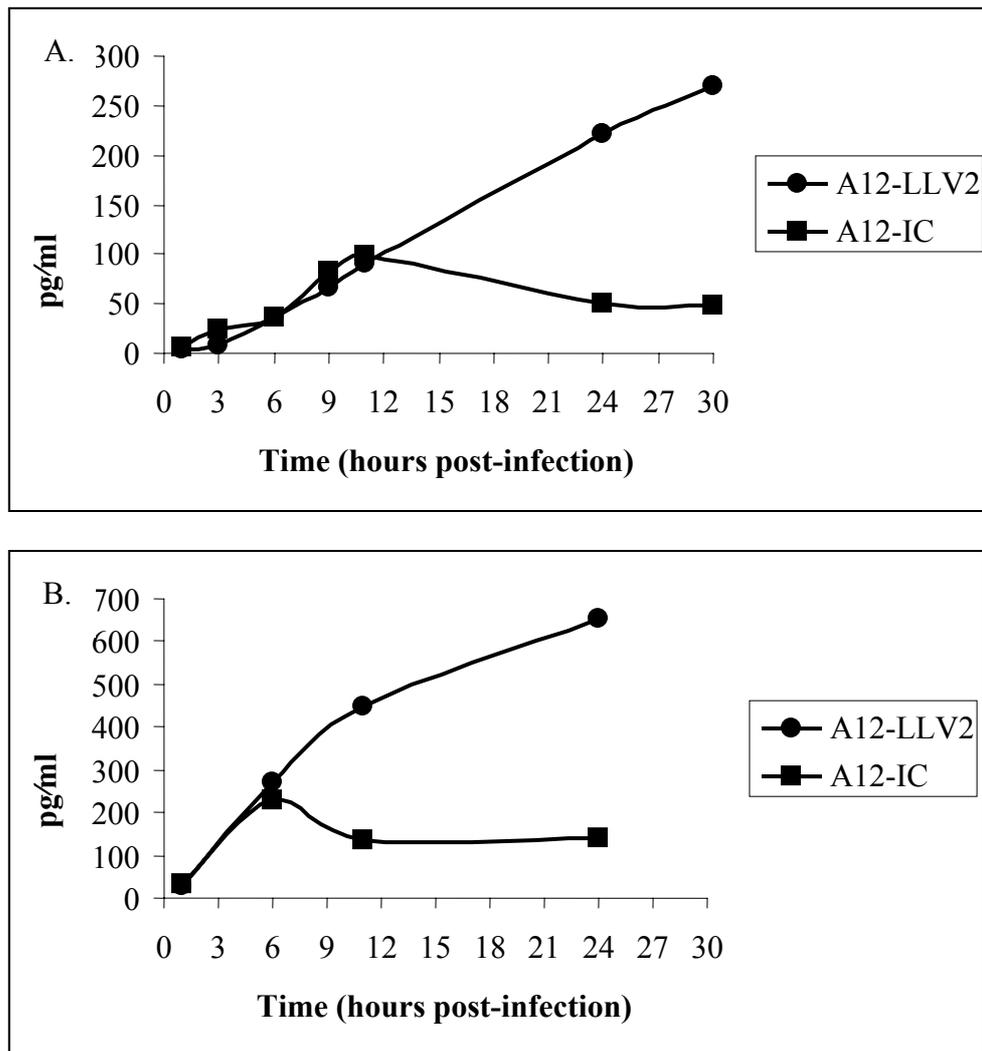
FIG. 4. Interferon response in porcine cells infected with A12-IC and A12-LLV2 at MOI 1. Supernatants were collected at different time post-infection and a tested for pIFN-α by ELISA. (A) Supernatants from SK6 cells (these data represent the mean from two experiments). (B) Supernatants from PK cells.
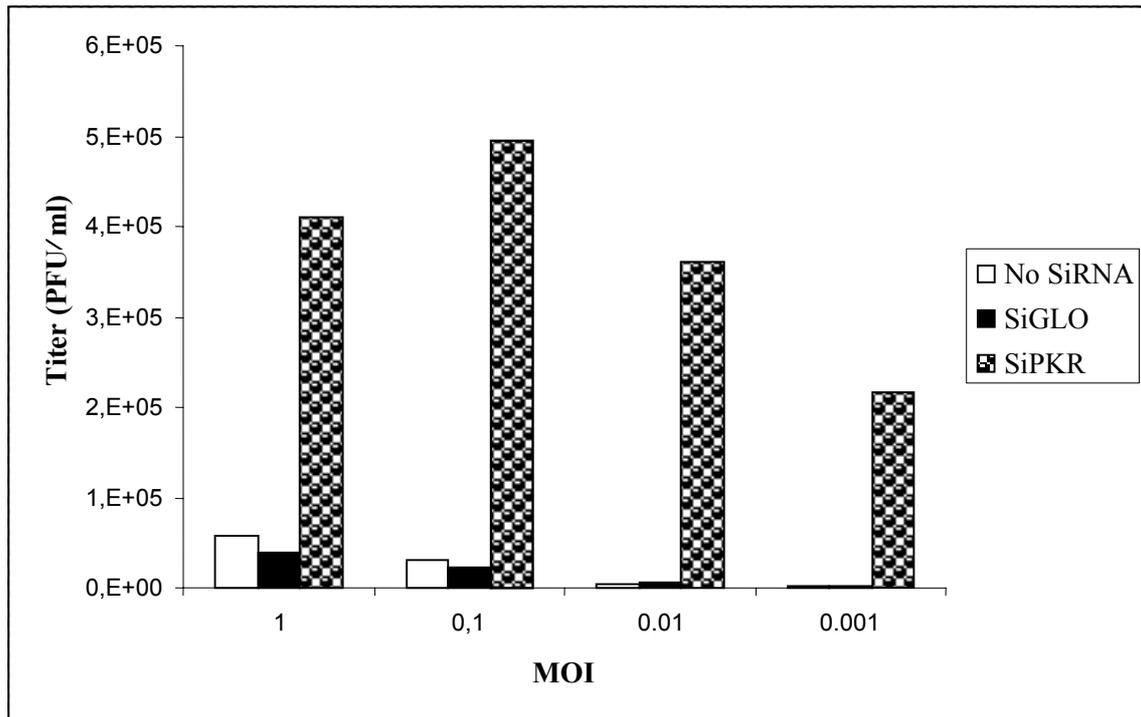
FIG. 5. SK6 cells transfected with SiPKR and challenged with A12LLV2 at different MOIs.

CONCLUSÃO

Os últimos surtos de febre aftosa (FMD) nas diferentes partes do mundo têm causado um profundo impacto sobre os procedimentos empregados no controle dessa enfermidade. As vacinas utilizadas apresentam problemas que limitam o seu uso em caso de surtos em muitos países livres da doença. O desenvolvimento de novas alternativas de vacinas e antivirais têm sido pesquisados por muitos países para contornar os problemas das vacinas disponíveis; além de permitirem o emprego como ferramentas em novas estratégias de controle da FMD.

Uma nova geração de vacinas contra a FMD, em estudo, emprega a tecnologia de vírus recombinante; utilizando como vetor o adenovírus humano tipo 5 defectivo (Ad5) expressando genes que codificam proteínas do capsídeo do FMDV. Tais vacinas têm sido testadas em animais e demonstraram ser eficazes na indução de proteção contra a doença. Também mostraram ser seguras, sem causar reações adversas e eliminação viral, além dos animais vacinados poderem ser diferenciados dos infectados por testes de diagnóstico baseado na detecção das proteínas não-estruturais do FMDV. No entanto, quando administrada em suínos não induz imediata imunidade ao FMDV. Para solucionar esse problema um tratamento profilático antiviral foi desenvolvido baseado no emprego do Ad5 contendo o interferon alfa suíno.

Este trabalho tem por objetivo avaliar o efeito adjuvante do interferon alfa suíno em animais imunizados com uma vacina recombinante de um adenovírus defectivo contendo as regiões codificadoras das proteínas do FMDV A24, bem como investigar alguns dos aspectos moleculares envolvidos na interação FMDV e a célula hospedeira em uma espécie susceptível.

Na primeira etapa deste estudo a resposta imunológica e a proteção contra o FMDV conferida pela vacina recombinante Ad5-A24 associada com o IFNα suíno são avaliadas em suínos. Através do uso de diferentes dosagens da vacina de Ad5-A24 em combinação com o antiviral. E a resposta imunológica conferida por este tratamento foi avaliada, sendo os animais desafiados aos 42 dias após a vacinação. Os resultados obtidos corroboram com estudos anteriores, demonstrando que a vacina recombinante de Ad5-A24 induz proteção da doença em suínos. O IFNα aumenta o nível de proteção induzido pela vacina de Ad5-A24 contra o FMDV homólogo, indicando que o IFNα pode ser empregado como um adjuvante em novas estratégias de vacinação anti-FMD.

Investigou-se os efeitos da infecção pelo vírus da FMD na indução da resposta de interferon tipo I (IFNα∕β) em células de origem suína. O aumento nos níveis de RNA mensageiros (mRNA) do IFNβ foi relacionado ao aumento nos níveis de indução dos mRNAs de proteína kinase dependente da dupla fita de RNA (PKR), 2'-5' oligoadenilato-sintetase (OAS) e da GTPase Mx (ou proteína Mx); assim como dos altos níveis de atividade antiviral. Foi possível confirmar o papel da PKR como um inibidor da replicação do FMDV. Os resultados obtidos, também, demonstram que a L^{pro} tem um importante papel na regulação da resposta imunológica inata do hospedeiro quando infectado pelo FMDV em vários níveis. Os dados obtidos neste trabalho indicam que a regulação também ocorre ao nível da transcrição e pela inibição da indução do IFNβ através de um mecanismo ainda não elucidado.

A resposta imunológica inata é importante para conter a replicação do FMDV, no momento em que for possível entender os mecanismos moleculares envolvidos nas interações do vírus com o hospedeiro ter-se-á possibilidade de desenvolver estratégias de vacinação mais efetivas para controlar esta importante enfermidade.

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