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EFEITO DO FATOR ESTIMULANTE DE COLÔNIAS DE GRANULÓCITOS E MACRÓFAGOS (GM-CSF) NA EFICÁCIA E POTÊNCIA DE UMA VACINA DE SUBUNIDADES DA FEBRE AFTOSA EM SUÍNOS

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Resumo

A febre aftosa é uma das doenças mais temidas nos rebanhos em todo o mundo. A vacinação tem sido uma arma eficiente no controle da doença, no entanto há preocupações com as vacinas atualmente utilizadas incluindo a necessidade de instalações de alta segurança para a produção dessas vacinas e a falta de um teste de diagnóstico aprovado que faça distinção precisa entre animais vacinados. Varias vacinas têm sido testadas contra a febre aftosa e uma dessas utiliza como vetor um vírus defectivo para replicação, derivado do adenovírus humano tipo 5 (Ad5), o qual contém as proteínas que compõe capsídeo do vírus da febre aftosa (P1-2A) e a protease 3C que protegeu suínos completamente contra o desafio de uma cepa homóloga (A12 e A24). Uma vacina com o Ad5-P1-2A+3C proveniente da cepa O1 Campos (Ad5-O1C), induziu um baixo título de anticorpos neutralizantes específicos em testes de potência vacinal em suínos. O fator estimulante de colônia de granulócitos e macrófagos (GM-CSF) tem sido utilizado com sucesso na formulação de vacinas para estimular a resposta imune contra inúmeras doenças, incluindo HIV, Hepatite C e B entre outros. Na tentativa de melhorar a resposta imune específica contra a febre aftosa induzida pelo Ad5-O1C, suínos foram vacinados com Ad5-O1C juntamente com Ad5-pGM-CSF. Entretanto, nas condições utilizadas nesse teste, o uso do Ad5 expressando o gene do pGM-CSF (fator estimulante de colônia de granulócitos e macrófagos de suíno) não melhorou a resposta imune do Ad5-O1C e adversamente afetou o nível de proteção de suínos desafiados com o vírus homólogo da febre aftosa.

Abstract

Foot-and-mouth disease (FMD) is one of the most feared diseases of livestock worldwide. Vaccination has been a very effective weapon in controlling the disease, however a number of concerns with the current vaccine including the inability of approved diagnostic tests to reliably distinguish vaccinated from infected animals and the need for high containment facilities for vaccine production, have limited its use during outbreaks in countries previously free of the disease. A number of FMD vaccine candidates have been tested and a replication-defective human adenovirus type 5 (Ad5) vector containing the FMDV capsid (P1-2A) and 3C protease coding regions has been shown to completely protect pigs against challenge with the homologous virus (FMDV A12 and A24). An Ad5-P1-2A+3C vaccine for FMDV O1 Campos (Ad5-O1C), however, only induced a low FMDV-specific neutralizing antibody response in swine potency tests. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been successfully used to stimulate the immune response in vaccine formulations against a number of diseases, including HIV, hepatitis C and B etc. To attempt to improve the FMDV-specific immune response induced by Ad5-O1C, we inoculated swine with Ad5-O1C and an Ad5 vector containing the gene for porcine GM-CSF. However, in the conditions used in this trial, pGM-CSF did not improve the immune response to Ad5-O1C and adversely affected the level of protection of swine challenged with homologous FMDV.

1. INTRODUÇÃO

1.1 Um breve histórico sobre a Febre Aftosa

A febre aftosa é uma enfermidade infecciosa aguda altamente contagiosa que acomete animais biungulados, selvagens ou domésticos (Donaldson, et al., 1989; Callis, et al., 1986). A febre aftosa é conhecida pela sua infecciosidade devastadora há centenas de anos. O primeiro relato da doença é de 1546 feito pelo monge italiano Girolamo Fracastoro (Hieronymi Fracastorii Veronensis: *in* Casas Olascoaga, et al., 1999). O agente da febre aftosa foi primeiramente descrito como sendo um vírus por Loeffler & Frosch em 1898. Já em 1922, Vallée e Carré descobriram que havia mais de um sorotipo da doença denominando-os de acordo com o local de origem de cada um, sendo "O" de Oise, França e "A" para Allemagne, Alemanha. Na mesma época, Waldmann e Trautwein (1926) trabalhando também com febre aftosa, descobriram os sorotipos e os denominaram como sendo A, B e C. Consequentemente, houve uma grande confusão até a OIE (Organização Internacional de Epizootia) estabelecer uma nomenclatura oficial (revisado por Brown 2003).

Bedson et al. (1927) demonstraram que 2 isolados do sorotipo "A" podiam ser diferenciados por neutralização cruzada, o que deu origem à classificação em subtipos. Nesta época também foram realizados os primeiros experimentos com vacinas para febre aftosa, as quais eram provenientes de vesículas de animais infectados e inativadas por formalina (Vallée e Carré 1925), sendo que muitos rebanhos foram vacinados na Europa e Argentina utilizando-se essa técnica.

A primeira vacina produzida com sucesso em cultivo celular, a partir de epitélio da língua foi produzida por Frenkel (1947) o que permitiu a produção de vacinas em larga

escala. No entanto, foi com o surgimento da produção de vacinas em células de rim de hamsters jovens em suspensão (BHK-21) (Capstick et al., 1962; revisado por Brown 2003) que permitiu a produção de vacinas suficientes para vacinar milhões de animais a cada ano possibilitando o controle da doença em varias regiões do mundo e sua posterior erradicação no oeste europeu (revisado por Sutmoller et al., 2003).

Entretanto, problemas tais como a infectividade residual nas vacinas devido a falhas na inativação por formaldeído, eram constantes. O advento da inativação por acetiltileneimina (Brown e Crick, 1959) e, posteriormente a substituição desta pela Etileneimina binária (BEI) (Banhemann, 1975), a qual é menos tóxica, tornou mais fáceis manipulação e transporte, mantendo mesma eficiência na inativação. Isso tornou as vacinas contra a febre aftosa mais seguras e baratas (Sutmoller et al., 2003)

Particular atenção tem sido dada à biologia molecular dos aftovírus, o que ocorreu principalmente a partir de 1960 (Bradish et al., 1960), quando se descobriu sua semelhança morfológica ao poliovírus, o que aumentou muito o conhecimento da biologia do vírus da febre aftosa (Brown, 2003). A descoberta de que há formação de cápsideos virais "vazios", ou sem RNA, porém contendo uma considerável quantidade de proteína que foi caracterizada como sendo a enzima RNA-polimerase do vírus que é encapsidada juntamente com o RNA. Essa descoberta foi considerada importante por suas implicações no diagnóstico diferencial entre animais vacinados e infectados através da presença de anticorpos contra as (NSP) proteínas não estruturais (Cowan e Graves, 1966). Estudos sobre a composição das partículas que compunham o capsídeo viral demonstraram a presença de uma fita simples de RNA e 60 cópias de cada uma das quatro proteínas do capsídeo (VP1, VP2, VP3 e VP4) bem como de uma enzima, o que possibilitou explicar a relativa labilidade do vírus, que à temperatura de 25^0C por 24 horas perde um $\log_{10}$ da sua

infectividade e até três $\log_{10}$ a 37^0C pelo mesmo período de tempo (Denoya et al., 1978). Neste processo a RNA polimerase cliva o RNA dentro dos capsídeos sem alterar sua morfologia (Denoya et al., 1978; Newman et al., 1994). Análises cristalográficas por raios X do vírion do aftovírus, diferentemente dos demais membros da família *Picornaviridae* revelaram uma região na superfície do capsídeo que contém um "loop" (Acharya et al., 1989), na proteína VP1. A seqüência deste "loop" tem sido usada como uma "impressão digital" do vírus para determinar sua origem, devido a sua variabilidade genética. Um exemplo do uso de tal método foi a identificação do vírus causador do surto na Inglaterra em 2001, o qual pôde ser identificado em poucos dias como relacionado ao causador dos surtos na África do Sul, Correia do Sul e Japão em 2000 (Brown, 2003).

1.2 Aftovírus

Geralmente os surtos de febre aftosa causam perdas econômicas extensas em países ou áreas livres da doença, devido aos custos diretos e principalmente indiretos, a maior parte das mesmas relacionadas a embargos alfandegários de animais e subprodutos. Os últimos surtos em 2000 no Japão e 2001 no Reino Unido, Argentina, Uruguai e Brasil mostraram a dimensão do risco da introdução e rápida disseminação da aftosa, em uma população susceptível (Mattheews, 2001).

O vírus da febre aftosa possui sete diferentes sorotipos que são A, O, C, *South African Territories* (SAT) 1, SAT 2, SAT 3 e Ásia 1 os quais são compostos de múltiplos subtipos. Há proteção cruzada entre subtipos do mesmo sorotipo, o que não ocorre entre os diferentes sorotipos, porém uma variação antigênica do subtipo pode reduzir a eficiência da vacina utilizada contra este no campo (Kitching et al., 1989).

1.3 O genoma dos aftovírus

O vírus da febre aftosa tem uma taxa de mutação elevada, que resulta do efeito de sua polimerase sem atividade de auto reparação (*proof-reading*) e da habilidade do genoma em acomodar considerável quantidade de mutações. Esta característica tem tornado este patógeno relativamente fácil de rastrear quando da ocorrência de surtos através da comparação da seqüência de nucleotídeos do genoma viral (Mason, et al., 2003c).

Nas extremidades não transcritas (UTRs) 5' e 3' do genoma dos aftovírus encontra-se um número distinto de elementos que têm sido identificados pela predição de sua estrutura secundária e, em alguns casos, pela sua função. A maioria das diferenças encontradas entre 8 diferentes isolados de "O" PanAsia, são mutações que ocorrem fora destas regiões UTRs ou que não afetam a estrutura terciária das proteínas expressas. Da mesma forma, a análise das porções codificantes poliproteína destes isolados, revelou que a maioria das mutações era silenciosa, não traduzidas na proteína. Também não houve casos de deleção ou inserção no segmento da poliproteína (Mason, et al., 2003c). No entanto, esse estudo também revelou que mesmo pequenas alterações no genoma do aftovírus, podem produzir dramáticas mudanças da virulência *in vivo*, aparentemente por provocar alterações em algum mecanismo não detectável de imediato. Porém, este estudo foi incapaz de identificar alterações genéticas específicas capazes de explicar porque estes isolados foram tão efetivos em sua disseminação através da Ásia, África e até na Europa, chegando a substituir isolados do tipo O enzoóticos em algumas regiões.

A febre aftosa é causada por um vírus RNA (8.5 kb) de fita positiva, não envelopado, apresentando um capsídeo icosaédrico de 25 - 30 nm de diâmetro, pertencente à família *Picornaviridae* e ao gênero *Aftovirus* (Murphy, et al., 1999).

O genoma do vírus da febre aftosa é composto por uma região não transcrita na extremidade 5' de seu genoma na qual se encontram várias regiões reguladoras do processo de replicação viral. Nesta extremidade do genoma está ligada a proteína 3B ou VPg que serve como um fator para a ligação direta da polimerase ($3D^{pol}$) ao RNA e tem a função de servir como co-fator replicação do mesmo (Hope et al., 1997; Xiang et al., 1998). No genoma do aftovírus são encontradas 3 cópias da 3B. Essas cópias têm funções na patogenicidade do vírus e estão relacionadas à sua capacidade de infectar diferentes espécies. O Fragmento-S dos aftovírus é o maior da família *Picornaviridae*, têm funções na replicação, bem como de estabilizar a molécula de RNA; em seguida encontra-se uma região rica em citosina que torna o RNA viral resistente à ação de RNAses, conhecida como Poli-C (Brown et al., 1974). Depois do poli-C está outra região peculiar denominada "Pseudo-nós" (*Pseudoknots ou PKs*), esta de função ainda desconhecida, também é encontrada em outros poliovírus como o vírus da encefalomiocardite suína (EMCV), (Duke et al., 1992). Após os PKs encontra-se uma estrutura em forma de "*loop*" que parece ser o *cre* do genoma do aftovírus. O *cre* é uma estrutura em forma de alça que possui uma região conservada composta por 5 nucleotídeos (AAACA) a qual é essencial para a replicação do genoma de todos os membros dessa família. A replicação do RNA não ocorre na ausência do *cre* no entanto a tradução ocorre normalmente (Mason et al., 2002).

O RNA do vírus da aftosa não possui na sua extremidade 5' um resíduo de 7-metil-guanina (Cap) envolvido na iniciação da tradução no ribossomo. Os aftovírus possuem uma estrutura secundária e terciária no RNA conhecida como IRES (*Internal Ribossomal Enter Site*), que no caso dos aftovírus é do tipo 2. O IRES reage com inúmeros fatores de iniciação celulares requeridos para a tradução Cap dependente. A ligação de um destes fatores de iniciação conhecido como eIF4G, que é clivado pela L^{pro}, serve como ponte de

ligação entre os mRNA Cap dependente com a unidade menor do ribossomo. Desta maneira, somente traduções IRES dependentes irão ocorrer, ocasionando a parada completa de tradução de mRNA celulares ou *shut-off* da tradução de proteínas do hospedeiro. O IRES também está envolvido na patogenicidade do vírus (revisado por Mason et al., 2003).

A região codificante das poliproteínas dos aftovírus inicia-se logo após o IRES. A primeira proteína a ser codificada é a proteína Leader (L^{pro}). Esta proteína está presente em todos os 7 sorotipos do vírus e possui duas versões, pois o vírus tem dois códons de iniciação nessa região que codifica a Lab e Lb respectivamente. Sugere-se uma maior síntese *in vivo* ao segundo AUG, pois o vírus viável pode ser recuperado de genomas contendo mutações no primeiro AUG, o que não acontece quando essa mutação é no segundo (Cao et a.l., 1995). A L^{pro} é uma proteinase que faz sua auto clivagem a partir da poliproteína nascente, além de clivar o fator de iniciação eIF4G. Além disso, a L^{pro} é um importante fator de virulência. Foi produzido em laboratório um vírus A12 com a ausência da L^{pro}, sendo que imediatamente ao segundo AUG codifica-se as proteínas estruturais (P1), o qual foi chamado de *Leaderless*, produzindo vírus viável com uma capacidade de replicação levemente reduzida em células BHK-21, sem diferença quando inoculado em camundongos lactentes, porém incapaz de produzir doença quando inoculado em bovinos ou suínos (Mason et al., 1997).

No entanto, quando foi produzido um *leaderless* a partir de sorotipo altamente virulento, O1 Campos, desencadeava um quadro leve da doença quando inoculado em suínos, inviabilizando o uso deste no campo, como uma vacina geneticamente atenuada (Almeida et al., 1998). Em outro estudo descobriu-se que o "*shut-off"* das proteínas celulares promovidas pelas L^{pro}, bloqueava a expressão de Interferon tipo I (IFN) que é a primeira resposta do hospedeiro contra o vírus (Grubman e Chinzangaram, 2000).

Depois da L^{pro} há uma região no genoma conhecida como P1 que codifica as proteínas estruturais que compõe o capsídeo do vírus. O capsídeo do aftovírus é composto por 60 cópias de cada uma destas proteínas 1A, 1B, 1C e 1D, sendo que as últimas três presentes na superfície do vírus enquanto a primeira fica internamente ao capsídeo mantendo as duas últimas unidas. Sugere-se que a ligação entre a 1B com a 1C se dá através de pontes de hidrogênio entre regiões ricas em histidina, particularidade que torna os aftovírus extremamente sensíveis ao pH, ocorrendo a dissociação do capsídeo em pH menor do que 6.5 (Acharya et al., 1989; Jacson et al., 2003), o que pode variar entre os diferentes sorotipos.

O processamento da poliproteína precursora P1/2A é efetuado pela $3C^{pro}$ levando a formação dos protômeros que contém uma cópia de cada uma das proteínas 1AB, 1C e 1D. Cinco protômeros formam um pentâmero e 12 pentâmeros formam um pró-vírion (Guttman e Baltimore, 1977). O último passo da formação do pró-vírion para o vírus maduro, é a clivagem da proteína 1AB que ocorre por mecanismos desconhecidos e é dependente da presença de RNA viral (Arnold et al., 1987).

Na proteína 1D encontra-se um fragmento móvel e saliente na superfície do vírus, ao qual se denomina G-H *loop*. Este *loop* foi descrito como um importante determinante antigênico, porém não é o único (Baxt et al., 1989). Neste G-H *loop* há uma região extremamente conservada entre os diferentes sorotipos e subtipos composta por arginina e asparagina (RGD). Essa região é a principal responsável pela ligação do vírus à célula hospedeira e, dependendo do sorotipo e conseqüentemente dos aminoácidos presentes nas regiões *cis* e *trans* do RGD, determina a afinidade do vírus às integrinas αvβ3, αvβ1, αvβ5 e αvβ6. O vírus O1 Campos, por exemplo, tem uma maior afinidade para com as integrinas αvβ1 e αvβ6, porém também pode utilizar com menor afinidade pelas outras 2 integrinas. Já

o A12 tem grande afinidade por αvβ6 e αvβ3 e praticamente nenhuma afinidade pelas demais (*in vitro*). A habilidade do vírus de utilizar múltiplas integrinas *in vitro* pode ser um reflexo do uso de múltiplos receptores durante o curso da infecção no hospedeiro susceptível (Duque e Baxt, 2003).

As proteínas derivadas das regiões P2 e P3 do genoma têm funções na replicação do RNA, formação e montagem das proteínas estruturais, sendo a 2C e 3A responsáveis pela interação entre o complexo de replicação do RNA com as membranas internas da célula. Estas proteínas têm a capacidade de ligação às membranas celulares (Bienz et al., 1987).

A região P2 do genoma do vírus da febre aftosa é composta pelas proteínas 2A, 2B e 2C, sendo 2A contígua a P1. A 2A possui apenas 18 aminoácidos e é responsável pela interrupção no processo de tradução do ribossomo. A tradução continua em seguida na 2B, deixando assim um fragmento com todas as proteínas estruturais para serem clivadas pela $3C^{pro}$ os quais irão formar os protômeros que por sua vez formam os capsomêros e a partir destes os cápsideos (Donnelly et al., 2001). Já as proteínas 2B e 2C estão implicadas no efeito citopático, processo que permite a liberação do vírus para o meio extracelular. A 2B também tem sido associada ao aumento de permeabilidade da membrana e bloqueio da secreção protéica (Doedens e Kirkegaard, 1995).

A proteína 3A é 66 aminoácidos maior nos aftovírus, do que nos poliovírus. Consecutivas passagens do vírus em embrião de pinto resultaram em atenuação do vírus em bovinos devido a uma deleção de 19 a 20 códons na extremidade carboxi-terminal, deleção idêntica foi observada no isolado O/TAW/97, que devastou a população de suínos em Taiwan em 1997, mas que não se disseminou entre a população bovina (Beard e Mason, 2000). A proteína 3A é parte de intermediários estáveis que incluem 3AB e 3ABB (O'Donnell et al., 2001). Nos poliovirus, a 3AB se liga à extremidade 5' do RNA viral e

funciona como co-fator para a ligação da $3D^{pol}$ (Lama et al., 1995). O aftovírus possui em seu genoma 3 cópias do gene codificante da 3B (ou VPg) e uma molécula da proteína é covalentemente ligada à extremidade 5' do genoma na encapsidação do RNA.

A proteínase 3^{pro} é responsável pela maioria das clivagens da poliproteína viral, exceto autoclivagens e da 2A. A $3C^{pro}$ faz as clivagens da P1 exceto a clivagem de maturação da 1AB (Bablanian e Grubman, 1993). As demais clivagens na P1 provavelmente permitem a formação dos capsídeos vazios, como é o caso das vacinas recombinantes de Ad5 (Mayr et al., 2001). Quando a P1 é administrada em animais sem a presença da $3C^{pro}$, há formação de anticorpos, porém não há proteção contra a doença clínica, em virtude da falta de processamento pela $3C^{pro}$, que promove um provável arranjo tridimensional, que expõe os epítopos neutralizáveis do vírus para a proteção vacinal (Mayr et al., 2001).

Assim como ocorre em outros picornavírus, a $3D^{pol}$ do aftovírus é uma RNA polimerase. Essa proteína tem uma seqüência de aminoácidos altamente conservada, quando comparada entre os diferentes sorotipos de vírus da febre aftosa. O complexo da polimerase quando incubado com soro de animal convalescente é inativado. Indicando que os animais que replicaram o vírus, desenvolvem anticorpos neutralizantes contra essa proteína, o que não acontecia com o soro de animais vacinados. Esta propriedade permitiu o desenvolvimento de um teste que diferencia animais vacinados de animais infectados pelo vírus conhecido como VIAA (antígeno associado à infecção), uma vez que a $3D^{pol}$ é uma proteína altamente imunogênica. A elongação do RNA nascente é catalisada pela $3D^{pol}$ num processo semelhante ao do poliovíus. Esse processo ocorre nos complexos de replicação os quais consistem de proteínas não estruturais e RNA e ocorrem nas membranas internas da célula, Golgi ou Retículo Endoplasmático (Polatinick e Wool, 1983).

Na extremidade 3' do RNA dos poliovírus, assim como dos aftovírus, há uma região não-traduzida rica em adenina, semelhante à cauda de poli(A) do mRNA celular. A diferença é que esta cauda poli(A) é genômica e não é adicionada pós-transcricionalmente como ocorre nos mRNAs celulares. Esta região é imprescindível para a transcrição viral dos aftovírus e é encontrada associada a 3CD, e também pode estar envolvida na formação de uma ponte com a extremidade 5', circularizando a molécula, o que pode ter importante papel na replicação viral (Barton et al., 2001).

1.4 Vacinas contra a febre aftosa

As vacinas existentes hoje no mercado contra a febre aftosa, cumprem papel primordial de qualquer vacina, que é proteger o hospedeiro dos efeitos debilitantes da doença clínica. Da mesma forma que a maioria das vacinas, esta não induz uma imunidade estéril contra o vírus, permitindo a replicação viral nas superfícies epiteliais o que leva a formação dos animais chamados carreadores após o desafio (Doel, 2003).

Para efetivo controle e erradicação da febre aftosa no mundo, se utiliza uma vacina de suspensão de vírus inativado por BEI e emulsificada com adjuvante oleoso e/ou hidróxido de alumínio (Brownlie, 2001). No entanto, estas vacinas convencionais requerem replicação do vírus e sua inativação, o que induz um risco potencial de escape, sendo necessárias, instalações de bioseguridade máxima, as quais tem um alto custo de manutenção, e um potencial risco biológico (Barteling & Vreeswijk, 1991).

Para se obter sucesso no controle da doença deve se obter uma cobertura vacinal de no mínimo 80%. Nos países da comunidade européia não se conseguiu um impacto significativo no controle a doença até este patamar ser atingido. No entanto, essa vacinação em massa estava relacionada ao rebanho bovino, pois poucos ovinos e suínos foram

vacinados (revisado por Doel, 2003). Da mesma forma, resultados semelhantes foram obtidos na América do Sul, apesar de as vezes o rebanho ovino ser mais que o dobro do bovino como é o caso do Uruguai, a doença foi controlada com a vacinação em maça apenas dos bovinos (Sutmoller et al., 2003).

As vacinas inativadas contra a febre aftosa existentes hoje no mercado, passam por um processo de purificação, para eliminar o máximo possível a presença de proteínas não estruturais. No entanto, uma pequena percentagem de animais irá reagir positivamente no teste VIAA mesmo não tendo sido expostos a replicação viral, principalmente após serem submetidos à várias vacinações. Das proteínas não estruturais dos aftovírus a $3D^{pol}$ é a mais imunogenica. Isso torna a $3D^{pol}$, a proteína mais adequada para uso em um teste para diferenciar animais vacinados de convalescentes. Assim, uma vacina como a de Ad5, que não possui a $3D^{pol}$, também teria a vantagem de permitir um o diagnóstico diferencial mais eficiente (Moraes et al., 2002).

O Adenovírus humano tem sido usado como um vetor para vacinas de aftosa, conferindo completa proteção em suínos contra o sorotipo A, após uma única dose com Ad5 defectivo em replicação, o qual contém as regiões codificantes de P1 (poliproteína, região estrutural) e de $3C^{pro}$, uma protease que tem a função de clivar a poliproteína formada após a tradução do RNA viral (Mayr, et al., 1999; Moraes, et al., 2002).

1.5 O vetor Ad5

O Adenovírus humano tipo 5 é um vírus DNA de fita dupla, linear no qual proteína TP está ligada covalentemente na extremidade 5', nesta extremidade existem repetições terminais invertidas associada a proteína básica VII e peptídeo "*um"* (Anderson et al.,

1989). O vírus também possui uma protease (Pr) a qual é necessária para o processamento de algumas proteínas durante o processo de maturação viral (Webster et al., 1989).

Os adenovírus pertencem a família *Adenoviridae*, os quais infectam uma grande variedade de células pós-mitóticas incluindo aquelas associadas a tecidos altamente diferenciados, como é o caso das células do músculo esquelético, pulmão, cérebro e coração. Devido ao vetor replicar-se seu DNA no núcleo e se replicar com alta eficiência, torna-os candidatos primordiais para carrear e expressar genes de interesse terapêutico (Russell, 2000).

O vetor de adenovírus utilizado nos experimentos aqui contidos pertencem a primeira geração. Os Ad5 de primeira geração possuem suas regiões do seu genoma deletadas, as quais correspondem a região E1 e E3 (Russell, 2000). Este vetores de primeira geração tem uma capacidade de expressar genes de até 6.5 kbp de DNA sob o controle de um promotor heterólogo, neste caso, o CMV (May et al., 1999). Assim, este vetor é incapaz de se replicar em células *in vitro* ou *in vivo*, a menos que a célula infectada contenha a região E1 que é indispensável para sua replicação. As células 293 (ATCC CRL-1573), as quais são utilizadas para replicar o Ad5 defectivo, possuem a região E1 em seu genoma (Grahan et al., 1977). As regiões E do genoma dos adenovirírus são expressas antes da replicação do DNA, já as L são expressas após (Grahan et al., 1977).

A expressão dos genes pelo Ad5 defectivo é apenas temporária, pois a ação do sistema imune contra a expressão do *trasgene*, bem como contra as proteínas do capsídeo viral do adenovírus são suplantadas pelo sistema imune (Grahan et al., 1977). Devido a deleção dos genes da região E1 onde estão se encontram as defesas pró-apoptóticas bem como da região E3 que tem funções semelhantes, tornando á célula hospedeira um alvo fácil para as defesas inatas (Poller et al., 1996) .

O pAd5 (plasmídeo de adenovírus 5 contendo origem de replicação, gene de resistência a Kanaminicina e Ampicilina, promotor CMV, seguido de um sítio único de restrição para a enzima de restrição ClaI e um sítio XbaI antes dos cóndons de terminação do trasngene (May et al., 1999).

1.6 Citocinas

A fase ativadora e efetora das respostas imunes inata e específica é, na maioria das vezes, mediada por proteínas chamadas citocinas. As citocinas servem para mediar e regular a resposta imune e inflamatória; neste sentido, citocinas têm função reguladora crítica no desenvolvimento da resposta imune (Deveroux & Linch 1998).

1.7 Susceptibilidade do aftovírus ao IFN-α e β

A primeira resposta do hospedeiro contra a infecção viral é a indução de Interferon Tipo I (IFN-α e β). A resposta imune inata, incluindo a produção de interferon IFN-α e β, é o resultado da síntese de moléculas provenientes da infecção viral, particularmente RNA de dupla fita. Expressão, secreção e ligação de IFN-α e β a receptores específicos na superfície da célula fazem a iniciação do sinal da rota de transdução e indução de um estado de resistência viral. Pela ativação de uma série de genes e com os produtos protéicos destes genes, poderão ser inibidos vários passos do ciclo de replicação viral. Para sobreviverem à resposta de IFN-α e β do hospedeiro, os vírus tiveram que desenvolver vários mecanismos que interferissem com a indução de IFN, síntese de genes induzidos por IFN ou na função dos produtos destes genes (Biron e Sen, 2001).

O vírus da febre aftosa desenvolveu um mecanismo para bloquear completamente a síntese de proteínas pela célula hospedeira, incluindo IFN-α e β, sem afetar a tradução dos seus próprios mRNA. Após a infecção pelo vírus da aftosa, a proteinase líder (L^{pro}), uma proteína viral não estrutural, é sintetizada e esta cliva o fator de iniciação de tradução eIF-4G, resultando na parada completa da tradução de mRNAs do hospedeiro, que são cap-dependentes. Como conseqüência da clivagem da eIF-4G, o RNA do vírus da aftosa inicia sua tradução por um processo cap-independente via IRES e somente requer o fragmento C-terminal da eIF-4G. Assim o vírus pode usar o mecanismo celular de síntese protéica para síntese de proteínas virais sem a competição de mRNA da célula (Devaney et al., 1988). A infecção por aftovírus induz a síntese de mRNA de IFN-α e β mas a L^{pro} inibe a tradução deste mRNA com cap, dessa forma ocorre um bloqueio rápido da resposta imune inata do hospedeiro. No entanto o vírus é altamente sensível à ação de IFN-α e β (Chinsangaran et al., 2001). A replicação do aftovírus em cultivo celular pode ser efetivamente bloqueada pelo produto dos genes induzidos por IFN-α e β, o que sugere seu uso potencial como agente anti-aftovírus *in vivo*, profilaticamente antes da infecção viral e terapeuticamente para reduzir a replicação e excreção viral em animais previamente infectados (Grubman, 2003).

1.8 Uso do GM-CSF como um adjuvante em vacinas

O uso de citocinas para modular a resposta imune produzida por vacinas de DNA e por imunização com vírus recombinantes, tem sido intensamente investigado. O GM-CSF (Fator estimulante de colônias de granulócitos e macrófagos) foi inicialmente descrito como um fator de crescimento para células tronco de granulócitos e linhagens de

macrófagos (Warren e Weiner, 2000). Atualmente este fator hematopoiético de crescimento, tem sido vastamente utilizado como adjuvante na indução de imunidade (Lee et al., 1998; Warren e Weiner, 2000).

O GM-CSF foi eleito como citocina para realização destes experimentos devido a suas características imuno-estimulantes. O GM-CSF atua como ativador de células apresentadoras de antígeno (APCs), tais como macrófagos e células dendriticas, aumentando a expressão de complexo de histocompatibilidade classe II (MHC), e conseqüentemente aumentando a capacidade destas células em apresentar antígenos e induzir a resposta primária por anticorpos. O GM-CSF também aumenta a maturação de células dendríticas e sua posterior migração para os linfonodos (Warren e Weiner, 2000), o GM-CSF também pode incrementar a resposta inflamatória no local de injeção, tudo isso aliado a reduzidos efeitos colaterais fazem do GM-CSF um atrativo componente para ser utilizado em vacinas (Jakob et al., 1998).

O *gm-csf* ativa neutrófilos, macrófagos, células dendríticas (DCs) e outras células mononucleares e também estimula as células progenitoras (*stem cells*) a maturarem e migrarem da medula óssea para a circulação periférica. Kusakabe et al. (2000) especularam que o *gm-csf* induz as DCs a se acumularem no sítio de vacinação com DNA e que as citocinas secretadas pelas DCs induzem uma resposta imune via Th2 mais forte, e o equilíbrio entre a resposta Th1/Th2 também são influenciadas pelo momento da administração do plasmídio contendo GM-CSF em relação ao momento da administração do plasmídio contendo o gene do antigeno vacinal.

Lee et al., 1998, observaram que as respostas imunes, humoral e celular contra as proteínas do envelope do HCV (Hepatite C vírus) em camundongos, foram incrementadas pela co-administração do gene do GM-CSF. As respostas de anticorpos e de

linfoproliferação para a proteína E2 foram acrescidas de 1.5 a 12.7 e 1.4 a 2.5 vezes respectivamente, quando o gene de GM-CSF foi co-transportado com o gene do antígeno. Injeções de plasmídeos bicistrônicos promoveram níveis de resposta de anticorpos e de linfoproliferação mais elevados do que quando o antígeno (HCV) e o GM-CSF se encontravam em plasmídeos diferentes . Estes resultados são semelhantes aos obtidos por Borauch et al., (2002), que observaram uma resposta melhor quando utilizados plasmídeos bicistrônicos do que quando os genes estavam em plasmídios diferentes.

Camundongos vacinados contra influenza A, com uma vacina lipossomal (INFLUSOMW-VAC) contendo a hemaglutinina e a neuraminidase associada ao GM-CSF ou IL-2 produziu altos títulos neutralizantes que persistiram por mais de 6 meses, além de oferecer proteção parcial cruzada contra outros subtipos testados em camundongos, já o mesmo preparado da vacina porém sem presença de nenhuma citocina produziu títulos de anticorpos menores e proteção por apenas 2 meses (Babai et al., 2002).

Lee et al, (1998) sugeriram que a concentração local de GM-CSF pode ter sido um fator crítico que contribuiu para melhorar a resposta imune quando co-expresso com o antígeno. Xiang et al., (1995) observaram que a inoculação separada de GM-CSF e do plasmídeo codificando o gene do antígeno administrado muitas horas mais tarde não apresentaram efeito sobre a magnitude da resposta de anticopos antígeno-específicos.

Co-injeção de um vetor plasmídeo expressando GM-CSF e a glicoproteína do vírus da raiva, aumentaram a atividade de linfócitos B e T auxiliares enquanto que a co-injeção com IFN-γ resultou na redução da resposta imune contra o antigeno viral em camundongos (Xiang & Ertl, 1995), dados semelhantes também foram observados por Somasundaram et al. (1999), com as glicoproteínas do vírus de aujeszky em suínos.

Devido aos seus efeitos imunomodulatórios e poucos efeitos colaterais o GM-CSF tem se tornado um atrativo adjuvante para vacinas usado só ou em combinação com outras moléculas de efeito adjuvante (Warren e Weiner, 2000).

1.9 GM-CSF em vacina para suínos

Somasundaram et al. (1999) testaram separadamente IFN- γ e IL-2 e em associação com GM-CSF em suínos como adjuvante em uma vacina DNA contra doença de aujeszky, utilizando-se como antígeno as glicoproteínas B e D deste vírus. A co-administração de *pgm-csf* nesta vacina de DNA aumentou a resposta imune induzida pelas gB e gD, sendo que esta resposta imune foi caracterizada pelo aparecimento mais precoce de imunoglobulinas IgG2 e um significativo aumento de anticorpos IgG1 e IgG2 anti-aujeszky, com uma conseqüente diminuição dos títulos da excreção e do tempo de excreção viral após o desafio com cepa virulenta do vírus da doença de aujeszky, já o IFN-γ e a IL-2 não tiveram efeito adjuvante.

Cedillo-Barrón et al., (2001), relataram um significativo incremento na resposta imune após 3 administrações em suínos vacinados contra febre aftosa, com uma vacina DNA contendo as proteínas da região P1+2A+3CD co-administrada com o gene do *pgm-csf*. No entanto, Cedillo-Barrón et al., (2003) relataram que o uso de um plasmídio contendo o gene do *pgm-csf* e proteínas do vírus da febre aftosa em suínos não teve diferença significativa quando comparado ao grupo de animais que receberam apenas o plasmídio contendo genes do antígeno.

O GM-CSF foi escolhido por ser capaz de recrutar e estimular DCs, as células apresentadoras de antígeno mais efetivas, o que o torna um adjuvante muito atrativo para vacinas (Tao e Levy, 1993).

No laboratório C do FMD unit de PIADC (New York – USA), onde este trabalho foi desenvolvido também outras citocinas como, Interferons alfa, beta e gama tem sido testadas como adjuvantes de vacinas recombinantes contra febre aftosa carreadas por Ad5.

2. Objetivos

2.1 Gerais:

Testar o pGM-CSF expresso em adenovírus como adjuvante para vacinas recombinantes de subunidades contra febre aftosa, com a finalidade de incrementar a resposta imune e obter proteção precoce e duradoura.

2.2 Específicos:

- Construir um Ad5 contendo o gene do *pgm-csf* ;

- Testar a expressão do *pgm-csf in vitro*;

- Testar a atividade do pgm-csf *in vitro* em células TF-1 que foi produzido pelas pelas células IB-RS-2;

- Testar a resposta imune humoral de suínos imunizados somente com Ad5-O1C ou em combinação com 2 doses diferente de Ad5-pgmcsf;

- Avaliar a resposta imune protetora do Ad5-O1C ou Ad5-O1C + Ad5-*pgmcsf* contra o desafio da cepa homóloga do vírus da febre aftosa O1 Campos;

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Este foi o principal trabalho desenvolvido pelo doutorando em PIADC

The Effect of Granulocyte-Macrophage Colony-Stimulating Factor on the Potency and Efficacy of a Foot-and-Mouth Disease Virus Subunit Vaccine

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The Effect of Granulocyte-Macrophage Colony-Stimulating Factor on the Potency and Efficacy of a Foot-and-Mouth Disease Virus Subunit Vaccine[1]

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3.1 ABSTRACT –. Luizinho Caron, Mario C.S. Brum, Mauro P. Moraes, William T. Golde, Clarice Weis Arns, & Marvin J. Grubman **[The Effect of Granulocyte-Macrophage Colony-Stimulating Factor on the Potency and Efficacy of a Foot-and-Mouth Disease Virus Subunit Vaccine]** Efeito do Fator Estimulante de Colônias de Granulócitos e Macrófagos (GM-CSF) na eficácia e potência de uma vacina de subunidades da Febre Aftosa em suínos. *Pesquisa Veterinária Brasileira (**(*)... .....*
(Corresponding author: Telephone Number: 631-323-3329, Fax Number: 631-323-3006, Email: mgrubman@piadc.ars.usda.gov)

Foot-and-mouth disease (FMD) is one of the most feared diseases of livestock worldwide. Vaccination has been a very effective weapon in controlling the disease, however a number of concerns with the current vaccine including the inability of approved diagnostic tests to reliably distinguish vaccinated from infected animals and the need for high containment facilities for vaccine production, have limited its use during outbreaks in countries previously free of the disease. A number of FMD vaccine candidates have been tested and a replication-defective human adenovirus type 5 (Ad5) vector containing the FMDV capsid (P1-2A) and 3C protease coding regions has been shown to completely protect pigs against challenge with the homologous virus (FMDV A12 and A24). An Ad5-P1-2A+3C vaccine for FMDV O1 Campos (Ad5-O1C), however, only induced a low FMDV-specific neutralizing antibody response in swine potency tests. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been successfully used to stimulate the immune response in vaccine formulations against a number of diseases, including HIV, hepatitis C and B etc. To attempt to improve the FMDV-specific immune response induced by Ad5-O1C, we inoculated swine with Ad5-O1C and an Ad5 vector containing the gene for porcine GM-CSF. However, in the conditions used in this trial, pGM-CSF did not improve the immune response to Ad5-O1C of swine challenged with homologous FMDV.

INDEX TERMS: Foot-and-mouth disease virus O1 Campos; Adenovirus; Granulocyte-macrophage colony-stimulating factor

3.2 RESUMO.- A febre aftosa é uma das doenças mais temidas nos rebanhos em todo o mundo. A vacinação tem sido uma arma eficiente no controle da doença, no entanto há preocupações com as vacinas atualmente utilizadas incluindo a necessidade de instalações de alta segurança para a produção dessas vacinas e a falta de um teste de diagnóstico aprovado que faça distinção precisa entre animais vacinados. Várias vacinas têm sido testadas contra a febre aftosa e uma dessas utiliza como vetor um vírus defectivo para replicação, derivado do adenovírus humano tipo 5 (Ad5), o qual contém o as proteínas que compõe capsídeo do vírus da febre aftosa (P1-2A) e a protease 3C que protegeu suínos completamente contra o desafio de uma cepa homóloga (A12 e A24). Uma vacina com o Ad5-P1-2A+3C proveniente da cepa O1 Campos (Ad5-O1C), no entanto, somente induziu um baixo título de anticorpos neutralizantes específicos em testes de potência vacinal em suínos. Fator estimulante de colônia de granulócitos e macrófagos (GM-CSF) tem sido utilizado com sucesso na formulação de vacinas para estimular a resposta imune contra inúmeras doenças, incluindo HIV, Hepatite C e B etc. Na tentativa de melhorar a resposta imune específica contra a febre aftosa induzida pelo Ad5-O1C, suínos foram vacinados com Ad5-O1C juntamente com Ad5-GM-CSFporcino. Entretanto nas, condições utilizadas nesse teste, o pGM-CSF não melhorou a resposta imune do Ad5-O1C em suínos desafiados com o vírus homólogo da febre aftosa.

TERMOS DE INDEXAÇÃO:. Febre Aftosa Vírus O1 Campos, Adenovirus, Fator Estimulante de Colônias de Granulócitos e Macrófagos

3.4 INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious, acute disease of domestic and wild cloven-hoofed animals (Callis & McKercher, 1986; Donaldson et al., 1989). The etiologic agent, FMD virus (FMDV), is a member of the Aphthovirus genus *Picornaviridae* family (Murphy et al., 1999) and contains a single-stranded, positive-sense RNA genome of approximately 8.5kb. The virus consists of seven serotypes (O, A, C, SAT1-3 and Asia) and multiple subtypes within each serotype.

FMD outbreaks can result in significant economic losses in disease-free countries due to direct and indirect costs, most of which are related to international trade embargoes of animals and animal products. Recent outbreaks in Taiwan and the United Kingdom have highlighted the major risk of introduction and rapid spread of FMD in a susceptible population (Mathews, 2001).

Currently, conventional binary ethyleinimine (BEI) inactivated vaccines emulsified with adjuvant have been widely used in effective control and eradication programs around the world (Brownlie, 2001). However, there are no approved diagnostic tests that can reproducibly differentiate vaccinated from infected/convalescent animals. Furthermore, conventional vaccines require growth and inactivation of live virus in containment facilities and introduce the potential for escape of live virus or incomplete inactivation (Barteling & Vreeswijk, 1991). As a result some FMD-free countries have not considered vaccination as part of their disease control strategy.

Several approaches are being used to develop alternative FMD vaccines that address these concerns, including construction of modified live-virus (Mason et al., 1997; Almeida

et al., 1998), biosynthetic proteins (Kleid et al., 1981; McKercher et al., 1985), synthetic peptides (Bittle et al., 1982; Clark et al., 1983; Brown, 1988; Blanco et al., 2001), naked DNA vectors (Ward et al., 1997; Chinsangaram et al., 1999; Wong et al., 2000; Benvenesti et al., 2001; Cedillo-Barrón et al., 2001), and recombinant viruses (Sanz-Parra et al., 1999a, b; Mayr et al., 1999, 2001; Berinstein et al., 2000; Moraes et al., 2002; Wu et al., 2003b). However, most of these approaches have either been unsuccessful in both swine and cattle or require multiple inoculations to induce protection.

Human adenovirus (Ad5) has been used as a vector for FMD vaccines encoding the capsid (P1-2A) (Sanz-Parra et al., 1999a, b) or P1-2A and 3C protease coding regions of FMDV (Mayr et al., 1999, 2001; Moraes et al., 2002; Wu et al., 2003b). Inoculation with one dose of Ad5 containing the A24 Cruzeiro P1-2A and A12 3C coding regions (Ad5-A24) protected swine from direct inoculation challenge with homologous virus (Moraes et al., 2002). More recently we have attempted to develop Ad5 vaccine vectors for other FMDV serotypes, in particular isolates of serotype O that are currently causing disease outbreaks throughout the world. We have constructed Ad5 and Ad2 vectors containing the P1-2A region of O1 Campos and the 3C of A12 (Ad5-O1C) and found that swine inoculated with these vectors developed a lower FMDV-specific neutralizing antibody response than Ad5-A24 inoculated animals (unpublished data). Likewise, swine inoculated with an Ad5-FMD bivalent vaccine containing the P1-2A regions from A24 Cruzeiro and O1C developed a higher neutralizing antibody response against A24 than against O1C (Wu et al., 2003b). It has been established that serotype O antigen induces a lower immune response as compared to serotype A antigen (Pay & Hingley, 1987; Doel et al., 1994). Because of this commercial vaccines usually contain about 4-5 times more O1 inactivated virus (146S antigen) than A 146S antigen.

The use of cytokines to modulate responses against immunization with DNA and recombinant virus vectors is being actively investigated. Granulocyte-macrophage colony-stimulating factor (GM-CSF), a hematopoietic growth factor, has been widely used as a molecular adjuvant to induce immunity. GM-CSF activates neutrophils, macrophages, dendritic cells (DCs), and other mononuclear cells, and also stimulates progenitor/stem cells to mature and migrate from the bone marrow to the peripheral circulation (Kusakabe et al., 2000). Intramuscular (IM) inoculation of the GM-CSF gene together with plasmids carrying viral genes, such as those encoding the glycoprotein of rabies virus and VP1 of encephalomyocarditis virus, increased antigen-specific immune responses and protective immunity in mice as compared to inoculation with the plasmid containing only the viral gene (Xiang & Ertl, 1995; Sin et al., 1997). Somasundaram et al. (1999) found an adjuvant effect of porcine GM-CSF (pGM-CSF) on a DNA vaccine containing the gD and gB glycoprotein genes of Aujeszky's disease virus (PRV) in pigs. This effect was characterized by an early appearance of anti-PRV IgG2, a significantly enhanced anti-PRV IgG1 and IgG2 antibody response, a significantly decreased and shortened period of virus shedding in nasal swabs and improved protection against viral challenge. In contrast, co-administration of porcine IFN-γ or IL-2 had no adjuvant effect.

Lee et al. (1998), observed that both humoral and cellular immune responses to hepatitis C virus (HCV) envelope proteins were augmented in rats by the co-delivery of the GM-CSF gene. Moreover, inoculation of bicistronic plasmids elicited higher levels of antibody and lymphoproliferative responses than did the co-inoculation of two independent expression plasmids that encoded the GM-CSF gene and each HCV envelope gene. Similarly Barouch et al. (2002) demonstrated that a bicistronic plasmid containing GM-CSF and HIV gp120 elicited a dramatic augmentation of vaccine-elicited $CD4^+$ T cell

responses in mice as compared to gp120 alone or individual plasmids containing each gene. These results suggest that the local concentration of GM-CSF may be a critical factor that contributes to the enhancement of the immune response to the co-expressed antigens. It has also been demonstrated that the time of administration of GM-CSF with respect to the DNA antigen vaccine can profoundly influence the nature of the Th1/Th2 balance of an antigen-specific immune response (Kusakabe et al., 2000).

We have tested pGM-CSF as an adjuvant for our Ad5-O1C vaccine and have delivered this cytokine with a recombinant Ad5 vector. We have previously utilized the Ad5 vector system to effectively deliver various cytokines including type I interferons (IFN-α/β) to swine (Chinsangaram et al., 2003; Moraes et al., 2003; Wu et al., 2003a) and cattle (Wu et al., 2003a). Swine inoculated with both Ad5-pGMCSF and Ad5-O1C, however, did not develop an enhanced immune response against O1C as compared to Ad5-O1C inoculated animal.

3.5 MATERIALS AND METHODS

3.5.1 Virus and cells

All adenovirus clones were generated, grown, and titered in human 293 cells (Graham, *et al*., 1977; Graham & Prevec, 1991), between passages 20 and 30. Plaque reduction neutralization assays with FMDV were performed in baby hamster kidney 21 cells (BHK-21) between passages 62 and 70. TF-1 cells, obtained from the American Type Culture Collection, are an indicator cell clone that is dependent on human GM-CSF or IL-3 for growth. These cells were used to analyze the biological activity of pGM-CSF obtained from the supernatants of IB-RS-2 cells (Instituto Biológico-Rim Suíno 2) infected with Ad5-pGMCSF, as pGM-CSF supports growth of this cell line as well.

3.5.2 Construction of recombinant adenoviruses Ad5-pGMCSF and Ad5-O1C

The pGM-CSF gene, containing the signal sequence, was obtained from plasmid p3CIa-pGM-CSF kindly provided by Dr. Steve Martin, Pharmacia-UpJohn. This plasmid was digested with HindIII and EcoRI, ligated to similarly digested pBluescript II KS (Stratagene, La Jolla, CA) and chemically transformed into Top 10 competent cells (Invitrogen, Carlsbad, CA) to generate the plasmid, pKSII-pGMCSF. The coding sequence of pGM-CSF was removed from pKSII-pGMCSF by digestion with ClaI and XbaI and ligated into similarly digested pAd5-Blue (Moraes et al, 2001) to generate the infectious clone pAd5-pGMCSF. Recombinant virus Ad5-pGMCSF was produced by transfection of 293 cells with PacI digested pAd5-pGMCSF following the protocol described (Wu et al., 2003b). The virus was isolated, propagated in 293 cells, and purified by CsCl gradient centrifugation (Moraes et al., 2002).

To construct Ad5-O1C, the infectious clone pCRM8 (Sá-Carvalho et al., 1997) a chimeric FMDV clone containing the P1-2A coding region of FMDV O1C in the background of FMDV A12, was digested with NcoI, ApaI to remove the O1C P1-2A coding region. Plasmid pP12X3C (Mayr et al., 1999), which contains the complete P1-2A and 3C coding regions and partial 2B and 3B coding regions of FMDV A12, was digested with the same enzymes to remove the A12 P1-2A coding region and ligated to O1C P1-2A to generate pP1-2A(O1C)X3C. This plasmid was digested with BglII and XbaI to remove the O1C P1-2A and A12 3C coding regions and ligated to similarly digested Ad5 transfer vector pShuttle (He et al., 1998). pShuttle-P1-2A(O1C)X3C was digested with ClaI and XbaI and the fragment containing the FMDV coding regions was ligated to similarly digested pAd5-Blue to generate pAd5-O1C. Recombinant virus Ad5-O1C was produced by

transfection of 293 with PacI digested pAd5-O1C and purified virus prepared as described above.

3.5.3 Expression of pGM-CSF and O1C capsid proteins synthesized in Ad5-pGMCSF or Ad5-O1C infected cells

IB-RS-2 cells were grown in 6 well plates and infected with Ad5-pGMCSF or Ad5-O1C at a multiplicity of infection (moi) of 20. At 5 or 23 h postinfection (hpi), the cells were preincubated for 1 h in methionine-free culture medium in the absence or presence of 5 μg/ml tunicamycin, followed by radiolabeling for 1 h with [^{35}S]methionine in methionine-free medium in the absence or presence of tunicamycin. The supernatants were harvested, frozen at -70^{o}C and the cells were washed twice with PBS, lysed, centrifuged and the cytoplasmic extracts frozen at -70^{o}C. Samples were immunoprecipitated with polyclonal goat anti-pGM-CSF antibody (R&D Systems, Minneapolis, MN) or various FMDV-specific antisera as described in the captions to Figs. 1 and 3 and the immune complexes were precipitated with *Streptococcus aureus* protein G (Calbiochem, San Diego, CA) and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel. The gels were dried and exposed to Kodak XDBF-1® X-ray film.

Alternatively IB-RS-2 cells were infected at a moi of 20 with Ad5-pGMCSF for 24 h. Supernatant fluids were harvested and centrifuged at 2000 rpm for 10 min. To remove the majority of virus inoculum, the supernatants fluids were filtered through a Centricon 100 filter (Millipore, Bedford, MA) and frozen in aliquots at -70^{0}C. Aliquots were examined for pGM-CSF expression by Western blot analysis on a 10% precast polyacrylamide gel, (NuPAGE, Invitrogen) and transferred to a PVDF membrane (Millipore). Protein was detected using a polyclonal goat anti-pGM-CSF antibody (R&D Systems), phosphatase

labeled rabbit anti-goat polyclonal antibody, and BCIP/NBT phosphatase substrate following the manufacturer's instructions (KPL, Gaithersburg, MD).

3.5.4 Biological activity of pGM-CSF synthesized in Ad5-pGMCSF infected cells

Biological activity was measured using a Cell Proliferation ELISA System RPN 250 (BIOTRAK® Amersham Pharmacia Biotech, Piscataway, NJ). A standard curve was generated by growth of TF-1 cells, in 96-well tissue culture plates, in the presence of dilutions of recombinant pGM-CSF (R&D Systems) (Kitamura et al., 1989). Dilutions of supernatant fluids from Ad5-pGMCSF infected IB-RS-2 cells were added, in duplicate, to a 96-well plate containing TF-1 cells. After 5 days incubation, bromodeoxyuridine (BrdU) was added and cells labeled for approximately 20 h. Cell proliferation was measured following the manufacturers recommended protocol (Bautista et al., 2002).

3.5.5 Animals

Eighteen out-bred Yorkshire gilts approximately 7-8 weeks old, weighting between 23-25 Kg, were divided into five groups and each group was housed in a separate room in the high-containment facilities at the PIADC. All animals were handled humanely according to protocols approved by the Animal Care and Use Committee (ACUC) of PIADC. The animals were observed for one week prior to the start of the experiment. The vaccine was administrated by IM inoculation in the neck with 2 ml of various doses of Ad5 vectors in PBS (Table 1). All animals were challenged 21 days post vaccination (dpv) and 35 dpv the remaining pigs were euthanised, following the ACUC protocol.

3.5.6 Serology and virus isolation

Serum samples were obtained at 0 and 4 days after inoculation and weekly thereafter. Sera were tested for the presence of neutralizing antibodies against FMDV O1C, in a plaque reduction neutralization (PRN) assay. Neutralization titers were reported as the serum dilution yielding a 70% reduction in the number of plaques (PRN_{70}) (Moraes et al., 2002).

Heparinized blood was obtained prior to challenge and daily for 7 days postchallenge (dpc) and examined for the presence of virus by a standard plaque assay in BHK-21 cells. Nasal secretions were obtained on the same days as heparinized blood, inoculated into IB-RS-2 cells, and observed for 48 h for cytopathic effects. Negative samples were frozen and a second passage performed. For positive samples, titration was performed from the original samples by a plaque assay on BHK-21 cells.

3.5.7 Challenge

The 18 animals were challenged with homologous FMDV O1C 21 days after receiving the respective vaccine combinations (Table 1). FMDV O1C was provided by the Instituto Pan-Americano de Febre Aftosa, Rio de Janeiro (PANAFTOSA). This virus was passed 5 times in BHK-21 cells, and once each in a bovine and a pig. Virus was harvested from vesicles of an infected pig, diluted with MEM, centrifuged at 2000g to remove debris, aliquoted, and frozen at -70^0C. An aliquot was titrated in BHK-21 cells and in 4 pigs to determine the "pig heel infectious dose 50%" ($PHID_{50}$) as previously described (Burrows, 1966). Pigs were infected with 100 $PHID_{50}$ by intradermal inoculation at 2 sites in the heel bulb of the rear feet.

3.6 RESULTS

3.6.1 Construction of Ad5 vectors

The Ad5-pGMCSF virus was constructed by insertion of the pGM-CSF gene into the E1 region of pAd5-Blue placing this gene under the control of the cytomegalovirus (CMV) immediate-early promoter (Moraes et al., 2001).

The Ad5-O1C virus contains the P1-2A capsid coding region of FMDV O1C and the FMDV A12 3C protease coding region all under the control of the CMV immediate-early promoter. We and others have previously shown that the A12 3C protease can efficiently process the P1-2A precursor from A24, O1C, and O1 Taiwan/99 (Sa-Carvalho et al., 1997; Almeida et al., 1998; Moraes et al., 2002; Wu et al., 2003b; unpublished).

3.6.2 Ad5-pGMCSF and Ad5-O1C expression

IB-RS-2 cells were infected with Ad5-pGMCSF and at 6 or 24 hpi were radiolabeled with [^{35}S]methionine in the absence or presence of 5 μg/ml tunicamycin. The cell culture supernatants were examined by SDS-PAGE. As can be seen in Fig. 1 (lane 2) multiple bands are present in the supernatant at 24 hpi, while in the supernatant from cells treated with tunicamycin a single band is present (lane 3). This band is immunoprecipitated by polyclonal serum that was produced against *E. coli*-expressed pGM-CSF (lane 6). These results are in agreement with published sequence data indicating that mature pGM-CSF has three potential N-linked glycosylation sites (Inumaru & Takamatsu, 1995). The inefficient immunoprecipitation of glycosylated pGM-CSF (lane 5) may be the result of interference with the antigen-antibody reaction by the extensive glycosylation of this protein. We further analyzed production of pGM-CSF in IB-RS-2 cells by Western blot analysis and

show glycosylated pGM-CSF is efficiently detected, suggesting that denatured protein is a better antigen for this polyclonal antibody (compare Fig. 2, lanes 6 and 7 to Fig. 1, lane 5).

To examine the expression of O1C capsid proteins by Ad5-O1C, IB-RS-2 infected cells were radiolabeled, cell lysates prepared at 5-7 hpi, immunoprecipitated with various FMDV-specific antibodies, and analyzed by SDS-PAGE (Fig. 3). Reaction with bovine convalescent serum precipitated the structural proteins VP0, VP3, and VP1 (lanes 2 and 4). Likewise monospecific polyclonal antibodies against FMDV A12 VP1 and VP2, which we have previously demonstrated immunoprecipitates O1C structural proteins (Grubman et al., 1987), each precipitates all 3 O1C structural proteins (lanes 5 and 6) suggesting that FMDV capsid protein complexes are assembled in Ad5-O1C infected cells. We have previously observed similar results in cells infected with other Ad5-FMDV viruses (Mayr et al., 1999; Moraes et al., 2002; Wu et al., 2003b).

The biological activity of pGM-CSF obtained from supernatant fluids of Ad5-pGMCSF infected IB-RS-2 was measured in vitro by a TF-1 cell proliferation assay. As shown in Fig. 4, Ad5 expressed pGM-CSF has biological activity as demonstrated by the support of TF-1 cell growth.

3.6.3 Immune response prior to challenge

To examine the potency and efficacy of Ad5-O1C in susceptible animals and determine if pGM-CSF can enhance the immune response of the FMDV antigens, 3 groups of swine, 4 animals per group, were inoculated IM with Ad5-O1C at $5x10^9$ pfu/ml in the absence (Group 3) or presence of $1x10^8$ or $1x10^9$ pfu/animal Ad5-pGMCSF (Groups 4 and 5; Table 1). Control groups, 3 animals per group, were inoculated with an Ad5 vector, Ad5-Blue (Group 1), or with Ad5-pGMCSF alone (Group 2).

After vaccination no adverse side effects, such as fever, apathy or inflammation at the inoculation site, were observed in any of the pigs. The FMDV-specific neutralizing antibody titers of the Ad5-O1C inoculated animals were detectable by 4-7 dpi, but were low and were not increased by co-administration of Ad5-pGMCSF (Table 1, Fig. 5). As expected the animals in the control groups did not develop an FMDV-specific neutralizing antibody titer.

3.6.4 Challenge

3.6.4.1 Clinical response

All animals were challenged 21 dpv. After challenge, swine in the control groups (1 and 2) developed a fever (temperature of 40°C or higher for 2 or more consecutive days) by 1-2 dpc and vesicular lesions by 1 dpc (Table 1). All of these animals had extensive disease by 2-3 dpc (Fig. 6). Between 3-5 dpc all animals in control group 1, that received the Ad5-Blue vector, died and on day 3 one animal in group 2, that received Ad5-pGMCSF, also died. Upon necropsy, these four animals all had extensive signs of heart necrosis (tiger heart) which was confirmed by histopathological examination. These cardiac complications are typical causes of death as a result of FMDV infection in young swine. The two control animals that survived each lost at least one hoof by one week postchallenge.

Nine of twelve of the vaccinated pigs (Groups 3, 4 and 5) developed fever by 2-5 dpc, while 1 pig in group 3 and two in group 4 never had fever. All vaccinated swine developed signs of infection, but the onset of disease was delayed 1-2 days compared to the controls, disease severity was reduced, and no animals in these groups died or lost their hoofs (Fig. 6). The group that received only Ad5-O1C (Group 3) had a slightly reduced clinical score

as compared to the groups that received both Ad5-O1C and Ad5-pGMCSF (Groups 4 and 5).

3.6.4.2 Serological response

The two surviving animals in the control groups became FMD positive by 7 dpc and all animals in the FMD vaccinated groups developed a substantial increase in FMDV-specific neutralizing antibody response after challenge (Table 1, Fig. 5).

In both control groups viremia was detected at 1 dpc and reached a peak of 1.5-3.5x10^6 pfu/ml at 2 dpc (Fig. 7A). In the FMD vaccinated groups, viremia was first detected in groups 4 and 5 (vaccine + pGMCSF) at 2-3 dpc and by 3-4 dpc in group 3 (vaccine alone). One pig in group 3 never developed viremia or fever and a second animal only had a low level of viremia for 1 day (Fig. 7A). Peak viremia was at 3 dpc in the dual-inoculated groups and was approximately 1,000-fold lower as compared to the controls (Fig. 7A). In the Ad5-O1C vaccinated animals, peak viremia was at 4 dpc and was approximately 10,000-fold lower than in the control pigs.

Virus present in nasal swabs reached a peak in both control groups by 2-3 dpc and at 4 dpc in all three FMD vaccinated groups (Fig. 7B). Group 3, vaccinated with only Ad5-O1C, had the lowest levels of virus shedding.

3.7 DISCUSSION

We have previously demonstrated that a single IM inoculation of Ad5-FMDV vectors containing the P1-2A coding regions from A12 (Mayr et al., 2001) or A24 (Moraes et al., 2002) and the A12 3C protease coding region can induce significant FMDV-specific neutralizing antibody titers in swine and protect most or all of the animals when challenged either by contact or direct inoculation. However, inoculation with an Ad5 vector containing the O1C capsid coding region or an Ad5 vector containing the O1C capsid coding region in a bicistronic expression cassette induces a significantly lower FMDV-specific neutralizing antibody response in swine (unpublished observations; Wu et al., 2003b). Similar results have been reported with the conventional inactivated FMD vaccine and therefore to be an efficacious vaccine, manufacturers include a higher dose of type O 146S antigen than type A in vaccine formulations (Pay & Hingley, 1987; Doel et al., 1994).

To enhance the immune response induced by Ad5-O1C, we tested a vaccination regimen that included co-administration of pGM-CSF, a cytokine that has been shown to enhance the immune response of animals to some vaccines (Xiang & Ertl, 1995; Lee et al., 1998; Somasudaran et al., 1999; Cedillo-Barrón et al., 2001; Barouch et al., 2002). We constructed an Ad5-pGMCSF virus that expressed biologically active pGM-CSF. Swine inoculated with Ad5-O1C and either a low or high dose of Ad5-pGMCSF developed a detectable FMDV-specific neutralizing antibody response prior to the animals administered only Ad5-O1C, ie., by 4 days, and the response was higher at 1 week postinoculation. However, by 2 weeks postinoculation and at the time of challenge the neutralizing antibody response of these 3 groups was equivalent (Fig. 5) and was considerably lower than the response to one inoculation of Ad5-A12 or Ad5-A24 (Mayr et al., 2001; Moraes et al.,

2002). Thus, the co-administration of Ad5-pGMCSF did not enhance the long-term FMDV-specific neutralizing antibody response.

After challenge the control groups (Groups 1 and 2) rapidly developed significant disease and all 3 animals in Group 1 and 1 of 3 animals in Group 2 died. The histopathology findings upon necropsy of the four animals that died after challenge revealed that they all had severe vesicular lesions on the mouth and all feet, resembling typical FMDV lesions, and moderate to severe myocardial necrosis resulting in heart failure, which was most likely the result of FMDV infection. In previous experiments with FMDV serotype A, control groups of swine rapidly developed significant disease after direct inoculation challenge, but did not die if they were healthy prior to the start of the experiment (Moraes et al., 2002; Chinsangaram et al., 2003; Moraes et al., 2003), suggesting that the challenge virus used in the present studies was either too virulent or the dose used was excessive. In subsequent titration of this virus, $1/100^{th}$ the dose used in this study still resulted in the death of 1 of 2 naïve animals and the animal that survived had severe disease including loss of hoofs. In either case the "overchallenge", in this experiment, masked the protective effects of the Ad5-O1C vaccine, since all vaccinated animals developed vesicular lesions and 3 of 4 animals had viremia. However, vaccination with Ad5-O1C clearly delayed and reduced the severity of disease. None of the animals inoculated with only Ad5-O1C died or lost their hoofs, the appearance of vesicular lesions was delayed for 1-2 days as compared to the control groups and was less severe. Furthermore, the peak of viremia was delayed 2 days, one animal never developed viremia, and virus titers were approximately 10,000-fold lower than in the controls, and the peak of virus shedding was delayed 2 days.

The addition of pGM-CSF did not enhance the efficacy of the Ad5-O1C vaccine. Although none of the animals given Ad5-pGMCSF died or lost their hoofs, all developed vesicular lesions that were somewhat more severe than the Ad5-O1C vaccinated animals. Furthermore, while viremia was delayed and reduced as compared to the control groups all of these effects can be attributed to the vaccine alone, as the Ad5-O1C inoculated animals had even lower levels of viremia.

We have subsequently repeated this experiment, and included a second route of inoculation, and obtained very similar results. Swine inoculated with Ad5-O1C IM with a needle or subcutaneously/IM by needleless delivery and challenged had no detectable viremia and clinical disease was delayed and less severe than control animals (data not shown). However, the addition of Ad5-pGMCSF reduced the level of protection afforded by the vaccine alone.

Cedillo-Barrón et al. (2001) demonstrated that the addition of plasmid encoded pGM-CSF along with a DNA based FMDV empty capsid vaccine resulted in a statistically significant increase in the antibody levels against FMDV and somewhat improved the protection of vaccinated swine as compared to swine only given the empty capsid vaccine. However, in these studies the swine were challenged after 3 DNA inoculations and the increase in FMDV-specific antibody levels was only detected after the second inoculation. Additional studies examining the adjuvant effect of GM-CSF for other antigens have suggested that the presence of this cytokine at the site of vaccine inoculation is important (Wang et al., 2002) and that plasmids co-expressing antigen and GM-CSF are more effective than co-administration of plasmids separately expressing each antigen (Lee et al., 1998; Barouch et al., 2002; Sun et al., 2002).

While our results do not agree with the findings of Cedillo-Barrón et al. (2001), it may be that factors including the level of expression of both the immunogen and the adjuvant, the type of cells expressing these proteins, as well as the proximity of antigen and adjuvant expression in the animal can have profound effects on induction of protective responses. Experiments to examine these factors as well as a more basic understanding of the host cell response to FMDV infection may help us identify cytokines that can potentially enhance the immune response to FMD vaccine candidates.

3.8 Conclusion

In previous work, we have demonstrated that swine given one dose of an Ad5-O1C vector developed only low FMDV-specific neutralizing antibody titers. To attempt to enhance the potency and efficacy of this vaccine, groups of swine were given either one inoculation of an Ad5-O1C vector or a combination of Ad5-O1C and an Ad5 vector containing the cytokine pGM-CSF at two different doses. Animals were challenged 3 weeks later by direct inoculation with virulent FMDV. Although the swine inoculated with only Ad5-O1C did not develop a significant FMDV-specific neutralizing antibody response and did develop clinical disease after challenge, disease signs and viremia were considerably reduced as compared to control groups.

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Table 1. Serum neutralization titers after vaccination and clinical scores

					$PRN_{70\%}$ [a]						
Animal	Group	Virus constructs	Dose (PFU/animal)	Clinical Scores[b]	Weeks post vaccination				Challenge ↓		
					0	½	1	2	**3**	4	5
2107	1	Ad5-Blue	$1X10^9$	16	<8	<8	<8	<8	<8	Dead	
2108	1			18	<8	<8	<8	<8	<8	Dead	
2109	1			18	<8	<8	<8	<8	<8	Dead	
2110	2	Ad5-pGMCSF	$1X10^9$	17	<8	<8	<8	<8	<8	160	1000
2111	2			15	<8	<8	<8	<8	<8	Dead	
2112	2			17	<8	<8	<8	<8	<8	640	1000
2104	3	Ad5-O1C	$5X10^9$	11	<8	<8	16	16	<16	2560	2000
2113	3			8	<8	<8	<8	8	<16	4000	1000
2114	3			11	<8	<8	32	64	32	4000	2000
2115	3			8	<8	<8	8	16	16	2560	2000
2105	4	Ad5-O1C+ Ad5-pGMCSF	$5X10^9+1X10^8$	9	<8	16	16	32	16	8000	2000
2116	4			12	<8	8	16	16	<16	4000	2000
2117	4			10	<8	16	64	16	<16	8000	8000
2118	4			15	<8	8	16	16	<16	2560	4000
2106	5	Ad5-O1C + Ad5-pGMCSF	$5X10^9+1X10^9$	12	<8	8	32	32	16	4000	4000
2119	5			11	<8	32	64	32	16	4000	4000
2120	5			9	<8	8	32	16	<16	1240	4000
2121	5			13	<8	8	16	16	<16	4000	2000

[a] Serum dilution that reduces the number of plaques by 70%; [b] Clinical scores were determined by the number of toes presenting FMD compatible lesions plus the presence of lesions in the snout and mouth, with a maximum score of 18;

Figure 1
Caron et al.

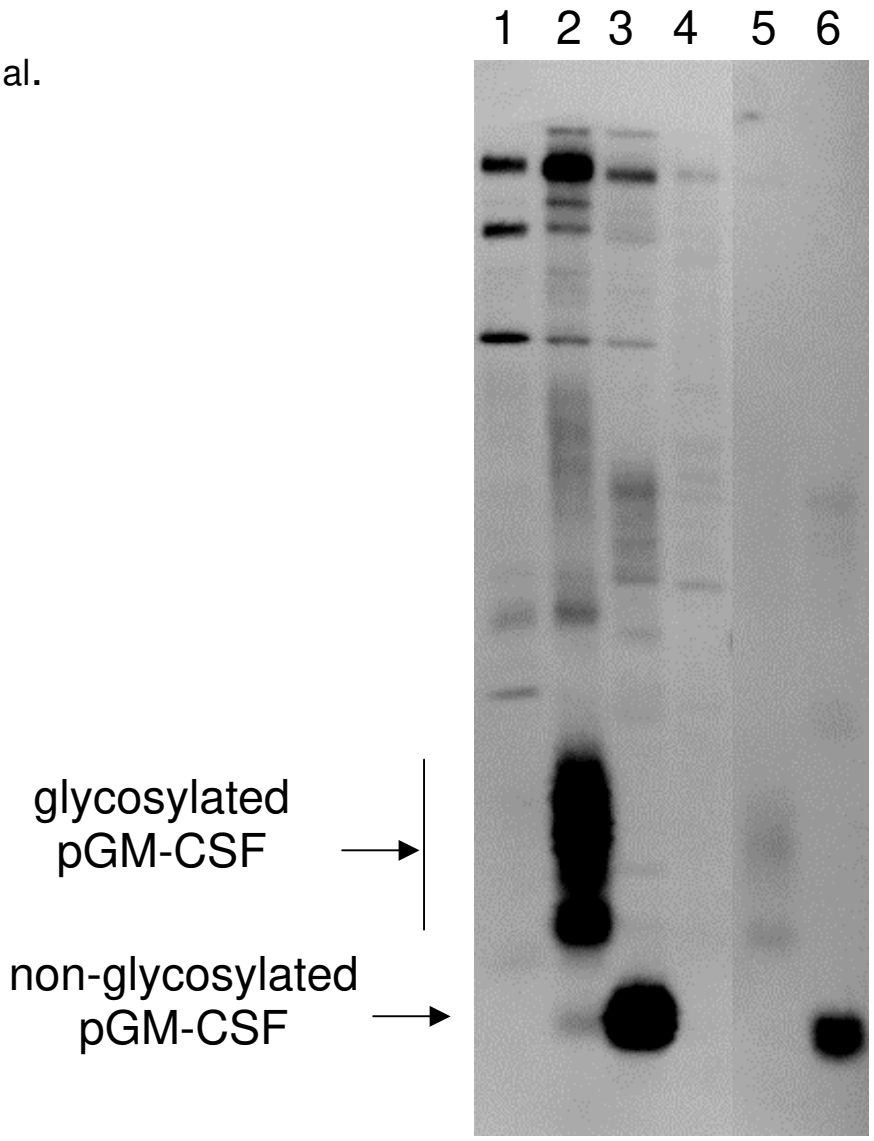

Fig. 1. Expression of pGM-CSF in Ad5-pGMCSF infected IB-RS-2 cells. IB-RS-2 cells were infected with Ad5-pGMCSF and radiolabeled with [^{35}S]methionine in the absence or presence of tunicamycin. Supernatant fluids were harvested and proteins were resolved by SDS-PAGE on a 15% gel or immunoprecipitated with a polyclonal antibody against pGM-CSF and subsequently resolved by SDS-PAGE. Lane 1: Supernatant fluid from cells infected for 6 hpi and radiolabeled in the absence of tunicamycin. Lanes 2-3: Supernatant fluids from cells infected for 24 hpi and radiolabeled in the absence or presence of tunicamycin, respectively. Lane 4: Immunoprecipitation of supernatant fluids from mock-infected, radiolabeled cells. Lanes 5-6: Immunoprecipitation of supernatant fluids from cells infected for 24 hpi and radiolabeled in the absence or presence of tunicamycin, respectively.

Figure 2
Caron et al.

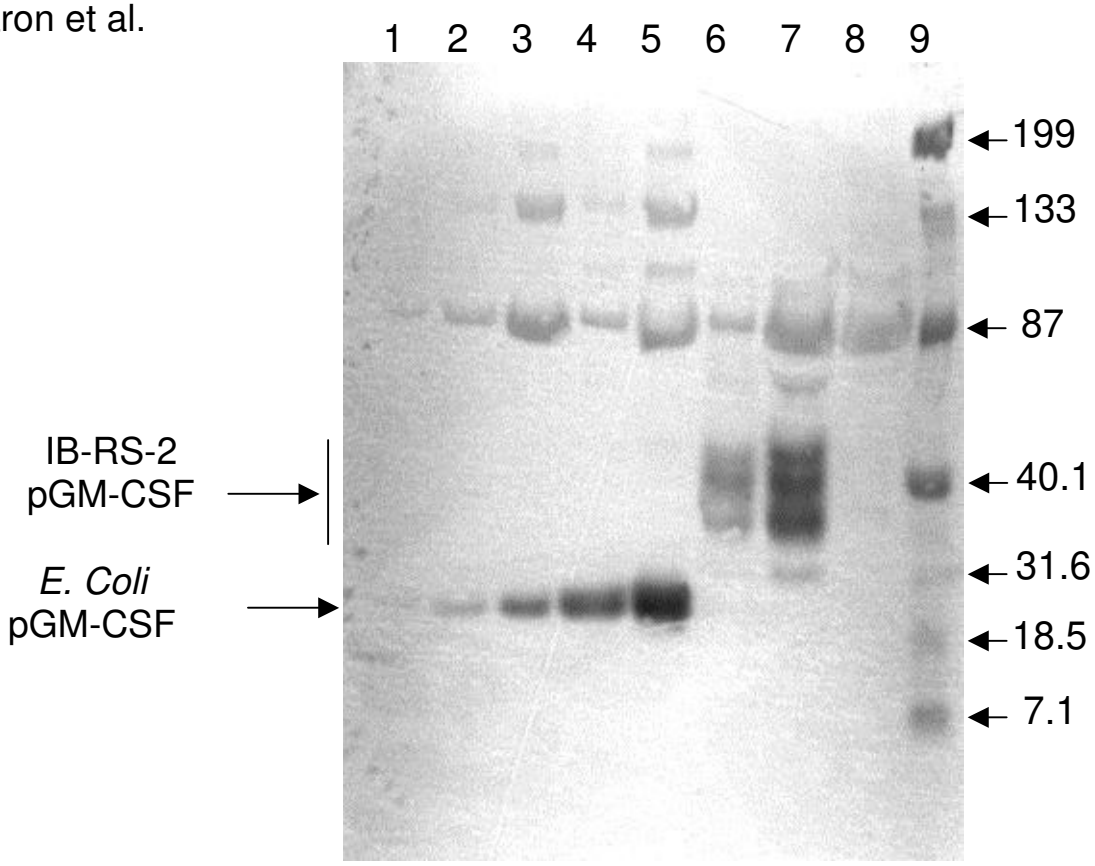

Fig. 2. Western blot analysis of supernatant fluids from Ad5-pGMCSF infected IB-RS-2 cells. Samples were resolved on a precast 10% polyacrylamide gel, transferred to a membrane and detected with a polyclonal goat anti-pGM-CSF antibody. Lanes 1-5: 0.5, 1, 5, 10, and 20 ng of recombinant pGM-CSF. Lanes 6-7: 2 and 9.8 µl supernatant fluids from Ad5-pGMCSF infected IB-RS-2 cells. Lane 8: 9.8 µl supernatant fluid from mock-infected IB-RS-2 cells. Lane 9: molecular weight marker. The numbers on the right represent molecular mass in kilodaltons.

Figure 3
Caron et al.

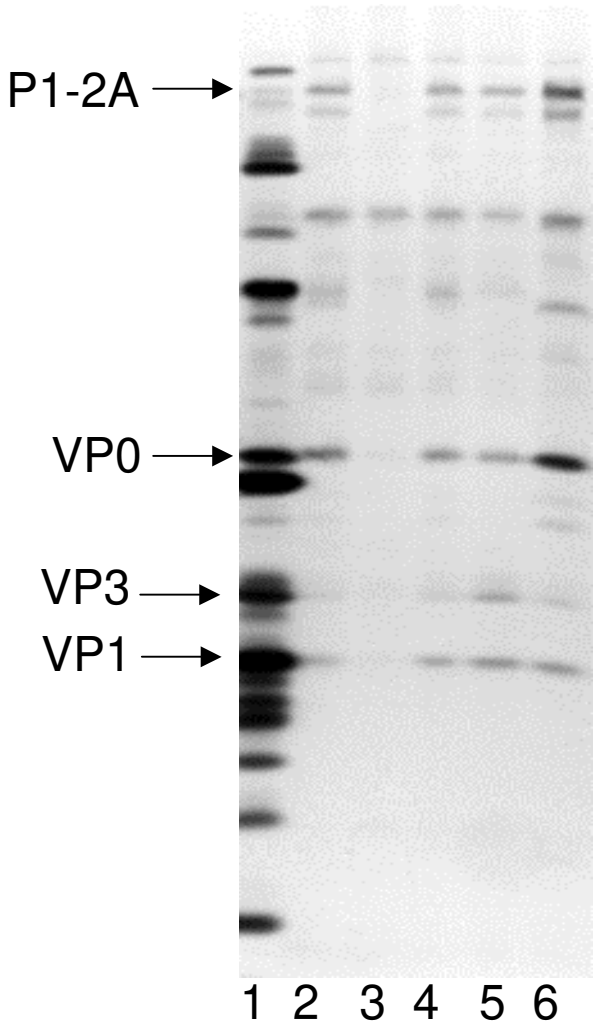

Fig. 3. Expression of O1C capsid proteins in Ad5-O1C infected IB-RS-2 cells. [^{35}S]methionine labeled lysates from Ad5-O1C infected IB-RS-2 cells were immunoprecipitated with various antibodies and proteins resolved by SDS-PAGE on a 15% gel. Lane 1: An FMDV O1C infected IB-RS-2 cell lysate was immunoprecipitated with convalescent serum from a bovine infected with FMDV. Lanes 2-6: cell lysates from Ad5-O1C infected IB-RS-2 cells were immunoprecipitated with convalescent serum from a bovine infected with several serotypes (lane 2), normal bovine serum (lane 3), convalescent serum from a bovine infected with FMDV O1C (lane 4), or polyclonal antibodies against FMDV A12 VP1 and VP2 (lanes 5 and 6, respectively).

Figure 4
Caron et al.

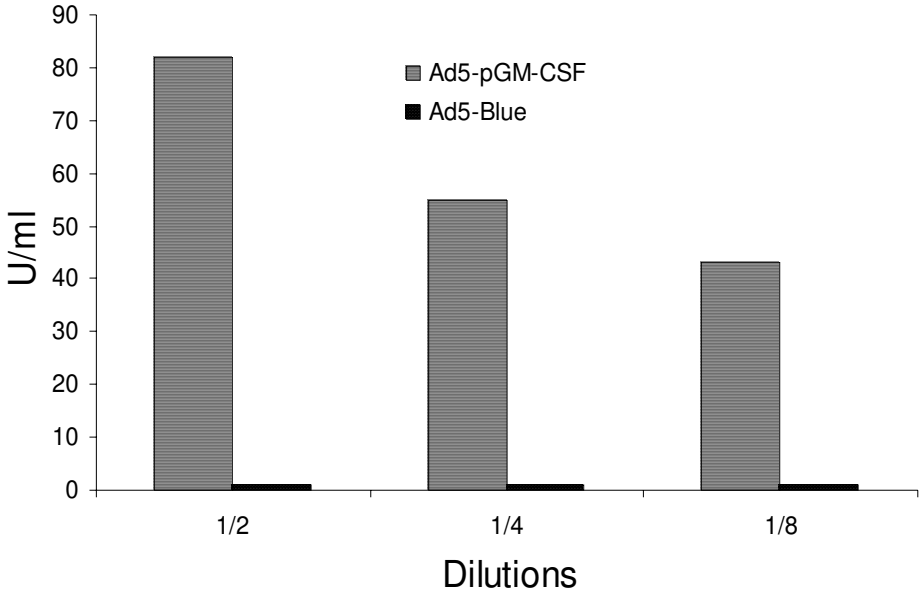

Fig. 4. BrdU proliferation assay. Recombinant pGM-CSF was added to TF-1 cells and examined for incorporation of BrdU to generate a standard curve. In the same assay supernatant fluids from Ad5-pGMCSF infected IB-RS-2 cells were titrated on TF-1 cells and examined for cell proliferation. The units/ml of pGM-CSF activity was determined from the standard curve (1 unit = 100 pg pGM-CSF).

Figure 5
Caron et al.

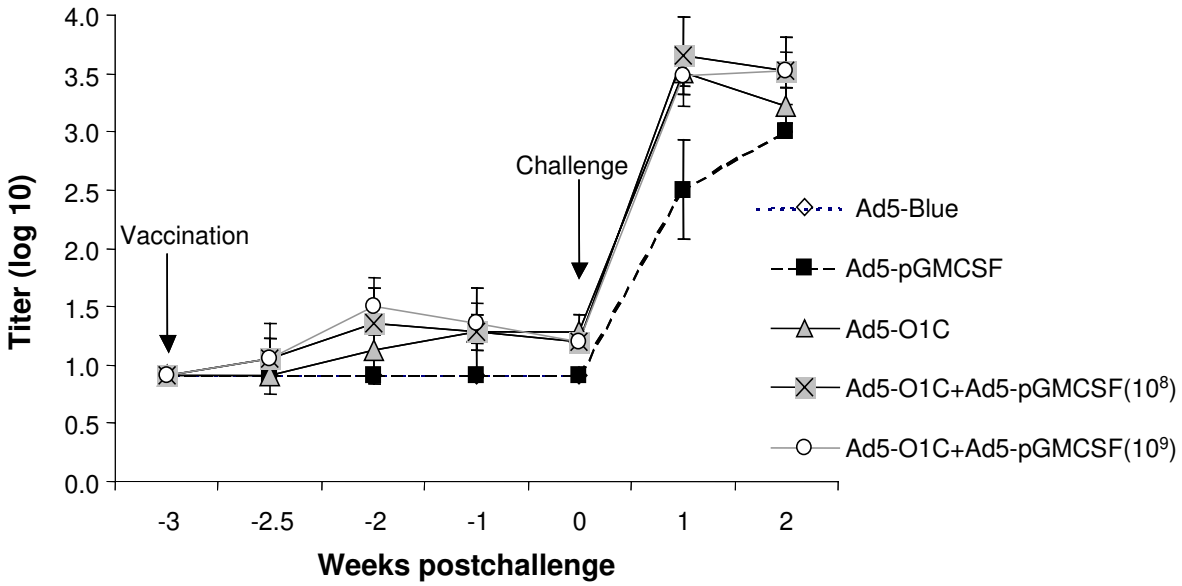

Fig. 5. Kinetics of induction of FMDV-specific neutralizing antibody titers after Ad5 inoculation. Serum samples were obtained weekly after Ad5 inoculation until 2 weeks postchallenge. The FMDV-specific PRN_{70} titers were determined and expressed as the mean of each group.

Figure 6
Caron et al.

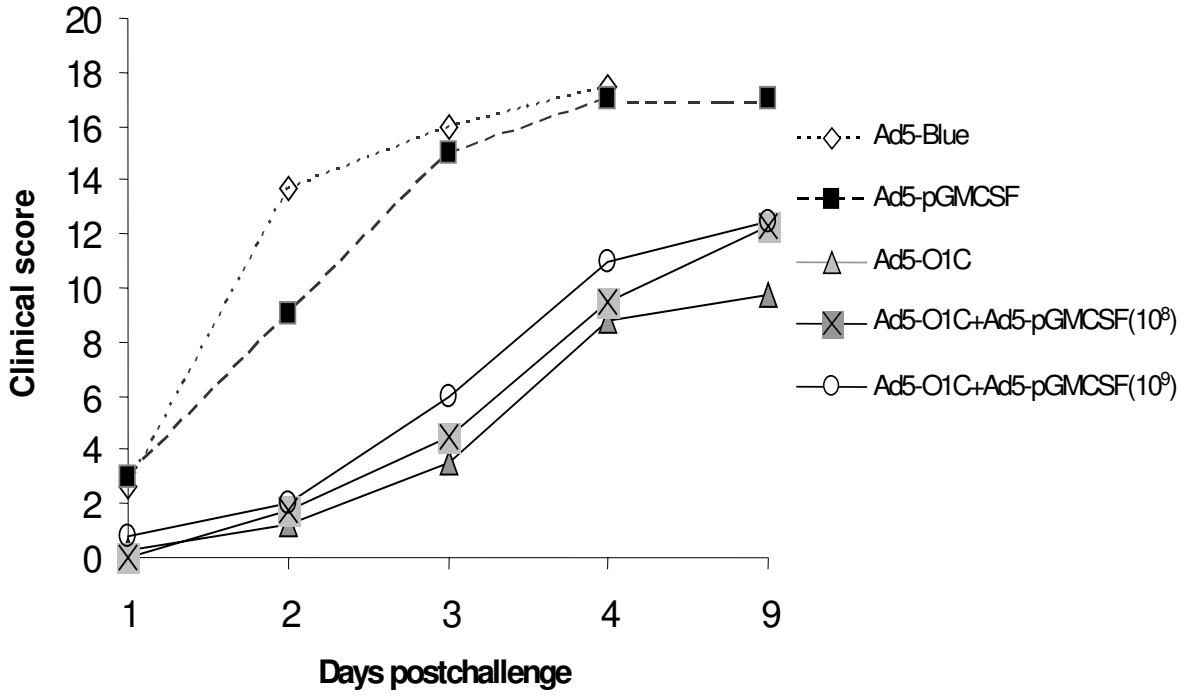

Fig. 6. Appearance of clinical disease after FMDV challenge. Animals were examined daily for vesicular lesions and the clinical score was determined as described in the legend to Table 1. The mean of each group is presented.

Figure 7A
Caron et al.

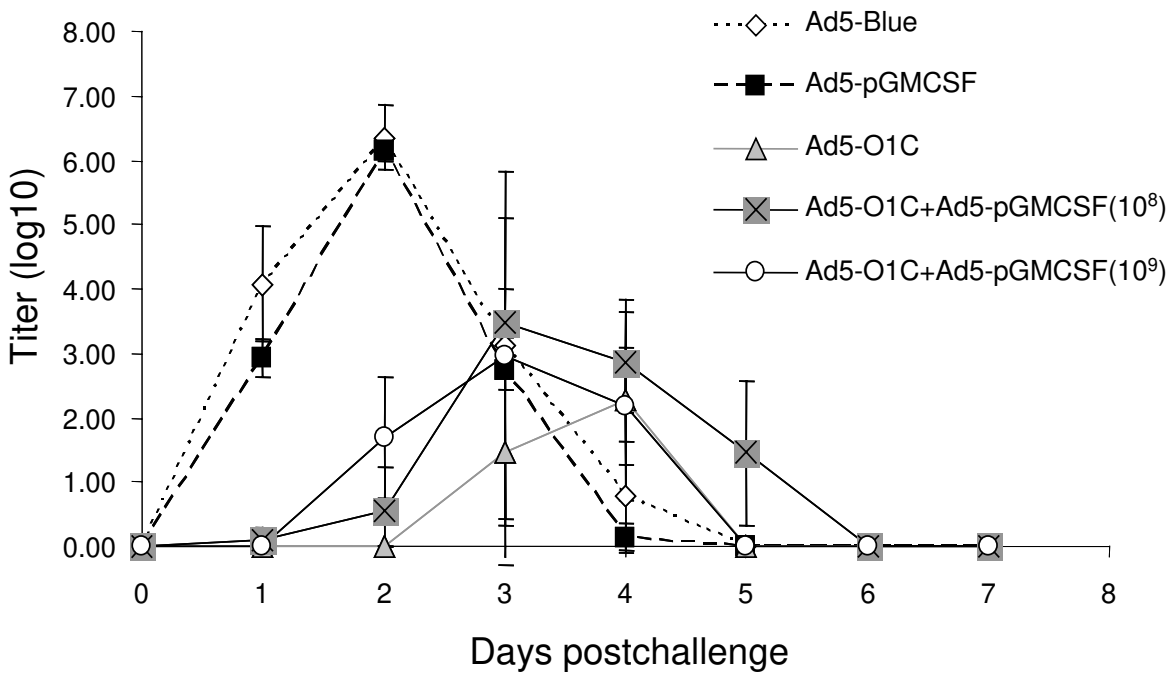

Fig. 7. Virus present in blood and nasal secretions after challenge. Panel A: Viremia in Ad5 inoculated pigs after FMDV challenge. Blood samples were obtained daily for 7 days postchallenge and virus titers, pfu/ml, determined by plaque assay on BHK-21 cells. The mean of each group is displayed.

Figura 7B

Caron et al.

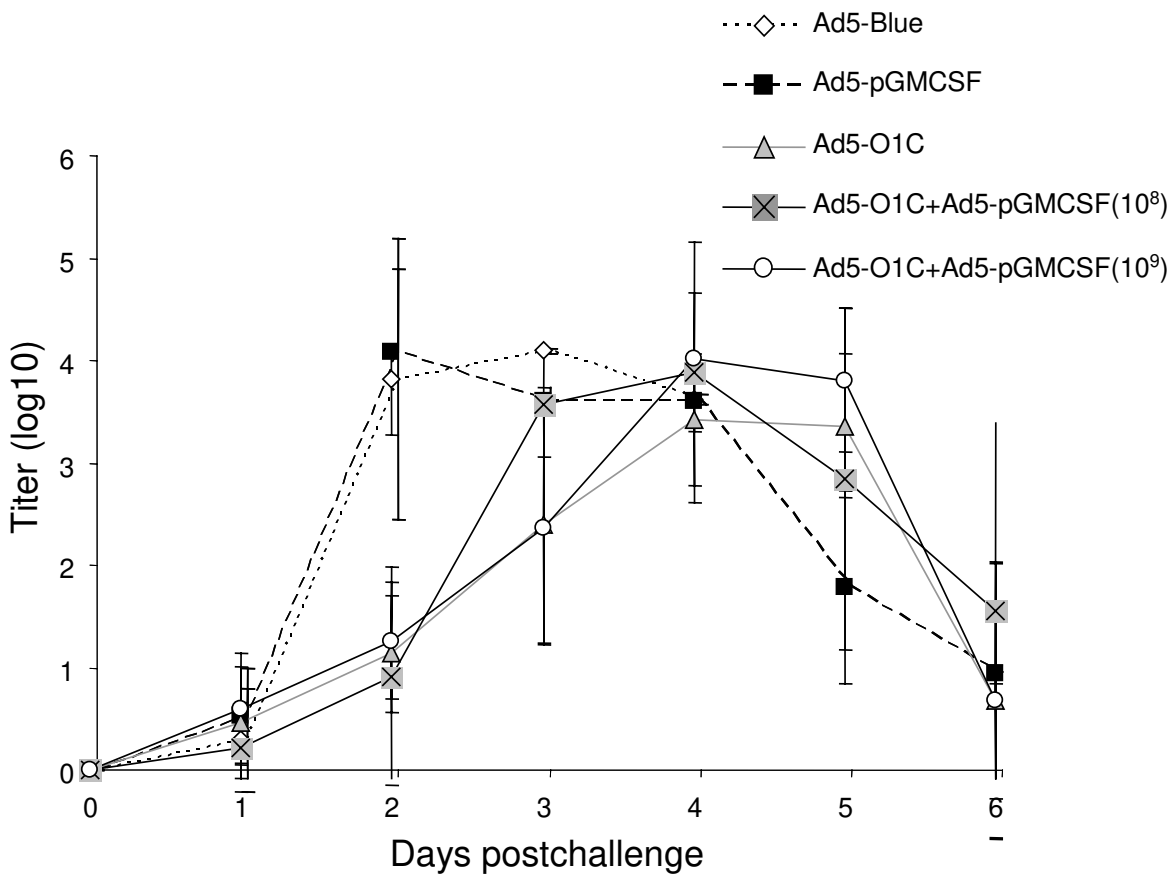

Fig. 7. Panel B: Virus in nasal swabs of Ad5 inoculated pigs after FMDV challenge. Nasal swabs were obtained daily for 6 days postchallenge and virus titers, pfu/ml, determined by plaque assay on BHK-21 cells. The mean of each group is displayed.

4. Discussão geral

Diferente de outros trabalhos nos quais o GM-CSF foi utilizado com sucesso incrementando a resposta imune de vacinas (Somasundaram et al., 1999; Cedillo-Barrón et al., 2001; Babai et al., 2002; Wang et al., 2002 etc..) utilizando-se diferentes formas de expressão ou injeção direta da proteína contra diferentes antígenos os quais forneceram resultados satisfatórios, neste o GM-CSF não produziu efeitos satisfatórios. Da mesma forma Cedillo-Barrón, em experimento utilizando plasmídios que codificavam peptídios do vírus da febre aftosa, não obtiveram incremento nos títulos de anticorpos específicos quando adicionados plasmídios expressando GM-CSF em suínos (Cedillo-Barrón et al., 2003.

Há várias hipóteses para explicar a ineficiência do GM-CSF de atuar como um adjuvante neste caso, como não era o objetivo deste trabalho essa questão fica pendente para ser estudada futuramente, o que sem dúvida é de grande interesse para a ciência na busca do entendimento de como o GM-CSF atua na resposta imune (Warren & Weiner, 2000). Porém com base em outros trabalhos podemos especular que a quantidade de GM-CSF utilizada, bem como a rota de administração tem efeito direto na resposta adjuvante (Sasaki et al., 2003). O tempo da administração do GM-CSF no local em relação ao antígeno também podem alterar profundamente o tipo de resposta imune (Kusakabe, et al., 2000). Como o GM-CSF modula a resposta imune (Kusakabe, et al., 2000) esta modulação pode estar desfavorecendo o tipo de reposta necessária para proteger contra a febre aftosa. Além disso, o aumento da resposta dos CTL aumentando a atividade citotóxica (Iwasaki et al., 1997) e consequentemente pode eliminar precocemente as células que receberam Ad5-O1C e estão expressando o antigeno da febre aftosa. Desta forma, o sistema imune não terá

tempo suficiente para montar uma resposta humoral adequada contra as proteínas da aftosa nas células que receberam o Ad5-O1C.

Em outro trabalho, ainda em andamento, a resposta imune a vacina Ad5-A24 em suínos foi melhorada quando da administração conjunta de Ad5- IFN-α (Botton, et al., 2003) o que é interessante, por ter a possibilidade de utilização do Ad5-A24 (antígeno) conjuntamente com o Ad5-IFN-α em caso de emergência de um surto de febre aftosa, além de implementar a resposta imune, irá atuar como terapia antiviral, inibindo atividade viral a partir de 24 horas após a vacinação com seu efeito perdurando por pelo menos 5 dias (Mason et al., 2003b)

A primeira medida profilática de combate a uma doença como essa é a vacinação, que provou ser altamente eficiente no oeste europeu, Uruguai, Argentina e região sul do Brasil. Porém as normas da Organização Internacional de Epizootias (OIE), ditam que um país não pode ser livre de febre aftosa se praticar a vacinação, permitindo o embargo de animais e produtos derivados provenientes destas regiões (www.oie.int). Os principais argumentos contra a vacinação são contraditórios e de pouco embasamento científico (Brown, 2003). O fato de os animais vacinados apresentarem a doença de forma subclínica, também pode ser verdadeiro para animais não-vacinados, o que pode ser exemplificado pelo surto de febre aftosa que ocorreu em 2001 na Inglaterra envolvendo ovinos. Há também poucas evidências de que os animais convalescentes possam transmitir a doença a outros (Brown, 2003; Sutmoller et al., 2003).

Esta política de não vacinação tem sido determinada por regulamentações de mercado, as quais especificam que países livres não aceitarão animais provenientes de países ou regiões que vacinam contra a doença (Brown, 2003; Sutmoller et al., 2003). A aplicação destas políticas trouxe graves conseqüências para a Inglaterra em 2001, cujas

áreas atingidas pela doença tiveram problemas de contaminação do ar devido à incineração de carcaças, o impacto negativo na população em geral e principalmente nos pecuaristas traumatizados pela perda dos animais gerou suicídios, depressão, etc. Desta forma a escolha pela não vacinação gerou o sacrifício e destruição de todos os animais infectados e possíveis contatos, levando ao extermínio de aproximadamente 8 milhões de cabeças do rebanho produtivo daquele país, com perdas na economia em geral, estimadas em 20 bilhões de libras. A maior parte das perdas foi provocada em outros setores da economia que não a agricultura, sendo o turismo o mais atingido (Sutmoller et al., 2003).

Já na Suíça, devido às altas concentrações de animais, houve sobrecarga na capacidade de sacrifício e queima de animais, obrigando as autoridades sanitárias a vacinar parte do rebanho. Desta forma nestas áreas ocorreu uma diminuição da quantidade de vírus circulante no ambiente. Posteriormente os animais das propriedades atingidas foram abatidos nas áreas onde ocorreram os surtos, ficando assim livres da doença. Esta prática diminuiu a disseminação da doença nas áreas vacinadas (Sutmoller et al., 2003).

A vacinação, quando aplicada em áreas endemicas de febre aftosa, permite proteção contra a doença clínica dentro de 7 dias, porém se for utilizado interferon tipo I (IFN-α e IFN-β), que serão expressos nos animais mediante a vacinação destes com vetor derivado de adenovirus humano tipo 5 (Ad5), que é defectivo em replicação, esta "janela imunológica" diminui para 24 horas e estende-se por mais de 5 dias, em suínos (Mason et al., 2003). Porém, o Ad5 carreando os interferons tipo I bovinos não foram tão eficientes a ponto de conferir uma proteção clínica completa, proporcionando, no entanto, proteção parcial com diminuição da doença clínica e conseqüentemente da excreção viral, o que é importante, pois permite uma redução da taxa de disseminação da doença (Wu et al., 2003a).

A taxa de disseminação da doença é a quantidade de animais que serão infectados a partir de cada animal doente. A epidemia encontra-se crescendo quando esta taxa e maior do que um e conseqüentemente extinguindo-se quando esta taxa e inferior a um (Eblé at al., 2004). Desta forma, num surto de aftosa, todos os mecanismos que contribuam para a redução da quantidade de vírus viável no ambiente podem diminuir a disseminação da doença. Assim, a vacinação perifocal conjuntamente com tratamento antiviral são armas importantes no combate à enfermidade (Grubman, 2003; Mason et al., 2003, Wu et al., 2003a).

Tendo em vista a rápida disseminação e a capacidade de causar graves perdas à agropecuária mundial, o vírus da febre aftosa torna-se o mais temido pelos pecuaristas em todo o mundo. A aftosa causa graves perdas na produtividade dos rebanhos e, dependendo da virulência da cepa, também morte de animais, como no surto que devastou o rebanho suíno de Taiwan em 1997.

A enfermidade tem causado perdas de bilhões de dólares nas regiões atingidas, disseminando-se de forma acidental. O que aconteceria em caso de disseminação proposital a rebanhos? Poderia causar graves prejuízos à agropecuária dos países ou regiões atingidas. Desta forma, seria prudente que os países estivessem preparados para este tipo de acontecimento, com estratégias de combate a surtos bem definidas, para que em quaisquer circunstâncias os danos fossem minimizados.

Fiscalização rigorosa da entrada de animais e produtos derivados, manutenção de pesquisas para buscar novas vacinas ou antivirais no combate a essa enfermidade, bem como manutenção de um banco de vacinas ou vírus purificado, concentrado, congelado para a imediata fabricação de vacinas, como o que é mantido na América do Norte e

Inglaterra, são medidas concretas e que podem evitar a infecção de milhões de animais, principalmente em países da América do Sul onde ainda há presença viral.

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6. Anexo I:

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Adenovirus-mediated type I interferon expression delays and reduces disease signs in cattle challenged with foot-and-mouth disease virus

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Foi um trabalho desenvolvido em paralelo com o trabalho principal em PIADC.

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Adenovirus-Mediated Type I Interferon Expression Delays and Reduces Disease Signs in Cattle Challenged with Foot-and-Mouth Disease Virus

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ABSTRACT

Foot-and-mouth disease (FMD) is an economically important disease of livestock. Eliminating FMD outbreaks in previously disease-free countries often relies on restriction of animal movement and massive slaughter of infected and in contact susceptible animals. To develop a more effective and humane FMD control strategy, we explored the possibility of using type I interferon (IFN-α/β) as a novel anti-FMD agent. We have demonstrated previously that swine inoculated with replication-defective human adenovirus type 5 (Ad5) vector expressing porcine IFN-α (Ad5-PoIFN-α) were completely protected from FMD virus (FMDV) challenge. To extend this approach to bovines, we constructed Ad5 vectors that express bovine IFN-α or IFN-β (Ad5-BoIFN-α and Ad5-BoIFN-β). Cells infected with these viruses produced high levels of biologically active BoIFN-α/β, but despite expression *in vitro*, no detectable IFN-induced biologic activity was found in cattle inoculated with Ad5-BoIFN-α. Because PoIFN-α inhibits FMDV replication in bovine cells, we evaluated the potential use of PoIFN-α against FMD in cattle. In cattle inoculated with Ad5-PoIFN-α, the appearance of vesicles was delayed after challenge with FMDV and disease was less severe than in control animals. One Ad5-PoIFN-α-inoculated animal never developed clinical disease. Similarly, although all the Ad5-PoIFN-α-inoculated animals developed viremia, it was delayed for 1 day as compared with the control group. These results suggest that *in vivo* expression of PoIFN-α partially protected cattle from FMD.

INTRODUCTION

FOOT-AND-MOUTH DISEASE (FMD) IS AN ACUTE, systemic vesicular disease affecting swine, cattle, and other livestock.[1] In cattle, vesicular lesions on the tongue and feet cause weight loss, and udder lesions decrease milk production. After FMD versus (FMDV) infection, most animals recover from the acute phase of disease, which lasts about 1 week. However, FMD causes high mortality among young animals when the heart muscle is affected.[2]

FMD is distributed worldwide. Countries where the disease is enzootic suffer severe economic loss as a result of decline in livestock production and international restrictions on exports of animals and animal products. FMD outbreaks in previously disease-free countries can be devastating. For example, the FMD outbreak in the U.K. in 2001 resulted in the slaughter of about 4 million animals (mainly sheep and cattle) and cost the government billions of dollars in compensation to farmers for slaughtered animals and in eradication measures.[3] In addition, the outbreak affected other areas of the economy, including tourism.

Conventional vaccines based on chemically inactivated viruses are used to control and eliminate FMD in many countries. Despite their success, however, FMD-free countries are often reluctant to vaccinate their livestock during an outbreak because (1) failure to completely inactivate the vaccine has led to outbreaks of the disease,[4,5] (2) no approved diagnostic test is available to reliably distinguish vaccinated from infected animals, and (3) vaccinated animals can become disease carriers following contact with FMDV.[6]

To overcome some of the problems associated with conventional FMD vaccines, FMD subunit vaccines have been developed in which viral proteins, peptides, or viral capsid structures are delivered directly[7–10] or expressed via plasmid DNA[11–14] or by recombinant viral fectors.[15,16] Like conventional FMD vaccines, however, none of these approaches are able to induce

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complete protection prior to 7 days postvaccination. Therefore, their use to rapidly control an FMD outbreak is limited.

Recently, we explored the possibility of using type I interferon (IFN-α/β) to rapidly induce protection against FMD in animals.[18] We constructed recombinant replication-defective human adenoviruses (Ad5) expressing porcine IFN-α/β (Ad5-PoINF-α and Ad5-PoIFN-β) and demonstrated that swine inoculated with Ad5-PoIFN-α are completely protected from FMDV infection when challenged 1 day postinoculation (dpi).[18] Additional studies indicate that protection can last for 3–5 days.[19] In this report, we extend our previous studies by adopting the IFN-based disease control strategy to cattle. We constructed Ad5 vectors containing bovine IFN-α or IFN-β genes (Ad5-BoIFN-α or Ad5-BoINF-β) and examined expression of BoIFN-α/β in cell culture as well as in cattle and swine. Cells infected with Ad5-BoIFN-α or Ad5-BoIFN-β produced high levels of biologically active BoIFN-α/β. Swine inoculated with Ad5-BoIFN-α or Ad5-BoIFN-β developed an antiviral response by 1 dpi. However, no detectable antiviral response was found in cattle after inoculation with Ad5-BoIFN-α.

Because PoIFN-α inhibits FMDV replication in bovine cell cultures,[20] we evaluated the efficacy of Ad5-PoIFN-α in cattle. Cattle inoculated with Ad5-PoIFN-α and challenged with FMDV had delayed and less severe clinical disease than control animals, and one inoculated animal never developed vesicles. Although all the Ad5-PoIFN-α-inoculated animals developed viremia, it was delayed for 1 day as compared with the control group. These studies demonstrate that cattle inoculated with Ad5-PoINF-α are partially protected from FMD.

MATERIALS AND METHODS

Cell lines and viruses

Human 293 cells (ATCC CRL-1573)[21] were used to generate recombinant adenovirus. Embryonic bovine kidney (EBK) cells and porcine kidney cells (IBRS2) were obtained from the Foreign Animal Disease Diagnostic Laboratory (Plum Island Animal Disease Center, Greenport, NY). Madin-Darby bovine kidney (MDBK, ATCC CCL-22) cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). All cells were maintained in Eagle's minimal essential medium (EMEM) containing either 10% calf serum or 10% fetal bovine serum (FBS) (EBK cells) supplemented with antibiotics. Baby hamster kidney (BHK-21, ATCC CCL-10) cells were used to propagate and titrate FMDV. The cells were maintained in EMEM containing 10% calf serum and 10% tryptose phosphate broth supplemented with antibiotics.

FMDV subtype A12 was generated from the full-length infectious clone $pRMC_{35}$[22] and used for the biologic assay of IFN. FMDV A24 Cruzeiro (a gift of Dr. Tanuri, Federal University of Rio de Janeiro, Brazil) was isolated from bovine tongue tissue, propagated several times in BHK-21 cells, and stored at −70°C. Virus used for challenge was obtained from the vesicular lesions of an FMDV A24-infected pig. The bovine infectious dose (BID_{50}) was determined by standard methods.[23] Vesicular stomatitis virus serotype New Jersey (VSV-NJ) was provided by Carol House and Jim House of the Foreign Animal Disease Diagnostic Laboratory, Plum Island Animal Disease Center. Recombinant Ad5 vectors expressing PoIFN-α or PoIFN-β (Ad5-PoIFN-α or Ad5-PoInf-β), VSV glycoprotein G (Ad5-VSVG), and *Escherichia coli* β-galactosidase (Ad5-Blue) were constructed as previously described.[18,24]

Construction of recombinant Ad5 expressing BoIFN-α or BoIFN-β

Cellular DNA extracted from EBK cells was used as a template for PCR amplification of BoIFN-α and BoIFN-β genes containing the signal sequence. PCR primers were designed based on GenBank sequences (BoIFN-α accession number M10953; BoIFN-β accession numbers M15477 and M15478). Primers used for amplification of BoIFN-α were 5′-CCGATGGCCCCAGCCTGGTCC-3′ (forward) and 5′-GGAT**GGATCC**TCAGTCCTTTCTCCTGAAWYTCTC-3′ (reverse). Primers used for amplification of BoIFN-β were 5′-CATCATGACCYACCGGTGCCTCCTCC-3′ (forward) and 5′-GGAT**GGATCC**TCAKTCACGGASGKAACCTGTTAG-3′ (reverse). To facilitate cloning, a *Bam*HI site (boldface) was added at the 5′-end of reverse primers following the TGA stop codon. The PCR product encoding BoIFN-α or BoIFN-β was cloned into linearized pCR-Bluntll-TOPO (Invitrogen, Carlsbad, CA) to produce plasmids pTOPO-BoIFN-α and pTOPO-BoIFN-β. The BoIFN-α and BoIFN-β genes were verified by DNA sequencing. An *Eco*RV-*Bam*HI fragment containing the coding sequence of BoIFN-α or BoIFN-β was isolated from pTOPO-BoIFN-α or pTOPO-BoIFN-β and inserted into compatible sites of pBluescript II KS (Stratagene, La Jolla, CA). The resultant plasmids, pKS-BoIFN-α and pKS-BoIFN-β, were digested with *Cla*I and *Xba*I, and the DNA fragments containing the coding sequences of BoIFN-α and BoIFN-β were ligated into *Cla*I-*Xba*I-digested pAd5-Blue[24] to generate full-length infectious clones pAd5-BoIFN-α and pAd5-BoIFN-β. Recombinant viruses Ad5-BoIFN-α and Ad5-BoIFN-β were produced by transfection of 293 cells with *Pac*I-digested pAd5-BoIFN-α or pAd5-BoIFN-β. The viruses were isolated, propagated in 293 cells, and purified by CsCl gradient centrifugation. Viral titer was determined by the method of tissue culture infectious dose 50 ($TCID_{50}$) and converted to plaque-forming units (pfu)/ml.

Immunoprecipitation

Immunoprecipitation was carried out as previously described.[25] Briefly, IBRS2 cells grown in 6-well plates were infected with Ad5-BoIFN-α or Ad5-BoINF-β at an moi of 20. At various times postinfection, the cells were preincubated for approximately 30 min in MEM free of methionine before labeling for 1 h with 50 μCi of ^{35}S-methionine. The cell culture supernatant was collected, centrifuged at 2000 rpm for 10 min to remove cells and cellular debris, and reacted with rabbit polyclonal antibody specific for BoIFN-α or BoIFN-β.[20] The immune complexes were then precipitated with *Staphylococcus aureus* protein A (Calbiochem, San Diego, CA) and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel.

IFN assay

IBRS2 cells were infected with Ad5-BoIFN-α or Ad5-BoIFN-β at an moi of 20. Cell culture supernatants were col-

lected at 24 h postinfection (hpi) and centrifuged at 2000 rpm for 10 min. To remove the majority of Ad5-BoIFN-α or Ad5-BoIFN-β inoculum, supernatant fluids were filtered through a Centricon 100 filter (Millipore, Bedford, MA), brought to pH 2 with HCl, incubated overnight at 4°C, and neutralized with NaOH. IBRS2 or MDBK cells were incubated overnight with dilutions of the treated supernatant fluids and infected with FMDV subtype A12 or VSV-NJ, respectively. Cells were overlaid with gum tragacanth at 1 hpi and stained for plaques at 24 hpi (A12) or 36–40 hpi (VSV-NJ). Antiviral activity of IFNs was reported as reciprocals of the highest sample dilution that caused 50% reduction in the number of plaques relative to the untreated cells. Plasma from swine or cattle inoculated with recombinant Ad5 viruses were directly assayed for antiviral activity without filtration and HCl treatment.

Quantitation of PoIFN-α by ELISA

No commercial ELISA is available to quantitate PoIFN-α/β or BoIFN-α/β. However, monoclonal antibodies (mAb) against PoIFN-α are commercially available, and we used these reagents to develop an ELISA to measure the amount of PoIFN-α produced in cattle or swine inoculated with Ad5-PoIFN-α. ELISA plates were coated overnight with an mAb against PoIFN-α (clone K9) (R&D Systems, Minneapolis, MN) and blocked with 5% normal goat serum. Plasma collected from cattle or swine inoculated with Ad5-PoIFN-α was diluted and added to the plate for 1 h at 37°C. The plates were washed and incubated with a biotinylated anti-PoIFN-α mAb (clone F17) (R&D Systems) for 1 h at 37°C. Peroxidase-conjugated streptavidin was then added to the plates. After the plates were incubated for 30 min at 37°C, the amount of bound peroxidase was determined by incubation with the TMB substrate (KPL) for 15 min. The reaction was stopped by adding 1 M H_2SO_4, and the plates were read at 450 nm on a Bio-Kinetics microplate reader. A standard curve was produced using recombinant PoIFN-α (R&D Systems).

Inoculation of cattle and swine with Ad5-BoIFN-α

The cattle and swine experiments were performed in disease secure isolation facilities at the Plum Island Animal Disease Center. Two animal experiments were carried out to determine the antiviral activity present in the plasma of cattle and swine inoculated with Ad5-BoIFN-α.

Three Holstein cattle (6–8 months old, 450–500 lbs each) were inoculated intramuscularly (i.m.) in the neck with 1×10^9, 2.5×10^9, 5×10^9 pfu/animal of Ad5-BoIFN-α, respectively. One bovine was inoculated with 5×10^9 pfu Ad5-VSVG as a control. Plasma was taken before inoculation and daily until 7 dpi and assayed for antiviral activity in MDBK cells. Similarly, three Yorkshire gilts (3 months old and about 35–40 lbs each) were inoculated i.m. in the neck with different doses of recombinant Ad5. Two animals were given either 1×10^9 or 5×10^9 pfu Ad5-BoIFN-α, and one animal was given 5×10^9 pfu Ad5-Blue as a control. Plasma was taken before inoculation and daily until 7 dpi and assayed for antiviral activity in MDBK cells.

Inoculation of cattle and swine with Ad5-PoIFN-α or Ad5-PoIFN-β

Five Holstein cattle (6–8 months old, 450–500 lbs each) were inoculated i.m. in the neck with different doses of recombinant Ad5. Two animals were given 1×10^9 or 5×10^9 pfu of Ad5-PoIFN-α, respectively. Another two were given 1×10^9 or 5×10^9 pfu of Ad5-PoIFN-β, respectively. One animal was given 5×10^9 pfu Ad5-VSVG as a control. Plasma was taken before inoculation and daily until 7 dpi and assayed for antiviral activity in IBRS2 cells. The level of PoIFN-α expression was also determined by ELISA. The identical experiments were carried out in five Yorkshire gilts (3 months old and about 35–40 lbs each).

Inoculation of cattle with Ad5-PoIFN-α and challenge with FMDV A24

Groups of Holstein cattle (about 200–250 lbs each) were housed in three separate rooms, each containing three animals. Group I was inoculated i.m. in the neck with 1×10^{10} pfu/animal of Ad5-Blue. Groups II and III were inoculated i.m. with 1×10^{10} pfu/animal of Ad5-PoIFN-α. One dpi, group I and group II were challenged with 2×10^4 BID_{50} FMDV A24 by intradermal inoculation in the tongue. Group III was challenged with FMDV A24 at the same dose 2 days after Ad5-PoIFN-α inoculation. Plasma was taken before inoculation and daily until 7 dpi and assayed for antiviral activity in MDBK cells. The level of PoIFN-α expression was also quantitated by ELISA. Nasal secretions and blood samples were collected every day for 7 days postchallenge (dpc). Serum samples were collected at 0, 4, 7, and 14 dpc. FMDV isolated from nasal secretions was quantified by plaque titration in 24-well plates. Animals were monitored every day for rectal temperature and tranquilized every second day for clinical examination. Clinical scores were determined by the following criteria: tongue lesion beyond inoculation site = 1, mouth lesion other than tongue = 1, lesion on nostril = 1, one lesion per foot = 1, two or more lesions per foot = 2. The maximum score is 11.

RESULTS

Characterization of Ad5-BoIFN-α and Ad5-BoIFN-β

Ad5 expressing PoIFN-α completely protects swine against FMDV infection.[18] In this study, we explored the possibility of using a similar strategy to control FMDV infection in cattle. The full-length genes encoding BoIFN-α and BoIFN-β were PCR amplified from EBK cells and inserted into the E1 deleted region of Ad5. Two recombinant viruses, Ad5-BoIFN-α and Ad5-BoIFN-β, were made in which the expression of BoIFN-α or BoIFN-β was driven by the cytomegalovirus (CMV) immediate-early promoter.

IBRS2 cells were used to examine the expression of BoIFN-α or BoIFN-β from Ad5-BoIFN-α or Ad5-BoIFN-β. These cells do not produce IFN mRNAs after viral infection.[20] Therefore, IFN detected after infection should be the result of expression from Ad5-BoIFN-α or BoIFN-β. IBRS2 cells were infected with Ad5-BoIFN-α or Ad5-BoIFN-β. Supernatant fluids were collected at various times postinfection and examined by SDS-PAGE and Coomassie blue staining. As shown in Figure 1A, BoIFN-α and BoIFN-β were detected by 23 hpi. The multiple BoIFN-β bands synthesized after infection were present as a single band in the tunicamycin (5 μg/ml)-treated sample (Fig. 1B), indicating they resulted from various levels of N-linked

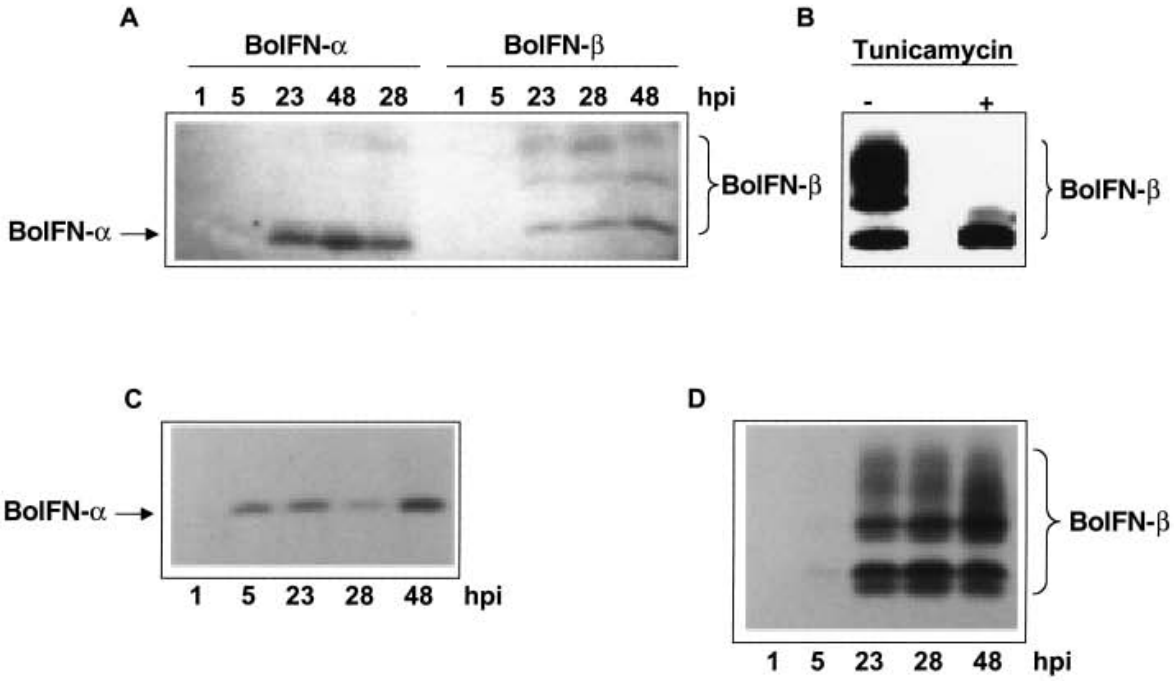

FIG. 1. Expression of BoIFN-α and BoIFN-β in Ad5-BoIFN-α or Ad5-BoIFN-β-infected IBRS2 cells. (**A**) IBRS2 cells were infected with Ad5-BoIFN-α or Ad5-BoIFN-β at an moi of 20. Supernatants were collected at 1, 5, 23, 28, and 48 hpi. Proteins were resolved by SDS-PAGE on a 15% gel and stained with Coomassie blue. (**B**) IBRS2 cells were infected with Ad5-BoIFN-β for 24 h and treated with cell culture medium or tunicamycin (5 μg/ml) for 3 h prior to radiolabeling with ^{35}S-methionine in the absence or presence of tunicamycin for 1 h. Supernatant fluids were collected, and proteins were separated by SDS-PAGE on a 15% gel and visualized by autoradiography. (**C** and **D**) ^{35}S-Methionine-labeled supernatants from Ad5-BoIFN-α and Ad5-BoIFN-β-infected IBRS2 cells, respectively, were collected at 1, 5, 23, 28, and 48 hpi and immunoprecipitated with a rabbit polyclonal antibody against BoIFN-α (**C**) or BoIFN-β (**D**). Proteins were resolved by SDS-PAGE on a 15% gel and visualized by autoradiography.

glycosylation. These results are in agreement with previous published data in which BoIFN-β has two predicted N-linked glycosylation sites, whereas BoIFN-α has none.[26,27] The synthesis of BoIFN-α and BoIFN-β was also confirmed by immunoprecipitation of radiolabeled supernatant fluids with rabbit polyclonal antibody against each protein. BoIFN-α and BoIFN-β were detected as early as 5 and 23 hpi, respectively, and synthesis continued for at least 48 h (Fig. 1C, D). We previously obtained very similar results on infection of IBRS2 cells with Ad5-PoIFN-α/β.[18]

To examine the biologic activity of BoIFN-α and BoIFN-β, supernatant fluids from Ad5-BoIFN-α and Ad5-BoIFN-β-infected IBRS2 cells were harvested at various times postinfection. Supernatants were examined for antiviral activity by a plaque reduction assay. Antiviral activity was detectable by 5 hpi and reached levels of about 204,000 U/ml 23–48 hpi (Table 1), sim-

TABLE 1. ANTIVIRAL ACTIVITY OF SUPERNATANTS[a] FROM AD5-BOIFN-α AND AD5-BOIFN-β-INFECTED IBRS2 CELLS ON BOVINE AND SWINE CELLS

	Ad5-BoIFN-α		*Ad5-BoIFN-β*	
Time postinfection (h)	*MDBK*[b]	*IBRS2*[c]	*MDBK*	*IBRS2*
1	<50	<50	50	<50
5	3,200	6,400	12,800	<50
23	51,200	>51,200	204,800	<50
28	102,400	>102,000	204,800	<50
48	204,800	204,000	51,200	100

[a]Supernatants were centrifuged through a Centricon 100 membrane, pH 2 treated, neutralized, and assayed for antiviral activity.
[b]Highest dilution that reduced VSV-NJ plaque number by 50%.
[c]Highest dilution that reduced FMDV A12 plaque number by 50%.

ilar to the levels of antiviral activity we have reported previously in supernatants from Ad5-PoIFN-α/β-infected cells.[18] Consistent with our previous observation,[20] BoIFN-α had equivalent biologic activity in both bovine and swine cells, whereas BoIFN-β was active only in bovine cells (Table 1).

Ad5-mediated expression of BoIFN-α in cattle and swine

We have shown previously that PoIFN-α was produced at a high level in swine inoculated with Ad5-PoIFN-α.[18] Having demonstrated that biologically active BoIFN-α was produced from Ad5-BoIFN-α-infected cells, we examined the level of *in vivo* expression of BoIFN-α in cattle. Three cattle were inoculated i.m. with 1×10^9, 2.5×10^9, or 5×10^9 pfu/animal of Ad5-BoIFN-α. As a control, an additional animal was inoculated with 5×10^9 pfu Ad5-VSVG. Animals were monitored for adverse clinical effects of IFN administration, and temperature was taken daily. Plasma samples were collected for 7 dpi and assayed for antiviral activity. None of the animals displayed abnormal behavior or had elevated temperatures. No antiviral response was detectable in any of the inoculated animals (data not shown). However, all animals developed a significant Ad5-specific neutralizing antibody response at 13 dpi, indicating that they were exposed to the recombinant virus.

To determine if Ad5-BoIFN-α can express biologically active BoIFN-α *in vivo*, we inoculated swine with different doses of Ad5-BoIFN-α. The animals were monitored, and plasma samples were assayed for an antiviral response. By 1 dpi, the swine inoculated with 1×10^9 pfu Ad5-BoIFN-α developed an antiviral response of 50 U/ml. The animal inoculated with 5×10^9 pfu had higher activity (100 U/ml) by 1 dpi, but in each case, the antiviral activity was detectable for only 1 day. These data demonstrate that Ad5-BoIFN-α can express biologically active BoIFN-α in animals.

Ad5-mediated expression of PoIFN-α and PoIFN-β in cattle and swine

Recently, we demonstrated that PoIFN-α and PoIFN-β inhibit FMDV replication in bovine cells,[20] and Ad5-PoIFN-α completely protected swine from subsequent challenge with FMDV.[18] We, therefore, reasoned that Ad5-mediated expression of PoIFN-α and PoIFN-β might protect cattle from FMDV challenge.

In a dose-response experiment, cattle (450–500 lbs) and swine (35–40 lbs) were inoculated i.m. with 1×10^9 or 5×10^9 pfu Ad5-PoIFN-α or Ad5-PoIFN-β. The animals were monitored for clinical signs. Plasma samples were collected and assayed for antiviral activity, and the amount of PoIFN-α in Ad5-PoIFN-α-inoculated animals was determined by ELISA. All the inoculated cattle behaved normally and did not develop fever. Only the bovine inoculated with the high dose (5×10^9 pfu) of Ad5-PoIFN-α developed an antiviral response (50 U/ml) and had a very low level of PoIFN-α (63 pg/ml), each detectable for only 1 day (Table 2). In contrast, swine inoculated with either 1×10^9 or 5×10^9 pfu of Ad5-PoIFN-α developed an antiviral response (400 and 800 U/ml, respectively) by 1 dpi, which lasted for an additional 3–4 days (Table 2). The amount of plasma PoIFN-α in low-dose and high-dose inoculated swine reached 10,747 and 77,630 pg/ml, respectively, by 1 dpi (Table 2). Similarly, the swine given the high dose (5×10^9 pfu) of Ad5-PoIFN-β developed a detectable, but lower, antiviral response.

Protection against FMDV A24 challenge in cattle inoculated with Ad5-PoIFN-α

Because Ad5-PoIFN-α induced higher levels of antiviral activity in swine and bovines than any of our other Ad5 vectors (Table 2), we examined the efficacy of administration of this vector against FMDV infection. Nine cattle (200–250 lbs each), smaller in size than in the previous experiments, were divided into three groups. Group I was inoculated i.m. with 1×10^{10} pfu per animal of a control virus, Ad5-Blue.[24] Groups II and III were inoculated i.m. with 1×10^{10} pfu per animal of Ad5-PoIFN-α. All animals were challenged intradermally in the tongue with 2×10^4 BID_{50} FMDV A24. Animals in groups I and II were challenged 1 dpi, and the animals in group III were challenged 2 dpi. Animals were monitored every other day for clinical signs of disease.

The cattle given Ad5-PoIFN-α developed an antiviral response of approximately 100–200 U/ml by 1–2 dpi that decreased over time but was still detectable for an additional 1–2 days. The amount of PoIFN-α in plasma reached between 746 and 4889 pg/ml by 1–2 dpi and was still detectable in some animals at 3 dpi. As expected, the Ad5-Blue-inoculated animals (group I) had no detectable antiviral activity or measurable PoIFN-α. Animals in groups II and III developed a fever (temperature $> 40°C$ for 1–2 dpi), but otherwise these animals appeared normal.

As summarized in Table 3, at 2 dpc all the Ad5-Blue-inoculated animals had vesicles on the tongue at sites distinct from the challenge sites and on the feet. Subsequently, some of the animals had vesicles on the gums, snout, and all four feet. The Ad5-Blue-inoculated animals developed low-level viremia 1 dpc, which increased and continued for an additional 2–3 days. Virus was detectable in nasal swabs in all animals in this group 2–5 dpc. Although vesicles appeared in all animals in group II by 2–6 dpc, disease was not as severe as in the control group. One animal (No. 16) in group III never developed vesicles, and in the other two animals, the appearance of vesicles was delayed until 4 dpc, and disease was not as severe as in the control group. Viremia was delayed until 2 dpc in the animals in groups II and III and continued for an additional 1–2 days. Compared with the control group, viral shedding from the animals in group II was not detectable until 3 dpc.

DISCUSSION

Mammalian cells, primarily leukocytes and fibroblasts, secrete IFN-α/β after viral infection. These cytokines bind to specific receptors on cells priming them to a virus-resistant state by activation of IFN-α/β-stimulated genes.[28] Among the best characterized gene products are double-stranded RNA (dsRNA)-dependent protein kinase (PKR), which globally inhibits protein expression; 2′-5′ A-synthetase, which induces expression of RNase L, resulting in degradation of RNAs; and MxA protein, which blocks transport of viral ribonucleoproteins to the nucleus.

TABLE 2. DOSE-RESPONSE OF AD5-POIFN-α AND AD5-POIFN-β IN CATTLE AND SWINE

Inoculum[a]	*Dose (pfu)*	*Species*	*Antiviral activity of plasma samples*[b]								*Amount of PoIFN-α (pg/ml) in plasma samples*[c]						
			0 dpi	*1 dpi*	*2 dpi*	*3 dpi*	*4 dpi*	*5 dpi*	*6 dpi*	*7 dpi*	*0 dpi*	*1 dpi*	*2 dpi*	*3 dpi*	*4 dpi*	*5 dpi*	*6 dpi*
Ad5-VSVG	5×10^9	Swine	<25	<25	<25	<25	<25	<25	ND[d]	ND	0	0	0	0	0	0	0
		Bovine	<25	<25	<25	<25	<25	<25	ND	ND	0	0	0	0	0	0	0
Ad5-PoIFN-α	1×10^9	Swine	<25	400	200	100	25	<25	ND	ND	0	10,747	6,905	1,713	0	0	0
		Bovine	<25	<25	<25	<25	<25	<25	ND	ND	0	0	0	0	0	0	0
	5×10^9	Swine	<25	800	800	400	100	25	25	<25	0	77,630	51,300	21,588	4,705	472	0
		Bovine	<25	50	<25	<25	<25	<25	ND	ND	0	63	0	0	0	0	0
Ad5-PoIFN-β	1×10^9	Swine	<25	<25	<25	<25	<25	<25	ND	ND	ND	ND	ND	ND	ND	ND	ND
		Bovine	<25	<25	<25	<25	<25	<25	ND	ND	ND	ND	ND	ND	ND	ND	ND
	5×10^9	Swine	<25	200	100	<25	25	<25	ND	ND	ND	ND	ND	ND	ND	ND	ND
		Bovine	<25	<25	<25	<25	<25	<25	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a]Animals were inoculated i.m. in the neck with 1 ml of the indicated dose of Ad5-vector.
[b]Highest dilution that reduced FMDV A12 plaque number by 50% in IBRS2 cells.
[c]Determined by ELISA (see Materials and Methods).
[d]ND, not done.

TABLE 3. CLINICAL DATA FROM CATTLE INOCULATED WITH Ad5-PoIFN-α AND CHALLENGED WITH FMDV A24

Group[a]	Animal no.	Clinical scores[b]					Viral titer (pfu/ml) in blood							Viral titer (pfu/ml) in nasal fluids							
		0 dpc	2 dpc	4 dpc	6 dpc	9 dpc	0 dpc	1 dpc	2 dpc	3 dpc	4 dpc	5 dpc	6 dpc	0 dpc	1 dpc	2 dpc	3 dpc	4 dpc	5 dpc	6 dpc	7 dpc
I	8	0	10	10	9	6	0	1100	10000	5250	0	0	0	0	0	925	17.5	110	239	0	0
	9	0	5	5	3	5	0	42.5	223	400	0	0	0	0	0	175	62.5	175	0	0	0
	10	0	7	7	4	3	0	120	1830	20	2.5	0	0	0	0	0	325	750	10	0	0
II	11	0	0	0	1	2	0	0	215	38.5	245	0	0	0	0	0	5	153	12.5	0	0
	12	0	1	3	4	1	0	0	1950	283	0	0	0	0	0	0	42.5	850	700	0	0
	13	0	0	2	3	2	0	0	10	1880	308	0	0	0	0	0	32.5	525	178	0	0
III	14	0	0	1	4	3	0	0	305	275	12.5	0	0	0	0	0	128	20	42.5	0	0
	15	0	0	3	2	6	0	0	1150	293	10	0	0	0	0	975	875	213	550	42.5	0
	16	0	0	0	0	0	0	0	253	318	480	0	0	0	0	10	130	500	185	25	0

[a]Group I was inoculated i.m. with 1×10^{10} pfu/animal of Ad5-Blue and challenged with 2×10^4 BID_{50} FMDV A24 by intradermal inoculation in the tongue at 1 dpi. Groups II and III were inoculated i.m. with 1×10^{10} pfu/animal of Ad5-PoIFN-a and challenged with 2×10^4 BID_{50} FMDV A24 at 1 (group II) or 2 dpi (group III).

[b]See Materials and Methods.

To successfully establish infection in the host, FMDV has developed a specific mechanism to evade the IFN defense system. FMDV encodes a papainlike proteinase, the L protein, that cleaves eIF-4G, a subunit of the cap-binding protein complex involved in initiation of translation of capped mRNAs.[29] Cleavage of eIF-4G results in shutoff of host cell protein synthesis, but production of FMDV proteins, which occurs by a cap-independent mechanism, is not affected. As a consequence of the shutoff of cellular protein synthesis, the virus also prevents the host from synthesizing IFN-α/β. This causes rapid onset of viral replication and spread of virus within infected animals in a short priod of time. In FMDV-infected cells, cytopathic effects can be seen as early as 3 hpi.[30] Similarly, in infected animals, it takes only 1–3 days for the appearance of oral and pedal vesicular lesions.[31,32]

IFN-α/β pretreatment of cells inhibits FMDV replication,[20,30,33,34] and the antiviral activity is mediated by PKR.[20] The rapid induction of an antiviral state by IFN-α/β could quickly contain an FMD outbreak and overcome the inherent limitations of FMD vaccines, which require approximately 7 days to elicit a protective immune response and for the vaccine to be matched to the outbreak strain.[35] For these reasons, we have proposed using prophylactic IFN treatment in combination with vaccination as a novel strategy to control FMDV infection in animals.[18,19]

Because of its short half-life in the blood[36] and adverse systemic effects,[37] direct administration of IFN-α/β to control FMD outbreaks may have limited utility. However, delivery of IFN via an Ad5 vector has a number of advantages. Recombinant Ad5 is easily produced at high titer in cell culture and can infect cells from several animal species susceptible to FMD.[38] Ad5-mediated delivery allows *in vivo* expression of IFN for a period of time, which can overcome rapid clearance of IFN from the host. In addition, systemic toxicity associated with direct administration of high doses of IFN may be minimized by controlling the amount of the recombinant virus inoculated.

We have demonstrated previously that swine inoculated with recombinant Ad5 expressing PoIFN-α were sterily protected when challenged 24 h later with FMDV.[18] Additional studies indicate that protection can last for 3–5 days.[19] In this report, we describe the generation and characterization of Ad5 vectors expressing BoIFN-α or BoIFN-β (Ad5-BoIFN-α or Ad5-BoIFN-β). Cells infected with these viruses produced high levels of biologically active IFN (Table 1). Despite expression of BoIFN-α/β *in vitro*, however, no antiviral activity was detected in cattle after inoculation of a high dose (5×10^9 pfu/animal) of Ad5-BoIFN-α, whereas swine inoculated with the same dose had detectable activity (100 U/ml). Thus, Ad5-BoIFN-α is able to express biologically active BoIFN-α *in vivo*. One likely explanation for the absence of detectable antiviral activity in cattle is the body size difference between the swine (35–40 lbs) and cattle (450–450 lbs) used in the experiments, resulting in dilution of the expressed IFN in the larger animal. It is also apparent that Ad5-BoIFN-α does not express as high levels of biologically active IFN in animals as does Ad5-PoIFN-α even though both vectors express essentially equivalent levels of IFN-induced biologic activity in cell culture (Table 1).[18] At present, we have no explanation for this inconsistency.

Of the Ad5-IFN vectors tested in animals, only Ad5-PoIFN-α induced a low but detectable antiviral response in cattle and the highest levels of antiviral activity in swine (Table 2). This suggests that PoIFN-α was expressed most efficiently in animals or more effective than the other IFNs in inducing an antiviral response *in vivo*. (As only an ELISA for PoIFN-α is available, the level of expression of the other IFNs *in vivo* is unknown.)

Compared with our previous swine experiment,[18] administration of Ad5-PoIFN-α failed to completely protect cattle from FMDV infection. However, disease was delayed and less severe in Ad5-PoIFN-α-inoculated animals compared with the control group, and one animal never developed clinical signs of disease. The lower level of protection in cattle compared with swine is directly correlated with the reduced levels of PoIFN-α detected. Thus, even at the same dose of Ad5-PoIFN-α administered, at least a 1000-fold lower level of PoIFN-α was detected by ELISA in cattle compared with swine (Table 2).

From experiments in swine, it appears that there is a threshold level of PoIFN-α, that is, 10,000–15,000 pg/ml in the blood, that is necessary to completely protect animals from direct inoculation challenge with FMDV.[19] This amount of PoIFN-α is necessary either on the day of challenge or 1–2 days prior to challenge. If this threshold level is not reached or if more than 2 days have passed since this level was attained, animals challenged at this time are either partially protected or not protected at all. In the current cattle experiment, the highest levels of PoIFN-α were only 4000–5000 pg/ml, well below the threshold level. These animals were not completely protected, but disease was delayed and less severe than in the control animals, mimicking the results we have obtained in swine.[19]

To induce complete protection in cattle with IFN, it will be necessary to enhance the level of IFN expressed and increase the duration of gene expression. One method to attain this goal is to develop Ad5 vectors with the foreign gene under the control of more efficient promoters or polyA signals or both. We have constructed new vectors that express 5–18-fold higher levels of a reporter gene than the current vector, which contains the CMV immediate-early promoter and the simian virus 40 polyA signal. We are currently using this new vector to produce recombinant viruses expressing IFN-α/β. It may also be possible to construct Ad5 vectors that have a modified tropism, allowing for more efficient infection of bovines, or use alternative routes of delivery.

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