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Use of recombinant modified vaccinia Ankara viral vectors for equine influenza vaccination

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Abstract

Recombinant modified vaccinia Ankara (MVA) vectors expressing equine influenza virus genes were constructed and evaluated for use in equine vaccination. Two strains of recombinant MVA, expressing either hemagglutinin (HA) or nucleoprotein (NP) genes were constructed. Each influenza virus gene was cloned from A/equine/Kentucky/1/81 (Eq/Ky) into an MVA construction plasmid, and was introduced to the deletion III locus of the wild type MVA genome by homologous recombination. Recombinant viruses were plaque purified, and antigen expression was confirmed by immunostaining.

Two ponies were primed by vaccination with 50 μg HA-DNA and two ponies were vaccinated with 50 μg NP-DNA using the PowderJect XR research device. Six and 10 weeks later, ponies were immunized with 2×10^9 infectious units of recombinant MVA encoding the homologous influenza antigen, equally divided between intramuscular and intradermal sites in the neck.

A marked rise in influenza virus-specific IgGa and IgGb serum antibody titers was detected following administration of MVA boosters with both HA and NP antigens. Influenza virus-specific lymphoproliferative responses and IFN-γ mRNA production were also strongly elicited by both antigens. This study demonstrates the facility with which recombinant MVA viruses expressing defined pathogen genes can be constructed, and provides preliminary evidence of the immunogenicity and safety of these vectors in the horse.

Keywords: Horses; Influenza virus; Vaccination; Modified vaccinia Ankara

1. Introduction

Modified vaccinia Ankara (MVA) is a highly attenuated strain of vaccinia virus which is frequently used in prime-boost vaccination protocols. Due to a series of genomic deletions incurred following extensive in vitro passage of parental vaccinia virus in chicken embryo fibroblasts, MVA is replication-deficient in virtually all mammalian cells (Moss, 1996). Its inability to replicate is caused by an inhibition of the processing of nascent viral structural proteins and subsequent viral reassembly within infected cells (Sancho et al., 2002; Sutter and Moss, 1992). Importantly, the expression of viral genes as well as recombinant genes introduced to the MVA genome is unaffected in non-permissive cells (Sutter and Moss, 1992). Hence, this virus is extremely safe, and is an effective tool for the delivery of foreign

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Abbreviations: MVA, modified vaccinia Ankara; HA, hemagglutinin; NP, nucleoprotein; Eq/Ky, A/equine/Kentucky/1/81 influenza virus; AMV, avian myeloblastosis virus; BHK-21, baby hamster kidney-21 cells; MMLV, Moloney's murine leukemia virus

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genes, leading to the endogenous expression of antigens of interest.

Prime-boost vaccination is a relatively novel immunization strategy which typically involves the consecutive delivery of a DNA vaccine and a recombinant virus vector, such as MVA, expressing the homologous antigen. This strategy has been shown to effectively immunize experimental animals against a wide variety of pathogens, frequently eliciting high levels of specific immunity (Allen et al., 2000; Amara et al., 2001; McShane et al., 2001; Schneider et al., 1999; Stittelaar et al., 2000). However, few experiments to determine its efficacy for the vaccination of large animals have been performed (Nam et al., 2002). The recombinant MVA viruses constructed in the current research provide tools with which the efficacy of ''prime-boost'' vaccination of horses for the prevention of influenza virus infection can be tested in the future.

Equine influenza virus is the leading cause of viral upper respiratory tract disease in horses (Mumford et al., 2003; Mumford et al., 1998). Infection of horses with this virus typically results in the establishment of immunity which can protect those individuals from reinfection for a year or more (Hannant et al., 1988). However, immunization with commercially available inactivated vaccines induces short-lived, sub-optimal immune responses which are qualitatively different from post-infection immunity (Nelson et al., 1998) and are frequently not protective (Morley et al., 1999; Mumford et al., 2003; Newton et al., 2000). The development of improved vaccines is critical for the control of this important and prevalent pathogen.

The equine influenza hemagglutinin (HA) gene is an excellent candidate antigen for use in equine vaccination strategies (Mumford, 1992). It has previously been shown that DNA vaccination with the Eq/Ky HA gene alone can induce protective immune responses in horses (Lunn et al., 1999). Furthermore, HA-specific immune responses are generally recognized as important for protection against influenza virus infection (Keitel et al., 1994; Tamura et al., 1990). These features made the HA gene an ideal immunogen for inclusion in this study. A second recombinant MVA strain, expressing equine influenza nucleoprotein (NP) was also constructed in the current research. Influenza NP is believed to play a role in cross-protective immune responses in other species by serving as a target for influenza virus-specific cytotoxic T lymphocytes (Christensen et al., 2000; Yewdell et al., 1985). However, little is known about the immunogenicity of this antigen in the horse. Therefore, the MVA-NP construct promises to be an excellent tool with which to induce, and subsequently evaluate the efficacy of high levels of NP-specific immunity in horses.

Here we describe the construction of two novel strains of recombinant MVA for influenza virus vaccination of horses. Preliminary data demonstrating the immunogenicity of MVA booster vaccination of horses against equine influenza is provided.

2. Materials and methods

2.1. Animals

Four 2-year-old male ponies were used in this study. These ponies were obtained from an unvaccinated feral herd which had no serological evidence of exposure to influenza virus. The maintenance and experimental protocols followed the animal care guidelines of the Research and Animal Resources Committee, University of Wisconsin.

2.2. Preparation of DNA vaccines

An existing plasmid construct (pWRG-HA; Olsen et al., 1997) encoding the hemagglutinin gene of influenza A/equine/Ky/1/81 (Eq/Ky) was used for HA-DNA vaccination of ponies. The Eq/Ky nucleoprotein (NP) gene was cloned into the WRG eukaryotic expression plasmid for NP-DNA vaccination of ponies. For this purpose, Eq/Ky genomic RNA was isolated (QiaAmp®; Qiagen, Valencia, CA) and the NP gene was reverse transcribed using avian myeloblastosis virus (AMV) reverse transcriptase (Fisher Scientific, Pittsburgh, PA) and a degenerate NP-specific primer (SZANP^{+}; Zou, 1997). The cDNA product was PCR amplified using the SZANP primer pair, and inserted into an intermediate vector (pCR-Script-Amp; Stratagene, La Jolla, CA). The NP insert was then sequenced. To our knowledge, this is the first determination of the Eq/Ky NP sequence, and it has been submitted to GenBank (accession no. AY291288). Finally, the NP gene was subcloned into the pWRG vector.

Both the pWRG-HA and pWRG-NP plasmids were propagated in *E. coli* DH5α cells (Invitrogen, Carlsbad,

CA), and purified by anion-exchange resin chromatography (Qiagen). Each plasmid was coated onto 2.5 μm gold beads at a concentration of 2.5 μg DNA/mg gold beads. Tefzel™ tubing was lined with the DNA-loaded gold beads at a rate of 1 mg gold/inch tubing. One half inch lengths of coated tubing, each containing 1.25 μg DNA, were used for each discharge of the PowderJect XR research device.

2.3. Generation of recombinant MVA strains

The Eq/Ky HA gene was subcloned from pWRG-HA into pCR-Script-Amp. Both the influenza HA and NP genes were then subcloned from pCR-Script-Amp into pLW-44 (generously provided by Dr. B. Moss (manuscript in preparation); (pLW-44HA, pLW-44NP)). The pLW-44 vector encodes a green fluorescent protein (GFP) reporter gene which lies upstream of the insertion site for the gene of interest. This cassette is flanked by a pair of MVA genomic sequences which allow homologous recombination and incorporation of both GFP and the gene of interest into the deletion III locus of the wild type MVA (wtMVA) genome.

BHK-21 cells (ATCC CCL 10) were propagated to near confluency in a six-well plate in minimal essential medium (MEM; Gibco, Grand Island, NY) supplemented with 10% FBS (Harlan Inc., Leicestershire, UK), and infected with wtMVA at an MOI of 0.05. One hour later, the monolayers were co-transfected with 4 μg pLW-44HA or pLW-44NP in the presence of Lipofectamine 2000 (Invitrogen) at a ratio of 1:5 (μg DNA:μl Lipofectamine), per manufacturer's instructions. After 48 h the monolayers were collected by scraping, and centrifuged at 500 × *g*. The infected cell pellets were rapidly freeze-thawed three times, sonicated on ice in a cup sonicator (3 × 30 s; Model 550 Sonic Dismembrator, Fisher Scientific), and used to infect near confluent BHK-21 cell monolayers in a six-well plate. A methylcellulose overlay was added after 1 h (1% methylcellulose (Sigma, St. Louis, MO) in MEM supplemented with 2% FBS, 2 mM Glutamax® (Gibco), 50 μg/ml gentamicin (Gibco), 0.25 μg/ml amphotericin B (Gibco)). Two days later, three fluorescent plaques for each *r*MVA strain were collected using sterile Pasteur pipettes into 0.5 ml aliquots of MEM-2. Each sample was freeze-thawed and sonicated as above, and replated on fresh BHK-21 monolayers. This plaque purification procedure was repeated at least six more times.

2.4. Preparation of rMVA stocks for vaccination

BHK-21 cell monolayers were propagated to near confluency in 50× T-150 tissue culture flasks and inoculated with *r*MVA-HA or *r*MVA-NP at an MOI of approximately 0.5. After a 72 h incubation, remaining adherent cells were dislodged using a cell scraper and the entire flask contents were pooled and centrifuged at 1000 × *g* for 10 min at 4 °C. The cell pellets were resuspended in 10 mM Tris–HCl (pH 9) and dounced (Duall-24®, Kontes Glass Co., Vineland, NJ) on ice to disrupt intact cells. This mixture was centrifuged at 500 × *g* for 5 min at 4 °C and the virus-laden supernatant was collected. The pellet was dounced again, and the procedure was repeated. The pooled virus-laden supernatant was overlaid on a 36% sucrose cushion and centrifuged in an SW-28 rotor at 13,500 rpm for 80 min at 4 °C. The viral pellets were resuspended and pooled in 1 mM Tris–HCl (pH 9), sonicated as above, and overlaid on a second 36% sucrose cushion. These tubes were centrifuged in an SW-41 swing bucket rotor at 15,000 rpm for 60 min at 4 °C. The virus pellets were pooled in 2 ml of 1 mM Tris–HCl (pH 9), aliquotted and stored at −80 °C. The vaccine virus strains were titered on BHK-21 cells in six-well plates and fluorescent plaques were enumerated after 48 h.

2.5. Immunizations

Each pony was DNA vaccinated on one occasion, and received MVA booster vaccinations 40 and 73 days later. Two ponies were immunized with Eq/Ky HA-DNA followed by MVA-HA, while the other two ponies received Eq/Ky NP-DNA followed by MVA-NP.

For DNA vaccination, ponies were sedated with detomidine HCl (30 μg/kg i.v.; Dormosedan®, Pfizer Animal Health, Exton, PA) and anaesthetized with ketamine (2.2 mg/kg, i.v.; Ketaset®, Fort Dodge Animal Health, Fort Dodge, IA). Fifty micrograms Eq/Ky HA- or NP-encoding plasmid DNA was delivered in 40 applications to skin and mucosal sites in each pony using the PowderJect XR research device. The applications were administered with 600 psi helium to 14 non-overlapping sites on inguinal skin and 6 on the

perineal skin, as well as 16 non-overlapping sites on the ventrum of the tongue and a total of 4 sites on the conjunctiva and third eyelid. Plasmid DNA to the latter 4 sites was delivered with 450 psi helium.

Each *r*MVA strain was sonicated and resuspended at 2×10^9 pfu/ml in sterile PBS for vaccination of ponies. Each pony received a single intramuscular (i.m.) dose of 0.5 ml *r*MVA (1×10^9 pfu) in the neck. A further 1×10^9 pfu *r*MVA was divided equally between five adjacent intradermal (i.d.) sites in the neck.

2.6. Experimental samples

Blood was collected by jugular venipuncture. Serum was prepared from samples collected from each pony prior to primary vaccination, and biweekly thereafter until 4 weeks after the second MVA booster vaccination. Heparinized blood for preparation of PBMC was collected from each pony prior to each vaccination, as well as 2 weeks after each MVA booster dose.

2.7. ELISA

Purified, sarcosyl-disrupted Eq/Ky virus was coated overnight at 4 °C onto 96-well polystyrene plates (Immulon-1®, Dynatech Laboratories Inc., Chantilly, VA) at 125 HA units per well. The plates were washed with PBS/0.05% Tween-20 (PBS-T) and blocked for 1 h at room temperature with 2% non-fat dried milk powder in PBS-T. Samples were diluted in PBS-T and incubated in triplicate wells for 90 min at 37 °C. Each plate included triplicate control wells containing a serum sample negative for influenza virus-specific antibodies, and a series of dilutions of a known Eq/Ky virus-specific antibody positive equine serum sample. Plates were washed with PBS-T and monoclonal antibodies specific for equine IgGa (CVS 45) or IgGb (CVS 39) antibodies (Lunn et al., 1998) were added to all sample wells. Again, plates were incubated for 90 min at 37 °C, then washed prior to the addition of peroxidase-conjugated goat-anti-mouse IgG antibody (ICN Biomedicals, Aurora, OH). After another 90 min incubation, the plates were washed and developed by addition of 3,3′,5,5′-tetramethylbenzidine substrate (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD). The optical density at 450 nm of each well was determined using a plate reader (EE 311SX Microplate autoreader, Bio-Tek Instruments, Winooski, VT). Relative serum antibody concentrations were calculated by comparison with the standard curve constructed using serial dilutions of the positive control serum, and expressed as titers.

2.8. Determination of influenza virus-specific lymphoproliferative responses

PBMC were isolated by overlaying leukocyte-rich plasma on Histopaque®-1077 (Sigma), centrifuging at $500 \times g$ for 30 min and harvesting the interface. The mononuclear cells were washed three times in PBS (pH 7.2) and resuspended at 2×10^6 ml^{-1} in cRPMI-10 (RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FBS (Harlan), 2 mM Glutamax® (Gibco), 50 μM 2-mercaptoethanol (Sigma), 50 μg/ml gentamicin (Gibco) and 0.25 μg/ml amphotericin B (Gibco)). Aliquots of 5×10^6 PBMC from each pony were stimulated with 5×10^6 EID_{50} influenza virus for 45 min at 37 °C, 5% CO_2, after which time the cells were pelletted to remove the influenza virus inoculum. All virus-stimulated and unstimulated cells were resuspended at 2×10^6 PBMC/ml in cRPMI-10.

Quadruplicate wells of virus- and medium-stimulated cells (2×10^5 per well) from each pony were set up in a 96-well round bottom culture plate. The plates were incubated for 72 h at 37 °C, 5% CO_2 and then pulsed with ^{3}H-thymidine (1 μCi per well) for 16 h. Radioactive thymidine uptake was measured using a microplate scintillation and luminescence counter system (Top Count, Packard, Meridien, CT). Lymphoproliferative data were expressed by subtracting the mean thymidine incorporation of unstimulated cells from that of influenza virus-stimulated cells.

2.9. Real-time PCR quantitation of influenza virus-specific IFN-γ mRNA responses

Aliquots of 1.6×10^6 medium or virus-stimulated equine PBMC samples, prepared as described above, were each placed in duplicate wells of a 24-well plate, and incubated for 48 h at 37 °C, 5% CO_2. After the incubation, the contents of the duplicate wells were pooled, centrifuged, and each cell pellet was resuspended in 300 μl RNA-STAT 60 (Tel-Test Inc., Friendswood, TX). Total RNA was extracted from each sample according to the manufacturer's protocol,

and RNA yield was calculated using a spectrophotometer. 1.2 μg of each RNA was brought to 52 μl in DEPC water (milli-Q water supplemented with 0.1% diethylpyrocarbonate), and heated to 65 °C for 10 min. The samples were then placed on ice, and 28 μl reverse transcription master mix (16 μl Moloney's murine leukemia virus (MMLV) buffer (Promega), 4 μl (800 U) MMLV reverse transcriptase (Promega), 0.8 μl oligo dT primer (0.5 mg/ml; Promega), 4 μl dNTP (10 mM; Promega), 2.4 μl RNAsin (40 U/μl, Promega) and 0.8 μl bovine serum albumin (10 mg/ml, Promega)) was added to each tube. The samples were incubated at 20 °C for 10 min followed by 40 °C for 1 h, in a thermocycler.

Real-time multiplex PCR was performed on each RT reaction product for the simultaneous measurement of equine IFN-γ and β-actin cDNA, using the iCycler iQ™ thermal cycling system (Bio-Rad Laboratories Inc., Hercules, CA). Specific primers and fluorescent probes for each template were manufactured by IDT Technologies Inc. (Coralville, IA). The primer and probe sequences used were as follows: IFN-γ forward primer: 5′-CGCAAAGCAATAAGTGAACTCATC-3′; IFN-γ reverse: 5′-CGAAATGGATTCTGACTCCTCTTC-3′; IFN-γ probe: 5′-FAM-TCTGTCGCCCAAAGCTAACCTGAGGAA-BHQ1-3′; β-actin forward primer: 5′-AGGGAAATCGTGCGTGACA-3′; β-actin reverse: 5′-GCCATCTCCTGCTCGAAGTC-3′; β-actin probe: 5′-HEX-CAAGGAGAAGCTCTGCTATGTCGCCCT-BHQ2-3′. In order to facilitate the simultaneous and independent measurement of both amplicons using a multiplex reaction, the probes were labeled with different reporter dyes as indicated in the probe sequences.

Separate plasmids encoding equine IFN-γ (pWRG1647-IFNγ) and β-actin (pCR3.1-βactin) were used to establish a standard curve of known template concentrations on each experimental plate. Each plasmid was propagated and purified as described above, and quantitated using a UV spectrophotometer. The plasmids were mixed at equal concentration, and log serial dilutions were made such that each plasmid was present at 10^{-15} to 10^{-23} mol/5 μl, corresponding to 6.02×10^{7} to 6.02×10^{0} copies of each plasmid/5 μl. Five microliters of each plasmid dilution was placed in triplicate wells of a 96-well iCycler iQ™ PCR plate (Bio-Rad). Milli-Q water was placed in triplicate wells as a negative control. Also, 5 μl of each reverse transcription reaction product to be analyzed was placed in triplicate wells. Twenty microliters master mix (12.5 μl quantitative PCR supermix (Invitrogen), 0.5 μl IFN-γ forward and reverse primers (10 μM), 0.5 μl IFN-γ probe (5 μM), 0.5 μl β-actin forward and reverse primers (5 μM), 0.5 μl β-actin probe (2.5 μM), 4.5 μl milli-Q water) was added to each well. Reactions were initially incubated at 95 °C for 3 min, followed by 45 cycles of 95 °C for 30 s and 60 °C for 60 s. Separate standard curves for IFN-γ and β-actin were established using the log dilutions of plasmid standards. The efficiency of PCR amplification of both plasmid standards was always 95–100%.

The IFN-γ and β-actin cDNA copy numbers present in each unknown sample was calculated by the system software using the standard curve. The β-actin copy number was used as an internal standard to normalize the data. The influenza virus-specific IFN-γ response of each pony was calculated and expressed as a stimulation index (SI), using the following equation:

$$\frac{\text{IFN-}\gamma\text{ copy number (virus-stimulated)}/\ \beta\text{-actin copy number (virus-stimulated)}}{\text{IFN-}\gamma\text{ copy number (medium-stimulated)}/\ \beta\text{-actin copy number (medium-stimulated)}}$$

3. Results

3.1. Confirmation of antigen expression by recombinant MVA-HA and -NP

Following several rounds of recombinant MVA plaque purification, the expression of HA and NP by the purified MVA strains was confirmed by immunostaining. Monoclonal antibodies specific for influenza virus HA and NP proteins (generously provided by Dr. Y. Kawaoka, University of Wisconsin) specifically stained *r*MVA-HA and *r*MVA-NP plaques, respectively, in infected BHK-21 cell monolayers (not shown). No cross-recognition of the heterologous antigens by the antibodies was observed.

3.2. Influenza virus-specific serum antibody responses

Primary DNA vaccination with the PowderJect XR research device did not induce detectable influenza

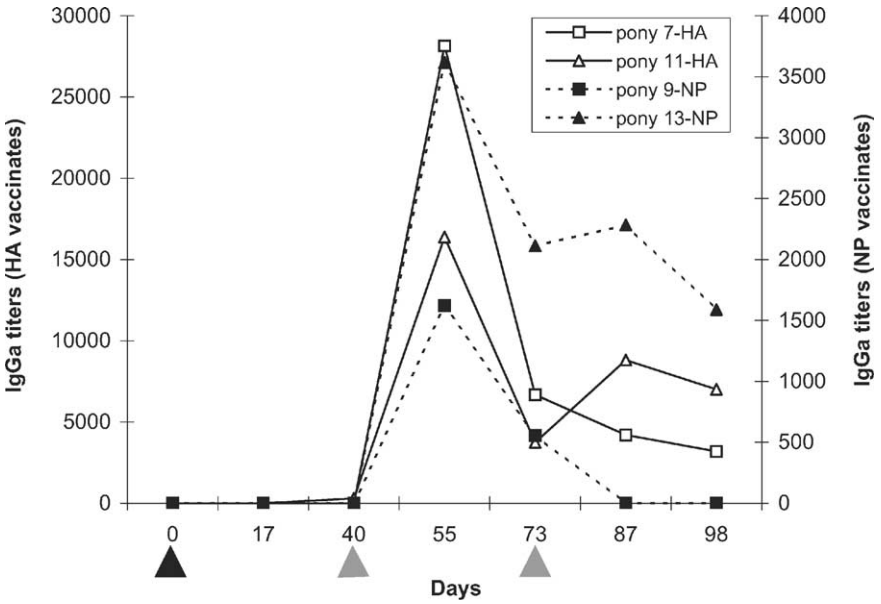

Fig. 1. Influenza-specific serum IgGa responses of each individual pony. Black arrowhead denotes primary DNA vaccination. MVA booster administrations are denoted by gray arrowheads.

virus-specific IgGa (Fig. 1) or IgGb (Fig. 2) antibody responses in any of the vaccinated animals. However, administration of the first MVA booster dose induced high levels of both influenza virus-specific antibody istoypes in all vaccinates (Figs. 1 and 2). Slight increases in influenza virus-specific IgGa antibodies were induced in 1 of 2 HA vaccinates and in 1 of 2 NP vaccinates by administration of the second MVA

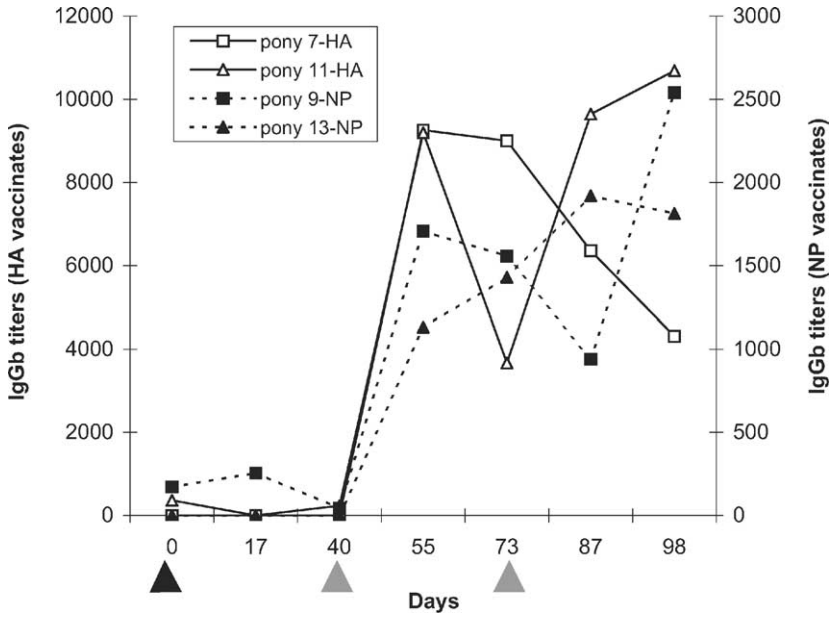

Fig. 2. Influenza-specific serum IgGb responses of each individual pony. Black arrowhead denotes primary DNA vaccination. MVA booster administrations are denoted by gray arrowheads.

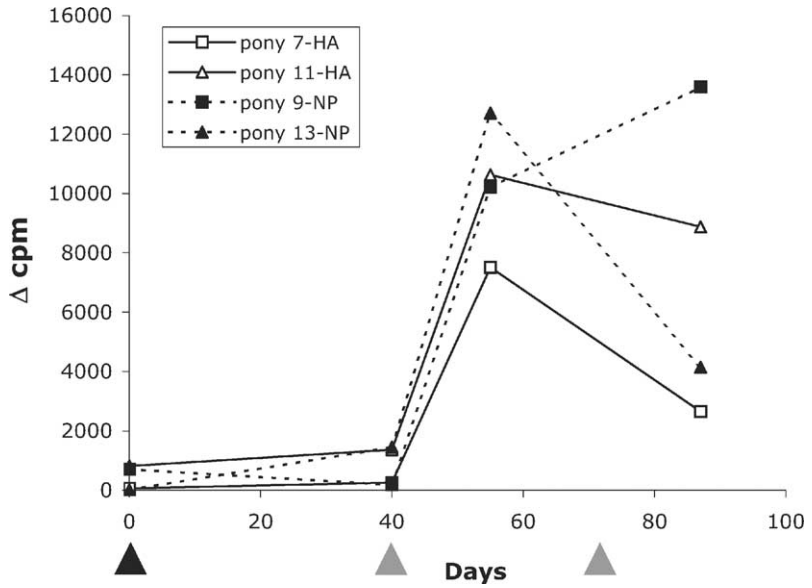

Fig. 3. Influenza-specific lymphoproliferative responses of each individual pony. Black arrowhead denotes primary DNA vaccination. MVA booster administrations are denoted by gray arrowheads.

booster dose (Fig. 1). This dose also elicited an increase in virus-specific IgGb antibodies in 1 of 2 HA vaccinates and in 1 of 2 NP vaccinates (Fig. 2). Four other ponies which were untreated and maintained at the same facility remained seronegative to influenza virus throughout this experiment (data not shown).

3.3. Influenza virus-specific lymphoproliferative responses

No detectable lymphoproliferative responses were induced in any pony by DNA vaccination alone (Fig. 3). Booster immunization with recombinant MVA induced high levels of influenza virus-specific

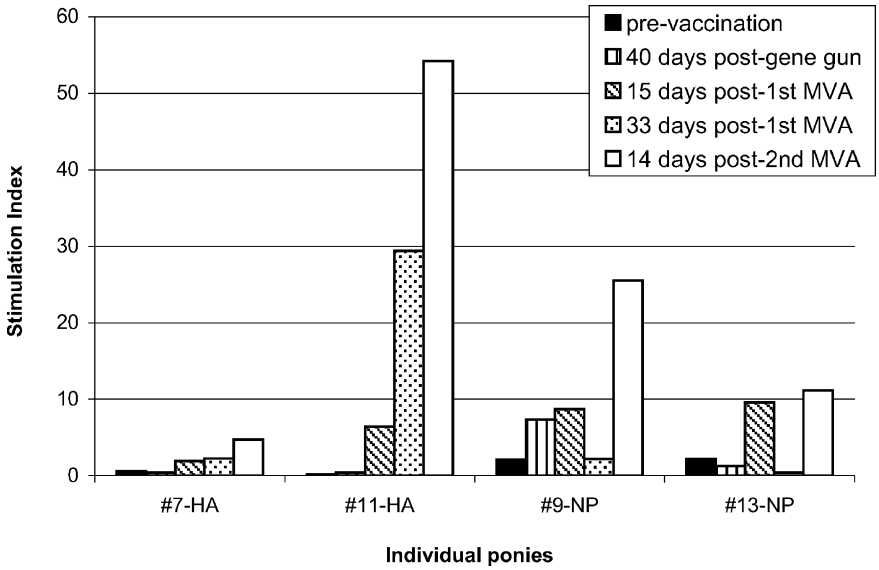

Fig. 4. Influenza virus-specific IFN-γ mRNA responses of each individual pony, prior to and following each vaccination.

cellular immunity in all vaccinated ponies. Both HA and NP antigens appeared to elicit comparable levels of virus-specific cellular immunity. Secondary boosting with MVA resulted in an increase in influenza virus-specific cellular immune responses in one vaccinate (pony 9-NP; Fig. 3).

3.4. Influenza virus-specific IFN-γ mRNA responses

Influenza virus-induced IFN-γ gene expression was upregulated in all four ponies following each MVA booster vaccination (Fig. 4). This was evidenced by an increase in the ratio of IFN-γ:β-actin mRNA detected in influenza-stimulated PBMC. Only one vaccinate (pony 9-NP) demonstrated an IFN-γ response to primary vaccination with the gene gun. However, influenza virus-specific IFN-γ production was increased in all four vaccinates following administration of the initial MVA booster dose, and this response was further augmented in all four vaccinates following the second booster administration.

4. Discussion

Recombinant strains of modified vaccinia Ankara (MVA) were constructed for the vaccination of horses against equine influenza, and the findings of this study provide preliminary evidence of their immunogenicity. Primary DNA vaccination of the influenza virus-seronegative experimental ponies did not induce detectable virus-specific humoral or cellular immune responses, which is consistent with our previous findings (Lunn et al., 1999). However, the first booster administration of recombinant MVA induced high levels of influenza virus-specific serum IgGa and IgGb as well as virus-specific lymphoproliferative responses in all vaccinated animals. Furthermore, influenza virus-specific IFN-γ responses were also induced in all four vaccinated ponies. While Eq/Ky HA is a well-known protective immunogen in horses (Lunn et al., 1999; Mumford, 1992), it is very interesting that this vaccination regimen also efficiently induced NP-specific immunity. Notably, the peak NP-specific antibody titers were of lower magnitude than the HA-specific responses. However, both antigens elicited comparable levels of influenza-specific lymphoproliferative (Fig. 3) and IFN-γ responses (Fig. 4). Interestingly, NP, which is an internal viral structural protein, is considered to be an important target of virus-specific cellular immunity (Townsend et al., 1984; Yewdell et al., 1985). The findings of the current study suggest that MVA-NP may prove to be an effective tool for the induction of NP-specific immunity in the horse, allowing future analysis of the role of NP as a protective antigen in this species.

The administration of a second MVA booster in this experiment resulted in an increase in antibody and lymphoproliferative responses in only a subset of the ponies. This apparent lack of response may have been the result of the booster being compromised by MVA-specific immunity induced by the prior vaccination (Sharpe et al., 2001). However, an alternative explanation is the 4-week interval between MVA vaccinations may have been too short to allow for the further amplification of antibody or lymphoproliferative responses in all vaccinated ponies. In this context it is noteworthy that the influenza virus-specific IgGb antibody titers in three of the four ponies were still increasing at the time of the second MVA vaccination. The results of the IFN-γ mRNA studies do show a strong increase in immune response after the second vaccination, suggesting that vector-immunity was not a factor and that in terms of this parameter there was a clear immune response to this second MVA booster dose. Nevertheless, it would be wise to consider a longer interval between vaccine administrations when using this technique in the horse.

The current research suggests that the construction of recombinant strains of MVA may be an efficient and effective way to induce and subsequently evaluate immune responses to selected antigens of important veterinary pathogens. The construction of recombinant MVA viruses expressing equine influenza virus antigens provides us with exciting tools for the study of equine influenza virus immunology and vaccinology. A preliminary investigation of these constructs in DNA prime-MVA boost immunization of ponies provides preliminary evidence of the immunogenicity of this vaccination regimen in the horse. Additionally, the findings demonstrate that MVA-NP can be used for the selective induction of NP-specific immunity in the horse, providing the opportunity to analyze the role of this antigen in protective immune responses. Further experiments are planned to establish the protective efficacy of this DNA prime-MVA boost vaccination

regimen, as well as to evaluate the role of NP as a protective antigen.

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