



Regional antibody and cellular immune responses to equine influenza virus infection, and particle mediated DNA vaccination

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Abstract

We have previously demonstrated that hemagglutinin (HA) gene vaccination and influenza virus infection generate protective antibody responses in equids. However, these antibody responses differ substantially in that particle mediated DNA vaccination does not induce an immunoglobulin A (IgA) response. A study was performed to investigate the regional immunoregulatory mechanisms associated with these different immune responses.

Ponies were either vaccinated with equine HA DNA vaccines at skin and mucosal sites, infected with influenza virus or left untreated and influenza-specific antibody responses and protection from challenge infection was studied. In a subset of ponies, lymphocytes from peripheral blood (PBLs), nasopharyngeal mucosal tissue, or lymph nodes (LNLs) were collected for measurement of influenza virus-specific lymphoproliferative responses, local antibody production and IL-2, IL-4 and IFN- γ mRNA production by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

DNA vaccination and influenza virus infection induced humoral immunoglobulin G_a (IgG_a) and immunoglobulin G_b (IgG_b) production and lymphoproliferative responses that were positively correlated with IFN- γ mRNA production. However, there were marked differences in immune response in that only influenza infection induced an IgA response, and the regional distribution of lymphoproliferation, IFN- γ and antibody responses. Responses to DNA vaccination occurred in PBLs and in lymph nodes draining DNA vaccination sites, while influenza virus infection induced responses in PBLs and hilar LNLs. In summary, common features of immune responses to either influenza virus infection or DNA vaccination were virus-specific IgG_a, IgG_b and IFN- γ responses, which are associated with protection from infection, even when the regional distribution of these immune responses varied depending on the site of immune encounter.

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Abbreviations: Eq/Ky, A/Equine/Kentucky/1/81; *E. coli*, *Escherichia coli*; EID₅₀, egg infectious dose 50%; HA, hemagglutinin; HBSS, Hank's buffered salt solution; HCMV, human cytomegalovirus; IgA, immunoglobulin A; IgG_a, immunoglobulin G_a; IgG_b, immunoglobulin G_b; IgG(T), immunoglobulin G(T); IL, interleukin; ISSs, immunostimulatory complexes; MHC, major histocompatibility complex; NA, neuroaminidase; qRT-PCR, quantitative reverse transcription-PCR; TH-1, T-Helper 1; TH-2, T-Helper 2

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1. Introduction

Equine influenza virus is one of the primary causes of infectious disease in the horse throughout the world (Mumford, 1992) and respiratory disease ranks second only to colic as the most common disorder requiring medical attention by equine practitioners in North America (Traub-Dargatz et al., 1991). Despite the importance of the disease, available vaccines remain unsatisfactory. Current inactivated vaccines fail to induce a full spectrum of immune responses, and studies evaluating formalin-inactivated influenza virus vaccines have demonstrated the failure of these vaccines to protect horses from infection (Morley et al., 1999; Nelson et al., 1998). Modified-live vaccines for equine influenza virus show more promising results (Lunn et al., 2001), however, the use of modified-live vaccines can be problematic in immunocompromised or pregnant animals (Oehen et al., 1991).

Previous experiments in our laboratory have established that the long lasting protective immunity following recovery from influenza virus infection is associated with mucosal immunoglobulin A (IgA) and serum immunoglobulin G₁ (IgG₁) and immunoglobulin G₂ (IgG₂) responses, while some inactivated vaccines produce only non-protective immunoglobulin G₁ (IgG₁) antibodies in naive ponies (Lunn et al., 1999a; Nelson et al., 1998). Furthermore, particle mediated DNA vaccination with the influenza virus hemagglutinin (HA) gene resulted in complete clinical and partial virological protection in ponies (Lunn et al., 1999b). This protection was associated with the same IgG₁ and IgG₂ antibody isotype responses as influenza virus infection, but without a mucosal IgA response. In spite of the fact that induction of the appropriate IgG responses can be sufficient to induce clinical protection from influenza virus, induction of a mucosal IgA response remains desirable. Mucosal IgA might help to prevent viral shedding following infection, and is thought to be important for heterotypic immunity (Renegar and Small, 1991; Tamura et al., 1990, 1991). A recent study also provided evidence that an important role of IgA may be to facilitate antigen presentation to mucosal T cells (Arulanandam et al., 2001). Despite its importance, it has proven extremely difficult to induce influenza virus-specific mucosal IgA responses in

naïve horses by vaccination (Lunn et al., 1999a,b; Nelson et al., 1998).

In order to develop more successful vaccination strategies it is critical to understand the immunoregulatory mechanisms involved in a protective immune response. The Th-1/Th-2 paradigm has proven useful for understanding immune responses to infectious disease in a number of species (Brenner et al., 1989; Mosmann, 1991; Mosmann and Sad, 1996), but such studies in horses have been limited by the lack of availability of reagents for equine cytokines. Recent development of quantitative reverse transcription PCR (qRT-PCR) assays for cytokine mRNA has allowed the examination of cytokine gene expression in equine immune responses to parasitic (Swiderski et al., 1999a), bacterial (Giguere et al., 1999), and viral infections (Folsom et al., 2001). We have previously demonstrated similarities as well as differences in the protection and antibody responses resulting from influenza infection or particle mediated DNA vaccination. The major aim of this study was to perform a comprehensive examination of immunological outcomes of equine influenza virus infection or particle mediated DNA vaccination by incorporating both humoral and cell-based assays in examining mucosal associated tissues and regional lymph nodes. Understanding the regulatory mechanisms involved in a protective immune response will help in future vaccine development.

A secondary goal of this study was to induce a mucosal IgA response by altering the immune response to particle mediated HA DNA vaccination through co-administration of IL-6 DNA. This cytokine was chosen because it has a wide range of activities in acute phase reactions and in the regulation of B and T cell functions. In a murine model of equine influenza infection, we have previously shown that particle mediated HA DNA vaccination coupled with an IL-6 cytokine expressing plasmid led to an increased level of protection compared to particle mediated HA DNA vaccination alone (Larsen et al., 1998).

To achieve these objectives, this study examined the regional and systemic influenza virus-specific immune responses resulting from particle mediated DNA vaccination or influenza virus infection. Parameters studied included the distribution and level of antibody production, lymphoproliferative responses, and cytokine mRNA expression throughout the body.

2. Materials and methods

2.1. Experimental ponies

A total of 25 influenza sero-negative ponies were used in this study. Ponies were of both sexes and their ages ranged from 1 to 6 years. Ponies were housed in individual isolation stalls, fed twice a day with a diet of hay and pelleted concentrate, and maintained according to the animal care guidelines of the Research and Animal Resources Committee, University of Wisconsin, Madison.

2.2. Influenza virus preparation and challenge infections

For the primary infection, and to estimate the protection associated with particle mediated DNA vaccination, the A/Equine/Kentucky/1/81 (Eq/Ky) virus was used. The virus was propagated and purified as described previously (Olsen et al., 1997). To assess virus shedding, viral titers in nasal swab samples were measured as egg infectious dose 50% (EID₅₀)/ml of viral transport media (Kendal et al., 1982). Influenza virus challenge infections were performed by intranasal installation of 10^{9.8} EID₅₀ of Eq/Ky diluted in 2 ml of PBS plus 1% bovine serum albumin, after sedation with 20–40 µg/kg Detomidine IV (Pfizer Animal Health, West Chester, PA).

2.3. Preparation of HA and IL-6 expressing plasmids

The preparation of plasmid DNA constructs expressing the Eq/Ky HA gene is described elsewhere (Olsen et al., 1997). Briefly, the HA gene was subcloned into a high copy number plasmid (pWRG7077) (PowderJect Vaccines, Madison, WI) containing the kanamycin resistance gene and the immediate early promoter and intron-A of human cytomegalovirus (HCMV). The preparation of plasmid DNA encoding the equine IL-6 gene (eqIL-6) (Genbank accession number U64794) was previously described (Swiderski et al., 2000). The eqIL-6 gene was subcloned into a plasmid suitable for DNA vaccination (pWRG1647) (PowderJect Vaccines, Madison, WI) containing the ampicillin resistance gene and the immediate early promoter and intron-A of HCMV. Both plasmids were purified for

PowderJect-XR1 particle mediated DNA vaccination administration by anion exchange chromatography (Qiagen, Chatsworth, CA) and coated onto gold beads at a concentration of 2.5 µg DNA per milligram gold beads as previously described (Lunn et al., 1999b). For preparation of the HA/IL-6 DNA vaccine, the HA and eqIL-6 plasmids were mixed and coated onto beads at a ratio of 1:1. To ensure that HA-DNA vaccine beads contained equivalent amounts of HA-DNA plasmid, the eqIL-6 plasmid was replaced with empty vector plasmid (pWRG1647). This also controlled for any transcriptional effect that the addition of a second plasmid might have on HA expression. In summary, each discharge delivered 0.625 µg of HA DNA and 0.625 µg of either empty vector or eqIL-6 DNA.

2.4. Experimental design

Twenty-five ponies were assigned to one of four groups. The first group was vaccinated with HA/IL-6 DNA (HA/IL-6 DNA vaccination group; *n* = 7) and the second group was vaccinated with HA DNA (HA DNA vaccination group; *n* = 7). The third group was infected with influenza virus (infection control group; *n* = 7), and the fourth group comprised influenza naïve control ponies (naïve control group; *n* = 4). Both DNA vaccination groups were vaccinated on three occasions on Days 0, 70 and 112. Each pony was vaccinated using the PowderJect-XR1 research device 24 times on inguinal skin, eight times on perineal skin, 24 times on the ventral tongue and four times on the conjunctiva and third eyelid. These target sites were chosen based on previous experiments performed in our laboratory (Lunn et al., 1999a,b). Total doses of DNA per vaccination were 37.5 µg of HA DNA, plus 37.5 µg of either eqIL-6 or empty vector DNA. Before DNA vaccination, ponies were sedated with xylazine HCl (1 mg/kg, IV; Miles, Shawnee, KS) and anesthetized with ketamine (2.2 mg/kg, IV; Ketaset; Fort Dodge, IA).

Three ponies from each DNA vaccination group were euthanized 14 days after the third DNA vaccination, on Day 126, in order to harvest regional lymphoid tissues for immunological studies. Euthanasia was performed using pentobarbital (60 mg/kg, IV; Schering-Plough Animal Health, Union, NJ). On the same day as the third DNA vaccination was administered to the DNA vaccination pony groups (Day 112),

four ponies from the infection control group were subjected to an influenza infection. These infected ponies were also euthanized on Day 126, together with the four influenza naïve control ponies.

The remaining four ponies in each DNA vaccination group, together with the three remaining (unchallenged) ponies from the infection control group, were challenge infected on Day 159 in order to measure protection after DNA vaccination. These ponies were then studied for a further 28 days. Physical examinations were conducted on all ponies throughout the course of the experiment at the same time as collection of serum and nasal secretions, and daily for 14 days after challenge infections.

2.5. Sample collection

Serum and nasal secretions for antibody assays were sampled at times indicated in the figures. Before sampling, ponies were sedated with 20–40 µg/kg Detomidine IV (Pfizer Animal Health, West Chester, PA). Blood was collected by jugular venipuncture into serum separator tubes (Becton Dickinson Vacutainer, Franklin Lakes, NJ) for preparation of serum. Nasal secretions for antibody measurement were collected by placing a narrow tampon (Tampax slender regular, Procter & Gamble, Cincinnati, OH) in the ventral nasal meatus for at least 15 min and subsequent centrifugation at 2000 × *g*. These samples were aliquoted and stored at –20 °C. Nasal secretion samples for virus isolation were collected daily for 10 days after challenge infections using Dacron swabs (Baxter Healthcare Corporation, McGaw Park, IL). Swabs were stored in 1 ml of virus transport medium at –70 °C.

For lymphoproliferation and cytokine mRNA experiments, 60 ml of blood was collected into 15 U/ml heparin (Elkins-Sinn, Cherry Hill, NJ) by jugular venipuncture. Tissues were collected immediately after euthanasia, and included the retropharyngeal, hilar, prescapular, and inguinal lymph nodes, and dorsal follicular nasopharyngeal tissues and adjacent caudal nasal mucosal tissues. Lymph nodes were collected using sterile technique into cRPMI (RPMI-1640 containing 0.01 M HEPES, 0.002 M sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FBS). Nasal and pharyngeal tissues were dissected and collected into cPBS (PBS plus 0.01 M

HEPES, penicillin, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% fetal bovine serum).

2.6. Antibody immunoassays

Serum and nasal secretions were assayed for Eq/KY virus-specific antibody using an ELISA procedure that has been extensively described (Lunn et al., 1999b; Nelson et al., 1998). This procedure allows for identification of influenza virus-specific antibody isotype and sub-isotype responses using monoclonal antibodies to equine IgGa, IgGb, IgG(T), and IgA (Lunn et al., 1996). Results are expressed as titers, which were determined by comparison of the test sample to a standard curve generated by serial dilution of a sample of known titer.

2.7. Preparation of PBMCs, mucosal and lymph node lymphocytes

Pony PBMCs were isolated over Histopaque-1077 (Sigma, St. Louis, MO). For preparation of lymph node lymphocytes, capsules were removed and lymph nodes were placed in sterile petri dishes and passed through a sterile metal screen (Sigma, St. Louis, MO) until only connective tissue remained. Pharyngeal and nasal mucosal tissues were washed in Hank's buffered salt solution (HBSS) (Gibco BRL, Grand Island, NY) plus 2 mM dithiothreitol for 30 min. The tissues were then digested in cRPMI containing 50 IU/ml collagenase and 50 IU/ml hyaluronidase (Worthington Biochemicals Corporation, Freehold, NJ) for 4 h at room temperature while stirring. The lymphocytes were pelleted at 800 *g*, re-suspended in 1.055 Percoll (Sigma, St. Louis, MO) and layered over 1.080 Percoll. A further 10 ml of RPMI was added as a top layer, and the gradient was centrifuged at 900 × *g* for 30 min and lymphocytes were recovered from the 1.055/1.080 Percoll interface. The purity of all sources was >95% as determined by a differential cell count on cytopspin preparations of each sample.

2.8. *In vitro* production of equine influenza-specific antibodies

For examination of local antibody production, 10⁷ lymphocytes in 1 ml cRPMI were stimulated with pokeweed mitogen (Gibco BRL, Grand Island, NY)

at a 1:100 dilution. The supernatant was collected after 14 days of culture and tested for equine influenza virus-specific antibody isotype and sub-isotype production by ELISA.

2.9. *Lymphoproliferation after in vitro re-stimulation*

For in vitro re-stimulation, 10^7 lymphocytes in 1 ml cRPMI were incubated at 37 °C for 45 min with or without the presence of $10^{6.75}$ EID₅₀ Eq/Ky. After incubation the cells were pelleted by centrifugation at $300 \times g$ for 10 min to remove the virus, and for lymphoproliferation experiments, lymphocytes were plated in triplicate in 96 well microtiter plate (Costar, Cambridge, MA), at a concentration of 2×10^5 cells in 200 μ l in each well. Plates were incubated in a humidified 37 °C, 5% CO₂ incubator for 72 h and subsequently each well was pulsed with 50 μ l of media containing 0.02 mCi/ml ³H thymidine for 10 h. Thymidine uptake was measured in counts per minute (cpm) using a microplate scintillation and luminescence counter system (Top Count, Packard, Meriden, CT). Lymphoproliferation was calculated as stimulation index, SI = virus-stimulated PBMCs (cpm)/un-stimulated PBMCs (cpm).

2.10. *Quantitative reverse transcriptase-polymerase chain reaction analysis of equine cytokines*

Lymphocytes from different lymphoid tissues were incubated with or without the presence of influenza virus for 45 min, as described for the lymphoproliferation experiments. After centrifugation, 3×10^6 lymphocytes in 1 ml cRPMI were plated into 24 well tissue culture plates (Corning, New York). The plates were incubated in a humidified 37 °C, 5% CO₂ incubator for 72 h. Lymphocytes were collected by centrifugation and lysed by addition of RNA STAT-60 (Tel-Test, Friendswood, TX). Lysates were frozen at -70 °C pending analyses. Production of equine IL-2, IL-4 and IFN- γ mRNAs was measured using a qRT-PCR procedure as described previously (Swiderski et al., 1999b). Briefly, cell mRNA was reverse transcribed into cDNA, and equine IL-2, IL-4, IFN- γ and β -actin-specific cDNAs were amplified using Taq thermostable polymerase and specific primers. The relative amounts of qRT-PCR products were

quantified using a Perkin-Elmer QPCR™ System 5000 (Zhao et al., 1996) and each cytokine value was corrected by the corresponding β -actin value. The ratio of mRNA levels in lymphocytes, stimulated with influenza virus, to mRNA levels from lymphocytes incubated in media alone was determined. Levels of cytokine expression were shown as fold increases of ratios in treatment groups over ratios calculated in naïve control ponies.

2.11. *Statistical analysis*

The clinical signs after challenge infection between groups of ponies were compared using Fisher's exact test. Differences between influenza-specific antibody titers among pony groups after challenge infection were analyzed by averaging the values of the 4 days after challenge infection and performing ANOVA and LSD analyses of the log-transformed data. In order to look for the effect of vaccination in each DNA vaccination groups paired *t*-tests were used to compare antibody titers on Days 2 and 83 and Days 2 and 124 after log transformation of the data. This test was used to look for the effect of vaccination in each individual group. In order to see if the DNA vaccination groups were different from each other, unpaired *t*-tests were used. The lymphoproliferative data and the qRT-PCR data were ranked and analyzed by ANOVA and LSD.

3. Results

3.1. *Clinical responses and protection from equine influenza virus infection*

After challenge infection, 6 of 7 ponies in the infection control group developed severe muco-purulent nasal discharge of 3–5 days duration and one pony developed a mucoid nasal discharge. All infection control group ponies exhibited spontaneous coughing and 6 of 7 ponies were pyrexic ($T > 101.5$ °F) for 1–4 days. In both DNA vaccination groups, a slight mucoid nasal discharge developed in 1 of 4 ponies post-challenge infection. No other abnormal clinical signs were seen in the DNA vaccinated ponies. Statistical analysis showed that the infection control group ponies demonstrated significantly more disease ($P = 0.015$) than either DNA vaccination group. No

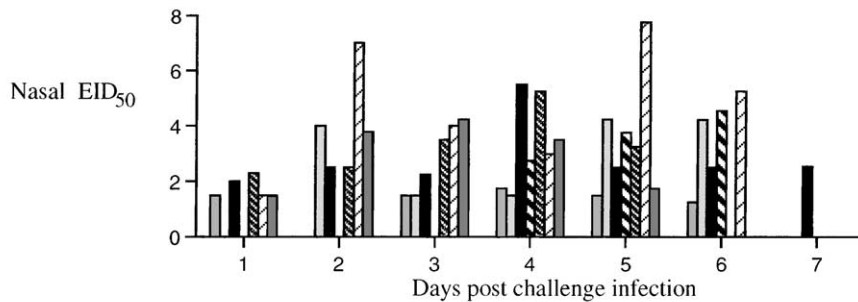
statistical difference in disease severity was found between the HA/IL-6 DNA vaccinates and the HA DNA vaccinates.

3.2. Virus shedding

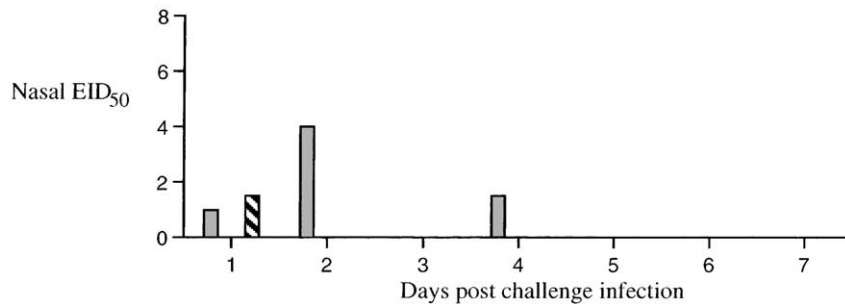
The results of virus isolation from daily nasal swabs of ponies post-challenge infection are shown in Fig. 1.

No virus was isolated beyond 7 days post-infection in any pony. In infection control group ponies virus shedding was seen in 7 of 7 ponies for durations of 3–5 days, with an EID₅₀ of 10⁴ or higher in 6 of 7 ponies. In both DNA vaccination groups, virus shedding occurred in 1 of 4 ponies for 3–4 days, but the EID₅₀ was below 10⁴. Another pony in each DNA vaccination group was virus positive on the first day

(a) Infection control group



(b) HA DNA vaccinates



(c) HA/IL-6 DNA vaccinates

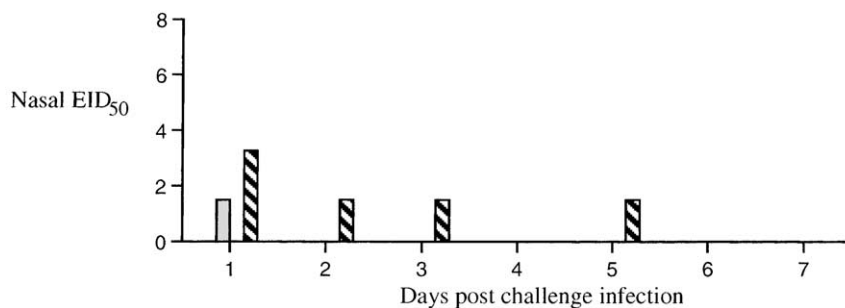


Fig. 1. Influenza virus shedding after challenge infection. Samples were collected into 1 ml of viral transport media from the nasopharynx using Dacron swabs. Results are expressed as EID₅₀/ml of transport media. Each bar represents an individual pony. (a) Infection control pony group; (b) HA DNA vaccination group; (c) HA/IL-6 DNA vaccination group.

post-infection but, since it was transient and low-grade, this was thought to represent residual challenge virus.

3.3. Anti-influenza virus antibody responses

Ponies in both DNA vaccination groups produced significant serum IgGa and IgGb responses following the second and the third DNA vaccinations (Fig. 2, Table 1), with no significant differences seen between the two DNA vaccination groups. Ponies in all groups produced IgGa and IgGb responses post-challenge, with the DNA vaccinated pony groups producing significantly higher titers than infection control group ponies ($P = 0.014$ for IgGa, $P = 0.0001$ for IgGb). Post-challenge infection IgGa titers were similar in both DNA vaccination groups, while IgGb titers were significantly higher in HA DNA vaccinates than in HA/IL-6 DNA vaccinates ($P = 0.001$). Low-level IgG(T) responses to DNA vaccination (Fig. 2) were not significantly different between the two DNA vaccination groups ($P = 0.18$). After challenge infection IgG(T) titers were significantly different in the three groups ($P = 0.001$), and were highest in the HA/IL-6 DNA vaccinates (Fig. 2).

Analysis of the IgG isotype responses in the nasal secretions demonstrated increases in both IgGa and IgGb following the second and third DNA vaccinations, although the two DNA vaccination groups were not significantly different from each other (data not shown). After challenge infection, nasal IgGa titers increased, but no significant differences could be

detected between the DNA vaccination groups or infection control group ponies ($P = 0.14$). Nasal IgGb titers after challenge infection were significantly different among the three groups ($P = 0.0014$). The HA DNA vaccinates produced the highest titers, followed by the HA/IL-6 DNA vaccinates, and the infection control group ponies, similar to the results observed for serum IgGb. Nasal IgG(T) was not detected in any group prior to challenge infection (data not shown). After challenge infection, there was a slight increase in nasal IgG(T) titers with no significant differences between the three groups ($P = 0.61$).

Analysis of nasal secretions demonstrated the presence of low titer nasal IgA responses in the DNA vaccination groups after the second and third vaccinations with no significant differences between the two DNA vaccination groups ($P = 0.67$) (Fig. 2). While these responses achieved significance compared to antibody levels prior to vaccination (Table 1), the titers were extremely low compared to nasal IgA titers achieved after challenge infection and of questionable significance. After challenge infection nasal IgA responses were highest in HA/IL-6 DNA vaccinates, but differences between groups were not statistically significant ($P = 0.13$). Serum IgA titers followed the pattern observed for nasal IgA but the titers were lower (data not shown).

3.4. In vitro viral re-stimulation experiments

Lymphocytes used for the in vitro viral re-stimulation experiments were harvested 2 weeks after the

Table 1

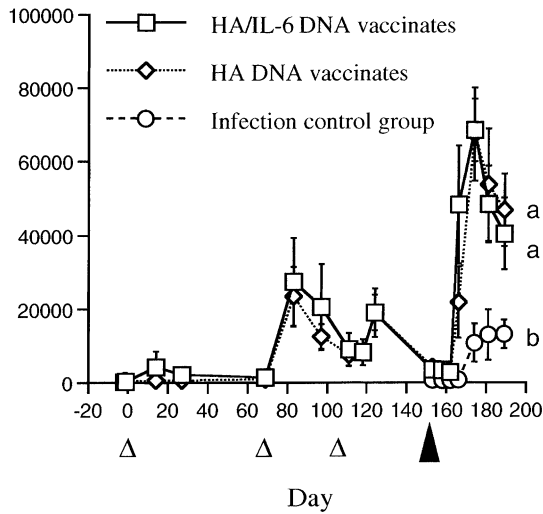
Statistical analysis of influenza-specific antibody titers in the two vaccination groups following the second (Day 83) and third vaccination (Day 124)^a

<i>t</i> -Test	Serum antibodies				Nasal antibodies			
	IgGa	IgGb	IgG (T)	IgA	IgGa	IgGb	IgA	IgG (T)
Days 2 and 83								
HA-IL6 vaccination	0.0004*	0.01*	0.07	0.02*	0.0002*	0.12	0.03*	0.22
HA only vaccination	0.0002*	0.008*	0.23	0.21	0.004*	0.04*	0.24	0.27
Days 2 and 124								
HA-IL6 vaccination	0.001*	0.005*	0.077	0.02*	0.001*	0.487	0.05*	0.26
HA only vaccination	0.0003*	0.002*	0.03*	0.02*	0.002*	0.006*	0.07	0.85

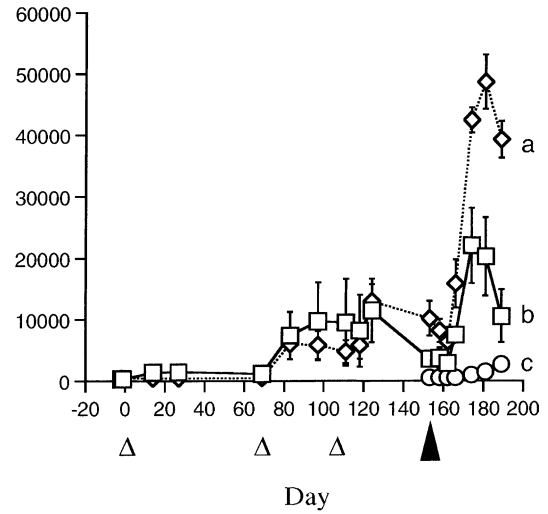
^a A paired *t*-test was performed comparing antibody titers on Days 83 and 124, respectively, with antibody titers on Day 2 and *P*-values are shown.

* Significant differences ($\alpha > 0.05$).

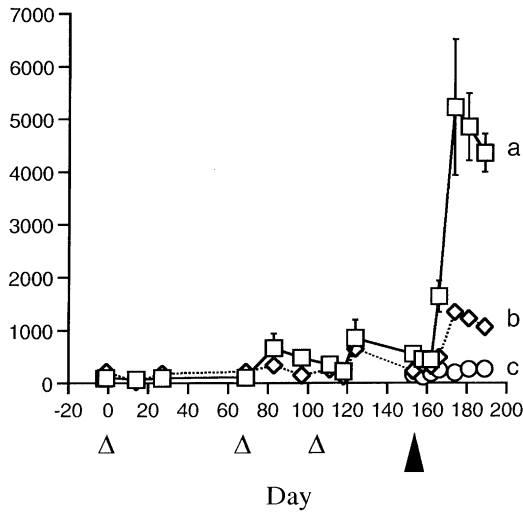
(a) Serum IgGa Titers



(b) Serum IgGb Titers



(c) Serum IgG(T) Titers



(d) Nasal IgA Titers

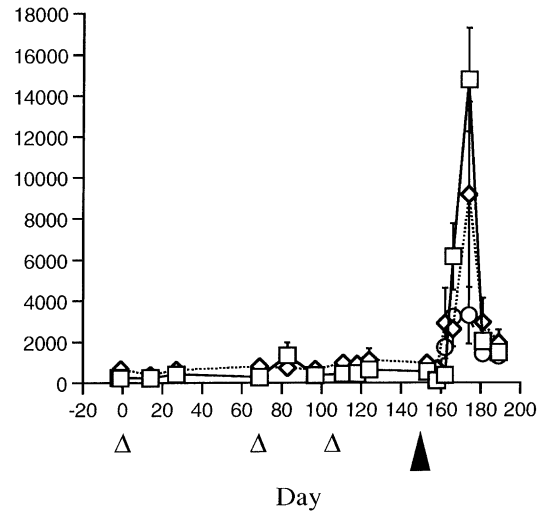


Fig. 2. Effect of DNA vaccination on serum and nasal antibody production. Anti-influenza virus antibody responses in serum and nasal secretions of ponies vaccinated with HA/IL-6 DNA (\square), HA DNA (\diamond), or infection control group (\circ). DNA vaccinations were administered on Days 0, 71, and 112 (open triangles below axis), and challenge infection on Day 159 (closed triangle below axis). Number of ponies in each DNA vaccination group were $n = 7$ until Day 124, and $n = 4$ from Day 124–189. In the infection control group $n = 3$. Individual graphs depict different antibody titers, all data points are mean \pm SEM: (a) serum IgGa antibody titers; (b) serum IgGb antibody titers; (c) serum IgG(T) antibody titers; (d) nasal IgA antibody titers. For statistical analyses the data of the last four time points post-challenge was averaged, log-transformed and analyzed by least significant differences. Different letters (a, b, or c) indicate significantly different groups ($P < 0.05$).

third DNA vaccination for the DNA vaccination groups, or 2 weeks after influenza virus infection for the infection control group. Lymphocytes were also collected from the naïve control group. Because

our experiments showed that there were no differences between HA DNA and HA/IL-6 DNA vaccinates prior to challenge infection, the DNA vaccination groups were treated as one group for all in vitro re-stimulation

experiments which increased the number of ponies in this group and therefore the statistical power of the analyses.

3.4.1. *In vitro* production of equine influenza virus-specific antibody responses

In vitro production of influenza virus-specific IgGa, IgGb and IgA is shown in Table 2. Ponies of the infection control group produced IgGa and IgGb in lymphocytes from the hilar lymph nodes. DNA vaccinated

ponies produced of IgGa and IgGb in lymphocytes from the inguinal lymph nodes, which drained a DNA vaccination site. The retropharyngeal lymphocytes in both infection control group ponies and DNA vaccinated ponies produced IgGa but not IgGb. Nasal tissue lymphocytes could only be collected from 2 of 4 infection control group ponies and cell yields in general were low. IgGa, IgGb and IgA production in nasal tissue lymphocytes could be detected in 1 of 2 ponies in the infection control group and 1 of 6 ponies

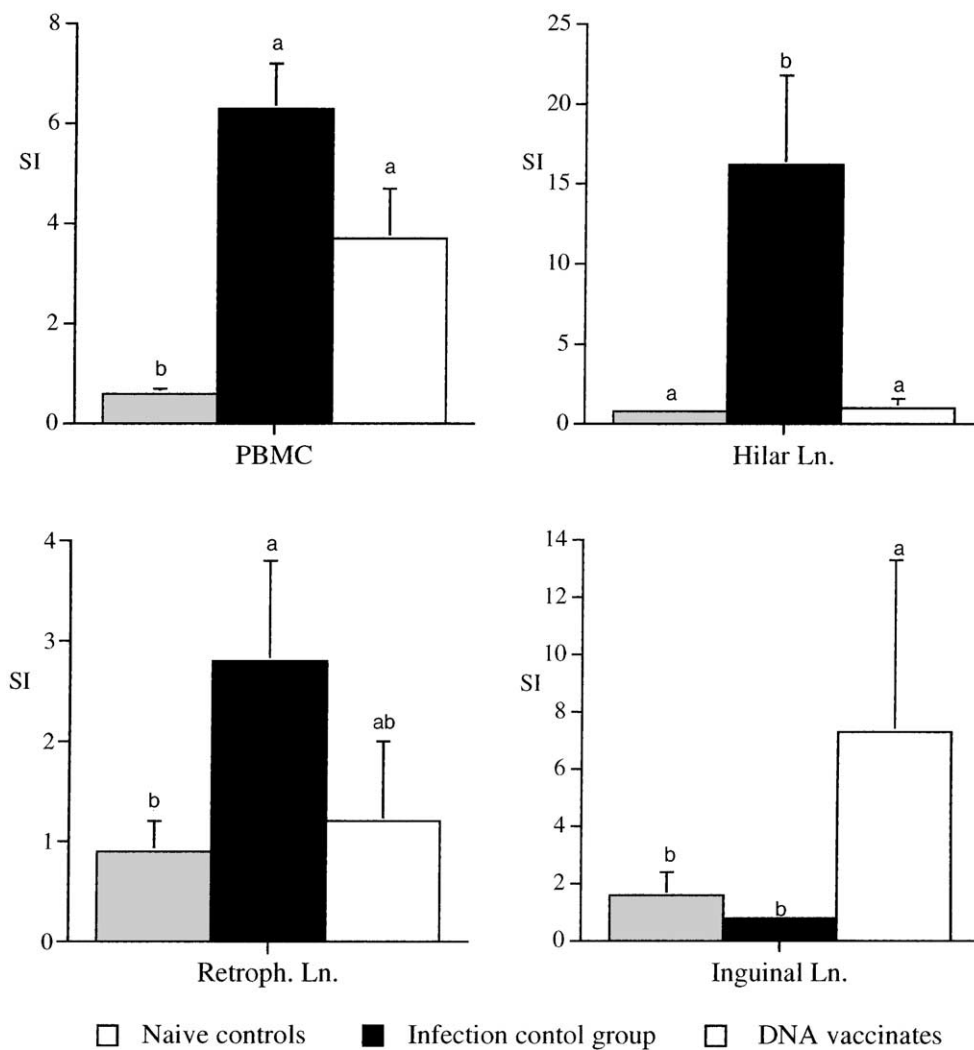


Fig. 3. Influenza virus-specific lymphoproliferation expressed as SI. Results are shown for PBMCs and lymphocytes prepared from retropharyngeal lymph nodes, hilar lymph nodes and inguinal lymph nodes. Groups included naïve control ponies ($n = 4$), infection control ponies ($n = 4$), HA DNA vaccinated ponies ($n = 6$). Results are represented as mean \pm SEM. Different letters (a or b) indicate significantly different groups ($P < 0.05$).

Table 2
In vitro influenza virus-specific antibody production in different lymphoid tissues collected 14 days after infection or vaccination^a

Tissue	IgGa				IgGb			
	HI	IN	RP	NT	HI	IN	RP	NT
Infection control ponies	3/4	0/4	2/4	1/2	3/4	0/4	0/4	1/2
DNA vaccinates	0/6	6/6	3/6	1/6	1/6	5/6	0/6	1/6

^a Hilar lymphocytes (HI), inguinal lymphocytes (IN), retropharyngeal lymphocytes (RP) and nasal tissue lymphocytes (NT). IgA was produced by one pony of the infection group and one pony of the DNA vaccination group in nasal tissue lymphocytes only. No antibody was produced in the naïve control group ponies.

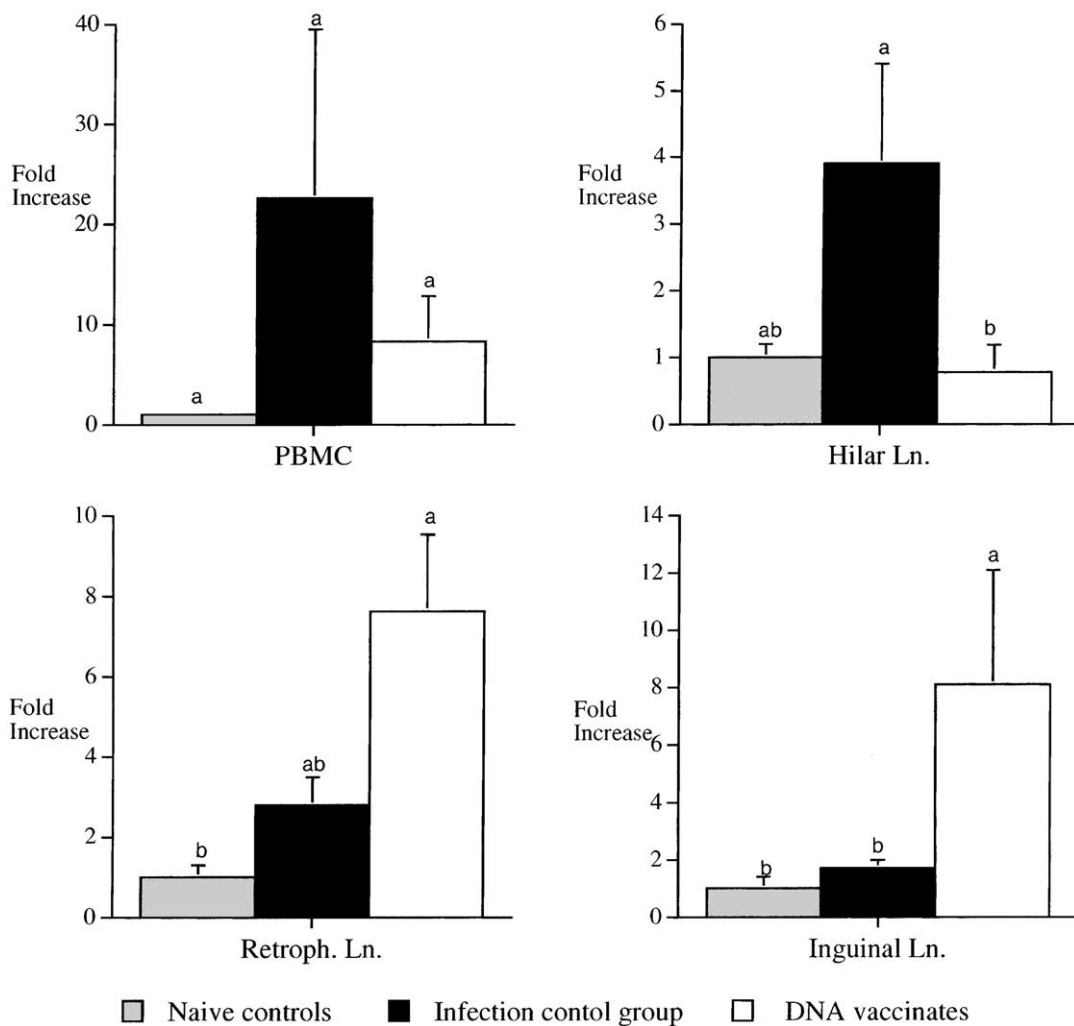


Fig. 4. Influenza virus-specific IFN- γ mRNA production determined by qRT-PCR and expressed as fold increase over levels in naïve ponies. Results are shown for PBMCs and lymphocytes prepared from retropharyngeal lymph nodes, hilar lymph nodes and inguinal lymph nodes. Groups included naïve control ponies ($n = 4$), infection control ponies ($n = 4$), HA DNA vaccinated ponies ($n = 6$). Results are represented as mean \pm SEM. Different letters (a or b) indicate significantly different groups ($P < 0.05$).

in the DNA vaccination groups. No antibody production was detected in any lymphocyte preparations from naïve control group ponies.

3.4.2. Influenza virus-specific lymphoproliferative responses

Influenza virus-specific lymphoproliferative responses are shown in Fig. 3. Both the DNA vaccination ponies as well as infection control ponies demonstrated significantly increased lymphoproliferative responses in PBMCs compared to naïve control group

ponies ($P = 0.0007$). In addition, infection control group ponies showed significantly increased lymphoproliferative responses in hilar lymph nodes compared to both naïve control group and DNA vaccinated ponies ($P = 0.011$). Similarly, in retropharyngeal lymph nodes, proliferation in infection control group ponies was significantly higher compared to naïve control group ponies, although infection control group ponies were not significant different from DNA vaccinated ponies. In inguinal lymph nodes, which drain a DNA vaccination site, DNA vaccination resulted in

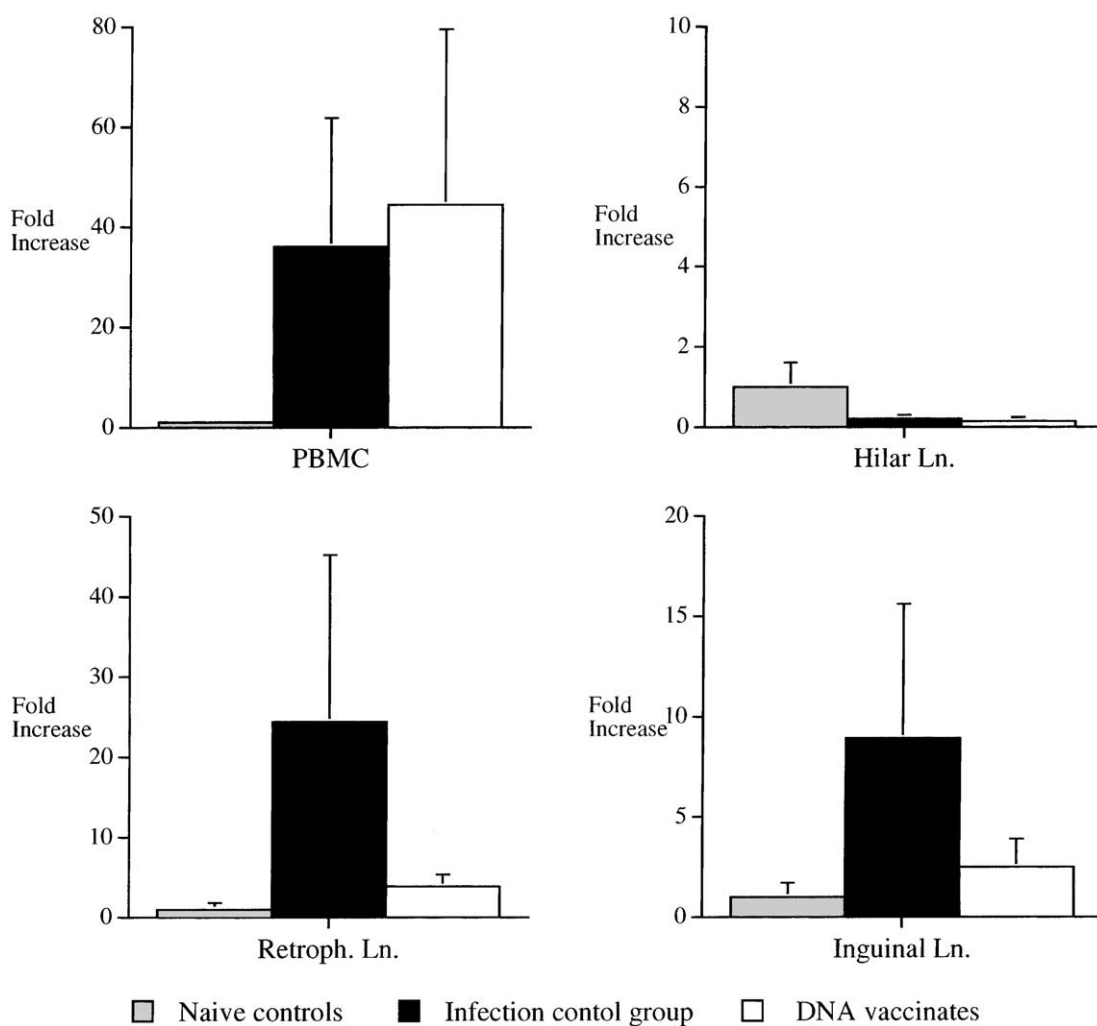


Fig. 5. Influenza virus-specific IL-4 mRNA production determined by qRT-PCR and expressed as fold increase over levels in naïve ponies. Results are shown for PBMCs and lymphocytes prepared from retropharyngeal lymph nodes, hilar lymph nodes and inguinal lymph nodes. Groups included naïve control ponies ($n = 4$), infection control ponies ($n = 4$), HA DNA vaccinated ponies ($n = 6$). Results are represented as mean \pm SEM.

significantly increased proliferative responses compared to both naïve and infection control group ponies ($P = 0.044$).

3.4.3. Cytokine mRNA levels after viral re-stimulation measured by qRT-PCR

IFN- γ responses are shown in Fig. 4 and followed a similar pattern to that seen for lymphoproliferative responses. IFN- γ mRNA responses in PBMCs and retropharyngeal lymph nodes were increased in infection control group ponies and DNA vaccinates when

compared to naïve control group ponies, but these differences were only statistically significant in retropharyngeal lymph nodes of DNA vaccinated ponies. Infection control group ponies also produced significantly increased IFN- γ mRNA responses in hilar lymph nodes when compared to DNA vaccinated ponies. In contrast, DNA vaccinates produced significantly increased IFN- γ mRNA responses in inguinal lymph nodes, while infection control group ponies showed no significant increases when compared to naïve control group ponies. IL-4 mRNA responses

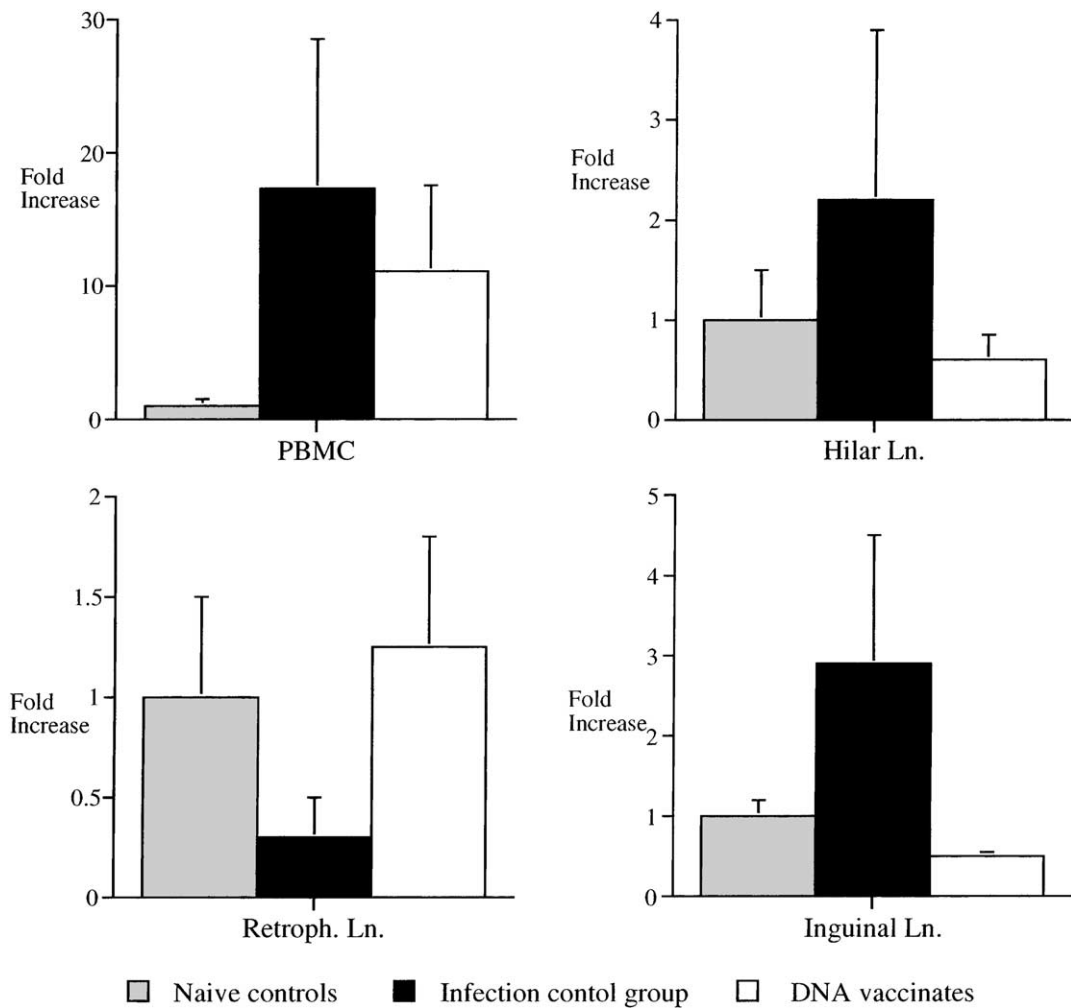


Fig. 6. Influenza virus-specific IL-2 mRNA production determined by qRT-PCR and expressed as fold increase over levels in naïve ponies. Results are shown for PBMCs and lymphocytes prepared from retropharyngeal lymph nodes, hilar lymph nodes and inguinal lymph nodes. Groups included naïve control ponies ($n = 4$), infection control ponies ($n = 4$), HA DNA vaccinated ponies ($n = 6$). Results are represented as mean \pm SEM.

were increased in PBMCs of both infection control group ponies and DNA vaccinated ponies and in retropharyngeal and to a lesser degree inguinal lymph node lymphocytes of infection control group ponies (Fig. 5). However, these responses were not statistically significant at a $P = 0.05$. Slight increases were observed for IL-2 mRNA responses, but these differences were not statistically significant (Fig. 6).

4. Discussion

This study provides an extensive examination of the distribution and type of immune responses following influenza virus infection or HA DNA vaccination. Previous work by our group (Lunn et al., 1999b; Nelson et al., 1998), and the experiments performed in this study, demonstrate that both influenza virus infection and DNA vaccination confer protection from homologous challenge infection. The current study shows that in both cases these protective immune responses are associated with influenza virus-specific antibody responses, and memory cells that proliferate and secrete cytokines or antibody upon *in vitro* re-stimulation with influenza virus. However, responses to infection and DNA vaccination differ markedly in terms of their regional distribution and mucosal antibody production.

In mice, immune responses to influenza virus infection are initiated in lymph nodes draining infection or vaccination sites (Klinman et al., 1998). The experiment reported here extends this observation to equids. Both influenza virus infection and DNA vaccination induced strong antigen-specific proliferative responses at sites of infection or vaccination. Influenza virus naturally infects the respiratory tract and proliferative responses were detected in PBMCs and the lymph nodes of the respiratory tract in infected ponies. In contrast, DNA vaccination was administered over skin and mucosal sites, including sites draining the inguinal lymph nodes. Fourteen days after vaccination proliferative responses were detected in PBMCs and inguinal lymph node lymphocytes, but not in respiratory tract lymph nodes.

When lymphoid tissue lymphocytes were examined for IFN- γ , IL-4, and IL-2 mRNA production following *in vitro* influenza virus re-stimulation, IFN- γ mRNA was the only cytokine that was significantly increased

compared to levels in the naïve control group ponies. This emphasizes the importance of this cytokine for protection from influenza virus in horses, and is consistent with findings in human studies (Tomoda et al., 1995). The distribution of IFN- γ mRNA production was similar to the distribution of lymphoproliferative responses. IFN- γ mRNA levels were increased in hilar lymph node lymphocytes of infected animals, while DNA vaccinates produced increased IFN- γ mRNA levels in retropharyngeal and inguinal lymphocytes, which drain sites of skin and mucosal vaccination. These results are consistent with studies in mice that showed that the vaccination route determines the location of influenza virus-specific cytokine producing cells (Novak et al., 1995; Okuda et al., 2001). Another study by our group, conducted in pigs, also demonstrated that after influenza virus infection IFN- γ production was increased at the systemic level (in the spleen) and in hilar lymph nodes (Larsen et al., 2000). There was a trend for increased IL-4 mRNA production in PBMCs of both infected and DNA vaccinated ponies, and in retropharyngeal lymph nodes of infection control ponies. However, these IL-4 changes were not significant, which may have resulted from selection of an inappropriate sampling time, or from high variability within experimental groups. Because of the importance of mucosal IgA in the upper respiratory tract, and the role of IL-4 in induction of IgA responses, an increase in IL-4 responses at these sites might have been anticipated. However, isolation of sufficient numbers of viable lymphocytes was difficult from this site.

As reported for proliferation and IFN- γ production, antibody production by local lymphocytes also differed dependent on the site of immune induction. Ponies of the infection group produced IgGa and IgGb in hilar lymph node lymphocytes, while in DNA vaccinates the inguinal lymph node lymphocytes produced IgGa and IgGb. Unfortunately, sufficient number of lymphocytes could not be collected at nasal and pharyngeal tissue sites from all animals, but data obtained from a limited number of ponies demonstrated local production of IgGa and IgGb in addition to IgA at these sites, which supports our previous observation of local IgG production in mucosal tissues (Lunn et al., 1999b; Soboll et al., 2003).

These results demonstrate that the induction sites of immune responses to influenza virus depend on the

route of administration. In the case of influenza virus infection the induction site and the site of immune defense are identical. However, DNA vaccination only directly targets some of the sites important for immune defense. Protection induced by DNA vaccination is associated with IgG_A and IgG_B responses and occurs in the absence of a primary nasal IgA response. This demonstrates that induction of only certain components of the complete immune response seen after influenza virus infection can provide protection. Consistent with this finding are studies in mice that show that passive transfer of either IgG or IgA are both capable of preventing infection (Tamura et al., 1990) and that mucosal IgA is not essential for protection from influenza virus infection (Mbawuike et al., 1999; Wong et al., 2001). Also, a number of recent studies have indicated that vaccines capable of inducing cytotoxic T lymphocytes or Th-1 cells protect mice from influenza virus challenge infection in the absence of any significant antibody (Johnson et al., 2000; Watabe et al., 2001). In light of these different immune responses, that are all associated with protection from challenge infection, a common denominator might be production of IFN- γ , which appears to be closely associated with all of these immune responses. We propose that, in the horse, protection from influenza virus infection is dependent on different immune mechanisms at the local and systemic level. At the systemic level, IgG_A and IgG_B antibody responses are associated with protection and may depend on an IFN- γ mediated TH-1 immune response. In contrast, at the mucosal level, production of secretory IgA is important, which typically depends on a TH-2 immune response.

Co-administration of IL-6 DNA with HA DNA failed to induce a primary IgA response but did influence the IgG isotype pattern following challenge infection, consistent with different priming of the immune responses at the systemic level. After challenge infection, the HA/IL-6 DNA vaccinates produced significantly higher levels of serum IgG(T) and lower levels of nasal IgG_B than the HA DNA vaccinates. Recent studies have indicated that the mechanisms regulating IgG_A/IgG_B responses differ from those regulating IgG(T), and that the IgG(T) isotype may be dependent on a Th-2 response (Slack et al., 1997). Also supportive of this proposal are results reported in a study examining the cytokine

profile of ponies vaccinated with irradiated L3 of *Strongylus vulgaris* (Swiderski et al., 1999a). Ponies vaccinated with the strongylus vaccine showed increased production of the TH-2 cytokines IL-4 and IL-5 that were associated with increased serum IgG(T) responses. In summary, our results indicate that DNA vaccination was inefficient in inducing local IgA production. Nevertheless, the co-administration of HA/IL-6 DNA may have caused a shift to a TH-2 like response in the systemic compartment, which was demonstrated by the IgG sub-isotype responses to challenge infection.

In conclusion, this study demonstrated that the induction of immune responses to influenza virus occurs at local sites of infection or vaccination. While influenza virus infection induces a complex set of immune responses resulting in protection, vaccine regimens like DNA vaccination, which induces only some of these immune responses, can also offer protection. The components of an immune response that protects from influenza virus infection seem to be different at the mucosal level and the distal airways. The common feature of different protective immune responses to equine influenza virus infection may be an IFN- γ response. Lastly, co-administration of IL-6 DNA and HA DNA failed to affect the immune response at the mucosal level, but it altered the IgG sub-isotype profile. Use of cytokine DNA as adjuvant offers means of manipulating immune responses, but the administration route and technique will be critical when trying to induce local immunity.

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