Safety, efficacy, and immunogenicity of a modified-live equine influenza virus vaccine in ponies after induction of exercise-induced immunosuppression

D. Paul Lunn, PhD; Steve Hussey, DVM; Randy Sebring, DVM; Keith E. Rushlow, PhD; Steve V. Radecki, PhD; Patricia Whitaker-Dowling, PhD; Julius S. Youngner, ScD; Thomas M. Chambers, PhD; Robert E. Holland Jr, PhD; David W. Horohov, PhD

**Objective**—To determine safety, efficacy, and immunogenicity of an intranasal cold-adapted modified-live equine influenza virus vaccine administered to ponies following induction of exercise-induced immunosuppression.

**Design**—Prospective study.

**Animals**—Fifteen 9- to 15-month-old ponies that had not had influenza.

**Procedure**—Five ponies were vaccinated after 5 days of strenuous exercise on a high-speed treadmill, 5 were vaccinated without undergoing exercise, and 5 were not vaccinated or exercised and served as controls. Three months later, all ponies were challenged by nebulization of homologous equine influenza virus. Clinical and hematologic responses and viral shedding were monitored, and serum and nasal secretions were collected for determination of influenza-virus-specific antibody isotype responses.

**Results**—Exercise caused immunosuppression, as indicated by depression of lymphocyte proliferation in response to pokeweed mitogen. Vaccination did not result in adverse clinical effects, and none of the vaccinated ponies developed clinical signs of infection following challenge exposure. In contrast, challenge exposure caused marked clinical signs of respiratory tract disease in 4 control ponies. Vaccinated and control ponies shed virus after challenge exposure. Antibody responses to vaccination were restricted to serum IgGa and IgGb responses in both vaccination groups. After challenge exposure, ponies in all groups generated serum IgGa and IgGb and nasal IgA responses. Patterns of serum hemagglutination inhibition titers were similar to patterns of IgGa and IgGb responses.

**Conclusions and Clinical Relevance**—Results suggested that administration of this MLV vaccine to ponies with exercise-induced immunosuppression was safe and that administration of a single dose to ponies provided clinical protection 3 months later. (J Am Vet Med Assoc 2001;218:900–906)

Equine influenza virus infection is a constant threat to the health of horses in North America. Control depends heavily on vaccination, but the currently available parenterally administered inactivated virus products frequently offer limited protection, resulting in recommendations that young susceptible horses be vaccinated as frequently as every 2 to 3 months. One reason for the limitations of currently available inactivated vaccines may be their failure to induce the appropriate spectrum of immune responses required for lasting and effective protection. We have recently demonstrated that influenza virus infection generates protective immune responses that depend on mucosal IgA and serum IgGa and IgGb responses, whereas a commercially available inactivated vaccine induced only a nonprotective serum IgG(T) response.

Modified-live virus (MLV) vaccines have the potential to induce all the appropriate components of an immune response necessary for protection, and a new MLV vaccine intended for nasal administration that contains a cold-adapted attenuated live influenza virus has recently been licensed for use to prevent equine influenza. The purpose of the study reported here was to determine the safety, efficacy, and immunogenicity of this vaccine in ponies. In addition, because reversion to virulence remains a concern with the use of MLV vaccines, we wanted to determine safety of this vaccine in ponies that were immunosuppressed prior to vaccination. An established protocol for exercise-induced immunosuppression was used.

**Materials and Methods**

**Ponies**—Fifteen unvaccinated 9- to 15-month-old ponies of mixed breed and either sex from a feral herd were used in the study. All ponies were seronegative for influenza virus infection prior to the study and were maintained in individual isolation rooms, fed a diet of hay and pelleted concentrates, and maintained in accordance with animal care guidelines of the University of Wisconsin Institutional Animal Care and Use Committee throughout the study.

**Experimental design**—Ponies were assigned to 1 of 3 groups with 5 ponies/group. Two groups were vaccinated by intranasal administration of a modified-live equine influenza virus vaccine by means of nebulization at the beginning of...
the study (day 0). The third group was maintained as an unvaccinated control group. One of the groups that was vacci-
nated was subjected to 5 days of strenuous exercise on a
high-speed treadmill prior to vaccination, with the final exer-
cise period ending several hours prior to administration of
the vaccine. The exercise regimen had previously been docu-
tmented to be immunosuppressive. Ninety-eight days after
vaccination, all ponies were challenge exposed to equine in-
fluenza virus and monitored for an additional 30 days.

After vaccination and again after challenge exposure, ponies
were examined, and nasal swab specimens were collected
daily for 14 days to detect viral shedding. Throughout the
study, clinical and hematologic responses were monitored,
and serum and nasal secretions were collected for determi-
nation of influenza-virus-specific antibody isotype responses.

Exercise regimen—Ponies in the exercise-vaccination

Exercise regimen consisted of exercising ponies on
the treadmill daily at incrementally increasing speeds and
incline as follows. Ponies were exercised for 3 minutes at a
speed of 1.5 m/s and then for 2 minutes at a speed of 3.5 m/s.
The treadmill was then inclined to 4°; and ponies were exer-
cised a further 2 minutes at 3.5 m/s, 2 minutes at 5.5 m/s, 2
min at 7.5 m/s, and 2 min at 9.5 m/s. The treadmill was
returned to level and briefly stopped for collection of postex-
ercise blood samples. The ponies were then cooled down by
walking for 2 minutes at 3.5 m/s and then for 5 minutes at
1.5 m/s. Heart rate was continuously monitored during exer-
cise, using electrocardiography and a telemetry device. The
objective of the exercise regimen was to increase heart rate to 200
beats/min or higher. The highest treadmill speed was not
used if ponies had already achieved the target heart rate of
200 beats/min or showed signs of exhaustion, and exercise
was instead continued for the same total time but at a lower
maximum speed (7.5 m/s).

On the first day of the exercise program, 60-ml jugular

venous blood samples were collected from each pony in the
exercise-vaccination program in syringes containing lithium
heparint (15 U/ml of blood) immediately before and after
exercise, for determination of lymphocyte responsiveness.
Similar paired samples were collected from ponies in the
nonexercise-vaccination group, with a similar time interval
between samples, to control for the effects of blood sample
collection on lymphocyte responsiveness. Each day of the
exercise program, jugular vein blood samples were collected
before and after exercise from ponies in the exercise-vaccina-
tion group into tubes containing sodium fluoride for mea-
surement of plasma lactate concentration by enzymatic
analysis.5 Samples were collected at the same times from
ponies in the nonexercise-vaccination group to control for the
effect of blood sample collection on plasma lactate con-
centration.

Measurement of lymphocyte proliferation—Peripheral

blood mononuclear cells were isolated from blood samples
by means of density gradient centrifugation and resus-
pended at a concentration of 106 cells/ml in medium consist-
ing of RPMI-1640 containing 0.2% sodium bicarbonate,
0.01M Hepes, 0.002M sodium pyruvate, penicillin, strepto-
mycin, and 10% fetal bovine serum. For determination of
lymphocyte proliferation, cells were plated (200 µl cells/well)
in triplicate into wells on 96-well microtitration plates6 to
which pokeweed mitogen antigen7 was added at final con-
centrations of 4, 2, 1, or 0 µg/ml, with or without a subopti-
mal dose (10 U/ml) of human recombinant interleukin-2.2
Plates were incubated in a humidified 5% CO2 atmosphere at

37 C for 72 hours. Fifty microliters of radioactive thymidine
(0.02 mCi/ml) were then added to each well, and 10 hours
later, thymidine uptake was measured (counts per minute;
cpm) by use of a microplate scintillation and luminescence
counter system.

Vaccination and challenge exposure procedure—On
day 0, ponies in the 2 vaccination groups were vaccinated
with a dose of the cold-adapted MLV vaccine strain virus
(A/equine/2/Kentucky/91) equivalent to that contained in a
single dose of the commercially available product. The virus
was suspended in phosphate-buffered saline solution and
administered by means of nebulization, using an open face-
mask and a disposable nebulizer unit8; nebulization took 20
minutes. Ponies were sedated for this procedure by adminis-
tration of xylazine (0.5 mg/kg [0.23 mg/lb] of body weight,
IV).

On day 98, all ponies were challenge exposed to a wild-
type isolate of equine influenza virus (A/equine/2/Kentucky/91).
Virus was grown in 11-day-old embryonated chicken eggs for
48 to 72 hours at 35 C, and allantoic fluid was harvested. For
challenge exposure, the virus was administered by means of
nebulization. Ponies were sedated, and 103 median egg infective
doses in 5 ml of phosphate-buffered saline solution was admin-
istered over a 15-minute period by use of a face-mask.

Sample collection—Venous blood samples were collect-
ed into tubes containing EDTA 2 days before, daily for 6 days
after, and again 10 and 14 days after vaccination and chal-
lenge exposure. Total WBC counts were determined by use of
an automated analyzer; differential WBC counts were deter-
mained manually. Serum was obtained and stored at –20 C
until analyzed for antibody responses.

Nasal secretions were collected, as described,1 by placing
a cotton tampon in the ventral nasal meatus for at least 15
minutes and subsequently centrifuging the tampon to recov-
er the nasal secretions. Narrow tampons that were easily
inserted and removed from the nasal meatus were used, and
hemorrhage was never associated with this procedure.

Samples of nasal secretions were stored at –20 C until ana-
lyzed for antibody responses.

For virus isolation, swabs9 were used to collect nasal
secretions. Swabs were stored in 1 ml of virus transport medi-
num at –70 C until tested. Samples were assayed for infectious
virus by inoculation (in triplicate) into the allantoic cavities
of 10-day-old embryonated chicken eggs. After 3 days of
incubation at 37 C, allantoic fluid was harvested and exam-
ined for hemagglutination activity.10

Determination of antibody responses—Nasal secre-
tions were assayed for influenza-virus-specific IgA titers,
and serum samples were assayed for IgGa, IgGb, and IgG(T)
titers by use of an ELISA, as described.1 An influenza virus
(A/Equine/2/Kentucky/91) isolated at and obtained from the
University of Kentucky was used for the ELISA. The virus
was concentrated from allantoic fluid by means of polyethyl-
ene glycol precipitation and purified by means of sucrose gra-
dient centrifugation, as described.13 Purified influenza virus
was coated onto wells on 96-well polystyrene plates14 at a con-
centration of 10 hemagglutinin (HA) units/well. Plates were
then incubated overnight at 4 C, washed with phosphate-
buffered saline solution containing 0.05% Tween, and
blocked with 1% telostean gelatin in phosphate-buffered
saline solution for 1 hour at 37 C. Serum samples were dilut-
ed in gelatin in phosphate-buffered saline solution and added
to the plates, and plates were incubated for 90 minutes at 37
C. Each plate included control wells consisting of a serum
tube negative for influenza-specific antibodies and a series of
dilutions of a serum sample with a known equine anti-
influenza virus titer. For assay of IgA concentration in nasal

JAVMA, Vol 218, No. 6, March 15, 2001 Scientific Reports: Original Study 901

Unauthenticated | Downloaded 07/31/24 10:09 PM UTC
secretions, samples were pretreated to reduce the mucin content by addition of 10 mM 1,4-dithiothreitol and incubated at 37°C for 30 minutes before being diluted with gelatin in phosphate-buffered saline solution.

After incubation, plates were washed, and equine immunoglobulin isotype-specific monoclonal antibody supernatants diluted in phosphate-buffered saline solution to a final antibody concentration of 0.01 mg/ml were added to the wells. Monoclonal antibodies had been previously characterized as specific for equine IgGa (WS29), IgGb (WS13), IgG(T) (WS30), and IgA (WS15). Plates were incubated for 90 minutes at 37°C and washed, and peroxidase-conjugated goat anti-mouse IgG and IgM(H+L) was added. Color was developed by addition of 3,3′,5,5′-tetramethybenzidine substrate, and the reaction was stopped by addition of H3PO4. Absorbance of each well was determined spectrophotometrically with an ELISA reader. Titors of samples were determined by comparison with a standard curve constructed using software supplied with the ELISA reader. Serum hemagglutination inhibiting (HI) antibody titers were measured, as described.

Statistical analysis—Severity of clinical signs was compared among groups by assigning ponies to 1 of 3 disease categories: no abnormal clinical signs, mild clinical disease (mucoid nasal discharge, infrequent cough), and marked clinical disease (severe mucopurulent nasal discharge, paroxysmal coughing). Differences among groups were analyzed by use of the Fisher exact test.

Other data were analyzed by use of a repeated-measures mixed-model approach in which horse was used as the experimental unit (random effect). Compound symmetry was assumed for the structure of the covariance matrix. Significance was defined as P < 0.05. The difference between post- and preexercise plasma lactate concentrations was calculated for each pony on each day, and these differences were compared across time. For analysis of lymphocyte proliferation data, cpm were log transformed prior to analysis. The relationship between exercise and concentration of mitogen was evaluated by use of a repeated-measures approach. Rectal temperature, total WBC count, lymphocyte count, antibody titers, and HI titers were analyzed by use of a repeated-measures approach. If the interaction between day and group was significant, pairwise differences between control and vaccinated groups were estimated by calculating least significant differences for each day. With the exception of rectal temperature, data were log transformed.

Results

Response to exercise regimen—The exercise regimen was successful in that ponies achieved their target heart rates each day. Plasma lactate concentrations after exercise were significantly (P < 0.01) higher than concentrations before exercise (Fig 1). Analysis of plasma lactate concentrations in ponies that did not exercise indicated that blood sample collection had no effect on plasma lactate concentrations.

Analysis of lymphocyte proliferation data indicated that exercise significantly reduced the mitogenic response to pokeweed antigen (Fig 2). However, when recombinant human interleukin-2 was included in the assay, mitogenic responses to pokeweed antigen after exercise were not significantly (P = 0.1) different from responses before exercise. In both assays, there was no significant interaction between pokeweed mitogen concentration and exercise. Analysis of lymphocyte proliferation data for ponies that did not

Figure 1—Mean plasma lactate concentration in 5 ponies before (gray bars) and after (white bars) exercise performed daily for 5 days prior to vaccination against equine influenza virus infection. Error bars represent SEM. *Significantly (P < 0.01) different from preexercise value.

Figure 2—Mean lymphocyte proliferation (measured as counts per minute [cpm] following tritiated thymidine incorporation) in 5 ponies in response to stimulation of lymphocytes with various concentrations of pokeweed mitogen before (gray bars) and after (white bars) exercise performed daily for 5 days. Error bars represent SEM. *Significantly (P < 0.05) different from preexercise value.

Exercise indicated that blood sample collection had no effect on results.

Clinical and hematologic responses—Vaccination did not result in abnormal clinical signs in any of the ponies, and no changes in total and differential WBC counts were detected. Challenge exposure on day 98 did not cause any abnormal clinical signs in ponies in either vaccination group (all 10 ponies were classified as disease category 1). However, 4 control ponies were classified as disease category 3 (marked clinical signs), and only 1 was classified as disease category 1, and the proportion of control ponies with marked clinical disease was significantly higher than the proportion of ponies in the 2 vaccination groups with marked clinical disease. Rectal temperature was also significantly higher in control ponies on days 100, 101, and 102 than in ponies in either of the vaccination groups.
Clinical signs of disease in the affected control ponies included copious mucopurulent nasal discharge for 5 to 8 days and coughing for 1 to 3 days after challenge exposure.

Challenge exposure resulted in leukopenia of 1 day's duration in both vaccination groups, compared with leukopenia of 5 day's duration in the control group (Fig 4). Control group ponies had significantly lower total WBC counts on days 101 through 104, compared with the 2 vaccination groups. This leukopenia was primarily the result of lymphopenia, and lymphocyte count for the control group ponies was significantly lower on days 100 through 104, compared with the 2 vaccination groups.

Viral shedding—After vaccination, virus was detected in nasal secretions from 3 of the 5 ponies that were vaccinated after exercise (1 pony shed virus for 3 days, 1 shed virus for 4 days, and 1 shed virus for 2 days) and 4 of the 5 ponies that were only vaccinated (2 shed virus for 7 days and 2 shed virus for 1 day). After challenge exposure, virus was detected in nasal secretions for 4 of the 5 ponies that were vaccinated after exercise (1 shed virus for 3 days, 2 shed virus for 2 days, and 1 shed virus for 1 day), 3 of the 5 ponies that were only vaccinated (1 shed virus for 3 days, and 2 shed virus for 2 days), and 4 of the 5 control ponies (1 shed virus for 5 days, 1 shed virus for 4 days, 1 shed virus for 3 days, and 1 shed virus for 2 days).

Antibody responses—Antibody responses to vaccination were restricted to the IgGa and IgGb subiso-
EQUINE

Titers were low (Fig 5); however, for both vaccination groups, serum IgGa titers on days 7 through 91 were significantly ($P < 0.01$) greater than titers obtained prior to vaccination (day 0), and serum IgGb titers on days 14 through 91 were significantly ($P < 0.01$) greater than titers obtained prior to vaccination. Titers were not significantly different between the 2 vaccination groups.

After challenge exposure, ponies in all 3 groups generated serum IgGa and IgGb and nasal IgA responses. Compared with day 91 values (ie, values obtained immediately before challenge exposure), serum IgGa and IgGb titers were significantly increased on days 105 through 126 in all 3 groups. Nasal IgA titers were significantly increased on days 105 through 126 in all 3 groups, and there were no significant differences among groups after challenge exposure. However, the control group had significantly lower serum IgGa and IgGb titers on day 91 (ie, immediately before challenge exposure) than did either vaccination group. There were no serum IgG(T) responses to vaccination or challenge exposure in any of the groups.

Patterns of serum HI titers were similar to patterns of serum IgGa and IgGb titers, with low titers following vaccination and high titers following challenge exposure (Fig 6). For both vaccination groups, HI titers on days 21, 28, and 91 were significantly ($P < 0.01$) higher than titers prior to vaccination. After challenge exposure, HI titers were significantly increased in all 3 groups, compared with titers on day 91, and significant differences among groups were not detected. On day 91, the control group had significantly lower HI titers than did either vaccination group.

Discussion

Results of the present study suggested that administration of a single dose of the MLV vaccine to ponies that had not previously been exposed to equine influenza virus provided clinical protection against challenge exposure with homologous equine influenza virus 3 months later. By contrast, administration of a commercially available killed-virus vaccine did not provide protection in previous experimental and field studies. However, use of this MLV vaccine did not prevent viral shedding following challenge exposure 3 months after vaccination. This demonstrates the continued importance of maintaining immunity in all members of a population of horses if spread of influenza virus infection is to be controlled by vaccination.

Although MLV vaccines are often successful in generating immunity, they can be dangerous in immunocompromised hosts and carry the risk of reversion.
pression to pathogenicity. Therefore, safety of this MLV vaccine was an important focus of this study. For this reason, a dose of the MLV equivalent to that in a dose of the commercial product was administered by nebulizer to ensure maximal exposure throughout the respiratory tract. Prior to vaccination, 1 group of ponies was subjected to several days of exercise stress to depress lymphocyte mitogenesis. However, ponies that were exercised prior to vaccination did not show any adverse effects of vaccination, shed vaccine virus for no longer than ponies that were only vaccinated, and developed the same protective immune responses to vaccination. Maximal exercise in unconditioned horses is an established model for inducing immunosuppression and provides a practical example of the adverse circumstances under which vaccination may be performed in the field. Using this exercise regimen, we were able to find no evidence of modulation of the immunogenicity or safety of the MLV vaccine.

In the present study, an immune response to vaccination was detectable 2 weeks after administration of the vaccine as an increase in serum IgGa and IgGb antibody titers and serum HI titers. However, these antibody responses were of a low level, compared with those resulting from subsequent challenge exposure. The IgGa response peaked 2 to 4 weeks after vaccination and then declined over the subsequent 2 months. The IgGb response peaked approximately 4 weeks after vaccination and was maintained until the time of challenge exposure. The HI response was similar to the IgGb response. Despite these low titers in response to vaccination, clinical protection 3 months after vaccination was complete. By contrast, the influenza-virus-specific serum antibody response to killed-virus vaccines, as determined on the basis of HI titer or, more quantitatively, by means of single radial hemolysis assay, has a strong correlation with protection from challenge exposure.

Long-lasting protection from influenza virus infection in naturally infected horses persists even when antibody titers have waned. In addition, vaccination of horses with a DNA-based vaccine containing the hemagglutinin gene resulted in low antibody titers but still provided protection from clinical disease. Thus, although high antibody titers are desirable, particularly after use of killed-virus vaccines, it is clear that the relationship between protection from equine influenza virus infection and serum antibody titers after vaccination is not always a simple correlation.

Another important factor is the isotype of antibody response induced by vaccination. The antibody response to MLV vaccine administration in the present study was restricted to the IgGa and IgGb subisotypes, and no IgG(T) response was detectable. This is similar to the pattern of IgG(T) response after influenza virus infection, which is associated with protection from subsequent challenge exposure.

Intranasal administration of modified-live influenza virus vaccines in people leads to generation of nasal secretory IgA responses. In the present study, on the other hand, intranasal administration of the MLV vaccine did not result in any detectable primary nasal IgA response or anamnestic response after challenge exposure. The reason for this is uncertain, but in a previous study involving vaccination of ponies with a DNA-based vaccine, it was shown that generation of nasal IgA responses required productive infection with the challenge virus. Therefore, we speculate that the degree of stimulation of the local mucosal immune system by a single dose of the MLV vaccine in ponies that had not previously been exposed to equine influenza virus may have been inadequate to induce a detectable IgA response. It is possible that additional booster doses of the MLV vaccine may result in primary nasal IgA responses and higher amplitude serum IgG responses.

Overall, the association of clinical protection with limited serum IgGa and IgGb responses in the present study further supports the association of these subisotype responses with protective immunity against equine influenza virus infection. In addition, results of the present study demonstrate that protection cannot be predicted simply on the basis of serum antibody titers after vaccination and other indices of immunologic response may need to be assayed to determine immune status.

References


JAVMA, Vol 218, No. 6, March 15, 2001
Scientific Reports: Original Study


