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

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**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, whereas a commercially available inactivated vaccine induced only a nonprotective serum IgG(T) response. A modified-live virus (MLV) vaccine has 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 vaccinated was subjected to 5 days of strenuous exercise on a high-speed treadmill prior to vaccination, with the final exercise period ending several hours prior to administration of the vaccine. The exercise regimen had previously been documented to be immunosuppressive.

Ninety-eight days after vaccination, all ponies were challenge exposed to equine influenza 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 determination of influenza-virus-specific antibody isotype responses.

**Exercise regimen**—Ponies in the exercise-vaccination group were acclimatized to a high-speed treadmill by walking them through the treadmill room for several days and then walking them on the treadmill for 5 minutes on 3 occasions.

The 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 exercised a further 2 minutes at 3.3 m/s, 2 minutes at 3.5 m/s, 2 minutes at 3.7.5 m/s, and 2 minutes at 4 m/s. The treadmill was returned to level and briefly stopped for collection of postexercise 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 exercise, using electrodes 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 vein blood samples were collected from each pony in the exercise-vaccination group into syringes containing lithium heparin (15 U/ml of blood) immediately before and after exercise, for determination of peripheral lymphocyte proliferation. 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-vaccination group into tubes containing sodium fluoride for measurement of plasma lactate concentration by enzymatic analysis.

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 concentration.

**Measurement of lymphocyte proliferation**—Peripheral blood mononuclear cells were isolated from blood samples by means of density gradient centrifugation and resuspended at a concentration of 10⁶ cells/ml in medium consisting of RPMI-1640 containing 0.2% sodium bicarbonate, 0.01M Hepes, 0.002M sodium pyruvate, penicillin, streptomycin, 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 plates to which pokeweed mitogen antigen was added at final concentrations of 4, 2, 1, or 0 µg/ml, with or without a suboptimal dose (10 U/ml) of human recombinant interleukin-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.

**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 unit; nebulization took 20 minutes. Ponies were sedated for this procedure by administration 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 33°C, and allantoic fluid was harvested. For challenge exposure, the virus was administered by means of nebulization. Ponies were sedated, and 10⁶ median egg infective doses in 5 ml of phosphate-buffered saline solution was administered over a 15-minute period by use of a face-mask.

**Sample collection**—Venous blood samples were collected into tubes containing EDTA 2 days before, daily for 6 days after, and again 10 and 14 days after vaccination and challenge exposure. Total WBC counts were determined by use of an automated analyzer; differential WBC counts were determined manually. Serum was obtained and stored at −20°C until analyzed for antibody responses.

Nasal secretions were collected, as described, by placing a cotton tampon in the ventral nasal meatus for at least 15 minutes and subsequently centrifuging the tampon to recover 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 analyzed for antibody responses.

For virus isolation, swabs were used to collect nasal secretions. Swabs were stored in 1 ml of virus transport medium 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 examined for hemagglutination activity.

**Determination of antibody responses**—Nasal secretions 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. 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 polyethylene glycol precipitation and purified by means of sucrose gradient centrifugation, as described. Purified influenza virus was coated onto wells on 96-well polystyrene plates at a concentration 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% teleostean gelatin in phosphate-buffered saline solution for 1 hour at 37°C. Serum samples were diluted 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 sample 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
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'-tetramethylbenzidine substrate, and the reaction was stopped by addition of H3PO4. Absorbance of each well was determined spectrophotometrically with an 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 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 days’ 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-
types in both vaccination groups. Titers were low (Fig 5); however, for both vaccination groups, serum IgGa titers on days 7 through 91 were significantly ($P < 0.01$) higher 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 rever-
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 subisotype 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 expo-

References


