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Comparison of protective efficacies between intranasal and intramuscular vaccination of horses with a modified live equine herpesvirus type-1 vaccine

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ABSTRACT

Immune responses were compared after intranasal (IN) and intramuscular (IM) vaccination of horses with a modified live equine herpesvirus type-1 (EHV-1) vaccine, and the protective effect after EHV-1 challenge was evaluated. IN- and IM-vaccinated groups (n = 5 each) showed significant rises in serum virus-neutralizing titers with increased levels of IgGa and IgGb antibodies after the first vaccination ($P < 0.05$). In nasal secretions, the IN group had significantly increased levels of IgA antibodies after vaccination ($P < 0.05$), whereas the response of the IM group was dominated by IgGa and IgGb subclasses. After challenge infection, the numbers of pyretic horses from 1 to 4 days post-inoculation were 3/5 in the placebo (PBO) group (n = 5), 0/5 in the IN group, and 1/5 in the IM group. The IN and IM groups had significantly lower levels of virus shedding than the PBO group ($P < 0.05$). There were no significant between-group differences in the numbers of viremic horses each day. Notably, two horses in the IM group had no virus shedding or viremia, whereas all horses in the other group did. Both IN and IM vaccination induced systemic humoral immunity and mucosal immunity, suppressing virus replication in the nasal mucosa, and partially protected horses from pyrexia, especially early in infection. This study showed a mucosal antibody response was induced, not only by IN vaccination but also by IM vaccination.

1. Introduction

Equine herpesvirus type-1 (EHV-1) infection occurs initially in the nasal cavity (Allen et al., 1999). After virus entry to the mucosal epithelial cells, virus-infected lymphocytes are transferred to the lymph nodes and the virus spreads to the whole body via the blood circulation (Allen et al., 1999). In this manner, horses undergo a viremic phase, and in some cases the infection results in serious outcomes such as abortion or neurological disorders (Allen et al., 1999). There are two time periods in which pyrexia is frequently observed after experimental infection of horses with EHV-1 (Heldens et al., 2001; Goodman et al., 2006; Goehring et al., 2010). The first is in the early phase of infection— generally within 4 days after virus inoculation, which corresponds to massive viral replication in the nasal mucosa. The second is in the viremic phase, generally from 5 to 10 days after virus inoculation. Because the nasal cavity is the initial site of infection, mucosal immunity plays an essential role in protecting horses from disease caused by EHV-1 infection.

Intranasal (IN) vaccination is a promising way to induce mucosal immunity for the control of respiratory viral infections (Neutra and Kozlowski, 2006). In the veterinary field, many vaccine products, including an equine influenza virus vaccine (FluAvert IN vaccine, Merck Animal Health, Intervet Inc., Madison, NJ, USA) and an EHV-1 vaccine (Calvenza EHV, Boehringer Ingelheim Vetmedica, Inc., Duluth, GA, USA), are licensed for use by the IN route. However, not many of them were supported by sufficient data showing the capacity to induce mucosal immunity and the protective effect. Many research groups have attempted IN vaccination for EHV-1 (Slater et al., 1993; Tewari et al., 1993; Breathnach et al., 2001; Patel et al., 2003a, 2003b, 2004), and some have demonstrated protective effects after challenge infection, in terms of infrequent occurrence of pyrexia and clinical signs and reduced virus shedding. However, the nasal antibody response that represents mucosal immunity was not measured in most of these studies, because they focused mostly on the serum antibody response and the clinical outcomes after challenge infection.

As reported previously, EHV-1 is a major cause of pyrexia in winter among Japanese racehorses in training facilities (Matsumura et al., 1992; Bannai et al., 2014). To control EHV-1 infection among racehorses, a modified live EHV-1 vaccine with deletion of the *glycoprotein E* gene (Equine Rhinopneumonitis Vaccine [Live], Nisseiken Co. Ltd., Tokyo, Japan) has been used via intramuscular (IM) administration. Its safety *in vivo* when used by the IN route was confirmed previously: vaccinated foals showed no

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obvious clinical signs or viremia (Tsujimura et al., 2009). However, the protective effect of this vaccine by the IN route has not yet been assessed. Here, we compared the immune responses after IN and IM vaccination of horses with Equine Rhinopneumonitis Vaccine (Live), and we evaluated the protective effects of the vaccine after challenge infection with EHV-1.

2. Materials and methods

2.1. Cell culture

A primary culture of fetal horse kidney (FHK) cells was used for propagation and titration of EHV-1. For virus-neutralization (VN) testing we used Madin-Darby bovine kidney (MDBK, ATCC #CCL-22) cells. For virus isolation we used rabbit kidney (RK-13, Sumitomo Dainippon Pharma, Tokyo, Japan) cells. Cells were cultured in minimum essential medium (MEM, MP Biomedicals, Irvine, CA, USA) containing 10% fetal calf serum (FCS, Sigma Aldrich Inc., St. Louis, MO, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma Aldrich Inc.). MEM containing 2% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin was used as a maintenance medium for VN testing and virus isolation. MEM containing 5% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% methylcellulose was used as an overlay medium for virus titration.

2.2. EHV-1 strains and EHV-1 vaccine

We used EHV-1 strain 10-I-224 for the challenge experiment, strain 89C25p for VN testing, and strain HH-1 for IgG subclass and IgA ELISAs. Strain 10-I-224 was isolated from a pyretic horse during an outbreak of respiratory disease at an equestrian facility in Japan in 2010. Strain 89C25p was isolated from a racehorse during an outbreak of respiratory disease at a training facility in Japan in 1989 (Matsumura et al., 1992); it is the parental strain of a modified live vaccine (Tsujimura et al., 2006). Strain HH-1 was isolated from an aborted fetus in Japan in 1967 (Kawakami et al., 1970). Equine Rhinopneumonitis Vaccine (Live) (Nisseiken Co., Ltd) was used for vaccination of horses. The vaccine does not contain adjuvant, and the modified live virus is suspended with a phosphate-buffered saline (PBS) provided by the manufacturer. It is licensed with a protocol that horses receive two doses of IM inoculation with a 4-weeks interval.

2.3. Horses

Fifteen female Thoroughbred horses (16–21 months old) were used. About 1 month before the start of the experiment, the horses were tested for antibodies with glycoprotein E1-ELISA for EHV-1 and glycoprotein G4-ELISA for EHV-4, as described previously (Andoh et al., 2013; Bannai et al., 2016). All horses had pre-existing antibodies to EHV-1 and EHV-4 at the starting time point, suggesting that they had undergone previous natural exposure to these viruses. They had no history of vaccination with EHV-1 vaccine before the experiment. The horses were allocated to three groups, namely IN vaccination, IM vaccination, and placebo (PBO) groups (n = 5 each); there were no significant differences in the geometric mean (GM) VN titers between the groups.

2.4. Vaccination and challenge experiment

Horses in the IN group (n = 5) were intranasally inoculated with the vaccine (one dose/head). Briefly, one dose of vaccine was suspended in 10 ml of MEM, and 5 ml was inoculated into each nostril via a syringe-connected nebulizer (a spray nozzle connected with a metal tube of 5 cm length and 1 mm diameter [own-made], which is attachable to a syringe). Horses in the IM group (n = 5) were inoculated intramuscularly with the vaccine (one dose/head) into the neck. Horses in the PBO group (n = 5) received intranasal inoculation of MEM. The second vaccination was performed 4 weeks later in the same manner as described above. Horses were challenged with EHV-1 strain 10-I-224 (4×10^6 plaque-forming units [pfu]/head) 4 weeks after the second vaccination, because this period was likely at the peak of immunity. Serum and nasal wash samples were collected at 1-week intervals from the time of the first vaccination to the end of the experiment. Nasal swabs and peripheral blood mononuclear cells (PBMCs) were collected every day after virus challenge. Rectal temperatures were recorded each afternoon after challenge, and clinical signs associated with EHV-1 infection, including pyrexia, nasal discharge, and swelling of submandibular lymph nodes, were monitored. Horses with temperatures ≥38.5 °C were regarded as pyretic. Nasal discharge was graded as follows: 0, no discharge; 1, serous discharge; 2, mucopurulent discharge; 3, severe mucopurulent discharge. Swelling of the submandibular lymph nodes was graded as follows: 0, hardly palpable nodes; 1, slightly palpable nodes; 2, easily palpable nodes; 3, enlarged painful nodes. Cumulative scores of each clinical sign were calculated. All experimental procedures were approved by The Animal Care Committee of the Equine Research Institute of the Japan Racing Association with the accession numbers 15-1, 15-7, and 17-1.

2.5. Virus neutralization testing for EHV-1

Virus neutralization (VN) testing for EHV-1 was performed with a focus-reduction method using MDBK cells and EHV-1 strain 89C25p, as described previously (Bannai et al., 2013). Serial two-fold diluted sera starting from a dilution of 1:10 were tested, and the antibody titer was expressed as the reciprocal of the highest dilution that reduced the number of foci by at least 50% compared with that of the virus control.

2.6. EHV-1-specific IgG subclass ELISA and IgA ELISA

An ELISA antigen was prepared as follows. EHV-1 strain HH-1 propagated in RK-13 cells was concentrated by treatment with 10% (w/v) polyethylene glycol (Sigma Aldrich Inc.) and 3% (w/v) sodium chloride (Wako Pure Chemicals Industries, Osaka, Japan), as described previously (Sugiura et al., 1987). The resulting product, which contained approximately 1×10^9 pfu/ml, was treated with 0.5% (w/v) N-lauroylsarcosine (MP Biomedicals, Inc., Aurora, OH, USA) for 30 min at 4 °C, and was then used as an antigen for IgG subclass and IgA ELISAs. Antigen diluted with 0.05 M carbonate–bicarbonate buffer (pH 9.6) at a protein concentration of 40 μg/ml was seeded onto a 96-well plate (Nunc Maxisorp, Thermo Scientific, Roskilde, Denmark). As blank controls, wells without antigen were also prepared. The plates were stored at 4 °C overnight for antigen adsorption. After being washed three times with PBS containing 0.05% Tween20 (Wako Pure Chemicals Industries) (PBST), the wells were treated with 100 μl of a diluent consisting of PBS containing 1% skim-milk (Wako Pure Chemicals Industries) for blocking. At each subsequent step, the plates were incubated at 37 °C for 1 h and washed three times with PBST. After reaction of the wells with diluted sera (1:200 for IgGa, IgG(T) and IgA; 1:2000 for IgGb, 50 μl/well), horseradish-peroxidase-conjugated goat anti-horse IgGa, IgGb, IgG(T), or IgA polyclonal antibodies (Bethyl Lab., Inc., Montgomery, TX, USA) diluted at 1:5000 (50 μl/well) were added. Color development was performed with TMB peroxidase substrate (Moss, Inc., Pasadena, MD, USA), and the optical density (OD) at a wavelength of 450 nm was measured. Final OD values were derived by subtracting the values for wells without antigen from those for wells with antigen.

For the nasal washes, PBS containing 2% skim milk and 0.4% skim milk was used as a blocking buffer and diluent, respectively. Total IgA concentrations in each nasal wash sample were determined with a Horse IgA ELISA Quantification Set (Bethyl Lab., Inc.); the samples were adjusted with the diluent to contain 20 μg total IgA/ml for application to ELISA.

2.7. Virus isolation from nasal swabs and PBMCs

Nasal swabs from both nostrils were suspended in 5 ml of FCS by