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A protective effect of epidermal powder immunization in a mouse model of equine herpesvirus-1 infection

Takashi Kondo,[a,b] Martha McGregor,[b] Qili Chu,[c] Dexiang Chen,[c] Taisuke Horimoto,[d,e] and Yoshihiro Kawaoka[b,d,e,*]

[a] *Epizootic Research Station, Japan Racing Association, Equine Research Institute, Tochigi 329-0412, Japan*
[b] *Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI 53706, USA*
[c] *PowderJect Vaccines, Inc., Madison, WI 53711, USA*
[d] *Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan*
[e] *Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, Saitama 332-0012, Japan*

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Abstract

To evaluate the protective effect of epidermal powder immunization (EPI) against equine herpesvirus-1 (EHV-1) infection, we prepared a powder vaccine in which formalin-inactivated virions were embedded in water-soluble, sugar-based particles. A PowderJect device was used to immunize mice with the powder vaccine via their abdominal skin. We found that twice-immunized mice were protected against challenge with the wild-type virus. This protective effect was equivalent to or better than that observed in mice immunized with other types of vaccines, including a gene gun-mediated DNA vaccine containing the glycoprotein D (gD) gene or conventional inactivated virus vaccines introduced via intramuscular or intranasal injections. These findings indicate that the powder vaccine is a promising approach for the immunological control of EHV-1 infection, either alone or as a part of prime-boost vaccination strategies.

Keywords: Equine herpesvirus-1; Epidermal powder immunization; Vaccine

Introduction

Equine herpesvirus-1 (EHV-1), which causes respiratory diseases, abortion, occasional neurological signs, and neonatal mortality, is enzootic in horses worldwide and a significant economic burden to the horse industry (Allen and Bryans, 1986; Crabb and Studdert, 1995). As with other herpesvirus infections, lifelong latency of the virus in the nervous system of the host occurs, and periodical reactivation of the virus can cause new outbreaks (Slater et al., 1994).

Because EHV-1 initiates its infection from the mucosal surface, a combination of mucosal and systemic antibody and cytotoxic T-lymphocyte (CTL) responses is required for successful immunological control of EHV-1 infection (Allen et al., 1999). Inactivated virus vaccines, which have been widely used for EHV-1 infection, are known to render only limited efficacy (Burrows et al., 1984; Gilkerson et al., 1997). Therefore, the development of more efficacious vaccines is essential to protect animals from both primary and reactivated EHV-1 infections. One approach has been DNA vaccination with glycoprotein (gp)-coding sequences because of its potential to induce both humoral and cell-mediated immunities (Fynan et al., 1993). Intramuscular (i.m.) inoculation of mice with glycoprotein D (gD) DNA has been shown to protect them from challenge with wild-type viruses and to induce higher levels of gD-specific antibodies in horses with pre-existing EHV-1/-4 antibody (Ruitenberg et al., 2000a). Moreover, a prime-boost immunization strategy of inoculation with gD DNA followed by purified gD protein has been shown to enhance both humoral and cell-mediated immune responses (Ruitenberg et al., 2000b).

Another promising technique is epidermal powder immunization (EPI). EPI is a new approach that delivers DNA or antigens via a needle-free delivery system directly to the epidermis, where Langerhans cells with antigen-processing

* Corresponding author. Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI 53706. Fax: +81-3-5449-5408.
E-mail address: kawaoka@ims.u-tokyo.ac.jp (Y. Kawaoka).

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and -presentation abilities are abundant (Chen and Payne, 2002). EPI is highly effective in inducing immune responses to viral agents, such as influenza, hepatitis B, and human immunodeficiency viruses (Chen et al., 2000, 2001a, 2001c, 2002a, 2002b; Osorio et al., 2003). In addition, EPI does not cause pain, injection-site reactions, or any severe adverse effects at the site of administration because of the low level of sensory nerve endings and vasculature in the epidermis. In this study, we compared the protective efficacy of whole virus-based EPI with that of a powdered gD DNA vaccine and that of inactivated virus vaccines introduced by i.m. or intranasal (i.n.) injection in a mouse model of EHV-1 infection, which mimics many of the features of infection in the natural host (Awan et al., 1990; Walker et al., 1999).

Results and discussion

Protective effect of a powder vaccine

Two doses of a powder vaccine (0.5 or 5 μg per mouse) were delivered to the abdominal skin of mice using a PowderJect device. A powder formulation that lacked the virus components was used as control. The test and control groups each consisted of 12 mice; in each group, six mice were immunized either once or twice after a 4-week interval. The six mice given a single immunization were then challenged with a wild-type virus (5×10^7 PFU/50 μl) i.n. 4 weeks after immunization. The six mice immunized twice were challenged with the same amount of the wild-type virus 2 weeks after the second immunization. Four days post-challenge, mice were euthanized and their lungs removed for virus titrations to evaluate the protective efficacy of the vaccine.

Before the challenge, the serum antibody response of each vaccinated mouse was measured by using an enzyme-linked immunosorbent assay (ELISA) with purified virus as an antigen. Significant induction of virus-specific IgG antibodies was detected irrespective of vaccine doses, even following the single immunization (Fig. 1). Post-challenge, the virus titers in the lungs were significantly reduced in the immunized mice compared with the titers in the lungs of the mock-immunized, control mice (Fig. 2). The booster immunization enhanced these protective effects. There was no significant difference in the efficacy between the two vaccine doses (0.5 vs. 5 μg). These results together indicate that the powder vaccine is effective against EHV-1 infection in this mouse model and provide the first evidence of effective EPI for herpesvirus infection.

Protective effect of a DNA vaccine

For comparison, we then prepared a DNA vaccine for epidermal immunization with a gene gun (Fynan et al., 1993). We constructed three gp-expression plasmids (pCEHV-1 gB, pCEHV-1 gC, and pCEHV-1 gD) and

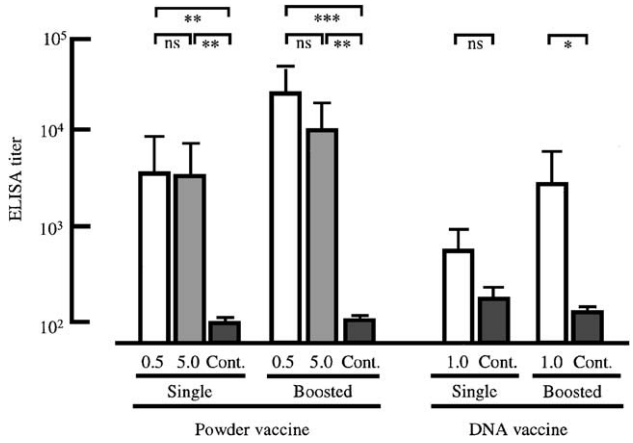

Fig. 1. Antibody responses to epidermal immunizations. A powder vaccine was prepared from whole virus and used to immunize BALB/cBYJ mice once (Single) or twice (Boosted) with either 0.5 or 5.0 μg of virus by use of a PowderJect device. A DNA vaccine was prepared from a plasmid expressing gD (1.0 μg) and used to immunize BALB/cBYJ mice once (Single) or twice (Boosted) by use of a gene delivery system. Sugar only- or vector alone-inoculated mice were used as controls (Cont.) for the powder or DNA vaccines, respectively. Sera were collected for ELISA titration of specific antibodies from immunized mice before challenge with the wild-type virus. The P values (Student t test) are shown as $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, or ns (not significant).

confirmed protein expression by transfecting each plasmid into COS-1 cells and immunostaining those cells with monoclonal antibodies to the gps. All three gps were expressed in the transfected cells (data not shown).

We next assessed the immunogenicity of gene gun-based DNA vaccination, the effectiveness of which has not previously been reported for EHV-1 infection. Previous studies have shown that gB, gC, and gD proteins are immunogenic, eliciting both neutralizing antibodies and cell-mediated immunity (cytotoxic T lymphocytes) (Alber et al., 2001; Packiarajah et al., 1998; Ruitenberg et al., 2001; Stokes et al., 1997; Tewari et al., 1994, 1995). To assess which gp-encoding DNA was the most immunogenic in our gene gun system, we injected mice with each DNA construct (1 μg) twice at a 2-week interval. Two weeks after the second injection, serum neutralizing antibody titers were measured by plaque-reduction assays. The sera from mice injected with pCEHV-1 gD contained specific neutralizing antibodies in agreement with previous reports (Ruitenberg et al., 1999; Walker et al., 2000); however, the sera from mice injected with pCEHV-1 gB and gC did not contain specific neutralizing antibodies (data not shown). For this reason, we used only the gD DNA vaccine for our further experiments.

To evaluate the protective effect of the gD DNA vaccine, we delivered pCEHV-1 gD (1 μg) to the abdominal skin of mice with a gene gun. As a control, plasmid vector was similarly delivered. Each group (test and control) consisted of 12 mice. Significant induction of a specific IgG antibody was not observed with a single immunization (Fig. 1), and in keeping with this finding, a single immunization did not protect the mice from challenge with the wild-type virus.

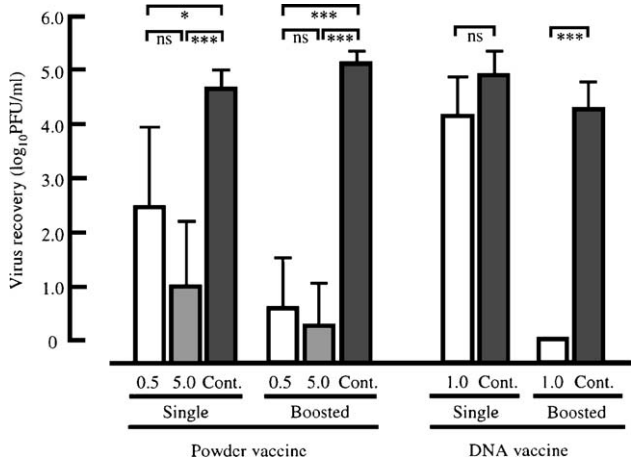

Fig. 2. Protective efficacy of epidermal immunizations against EHV-1 infection in mice. Preparation and immunization protocols of powdered inactivated and DNA vaccines were as described in the legend for Fig. 1. Lungs were removed 4 days post-challenge with the wild-type virus, and homogenates were titrated for virus. The *P* values (*t* test) are shown at the top, as in Fig. 1.

Virus titers in the lungs of these immunized mice were not significantly reduced compared with the control (Fig. 2). By contrast, no virus was recovered from the lungs of any mice given the booster immunization, which suggests that the vaccine can be effective under the right conditions and corroborates previous studies of i.m. injection of gD DNA (Ruitenberg et al., 1999; Walker et al., 2000). These data indicate that gene gun-mediated DNA epidermal immunization for EHV-1 infection may be useful; however, the failure of this approach to protect mice given a single immunization suggests that the protein-based epidermal immunization with the powder vaccine may be more effective than DNA-based epidermal immunization.

Protective effect of an inactivated vaccine

To compare the efficacy between protein-based vaccines, we next examined the protective efficacy of the traditionally inactivated virus vaccines. Vaccines were administrated once (single) or twice (boosted) via the i.m. route with Freund's complete adjuvant (FCA) or via the i.n. route dosed at 0.5 or 5 μg per mouse. Four weeks after the primary immunization, six mice in each group were given booster immunization under the same conditions as before. Before virus challenge, the serum IgG antibody responses were significantly higher in mice immunized i.m. with the inactivated vaccine than those epidermally immunized with the powdered inactivated vaccine (Fig. 3). However, no significant differences in protection were observed between these two vaccination strategies, with the exception of one experimental condition where mice received a lower dose of the vaccine twice (Fig. 4). These results suggest that the powdered inactivated vaccine may induce better CTL responses to the viral antigens than those induced by inactivated vaccines. Together, these data suggest that the powdered inactivated vaccine is at least as efficacious as inactivated vaccines in protecting against EHV-1 infection.

Live, attenuated vaccines offer another option for the control of EHV-1 infection. Commercial live, attenuated vaccines and vaccine candidates include tissue culture-selected thymidine kinase-deficient, temperature-sensitive, and gE/gI-deficient viruses (Bürki et al., 1990; Cornick et al., 1990; Matsumura et al., 1998; Patel et al., 2003a, 2003b; Slater et al., 1993). Their protective efficacies appear to vary depending on the experimental conditions. Of these vaccines, the recently developed vaccine candidate derived from a temperature-sensitive mutant showed remarkable efficacy for protection against abortion and respiratory

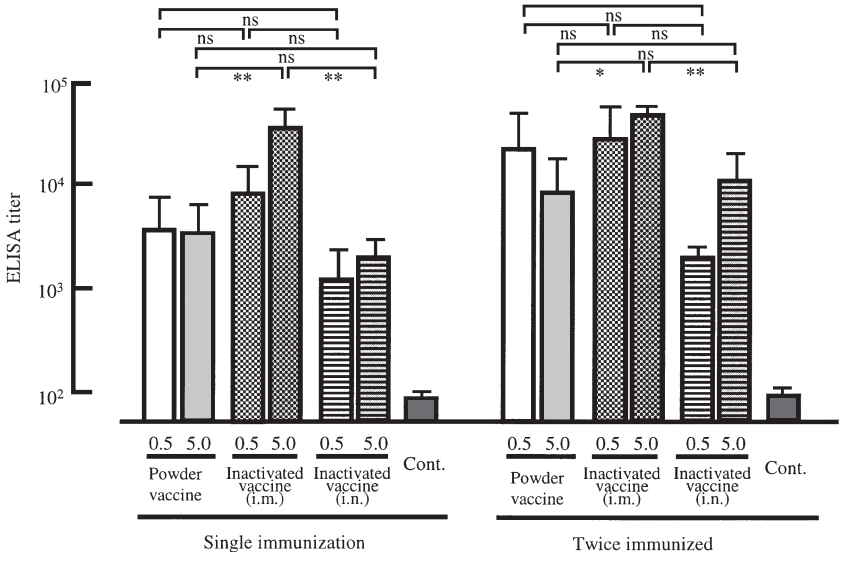

Fig. 3. Antibody responses to whole virus vaccines. A powdered inactivated vaccine was prepared as described in the legend for Fig. 1. Inactivated vaccines were prepared from whole virus and BALB/cBYJ mice were immunized once or twice, intramuscularly (i.m.) or intranasally (i.n.), with either 0.5 or 5.0 μg of virus. Untreated mice served as controls (Cont.). ELISA was done as described in the legend for Fig. 1. The *P* values (*t* test) are shown at the top, as in Fig. 1.

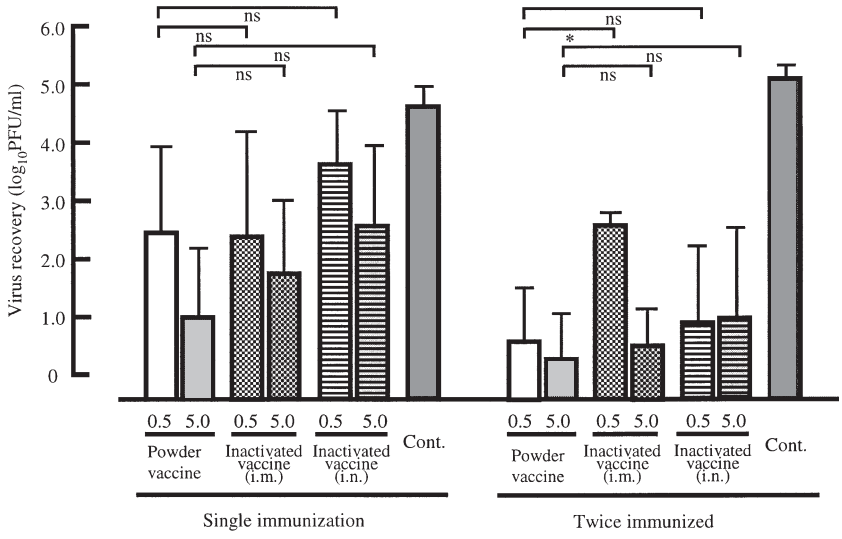

Fig. 4. Protective efficacy of whole virus vaccines against EHV-1 infection in mice. The powder and the inactivated vaccines were prepared as described in the legends for Figs. 1 and 3. Challenge with the wild-type virus and virus titration protocols were as described in the legend for Fig. 2. The *P* values (*t* test) are shown at the top, as in Fig. 1.

disease by EHV-1 infection (Patel et al., 2003a, 2003b). Although long-term immunity might be anticipated by these live vaccines, the possibility of a vaccine virus regaining its virulence and causing persistent infection is a major concern. Therefore, extensive safety evaluation is required for live vaccines.

Because a combination of mucosal and systemic antibody and CTL responses is thought to be required for successful immunologic control of EHV-1 infection (Allen et al., 1999), a single type of vaccine alone may not be sufficient to achieve such control. Accordingly, combined vaccinations involving prime-boost strategies may be more effective, as exemplified by the study of Ruitenberg et al. (2000b), in which they showed that a combination of gD-based DNA and purified gD protein immunizations could enhance virus-specific antibody titers relative to those achieved in mice immunized with DNA or protein only. Although the titers of virus-specific serum IgG were higher in mice administrated inactivated vaccine via i.m. than via i.n. (Fig. 3), both provided equivalent protective effects (Fig. 4), suggesting the induction of mucosal IgA antibody by i.n. immunization of the inactivated vaccine. Given that EPI induces CTL and humoral responses (Chen et al., 2001a), a prime-boost vaccination with i.n.-inactivated vaccine (for the mucosal immune response) and EPI (for both humoral and cellular immune responses) may be more effective in preventing EHV-1 infection. Moreover, using adjuvants (such as alum, inactivated cholera toxin, or CpG DNA) or coating gold particles with antigens can enhance both the CTL and antibody responses of powder vaccines to some virus infections (Chen et al., 2001b; Maa et al., 2003). Thus, it would be of interest to test whether such adjuvants enhance the efficacy of the EHV-1 powdered inactivated vaccine and to assess which prime-boost combinations (among powdered inactivated, DNA, and inactivated vaccines) are the most effective for the prevention and control of EHV-1 infection.

Materials and methods

Cells and virus

Equine derm cells, Madin–Darby bovine kidney (MDBK) cells, and COS-1 cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. EHV-1, strain 89 c25 (Matsumura et al., 1998), was used for all experiments. Virus-infected cells were maintained in MEM with 2% FCS for 4 days.

Preparation of and immunization with a powder vaccine

Virus, propagated in equine derm cells, was concentrated by centrifugation (100,000 × *g*), resuspended in TNE buffer (20 mM Tris–HCl, pH 7.4, 0.14 M NaCl, 1 mM EDTA), and purified through a 20–60% (w/v) sucrose gradient. Virus-containing fractions were pelleted, resuspended in 20 mM phosphate buffer, and inactivated with formalin. Briefly, formalin (0.01%) was added in equal volume to the virus suspension and samples were incubated at 4 °C for 72 h, then subjected to four cycles of freeze (in liquid nitrogen)-and-thaw (at 37 °C). Inactivation of the virus was confirmed by plaque assay with MDBK cells. To create a sugar-based powder vaccine, D(+)-trehalose (Sigma) was added to the inactivated virus preparation to a final concentration of 20% (w/v). This sugar–virus mixture was then spread by droplets onto a glass petri dish, dried in a desiccator for 48 h, scraped from the plates, and sieved to make particles of 35–38 nm in diameter. This formulation

was stored under desiccation at 4 °C. For the PowderJect ND device (PowderJect Vaccines), the powder vaccine (1 mg; containing 5 or 0.5 μg of virus components) was loaded into a trilaminate cassette, sealed with membrane, and stored in a desiccator until use.

We used BALB/cBYJ mice (5-week-old female) to evaluate the protective effect of the powder vaccine. After the hair on the abdomen of these mice was shaved, the powder vaccine (0.5 or 5 μg per mouse) was delivered to the abdominal skin by use of the PowderJect device with helium (He) gas at a pressure of 50 bar.

Preparation and immunization of DNA vaccines

The full-length gB, gC, and gD genes were amplified by PCR from EHV-1 (strain 89 c25)-infected cells and cloned into the pCAGGS/MCS plasmid (Kobasa et al., 1997; Niwa et al., 1991) (designated pCEHV-1 gB, pCEHV-1 gC, and pCEHV-1 gD, respectively). All constructs were sequenced; the nucleotide sequences of these gps were identical to those of strain Ab4 in GenBank, except for one silent substitution in the gC gene. The sequence of the PCR and sequencing primers will be provided upon request. For immunization with the PowderJect XR delivery system (PowderJect Vaccines), the gp plasmids and the vector alone (included as a control) were purified with a commercial kit (QIAGEN), combined with gold beads in 0.05 M spermidine by sonication, washed three times with 100% ethanol, and then resuspended in ethanol. The adhesive 0.1% PVP (in ethanol) was then added and the DNA-gold bead suspension was placed in tefzel tubing in a tube turner and dried in nitrogen gas. The tubes were cut into cartridge-size sections and stored in a desiccator at 4 °C until use. To determine the amount of DNA on the beads, a small amount of TE buffer was placed in the cartridges and shaken for 10 min. The buffer was then centrifuged to deposit the beads and the supernatant run on an agarose gel; the plasmid DNA concentration was 1 μg per cartridge. To investigate the immunogenicity of the DNA vaccines, we delivered gp-expression plasmids (1 μg) to the abdominal skin of mice with a gene gun at 400 psi He gas pressure.

Immunization with an inactivated vaccine

The formalin-inactivated purified virus, used for the powder vaccine, was also used for an inactivated virus vaccine. The vaccine was administrated via the i.m. route with Freund's complete adjuvant (FCA) or via the i.n. route dosed at 0.5 or 5 μg per mouse.

Detection of virus-specific antibodies

Blood was taken via retro-orbital bleeding under anesthesia from each vaccinated mouse and the serum antibody response measured by ELISA with purified virus as an antigen. Briefly, Immulon II plates (Thermo Labosystems) were coated with purified virus (0.5 μg/well) in PBS. Plates were blocked with 5% skim milk for 1 h and washed three times with PBS–0.05% Tween 20. Mouse sera dilutions (50 μl) were then added to each well and incubated for 1 h. Plates were then washed three times and 50 μl of conjugate (goat anti-mouse Ig horseradish peroxidase) (Zymed; 1:5,000) was added and incubated for 1 h. Plates were then washed three times and 100 μl of substrate (ABTS) was added for 15 min. Color development was stopped with 1% NaF and absorbance was read (optical density at 405-nm wavelength). The highest dilution of test sera to yield an absorbance of 0.1 units higher than that of the control normal mouse serum was considered to be the virus-specific titer.

Challenge with a wild-type virus

Mice were immunized once with each vaccine and challenged with 5×10^7 PFU of wild-type virus (in 50 μl) i.n. 4 weeks after immunization. Mice immunized twice were challenged with the same amount of wild-type virus 2 weeks after the second immunization. Four days post-challenge, virus titers in lung homogenates from the mice were determined by using plaque assays with MDBK cells.

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