Vaccine xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Antibody and cellular immune responses of naïve mares to repeated vaccination with an inactivated equine herpesvirus vaccine

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ARTICLE INFO

Article history:
Received 4 May 2015
Received in revised form 30 August 2015
Accepted 2 September 2015
Available online xxx

Keywords:
Horse
Equine herpesvirus
EHV Multiplex assay
IgG isotype
Interferon-gamma
Th1-cell

ABSTRACT

Equine herpesvirus type 1 (EHV-1) continues to cause severe outbreaks of abortions or myeloencephalopathy in horses despite widely used vaccination. The aim of this work was to determine the effects of frequent vaccination with an inactivated EHV vaccine on immune development in horses. Fifteen EHV-1 naïve mares were vaccinated a total of 5 times over a period of 8 months with intervals of 20, 60, 90 and 60 days between vaccine administrations. Total antibody and antibody isotype responses were evaluated with a new sensitive EHV-1 Multiplex assay to glycoprotein C (gC) and gD for up to 14 months after initial vaccination. Antibodies peaked after the first two vaccine doses and then declined despite a third administration of the vaccine. The fourth vaccine dose was given at 6 months and the gC and gD antibody titers increased again. Mixed responses with increasing gC but decreasing gD antibody values were observed after the fifth vaccination at 8 months. IgG4/7 isotype responses mimicked the total Ig antibody production to vaccination most closely. Vaccination also induced short-lasting IgG1 antibodies to gC, but not to gD. EHV-1-specific cellular immunity induced by vaccination developed slower than antibodies, was dominated by IFN-γ producing T-helper 1 (Th1) cells, and was significantly increased compared to pre-vaccination values after administration of 3 vaccine doses. Decreased IFN-γ production and reduced Th1-cell induction were also observed after the second and fourth vaccination. Overall, repeated EHV vaccine administration did not always result in increasing immunity. The adverse effects on antibody and cellular immunity that were observed here when the EHV vaccine was given in short intervals might in part explain why EHV-1 outbreaks are observed worldwide despite widely used vaccination. The findings warrant further evaluation of immune responses to EHV vaccines to optimize vaccination protocols for different vaccines and horse groups at risk.

1. Introduction

Equine herpesvirus-1 (EHV-1) causes respiratory disease, abortion and neonatal illness, as well as neurologic disease known as equine herpes myeloencephalopathy (EHM). In recent years, EHV-1 has been responsible worldwide for a number of neurological outbreaks at race tracks, equine competitions, and veterinary teaching hospitals causing fatal disease in some horses and resulted in quarantines and closures [1,2]. In addition, large breeding farms have experienced outbreaks of EHV-1 abortion, several of which also involved signs of EHM during the same time [3,4]. These EHV-1 outbreaks have major animal welfare and also economic impacts on the equine industry.

Recommended prevention strategies against EHV-1 infection include vaccination and biosecurity practices. While vaccines reduce clinical disease and shedding [5,6] many EHV cases have occurred in frequently vaccinated horses [3,4]. Available inactivated vaccines are licensed for prevention of respiratory disease, or both respiratory disease and abortion. None of the currently

Abbreviations: AAEP, American Association of Equine Practitioners; CHO cells, Chinese hamster ovary cells; CTL, cytotoxic T-lymphocyte; ELISA, enzyme-linked immunosorbent assay; EHM, equine herpes myeloencephalopathy; EHV-1, equine herpesvirus type 1; gC, glycoprotein C; gD, glycoprotein D; IFN-γ, interferon-gamma; Ig, immunoglobulin; IgG, immunoglobulin G; IL-4, interleukin 4; MFI, median fluorescence intensity; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; SN, serum neutralization; IM, intramuscular; USDA APHIS, United States Department of Agriculture: Animal and Plant Health Inspection System.

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http://dx.doi.org/10.1016/j.vaccine.2015.09.009
0264-410X/

available EHV vaccines are approved or have been shown to prevent EHM. In addition to inactivated vaccines, a modified-live vaccine is available in North America and Europe and provide protection against fever and other clinical signs of disease [5,6]. The modified-live vaccine is not approved for use in pregnant mares. For prevention of abortion, repeated vaccination with an inactivated EHV vaccine is recommended by vaccine suppliers and in the EHV vaccination guidelines of the American Association of Equine Practitioners (AAEP), which for many brood mares can mean 3–4 EHV vaccinations per year for many years. For competition horses, EHV vaccination is recommended every 6 months by AAEP [web1], but many horses are vaccinated as often as 4 times annually [1]. In addition, equine events may require frequent EHV-1 vaccination with documentation that the horse has been vaccinated within a certain time frame preceding the event (often less than 30 days). However, data to support that frequent administration of EHV vaccines improves immunity and protection from disease and studies evaluating the effect of repeated vaccination within short time intervals on antibody and cellular immunity are widely missing. Moreover, retrospective data analyses from neurological EHV outbreaks suggest that frequent vaccination is a potential risk factor for the development of EHM [1,2].

Antibody responses to EHV-1 vaccination or exposure to the virus are commonly measured by serum neutralization (SN) assays, complement fixation (reviewed by [7]), or ELISA [6,8,9]. Herpesviruses have a number of highly antigenic envelope glycoproteins (reviewed by [10]). EHV-1 glycoproteins, including gB, gC, gD, gG, and gp2, elicit immune responses in mouse models and the natural host [11,12]. The gC and gD proteins are involved in cell entry [13,14], are targets for vaccine development [15,16], and have been shown to induce B-cell responses and neutralizing antibodies against human herpes viruses [17]. Besides their function in cell entry, EHV gD plays a major role in defining the different cellular host ranges of EHV-1 and EHV-4 [18], while EHV gC is able to interfere with the activation of the complement cascade by binding to the complement component C3 [19].

The purpose of this work was to provide comprehensive, longitudinal data on EHV-1-specific immunity of horses after repeated vaccination by using a commercial inactivated EHV vaccine as a model vaccine to simultaneously evaluate antibody and cellular immune responses of the host. We established a novel, quantitative EHV-1 Multiplex assay based on two major EHV-1 antigens (gC and gD) as a tool to quantify antibody responses in horses. Continuous EHV-1 Multiplex assay results were first compared to the EHV SN titers of archived sera to validate the new assay. Serum samples from vaccinated horses were then used to quantify total antibody values and Ig isotype responses after repeated vaccination. Samples from vaccinated horses were also used to evaluate EHV-1-specific cytokine and T-cell responses in the peripheral blood.

2. Materials and methods

2.1. Archived horse sera and serum neutralization assay

Archived serum samples from 58 adult horses were used to validate the EHV-1 gC and gD Multiplex assay described below. The serum samples originated from horses at three equine research facilities at Cornell University representing naturally EHV-1 infected, vaccinated or non-exposed horses. The horses were of mixed breeds including Thoroughbreds, Standardbreds, Warmbloods, Draft Horses, Icelandic Horses and Ponies. There were either geldings ($n = 9$) or mares ($n = 49$) with a median age of 8 years (range 4–23 years) at the time of sampling.

Sera were tested for EHV-1 neutralization antibodies by a standard micro serum neutralization (SN) assay at the Animal Health Diagnostic Center at Cornell University. In brief, this assay used two-fold serial dilutions of heat inactivated serum samples or controls incubated with a constant concentration of virus. After 1.5 h of incubation, RK13 cells were added in suspension. The 50% neutralizing dilution was determined after 3 days based on the presence or absence of cytopathic effects in the wells.

2.2. Cloning and expression of EHV-1 gC and gD

RNA was purified from equine peripheral blood mononuclear cells infected with EHV-1 strain Ab4 (NCBI accession AY665713) and reverse transcribed using SuperScript III (Invitrogen, Waltham, MA). The extracellular regions of gC (corresponding to amino acid (aa) residues 30–431) and gD (aa residues 31–355) were cloned into a mammalian pcDNA3.1-based vector (Invitrogen, Carlsbad, CA) containing the equine IL-4 gene as a tag for detection and purification as previously described [20]. Chinese hamster ovary (CHO) cells were transfected with purified linear DNA from each construct using the Geneporter II transfection reagent (Gene Therapy Systems, San Diego, CA, USA). Transfected CHO cells were subsequently plated into 96-well plates for neomycin selection of stable clones. Expression of the fusion protein was monitored by intracellular anti-IL-4 staining of cells by flow cytometry [20] and by IL-4 multiplex analysis of supernatants [21]. Clones with the highest secretion of the fusion protein were further purified by 2–3 rounds of limiting dilution. To collect serum free supernatants, stable transfectants were grown until 60–70% confluent, washed with medium without FCS, and maintained for 2 weeks or until cells detached. Supernatants were collected and purified using an FPLC instrument (GE Healthcare, Piscataway, NJ) and an anti-IL-4 affinity column [20].

2.3. Detection of antibodies by a fluorescent bead-based EHV-1 Multiplex assay

The EHV-1 Multiplex analysis was performed using fluorescent beads and a Luminex system (Luminex Corp., http://www.luminexcorp.com). The purified EHV-1 gC and gD antigens were coupled to beads 34 and 36, respectively, using a bead coupling procedure previously described in detail [21]. A total of 40 μg of recombinant gC or gD antigen was used per 5×10^6 beads.

For antibody detection in the Multiplex assay, all serum samples and controls were diluted 1:400 in PBN blocking buffer (PBS with 1% (w/v) BSA and 0.05% (w/v) sodium azide). Three control serum samples from horses of known EHV-1 status were included in each assay. The negative serum control typically resulted in median fluorescent intensities (MFIs) of 120 (gC) and 140 (gD). The moderately positive serum control had MFIs of 4500 (gC) and 3200 (gD) and the high positive serum resulted in MFIs of around 13,000 (gC) and 10,000 (gD). Millipore Multiscreen HTS plates (Millipore, Danvers, MA) were soaked with PBST (PBS with 0.02% (v/v) Tween 20) for at least 2 min. After aspirating the PBST, 50 μl of the diluted serum samples and control sera were added. Wells with plain PBN were used as blank control values for the assay. The gC and gD beads were vortexed and sonicated for 20 s, and 50 μl bead solution containing 5×10^3 of each gC and gD beads were added per assay well. The plate was covered to protect it from light and was incubated at room temperature with shaking for 30 min. Plates were washed using the Biotek ELx50 plate washer (Biotek Instruments Inc., Winooski, VT). For total Ig detection, 50 μl of a biotinylated goat anti-horse IgG(H+L) antibody (Jackson Immunoresearch Laboratories, West Grove, PA) was added to the plate at 1:10,000 dilution in PBN. The plate was incubated as above and washed afterwards. Another 50 μl of streptavidin-phycoerythrin (PE) (Invitrogen, Carlsbad, CA) were added to each well at a dilution of 1:100 in PBN and were incubated as above and washed afterwards. Finally, 100 μl of PBN were

Please cite this article in press as: Wagner B, et al. Antibody and cellular immune responses of naïve mares to repeated vaccination with an inactivated equine herpesvirus vaccine. Vaccine (2015), http://dx.doi.org/10.1016/j.vaccine.2015.09.009