Vaccine xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

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

Genetic and subunit vaccines based on the stem domain of the equine influenza hemagglutinin provide homosubtypic protection against heterologous strains

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ARTICLE INFO

Article history:
Received 21 July 2017
Received in revised form 27 January 2018
Accepted 2 February 2018
Available online xxxx

Keywords:
Equine influenza virus
Hemagglutinin stem region
Conserved epitopes
Lethal challenge
Cross-protection

ABSTRACT

H3N8 influenza virus strains have been associated with infectious disease in equine populations throughout the world. Although current vaccines for equine influenza stimulate a protective humoral immune response against the surface glycoproteins, disease in vaccinated horses has been frequently reported, probably due to poor induction of cross-reactive antibodies against non-matching strains. This work describes the performance of a recombinant protein vaccine expressed in prokaryotic cells (ΔHAp) and of a genetic vaccine (ΔHAe), both based on the conserved stem region of influenza hemagglutinin (HA) derived from A/equine/Argentina/1/93 (H3N8) virus.

Sera from mice inoculated with these immunogens in different combinations and regimes presented reactivity in vitro against highly divergent influenza virus strains belonging to phylogenetic groups 1 and 2 (H1 and H3 subtypes, respectively), and conferred robust protection against a lethal challenge with both the homologous equine strain (100%) and the homosubtypic human strain A/Victoria/3/75 (H3N2) (70–100%). Animals vaccinated with the same antigens but challenged with the human strain A/PR/8/34 (H1N1), belonging to the phylogenetic group 1, were not protected (0–33%). Combination of protein and DNA immunogens showed higher reactivity to non-homologous strains than protein alone, although all vaccines were permissive for lung infection.

1. Introduction

Equine influenza (EI) is a highly contagious infectious disease in horses [1]. Although H7N7 isolates have not been reported in recent years [2], the H3N8 subtype still poses a serious threat to horse welfare and the disease has not yet been successfully controlled by different vaccination strategies [3]. Partially protected horses may become subclinically infected and shed virus [4]. In Argentina the first reported outbreak was in 1976 (H7H7 subtype), and the most recent outbreak occurred in 2012, caused by a subtype H3N8 virus [5].

The Influenza virus HA glycoprotein is the primary target of neutralizing antibodies induced after vaccination or infection. A precursor polypeptide HA_0 is cleaved into disulfide-linked HA1 and HA2 subunits. The highly variable globular head domain consists of a part of HA1 only (including the receptor-binding domain), whereas the stem domain contains parts of both HA1 and HA2 and is more conserved. It possesses a short sequence (fusion peptide) responsible of the fusion of the viral and endosomal membranes that allows the release of the viral genome into the cytoplasm [6,7]. During natural infection or vaccination with conventional influenza vaccines, the stem domain is believed to be masked from the immune system by the highly immunogenic globular head [8].

Influenza HA proteins have been divided into two major phylogenetic groups, based on the primary sequence and the structural features of the stem region: group 1 (subtypes H1-2, H5-6, H8-9, H11-13, and H16) and group 2 (subtypes H3-4, H7, H10, and H14-15) [9]. Different monoclonal antibodies (mAbs) cross-react specifically with group 1 HAs [10–13] or group 2 HAs [14–17]. However, mAbs that recognize both groups have also been described [18,19].

Several studies have reported that a truncated HA molecule lacking most of the globular head ("headless" HA), offered protection after challenge with influenza virus in a mouse model [20–22,8,23,24]. Although the induced antibodies did not prevent infection, they might neutralize influenza virus by preventing

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https://doi.org/10.1016/j.vaccine.2018.02.019
0264-410X/

fusion of viral and host membranes in the late endosomes [25]. Vaccination of mice with such "headless" HA immunogens was shown to elicit antisera that cross-react with multiple subtypes of influenza virus and provided full protection against death after challenge [26].

Over the past decade, many studies have shown that immunizations done using different types of vaccines containing the same antigens may be more effective than the classical approach of multiple homologous boosts [27]. While the immunogenicity of DNA vaccines is generally too low when used alone, it has been shown that combined immunization in a heterologous prime-boost format may be more effective than homologous prime-boost, as it generates both T cell responses and protective cross reactive antibodies [27,28].

There are currently three types of EI vaccines in the market: an inactivated vaccine, a modified-live (MLV) cold-adapted equine influenza vaccine and a Canarypox vectored vaccine [3]. Experimental DNA vaccines expressing the complete viral HA have also been described, they were able to generate homologous and heterologous immune responses against EIV belonging to different lineages, and protected ponies against homologous challenge [29–31].

In this work we explored the use of "headless" HA immunogens in order to enhance the induction of broadly reactive antibodies that could improve the spectrum of protection of equine influenza vaccines. We found homosubtypic protection using protein- and DNA-based vaccines encoding a "headless" HA subunit of A/equine/Argentina/1/93(H3N8) virus strain.

2. Materials and methods

2.1. Virus strains

Type A influenza viruses used in this study include the mouse adapted influenza strains PR8, A/PR/8/34 (H1N1); X47, which is a laboratory strain containing the genomic segments encoding the HA and NA proteins from A/Victoria/3/75 (H3N2) strain and the other 6 genes from PR8; Pan, the pandemic strain A/pdm09 (H1N1), kindly provided by Dr. Xavier Saelens from Ghent University; Eq/93, A/equine/Argentina/1/93 (H3N8) mouse-adapted in our laboratory after 23 serial passages in lungs [32]; and Eq/12, A /equine/Argentina/E2345-1/2012 (H3N8) [5], used only for in vitro experiments.

2.2. Production of "headless" HA

A DNA fragment encoding the HA stem domain ("headless" HA, ΔHAp) of the A/equine/Argentina/1/93 strain (accession number L39913) was chemically synthesized (Genscript, USA) using codons optimized for *Escherichia coli*. The construct consisted of the complete HA2 subunit, two small portions of HA1 and a C-terminal hexa-histidine tag (Fig. 1A, Supplementary Fig. 1). The design of the construct was based on a previously reported strategy, which has been used to express HA2 in its neutral pH conformation, provided that regions of HA1 are in close spatial proximity to HA2 [22,23]. For this purpose, the HA1 fragments which interact with HA2 were identified (7–46 and 290–321), and some hydrophobic residues were changed in order to destabilize the low-pH conformation of HA2, as it has been suggested [22]. The DNA fragment containing the synthetic gene was cloned into the expression vector pET-22b (Novagen) between the *BamH*I and *Not*I restriction sites and transformed in *E. coli* BL21(DE3) cells. Cells were lysed using a French press and the inclusion bodies were solubilized in binding buffer containing 100 mM phosphate buffer, 150 mM NaCl, 10 mM imidazole and 6 M Urea, and loaded into a Ni-NTA column (HisTrap HP, GE HealthCare). The protein was eluted with 200 mM imidazole and dialyzed overnight against phosphate-buffered saline (PBS), using a permeable cellulose membrane (molecular cut-off: 15 kDa, Spectrum Laboratories, Auckland).

A DNA fragment encoding a second "headless" HA (ΔHAe) based on the A/equine/Argentina/1/93 HA coding sequence, containing the heterologous trimerization domain GCN4 [33], was chemically synthesized (Genscript, USA) using codons optimized for efficient expression in eukaryotic HEK293T cells (Human Embryonic Kidney 293 cells containing the SV40 T-antigen) (Fig. 1A, Supplementary Fig. 1). The trimerization domain was introduced to stabilize the native trimeric structure of the HA protein, as previously reported [33]. The synthetic DNA fragment was cloned in the modified expression vector pCAGGS (designated as pXL), between *Sal*I and *Not*I restriction sites [34,35]. Full-length HA sequences from X47 and A/equine/Argentina/E2345-1/2012 viruses were also cloned in the pXL vector. HEK293T cells, maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum, were transfected by the calcium phosphate method. Twenty four or 48 h after transfection, the cells were scraped in lysis buffer containing 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% Nonidet P-40 and 1 mM PMSF (phenylmethylsulfonyl fluoride), separated in soluble and insoluble fractions and analyzed by Western blot.

2.3. Assessment of protection after challenge

All animal procedures were in accordance with the Committee for the Evaluation of Experiments with Animals (Spanish acronym CIEMAE) guidelines. Efforts were made to minimize the suffering of the animals. Female BALB/c mice were obtained from ICIVet (UNL, Santa Fe, Argentina) and immunized at the age of eight weeks. The animals were housed in a temperature-controlled room (22–25 °C) with 12/12 h light/dark cycles and received food and water *ad libitum*. When necessary, mice were anesthetized by intraperitoneal (ip) injection of a mixture of ketamine (60 mg/kg) and xylazine (12 mg/kg).

Doses of 50 μg of the DNA vaccine (ΔHAe) without adjuvant were injected intramuscularly (im), while 10 μg of recombinant protein (ΔHAp) in incomplete Freund's adjuvant (Sigma-Aldrich) were injected ip. The interval between doses was 3 weeks. Group 1 and group 2 received only the DNA vaccine (DDD group) or recombinant protein (PPP group), respectively (Table 1). Group 3 received two doses of DNA vaccine and a dose of recombinant protein (DDP group); group 4 was primed with protein and boosted with two doses of DNA (PDD group). Mice in group 5 received a DNA vaccine based on the full-length HA of X47 or Eq/93, as described in each experiment (FFF group), and group 6 received only PBS. Mice were challenged three weeks after the third vaccination with 2 LD_{50} of mouse-adapted virus strains Eq/93 (H3N8), X47 (H3N2) or PR8 (H1N1) according to the experiment, in a volume of 50 μl administered intranasally (in). Survival and body weight were monitored for 18 days after challenge (Supplementary Fig. 2).

2.4. Serological analysis

The titers of "headless" HA-specific antibodies were determined by ELISA (enzyme-linked immunosorbent assay) in 96-well plates (Maxisorp, Greiner) coated overnight with ΔHAp recombinant protein (2 μg/ml in carbonate buffer). Three-fold serial dilutions of mouse serum were added to each well and incubated for 2 h at room temperature (RT). Peroxidase-conjugated goat anti-mouse antibody (Promega, W4021) was then added and incubated for 1 h, followed by addition of TMB (tetramethylbenzidine reagent, BD). The reaction was stopped by 1 M sulfuric acid and the absor-

Please cite this article in press as: Ibañez LI et al. Genetic and subunit vaccines based on the stem domain of the equine influenza hemagglutinin provide homosubtypic protection against heterologous strains. Vaccine (2018), https://doi.org/10.1016/j.vaccine.2018.02.019