Veterinary Microbiology 212 (2017) 7–15

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Immunogenicity of a recombinant adenovirus expressing porcine reproductive and respiratory syndrome virus polyepitopes

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ARTICLE INFO

Keywords:
PRRSV
Polyepitope
IFN-γ
Vaccine
Swine
Adenovirus

ABSTRACT

The objective of this work was to evaluate the immunogenicity of a chimeric antigen containing characterized PRRSV epitopes. A synthetic gene, designated HEJ, encoding defined epitopes was used to generate a recombinant adenovirus designed Ad-HEJ. The chimeric antigen included T-cell epitopes from structural and nonstructural proteins, and a neutralizing B-cell epitope. Following a homologous prime-boost immunization, the Ad-HEJ virus elicited significant ($p < 0.05$) epitope-specific IFN-γ responses compared to sham-treatment. Two weeks post-challenge, this response was significantly ($p < 0.05$) higher compared to the negative control treatment. IFN-γ response to PRRSV stimulation *in vitro* were observed in both groups only after challenge. Antibodies against PRRSV and peptides were detectable following prime-boost immunization in the Ad-HEJ treatment group and the responses increased post-challenge against the virus and against most of the peptides. All the swine were viremic one week post-challenge, but four weeks later, five out of the seven Ad-HEJ vaccinees had cleared the PRRSV, whereas only two of the six negative controls had cleared the virus. The outcome suggests that the adenovirus expressing defined epitopes induced a strong immune response against the peptides, but this response was not sufficient to confer protection against PRRSV challenge.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) disease causes reproductive failure and respiratory distress in sows, and growth reduction and high mortality in young swine (Collins et al., 1992). Currently, PRRS, which is caused by PRRS virus (PRRSV), represents one of the most important diseases affecting swine due to economic losses estimated at 644 million USD annually (Holtkamp et al., 2013). The PRRSV belongs to the *Arterivirus* genus, *Arteriviridae* family, and order of *Nidovirales*, along with other viruses such as equine arteritis virus, lactate dehydrogenase elevating virus, and simian hemorrhagic fever virus (Snijder et al., 2013). The genome of PRRSV is about 15 kb in length and contains 10 open reading frames (ORFs) flanked by two untranslated regions at 5′ and 3′ ends. The ORFs1a and 1b comprise about 80% of the viral genome and encode two polyproteins, which after enzymatic cleavage result in 14 non-structural proteins (nsp1-nsp14). The ORF2a, ORF2b, ORFs 3–7, and ORF5a genes encode for eight structural proteins: GP2, E, GP3, GP4, GP5, M, N, and GP5a, respectively (Snijder et al., 2013).

Several studies have described the participation of different structural and non-structural proteins in the induction of an immune response against PRRSV, but only a few studies have attempted to identify the protective epitopes from these antigens. These epitopes have been described by using computational predictions (Burgara-Estrella et al., 2013; Diaz et al., 2009), screening peptide libraries (Mokhtar et al., 2014; Ostrowski et al., 2002), overlapping peptides (de Lima et al., 2006; Parida et al., 2012; Vashisht et al., 2008; Wang et al., 2014, 2011) or by other methods (Chen et al., 2013; de Lima et al., 2008; Oleksiewicz et al., 2005; Vashisht et al., 2008; Wang et al., 2014). These studies suggest that it is possible to identify immunogenic regions that are able to induce protective immunity.

The evaluation of epitope-based antigens for induction of protective immunity against PRRSV has been poorly explored. Charerntantanakul et al., immunized pigs with three doses of a mixture of peptides derived from GP5 ectodomain, but the pigs were not protected against PRRSV after challenge. However, when the peptides were formulated with IL-

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http://dx.doi.org/10.1016/j.vetmic.2017.10.020
Received 11 July 2017; Received in revised form 25 October 2017; Accepted 26 October 2017

12 and used in the booster dose, an increase in the cellular immune response to MLV vaccine was observed (Charerntantanakul et al., 2006). In another study, Chen et al., showed that an epitope-based vaccine could induce PRRSV-specific humoral and cellular immune responses by immunization of pigs with synthetic peptides containing conserved B- and T-cell epitopes in combination with Gp96N, a heat shock protein that has previously been described as a potent adjuvant (Chen et al., 2013). Murthy et al., evaluated conserved protective epitopes fused to HBcAg to form VLPs and showed that the VLPs were able to block infection of MARC-145 cells by PRRSV. However, high level of endotoxins hindered the evaluation of immunogenicity and ability of the VLPs to confer protection in animal models (Murthy et al., 2015). Immunization of pigs with a non-pathogenic PCV1-2a virus expressing four linear PRRSV B-cell epitopes showed that two epitopes elicited neutralizing antibodies (Piñeyro et al., 2015). A recent study showed that immunization of pigs with protein M- and nsp5-derived peptides encapsulated in chitosan particles in combination with a synthetic multi-TLR2/TLR7 agonist revealed that epitopes in the M and nsp5 antigens are potential vaccine candidates (Mokhtar et al., 2017). Thus, data from previous studies suggest that it is possible to use defined epitopes to induce protective immunity against PRRSV.

The adenovirus vector is an attractive antigen delivery platform since expression of the encoded antigen can be improved by using single cycle adenovirus vector and the vector can also override pre-existing immunity to prime $CD8^+$ T-cell memory responses thus allowing for a homologous prime-boost immunization strategy (Crosby and Barry, 2017; Steffensen et al., 2012). In addition, the adenovirus vector has previously been shown to elicit protective immunity against infectious diseases in pigs (Ferreira et al., 2005; Sun et al., 2011, 2010; Wesley et al., 2004). Notably, immunization of pigs with a recombinant adenovirus expressing PRRSV GP5 antigen induced high titers of anti-Gp5 antibodies and conferred memory that was rapidly recalled upon PRRSV infection (Gagnon et al., 2003b). Recombinant adenovirus expressing multi-epitopes have also been shown to induce protective immunity in pigs (Du et al., 2008). The objective of this study was to evaluate the immunogenicity and protective efficacy of an adenovirus-vectored chimeric antigen containing one neutralizing B-cell epitope and multiple T-cell epitopes previously reported as strong IFN-γ inducers.

2. Material and methods

2.1. Generation of a recombinant adenovirus construct

Defined PRRSV T-cell epitopes (Burgara-Estrella et al., 2013; de Lima et al., 2006; Diaz et al., 2009; Parida et al., 2012; Vashisht et al., 2008; Wang et al., 2014) and a B-cell epitope (Ostrowski et al., 2002) were used to design a novel chimeric polypeptide fused in-frame to a flag tag at the C-terminal. The resultant polypeptide sequence, designated HEJ, was used to generate a synthetic gene which was codon-optimized, synthesized, cloned in pUC57 and sequence-validated by GenScript (GenScript Inc., NJ, USA). The gene encoding the HEJ chimera was subcloned into Gateway pDonR221 vector following PCR amplification by attB1 forward and attB2 reverse primers as per manufacturer's instructions (Invitrogen). Positive clones were selected by PCR screening and validated by sequencing. One pDonR-HEJ clone was then selected to transfer the gene into the pAd/CMV/V5 vector (Invitrogen) via homologous recombination. Positive clones, designated pAd-HEJ, were identified as above and protein expression was confirmed by immunocytometric analysis of transfected human embryonic kidney (HEK)-293A cells (Invitrogen). To generate replication-deficient recombinant adenovirus, six positive pAd-HEJ clones were Pac-I digested and transfected into HEK-293A cells. Protein expression by the resultant adenoviruses was evaluated by immunocytometric analysis of infected HEK-293A cells and one clone, designated Ad-HEJ, was selected for scale up to generate bulk virus stock for immunization. The titer of the Ad-HEJ virus was determined using QuickTiter™ Adenovirus Titer Immunoassay Kit (Cell Biolabs, VPK-109). The HEK-293A cells were plated and infected as per manufacturer's protocol, however cell staining was performed with the following modifications: Primary antibody used was rabbit anti-adenovirus polyclonal IgG (1:500 dilution, purified from an in-house generated polyclonal serum). Secondary antibody used was Alkaline-Phosphatase-conjugated anti-Rabbit IgG (1:1000) (Jackson ImmunoResearch) and the substrate used was FastRed TR–Naphthol AS-MX (Sigma). A recombinant adenovirus expressing luciferase, designated AdLuc, was also scaled up and titrated similarly to serve as a negative control immunogen.

2.2. Validation of protein expression

Protein expression by the pAd-HEJ plasmid construct and the Ad-HEJ recombinant adenovirus was validated by immunocytometric analysis of HEK-293A cells as previously described (Njongmeta et al., 2012). Briefly, HEK-293A cells plated in 12-well tissue culture plates were transfected with the pAd-HEJ construct or infected with the Ad-HEJ recombinant adenovirus and incubated for 48 h. in a humidified atmosphere at 37 °C and 5% CO_2. The cells were then fixed with ice-cold methanol for 10 min, rinsed with 1X Phosphate Buffered Saline (PBS) and probed with either mouse anti-FLAG M2–alkaline phosphatase conjugate (Sigma, St. Louis, MO) diluted 1:1000 in blocking buffer (PBS with 5% fetal bovine serum) or with convalescent PRRSV serum (Rascon-Castelo et al., 2015) for 1 h. The cells were then washed 3X with blocking buffer and the cells probed with positive serum were then further incubated with a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-swine IgG (Southern Biotech) for 1 h. The cells were washed again as above and incubated with FastRed TR–Naphthol AS-MX substrate (Sigma). Mock transfected/infected cells served as negative controls. The stained cells were visualized and images were recorded by an inverted phase-contrast microscope (Olympus IX70).

2.3. Immunogenicity and protective efficacy of the recombinant adenovirus construct

Thirteen, three-week-old, conventional swine were allocated in the animal facility unit of the Centro de Investigación en Alimentación y Desarrollo, A.C. with ad libitum access to water and commercial food. The swine were obtained from a breeding farm with no history of PRRSV infections: PRRSV negative status was confirmed by ELISA analysis (IDEXX Laboratories, Westbrook, Maine, USA) and also by real-time PCR detection. After arrival at the animal facility, the swine were allowed to acclimatize for a week. For immunizations, the swine were divided into two groups: Ad-HEJ vaccine (treatment) group (n = 7) and sham vaccine (negative control) group (n = 6). The swine in the treatment group were inoculated intramuscularly with the Ad-HEJ adenovirus (1×10^{11} ifu), whereas the negative controls were similarly inoculated, but with the AdLuc adenovirus (prime, week −7). At week-3, the swine received booster doses of the cognate construct and at week 0 they were challenged intranasally with 5 mL of 10^5 $TCID_{50}$ of PRRSV (strain NVSL 97-7895). The swine were bled weekly, starting from week-7, up to four weeks after challenge. After euthanasia, lung and tonsils samples were collected to evaluate viral loads.

2.4. Virus

PRRSV (strain NVSL 97–7895; GenBank accession no. AY545985) was propagated in MARC-145 cells using Dulbecco's modified Eagle medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO, Grand Island, NY), 100 IU penicillin mL^{-1} and 100 µg streptomycin mL^{-1} (Sigma, St Louis, MO) (complete DMEM). When characteristic cytopathic effect was observed, cell cultures were freeze-thawed twice and the cell lysates were centrifuged at 650 × *g* at 4 °C for 20 min. The supernatant