Vaccine 26 (2008) 3594–3600

Contents lists available at ScienceDirect

Vaccine

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

Development of a porcine reproductive and respiratory syndrome virus differentiable (DIVA) strain through deletion of specific immunodominant epitopes

Marcelo de Lima[a,b], Byungjoon Kwon[a], Israrul H. Ansari[a], Asit K. Pattnaik[a], Eduardo F. Flores[b], Fernando A. Osorio[a,*]

[a] *Nebraska Center for Virology and Department of Veterinary and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583-0900, USA*
[b] *Departmento de Medicina Veterinaria Preventiva, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil*

ARTICLE INFO

Article history:
Received 14 March 2008
Received in revised form 24 April 2008
Accepted 30 April 2008
Available online 19 May 2008

Keywords:
PRRSV
B-cell epitopes
Peptides
Infectious cDNA clone
DIVA
Marker vaccines

ABSTRACT

The availability of a DIVA (differentiating infected from vaccinated animals) vaccine is very important for the control and eradication of endemic infectious diseases such as porcine reproductive and respiratory syndrome (PRRS). Previous studies in our laboratory identified several B-cell linear epitopes consistently recognized by convalescent sera obtained from pigs infected with a North American porcine reproductive and respiratory syndrome virus (PRRSV) strain. To ascertain if one or more of these immunodominant epitopes can be used as the basis of DIVA differentiation, we selected two epitope markers previously identified on the non-structural protein 2 (PRRSV NSP2, predictably the viral protein most likely to tolerate large deletions). The choice of these epitopes was primarily based on their immunodominance and their deletion were performed along the backbone of the wild-type cDNA infectious clone (FL12). We were able to successfully rescue a mutant that fulfilled the requirements for a DIVA marker strain, such as: efficient growth of the deletion mutant *in vitro* and *in vivo* and induction of specific seroconversion as measured by a commercial ELISA kit, with absence of a marker-specific peptide-ELISA response in 100% ($n = 15$) of the inoculated animals. In summary, our results provide proof of concept that DIVA PRRSV vaccines can potentially be developed by deletion of individual "marker" immunodominant epitopes.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a small enveloped, positive-strand RNA virus associated with reproductive failure in pregnant sows and severe pneumonia in neonatal pigs [1]. Porcine reproductive and respiratory syndrome (PRRS) is currently one of the most important infectious diseases of swine causing significant economic losses to the pig industry worldwide [2]. PRRSV, along with lactate dehydrogenase-elevating virus (LDEV), equine arteritis virus (EAV) and simian hemorrhagic fever virus (SHFV), is classified into the order *Nidovirales*, family *Arteriviridae* [1]. Based on significant antigenic and genetic differences reported among North American and European PRRSV strains [3,4], two distinct genotypes of the virus have been recognized: European (type I) and North American (type II). Although isolates from both genotypes can cause disease with similar clinical signs, their genomes exhibit divergences of approximately 40% [5].

Vaccination against PRRSV infections is being carried out since 1995 in the United States. The most commonly used vaccine consists of a North American PRRSV strain attenuated by multiple passages in cell cultures. The efficacy of these currently used attenuated vaccines is somewhat controversial and is generally accepted that there is a need for improvements on their safety and efficacy. In this context, the availability of a DIVA (differentiating infected from vaccinated animals) vaccine would be of great value for the control and eventual eradication of PRRS. Epidemiological as well as regulatory considerations advise that a PRRSV DIVA vaccine should be designed based on a negative marker (i.e., a marker absent from the vaccine strain but consistently present in wild-type (wt) strains). Classical examples of modified-live vaccines carrying deletions of non-essential and immunogenic structural proteins have been produced for large DNA viruses such as pseudorabies virus (PRV) and bovine herpesvirus-1 (BHV-1) [6–8]. While technically

* Corresponding author at: 111 K. Morrison Life Sciences Research Center, 4240 Fair Street, East Campus, Lincoln, NE 68583-0900, USA. Tel.: +1 402 472 7809; fax: +1 402 472 9690.
E-mail address: fosorio@unl.edu (F.A. Osorio).

0264-410X/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2008.04.078

straightforward in the case of some double-stranded DNA viruses, deleting antigen-coding sequences from the genome of a small RNA virus like PRRSV, which encode only a few proteins with essential functions, seems to be a more difficult task [9–11]. Thus, the generation of a mutant virus carrying a deletion of an immunodominant and conserved protein segment (or a combination of deletions within the same protein or even in different proteins) would be an attractive alternative to generate a live-attenuated marker vaccine strain.

Using a systematic and detailed approach, we previously demonstrated by Pepscan analysis the presence of B-cell linear epitopes in the non-structural protein 2 (NSP2) and structural proteins of a North American strain of PRRSV, consistently recognized by the humoral immune response elicited by PRRSV-infected pigs [12]. In this context, the selection of immunodominant epitopes and deletion of these regions in a full-length infectious cDNA clone would be an alternative approach for the development of marker live-attenuated PRRSV vaccines since the genome of small RNA viruses, like PRRSV, generally does not tolerate less subtle changes such as deletions of entire genes [9–11]. Based primarily on the immunodominance and level of amino acid conservation observed for some of the peptides distributed along the different proteins, we selected target epitopes (serological markers candidates) to be deleted in the wt infectious cDNA clone (FL12) by reverse genetics.

The approach of epitope deletion has proved to be feasible for arteriviruses through deletion of a 46 amino acid immunodominant region from the ectodomain of the glycoprotein L (gL) of EAV without deleterious effects on the replication and immunogenicity of the virus [13]. Furthermore, a peptide ELISA based on this particular domain enabled serological discrimination between vaccinated and wt virus-infected animals [13].

The present study describes the generation of a PRRSV deletion mutant, named FLdNsp2/44, which lacks amino acid residues 431–445 within the sequence of the NSP2 derived from a US-type strain of PRRSV. In order to explore the potential of this approach to generate a live-attenuated marker vaccine against PRRSV, we evaluated the replication efficacy *in vitro* of the epitope deletion mutant and its biological properties *in vivo*, such as replication efficiency and immunogenicity in pigs as well as virulence in a pregnant sow model.

2. Material and methods

2.1. Cells, viruses and antibodies

The virus used for animal inoculation was recovered from MARC-145 cells transfected with RNA transcripts produced *in vitro* from the full-length infectious cDNA clone (FL12) derived from PRRSV NVSL 97-7895 type II strain (GenBank accession no. AY545985) [14]. MARC-145 cells were propagated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 units/ml of penicillin, 20 μg/ml of streptomycin, and 20 μg/ml of kanamycin) and used for RNA electroporation, viral infection and amplification, virus titration and experiments of growth kinetics. Indirect immunofluorescence assays (IFA) were performed as previously described [15], using N protein-specific monoclonal antibodies (MAbs): SDOW17 (National Veterinary Services Laboratories – NVSL, Ames, IA) and SR30 [kindly provided by Dr. Eric Nelson (South Dakota State University, SD, USA)]. The secondary antibody used was a goat anti-mouse IgG antibody (Alexa Fluor – 488, Molecular Probes, Eugene, OR).

2.2. Introduction of deletions into the full-length PRRSV cDNA infectious clone

In order to explore deletion of possible markers, two regions were previously selected for deletion in the NSP2. Each region spans 15 amino acids residues which were found to be highly immunogenic, relatively conserved among US-type PRRSV strains and most importantly, consistently recognized by antibodies of PRRSV-infected pigs [12]. The regions selected as serological marker candidates were P^{431}PPPPRVQPRKTKSV^{445} and K^{441}TKSVKSLPGNKPVP^{455} within NSP2 amino acid sequence. All deletions were introduced into the pFL12 plasmid which contains the full-length cDNA of NVSL 97-7895 PRRSV strain [14], by overlap extension method as previously described [16]. Sequences from either side of the point of deletion were amplified by using specific primers designed such that their 3′ ends hybridize to template sequence on one side of deletion and the 5′ ends are complementary to template sequence on the other side of the deletion (Table 1). Using this approach the products generated from the PCR reaction using the reverse and forward primers with overhang regions are therefore overlapping at the deletion point. After amplification of the flanking regions, both amplicons were recovered by phenol/chloroform extraction and precipitated with ethanol using standard protocol as described elsewhere [17]. The equimolar amounts of the two amplicons were mixed and subjected to 4–5 cycles of PCR followed by the additional rounds of PCR amplification with external primers. After gel purification and precipitation the DNA was digested with SpeI and SphI or XhoI and the fragment was cloned directionally into the pFL12. Confirmation of the deletion and absence of any other mutations within the region was confirmed by nucleotide sequencing.

2.3. In vitro transcription, RNA electroporation, and recovery of epitope deletion mutants

The full-length plasmid (pFL12) was digested with AclI and linearized DNA was used as the template to generate capped RNA using the mMESSAGE mMACHINE Ultra T7 kit according to manufacturer's (Ambion) recommendations. Briefly, after *in vitro* RNA transcription, the reaction mixture was treated with DNaseI to digest the DNA template, extracted with phenol/chloroform and finally precipitated with isopropanol. Following electrophoresis through a glyoxal agarose gel, the integrity of the RNA transcripts was analyzed upon ethidium bromide staining of the gel.

Sub-confluent monolayers of MARC-145 cells were used for electroporation with approximately 5 μg of *in vitro* produced transcripts along with 5 μg of carrier RNA isolated from uninfected MARC-145 cells. About 2×10^6 cells in 400 μl of DMEM containing 1.25% DMSO were pulsed once using Bio-Rad Gene Pulser Xcell at 250 V, 950 μF in a 4.0-mm cuvette. After this treatment, the cells were diluted in normal growth media containing 10% of fetal bovine serum and placed into a 60-mm cell culture plate. The

Table 1
Primers used for amplification of specific fragments in each of the selected targets for deletion

Protein	Primer	Nucleotide sequence (5′–3′)
NSP2	d44Rev	GTTCCCTGGCAAGCTCTTCGGTGTCCACCGTGG
NSP2	d44For	CCCACGGTGGACACCGAAGAGCTTGCCAGGGAAC
NSP2	d45Rev	CTGACCTTCCTGCGTGGAGCTCGAGGCTGAACTCTTGG
NSP2	d45For	CCAAGAGTTCAGCCTCGAGCTCCACGCAGGAAGGTCAG

Primers were designed such that their 3′ ends hybridize to template sequence on one side of deletion and the 5′ ends are complementary to template sequence on the other side of the deletion. The selected protein and primers with its 5′–3′ nucleotide sequence are shown.