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Immune response to ORF5a protein immunization is not protective against Porcine Reproductive and Respiratory Syndrome Virus infection

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped RNA virus responsible for PRRS in swine, a disease with globally significant animal welfare and economic concerns. There is no specific treatment and variably effective immune protection. Molecular mechanisms responsible for virulence, pathogenesis and protective immune response remain poorly understood. These factors limit progress towards development of effective measures for prevention and treatment of PRRS. A novel PRRSV ORF5a protein, encoded in an open reading frame (ORF) that overlaps the major envelope glycoprotein GP5 ORF, was recently identified. Because ORF5a is highly conserved in diverse PRRSV isolates, is a structural protein in the virion, and elicits a specific antibody response in infected pigs, we investigated its potential role in immune protection against PRRSV infection. Pigs immunized with ORF5a protein had robust serologic responses. However, the antibodies did not neutralize virus, and immunity did not protect against challenge infection. We conclude from these findings that the ORF5a antibody response is neither neutralizing nor protective.

Keywords

Swine; immunology; arterivirus; PRRSV; neutralizing antibody

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an RNA virus responsible for PRRS in swine; a disease with globally significant animal welfare and economic concerns. There is no specific curative treatment, and variably effective immune protection from previous exposure or vaccination (Benson et al., 2000; Cano et al., 2007a; Cano et al., 2007b; Opriessnig et al., 2007). Despite widespread use of vaccines and live virus inoculation with circulating field strains as a means to promote immune protection against PRRSV, outbreaks of PRRS continue to occur in naïve and PRRSV-exposed populations,

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resulting in significant losses to producers and the swine industry (Holtkamp et al., 2011; Neumann et al., 2005). While viral genetic and antigenic diversity likely contribute to variable immune protection, precise molecular mechanisms responsible for protective immunity also are poorly understood.

Recently, a novel ORF5a protein, encoded in an alternative open reading frame (ORF) of subgenomic mRNA 5, was discovered in all Arterivirus family members (Firth et al., 2011; Johnson et al., 2011). ORF5a protein is a structural protein in PRRSV virions, is present in PRRSV-infected cells, and elicits antibody production in infected pigs (Johnson et al., 2011). This, along with its evolutionary conservation indicates an important biological role. However the function of ORF5a protein and its contribution to host immunity to PRRSV is unknown. Here, we examined the role of ORF5a protein in the immune response to PRRSV by immunization of pigs with ORF5a protein and evaluation of the response to virulent viral challenge, with the hypothesis that ORF5a immunization would reduce viral infection.

Materials and Methods

Animals

Twenty-two, four week old male and female cross-bred pigs sourced from a commercial PRRSV-negative swine herd were randomly assigned to four groups (Table 1). Pigs had been weaned one week prior and were acclimated for 5 days in groups of 5 or 6 pigs, housed in separate rooms of the University of Minnesota College of Veterinary Medicine animal isolation facility. Pigs were tested for viremia and antibodies (assays described below) to PRRSV to ensure they were negative at the beginning of the study. Pigs were immunized on days 0, 10, and 20, following a standard protocol (Li and Murtaugh, 2012). Viral challenge was performed by intramuscular inoculation on day 28 with 1 ml cell culture media containing 3×10^5 TCID₅₀ of PRRSV strain VR2332 (Genbank accession number U87392) (Yoon et al., 1999).

Blood was collected on days 0, 10, 20, 28, 35, 42, and 49 in serum separator tubes and serum was stored frozen at -20°C for determination of antibody levels and viremia. Clinical response to challenge was evaluated by daily measurement of rectal temperature for 7 days from day of challenge, and observation for disease symptoms such as coughing, sneezing or lethargy. On day 49, pigs were anesthetized with an intramuscular combination of tiletamine, zolazepam and xylazine for blood collection and euthanized by intravenous barbiturate overdose. Necropsies were conducted to evaluate gross lung pathology.

The study was approved by and conducted under the guidelines of the University of Minnesota Institutional Animal Care and Use Committee.

Immunogens

Synthetic full length PRRSV VR2332 ORF5a protein (51 amino acid) was provided by the University of Minnesota Biomedical Genomics Core Peptide Synthesis Facility (Minneapolis, MN) for use as the primary immunogen. Immediately prior to use, lyophilized ORF5a protein was suspended in phosphate buffered saline (PBS) and emulsified in an equal volume of two different adjuvant preparations; (a) incomplete Freund's adjuvant (IFA) (Sigma-Aldrich, St. Louis, MO), and (b) equal volumes of IFA and a liposome preparation containing monophosphoryl lipid A (MPL) (Ernst et al., 2006) (Molecular Express Inc., Rancho Dominguez, CA) using the double-hubbed needle method (Berlin and McKinney, 1958; Freund and Thomson, 1945). MPL is a low-toxicity derivative of lipopolysaccharide, a TLR4 agonist, shown in other systems to aid robust adaptive immune responses without the proinflammatory activity of LPS (Ernst et al., 2006; Mato-Haro et al., 2007; Olson et al., 2010). The dose of synthetic ORF5a in 1 ml preparations for each of the three

immunizations at day 0, 10 and 20 of the study was 50 ug, 20 ug and 200 ug respectively per pig.

PRRSV nonstructural protein 7 (nsp7) ELISA

Nsp7 was expressed and purified for antibody capture ELISA as described (Brown et al., 2009; Johnson et al., 2007). Plates were coated with 100 ng nsp7 per well, and serum samples were diluted 1:50 in 5 % non-fat dry milk (NFDM) in phosphate-buffered saline containing 0.05 % Tween 20 (PBST). Detection antibody, horseradish peroxidase-conjugated goat anti-pig IgG (Bethyl Laboratories Inc. Montgomery TX), was used at a 1:100,000 dilution in 5 % NFDM in PBST. Immune complexes were revealed by oxidation with TMB peroxidase substrate (KPL, Gaithersburg MD) for 15 min and stopped with 1 M phosphoric acid. Absorbance was read at 450 nm in a ThermoMax Microplate Reader (Molecular Devices, Sunnyvale CA). Controls for the assay included known PRRSV-positive and negative serum.

ORF5a protein ELISA

ORF5a protein containing myc- and 6xhis- tags was expressed and purified as described (Johnson et al., 2011) for antibody capture ELISA. ELISA was performed as described and plates were coated with 100 ng ORF5a per well (Johnson et al., 2007). Serum samples were diluted 1:50 in 5 % non-fat dry milk (NFDM) in phosphate-buffered saline containing 0.05 % Tween 20 (PBST). Detection antibody, horseradish peroxidase-conjugated goat anti-pig IgG (Bethyl Laboratories Inc. Montgomery TX), was used at a 1:25,000 dilution in 5 % NFDM in PBST. Controls included buffer only, known ORF5a-positive serum and known PRRSV-negative serum. Absorbance values are represented as the mean of the duplicate wells or the group average $\pm$ standard error. Cutoffs were assigned as twice the value of the background level absorbance, determined as the mean absorbance of the negative control wells.

Viral RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

Viral RNA was isolated from serum using a QIAmp Viral RNA Mini kit (Qiagen, Valencia CA) eluted into 50 ul of RNase-free water and stored at -80°C . Complementary cDNA was synthesized with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad CA) with random hexamer primers. Primer sequences GATAACCACGCATTTGTCGTC (forward) and TGCCGTTGTTATTTGGCATA (reverse) amplified a 149 bp PRRSV ORF6 fragment. Primer specificity for Type II PRRSV viruses was confirmed by BLAST analysis against the NCBI nonredundant nucleotide database. PCR reactions were performed in a total volume of 20 ul, containing 5 ul of cDNA, 10 ul of SYBR Green PCR Master Mix (PerfeCTa SYBR Green FastMix, Quanta Biosciences, Gaithersburg MD) and 200 ng of each primer. Reactions were run in a Stratagene Mx3000P thermal cycler (Agilent Technologies, Inc. Santa Clara CA), with activation at 95°C for 1 minute, 35 cycles of denaturation at 95°C for 3 seconds and annealing/extension at 60°C for 25 seconds, followed by a dissociation step. All samples were run in duplicate. PRRSV was quantified using the standard curve method similar to (Wasilk et al., 2004). A standard curve was generated from gel purified ORF6 PCR product from the same stock of PRRSV VR2332 used to infect the pigs using the same method of viral RNA isolation, cDNA synthesis and primers for PCR described above. After purification, cDNA concentration was estimated by spectrophotometry and number of copies of the template calculated (Staroscik, 2004). Serial 10-fold dilutions were used to construct a standard curve, ranging from $>10^5$ to <1 copy per reaction. Identification of positive samples was determined by the presence of a quantification cycle value and analysis of the dissociation product. Negative controls without cDNA were used in all PCR plates.

Serum neutralization assay

MARC145 cells were plated at 5000 per well in ninety six-well Nunc™ tissue culture plates (Thermo Scientific, Rochester NY) in Minimal Essential Medium (MEM) (Gibco, Grand Island NY) supplemented with sodium bicarbonate, non-essential amino acids, HEPES buffer (Sigma, St. Louis MO) and gentamycin sulfate (Cellgro Mediatech, Manassas VA) with 10 % fetal bovine serum (FBS) (Sigma, St. Louis MO) and cultured for 48 hours at 37 °C in 5 % CO₂ before use. Porcine pulmonary alveolar macrophages were similarly plated at 80,000 per well in RPMI 1640 (Cellgro Mediatech, Manassas VA) with sodium pyruvate (Cellgro Mediatech, Manassas VA) in place of sodium bicarbonate, supplemented with 5 % FBS and cultured for 1 day at 37 °C in 5 % CO₂ before use.

Serum samples were heat-inactivated at 56 °C for 30 min, two-fold serially diluted in MEM or RPMI without FBS, and mixed with an equal volume of 3×10^5 TCID₅₀/ml VR-2332 for 1 hour at 37 °C. After incubation, 50 ul of the serum-virus mixture was added to MARC145 and PAM cells and incubated for 1 hour at 37 °C. Inoculates were then removed and cells were cultured with MEM or RPMI containing FBS for 1 day. Controls included serum with previously determined PRRSV neutralizing activity, virus alone with no serum, and uninfected cells.

After culture, cells were fixed with 3.7 % formaldehyde (Sigma, St. Louis MO) in PBS for 30 min at room temperature, permeabilized with 0.1 % Triton X-100 (Sigma, St. Louis MO) in PBS for 5 min, and blocked with NFDm in PBS, pH 9.6, for 1 hour at 37 °C. After washing with PBS, 1:10,000 diluted anti-nucleocapsid mAb SR30-A (Rural Technologies, Brookings SD) was added to cells for 1 h at 37 °C. After washing, the secondary antibody HRP-conjugated goat-anti-mouse IgG (H+L) (KPL, Gaithersburg MD) was diluted 1:500 and then added to cells for 1 h at 37 °C. Finally color was developed with TMB peroxidase substrate (KPL, Gaithersburg MD) for 15 min and stopped with 1 M phosphoric acid. Absorbance was read at 450 nm in a ThermoMax Microplate Reader (Molecular Devices, Sunnyvale CA). Results were calculated as the percent inhibition of viral infection at each dilution compared with virus only controls (with background absorbance from uninfected cells subtracted).

Statistics

Pairwise comparison of viremia between groups was conducted using the nonparametric Wilcoxon rank sum test. Fisher's exact test was used to examine pairwise comparisons of proportion of viremic pigs per group. As no statistical or biological differences in viremia or antibody titers were observed between the immunized groups regardless of adjuvant preparation (group 2 and 3 in Table 1), data from these two groups were combined for graphical presentation in Figures 1 and 2.

The difference in distribution of virus neutralizing capacity between the paired pre-immune and immune sera was investigated for each serum dilution using a paired two-tailed t test. Correlation coefficients were calculated between serum neutralizing capacity and ORF5a antibody level, and tested with the hypothesis that they were correlated by a two tailed t test with n-2 degrees of freedom.

Results and Discussion

Pigs were determined to be naïve to PRRSV at the beginning of the study as antibody responses to PRRSV ORF5a (Figure 1) and nsp7 (data not shown) were negative indicating absence of prior exposure, as well as by absence of viremia in the RT-qPCR assay. All pigs immunized with ORF5a protein had seroconverted by the day of challenge (day 28) (Figure 1). At this time, the antibody response (as evidenced by a plateau in the antibody response

curve for each individual pig) was fully developed. Following challenge, all pigs developed viremia, with no quantitative differences between immunized and non-immunized groups (Figure 2, $p > 0.05$). There was no significant difference between the proportion of viremia-positive pigs per group in challenged groups irrespective of immunization as determined by Fisher's exact test, showing that the results were not skewed by individual pig responses.

Despite viremia in all challenged animals the response of pigs to the PRRSV VR2332 challenge of 3×10^5 TCID₅₀ was subclinical. No coughing or sneezing was observed in the challenged animals, nor were there differences in body temperature between groups or evidence of fever in individual pigs (data not shown). Similarly, gross lung lesions were minimal or absent.

To determine whether ORF5a immune sera has virus neutralizing capacity, an ELISA-based assay was used to quantify PRRSV infection of MARC 145 cells and porcine alveolar macrophages after pre-incubation with sera (Li and Murtaugh, 2012). Sera from a sow with PRRSV neutralizing activity of greater than 50 % at a 1:16 dilution was used as a positive control for the assay. Neutralizing capacity of immune sera at challenge on day 28 were compared to day 0 baseline for all pigs. There was less than 50 % neutralizing activity of ORF5a immune sera at a 1:2 dilution and no difference in neutralizing capacity between paired pre-immune and immune sera across all animals ($p = 0.38$) (Table 2). Furthermore, there was no correlation between the level of anti-ORF5a antibodies in serum and virus neutralizing capacity. Similarly, serum level of ORF5a antibody was not correlated with the level of viremia.

ORF5a protein is a predicted membrane-spanning structural protein in the virion (Johnson et al., 2011). Therefore, we examined its potential role in anti-PRRSV immunity, particularly with respect to induction of a neutralizing antibody response. It was observed that in response to PRRSV infection the antibody response to ORF5a is relatively late in onset (4 weeks after PRRSV infection) and low level (Johnson et al., 2011), consistent with that described for PRRSV-neutralizing antibodies (reviewed in (Lopez and Osorio, 2004)). Pigs had a robust antibody response to ORF5a protein immunization, showing that it is highly antigenic in swine. However, ORF5a protein seroconversion did not translate to protection against PRRSV viremia. Immunized pigs had equivalent levels of viremia which was resolved at the same rate as in the non-immunized group. As PRRSV infection was subclinical in this study, it remains unknown whether ORF5a immunization would alter the course or severity of clinical disease. Immune serum did not significantly neutralize PRRSV, and the level of ORF5a protein antibodies was not correlated with the virus neutralizing capacity of sera. No effects of MPL-liposome inclusion in the adjuvant emulsion with IFA were observed. Collectively, the data show that ORF5a protein immunization elicits antibodies that do not have virus neutralizing activity, and the immune response is not protective to PRRSV challenge.

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Figure 1.

Antibody response to ORF5a protein immunization. Serum samples were stored and tested on the same day by ELISA as described in Materials and Methods.

Figure 2.

Effect of immunization on PRRSV viremia. Quantitative RT-PCR was performed as described in Materials and Methods using the standard curve method with a gel-purified PCR product.

Table 1

Treatment groups

Group	Treatment	N
1a	Untreated negative control	3
1b	ORF5a protein-IFA immunization, non-challenged	3
2	ORF5a protein-IFA immunization, PRRSV challenged	6
3	ORF5a protein-liposome-IFA immunization, PRRSV challenged	5
4	Non-immunized, PRRSV challenged	5

Table 2

Comparison of pre- and post-ORF5a immunization 50% serum neutralization titers

Group	<u>pigs with 50% SN titer</u> <u>1:2 / total pigs</u>	
	pre-immunization	post-immunization
1a	0/3	0/3
1b	0/3	0/3
2	0/6	0/6
3	0/5	0/5
4	0/5	0/5