RESEARCH REPORT
SWINE HEALTH

Title: Mechanism of Antibody-Mediated Neutralization of PRRSV**, NPB #14-222**

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Industry Summary:
One of the challenges in developing effective vaccines for PRRSV is the limited understanding of how neutralizing antibody inhibits PRRSV replication, and how variation in PRRSV leads to escape from neutralizing antibody. In this study, we examined how genetic changes in specific virus proteins leads to increased resistance to neutralizing antibody. We identified genetic variants in PRRSV-infected pigs with high virus levels at 4-6 weeks post-infection. The predominant genetic variants were inserted into an infectious molecular clone and tested in virus neutralization assays to identify genetic changes in PRRSV that led to increased resistance to neutralizing antibody. We identified a combination of amino acid changes in both GP2/3/4 and GP5 that led to escape from neutralizing antibody. The specific amino acid changes responsible for resistance to neutralization were different in each pig, and we could not identify a single amino acid change that could predict susceptibility or resistance to neutralizing antibody. To determine how neutralizing antibody inhibits PRRSV replication, virus was incubated in the presence or absence of neutralizing antibody and PCR was used to quantify viral RNA at early steps in the virus replication cycle. Results indicated that neutralizing antibody inhibits attachment of virus to cells and also inhibits a second, post-entry step in replication that occurs between 4-8 hours after virus has entered the cell. Together, these results indicate that neutralizing antibody targets multiple virus proteins to inhibit replication at two different steps in the virus replication cycle. Multivalent vaccines designed to target these distinct steps in PRRSV replication could enhance efforts to control this important swine pathogen.
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Scientific Abstract:

Genetic diversity of porcine reproductive and respiratory syndrome virus (PRRSV) challenges efforts to develop effective and broadly acting vaccines. Although genetic variation in PRRSV has been extensively documented, the effects of this variation on virus phenotype are less well understood. In the present study, PRRSV ORF2-6 variants predominant at early times after experimental infection were identified and chimeric viruses containing all or part of predominant ORF2-6 haplotypes were constructed and tested in virus neutralization. In two pigs, genetic variation in ORF2-6 resulted in increased resistance to neutralization by autologous sera. Mapping studies indicated that variation in either ORF2-4 or ORF5-6 could confer increased neutralization resistance, but there was no single amino acid substitution that was predictive of neutralization phenotype. Detailed analyses of the early steps in PRRSV replication in the presence and absence of neutralizing antibody revealed both significant inhibition of virion attachment and, independently, a significant delay in the appearance of newly synthesized viral RNA. These data reveal that limited variation appearing early after PRRSV infection alters important virus phenotypes and contributes to antigenic diversity of PRRSV.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) continues to be an economically important disease of swine. One of the major challenges in developing effective vaccines to PRRSV is overcoming the multiple and diverse strategies the virus employs to evade both innate and adaptive host immune responses. Genetic variation is thought to play an important role in immune evasion of PRRSV. Despite intense research over the past twenty years, however, there remain large knowledge gaps regarding correlates and mechanisms of protective immunity. A PRRSV-specific antibody response is observed in infected pigs, but the virus uses decoy epitopes and/or glycan shielding to protect itself from these antibodies. Neutralizing antibody (NAb) against PRRSV has been shown to be important for PRRSV clearance, but the mechanism(s) by which neutralizing antibody acts to inhibit virus replication is not known. Adding to the complexity of the problem, PRRSV utilizes different glycoproteins for attachment, entry, and uncoating. This suggests that neutralizing antibody may act at multiple steps during the early stages of PRRSV replication.

As a result of the complex nature of PRRSV entry, there are many opportunities for neutralizing to block virus infection at early stages of infection, including attachment, internalization, and uncoating. While the virus appears to present many targets for neutralizing antibody, the mechanism(s) by which antibody acts to inhibit replication PRRSV is not known. Consequently, it is not known if/how specific genetic variation in viral envelope genes result in evasion of neutralizing antibody. Such information would be valuable in predicting the neutralizing phenotype (i.e. sensitivity or resistance to neutralization) of new viral strains and the potential for vaccine failure. Further knowledge as to the mechanisms by which neutralizing antibody targets specific amino acids to inhibit specific steps in PRRS replication can aid in design and evaluation of potent and effective PRRSV vaccines.

Objectives:

The specific objectives were to:

1. Identify specific amino acids in PRRSV envelope proteins targeted by neutralizing antibody;
2. Determine the specific step in the virus replication cycle blocked by neutralizing antibody.

Materials & Methods:

Overall Experimental Design.

In previous studies funded by the National Pork Board, sera samples from five pigs with detectable viremia at 5-6 weeks following experimental infection with PRRSV strain NVSL97-7895 were used to analyze genetic variation in ORF2-6 at early times after infection. Viral RNA was isolated from the NVSL97-7895 inoculum and from sera samples collected at 7 dpi and at a late viremic day from each pig. We identified predominate ORF2-6 haplotypes in each of the five pigs, and in two of the 5 pigs variation in ORF2-6 resulted in increased resistance to autologous neutralizing antibody. In this proposal, we extended those finding to identify the specific envelope protein(s) that contributed to increased resistance to neutralization by autologous sera. The variable regions ORF2-4, which encodes the minor envelope proteins GP2/3/4, or ORF5-6, which encodes the major envelope proteins GP5/M were isolated from neutralization resistant haplotypes and used to generate chimeric PPRSV in the pFL12 backbone. Chimeric viruses were tested for sensitivity to neutralization using day 42 sera collected from each pig. Based on these results, we evaluated the ability of single amino acid substitutions to confer resistance to autologous neutralizing antibody. To identify the step in virus replication inhibited by neutralizing antibody, PRRSV was incubated in the presence or absence virus neutralizing antibody and virion RNA quantified at successive steps in the virus replication cycle, including attachment, entry, and synthesis of viral RNA. Comparison of viral RNA levels in the presence and absence of antibody allowed us to determine what step(s) in the replication cycle were inhibited by the presence of neutralizing antibody.

Experimental Methods

Generation of Chimeric PRRSV

Chimeric viruses containing the predominant ORF2-6 haplotypes were generated in the backbone of the infectious molecular clone pFL12 using shuttle plasmids to facilitate swapping regions of pFL12 and the ORF2-6 haplotypes. The

ORF2-6 sequences were selected from haplotypes existing in our library or synthesized (GeneArt, Thermo Fisher Scientific), inserted into the pFL12 backbone and transformed in DH5α *Escherichia coli* cells. Chimeric viruses were generated from the chimeric infectious clones via *in vitro* transcription and electroporation into MARC-145 cells. Briefly, plasmid DNA was linearized by digestion with AclI and viral RNA was synthesized using the T7 Ultra mMESSAGE mMACHINE *in vitro* transcription kit (Ambion, Life Technologies). Five μg of *in vitro* transcripts and 5 μg naïve MARC-145 cellular RNA were added to $2x10^6$ MARC-145 cells in 400 μl DMEM containing 1.25% DMSO and electroporated at 250V and 950uF (GenePulser Xcell, Bio-Rad). Electroporated cells were plated in a single well of a 6-well plate in5 ml DMEM supplemented with 10% FBS, antibiotics, and 1.25% DMSO. At 18 hours post transfection (hpt), media was replaced with 5 ml DMEM supplemented with 5% FBS and antibiotics. At 96 hpt, supernatants were harvested and cells were stained by immunocytochemistry to verify virus replication. Supernatants were passaged two to three times in MARC-145 cells to produce high titer chimeric virus stocks. All stocks were sequenced through ORF2-6 to confirm the correct haplotype sequence.

Virus Neutralization Assays

Neutralizing antibody assays were performed using a focus-reduction assay. Briefly, sera was heat-inactivated, diluted, incubated for 1 hour at 37°C with 200 focus-forming units (FFU) of virus, and inoculated in duplicate or triplicate onto MARC-145 cells seeded the previous day in a 12-well plate at $3x10^5$ cells/well. Cells and virus were incubated an additional 24 hours at 37°C in 5% CO_2, then the cells were fixed in ice-cold acetone:methanol and stained for PRRSV N protein by immunocytochemistry using the monoclonal antibody SDOW17 (Rural Technology) as the primary antibody and sheep anti-mouse IgG conjugated HRP (Jackson ImmunoResearch) as the secondary antibody. Following addition of the HRP substrate, cells were rinsed with distilled water, air-dried, and foci of infected cells enumerated by light microscopy. The percent reduction in FFU compared to a virus-only control was calculated as the percent neutralization. Assays were done in duplicate and repeated at least twice.

PRRSV Binding and Entry Assays

To assess binding/attachment of PRRSV to MARC-145 cells, PRRSV strain vFL12 was incubated in the presence or absence of neutralizing antibody for 1 hr at 37°C. The antibody source was a 1:2 dilution of pooled sera collected at 42 days post-infection from ~200 pigs experimentally infected with NVSL97-7895. The pooled sera sample was found to neutralize 86% of vFL12 at 1:8 dilution. The samples were chilled on ice and inoculated onto MARC-145 cells at an MOI of 1, and cells were incubated at 4°C for one hour to facilitate virion attachment, but not uptake into the cells. Following incubation, media was removed, cells were washed six times with media. Bound virus was eluted by incubating cells in 300 μl 1X trypsin-EDTA for 10 min at room temperature. Following the addition of 50 μl FBS, cells and supernatant were collected and separated by centrifugation. Virion RNA was isolated from the supernatant fraction using the QIAamp Viral RNA Mini kit (Qiagen) and RNA was quantified by RT-qPCR using primers specific for PRRSV ORF7 RNA. For entry and replication assays, PRRSV was incubated in the presence or absence of antisera and inoculated onto MARC-145 cells at 4°C as described above. Following washing to remove unattached virions, fresh media was added and the cells were shifted to 37°C, designated as time 0 hr. At 1, 4, 8, 12, or 24 hr, cells were treated with trypsin and pelleted by centrifugation as described above, and total RNA was isolated from the cell fraction with the RNeasy mini kit (Qiagen) and quantified by RT-qPCR as above. All assays were done in duplicate and repeated 2-3 times and results are reported as mean copy number of viral RNA/well.

Results:

Objective 1: Identify specific amino acids in PRRSV envelope proteins targeted by neutralizing antibody.

Identification of ORF2-6 Haplotypes that Increase Resistance to Neutralizing Antibody

To identify the viral targets of neutralizing antibody, ORF2-6 haplotypes from experimentally infected pigs were used to generate ORF2-6 chimeric viruses in the background of vFL12, which was derived from the NVSL97-7895 inoculum (Figure 1A). Chimeric viruses were tested for sensitivity to neutralization by autologous sera. Predominant ORF2-6 haplotypes from two PRRSV-infected pigs (3197 and 1115) showed increased resistance to neutralization by autologous sera (Figure 1B). Chimeric viruses containing either haplotype A or haplotype B from pig 3197, designated v3197A or v3197B, were significantly more resistant to neutralization by autologous sera than was vFL12, with average

neutralization of 11% (*P*=0.0002) and 7% (*P*=<.001), respectively. Chimeric virus containing haplotype A from pig 1113, v1113A, was also significantly more resistant to neutralization than vFL12 (average neutralizations of 18%, *P*=0.009). Chimeric virus containing haplotype B from pig 1113, v1113B, showed increased resistance to neutralization, however this difference was only close to being significant (average neutralization of 29%, *P*=0.059).

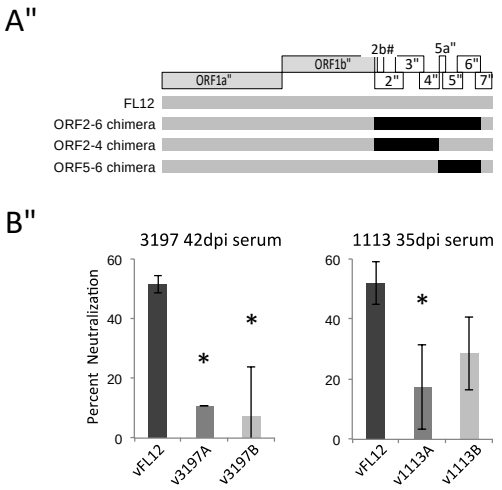

Fig 1 Neutralization phenotype of ORF2-6 haplotypes (**a**) Schematic of chimeric viruses containing variable regions of PRRSV. (**b**) Susceptibility of vFL12 and ORF2-6 chimeric viruses to neutralization by autologous sera diluted 1:4 (1113) or 1:8 (3197). Asterisks indicate significant difference (*P*<0.05) in neutralization compared to vFL12.

Variation in either major or minor envelope glycoproteins can mediate escape from neutralizing antibody

To identify the envelope protein(s) that contributed to increased resistance to neutralization by autologous sera, we generated a set of chimeric viruses in which the predominant haplotypes from pigs 3197 and 1113 were separated into their oligomeric units (Figure 2). None of these haplotypes contained amino acid changes in M, so the chimeric virus designations include either 2-4, or 5 (Figure 2). As before, each of the chimeric viruses was tested for sensitivity to neutralization by autologous sera, using vFL12 as a reference in all neutralization assays.

In pig 3197, both v3197A-5 and v3197B-5 viruses were significantly more resistant to neutralization by autologous sera than vFL12, with average neutralizations of 29 and 6% (*P*=0.035 and <0.001), respectively (Figure 2A). In contrast, chimeric viruses containing the ORF2-4 region (v3197A-2-4 and v3197B-2-4) were neutralized at levels similar to vFL12. Thus, increased resistance to neutralization in both v3197A and v3197B mapped to ORF5. In pig 1113, haplotypes A and B differed in respect to the region that mediated increased resistance to neutralization (Figure 2B). Chimeric virus v1113A-2-4 was neutralized at 18%, (*P*=0.016) whereas v1113A-5 was neutralized at levels similar to vFL12, indicating that resistance to neutralization in the 1113A haplotype mapped to ORF2-4. In contrast, increased resistance of v1113B was mediated through ORF5: v1113B2-4 chimeric virus was neutralized at similar levels as vFL12 (41%, *P*=0.2), but v1113-B-5 chimeric virus was significantly more resistant to neutralization (19%, *P*=0.025) (Figure 2B). These mapping studies indicate that targets of virus-neutralizing antibody include both the major and minor glycoprotein complexes.

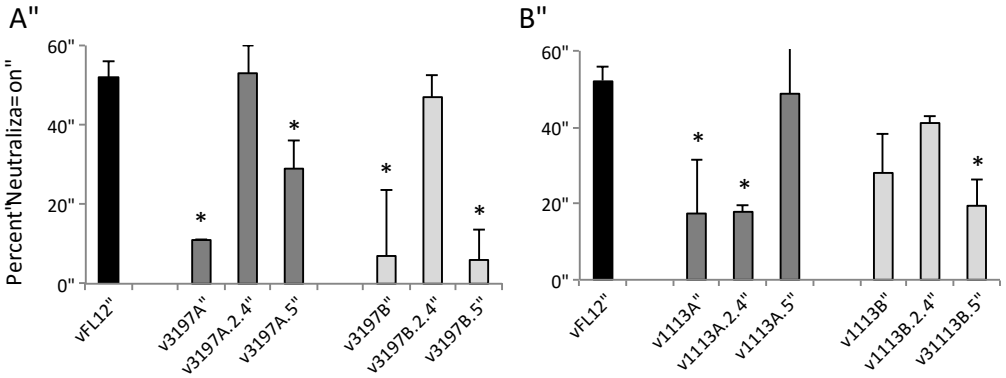

Figure 2. Mapping regions of ORF2-6 that confer resistance to autologous neutralizing antibody. Two hundred FFU of vFL12 or chimeric viruses containing either ORF2-4 of ORF5 haplotypes were tested in neutralization assays using sera from pigs (**a**) 3197 or (**b**) 1113. Results denote the mean percent neutralization ± SD compared to a no-serum control. Asterisks indicate significant difference (P<0.05) in neutralization compared to vFL12.

No Single Amino Acid Substitution is Predictive of PRRSV Neutralization Phenotype

Because of the limited variation in ORF2-6, it was of interest to determine if other single amino acid changes conferred resistance to autologous neutralizing antibody. Viruses were generated that contained unique amino acid changes found only in neutralization resistant haplotypes: GP3 P96S, GP5 N32K, or GP5 K57E. On their own, none of these single amino acid changes resulted in increased resistance to autologous neutralizing antibody (not shown). The GP5 A27V substitution was found in predominant genotypes from both pigs that had neutralizing antibody to the inoculum virus (1113 and 3197). The GP5 A27V substitution alone was sufficient to confer resistance to neutralization by pig 1113 autologous sera; however, this was not true for 3197 autologous sera (data not shown). Also, the presence of GP5 A27V in the predominant haplotype of a third pig (3068) did not confer resistance to 3068 neutralizing sera (Not shown). In most cases, therefore, resistance or sensitivity to neutralization depended on a combination of amino acid changes that were unique to each haplotype, and to each pig. Importantly, there was no single amino acid change that was predictive of neutralization phenotype.

Overall, results of studies in Objective 1 revealed that variation in ORF2-4 or ORF5 could, independently, confer increased resistance to neutralization. Because the minor and major glycoproteins are believed to play different and/or distinct roles during early stages of virus replication, these findings raise the possibility that PRRSV is susceptible to neutralization at multiple steps in the virus replication cycle.

Objective 2. Determine the specific step in the virus replication cycle blocked by neutralizing antibody.

Mapping studies indicated that targets of virus-neutralizing antibody include both the major and minor glycoprotein complexes. To better understand how variation in PRRSV glycoproteins contributes to increased resistance to neutralization, we quantified the effects of neutralizing antibody at early steps in PRRSV replication (Figure 3). Binding/attachment of PRRSV to MARC-145 cells was significantly reduced in the presence of virus-neutralizing antibody (P<0.05) (Figure 3A). Following entry, detectable virus RNA decreased in both treatment groups from 1-4 hours post-entry, indicative of the eclipse phase of virus replication. In the absence of antibody, production of new virion RNA occurred between 4 and 8 hours post-entry and rapidly increased through 24 hr post-entry. In the presence of neutralizing antibody, however, newly synthesized viral RNA was not detected until after 8 hours post-entry, after which time the rate of increase in viral RNA was similar to that seen in the absence of antibody. The delayed appearance of newly synthesized RNA was not merely a consequence of reduced virion attachment, as the cells that were infected at different MOIs showed similar kinetics during the eclipse phase, and synthesis of viral RNA was always initiated between 4 and 8 hr post-entry (Figure 3B).

In addition to blocking attachment, therefore, neutralizing antibody also targets a post-entry step in virus replication that occurs between 4 and 8 hours post-entry. It is not clear from this data which of the viral proteins are targeted at the attachment and/or post-entry steps in virus replication. Nonetheless, these data provide support for the mapping results indicating that virus-neutralizing antibody may target both the major and minor glycoproteins to inhibit multiple steps during early stages of PRRSV infection.

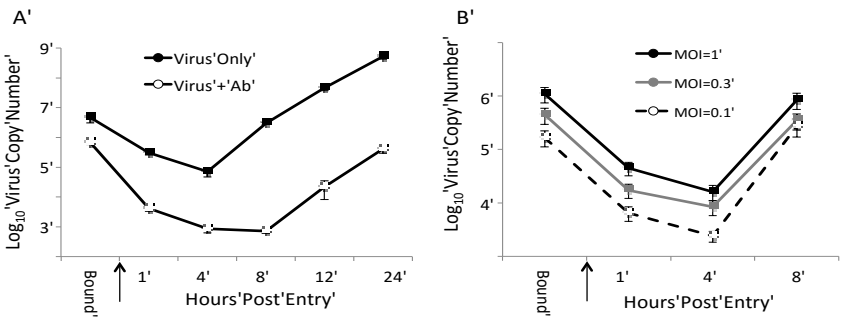

Figure 3. The effect of neutralizing antibody at early stages of PRRSV replication. Quantitation of PRRSV binding and entry (**a**) in the presence and absence of neutralizing antibody, and (**b**) in cells inoculated at varying MOI. Bound represents the RNA copy number present in attached virions after incubation at 4°C, and the arrow indicates time 0, when cells were shifted to 37°C. Results denote the mean virus copy number per well ± SEM.

Discussion

Genetic diversity of porcine reproductive and respiratory syndrome virus (PRRSV) confounds efforts to develop effective and broadly acting vaccines. Genetic variation may lead to changes in virus phenotypes that are important in replication, immune evasion, host cell tropism and/or transmissibility; however, few studies directly link PRRSV genotype and phenotype. Here, sera samples from five experimentally infected pigs collected at early times after infection were used to characterize ORF2-6 variation and determine the effects of variation on the antigenic phenotype of PRRSV. Limited genetic variation was observed during the first six weeks after infection. However, predominant ORF2-6 haplotypes were identified in each pig which, using reverse genetics, were found to vary in antigenic phenotype. In some but not all pigs, genetic changes in ORF2-4 and/or ORF5 resulted in increased resistance to autologous, type-specific neutralizing antibody. Resistance or sensitivity to neutralization depended on a combination of amino acid changes that were unique to each pig, and there was no single amino acid change that was predictive of neutralization phenotype. Rather, results suggest that virus-neutralizing antibody may target both the major and minor glycoproteins to inhibit multiple steps at early stages of PRRSV replication. These data reveal that variation appearing early after infection alters important virus phenotypes and contributes to antigenic and biologic diversity of PRRSV. The finding that PRRSV-infected pigs generate a neutralizing antibody response that targets both GP5 and GP2/3/4, and one that inhibits multiple stages in early virus replication, may motivate additional studies to delineate mechanism(s) by which neutralizing antibody inhibits PRRSV replication.