RESEARCH REPORT
SWINE HEALTH

Title: Enhancement of efficacy of PRRSV vaccines by altering the glycosylation pattern of viral glycoproteins - NPB # 05-194

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Industry Summary:

In previous studies, we have demonstrated that virus-neutralizing antibodies are important for protective immunity against PRRSV. These neutralizing antibodies constitute a significant correlate for evaluating the efficacy of a vaccine. We also know that the higher and the more cross-reactive is the titer of PRRSV-neutralizing antibodies invoked by a vaccine, the better is its immunogenic potential against infection. Studies conducted by us and others have unambiguously demonstrated that the glycoprotein GP5 is a major inducer of protective neutralizing antibodies. Through genetic manipulation of PRRSV genome, we have recently demonstrated that elimination (through a process called "hypoglycosylation") of selected sugar moieties present on the surface of GP5 dramatically enhances the ability of a PRRSV strain to invoke a more robust response composed by PRRSV-neutralizing antibodies. As evidence indicates that other glycoproteins that make up the PRRSV may also be involved in the PRRSV-neutralizing response, we had proposed to generate PRRSV containing hypoglycosylated forms of all the remaining glycoproteins (GP2, GP3, and GP4) and assess the cumulative effect of these changes on the ensuing PRRSV-neutralizing antibody response. Mutations in the

glycosylation sites of GP2, GP3, and GP4 proteins were introduced individually and mutant PRRSVs were generated from infectious clones containing these mutant glycoproteins. When inoculated into pigs and antibody response in the infected pigs were analyzed, we observed that there was a general down-regulation of neutralizing antibody response in pigs infected with the GP2 and GP4 glycosylation mutants. This result is contrary to our expectation of obtaining higher levels of neutralizing antibody response in these infected pigs. Overall, our results suggest that hypoglycosylation of the minor glycoproteins of PRRSV does not enhance neutralizing antibody response in pigs.

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Scientific Abstract:

PRRSV, the causative agent of PRRS is of major economic significance to the pork industry in the USA and around the world. Current commercial vaccine does not provide adequate protection against PRRSV outbreaks. Therefore, there is an urgent need for development of more efficacious vaccine to combat PRRS. Our previous studies have suggested that (i) induction of neutralizing antibody response is an important correlate of evaluating the efficacy of a vaccine; (ii) neutralizing antibodies can be enhanced by hypoglycosylation of the major surface glycoprotein (GP5). While it is known that GP5 plays a prominent role in neutralizing antibody induction, it has been suggested that other PRRSV glycoproteins, such as GP4 (which is known to be the target of a PRRSV-neutralizing monoclonal antibody) may also have a role. On the other hand, nothing is known about the possible role of PRRSV GP2 and GP3 in neutralization of PRRSV. We hypothesized that PRRSV neutralizing antibody response can be enhanced by hypoglycosylation of GP2, GP3, and GP4 proteins. Towards this goal, using the infectious cDNA clone (FL12) of PRRSV prepared in our laboratory, we generated a series of mutant PRRSVs containing hypoglycosylated forms of these minor glycoproteins. These viruses possessed different growth potential in vitro. When these viruses were inoculated

into pigs and their neutralizing antibody response was examined, we observed that neutralizing antibody response in most of the mutant virus-infected pigs was lower than the wt PRRSV infected pigs. These results indicate that interactions of the wild-type minor glycoproteins with GP5 may be critical for neutralizing antibody response and that altering the glycosylation pattern of the minor glycoproteins may have negatively affected their interactions with GP5 resulting in lower neutralizing antibody response.

III. Introduction

The need for a new generation of PRRSV differential vaccine is well exemplified by the importance given by the pork industry to this topic. Since the launching of the PRRSV initiative in 2003, development of an efficacious and differential vaccine has been high on the list of research priorities for NPB research grant program. In addition, a joint review of PRRSV Initiative components (NPB and USDA-CAP programs) held in 2006 suggested that development of a new generation of PRRSV with higher safely and efficacy is very important. The research proposed in this project explores a way to significantly enhance the immunogenic efficacy of a potentially new generation of vaccines. The main finding that originates this proposal is our recently provided evidence that the poor, meager and sluggish neutralizing immune response invoked by PRRSV in vivo is, in a great part, due to the phenomenon of "glycan shielding" caused by the sugars that surround the antigenic sites of the surface glycoprotein GP5 of PRRSV. This shielding by the sugar moieties would preclude the host's antibodies to reach and neutralize the inmunogenic epitopes, which like the epitope B described on the GP5 of PRRSV, interact with the viral receptor on the host cell. The phenomenon of glycan shielding has been well described for HIV and SIV, and is possible that it occurs with other viruses such as influenza, hepatitis B virus and the arterivirus LDV. In the particular case of PRRSV we verified that mutants of wt PRRSV that have been deprived of one or two sugar moieties on GP5 exhibited dramatically enhanced antigenicity, expressed as a sensibly enhanced capacity of being neutralized by regular sera from wt PRRSV-

convalescent pigs. This enhancement of "neutralizability" is evidenced by the significantly increased end-point titer of these sera against the mutants, as compared with the regular end-point that is reached by the sera against wt PRRSV. This observation clearly suggests that the removal of one, and particularly two, of the sugar residues on GP5 increases the accessibility of the neutralizing epitope to specific antibodies. Most importantly, these results appear to indicate the presence of a significant amount of PRRSV-neutralizing antibodies in the wt PRRSV-infected convalescent sera that would otherwise be undetectable because of the typical use of wt PRRSV containing fully glycosylated ("shielded") GP5 in SN assays. It is conceivable that these highly abundant, yet unnoticed antibodies in infected animals do not really contribute to protection because the wt PRRSV is fully glycosylated (thus shielded from neutralization). Certainly our most significant piece of new information for the future of PRRSV vaccinology and for this proposal, centers on the fact that the sugar-deprived mutants of PRRSV exhibited enhanced immunogenicity upon inoculation of pigs, to the point that the mutants can outperform the wt PRRSV in their ability to mount a sizable wt PRRSV-neutralizing response at 48 days PI. The mutants also developed an early and more robust homologous neutralizing antibody response than that induced by wt PRRSV, to the point where, in the case of the mutants, the characteristically sluggish and meager nature of PRRSV-neutralizing antibody response appears to have been corrected.

In this proposal, we continued these studies by assessing whether this effect of enhanced neutralizing antibody response against wt PRRSV can be further enhanced if deglycosylation is additionally conducted on the other surface glycoproteins of PRRSV. While it is known that GP5 plays a prominent role in neutralizing antibody induction, it has been suggested that other PRRSV glycoproteins , such as GP4 (which is known to be the target of a PRRV-neutralizing monoclonal antibody) (6) may also have a role. On the other hand, nothing is known about the possible role of PRRSV GP2 and GP3 in neutralization of PRRSV.

IV. Objectives

No.	Objective
1.	Examine the effects of alteration of the glycosylation pattern of PRRSV GP2, GP3, and GP4 on antigenicity and immunogenicity of PRRSV.
2.	Study the cross-reactive capacity of the hypoglycosylation-enhanced neutralizing antibody response against diverse viral isolates.

V. Materials and Methods

(i) Generation of mutant glycoproteins and examination of their expression in vitro

An intermediate pBR322-based plasmid containing a DNA fragment of ~4.9 kbp encompassing ORF2-7, and the entire 3'UTR of PRRSV from the PRRSV infectious cDNA clone (FL12) (EcoRV to BstZ17I fragment) was generated. Individual GP2, GP3, and GP4 coding sequences were also cloned in pGEM vector by PCR amplification of the corresponding sequences with gene-specific primers under the control of T7 RNA polymerase promoter so that expression of these proteins in transfected cells can be studied. The intermediate plasmid served as the template for mutagenesis to introduce mutations at the glycosylation sites in the glycoproteins. The following mutants were generated by PCR mutagenesis: GP2 (N178A, N184A, N178/184A), GP3 (N29A, N42A, N50A, N131A, N152A, N160A, and N195A), and GP4 (N37A, N84A, N120A, and N130A). In each of these mutants, the asparagine residue (N) of the glycosylation site at a particular position (identified by a number) in the protein backbone was altered to alanine (A). All mutant clones were sequenced to confirm the presence of the desired mutation and to make sure that unwanted changes were not introduced into the coding regions during mutagenesis. The mutant coding sequences were further subcloned into pGEM vector for protein expression studies. For protein expression studies, BHK-21 cells infected with vTF7-3 (which expresses T7 RNA polymerase in infected cells) were transfected with plasmids encoding the wild-type or mutant proteins under the control of T7 RNA polymerase promoter in pGEM vector. Transfected cells were radiolabeled with ^{35}S-amino acids and the viral proteins were immunoprecipitated from cell lysates with specific antibodies to GP2, GP3, and GP4. These monospecific rabbit antibodies were generated using peptides specific to these glycoproteins and they specifically immunoprecipitate the corresponding proteins from the transfected cells. The expressed proteins were detected by SDS-polyacrylamide gel electrophoresis and fluorography.

(ii) Construction of full-length cDNA clones encoding the mutant glycoproteins

The mutant coding sequences from the intermediate plasmid vector were then moved to the full-length infectious clone FL12 using the restriction enzyme sites EcoRV and BstZ17I. Again, the identity of the mutants

was confirmed by sequencing of the clones. Since we experienced unknown technical difficulties in generating the GP3 mutants at the initial stages of the project, so far we have not generated the full-length infectious clones containing the mutant GP3 coding sequences.

(iii) Rescue of mutant viruses and examination of their in vitro growth properties

The full-length plasmids encoding various glycoprotein mutants were digested with AclI and the linearized DNAs were used as the template to generate capped RNA transcripts using the mMESSAGE mMACHINE Ultra T7 kit as per manufacturer's (Ambion, Austin, TX) recommendations and as described previously from our laboratory. The integrity of the in vitro transcripts were examined by glyoxal agarose gel electrophoresis followed by ethidium bromide staining.

Subsequently, MARC-145 cells were electroporated with approximately 5.0 μg of in vitro transcripts along with 5.0 μg of total RNA isolated from MARC-145 cells as described previously. About $2x10^6$ cells in 400 μl of DMEM containing 1.25% DMSO were pulsed once using Bio-Rad Gene Pulser Xcell at 250V, 950μF in a 4.0 mm cuvette. The cells, diluted in normal growth media, were plated in a 60-mm cell culture plate. A small portion of the electroporated cells were plated in a 24-well plate to examine expression of N protein at 48 hrs post-electroporation, which would indicate genome replication and transcription. Once expression of N protein was confirmed using indirect immunofluorescence assay (IFA), the supernatant from bulk of the electroporated cells in 60-mm plates were collected at 48 hrs post-electroporation, clarified and passed onto naïve MARC-145 cells. The infected cells were observed for cytopathic effect (CPE) along with the expression of N protein using IFA. The supernatants from infected cells showing both CPE and positive fluorescence were considered to contain infectious virus. After confirmation, high titer virus stocks were prepared in MARC-145 cells, titrated, and frozen at -80°C in small aliquots for further studies. In all the experiments, FL12 containing wt PRRSV genome and FL12pol$^-$ containing polymerase-defective PRRSV genome (15) were used as controls.

Wt and mutant virus growth kinetics in MARC-145 cells were determined as described previously from our laboratory by plaque assay.

(iv) Animal experiments

Twenty-one-day old, recently weaned pigs were purchased from a specific-pathogen-free herd with a certified record of absence of PRRSV infection. Four pigs per group were infected with either FL12 wt PRRSV or mutant viruses. In all cases, the inoculum consisted of 10^5 $TCID_{50}$ diluted in 2 ml and were administered intramuscularly in the neck. The rectal temperatures of the inoculated animals were monitored for 15 days post-inoculation (PI). Viremia was measured by regular isolation on MARC-145 cells at days 4, 7, and 14 post-inoculation. Serum samples were drawn weekly for a total period of 46 days post-inoculation. The serum samples were used to detect homologous neutralization titers for each of the mutants and wt PRRSV.

The titer of PRRSV-neutralizing antibodies in a serum sample was determined using the fluorescence focus neutralization assay. Serial dilutions of test sera were incubated for 60 min at 37ºC in the presence of 200 $TCID_{50}$ of the challenge virus, which consisted of either FL12 (wt PRRSV) or any of the mutant glycoprotein-containing viruses in Dulbecco's modified Eagle's medium containing 5% FBS. The mixtures were added to 96-well microtitration plates containing confluent MARC-145 cells which had been seeded 48 hrs earlier. After incubation for 24 hrs at 37ºC in a humidified atmosphere containing 5% CO_2, the cells were fixed for 10 min with a solution of 50% methanol and 50% acetone. After extensive washing with PBS, the expression of N protein of PRRSV was detected with monoclonal antibody SDOW17 using a 1:500 dilution, followed by incubation with FITC-conjugated goat anti-mouse IgG (Sigma) at a 1:100 dilution. Neutralization titers were expressed as the reciprocal of the highest dilution that inhibited 90% of the foci present in the control wells.

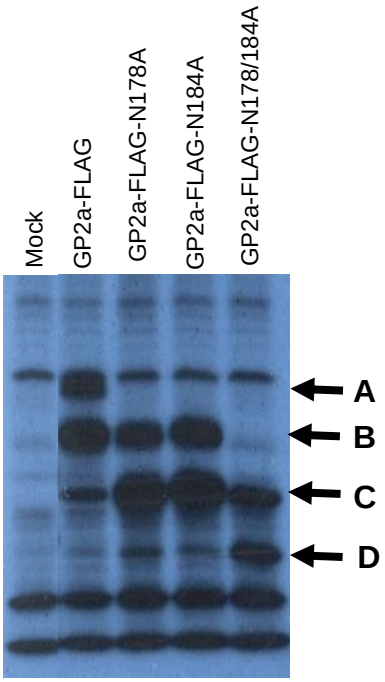
VI. Results

Objective 1: Examine the effects of alteration of the glycosylation pattern of PRRSV GP2, GP3, and GP4 on antigenicity and immunogenicity of PRRSV

(i) Expression and characterization of mutant glycoproteins in transfected cells

(a) GP2 Protein: The two potential glycosylation sites N178 and N184 were mutated in GP2 and expression of these mutants along with wt GP2 was examined in transfected cells. For these studies, we used a carboxy-

terminal FLAG-tagged GP2 protein and their



mutants and expression of these proteins (Fig. 1) revealed that both of the glycosylation sites are used for addition of glycan moieties onto GP2 protein. The results indicate that GP2 has two glycosylation sites in the protein and both sites are modified in the mature protein expressed in transfected cells.

(b) GP3 Protein: This protein contains as many as seven potential glycosylation sites at amino acid positions 29, 42, 50, 131, 152, 160, and 195. Each of these potential glycosylation sites were mutated to alanine and expression of the mutant proteins were examined in transfected cells. Transfected cells were radiolabeled with ^{35}S-amino acid precursors and the proteins were immunoprecipitated with anti-GP3 peptide antibody. The

proteins were separated in SDS-PAGE and detected by fluorography. As shown in

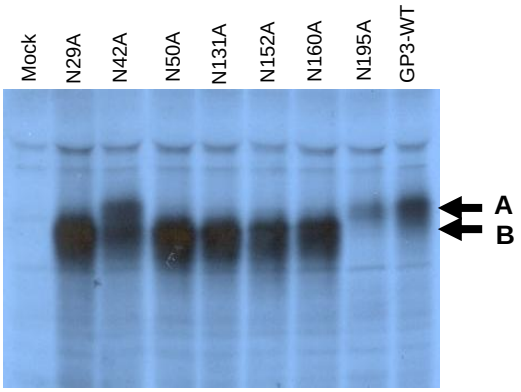


Figure 2. Expression of wt and single site glycosylation mutants in transfected cells. Protein bands identified as A and B represent the fully glycosylated mature GP3 and mutant GP3 in which just one potential glycosylation site is mutated.

Fig. 2, it appears that only five (at positions 29, 50, 131, 152, and 160) out of the seven potential glycosylation sites are used to generate the mature and fully glycosylated GP3 protein. The potential sites at positions 42 and 195 are not used for addition of glycan moieties in GP3.

(c) GP4 Protein: The GP4 protein possesses four potential glycosylation sites. To determine which of these sites are glycosylated in the mature GP4 protein, expression of the mutant GP4 with individual amino acid substitutions at each of these was examined in transfected cells. Immunoprecipitation of cell extract with anti-GP4 peptide antibody resulted in detection of GP4 in transfected cells. Expression of each of the mutant proteins was also detected. Our results (Fig. 3) revealed that all four potential glycosylation sites in GP4 are used for addition of glycan moieties to generate the mature GP4 protein. One of the mutant proteins (GP4-

N120A) was consistently detected at

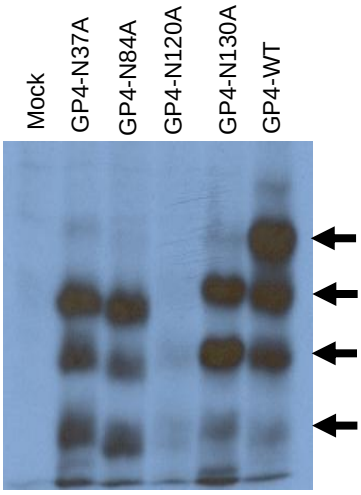


Figure 3. Expression of GP4 wt and mutant proteins in transfected cells. Protein bands identified represent fully glycosylated (A) or partially (B and C) or unglycosylated (D) GP4 proteins.

levels significantly lower than the other mutant proteins. It is possible that reactivity of this mutant protein with the antibody may have been altered. Alternatively, the protein may be relatively unstable compared to the wt or other mutant GP4 proteins.

(ii) Recovery of mutant PRRSV encoding hypoglycosylated forms of viral glycoproteins

The coding sequences of the mutant GP2 (N178A, N184A, and N178/184A) and GP4 (N37A, N84A, N120A, and N130A) proteins were transferred to full-length infectious cDNA clone (FL12) of PRRSV separately. In vitro transcripts were generated from the corresponding plasmids and transfected into MARC-145 cells. Viruses encoding GP2-N178A, GP4-N37A, GP4-N84A, GP4-N120A, and GP4-N130A could be readily recovered from the transfected cells. Multiple attempts to recover viruses encoding GP2-N184A and GP2-N178/184A failed, indicating that glycan addition at position 184 in GP2 is important for recovery of infectious PRRSV. Most of the mutant viruses had similar growth kinetics as the wt PRRSV and all grew to final titers that were within 2- to 3-fold of the wt virus. Overall, the results indicate that mutations of single glycosylation sited in these mutant viruses did not adversely affect the growth of the mutant viruses in cell culture.

(iii) Neutralizing antibody response in pigs infected with glycosylation mutant PRRSVs

Groups of four pigs each were infected with various mutant viruses intramuscularly. Serum samples from each of the animals were collected at 7, 14, 21, 35, and 46 days post-infection (dpi) and neutralizing antibody titers in the sera were determined as previously reported from out laboratory. Results (shown in Table 1) revealed that in all mutant virus infected pigs, the neutralizing antibody response was significantly down-regulated as compared to the wt PRRSV. This was surprising given our previous observation that pigs infected with PRRSV containing hypoglycosylated forms of GP5 produced significantly higher levels of neutralizing antibody response not only to the homologous mutant viruses but also to the wt PRRSV. Only one animal (# 086975) in the group infected with GP4-N84A produced neutralizing antibody titers that were close to the wt PRRSV. The reason(s) for this higher response is unclear at this time but in most other animals, the neutralizing antibody response was down-regulated.

Table 1:

NEUTRALIZING ANTIBODY ACTIVITY AGAINST FL12 WT AT DIFFERENT TIMES PI

Group infected with	Animal ID	7dpi	14dpi	21dpi	35dpi	46dpi
GP4-N37A	086968	0	ND	0	1/8	1/16
	086969	0	0	1/2	1/2	1/8
	087290	0	0	1/2	1/16	1/32
	087292	0	0	0	1/4	1/8
	Geometric mean	**1**	**1**	**1.41**	**5.66**	**13.45**
GP4-N84A	086731	0	0	1/2	1/8	1/16
	086975	0	1/2	1/2	1/16	1/128
	087289	0	0	1/2	1/8	1/16
	087299	0	0	0	1/4	1/16
	Geometric mean	**1**	**1.19**	**1.68**	**8**	**26.9**
GP4-N120A	086951	0	0	0	1/16	1/64
	086964	0	0	0	1/2	1/16
	086970	0	0	0	1/8	1/32
	087295	0	0	0	1/4	1/16
	Geometric mean	**1**	**1**	**1**	**5.66**	**26.91**
GP4-N130A	086965	0	0	0	1/4	1/64
	086971	0	0	1/2	1/8	1/32
	086972	0	0	0	1/4	1/16
	086974	0	0	0	1/16	1/64
	Geometric mean	**1**	**1**	**1.19**	**6.73**	**38.06**
FL12 wt	086953	0	0	0	1/16	1/128
	086954	0	0	0	1/16	1/64
	086963	0	0	1/2	1/32	1/128
	086973	0	0	1/2	1/32	1/128
	Geometric	**1**	**1**	**1.41**	**22.63**	**107.63**

	mean					
GP2-N178A	086952	0	0	0	1/2	1/4
	086967	0	0	1/2	1/16	1/32
	087294	0	0	0	1/8	1/16
	087296	0	0	0	1/8	1/64
	Geometric mean	**1**	**1**	**1.19**	**6.73**	**19.03**

Objective 2:

This objective deals with the study of cross-reactive capacity of the hypoglycosylation-enhanced neutralizing response against diverse viral isolates. Since, we could not detect higher levels of neutralizing antibody response in pigs infected with the hypoglycosylated mutant PRRSV, we were unable to carry out further studies on this objective.

VII. Discussion

PRRSV encodes four structural glycoproteins (GP2, GP3, GP4, and GP5) which are necessary for morphogenesis of infectious virus particles. Results presented in this report reveal that GP2 has two glycan moieties, GP3 has five glycan moieties and GP4 possesses four such moieties. Glycan addition at residue 184 of GP2 is required for recovery of infectious particles.

In previous studies, we had shown that the glycosylation of certain sites in the major surface glycoprotein, GP5 is important for recovery of infectious PRRSV from cells. Furthermore, we had observed that pigs infected with viruses encoding hypoglycosylated forms of GP5 could mount significantly higher levels of neutralizing antibody response against not only the homologous mutant viruses but also against the wt PRRSV. In the light of these observations, we speculated that it may be possible to further enhance the neutralizing antibody response by hypoglycosylation of other minor glycoproteins present on the surface of PRRS virions. However, our results show that hypoglycosylated forms of the minor glycoproteins do not induce higher neutralizing response, rather, a significant down-regulation of the response was observed in pigs infected with these mutant viruses. At this time, it is unclear how the hypoglycosylated forms of GP2 and GP4 down-regulate neutralizing response. It is possible that these mutant glycoproteins interact with GP5 in a manner that affect the detection of the neutralizing epitope on GP5 without affecting interactions that are required for virus assembly and infectivity.