RESEARCH REPORT
SWINE HEALTH

Title: Development of a Broadly Protective PRRS Vaccine – **NPB #05-205**

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Abstract

Current PRRS vaccines provide limited protection against heterologous strains of viruses. The sub-optimum level of protection is likely caused by 1) a high degree of sequence variation in structural proteins and 2) deceptive imprinting due to the presence of immunodominant non-protective epitopes (IDNPEs). We hypothesize that variable IDNPEs act as decoys to mislead the host from mounting humoral or cellular immune responses against more highly conserved epitopes that may otherwise induce cross-strain protection. We have proposed to apply immune refocusing technology to the development of a broadly protective PRRS vaccine. Our review of available sequence databases, epitope discovery data, and published literature has led us to focus on the design of new vaccines containing immune refocused GP5 glycoproteins. We have identified two GP5 domains that appear to contain IDNPEs. By site-directed mutagenesis, we have introduced a series of mutations in the N-terminal ectodomain and the C-terminal endodomain of GP5 for expression in recombinant vaccinia virus vectors. A subset of the GP5 mutants have been co-expressed with the viral M protein to determine whether the formation of GP5-M heterodimers result in improved immunity. A preliminary antigenicity study in mice has been completed. Serum samples have been analyzed for neutralization of PRRS virus using a newly developed microneutralization assay. Results indicate that some GP5 mutants induced sera with reduced neutralization activity (as compared to wild-type GP5): these mutants are helpful in mapping important viral epitopes. The results also indicate that some of the mutants induced sera with increased neutralizing activity: these mutants appear to refocus the immune system to recognize protective viral epitopes and will be the focus of future studies. Taken together, the results from the current work suggest that several of the mutants represent enhanced vaccine candidates. In a future study, we propose to incorporate these immune refocused GP5 genes into molecular clones of vaccine strains for immunogenicity and protection studies in swine.

III. Introduction

Major challenges in the design of a broadly protective vaccine against porcine respiratory and reproductive virus (PRRSV) include overcoming the problems of ineffective immune responses and antigenic variation. Many pathogens that are resistant to conventionally designed vaccines appear to have immunodominant B and T cell epitopes that either do not induce neutralizing immunity or induce only strain-specific immunity. Immunodominant, non-protective epitopes (IDNPE), serve to misdirect or block the induction of more protective or cross-strain immunity. This phenomenon, termed "deceptive imprinting", assists the pathogen in the evasion of the host immune system. Using site-specific mutagenesis, we have altered the IDNPEs in other pathogens (e.g., HIV, CAEV, NTHI, and malaria) and observed a refocusing of the immune system towards more broadly protective epitopes. We have completed the second year of our efforts to identify and immune dampen IDNPEs in PRRSV. We, and others, seek to determine whether the immune refocused antigens can induce immunity against a broader range of viral strains.

Our earlier work with HIV lead to the discovery of both deceptive imprinting and immune refocusing technology. Several scientists in other fields, including PRRS vaccine development, understood the implication of the HIV discoveries and were among the first to apply these ideas to map and characterize decoy epitopes on PRRSV. The work being funded in this grant is both unique and complementary to others currently funded. We contribute our expertise and knowledge of acute phase mapping, epitope discovery methods, and the design and platform technology of immune refocusing viral antigens for PRRS vaccine development.

III. Stated Objectives from original proposal

Objective #1 *Improve upon the availability of native, whole PRRSV preparations and further develop eukaryotic systems for the expression of more authentically processed GP5, M, GP5-M heterodimers, GP4 and other viral proteins for Immunodominant Non-Protective Epitope (IDNPE) mapping and analysis.*

Objective #2 *Identify IDNPEs on vaccine target proteins. Serological reagents that have been produced in the first year of funding will be used to map dysregulating and strain-specific epitopes on PRRSV proteins*.

Objective #3 *Engineer and produce recombinant PRRSV proteins in which the IDNPEs have been immuno-dampened.*

Objective #4 *Immunize animals with the immune dampened vaccine candidates and characterize the immune responses.*

IV. Materials and Methods

Cells and virus

VR2332 and MN184 strains were obtained from Michael Murtaugh (U. Minnesota), NSU 97-7895 strain was obtained from Fernando Osorio (U. Nebraska), the MLV modified live vaccine derived from VR2332 was purchased from Boherineger Ingleheim. MA104 and MARC-145 cell lines were a kind gift from Dr. Murtaugh.

Peptides and recombinant proteins

An overlapping set of 14mer peptides representing the VR2332 GP5 protein were synthesized in solid phase for immunological mapping. Remaining aliquots of the peptides are available to the greater PRRS research community.

A set of proteins and protein fragments engineered by Dr. Murtaugh and expressed under contract were obtained for the purpose of generating protein-specific PRRS sera. The recombinant proteins were mixed with Freund's adjuvant and used to immune mice. Mouse sera was found to react with both the recombinant and native PRRS proteins.

Serological materials and immunological methods

Sera produced from PRRS-infected pigs in the "Big Pig Study" was obtained from Jeff Zimmerman (Iowa State University). The sera was used to map protective and strain-specific epitopes using ELISA and Western blots.

Protein-specific sera was raised against bacterially expressed recombinant PRRS proteins as noted above.

For mapping linear epitopes, ELISA plates were coated with the overlapping GP5 peptide library, blocked in a solution of 10% dry milk in PBS, and reacted with diluted sera from infected and immunized animals. After washing with a PBS solution containing 0.1% NP40 and 0.1% Tween 20, bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and appropriate chemophores. All assays were done in triplicate and repeated at least three times.

Western blot detection was done using standard methods. Briefly, protein samples were denatured in SDS and 2-mercaptoethanol, separated in polyacrylamide gels, and transferred to nitrocellulose membranes. The blots were blocked in 5% milk, reacted with diluted sera from infected and immunized animals, and detected with horseradish peroxidase-conjugated secondary antibodies. Bound antibodies were visualized by chemiluminescence and X-ray film.

Molecular techniques

Standard molecular cloning techniques were used. At the outset of the study, the GP5 gene was synthesized using codons optimized for expression in mammalian cells. Mutations were introduced using Quick-change methods. All clones were sequenced prior to use.

GP5, M, and derived genes were cloned into a derivative of pSC11 for engineering recombinant vaccinia viruses. The pSC11 constructs were transfected into mammalian cells lacking thymidine kinase. The cells were infected with the WR strain and recombinant viruses selected with bromodeoxyuridine. Virus plaques were purified to homogeneity through at least six rounds of isolation and shown to express GP5 and/or M proteins. Vaccinia viruses were amplified in HeLa cells and purified through sucrose gradients for use as immunogens.

Animal studies

Protein-specific serological reagents were raised using mice housed at BioCon, Inc., in Rockville, MD. All manipulations were performed humanely by appropriately trained and certified animal care technicians. Protein immunogens were mixed with complete Freund's adjuvant and injected into multiple subcutaneous sites. Boosts were performed using incomplete Freund's adjuvant.

Immune refocused serological reagents were raised using groups of four mice each at BioCon, Inc. Sucrose purified recombinant vaccinia virus engineered to express mutated GP5, alone or in combination with M protein, were injected in 0.1 cc volumes into two intradermal sites. Mice were boosted with recombinant vaccinia virus 3 and 4 months after the initial inoculations.

V. Results

Objective #1 *Improve upon the availability of native, whole PRRSV preparations and further develop eukaryotic systems for the expression of more authentically processed GP5, M, GP5-M heterodimers, GP4 and other viral proteins for Immunodominant Non-Protective Epitope (IDNPE) mapping and analysis.*

We have optimized the production of highly purified PRRSV virions for protein analysis using chemical precipitation, rate-velocity sucrose gradient centrifugation, and cesium chloride density gradient centrifugation. The purified virions are needed for immunological analysis, positive controls in gene expression assays, assessment of serological samples, and analyses of virion proteins. In addition, the purified virions serve as a source of native viral proteins, GP5, M, GP5-M heterodimers, GP4, and all other viral proteins. "Mock infected" materials for negative control have also been produced by processing samples from uninfected cells.

After assessing multiple vector systems, we have successfully expressed GP5 using recombinant vaccinia virus in mammalian cells. The use of a mammalian system results in the expression of recombinant GP5 that closely resembles the native glycoprotein. Several studies have showed that the GP5 and M proteins are assembled as heterodimers. Because it is possible that the heterodimers present nonlinear epitopes that are not present in the separate proteins, we have engineered vaccinia virus vectors that co-express GP5 and M proteins. Due to the interest in GP5, M, and the GP5-M heterodimers as leading targets for vaccine candidates, we are currently concentrating our attention on these proteins at the expense of other viral proteins. Figure 1 shows a Western blot analysis of denatured and non-denatured virus particles. The results demonstrate the dimeric nature of the GP5-M interaction and the recognition of these proteins with anti-M, anti-GP5 and sera from infected swine.

Although many researchers have published data using anti-PRRS monoclonal and polyclonal antibodies, we were unable to obtain these antibodies. Therefore, we produced reagent volumes of PRRS antibodies to assist in identification of native and recombinant proteins. We obtained a panel of recombinant PRRSV proteins and polypeptides expressed in bacterial systems by Drs. Murtaugh and Johnson (U. Minn.). The panel included proteins and protein fragments representative of NSP-2P, NSP-4, gp5, N, M, and some interesting gp5+M combinations. The proteins were mixed with adjuvant and used for inoculation of groups of mice. The resultant mouse sera were instrumental in helping us identify native and recombinant PRRS proteins and develop serological assays. Small volumes of these sera are available to PRRS researchers upon request.

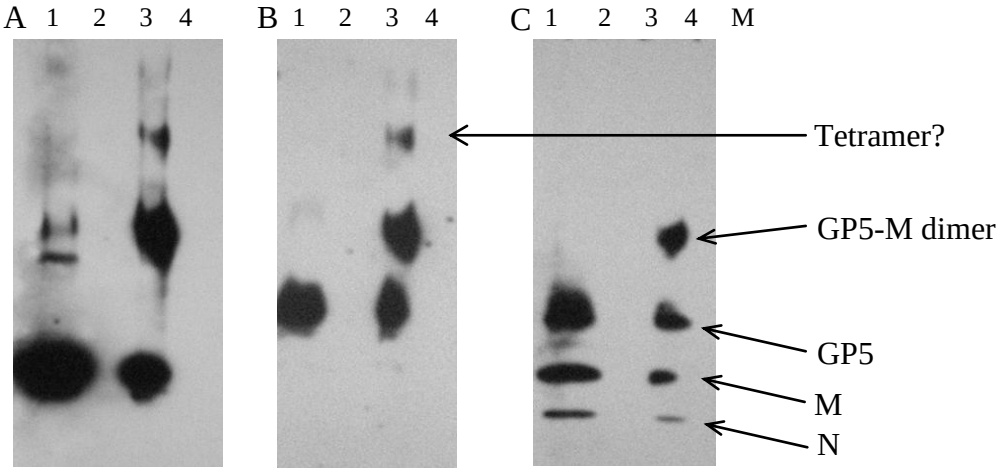

Figure 1. Western blot analysis of purified PRRSV. VR2332 virions were purified from the supernatant of infected cells, electrophoresed, transferred to membranes, and probed with antisera. Lanes 1: virions, reduced; Lanes 2: mock virions, reduced; Lanes 3: Virions, non-reduced; Lanes 4: mock virions, non-reduced. Panels A, B, and C were probed with mouse sera after immunization with bacterially expressed M fragment, mouse sera after immunization with bacterially expressed GP5 fragment, and swine sera 84 days following infection with VR2332, respectively. Arrows indicate putative identification of virus protein bands.

Objective #2 *Identify IDNPEs on vaccine target proteins. Serological reagents that have been produced in the first year of funding will be used to map dysregulating and strain-specific epitopes on PRRSV proteins*.

Published reports of the GP5 of PRRSV and related viruses, suggest that the glycoprotein has three membrane spanning domains that result in an N-terminal ectodomain (Ecto-1) and a C-terminal endodomain (Endo-2) (Fig. 2). The drawing below shows the relative locations of the major protein domains as well as the regions that we have targeted for mutagenesis (boxed).

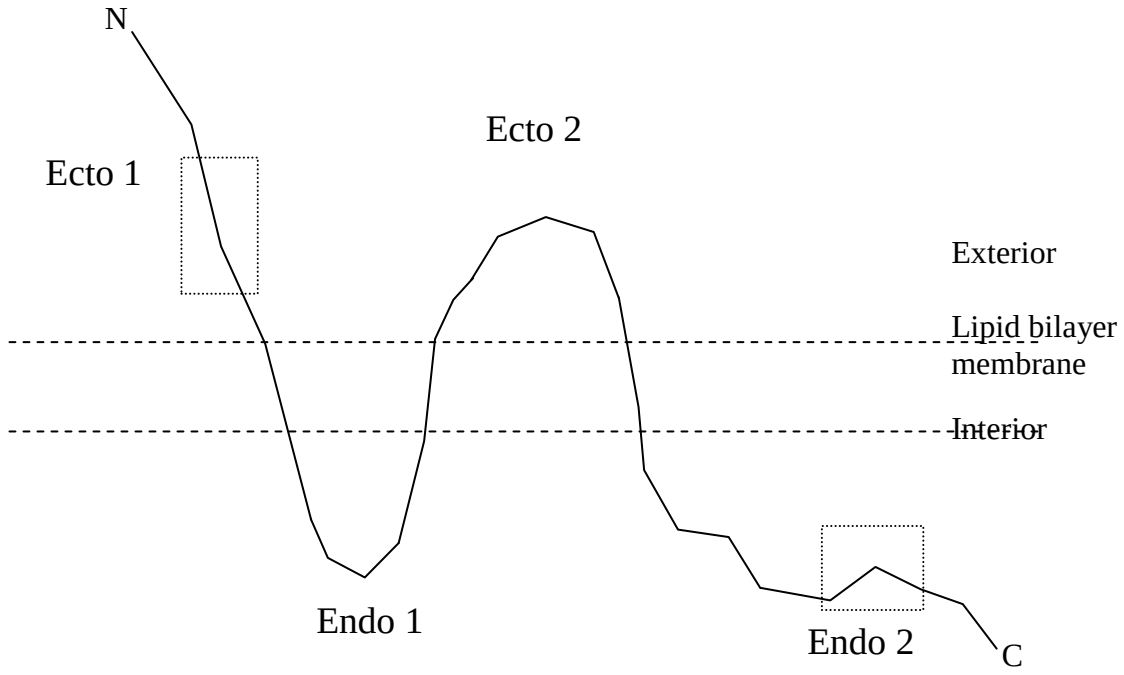

Figure 2. Representation of PRRSV GP5 glycoprotein showing approximate positions of two putative ectodomains and endodomains. Boxes indicate localization of putative dysregulating or decoying epitopes that have been targeted for immune refocusing. Figure is not drawn to scale. Note that the GP5 protein appears to interact with the viral M Protein through cysteine residues in the ectodomains.

Numerous published studies have suggested that the major neutralizing and the decoy epitopes are juxtaposed in Ecto-1. Based upon these data, alignments of multiple sequences, and our prior experience with Immune Refocusing Technology, we engineered a series of mutations in the Ecto-1 domain (Fig. 3). These mutations were designed to dampen the immunogenicity of decoy epitopes and to further characterize the protective epitopes in the Ecto-1 domain. Previously, we engineered the mutations into the complete codon-optimized GP5 gene. In the current grant period, we have cloned the mutated GP5 genes into a transfer plasmid that contains sequences that promote recombination into the vaccinia virus genome.

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VR2332  -PFCF A V L A N A S N D S S S H L Q L I Y N L T L CELNGTDWL..
C1      -PFCF A n L s N A S N D S S S H L Q L I Y N L T L CELNGTDWL..
C2      -PFCF A V a A N A S N D S S S H L Q L I Y N L T L CELNGTDWL..
C3      -PFCF A V L A N A S N D S S S H L Q L I Y N L T L CELNGTDWL(stop)
C4      -PFCF A - - - N A S N D S S S H L Q L I Y N L T L CELNGTDWL..
C5      -PFCF A V L A N A S N D S - - - - - - - - - N L T L CELNGTDWL..
C6      -PFCF - - - - - - - - - - - - - - - - - - - - - - - -------WL..
C7      -PFCF A V L A N A S N D S S S l L Q L I Y N L T L CELNGTDWL..
C8      -PFCF A V L A q A S q D S S S H L Q L I Y q L T L CELNGTDWL..
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Figure 3. Immune refocusing mutations of the GP5 ectodomain. The figure shows a portion of the GP5 sequence that has been altered. C3 is truncated after –DWL and approximates the Endo-1 domain.

Our peptide mapping and Western blot data suggest that the Endo-2 domain contains an immunodominant epitope in a fragment represented by the synthetic peptide IEKRGKVEVEGHLIDL. Since this time, Dr. Osorio has presented independently derived data that appears to confirm the immunodominant epitope in Endo-2. To determine whether this segment of the GP5 glycoprotein contains a decoying or otherwise dysregulating epitope, we have designed a series of endo-domain mutants to immune dampen the dominant epitope (Figure 4). We have designed and engineered the second set of mutations into a transfer vector for the derivation of recombinant vaccinia viruses.

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VR2332 -WRSPVI I E K R G K V E V E G H L I D L KRVVLD-
D1     -WRSPVI I a g a G K V E V E G H L I D L KRVVLD-
D2     -WRSPVI I E K R G a V g V a G H L I D L KRVVLD-
D3     -WRSPVI I E K R G K V E V E G a L I g L KRVVLD-
D4     -WRSPVI I a g a G a V g V a G a L I g L KRVVLD-
D5     -WRSPVI I E K R G K V n V t G H L I D L KRVVLD-
D6     -WRSPVI - - - - - - - E V E G H L I D L KRVVLD-
D7     -WRSPVI I E K R G K V - - - - - - - - - KRVVLD-
D8     -WRSPVI - - - - - - - - - - - - - - - - KRVVLD-
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Figure 4. Immune refocusing mutations of the GP5 endodomain. The figure shows a portion of the GP5 sequence that has been altered based upon immune mapping data.

Objective #3 *Engineer and produce recombinant PRRSV proteins in which the IDNPEs have been immuno-dampened.*

We have engineered full-length GP5 genes to incorporate the mutated amino acids. After assessing the expression of GP5 in several mammalian systems, we observed good expression using a codon-optimized gene in a vaccinia virus vector. Figure 5 shows Western blots of two independent assays in which recombinant GP5 species were expressed by recombinant vaccinia vectors and detected using sera from mice immunized with a bacterially expressed GP5 fragment. Panel A contains extracts from cells infected with a vaccinia recombinant containing the wild-type (unmodified) GP5 gene and non-recombinant Western Reserve vaccinia. Panel B contains extracts from two isolates each of GP5 ecto-6 (large deletion), ecto-8 (hyper-glycosylated), and endo-3 (charge dampened). The figure shows that the codon-optimized GP5 gene can be expressed in mammalian cells by recombinant vaccinia virus.

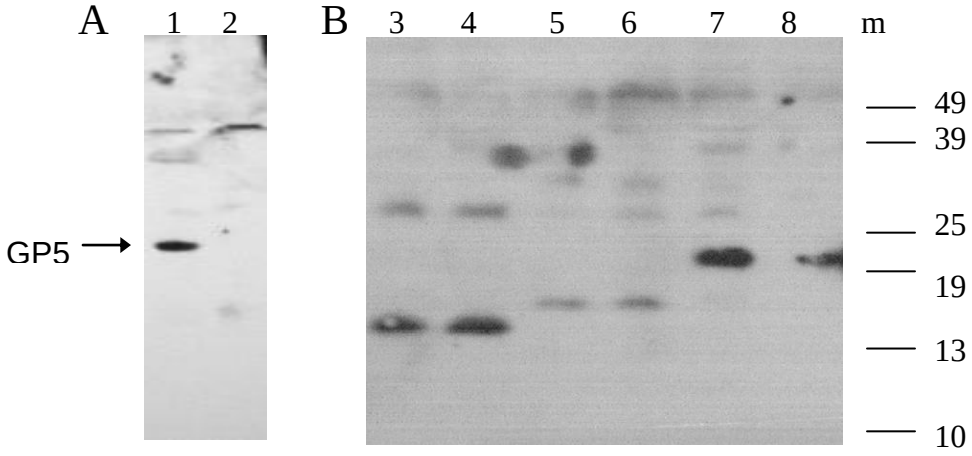

Figure 5. Western blot detection of recombinant GP5. Detergent extracts from HeLa cells infected with vaccinia recombinants were denatured, electrophoresed, transferred to membranes and probed with anti-GP5 mouse sera. Panel A contains extracts from GP5-WT and WR vaccinia (Lanes 1 and 2). Panel B contains extracts from duplicate isolates of vaccinia expressing Ecto-6, Ecto-8, and Endo-3 mutants (lanes 3 and 4, 5 and 6, and 7 and 8).

Based upon our previous experiences demonstrating that the use of heterologous signal sequences can result in increased expression of viral glycoproteins, we engineered the codon-optimized signal sequences of two secreted proteins into the GP5 vaccinia virus transfer vector. However, we did not observe expression of GP5 from recombinant vaccinia with either the human tissue plasminogen activator or the murine interferon-gamma leader peptides (data not shown) and therefore the native signal peptide will be used for all recombinant vaccinia for immunization.

In addition to our work with GP5, we have studied the interactions between GP5 and M protein. We observed the presence of higher ordered species of proteins (heterodimers and, potentially, tetramers shown in Figure 1) that may shed light on the structure of the glycoprotein spike that the virus assembles in order to bind cells for infection. We propose that the GP5-M protein dimer or tetramer functions as the receptor binding molecule and that, as is the case with HIV and influenza, non-linear, conformational epitopes may be formed that play major roles in immunity and immune evasion. By co-expressing the M protein with various GP5 mutants, it may be possible to map the determinants that permit GP5-M interactions and further characterize this viral structure. Therefore, we have devised a vaccinia expression system for the co-expression of GP5 and M proteins in mammalian cells (Figure 6). The constructs contain dual promoters to drive expression of each of the two genes. We have engineered recombinant vaccinia viruses for co-expression of M with a small set of the GP5 mutants. If we can show that the recombinant GP5 and M proteins produce dimers or tetramers, we will

make a complete set of the dual expression vectors for each of the GP5 mutations and assess their immunogenicity in mice.

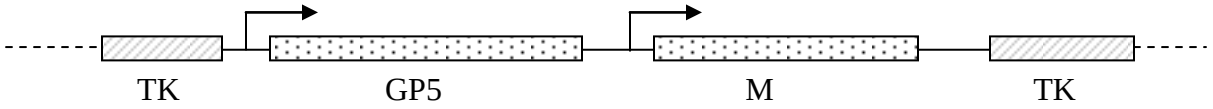

Figure 6. Structure of vaccinia transfer vector for the co-expression of VR2332 GP5 and M. The codo-optimized GP5 genes were cloned into a derivative of the vaccinia vector, pSC11. The M gene was cloned into pCR2.1 with a vaccinia virus promoter in the 5' flank. All upstream translation start sites removed and the modified M gene subcloned into the each of the pSC-GP5 constructs. The drawing shows the location of the two thymidine kinase sequences that promote homologous recombination into the vaccinia genome. The arrows represent the locations of the vaccinia virus transcriptional promoters.

After design and construction of the necessary plasmid vectors, we have derived recombinant vaccinia viruses for the expression of the immune refocused GP5 proteins. The vaccinia are to be used for production of recombinant protein and for vaccination of test animals. The full set of recombinant vaccinia includes:

Wild-type, unmodified GP5	WT GP5 expressed with M
Eight ecto-1 mutations	M expressed alone
Eight endo-2 mutations	M with GP5-Ecto6
Empty vaccinia vector	M with GP5-Ecto8
	M with GP5-Endo4

Objective #4 Immunize animals with the immune dampened vaccine candidates and characterize the immune responses.

Numerous studies of PRRSV epitopes, especially those contained within GP5 have been conducted in mice. In addition, mouse monoclonal antibodies to both neutralizing and decoy epitopes have been described. Although mice can not be infected with the VR2332, they present an attractive model for the immunological studies, especially those associated with the analysis of immunogens. Advantages of mice over natural hosts such as swine include 1) mice usually require less immunogen than larger animals, 2) mice are small and large numbers can be housed in colonies, 3) mice are not infectible with PRRSV and, therefore, do not have an immunological background associated with PRRSV infection that could interfere with or confound an immunization study, and 4) mice are relatively inexpensive and, therefore, can be used to study larger numbers of test immunogens. After consultation with established investigators in the PRRSV field, we initiated a mouse immunization study to assess the ability of our panel of 23 vaccinia recombinants to elicit broadened neutralizing antibody responses.

Groups of four outbred mice each were immunized intradermally with approximately 2 x 10^7 plaque-forming units of recombinant vaccinia virus (Figure 7). After fourteen and seventeen weeks, the mice were boosted with a subsequent immunization of recombinant vaccinia. Twenty-one weeks after the initial immunizations, the mice were euthanized and bled out for sera. Approximately 1 mL of sera was obtained from each mouse. Serum pools were made to average out the responses for the individuals in each group. We had originally intended to boost the mice with recombinant GP5 produced in mammalian cells. GP5 mutants were engineered to encode a polyhistidine tract in the 3' end of the genes. HeLa cells infected with the recombinant vaccinia viruses expressing the modified GP5 proteins produced adequate quantities of recombinant GP5. However, the extracted GP5-His would not bind to metal-chelated resins used for standard His-tag purifications. We hypothesize that the C-terminal cytoplasmic tail of the glycoprotein was folded in a manner that prevented exposure of the histidine tag to the resin. For this reason, we used recombinant vaccinia viruses for all immunizations.

Group	Antigen	Group	Antigen	Group	Antigen
P1	C1	P11	D1	P21	WT and M (two separate vectors)
P2	C2	P12	D2		
P3*	C3	P13	D3		
P4	C4	P14	D4	P22	D4+M
P5	C5	P15	D5	P23	C6+M
P6	C6	P16	D6	P24	C8+M
P7	C7	P17	D7	P25	VR2332 virion
P8	C8	P18	D8	P26	97-7895 virion
P9	GP5 WT	P19	M		
P10	WR	P20	WT+M (dual expression)		

Week –1	Prebleed	Week 16	Test bleed
Week 0	Immunize	Week 17	Boost
Week 5	Test bleed	Week 19	Test bleed
Week 14	Boost	Week 21	Bleed out

Figure 7. Details of GP5 antigenicity experiment. Groups of four mice each were inoculated with recombinant vaccinia viruses engineered to express immune refocused GP5 antigens. Antigens designated with C or D prefixes contain modified ectodomains or endodomains, respectively as detailed in Figures 3 and 4.

We have completed the initial serological analysis of the immune responses induced by immunization of the mice with immune refocused GP5 genes. Sera from individual mice in each group were pooled to normalize the variations in immune responses between individuals and to produce a large enough volume of serum for assays. Several dilutions of the sera were incubated with homologous (VR2332) and heterologous (97-7895) viruses for 1 hour and then used to inoculate in triplicate monolayers of MARC145 cells in 96-well plates. After another 1 hour at 37C, the inocula were rinsed off, 200 ul of complete media was added to each well, and the plates were returned to the incubator. After 48 hours, the cells were washed with PBS and solubilized in detergent. Samples of the detergent extracts were used to coat ELISA plates as summarized in the Methods section above. The amount of viral antigen present, a measurement of viral infection, was quantitated by reaction with anti-N antibody and detected in an ELISA plate reader. Data from several assays were analyzed for consistency and effectiveness of presentation methods. The mean values of the OD readings from the replicate wells were tabulated in a bar graph using a standard antibody dilution of 1:20 (Figure 8). A red dotted line corresponding to the signal produced by neutralization with serum from WT-GP5 was drawn across the graph to assist in comparison between immune responses induced by the various mutant constructs. Data points that exceed the red lines result from sera that do not neutralize virus as well as that from immunization with WT-GP5. Data points that are below the red line identify constructs that induce greater neutralizing responses. Thus, those that do not neutralize virus assist in the identification of important viral epitopes and those that do neutralize virus identify putative vaccine candidates for further study. Negative controls include normal mouse sera, prebleed serum pool, no serum, and serum from mice immunized with empty vaccinia virus vector (WR). Positive controls include swine serum sampled 84 days after infection with VR2332. It is interesting to note that not all of the constructs that result in enhanced homologous neutralization activity induce enhanced heterologous neutralization and vice verse. In addition, the data suggest that M protein contains no neutralizing epitopes and that some constructs may induce virus-enhancing activities. Therefore, as expected, this study has helped us to identify epitopes that are important in both strain-specific and broadened immunity.

Although we are continuing to analyze the sera for broadened neutralizing activity against other isolates, we recognize the limitation of the reagents that we have produced. Most importantly, the use of vaccinia virus immunization induced relatively low-level humoral responses as measured by whole-virus ELISA and Western blot analyses. We anticipate that enhanced antigen presentation, preferably in the context of a virus particle, would induce higher levels of antibodies which would facilitate more in-depth and exact analyses of the immune responses. For this reason, we propose a follow-up study in which, in collaboration with Drs. Osorio and Pattnaik at the University of Nebrasksa, we insert the immune refocused GP5 genes into a molecular clone of a vaccine strain. We anticipate that the immunization of pigs with these newly derived vaccine candidates will facilitate the identification of the most promising vaccine candidates and help advance the development of a more broadly protective PRRS vaccine.

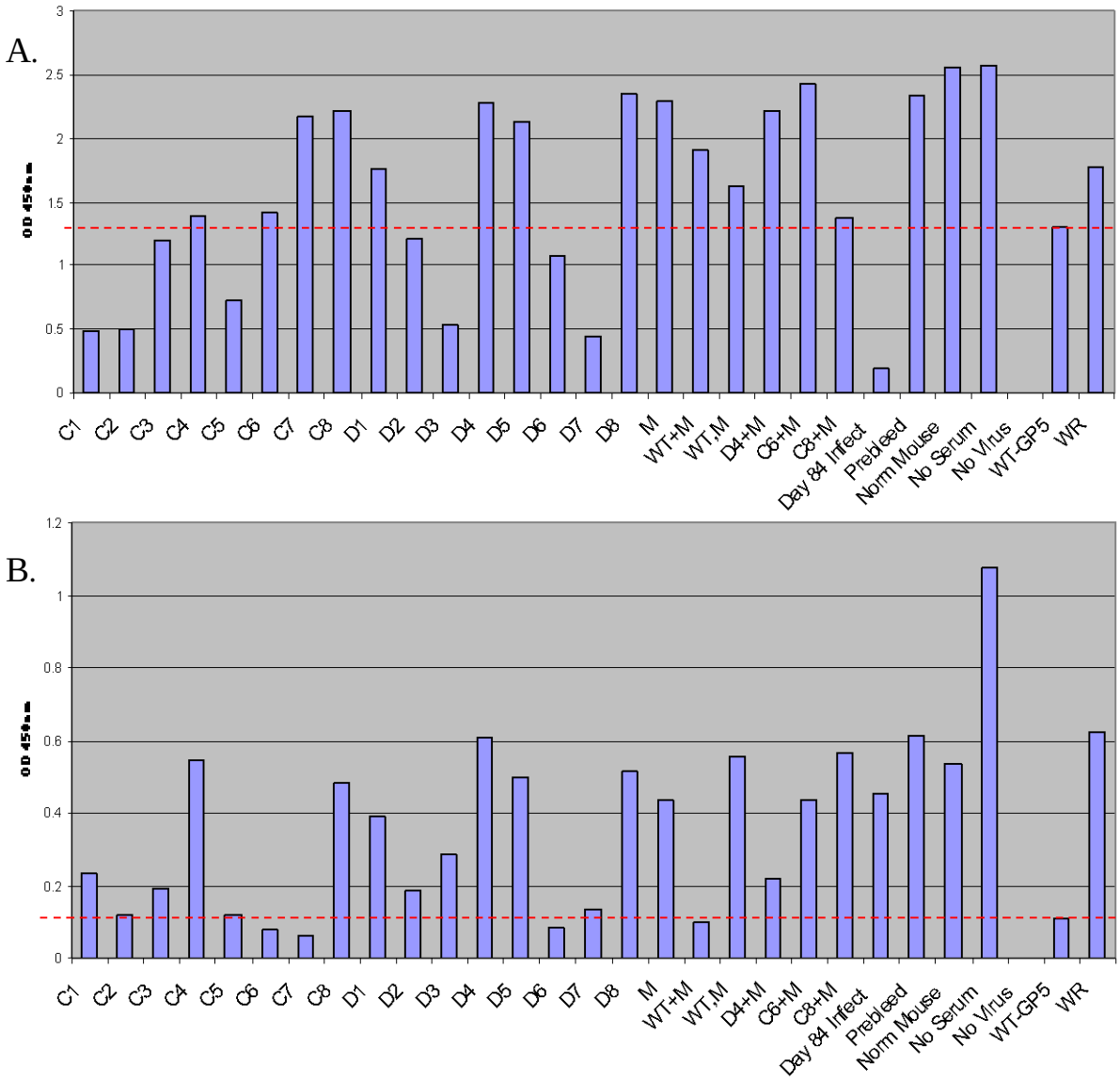

Figure 8. Neutralization ELISA. Mice were immunized with recombinant vaccinia viruses designed to express immune refocusing the GP5 mutations as listed on the horizontal axis. Sera were pooled within groups and tested for neutralization of PRRS strains VR2332 (Panel A) and N-9767 (Panel B) at a standard serum dilution of 1:20. Two days following inoculation of monolayers, detergent extracts were used to coat ELISA plates which were detected with anti-N antibody and read by ELISA. Mean values of triplicate assays were graphed above. Higher OD values within an assay indicate greater virus antigen expression. A dotted red line was drawn across the graph at the level of WT-GP5 serum neutralization. Mutants with values below the red line appear to induce greater levels of neutralizing antibody in comparison to unmodified, WT GP5 immunization. WR = serum from mice immunized with non-recombinant WR vaccinia virus (negative control); WT-M indicates GP5 and M co-expressed in a dual expression vaccinia virus; WT+M indicates co-immunization with vaccinia expressing GP5 and M as separate vectors.

Future Aims

Modified GP5 constructs that induce higher heterologous neutralization titers when compared to unmodified (WT) GP5 will be selected for further study. We intend to collaborate with PRRSV researchers having access to large animal facilities to perform follow-up vaccine efficacy studies in swine.

We plan to design and engineer second-generation modified GP5 constructs. We had not anticipated the discovery of an immunodominant epitope in the C-terminal endodomain of GP5. A set of combination constructs will be made that incorporate the two or three Endo- and Ectodomain mutations that induce broadened protection.

Initial analyses of serum samples from the immunization study suggest that the GP5-M dual expression constructs produced higher anti-GP5 titers than the GP5-alone recombinant vaccinia. If these results are confirmed, we will examine this novel system more fully for expressing second-generation GP5 mutants.

We are currently studying a set of immune refocused foot-and-mouth disease mutants in collaboration with USDA researchers at the Plum Island Animal Disease Center. Together, we have engineered the FMDV mutants into a recombinant adenovirus system for vaccination of cattle and swine. We will consider vectoring the PRRSV GP5 refocused antigens in the adenovirus system for vaccine-challenge studies. In addition, we have developed strategies to insert the modified GP5 genes into molecular clones of the Dr. Kay Faaberg's Ingelvac vector and Dr. Fernando Osorio's attenuated proviral vector.

VII. Discussion:

Our results confirm and extend several previous findings. First, we discovered an immunodominant epitope in the C-terminal endodomain, or cytoplasmic tail, of the GP5 glycoprotein. Because we expected that this portion of GP5 would be on the interior of virions and cells (and therefore less accessible to the immune system), we did not anticipate that sera from infected or immunized animals would focus on this region. This result demonstrates the value of epitope mapping and the neutralizing data should define the significance these immunodominant epitopes. Thus, it is of immediate importance to map three-dimensional epitopes on the virion so that additional immunodominant (and potentially dysregulating) epitopes can be discovered.

Careful analysis of viral proteins using both denaturing and nondenaturing electrophoresis methods confirmed the association between GP5 and M proteins. Because many of our nondenaturing gels contain higher molecular weight species that react to both GP5 and M proteins, we propose that the GP5 and M proteins exist as heterotetramers with two copies of each protein present. Follow-up studies will be required to determine whether the putative heterotetramers are used as the functional cell-binding receptor structure.

The study produced several unique reagents and methods. Using bacterially expressed proteins and polypeptides obtained from Dr. Murtaugh's laboratory, we raised gene product specific antisera. The antibodies react to both native and recombinant viral proteins. These important reagents assist us in identifying and discriminating between the many virion proteins. In mapping the linear epitopes of GP5, we synthesized an overlapping peptide library representing the glycoprotein. Remaining peptides are available to the PRRS research community. After discussions with several researchers, we became aware of the previous difficulties in expressing the GP5 in mammalian cells. Because GP5 must be expressed in mammalian systems to ensure accurate glycosylation and folding of the protein, we codon-optimized the gene. In addition, we developed a recombinant vaccinia virus system that expresses significant quantities of GP5. Prior methodologies for the production of PRRS virions result in low yields of impure material. We applied the experience gained through our work with many other mammalian viruses to develop a production and purification protocol that results in high-titered virus and highly purified virions. In addition, we developed a high-throughput method for deriving the virus neutralization titer of large numbers of sera. This protocol, which uses an ELISA readout for infected cells, will be useful in assessing neutralizing titers from larger immunization and vaccine studies.

Although the final determination of the success in applying the immune refocusing technology to PRRSV awaits testing in swine using immune-refocused GP5 genes incorporated into attenuated vaccine constructs, the data generated in this study has helped to identify important GP5 epitopes and point the way for future immune-refocusing studies. Specifically, several of the immune refocused GP5 constructs induced enhanced homologous neutralizing activities (e.g., C1, C2, C5, D3, and D7) and others induced better heterologous neutralization (e.g., C6, C7, D6). The significance of the disparity in the induction of homologous vs. heterologous neutralization is not clear and will require further study. The identification of multiple immune refocused ectodomain (the C series) and endodomain (the D series of mutants) suggests that further improvements can be made by combining the mutations. Hence (C1, C2, or C5) + (D3 or D7) combinations may be expected to induce enhanced homologous neutralization where (C6 or C7) + (D6) combinations may induce better heterologous neutralization.

VIII. Lay Interpretation:

The current vaccines against PRRSV protect pigs exposed to the strain used to make the vaccine, but not to the many field strains that herds encounter. The lack of protection against heterologous challenge may be caused by genetic variability of the virus and by dysregulating epitopes (portions of the virus against which the host immune system reacts) on the virus that misdirect the pig immune system. We have applied our immune refocusing technique to improve protection against many strains of PRRSV. Thus, we have attempted to redirect

the immune system away from strain-specific or non-protective portions of the virus (decoy epitopes on GP5) and towards parts of the virus that were previously hidden yet may induce broader protection. To test this method, we engineered recombinant vaccinia viruses that express two sets of mutated GP5 glycoproteins and then used these viruses to immunize laboratory animals. Some of the modified GP5 glycoproteins induced higher levels of neutralizing activity and suggest that the immune refocusing technology may result in improved PRRS vaccines. We have submitted a follow-up proposal designed to investigate these mutations in the context of an existing PRRS vaccine strain. These studies would lead to second-generation vaccines which may be capable of broadened protection against multiple strains of PRRSV.