Identification of PRRSV epitopes associated with antibody-dependent enhancement and neutralization of virus infection

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Identification of PRRSV epitopes associated with antibody-dependent enhancement and neutralization of virus infection

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This is to certify that the Master's thesis of

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I dedicate this work to Pedro, my husband, whose encouragement and support have helped me grow as a person and as a professional, and to my daughter Aurora del Sol for her delightful dimpled smile and contagious healing giggle. I want to mention my mother for teaching me the value of hard work and perseverance, and my father for his joy for life. Last but not least I dedicate this to the Lord Jesus, who gave me life, strength and wisdom to fulfill my dreams.
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Porcine reproductive and respiratory syndrome (PRRS) is an economically significant disease of swine, caused by a small, enveloped RNA virus belonging to the family *Arteriviridae*. PRRS virus preferentially replicates in macrophages and is capable of establishing persistent infection. While the mechanism by which PRRS virus persists in infected animals is unknown, enhanced infection and replication of PRRS virus in the presence of virus-specific antibody has been demonstrated *in vitro* and *in vivo*. This phenomenon, in which virus-specific antibody facilitates the entry of virus into susceptible cells resulting in increased severity of the disease, has been described as antibody-dependent enhancement (ADE) of virus infection. ADE also is considered to be a significant obstacle to developing effective vaccines for many viruses for which ADE has been reported. In this study, the role of specific PRRS viral epitopes in ADE and/or virus neutralization (VN) was assessed *in vitro*, using 18 monoclonal antibodies (MAbs) to 4 PRRS viral proteins: 15kD nucleocapsid (N), 19kD matrix (M), 25kD envelope glycoprotein (GP5), and 45kD GP3, each of which represents a distinct epitope. One-way ADE and VN assays were performed using homologous and heterologous PRRS virus isolates in the presence or absence of each MAb. ADE activity was determined by assessing the increase of progeny virus yield in porcine alveolar macrophage cultures in the presence of individual MAbs. Neutralizing activity was determined by the assessment of reduction or blocking of virus replication in MARC-145 cells in the presence of individual MAbs. MAbs could be categorized into 3 groups: enhancing, neutralizing and neither. Neutralizing epitopes appeared to reside on the M, GP3 and GP5 proteins. ADE epitopes were associated with the N, and GP5 proteins. Identification of the epitopes responsible for ADE and VN may provide the basis for developing efficacious second-generation vaccines for the control of PRRS virus.
GENERAL INTRODUCTION

A. Introduction

As is widely recognized, porcine reproductive and respiratory syndrome (PRRS) is an economically significant disease of swine caused by the PRRS virus (PRRSV). In acute outbreaks, economic losses from PRRSV have been estimated to range from $236 to $502 per sow in farrow-to-finish and breeding stock operations. In response to the economic effects of PRRS, various management strategies and vaccination protocols have been tested for controlling PRRS. At present, the definitive solution to the prevention and control of PRRS has not been found. A modified live virus (MLV) vaccine and a killed vaccine are currently available to swine producers, but it has been observed that PRRS outbreaks occur even in vaccinated herds. Several explanations have been proposed to account for this fact:

1. The currently available vaccines do not induce solid protective immunity;
2. Field isolates of PRRSV vary genetically and antigenically from vaccine strains to the degree that they cannot be controlled by the available vaccines; and/or
3. Antibody dependent enhancement (ADE) interferes with the protection conferred by PRRSV vaccines.

Enhancement of PRRSV infection in the presence of antibody is the subject of the present study. Antibody-dependent enhancement of virus infection is a phenomenon in which virus-specific antibodies actually enhance the entry and possibly the replication of virus in monocytes/macrophages and other cells that have Fc receptors on their surfaces. ADE has been described for viruses representing 12 different families, including the Arteriviridae to which PRRSV belongs. These effects are responsible, in part or in total, for the difficulties encountered in developing effective vaccines for a wide variety of viruses including Aleutian mink disease virus, dengue virus, equine infectious anemia virus, feline infectious peritonitis virus, and human immunodeficiency virus type 1.

To minimize the risk associated with ADE in controlling disease by vaccination,
efforts have been made to formulate vaccines that induce a balanced immune response or minimize ADE\textsuperscript{13,14}. The objective of the study was to characterize the role of PRRS viral proteins in ADE and virus neutralization (VN) and identify responsible epitopes, as the first step towards the development of a subunit vaccine(s) enriched with components associated with VN that would offer solid protective immunity against PRRSV.

**B. Thesis organization**

This thesis is organized in three main chapters. The first is the literature review on porcine reproductive and respiratory syndrome (PRRS) and its causative agent, PRRSV. The second is a review article on the subject of antibody dependent enhancement of viral infection to be submitted for publication in Viral Immunology. The third chapter is a manuscript of the experimental work on the identification of PRRSV epitopes involved in antibody dependent enhancement and neutralization of infection. This work will be submitted for publication in Archives of Virology. This thesis also contains a general conclusions section followed by the list of references for the first chapter and the general conclusions.
PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (PRRS)

A. Clinical presentation and epidemiology of PRRS

Porcine reproductive and respiratory syndrome (PRRS) emerged late in the decade of the 1980's as a devastating new disease in swine. Several names were given to the disease in different regions of the world. Some of those are: mystery swine disease \(^{155}\), blue-eared pig disease \(^{4,67}\), swine infertility and respiratory syndrome (SIRS) \(^{41,58}\), syndrome disgrénesique et respiratoire du porc \(^{256}\), porcine epidemic abortion and respiratory syndrome (PEARS) \(^{174}\), and SMEDI-like syndrome \(^{57,104}\). In 1992 it was officially designated PRRS in the First International Symposium on SIRS/PRRS, in St. Paul, Minnesota, USA \(^{44,132}\).

The PRRS outbreaks were first recognized in North America in 1987, and reported in Germany in 1990 and later in The Netherlands, France, Belgium, England and Denmark in 1992 \(^{4,12,24,104}\). Seropositive herds were also found in Italy and Poland, although virus had not been isolated. Korea and Japan have also been affected by this disease \(^{37,151,195}\). Currently, PRRS is highly prevalent in all pork-raising countries \(^{2,258}\).

In 1991, the etiology of PRRS was established when a previously unrecognized, small, enveloped RNA virus was isolated from animals affected by PRRS in the Netherlands. This agent was named Lelystad virus \(^{233}\). A virus with similar physico-chemical characteristics to Lelystad virus was identified later in United States and designated ATCC VR-2332 \(^{19,46}\). Retrospective studies found seropositive samples that date as far back as 1984, in the US \(^{146}\), and 1979 in Canada \(^{30}\). Other retrospective studies identified seropositive pigs in South Korea during 1985 \(^{196}\) and in Japan in 1988 \(^{151}\).

1. Clinical manifestation

Clinical signs in pigs affected by PRRS are highly variable in incidence and severity. Clinical manifestations also vary with the age of the pig, overall health and vaccination status. In general the early signs of PRRS include “flu”-like illness, conjunctivitis,
depression, lethargy and inappetence, which can last from a few days to 2 weeks. The European Commission (Directorate-General for Agriculture) released, in 1991, the case definition for PRRS in breeding stock. This syndrome was defined by an abnormal rate of abortion in the late term of gestation, or prolonged parturition producing mixed litters of live, dead and mummified fetuses. In pregnant sows, the rate of stillbirths may increase to 50-70%, in addition to increased abortion rate. Premature farrowing by 5-7 days, poor conception rate and slow return to heat are common signs. Infected boars may exhibit transient respiratory distress, with subsequent decrease in libido, and in the motility and quantity of sperm.

In young pigs, the syndrome is presented as respiratory distress. The symptoms may vary and can be complicated by secondary respiratory infections. Gastrointestinal or CNS symptoms are not usually associated with PRRS, however, vomiting and diarrhea, and CNS signs in experimentally inoculated young pigs have been reported.

Affected grow-to-finish pigs usually show mild clinical signs, although, anorexia, listlessness, high respiratory rate and fever may be observed. These pigs may have reduced growth rates.

Within an affected herd PRRS can be categorized into subclinical, acute or epidemic, and chronic or endemic forms. In the subclinical form the pigs do not show observable signs of disease except seropositivity to PRRSV. Both the epidemic and endemic forms of the disease show symptoms related to respiratory distress and reproductive failure. The epidemic form shows all of the symptoms related to the disease. The duration of the epidemic phase can be of weeks to several months. An acute form of the disease was observed in 1996 in Southeastern Iowa and other states of the Midwest and North Carolina. Ten to fifty percent of sows aborted over a 3-6 week period and sow mortality ranged from 5% to 10% of inventory in a period of one to two weeks. Severe PRRS outbreaks were also reported in vaccinated herds in Denmark implicating that current vaccine virus may spread through out a naïve herd and cause disease. Endemically infected herds may not go back to the same production level prior to the outbreak because of continued reproductive failure in sows and secondary respiratory infections that prevail in young piglets.
2. Prevalence and economic impact

PRRSV infection is highly prevalent in swine producing regions throughout the world except in Sweden, Norway, and Australia.\textsuperscript{258} In 1995 the United States' Department of Agriculture performed a serologic survey, as part of the National Animal Health Monitoring System, to estimate the prevalence of PRRSV infection. The study revealed 68\% prevalence rate, which included vaccinated herds. Prevalence in unvaccinated herds was found to be 59\%\textsuperscript{1}. Other researchers report a prevalence of 60 to 80\%\textsuperscript{259}.

A nationwide survey in Denmark showed a PRRSV prevalence in finishing herds of 33\% in 1996\textsuperscript{147,149}. In the region of Pays de la Loire of France prevalence was estimated to be 2.7\% a year after the first PRRSV outbreak and after a control program was put to effect to avoid introduction of the virus through infected boars, semen used for artificial insemination, and possible environmental sources (e.g. contaminated fomites)\textsuperscript{115}. In the Federal State of Sachsen-Anhalt two wild boars were found to be seropositive for PRRSV during the hunting season in 1994\textsuperscript{164}. Seropositive rates varying from 8.8 to 67.4\% were reported in Korea in 1993, however retrospective studies indicate the presence of PRRS in this country since 1985\textsuperscript{197}.

The economic impact of PRRS has been calculated to amount losses of $236 per female during the acute stage and up to $502 per sow during chronic stage of disease\textsuperscript{177}. This represents a decrease in annual production of 5-20\% (due to slaughtering which increases to 10\%-15\%), and reduction of annual production of 1-1.5, 2-2.5 and 3.8 pigs per sow per year\textsuperscript{39}.

PRRS outbreaks also increase other expenses related to production such as diagnostic tests, surveillance, and treatment. Treatment to prevent secondary infections can increase the production costs by up to 60\%\textsuperscript{167}.

In concert with the infertility problems that arise with the persistence of PRRS, young pigs fail to thrive, increasing the costs of production.
B. Porcine reproductive and respiratory syndrome virus

1. Taxonomy

Porcine reproductive and respiratory syndrome virus (PRRSV) is an Arterivirus member of the Family Arteriviridae in the Order Nidovirales \textsuperscript{32,33}. This order includes one more family: Coronaviridae. Among the main characteristics of the order Nidovirales is a linear, non-segmented, single-stranded, positive-sense RNA genome, which is expressed by a nested set of transcripts sharing the 3’ end. The 5’ end of the viral genome possesses two open reading frames (ORFs) translated by frame-shifting mechanism, which encode for the viral replicase. This replicase contains a polymerase and a helicase domain and is upstream of structural and non-structural genes that vary in number and kind. Other characteristics include the presence of an envelope and polyadenylation of the genomic RNA \textsuperscript{32}.

The Arterivirus family includes equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) of the mouse, and simian hemorrhagic fever virus (SHFV), in addition to PRRSV \textsuperscript{32,48}. The host cell for all the viruses in this genera are macrophages or monocytic lineage cells of host species. These viruses also share morphologic and genomic similarities as well as the establishment of asymptomatic persistence.

2. Genomic structure and gene expression

PRRSV contains a non-segmented, linear, single stranded, positive-sense RNA genome of 15 kilobases in length, which is divided into seven (ORFs) \textsuperscript{137}. ORF 1a and 1b encode for the viral replicase (further described below) and occupies about two thirds of the entire genome \textsuperscript{133}. ORF2, ORF3, and ORF4 encode minor glycoproteins (GP) GP2, GP3 and GP4, respectively, which are postulated to be membrane associated proteins \textsuperscript{14,133,137,138}. ORFs 5 through 7 encode the major structural proteins: major envelope glycoprotein (GP5, which was previously designated E), matrix (M), and nucleocapsid (N), in that order \textsuperscript{139}. There have been reports of an additional putative structural protein encoded by ORF2 \textsuperscript{136} and of a smaller ORF- called ORF X- within the ORF5 \textsuperscript{48}.

Virus genomic RNA is transcribed through the generation of 6 or 7 subgenomic
messenger RNAs (sgmRNAs) by the viral RNA-dependent-RNA polymerase (RdRp) in a nested set that share the 3’ end \(^48\). Each one of the sgmRNAs have the same 5’ leader sequence that is derived from the 5’ end of the viral genome \(^129,134\). Subgenomic mRNAs are translated independently \(^62,135\).

Expression of the viral replicase polyprotein genes (ORF 1a and 1b) is achieved by a minus-one (-1) ribosomal frame shifting mechanism which has been described by Allende (1999) \(^10\). This mechanism is very similar to that present in other arteriviruses namely LDV and EAV \(^135\). Various domains including helicase, cystein protease and serine proteases have been described for the replicase of PRRSV \(^10,156,199\). Post-translational self-cleavage of the polyprotein produces 12 nonstructural proteins (Nsps) that are responsible for virus replication and transcription as demonstrated for EAV \(^225-227\).

EAV studies have shown that two non-structural proteins (Nsps) can interact with each other and insert themselves in double membrane vesicles in the perinuclear region during virus replication. Nsps 2 and 3 contain hydrophobic domains that may allow them to localize or embed themselves in the double membrane vesicles \(^201\). These proteins are involved in the control of proteolysis of replicase precursors and membrane association of the virus replication complex. It has been shown that EAV Nsps 1 & 2 are self-cleaved quickly after translation \(^201\). Other EAV Nsps in both ORF1a and ORF1a/b polyproteins are cleaved by Nsp4 \(^226\). EAV RdRp domain is found in Nsp9 and the helicase domain is located in Nsp10 \(^192\). A metal binding domain was also found in Nsp10 \(^53\).

Recent studies have demonstrated that PRRSV undergoes genetic recombination during infection. Usually defective interfering RNAs (diRNAs) are formed during this type of event in high multiplicity of infection (MOI) and are of short duration in persistent infection of cells. However, diRNAs were not found for PRRSV \(^251\). Further studies revealed the production of atypical mRNAs, termed heteroclite mRNAs \(^134,250\). The heteroclite mRNAs are formed by transcription of the 5’ and 3’ ends of the genome with have a large internal deletion and aberrant 5’-3’ junction sites. Heteroclite mRNAs were observed both \textit{in vivo} and \textit{in vitro}, and could be obtained from virus-like particles. Some of these transcripts could produce proteins in frame with ORF6, including one that potentially
encodes for a fusion protein of ORF1a Nsp5 with the M protein. Yet, others produce relatively short peptides. Their function, if any, remains to be determined.

3. Morphology and structure

Porcine reproductive and respiratory syndrome virus is an enveloped virus 45 to 80nm in diameter with an icosahedral nucleocapsid core about 25-35nm in diameter. The nucleocapsid core contains genomic RNA. The virus may consist of as many as 6 structural proteins; however, 3 structural proteins, i.e. N, M and GP5, have been consistently demonstrated.

a) Major structural proteins

GP5, encoded by ORF5 (603 bases), is 25-26 kilodaltons (kD) and the major envelope protein. It has membrane signal peptide with a cleavage site between amino acids (AA) 32 and 33 for the European strain, Lelystad virus, and between 25 and 26 for various North American strains. GP5 possesses two putative transmembrane alpha helices located between amino acids 65-130 and 170-190. GP5 also exhibits a highly glycosylated putative ectodomain located at the N terminus of the protein. Glycosylation, which occurs in heterogeneous and homogeneous forms, containing complex glycans as well as high-mannose oligosaccharides or only complex carbohydrates, respectively. Glycosylation in PRRSV takes place as a rather slow process in the premedial Golgi compartment, compared to its counterpart in EAV and HA protein of influenza virus.

GP5 has been found to possess epitopes related to neutralization of the virus. Most of these epitopes are associated with, or potentially located in, the proposed ectodomain of the protein. In contrast, Rodríguez et al. reported the presence of an immunodominant epitope present at the C terminus of GP5. Even though this region was recognized in immunoblotting by antisera to PRRSV, its involvement in virus neutralization is unlikely since MAbs produced against a recombinant protein containing GP5 AA30-67/130-201 did not show neutralizing activity.

Overall, GP5 shows high sequence variation between North American isolates and
the European prototype, Lelystad virus, with only about 55% AA identity. Aminoacid identity ranges from 88-97% among US field strains and 88 to 99% when compared to the sequence of a modified live US vaccine strain. Many of the aminoacid differences among strains are localized in the hypervariable region of the ectodomain, which comprises AA 26 and 39 and was found to be highly antigenic.

GP5 also forms heterodimers through a cystine residue with the matrix (M) protein. It has been shown that M is bonded to GP5 by a cystine residue located at the N terminus end of each protein. This bonding occurs after accumulation of M protein in the endoplasmic reticulum of infected cells and prior to Glycosylation of GP5. However, like for EAV M protein, it can form homodimers early in transcription but which are not present in the virion. These MM dimers function is unknown and fade as M-E heterodimers appear. M is encoded by ORF6 (525 bases); and it is a 19kD non-glycosylated protein that has three potential membrane spanning domains. M has been demonstrated to strongly stimulate T-cell proliferation in vitro but the significance of these findings in vivo has yet to be studied.

The core of PRRSV is formed by the capsid, which contains the viral genome. It is a non-glycosylated 15kD polypeptide encoded by ORF7 (372 bases) located at the 3' end of the genome. The capsid protein constitutes about 20 to 40% of the protein in a PRRSV particle. A highly negative region in its N terminus is believed to interact with the viral RNA, forming the nucleocapsid. Likewise, C termini from different N proteins have been shown to interact with each other in a non-covalent fashion to form homodimers that would multimerize to produce an icosahedron.

The N protein possesses four regions that are highly conserved, when compared to nucleocapsid sequences from all other arteriviruses, perhaps due to functional constraints. N has also been reported to have hemagglutinin (HA) activity with mouse erythrocytes that can be enhanced by treating the virus with Tween 80 and ether, but was abolished by treating the virus with virus-specific antiserum. Mercaptoethanol treatment terminated HA activity. Pepsin, formalin and dithiothreitol (DTT) were gradual inhibitors of the HA activity by the virus. Fractionation by cesium chloride (CsCl) gradient and subsequent SDS-
PAGE analysis revealed that this protein co-migrates with the 15kD nucleocapsid protein\textsuperscript{97}.

PRRSV N protein has been demonstrated to be highly immunodominant since it is transcribed in higher levels than other sgRNAs and consequently translated more, thus, antibodies specific to N are produced in high quantities during early humoral immune responses, but are non-neutralizing in nature\textsuperscript{54,157}. Aminoacid identities of 96-100% between sequences from North American isolates and of 94-100% between North American and European isolates have been found\textsuperscript{127}. For that reason, it has been a major target for detection of PRRSV in tissues, as well as major antigen for serological assays.

b) Minor proteins

PRRSV ORF4 (537 bases) encodes for a structural glycosylated apoprotein of 20-35kD depending on the level of N-glycosylation and its localization, i.e. the intracellular form is of 20-28kD whereas the virion associated protein is about 31-35kD. Certain monoclonal antibodies to LV GP4 (178 AA) had neutralizing activity \textit{in vitro} but did not react with the VR2332 US isolate\textsuperscript{140}. The neutralizing epitope was mapped to a region comprising AA 40 to 79 which is part of a seemingly hypervariable region of GP4\textsuperscript{231}. Weiland et al suggested that GP4 antibodies may not be as efficient as anti-GP5 antibodies in neutralizing PRRSV since the former required a high antibody titer to have the same level of neutralization as anti-GP5 antibodies at high dilution\textsuperscript{231}. Nevertheless, \textit{in vivo} tests indicated that immune sera had high antibody titers against GP4 epitopes demonstrating its immunogenicity during PRRSV infection\textsuperscript{162}.

GP3 is a highly glycosylated protein that is expressed in similar rate as the N, M and GP5\textsuperscript{76}. It has been described as a structural protein in the European prototype Lelystad virus\textsuperscript{136}. In contrast, it has not been found in the virion of North American strains, but in cell culture supernatants at high concentrations\textsuperscript{120}. Hence, GP3 is considered a non-structural protein with unknown function\textsuperscript{76}. It has been reported that GP3 does not elicit a neutralizing antibody response\textsuperscript{77}. Its antigenicity is highly variable from one generation to the next\textsuperscript{113} and to the point of the generation of deletion mutants viruses that outcompete their non-deleted counterparts\textsuperscript{161}. 
Snijder et al. (1999) found that, in EAV, a region of 76 nucleotides, believed to be untranslated, contains the 5' end of an ORF of 201 nucleotides in length, which they designated ORF 2a. ORF 2a overlaps the previously described ORF2 (now designated ORF2b) which encodes the Gs protein and both are located in the second subgenomic mRNA (sgmRNA 2). Homologous genes were also found for other arteriviruses. The predicted protein (67AA) is associated with intracellular membranes, was demonstrated to be present in purified virions and is essential for virus infectivity 200. Confirming these findings, Wootton et al (2000) also described a second ORF 2 in a Canadian PRRSV isolate. This smaller ORF (designated ORF 2b, 222 nucleotides) encodes for a 73AA protein and lies within ORF 2a (771 nucleotides) which encodes a 256AA protein. This is the only proof of bicistronic genes in an arterivirus 239.

As described earlier the viral replicase is a polyprotein encoded by two contiguous ORFs, ORFla (7509 bases) and ORFlb (4371bases) that are expressed by a minus one ribosomal frame shifting mechanism. These two ORFs are proposed to encode a total of 12 Nsps. Nsps 1α to 8 are encoded by ORFla and Nsps 9 to 12 by ORFlb 239. The function of several of these Nsps is unknown, however, both Nsp1α and Nsp1β contain a papain-like cystein protease motif 60, Nsp2 has a chymotrypsin-like cystein protease, Nsp3 and 5 contain hydrophobic domains 202, and Nsp4 encodes a serine protease proposed to cleave the ORFla/b polyproteins 203,226. Nsp9 encodes for the RNA-dependent RNA polymerase (RdRp) 26, Nsp10 encodes for the helicase protein 53,105, and Nsp11 has a Coronavirus-like motif 99. All other Nsps not mentioned here have not been characterized yet.

Sequence analysis of three independent vaccine-derived PRRSV strains has shown that parallel mutations on ORF1 helicase and papain-like cystein protease domains were reverted to the parental strain 159. These findings suggest that this polyprotein has an important role in virus virulence and/or pathogenesis.
4. Characteristics

a) Tropism for macrophages

PRRSV exhibits preferential replication in macrophages. Studies using the *in situ* hybridization technique have demonstrated that porcine macrophages from the lung, spleen, liver, thymus, lymph nodes and Peyer’s patches, and microglial cells are permissive to PRRSV. In infected animals 80 to 94% of infected cells collected from lungs were alveolar macrophages, although only 2% of the total alveolar macrophages were found positive. Other porcine cells that can be infected with PRRSV are type II pneumocytes and spermatids, however, peritoneal macrophages and bone marrow’s progenitor cells were reported to be refractive.

Although freshly-collected peritoneal macrophages and peripheral blood monocyte cells (PBMC) do not support PRRSV replication, cultivation for 24-48 hours can produce a low virus yield. Isolation or detection of PRRSV from PBMC has been documented in experimentally infected animals. *In vitro* maturation of PBMCs to macrophages by the addition of macrophage colony-stimulating factor (M-CSF), activation by adhesion to endothelial cells, or by addition of L929 cell supernatant makes them permissive.

The use of permissive cell lines has been a breakthrough in the study of PRRSV, however very little is known about the viral or cellular receptor required for entry into both the natural host cell and/or cell lines. A porcine alveolar macrophage (PAM) membrane protein with molecular mass of 210 kD has been postulated to be the viral receptor. Monoclonal antibodies (MAbs) to this protein inhibited PRRSV binding to, and infection of PAM. However, the same MAbs failed to find the same or a similar protein on a permissive cell line (i.e., MA104). Recent studies have reported that PRRSV can attach to certain non-permissive cells, e.g. rabbit kidney cells, human carcinoma cells, porcine testicular and porcine kidney cells, without entry and replication of the virus. This led to speculate that another receptor or a yet unknown mechanism was required for the events following attachment. Binding studies with various cell types have indicated that, besides binding, PRRSV can be internalized through endocytosis into certain non-permissive cells (i.e. Vero...
cells) and, yet, not cause infection even when the viral RNA is capable of producing infectious progeny by itself in the same cell types \(^{106}\). Endocytosis of PRRSV occurs via clathrin pit formation \(^{154}\) but multiple cellular as well as viral proteins may be needed for uncoating of the virus and subsequent infection \(^{106}\).

### b) Antigenic variation

Difficulty in the development of efficacious vaccines and diagnostic tests, and changes in virulence are a few issues that have been attributed to the remarkable genetic and antigenic variability demonstrated among PRRSV field isolates \(^{131}\).

Genetic analyses have shown the existence of at least two major virus genotypes, the European and the North American, with extensive genetic variation both within and between these genotypes. Nelsen, et al. (1999) found differences between the prototypic North American (VR-2332) and European (Lelystad) viruses in the 5' leader sequence and parts of ORF 1a \(^{156}\). Marked differences were also found between European and North American isolates in some structural genes \(^{102;152}\). ORF 7 is highly conserved among North American isolates with 95 to 100 percent amino acid homology, but a comparison of North American viruses and Lelystad virus found only 57 to 59 percent amino acid homology \(^{128;152}\). ORF 6 is the most conserved gene among North American isolates with up to 100 percent amino acid identity and the most conserved between North American and European isolates with 70 to 81 percent identity \(^{102;127;152}\). GP5, the major envelope protein encoded by ORF 5, showed the highest variability among various isolates, which affects the potential level of glycosylation \(^{169}\). This hypervariability localizes on a highly antigenic region \(^{128}\). The amino acid sequence homology of GP5 varies from 88 to 97 percent among North American isolates and from 51 to 59 percent when comparing North American viruses to the Lelystad virus \(^{11;102;126;152}\). ORF2 and ORF4 encoded proteins show antigenic variation \(^{128}\). A comparison of the Lelystad virus with isolate ATCC VR-2332, the North American prototype virus, found an amino acid identity of 63, 58, and 68 % for ORFs 2, 3, and 4, respectively \(^{152}\). Similar results were reported when comparing the Lelystad virus with U.S. isolate VR-2385 \(^{145}\).
Genetic diversity is mirrored by antigenic diversity among PRRS virus isolates. Antigenic variation was initially demonstrated in a comparison of European and North American isolates. Using an immunoperoxidase monolayer assay (IPMA), Wensvoort et al. (1992) evaluated the reactivity of polyclonal porcine antibodies raised against either Lelystad virus or North American isolate ATCC VR-2332 with PRRS virus isolates from around the world. The investigators were able to differentiate European from North American isolates on the basis of differences in IPMA antibody titers. That is, significantly higher antibody titers were obtained in the homologous assay system. Later studies found even greater antigenic diversity among PRRS virus isolates than initially suspected.

Yoon et al. (1995) examined 22 PRRSV isolates from 8 different U.S. states recovered from samples collected between 1989 and 1993. Using a panel of 5 MAbs specific for the N protein, the 22 virus isolates fell into one of 3 groups based on their reactivity pattern. Yang et al. (1999) expanded this study to include 70 North American isolates recovered from samples collected between 1989 and 1995 using a panel of 23 MAbs against the N protein. These investigators found 5 antigenic groups, with the European Lelystad virus representing an antigenic group distinct from any of the North American groups identified.

Furthermore, using antibodies against discontinuous epitopes of the N and M proteins and continuous epitopes of the GP5 and GP3 proteins, the 65 North American isolates in the first and second antigenic groups were further subdivided into 9 and 4 antigenic subgroups, respectively. Other researchers have also found great divergence between European isolates and North American isolates, the major differences are among envelope proteins, however N protein presents the lesser diversity and the sharing of two epitopes. A study conducted in Korea showed that 92% of the isolates shared a common N epitope, recognized by MAb SDOW17, with the VR-2332-derived vaccine strain and all isolates were categorized as North American genotypes by differential PCR.

That field isolates of PRRSV show a remarkable degree of genetic and antigenic variability has become abundantly evident. Although the assumption is that these changes occur during the course of in vivo replication in swine and arise, in large part, because of the errors that occur during RNA replication, the degree and rate of mutation of PRRSV in
infected pigs over time is not known. Furthermore no correlation between the severity of lesions and genetic differences among the isolates studied has been found.

c) High infectivity

PRRSV has been found to be highly infectious, i.e. only a minimum infectious dose is required compared to other viruses. As little as $10^1$ fluorescent foci units per milliliter could establish infection as determined by viremia and seroconversion when the virus was given intranasally or intramuscularly. Onset of respiratory symptoms appeared to be correlated with challenge dose independent of the route of inoculation; however no link was found between the severity of clinical manifestation and virus dose given. Yet, minimum infectious dose and clinical effect remains to be assessed for other routes of challenge, as PRRS virus is known to establish its infection via many different routes.

d) Persistence

PRRS virus produces a persistent infection despite an active immune response. The PRRS virus carrier state was first recognized following transmission of virus from animals infected 99 days earlier to commingled sentinel pigs under experimental conditions. Subsequently, Wills et al. (1997) reported isolation of virus up to 157 days post inoculation. In an experimentally inoculated population, approximately 90% of inoculated animals were found to bear the virus at 105 days post inoculation.

Within herds, three factors contribute to the persistence of PRRSV: infection of sows during pregnancy, infected growing pigs and the frequent introduction of seronegative animals. Other risk factors include large herd size (>50 sows), total indoor confinement and the use of only one building, storage of slurry under floor, lack of disinfecting procedures, fumonisin mycotoxin in feed, rodent problems and, possibly, wild bird species.

Passive immunization of piglets with maternal antibodies against PRRSV is an important part of maintaining reduced levels of persistence in herds by reducing the number of susceptible animals. Chung et al. found that piglets whose mothers had low antibody
titers had more viremia than piglets nursing from sows with higher levels of anti-PRRSV antibodies. So, as the maternal antibodies dwindle between 6 to 8 weeks after birth, these piglets become major PRRSV reservoirs in a farrow-to-finish farm setting.

The exact mechanism(s) by which the virus persists in the host is (are) not known, but PRRSV isolates are characterized by a high degree of genetic and antigenic variation as reviewed above. Feasibly, persistence in the host and viral diversity could be two sides of the same issue. Another possibility was suggested by Allende et al (2000). Their findings indicate that PRRSV persists through a slow rate of viral replication that extends up to 150 days post infection as demonstrated by the presence of negative RNA in examined samples. This persistent state could be linked to changes in the glycoprotein (GP3 and GP5) and M protein genes. Similar observations have been made with LDV, an arterivirus closely related to PRRS virus. Nevertheless, this is an area deserving further studies since persistence is an important epidemiological feature that provides a ready means for PRRS virus to perpetuate itself through a cycle of transmission from carrier to susceptible animals. As a consequence, elimination of PRRS virus from herds is difficult and cyclic bouts of PRRS virus-associated health problems are commonplace.

C. Pathogenesis

1. PRRSV pathogenesis

Transmission of PRRSV occurs by direct contact. Once in the pig, the virus binds to-, is ingested by- and replicates mainly in macrophages. Binding to alveolar macrophages has been shown to occur in a dose dependent manner and reaches a maximum at 1 hour post exposure in vitro. In vitro studies demonstrated that internalization occurs by receptor mediated endocytosis through clathrin coated vesicles. Gnotobiotic pigs show typical clinical signs upon infection with PRRSV. Edema in infected tissues may be induced by the accumulation of immune complexes and consequential attraction of inflammatory cells that release vasoreactive cytokines, or by direct vascular damage due to viral replication. Viremia that can last up to 8 weeks occurs prior to the development of lung lesions, virus
may gain access to the serum via its replication in pulmonary intravascular macrophages or pulmonary endothelium. PRRSV replicates for a long time in porcine alveolar macrophages and can be found in bronchoalveolar lavage fluid (BALF) up to 49 DPI.

Interstitial pneumonia is a lesion commonly observed in pigs infected with PRRSV. It consists of the enlargement of the alveolar septa induced by the infiltration of leukocytes, principally macrophages. Clinical manifestations such as hyperpnea and dyspnea, associated to interstitial pneumonia, correspond to microscopic lesions and high virus titers in the lungs of affected pigs. Virus replication on lymph nodes results in hyperplasia and necrosis in the germinal centers. Nevertheless, PRRSV replication in lymph nodes is transient and limited to a certain cell population of unknown identity.

Heart lesions have been observed late in infection and may be correlated to enlargement of the heart, however, virus titers are low. Kidney lesions are not commonly described for PRRSV infection but, when they occur, can be severe and may be related to virulence of the PRRSV strain. In a study conducted by Rossow et al. vasculitis, and not necrosis, was observed in kidney at 21 days post exposure with the virus.

PRRSV has also been reported to cause brain lesions. In neonatal pigs from PRRSV-positive and vaccinated herds, meningoencephalitis has been observed in the absence of other pathogens. Immunohistochemistry of brain tissue revealed PRRSV-infected macrophages and microglial cells, but other infected cells that did not react with macrophage-specific monoclonal antibody were also observed, suggesting the presence of other permissive cells. Infection in these piglets may have occurred in utero since fetuses per se are susceptible throughout their gestation, but vertical transmission seems to only occur during the last trimester of gestation.

Study of the expression of macrophage genes induced upon infection is an excellent tool to examine virus pathogenesis. Downregulation of the expression of various genes including a 2'-5' oligoadenylate (2-5A) synthetase and a putative dual-specificity phosphatase was observed using differential display reverse transcription (DDRT)-PCR to detect expressed sequence tags (EST) of alveolar macrophages after infection with PRRSV. 2-5A synthetase is an intracellular enzyme produced by monocytes, and B and T
lymphocytes, and is induced by IL-6, in murine 21, by IFNβ and in much lower levels by IFNγ, but not by TNF, in humans 238. Increased expression of 2-5A synthetase promotes oligomerization of ATP which activates RNase L, an enzyme responsible for cleavage of ssRNA, thus conferring resistance to virus infection 23,35.

The upregulation of the expression of Mx1, ubiquitin-specific protease (UBP) 252, and porcine RNA helicase induced by virus (RHIV-1) 253 was demonstrated in alveolar macrophages of pigs inoculated with PRRSV. Mx1 is member of an IFN-inducible family of proteins and is associated with intracellular protein transport 150. UBP is also associated with protein trafficking 254. In PRRSV infected pigs, transcript levels were highest in lungs and tonsils for both Mx1 and UBP proteins. However, only UBP was found in higher than normal levels in tracheobronchial lymph nodes of affected pigs. These sites are associated with persistence of infection in vivo 253. Elevated RHIV-1 transcript level was also found in tissues of persistent PRRSV infection.

Recent studies by Girard et al. indicate that infection with PRRSV increases the secretion of two matrix metalloproteases that aid in the infiltration of lymphocytes, macrophages and neutrophils into the lungs. Such proteolytic activity reached its peak between 7 to 14 DPI, went down to normal after 42DPI, and was correlated with the interstitial pneumonia lesions observed during leukocyte migration 74.

Programmed cell death has been proposed to contribute to the pathogenesis of diseases induced by various viruses 47,142,220. Apoptotic activity of PRRSV has been associated with the major envelope glycoprotein GP5, in transfected COS-1 cells and cultured porcine alveolar macrophages 207, suggesting that lung lesions characteristic of the disease were not caused by necrosis as previously believed 84,182,184. Questions on the in vivo relevance of this phenomenon led to the investigation of apoptosis in tissues of infected pigs 210. Virus-induced apoptosis was demonstrated in testicular germ cells 209, lung and lymphoid tissues but not always colocalized with infected cells 210, suggesting that apoptosis of the bystander cells could be indirectly induced 198.
2. Factors influencing/contributing to pathogenesis of disease

a) Immune modulation

It has been proposed that PRRSV can suppress the development of normal humoral and cellular immune responses. Since alveolar macrophages are the host cell for PRRSV, the effect of infection on their number and function has been proposed as the principal means by which the virus modulates immune responses. The effects of PRRSV infection on macrophages and other cells of the immune system are discussed in another section (page 24). However, it should be noted that downregulation of antigen expression on both alveolar and peripheral macrophages has been observed upon infection with PRRSV, and this may be an explanation for the curtailment of virus clearance. Experiments conducted by Albina et al. to monitor immune responses to PRRSV did not demonstrate systemic immune suppression but stimulation. After day 21 post-infection, production of antibodies increased and the numbers of leukocytes including CD8+, CD2+ and IgM+ cells were raised. Others have confirmed these findings and also found decreased CD4+ cell numbers and lower CD4+/CD8+ ratios. Despite these observations, the depletion of lymphocytes from lymphoid tissues has been reported and proposed to promote susceptibility to secondary bacterial infections.

PRRSV infection may affect the lymphocyte responses to other pathogens or vaccination against other pathogens. In this study, PRRSV-infected pigs were vaccinated against pseudorabies virus (PRV) and their T cell responses were measured after PRV challenge. The detection of T cell responses in PRRSV infected pigs was delayed by a week when compared to PRRSV-negative pigs. The peak of these responses decreased two weeks sooner in PRRSV infected pigs than non-infected pigs. Their study also revealed that these responses in PRRSV-infected pigs were not as high as in those infected only with PRV, confirming previous findings. However, humoral responses against PRV developed equally in both groups.
b) Difference in virulence

Differences in virulence among PRRSV isolates have been extensively described \(^{33,35,234}\). More virus antigen was detected in tissues typically affected by PRRSV, in pigs inoculated with highly virulent isolates than with isolates with lower virulence \(^{85}\). Virulence did not affect the cell types infected or the distribution of antigen in those tissues \(^{85}\). In other studies, differences in virulence were not linked to the ability of the virus to cross the placenta of experimentally infected sows, but to its intrinsic pathogenicity \(^{130,131}\).

Variations in the sequences of viral envelope proteins, can result in the development of neurovirulence as is the case of Sindbis virus \(^{62}\), dengue virus \(^{81}\), certain murine retroviruses \(^{180}\) and encephalitogenic flaviviruses \(^{124}\). Sequence analysis of PRRSV strains of varying virulence revealed that N and M proteins from US and Canadian strains share 96 to 100% aminoacid identity as compared with to 57 to 81% between North American strains and European isolates, regardless of their virulence \(^{127}\). However, when comparing US isolates only, the low virulence strain revealed higher variation in the sequences of ORF2 and 4 encoded proteins \(^{128}\).

c) Pig breed

Susceptibility to PRRSV infection is mostly age-dependent \(^{217}\) and predisposition due to pig breed has been ruled out by some researchers \(^{51}\). However, experiments using Meishan, Hampshire and Duroc pigs demonstrated that Hampshire pigs had more severe lesions than the other breeds. Meishan pigs would develop myocarditis and Duroc pigs had lower PRRSV-specific antibody titers. Thus, researchers concluded genetic differences between these breeds were accountable for the disease level outcome \(^{86}\).

d) Co-infection

Porcine reproductive and respiratory syndrome is usually complicated due to co-infection with respiratory tract pathogens. The most commonly isolated secondary pathogens during PRRS outbreaks are: *Haemophilus parasuis*, *Actinobacillus pleuropneumonia*, *Streptococcus suis*, and *Salmonella* species. Enzootic pneumonia as well
as atrophic rhinitis are other diseases associated with this syndrome.31,183

It is commonly thought that macrophage depletion in lungs or tonsils due to PRRSV infection has an effect in predisposing the animals to bacterial infections. In 1995 Cooper et al performed studies that could not demonstrate the potentiation of secondary bacterial infections (H. parasuis, S. suis, Sa. choleraesuis, and P. multosida) by primary PRRSV infection.49 They postulated that stress due to environmental changes or management, and differences in virus strains (some produce different degrees of clinical disease, severity of lesions and immunosuppression) as well as chemical and bacterial agents present in the pigs system can contribute to the potentiation of infection by respiratory bacterial pathogens in field cases. Other investigators also corroborated the lack of a predisposing effect by PRRS virus for secondary bacteria such as H. parasuis.190

In contrast, other investigators have been successful in demonstrating a synergistic effect between PRRS virus and bacterial agents.205 Studies performed by Galina et al demonstrated an increase in the number of animals with suppurative meningitis in pigs infected with PRRS virus followed by challenge of S. suis. In utero infection with PRRSV demonstrated that piglets become more susceptible to S. suis type II infection.70 Dually infected piglets had higher mortality rates (20 out of 22 piglets or 91%) than single infected pigs (1 out of 18, or 5%, PRRSV singly infected, and 5 out of 23, or 21%, S. suis type II singly-infected pigs) and showed reduced numbers of leukocytes, particularly lymphocytes, as a result of thymocyte depletion. The thymus in infected piglets were involuted and the lymph nodes showed hypertrophy, hyperplasia, necrosis and apoptosis. Bone marrow exhibited hypoplasia characterized by a lack of myeloid and erythroid precursors. Dually infected pigs not only had higher mortality, but the severity of meningeal disease was increased as well. Based on organ lesions, the higher mortality rate and the severity of meningitis observed in euthanized infected and control piglets, it was proposed that PRRSV infection in utero results in underdevelopment of immune organs and that this predisposes the piglets to secondary bacterial infections.

Due to conflicting observations on the interaction between PRRS virus and bacterial agents among studies, a different challenge scheme (i.e. bacteria first followed by PRRS
virus inoculation) was explored. Using this approach, Wills et al (2000) investigated *S. choleraesuis* and PRRS virus dual infection with and without stress factors and demonstrated synergism between the two agents. Singly infected pigs did not exhibit clinical signs, but dual-infected piglets and those that were also treated with a stressing agent (dexamethasone), did show severe clinical signs similar to those seen in the field, and had a mortality rate of 43%. This group of piglets also shed both PRRSV and *S. choleraesuis* for a longer period of time and the distribution of *S. choleraesuis* in the tissues was increased. A similar approach was applied to studying the interaction between *Mycoplasma hyopneumoniae* and PRRS virus. *M. hyopneumoniae* infected pigs developed more severe pulmonary lesions when subsequently exposed to PRRSV. Nevertheless, PRRSV primary infection did not influence the course of Mycoplasma secondary infection, except that microscopic lesions characteristic of *M. hyopneumoniae* were more severe in these animals. The lack of exacerbation of *M. hyopneumoniae* infection has also been reported before by others.

Interaction between PRRSV and other viral agents has been studied as well. Simultaneous inoculation of pigs with PRRSV and porcine circovirus type 2 (PCV2) showed an enhanced distribution and replication of PCV2. However PRRSV infection was not different in dually infected pigs from the PRRSV-only infected ones. In contrast, PRRS virus did not affect clinical signs of swine influenza virus (SIV) infection. Neither did it increased the pathogenicity of *Pasteurella multosida* secondary infection in SIV-infected animals.

e) ADE

Independent studies by Yoon and Choi have pointed out the role of antibodies in the pathogenesis of PRRSV. Treatment of PRRSV with subneutralizing titers of specific antibodies produce increased viral titers both *in vivo* and *in vitro*. The implications of this phenomenon for the maintenance of persistence may be related to the facilitation of entry into cells that express Fc receptors on their membranes. In pigs with low PRRSV-specific antibody titers, whether because these are of maternal origin, from natural
infection or from vaccination, ADE could be responsible for exacerbation of disease \(^{40,246}\).

\textbf{D. Immunology}

Studying the immune responses to PRRSV has been described as challenging since the virus induces unconventional responses and is able to persist in the presence of neutralizing antibodies.

\textbf{1. Humoral immune response to PRRSV.}

Nelson and colleagues \(^{157}\) studied the humoral response of intranasally inoculated, young gnotobiotic pigs as well as adult pigs. PRRSV-specific antibodies were detected in pig sera between 14 and 15 days postinfection (DPI) by immunofluorescence assay. Their study also showed that humoral response of adult boars was delayed by a week, since detection of antibodies in serum did not occur until 21 DPI. Albina et al demonstrated an increase in antibodies in serum beginning on the third week post infection (WPI) up to the 8\(^{th}\) week post infection with PRRSV \(^7\). Several other researchers have reported that anti-PRRSV antibodies arise between the end of the first through the second week post infection \(^{98,117,157,166,233,249}\), yet these do not have neutralizing activity \(^{249}\).

In other studies, PRRSV-specific antibodies were detected in serum of experimentally infected pigs as early as 5-9 DPI by immunoperoxidase monolayer assay (IPMA), and between 9 and 28 DPI by indirect fluorescent antibody (IFA), enzyme-linked immunosorbent assay (ELISA) or serum virus neutralization (SVN) assays. Peak levels of antibodies were found between 10 and 11 WPI as measured by SVN, but sooner, between 4 and 6 WPI, by the other assays. Western blot analysis of these sera indicated that an immune response to the nucleocapsid protein is mounted first \(^{249}\). However studies by Loemba et al. \(^{117}\) have failed to show that the first antibodies produced are specific to the nucleocapsid. In their studies they found that antibodies specific to the envelope protein (E) appeared at 7 DPI and that anti-15 and -19KD immunoglobulins appeared after two weeks post infection. Despite of these differing results, the pattern and onset of the generation of a humoral response is in accordance with previous findings.
Recent studies in gnotobiotic pigs show that the first immunoglobulins detected, both in sera and BALF, were IgM and IgG at 9 DPI. After this point, IgA was detected in BALF and increased to a titer of 6 log2 by 14 DPI. This titer was maintained until 35 DPI when it started to decline and disappeared by 40 DPI. In contrast, IgA levels in serum peaked to a titer of 10.3 log2 at 25 DPI, even when their appearance and disappearance occurred at the same time points as for BALF. Only two pigs had neutralizing Abs, one at 35 DPI and the other at 52 DPI but remained at low titer levels (1 to 3.6 log2)\(^{110}\).

Neutralizing antibodies to PRRSV were detected between 51 and 70 DPI in gnotobiotic pigs that received two subcutaneous inoculations of PRRSV plus Freund's adjuvant at 14 and 28 days after the first intranasal inoculation. This study also showed that many pigs would produce antibodies specific to both 15 and 19 kD first, but failed to prove a correlation between the specificity of the antibody and its time of appearance \(^{157}\). Neutralizing activity has been associated with GP5, M protein \(^{40;168;249}\), and with GP4 \(^{228}\). Despite the high immunogenicity of the N protein, neutralizing activity has not been associated with N-specific antibodies \(^{244;249}\).

Epitope mapping studies demonstrated that ORF 4's protein is immunodominant \textit{in vivo} and that IgA response varies depending on length of viremia. \(^{162}\)

2. **Cellular immune response**

Cellular immune responses to PRRSV infection have been shown to be delayed and barely detectable after 3 WPI \(^{125}\). Macrophages will be discussed in detail since they are the host cell for PRRSV.

a) **Macrophages**

Normal phagocytic activity in macrophages is affected by various factors including inflammatory processes in lungs, acute and chronic systemic diseases and air pollutants. In the same way, complement receptors (C’R) and receptors to the constant fraction of immunoglobulins (FeR) directly influence the effectiveness of phagocytosis. The pathogenic and activation states of the macrophages change the receptor density and affinity,
respectively. Harmsen et al\textsuperscript{87} studied porcine alveolar macrophages (PAM) collected from bronchoalveolar lavage fluid (BALF). BALF of healthy pigs is composed primarily by alveolar macrophages (AM), lymphocytes and heterophilic cells, which amount for 62\%, 24\% and 5\% of the cells, respectively. Ninety percent (90\%) of AM have FcR and 23\% have C’R. C’R levels increased when macrophages were activated, however, it was not clear if this was an increase in the receptor affinity, the number of receptors per cell or in the number of macrophages bearing C’R due to chemotaxis. They also found that FcR are directly involved in endocytosis of opsonized particles, whereas C’R cannot trigger endocytosis by itself, but it does in synergism with IgG. However, their findings demonstrate that AM population is heterogeneous, since only some cells could form rosettes with opsonized bacteria, others could form rosettes and endocytose, and, yet, some would do neither.

Labarque\textsuperscript{110} reported that BALF from gnotobiotic piglets infected with PRRSV showed increased numbers of macrophage/monocytic cells ranging from 2- to 5-fold from 9DPI through 52DPI, peaking at 25DPI. Dwindling of macrophage numbers occurred between 9 and 20DPI while monocytes and non-phagocytic cells entered the alveolar lumen.

PRRSV infection can reduce the ability of porcine alveolar macrophages (PAM) and pulmonary intravascular macrophages (PIM) to kill bacteria at 24 hours post infection, by 8.3 and 11.9\% respectively, as observed \textit{in vivo} by Thanawongnuwech and colleagues\textsuperscript{216}. Their studies also demonstrated that the percentage of bacteria killed was not significantly different between experimental and control (uninfected) groups. Also, there was an increase in internalization of opsonized bacteria, although this was not statistically significant. There was, however, a significant reduction on the production of superoxide anion in both macrophage populations after infection with PRRSV.

Oleksiewicz et al (1999) also demonstrated that the phagocytic activity of alveolar macrophages is not affected after PRRSV infection \textit{in vitro}\textsuperscript{163}. Alveolar macrophages were viable at 24 hours post infection and there were no significant changes in their ability to phagcytose after infection with PRRSV; moreover, MHC II production is not affected. However, at 48 hours post infection there was 40\% fewer macrophages. This cell death was
attributed to virus replication and its apoptotic capacity. Despite this, phagocytosis was similar to control cells when only considering live cells, whether PRRSV-infected or not. These findings support the idea that the dwindling numbers of phagocytes is the lone cause for the reduction in phagocytic activity and not to the production of a soluble anti-phagocytic virokine, or virus-induced cytotoxic agent, as seen in Dengue virus infection.

Macrophages have been shown to exhibit low efficacy in phagocytosing *Haemophilus parasuis*. Hence, Segales et al propose that PRRSV may indeed activate alveolar macrophages instead of causing their demise. Solano et al also proved that clearance of *H. parasuis* is increased early in PRRSV infection *in vitro*. However, when alveolar macrophages of PRRSV-infected pigs were cultured and treated with *H. parasuis* their phagocytic activity was slightly reduced at 168 and 216 hours post infection with PRRSV. The macrophages capability of killing bacteria through the production of superoxide anion was impaired during this time as well.

### b) Lymphocytes

An antigen-specific, cell-mediated immune response to PRRSV has been shown in pigs following infection. This response was blocked by anti-CD4 and anti-MHC class II antibodies, and was initially detected in virus-infected animals at 4 weeks following exposure and continued to be detected through 11 weeks after exposure. ORFs 2, 5 and 6 products induced higher responses, with M protein (ORF 6 product) inducing the highest response. A skin test revealed that infected animals also developed a virus-specific delayed type hypersensitivity reaction.

An increase in the number of CD8+ cells after 21 days post infection of pigs has been reported. This increase was observed for three weeks. Shimizu et al also demonstrated the increase in CD2+, CD8+ and CD4+ cells preceded by a reduction on the CD2+ and CD8+ populations between 3 and 28 DPI. However numbers of thymocytes were not affected throughout the course of infection. They also reported that there was no evidence of virus-induced CD4+ cells depletion or mitogenic effect on CD8+ cells, *in vitro*.
Further *in vivo* studies revealed that this increase in the number of CD8+ cells is localized to the systemic lymphoid tissues, whereas an increase in B lymphocytes is observed in lymphoid tissues associated with the mucosa.

Studies performed with an American strain of PRRSV revealed an increase of lymphocyte proportion in BALF of specific pathogen free (SPF) pigs after 21 DPI, reaching maximum at 28 DPI. During this time period (14 to 35 DPI) the macrophage proportion decreased and increased after the lymphocytes ratio dwindled. Recent studies confirm the findings described above. The overall number of macrophages in BALF does not decrease during infection with the European strain of PRRSV (LV) but that the percentage of these is affected by the infiltration of lymphocytes. These lymphocytes were of the cytotoxic phenotype (CD8+) as well as natural killer (NK) cells and started to show up on BALF between 10 to 21 dpi increasing the total number of cells to 10 times its original number during this time period. T-helper cells (CD4+CD8-) or CD4/CD8 double positives were not detected among the infiltrated cells.

Zuckerman et al. (1998) compared the cell-mediated immune response of pigs to PRRS virus infection versus vaccination with either pseudorabies (PRV) or PRRS virus vaccines using a lymphocyte proliferation assay and the ELISPOT test. The ELISPOT test measures virus-specific interferon gamma-producing cells. Infection with a PRRS virus field isolate induced a long lasting (> 1 year) and strong CMI response in pigs, which was comparable in response to the CMI response following PRV vaccination. In contrast, a modified-live PRRS virus vaccine was far less effective at inducing CMI when compared to the CMI response arising from a highly protective PRV vaccine. Nonetheless, induction of virus-specific cytotoxic T-lymphocytes by PRRS virus infection has not been documented. Furthermore, the role of CMI in the control of PRRS virus infection is generally not understood.
3. Effect on cytokines

a) IFN

Buddaert et al. performed both in vitro and in vivo studies to determine the effect of interferon alpha (IFNα) on PRRSV replication. When exogenous IFNα was used to pretreat cultured alveolar macrophages, PRRSV yield from infected macrophages was reduced up to 2.6 log10 TCID₅₀ in comparison with 3.7 log10 TCID₅₀ reduction of vesicular stomatitis virus (VSV), a virus highly sensitive to porcine IFNα. The in vivo study, demonstrated that primary infection with porcine respiratory coronavirus (PRCV), which induces natural IFNα in the lungs, reduced PRRSV yield in alveolar macrophages. Dual infection also caused a dramatic increase in IFNα production, compared to PRRSV singly infected pigs. In contrast, no effect on PRRSV yield was observed in pigs exposed to PRRSV followed by PRCV. Even though PRRSV exhibits low capability to induce IFNα production, a secondary infection with PRCV increases total IFNα to a level similar to that of a PRCV single infection. The later results are in disagreement with the findings of Albina et al. (1998), where PRRSV infection affects IFNα production by secondary infection with transmissible gastroenteritis virus (TGEV).

In vivo studies performed by Meier et al. showed that virus specific IFNγ responses are not detected for the first 9-10 weeks after infection in comparison to IFNγ response against other pathogens, such as pseudorabies virus (PRV). They found it takes 34 weeks for the PRRSV-specific IFNγ response to reach the same level that a PRV-specific IFNγ response will attain in two weeks by vaccination. Likewise, the aforementioned researchers reported that the addition of IL12 or the blocking of IL10 with specific antibodies could enhance the IFNγ response of PBMC cultures. The addition of IL10 suppresses the IFNγ production under the same culture conditions.

Suppression of PRRS virus replication by IFNγ as well as type I IFN was also demonstrated by other investigators. Their study also showed that IFNγ production occurs mainly in lymph nodes and lungs of infected pigs, however there may be certain populations of infected cells located in lymph nodes and tonsils, that could be resistant to the
effects of IFNγ during the asymptomatic stage of infection.

b) Other cytokines

In dual infection studies with PRRSV and Mycoplasma hyopneumoniae, PRRSV infected pigs showed the tendency to have increased expression of IL1alpha, IL1beta and IL8 mRNA. Similarly, other studies have demonstrated the increase in expression of IL-1β mRNA in BALF cells from experimentally infected pigs although TNF was not detected.

4. Maternal immunity

Very little is known about the role of colostrum-derived immunity in PRRS virus infection, and the protection conferred by colostral antibody is presently an area of investigation. Specific antibodies have been demonstrated in colostrum from experimentally infected sows. Albina et al. (1994) reported that passive maternal antibody was detected in the serum of piglets tested 4 days after birth and disappeared by 3 weeks of age. In some instances, no maternal antibody was detected in sera of piglets born to infected dams. Alternatively, maternal antibody specific for PRRS virus has been reported to persist as long as 4 to 10 weeks of age and occasionally up to 16 weeks of age in pigs nursing immune dams. Senn et al. (1998) estimated the mean half-life of maternal antibody to be 16.2 days (95% confidence interval: 13.7 - 18.7 days) and 8.1 days (95% confidence interval: 6.3 - 10.0 days) for ELISA and SVN antibodies respectively.

Although passive immunity is believed to be protective against infection, passive maternal antibodies may play a limited role in preventing infection or decreasing the severity of the disease in young animals. Molitor (1993) reported that pigs from non-immune dams were not protected following challenge when they were passively given anti-PRRSV antibodies, while challenged pigs born to immune dams were protected. Yoon et al. (1996) demonstrated enhanced infection and replication of PRRS virus in pigs with passively transferred antibody. These observations suggest that antibody alone may not be able to protect pigs from disease and that cell mediated immunity might play an important role.
role in protecting pigs from PRRS virus infection. It is known that mammary secretions contain high numbers of leukocytes \(^{188}\). These cells are absorbed by the neonatal pig from the digestive tract and distributed via the lymphatic vessels to the lymph nodes \(^{221}\). Acquisition of maternal leukocytes by the piglet confers measurable cell-mediated immune responses \(^{235}\). It is possible, but unproven, that maternal-derived protection against PRRS virus infection requires both cell mediated and humoral aspects.

5. Immunity conferred by natural exposure

The return of herd reproductive performance parameters to acceptable levels following a clinical episode of PRRS is evidence that some type of protective immunity develops following exposure. Freese and Joo (1994) investigated 2 herds with a previous history of clinical PRRS by serological monitoring and virus isolation 6 months after the initial outbreaks and observed that transmission of PRRS virus had spontaneously ceased in one of the 2 farms investigated. However, herd immunity against PRRS may be difficult to achieve and maintain. Mishchenko et al. (1997) reported that clinical signs of PRRS recurred in 20 to 30% of sows at 12 to 14 months after recovery \(^{141}\).

In individual animals, the development of protective immunity to PRRS virus, particularly with respect to reproductive disease, has been shown to occur both in naturally and in experimentally infected pigs \(^{17; 29; 59; 71; 78; 111; 172; 224}\). In an experimental challenge study, 8 gilts were initially exposed intranasally to PRRS virus at 86 to 96 days of gestation and gave birth to an average of 5.8 live pigs, 0.6 stillborn pigs, and 2.1 mummified fetuses \(^{79}\). Five months after initial exposure, the same females were bred and subsequently re-challenged at 93 days of gestation (7 to 8 months after first challenge). These sows gave birth to an average of 10.8 live pigs, 0.5 stillborn pigs, and 0.3 mummified fetuses, indicating that they had recovered from the initial infection and had acquired immunity to subsequent reproductive losses. Lager et al. (1997) reported that gilts exposed to PRRS virus isolate NADC-8 at the time of breeding (day 0) were protected against homologous challenge, but only partially protected against heterologous challenge, at day 90 of gestation. Protection against subsequent reproductive losses appears to be of long duration in individual animals,
at least against challenge with homologous virus. Experimentally infected sows were protected against reproductive losses when challenged with homologous virus as long as 604 days after the initial exposure. 

In endemically infected herds, chronic or cyclic PRRS problems, primarily manifesting as respiratory disease in groups of animals, have frequently been reported. In individual pigs, several investigators have shown that PRRS virus persists even in the face of an active immune response. In one study, virus was isolated from experimentally infected pigs for up to 157 days after challenge. Benfield et al. (1997) were able to detect viral RNA in pigs born to sows challenged with PRRS virus at 80-95 days of gestation for up to 210 days after birth. Those pigs shed and transmitted the virus to commingled sentinels for up to 112 days after birth. These observations raise questions regarding the protective role of humoral and/or CMI responses, questions for which we currently have no answers.

6. Vaccine-induced immunity

In recent years commercial vaccines have been produced and utilized to control PRRS in herds worldwide. Currently, vaccines for PRRS are available to swine producers either as modified live virus (MLV) or killed products. Vaccination of animals in endemic areas is relatively effective for the prevention of reproductive failure. Vaccination of boars may also reduce shedding of vaccine or challenge virus in the semen, indicating some level of protection.

Despite the wide use of commercially available vaccines, PRRS still abides in many herds. The efficacy and safety of current vaccines do not appear to be satisfactory. Osorio et al. tested both modified live and the inactivated vaccines commercially available. The survival percentage was of 50% for pigs vaccinated with the modified life vaccines and 10% with the inactivated virus vaccine. Severe outbreaks of PRRS in so-called well vaccinated swine operations in southeast Iowa, Illinois, Minnesota, Nebraska and North Carolina has been documented. MLV vaccine virus is shed in semen of vaccinated boars and may be transmitted to naïve animals and herds. In some instances, reversion to virulence of
modified-life vaccine virus has been demonstrated in herds in Denmark and Canada\textsuperscript{25,56,118}. Sequence analysis of three independent vaccine-derived PRRSV strains has shown that parallel mutations on ORF1’s helicase and papain-like cystein protease domains were reverted to the parental strain\textsuperscript{159}.

The control of respiratory problems through vaccination appears to be more problematic. Recently, the general trend among many USA swine producers and veterinarians has been to limit the use of vaccines to the control of clinical outbreaks and for use in the acclimatization of purchased seedstock. Recent outbreaks of PRRS in Danish herds after the introduction of a modified-live virus vaccine suggest that we know little about protective immunity from PRRSV\textsuperscript{25}. Observations of this type emphasize the need for basic research on the immunobiological properties of viral proteins and their roles in immunity and pathogenesis. There are indications of progress in these areas. For example, Plana-Duran et al. (1995) reported that PRRS virus neutralizing antibodies were detected in pigs inoculated with baculovirus-expressed GP5 protein. Immunization with baculovirus-expressed GP3 induced even higher protection than immunization with GP5 protein\textsuperscript{171}. The development of “second” generation vaccines such as DNA vaccines is also in progress. Kwang et al studied the immune response to various DNA vaccines encoding for PRRSV ORF4, 5, 6 and 7 proteins. PRRSV-specific antibodies were detected in 71% of the vaccinated pigs. Cell mediated immune responses were elicited in 86% of vaccinated pigs as assessed by lymphocyte proliferation and IFN\gamma production\textsuperscript{109,109}. These studies also revealed the presence of neutralizing antibodies for both ORF4 and ORF5 envelope glycoproteins.
ANTIBODY DEPENDENT ENHANCEMENT OF VIRUS INFECTION

A review paper to be submitted to Viral Immunology

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A. Abstract

In general virus-specific antibodies are considered antiviral and play an important role in the control of virus infections in a number of ways. However, in some instances, the presence of specific antibodies can be beneficial to the virus. This activity is known as antibody dependent enhancement (ADE) of virus infection. The ADE is a phenomenon in which virus-specific antibodies enhance the entry of virus, and in some cases the replication of virus, into monocytes/macrophages and granulocytic cells through interaction with Fc and/or complement receptors. This phenomenon has been reported in vitro and in vivo for viruses representing numerous families and genera of public health and veterinary importance. These viruses share some common features such as preferential replication in macrophages, ability to establish persistence, and antigenic diversity. For some viruses, ADE has become a great concern to disease control by vaccination. Consequently, numerous approaches have been made to the development of vaccines with minimum or no risk for ADE. Identification of viral epitopes associated with ADE or virus neutralization is important for this purpose. In addition, clear understanding of the cellular events after virus entry through ADE has become crucial for developing efficient intervention. However, the mechanisms of ADE remain to be better understood.

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B. Introduction

Animals have various mechanisms to prevent invasion of potentially pathogenic foreign agents. Due to anatomic location, both gastrointestinal and respiratory passages of higher order animals are more likely to come in contact with pathogens. The intricate structure of the airways and the presence of physiological barriers, such as mucosal surfaces and cilia in the lumen surface, compose the first line of defense against respiratory pathogenic agents. Even when pathogens could overcome these non-specific defenses, and reach the innermost branches of the bronchioles and the alveoli, physiologic inflammatory responses take place to stop them. Specialized, immune cells called macrophages are resident in the alveolar spaces and are capable of ingesting foreign bodies in a non-specific as well as specific manner, and destroy them. As proteins of ingested agents are degraded by the now activated macrophage, these protein segments or peptides are transported to the cell surface to be presented to other cells of the immune system that have been called through the production of chemical signals, or cytokines. Among the attracted cells are lymphocytes, which are composed of two main populations: T cells and B cells. The T cells are divided into cytotoxic T cells (Tc) and helper T cells (Th). The later are subdivided into various subpopulations with functions that can be modulated by the cytokines present in their microenvironment and by each other. Th cells determine the course of action of the overall immune defense strategy, whether it is mainly a humoral response (by antibody production) or cellular-mediated cytotoxicity (by Tc). B cells are responsible for the production of specific antibodies, through activation by interaction with Th. Antibodies are especially effective in agglutinating or opsonizing, and neutralizing soluble antigens such as bacteria or toxins, but are less so against viruses or other intracellular pathogens. Cell-mediated cytotoxicity by Tc or other cells such as natural killer (NK) and lymphokine-activated killer (LAK) cells, on the other hand, play a major role in the clearance of such pathogens by killing the infected cells. However a humoral response is important to neutralize cell-free and circulating virus during primary infection and plays a central role in preventing reinfection by the viral pathogen 85.

Despite the efficacy and specificity of the immune system, there are many pathogens
capable of evading it and establishing infection that leads to disease. For example, both human and murine cytomegaloviruses exhibit resistance to killing by NK cells and interfere with the major histocompatibility complex type I (MHC I) antigen processing. Also, the murine cytomegalovirus can alter surface expression of MHC I molecule. Measles virus inhibits interferon (IFN) α and β production, while Vaccinia virus produces an IFN receptor homologue and simultaneously interferes with IFN intracellular signaling. Other homologues produced are the tumor necrosis factor receptor II (TNFRII) by both Myxoma and Cowpox viruses. Cowpox virus can also inhibit interleukin (IL)-1β converting enzyme, which tends to attenuate the acute phase of infection and reduces mortality of infected cells providing the virus with more time for production of progeny viruses. Molluscum contagiosum virus and bovine herpesvirus type 4 each encode a protein that prevents receptor-induced apoptosis. Herpes simplex virus inhibits complement function, interferes with antigen processing for MHC I presentation, protects infected cells from apoptosis and expresses immunoglobulin constant-fraction receptor (FcR) homologues that prevent neutralization by complement and antibody-dependent cell-mediated cytotoxicity. Human immunodeficiency virus type 1 (HIV-1) also utilizes several strategies to evade the immune response. In addition to infect CD4+ lymphocytes and macrophages, this virus is capable of downregulation of CD4 and MHC class I molecule. Latency of HIV infection is achieved by hiding in glial cells and resting T cells. HIV can also generate escape mutants, and can cause T lymphocyte apoptosis by upregulation of Fas (CD95) and Fas ligand.

Antigenic variation is another less aggressive but highly effective method by which viruses can evade the immune response. Variability in antigenic sites can generate high heterogeneity in the viral populations (i.e. quasi-species) even within serotypes. Epitopes present in one generation of viruses can be altered in succeeding generations, making them less immunogenic or, can be eliminated through mutations. However, some of these mutations could be silent, not affecting neutralizing epitopes. When an antibody response specific to a particular epitope on the parental strain is elicited, these antibodies will be less likely to confer protection when a second infection with a variant progeny strain
occurs. Antigenic variation may also affect the virulence of the virus. Development and efficacy of vaccines can be affected by antigenic variation since field strains may have evolved and lost epitopes present in the vaccine strain, particularly epitopes associated with virus neutralization.

Other viruses can utilize preexisting antibodies, which potentially neutralize their capability of infecting through their natural receptor-ligand route, and bind to the FcR on phagocytes, to facilitate infection of their target cells. These viruses usually can replicate in the macrophages or monocytes, and may use them as reservoirs or cellular trampolines in order to reach other body tissues. Consequently, increased chance to infect target cells results in increased production of viral progeny and often exacerbation of the disease caused by them. This phenomenon has been known as “antibody-dependent enhancement” (ADE) or “immune enhancement of disease.” This review article focuses on those pathogens of public health or veterinary importance that are capable of utilizing antibodies to enhance their infection, which may result in exacerbation of clinical manifestations.

C. Antibody-dependent enhancement of viral infection

Antibody dependent enhancement (ADE) of infection is a phenomenon in which virus-specific antibodies enhance the entry of virus, and in some cases the replication of virus, into monocytes/macrophages and granulocytic cells through interaction with Fc and/or complement receptors. This phenomenon was first described by Hawkes in 1964. He reported that it was possible to increase the total yield of a variety of flaviviruses—including Japanese encephalitis virus, Murray Valley encephalitis virus, and Getah virus—in chick embryo cell cultures by first exposing the viruses to high dilutions of homologous antibody which was antiviral at low dilutions. The scientific community considered his findings artifacts albeit he could reproduce the results. It was not until 1977 that Halstead linked the concept of enhancement with severe dengue disease.

Subsequent to Hawkes’s observation on the aforementioned flaviviruses and some arboviruses, ADE has been described for numerous viruses that belong to different families and orders. Some examples are: yellow fever virus, dengue virus (DV), HIV-1
respiratory syncytial virus (RSV) 40, Hantavirus 171,307, Ebola virus 154, Getah virus, Sindbis virus 21, Bunyamwera virus 104, influenza virus 114,115, West Nile virus (WNV), Japanese encephalitis virus B (JEB), rabbitpox virus 61,62, feline infectious peritonitis virus (FIPV) 170, lactate dehydrogenase elevating virus (LDV) of mice,18,19,69, reovirus 16, rabies virus 82, murine cytomegalovirus 68, foot-and-mouth disease virus (FMDV) 8, porcine reproductive and respiratory syndrome virus (PRRSV) 175,27,28, simian hemorrhagic fever virus (SHFV) 123, and Aleutian disease virus (ADV) of mink. 77. Some of these viruses represent a significant human health threat; yet others are of veterinary importance. Common features among the viruses described above are that: a) they replicate, in part or exclusively, in macrophages 51,71,97,117,177, b) they induce the production of large amount of antibodies that neutralize, even homologous virus, poorly 7,51,97,111,177 and c) they cause persistent infections which are commonly characterized by viremia of long duration. Antigenic diversity among isolates is also a common feature of these viruses, which renders them partially resistant to neutralization by antibodies raised against heterologous isolates 51,97,128.

D. Mechanism of ADE

Although the precise mechanism of ADE is not completely understood, it is generally assumed that increased yields of virus are primarily due to a greater number of susceptible cells being infected.30,40,40,51,77,117,175. This increase in the infection rate of cells is shown to be mediated by receptors, most notably FcR, which facilitate the uptake of virus-antibody complexes. However, studies have also suggested that other mechanism(s) could also account for increased virus yields. Antibody may also increase the efficiency of virus replication, either by facilitating the uptake of infectious antibody-virus complexes or by increasing the synthesis of viral protein and nucleic acid. For example, Gollins et al demonstrated that antibodies can increase the number of WNV particles attached to mouse macrophage-like cells by comparing radioactivity counts associated with cells infected with radiolabelled WNV complexed to virus-specific antibodies with counts in the absence of antibody 41,42. Robinson et al. demonstrated that replication of HIV-1 was initiated sooner
when the virus was pretreated with HIV specific antibodies, as opposed to untreated virus. Progeny virus was released sooner from treated cells than from non-treated cells, as well. They also found that protein and RNA synthesis were increased in cells that were infected with HIV-1 treated with antibody. In contrast, Olsen and Scott studied the kinetics of FIPV infection in individual feline peritoneal macrophages in the presence and absence of antibody utilizing in situ hybridization. They demonstrated that the number of infected cells was increased in the presence of antibody. However, based on the relative intensity of radiograms of individual cells, they did not find any evidence that the efficiency of virus replication within the cells was enhanced.

Generally, interaction between virus-antibody complexes and FcR on monocytes/macrophages or granulocytes induces signal transduction, resulting in phagocytosis, release of cytokines, a superoxide burst, and antibody-dependent cell-mediated cytotoxicity. These responses are considered antiviral. It is not known how this interaction results in enhanced infection. However, since these viruses are known to replicate in part or exclusively in these cells, it is assumed they have the ability to modulate antiviral mechanisms of the cells either by utilizing their own products or by interfering with metabolic pathways of cells. It is also possible that infections by virus-antibody complexes are restricted to immunologically immature subpopulations of the cells. Halstead and his associates found that human monocytes cultured more than 1 day prior to being infected with DV-antibody mixture became increasingly less permissive to infection. This loss of permissiveness may have been due to increased lysosomal activity. These observations explain why high virus titers are produced in bone marrow explant cultures in which young monocytes are continuously produced. Restriction of virus infection to immunologically immature cells was also demonstrated in mice that were persistently infected with LDV.

1. Antibodies mediating ADE

Enhancement of virus infection has been demonstrated using various sources of antibodies. These sources include polyclonal antisera generated in natural host or other animals, mouse ascitic fluids containing MAbs to the virus of interest, and immunoglobulins.
isolated from antiserum. The mechanism by which ADE is mediated is known to be primarily through the interaction of the Fc region of virus-specific IgG and Fc receptors on the surface of monocytes/macrophages and granulocytic cells. Halstead and O'Rourke fractionated IgG and IgM from antiserum of DV-immunized monkeys and evaluated which fraction increased the yield of progeny virus. Enhancement was observed only with the IgG fraction, while virus that was exposed to the IgM fraction was neutralized. Similar observations were made by Olsen et al. who evaluated the ability of mouse MAbs specific for the spike (S) protein of FIPV to mediate ADE in feline peritoneal macrophages. Only IgG class MAbs enhanced FIPV infection, while IgM class MAbs did not mediate ADE. To date, it is not known whether or not other subtypes of antibodies (IgA, IgD and IgE) can mediate ADE.

Different isotypes (subclasses) of IgG have also been evaluated for their ability to mediate ADE. In the case of DV, murine IgG1, IgG2a, and IgG2b monoclonal antibodies specific for the E envelope protein of DV serotypes 2 and 4 are reported to enhance infection of the virus when cells with compatible Fc receptors were used as targets. No information is available about the role of IgG3 in ADE of DV infection. In contrast, Corapi et al. evaluated the ability of 19 mouse monoclonal antibodies specific for the S envelope protein of FIPV to induce ADE in feline peritoneal macrophages. All MAbs were capable of neutralizing the ability of virus to infect a permissive cell line. Fifteen of 19 MAbs induced ADE of infection in macrophages and all but one were of the IgG2a subclass. The remaining 4 MAbs that did not induce ADE were IgG1. The difference in the isotypes between neutralizing MAbs that induced ADE and those that did not induce ADE suggested that there might be a restriction in the subclasses capable of mediating ADE. It is also possible that the difference in the ability of FIPV-specific murine IgG isotypes to mediate ADE is due to differences in the binding affinity of murine isotypes to FcR on feline macrophages. The ability of the different isotypes of human and other mammalian IgG to enhance virus infection has not been evaluated.
2. Receptors involved in ADE

Several cell surface molecules, including the FcR, complement receptor (CR), \( \beta_2 \)-microglobulin, and some “Cluster Designation” (CD) molecules, have been reported to play a role, or at least to be involved, in mediating ADE of virus infection \(^{86,97} \). Antibody-FcR interaction is known to play a key role in ADE. The FcR-mediated mechanism of ADE was first suggested by Halstead et al. who reported that F(ab')\(_2\) fragments prepared from IgG did not enhance infection of DV in human peripheral blood leukocyte cultures while whole IgG did so \(^{56} \). This was indirect evidence which suggested that interaction of virus-IgG complexes with FcR on the cell surface may be necessary for ADE of virus infection. Other indirect evidence for this interaction was shown by Daughaday et al. \(^{31} \). These investigators found that ADE of DV infection in monocytes was inhibited by first treating the cells with immunoglobulin specific to cellular receptors prior to exposing cells to virus-antibody mixtures. Yang et al. has shown that antisera specific for DV serotype 1 (DV-1) can neutralize DV-2 infection into BHK-21 cell line but does not neutralize infection of human monocytes \(^{173} \). Peiris et al. conclusively demonstrated that the interaction between virus-antibody and FcR is essential for the ADE of virus infection \(^{118} \). They were able to block ADE of WNV infection in a macrophage-like cell line (P388D1) by pretreating the cells with anti-FcR MAb prior to exposing cells to a virus-antibody mixture. Other investigators were also able to block infection of cells by virus-antibody complexes by first treating virus-antibody mixture with Protein A which binds to the Fe portion of antibody \(^{77,117} \). Necessity of FcR in ADE of virus infection was also demonstrated by the observation that FMDV complexed to antibody could infect an engineered cell line expressing murine FcγR which otherwise would not be permissive to the virus. \(^{98} \) Similarly, a human cell line (K562) expressing FcR, normally is not permissive for ADV, supported viral replication when the cells were exposed to ADV complexed to antibody \(^{34} \).

In humans, there are 3 types of Fc receptors which bind human IgG: FcγRI, FcγRII, and FcγRIII \(^{6,133,164} \). The FcγRI is present exclusively on monocytes/macrophages and binds human IgG with high avidity. It has higher specificity for IgG1 and IgG3 isotypes than for IgG2 and IgG4 isotypes. The two other receptors, FcγRII and FcγRIII, are found on
monocytes, macrophages, eosinophils, neutrophils, natural killer cells, B lymphocytes, and T lymphocytes. These two receptors have relatively low avidity for IgG compared to FcγRI. Kontny et al. showed that FcγRI mediated ADE of DV infections in U937 cells. In a related study, FcγRII was also reported to mediate ADE of DV infection in a human erythroleukemic cell line (K562), which has only FcγRII. The role of FcγRIII in ADE of DV infection is not known.

Mouse macrophages are known to have two types of Fe receptors, designated FcRI and FcRII, which bind IgG. The FcRI is trypsin-sensitive and binds IgG2a, while the FcRII is trypsin-resistant and binds IgG2b and IgG1 complexes. Peiris et al. studied the inhibitory effect of anti-mouse FcRII antibody on the ADE of WNV infection mediated by anti-WNV MAbs of subclasses IgG1 or IgG2a. Pretreatment of P388D1 cells with anti-FcRII antibody completely inhibited enhancement of virus infection mediated by both IgG1 and IgG2a anti-WNV MAbs. Enhancement of WNV infection, however, was independently achieved with anti-WNV MAbs of both subclasses. Other investigators found that intact anti-FcRII antibody interfered with both FcRI and FcRII in a macrophage rosetting assay, suggesting that both Fc receptors on mouse macrophages can mediate ADE of virus infection.

Since ADE of virus infection results from the interaction of virus, antibody, and FeR, changes in any of these three components may modulate the ADE. Of the three components, the FeR can be most easily modulated in vitro within relatively short periods of time by treating FeR-bearing cells with certain cytokines or proteolytic enzymes. A quantitative (i.e., number) change or a qualitative (i.e., avidity for Fe portion of IgG) change in FeR expressed on cells may influence the ADE of virus infection. For example, gamma interferon (IFNγ) is known to increase the number of FcγRI without changing in the affinity of each FcγRI for the Fe portion of IgG. Kontny et al. reported that pretreatment of human monocytic cells with IFNγ augmented ADE of DV infection and that the level of enhancement correlated with the increase in the number of FcγRI on the cells. In another case, Halstead and O'Rourke found that pretreatment of monocytes with pronase, trypsin, and protease augmented ADE of DV infection. Zoellner et al. have also suggested that
protease may play a role as a cofactor in ADE of HIV infection \(^{178}\). More recently, Mady et al. examined the effects of neuraminidase on ADE of DV infection mediated by the low-affinity FcγRII \textit{in vitro} \(^{94}\). They found that neuraminidase treatment of the K562 cells that have only FcγRII increased the degree of ADE of DV infection by human anti-DV antibodies. It is known that treatment of FcγRII with enzymes such as pronase, trypsin, elastase, and neuraminidase increases the avidity of receptors for IgG but does not increase the number of the receptor expressed on cell surface \(^{32;165}\).

Besides FcR, complement receptors have also been implicated in ADE of virus infection \(^{20;142}\). Cardosa et al. found that infection of P388D1 cells by WNV is enhanced in the presence of virus-specific IgM by supplementing fresh mouse serum containing complement to virus-IgM mixtures prior to inoculation. \(^{20}\) However, the magnitude of enhancement of WNV infection mediated by complement was less than IgG-mediated ADE of virus infection. Complement-dependent ADE of HIV infections has also been reported \(^{44;75;105;140;142}\). Subneutralizing levels of HIV-specific antibody enhance virus replication in several human neoplastic cell lines that express CR and CD4, as well as FcR, in the presence and the absence of complement. The magnitude of the enhancing effect was greater in complement-mediated ADE of HIV replication than enhancement mediated by the presence of antibody only. \(^{97}\) Furthermore, Robinson et al. reported that replication of HIV was initiated sooner and the efficiency of replication (e.g., protein and RNA synthesis) was enhanced through the mechanism of complement-mediated ADE \(^{139}\).

Several cell surface molecules are known to enhance virus infections or to be involved in ADE \(^{93;97}\). Takeda et al. demonstrated that ADE of HIV infection in monocyctic cells via FcR was blocked by pretreatment of cells with monoclonal antibodies to CD4 molecule, as well as to FcγRI \(^{155}\). Robinson et al. also reported that enhanced infection of HIV through complement-mediated ADE required not only complement receptors, but also CD4 molecules on the surface of cells \(^{142}\). These observations indicate that the presence of the CD4 molecule on the cell surface may be a requirement for both the FcR-mediated and complement-mediated ADE of HIV-1 infection. In the case of DV infection, Mady et al. used bispecific antibodies which were prepared by chemically cross-linking anti-DV
antibodies to antibodies specific for one of three Fc receptors or non-FcR molecules, and demonstrated that DV infection could be enhanced by non-FcR molecules such as β2-microglobulin, CD15 or CD33.

The critical role of natural viral receptor(s) on the membrane of target cells in ADE is uncertain. Chinese hamster ovary cell line engineered to express murine FcγR was permissive to FMDV coupled with antibody whereas it was not permissive to poliovirus-antiVP1 antibody complexes. This lead investigators to the conclusion that the natural receptor for FMDV is only involved in attachment and not in subsequent steps of replication such as uncoating. This conclusion was ratified when FMDV with mutated RGD sequences of the G-beta H loop of VP1 protein of the nucleocapsid was unable to bind susceptible cells. However, when these mutants were complexed with antibody against the VP1 protein, virus infection was established in the cell line expressing FcγR on the cell membrane, revealing that the mutations only affected the binding capability of the virus. From these observations, one can speculate that naturally occurring mutations during infection could be bypassed in the presence of antibodies specific to epitopes from this receptor protein and would enhance infection instead of neutralizing it.

3. Viral proteins/epitopes associated with ADE

Viral antigenic determinants associated with envelope protein(s) induce antibodies which mediate ADE. Scott and his associates conducted extensive studies utilizing monoclonal antibodies specific for the nucleocapsid protein, matrix protein (M), and the S protein of FIPV. ADE-associated epitopes were only found on the S protein. More recently, an in vitro experiment using vesicular stomatitis virus (VSV) vector expressing surface glycoprotein of Ebola virus (Zaire strain), which is associated with cell penetration, demonstrated that antibody generated against this protein could enhance infection of the VSV vector and of a less virulent strain, Ebola-Reston in human kidney 293 cells. The same observations have also been made for envelope proteins of other viruses for which ADE has been reported. Specific examples are: the E protein of DV, gp120 and gp41 of HIV, HA protein of influenza virus, G1 and G2 proteins of hantavirus.
To date, no internal proteins of enveloped viruses, for intact viruses, have been reported to be associated with the induction of enhancing antibody, with the exception of pre-M protein, an immature matrix protein of dengue virus. However, it is plausible that completely or partially naked virus containing infectious genetic material could establish infection and replicate in a target cell if it is internalized through antibody-FcR interaction (i.e. ADE), as is the case for transfection of infectious viral RNA to non-permissive cells.

Reovirus is the only non-enveloped virus for which ADE was reported. Enhancement of reovirus infection in the P388D1 cells was demonstrated to be mediated mainly by monoclonal antibodies specific for the σ-1 protein, a major outer capsid protein which determines the serotype of reoviruses. In addition, monoclonal antibody specific for other capsid proteins, such as μ1c protein, was also reported to mediate the ADE of reovirus. No protein of the inner capsid of reovirus was found to be associated with ADE.

Since viruses in the same genus or family may share common antigenic determinants, ADE of virus infections can be mediated by antibodies raised not only against heterologous strains but also different serotypes of the viruses or even against closely related viruses in the same genus or family. This observation suggests that enhancing antibodies may not be highly specific for a specific virus. For example, studies using polyclonal antibody revealed that DV infection can be enhanced by antisera raised against heterologous serotypes of DV and also by antisera specific for other flaviviruses, suggesting that not only serotype-specific but also serotype- and flavivirus-cross reactive epitopes are associated with ADE. There was a difference in the magnitude of enhancement mediated by homologous sera as compared to heterologous sera. A similar observation has also been made utilizing monoclonal antibodies. These studies revealed that infections of DV type 2 (DV-2) could be enhanced by monoclonal antibodies directed against heterologous DV-2 isolates and against DV type 4. Likewise, Tamura et al. found that infection by influenza A virus was augmented by pretreating the virus with antisera raised against different subtypes of the virus. In the case of FIPV, virus infection in feline peritoneal macrophages was enhanced by monoclonal antibodies generated against transmissible
gastroenteritis virus (TGE) of swine which, like FIPV, belongs to the family Coronaviridae. A similar observation was made with African flaviviruses, such as West Nile, Zika, Wesselsbron, Dakar bat, Potiskum, Uganda S, and yellow fever viruses. For example, anti-Potiskum virus antibodies enhanced other flaviviruses but most of antibodies to the other flaviviruses did not enhance infection of Potiskum virus (in mouse macrophage like cell line, P388D). On the contrary, all heterologous antibodies enhanced West Nile, Uganda S, Wesselsbron and Zika viruses. Moreover, homologous antibody induced higher levels of ADE of virus infection (as calculated by enhancement ratio of virus plaque counts) than heterologous antibody. This could be related to the amount of “enhancing” epitopes shared among species. Cross enhancement could have important epidemiological relevance in areas where these viruses are endemic since cross-reactive “enhancing” antibodies permits co-existence of multiple serotypes in the population and promotes their persistence.

Since antigenically distinct strains of virus may have different quantitative and/or qualitative profiles of epitopes associated with ADE, differences in epitopic profiles may influence augmentation of virus infection in the presence of antibodies directed against heterologous strains or viruses. Consequently, strains vary in their susceptibility to ADE and/or ability to induce ADE. Halstead and others evaluated anti-DV sera from naturally infected humans or produced in various species of animals (mouse, rabbit, monkey) against 4 different serotypes of DV for their ability to cross-neutralize DV-2 and to mediate ADE of DV-2 infections. Their studies revealed that heterotypic antisera poorly neutralized DV-2 infectivity for continuous cell lines, but both homotypic and heterotypic antisera enhanced DV-2 infection in human peripheral blood leukocyte cultures. The degree of ADE of DV-2 infection mediated by either homotypic or heterotypic antisera varied. Higher ADE activity for DV-2 infection was detected in the heterotypic antisera, most notably DV-1 antisera, than in the homotypic serum. Moreover, the magnitude of maximum ADE of DV-2 infections mediated by heterotypic antisera varied among the antisera, as well as the serum dilution at which the maximum ADE activity for DV-2 was observed. These results suggested that DV serotypes and field isolates varied in their susceptibility to ADE mediated by antibody raised against heterologous serotypes or
isolates, and probably also varied in their ability to induce enhancing antibodies. The variability in ADE response among the DV-2 isolates was attributed to differences in the epitopic profiles of the isolates. A difference in the susceptibility to ADE among isolates has also been reported for the porcine arterivirus, PRRSV. Investigators demonstrated that some isolates produced higher progeny virus titers than others when inoculated to porcine alveolar macrophages after treated with the same PRRSV antiserum\textsuperscript{176}.

In similar studies with FIPV, Olsen et al. evaluated the biological function of MAbs raised against the S protein of the virus and discovered that specific epitopes of the S protein vary in their ability to induce ADE-associated antibodies\textsuperscript{117}. They were able to categorize the monoclonal antibodies that represent the epitopes of the envelop S protein into 3 groups according to their ability to: a) only neutralize, b) both neutralize and enhance, and c) only enhance FIPV infection. Furthermore, monoclonal antibodies with specificity for different FIPV antigenic determinants varied in their ability to enhance virus infection, suggesting that epitopes are either strongly or weakly associated with ADE\textsuperscript{30,117}. These finding are particularly noteworthy because they suggest that it may be possible to develop vaccines with strong neutralizing and weak ADE inducing characteristics. HIV-1 isolates similarly vary in their susceptibility to ADE and/or in the ability to induce enhancing antibodies. It is believed that susceptibility is due to the great genomic diversity that has been demonstrated among HIV isolates\textsuperscript{97}. Currently, variation in the susceptibility of isolates to ADE and in the ability to induce ADE are of great concern for developing vaccines against viruses for which ADE and antigenic diversity have been reported.

4. Cellular events

It has been shown that for some viruses binding is not sufficient for infection, suggesting the involvement of other viral and host cell proteins in the internalization process\textsuperscript{160}. In such cases, the presence of antibodies that prevent binding will not neutralize but facilitate infection, as seen with attachment-defective FMDV mutants\textsuperscript{99}. Thus, internalization may occur through other pathways instead of the endocytic pathway, commonly attributed to enveloped viruses\textsuperscript{43,110}. However, it has also been shown that the
presence of enhancing antibody does not affect the internalization pathway of WNV \(^{42}\).

While enhanced entry and increased production of virus is documented in association with ADE, it is not well understood how cells like macrophages, whose internal microenvironment should be antiviral, become favorable to virus replication when ADE of viral infection occurs. Some limited studies have suggested that virus entry by ADE follows a pathway which may modulate normal immune functions of infected cells such as macrophages. In an early study with DV, the production of high level of prostaglandin E2 (PGE2) but not IL-4 from peripheral mononuclear leukocytes was observed when the cells were inoculated with DV-2 pre-treated with DV-1 antiserum \(^{25}\). The PGE2 is a lipid cytokine produced by macrophages that has anti-inflammatory properties and is responsible for increasing the production of IgG2 in humans \(^{70}\). In bovine, PGE2 also has important regulatory functions in type 1/type 2 immune responses and can regulate virus expression and disease progression in BLV infection \(^{130}\).

A recent study by Lidbury et al (2000) has brought some insight to the cellular events of ADE when they studied Ross river virus (RRV), the causative agent for epidemic polyarthritis \(^{89}\). When murine macrophages were infected with RRV through antibody-FcR interaction instead of the natural viral receptor on the cell, LPS stimulation of cells did not produce successful antiviral response. Instead, the production of IRF-1 and NF-kappaB transcription factors for antiviral genes such as TNF and inducible nitric oxide synthase genes was inhibited. Thus RRV replication was not affected. Despite this, transcription of cellular genes unrelated to antiviral activity and overall mRNA translation was not downregulated.

**E. Biological significance of ADE**

1. **Disease enhancement**

Antibody dependent enhancement of virus infection has been suggested as a disease-enhancing factor for several human and animal viral diseases \(^{97}\). Specific examples include Aleutian mink disease virus, dengue virus, feline infectious peritonitis virus, and respiratory
syncytial virus. In addition, ADE has also been implicated as a major obstacle to the development of vaccines for such viruses as ADV, bluetongue virus, DV, FIPV, influenza virus, lentiviruses, measles virus, rabies virus, and RSV. In all cases, the presence of antibodies induced by vaccination increased the susceptibility to subsequent virus infections and/or exacerbated the severity of clinical disease by virus challenge in vaccinated individuals.

a) Dengue virus

Dengue viruses belong to the genus Flavivirus of the family Flaviviridae. There are 4 serotypes: DV types 1, 2, 3, and 4. Dengue virus infections are considered a serious human health problem in many areas of the world. Dengue virus infection can be asymptomatic or cause two forms of disease. In most cases, DV infection causes a febrile disease referred to as 'dengue fever', which is characterized by fever, retro-orbital pain, muscle aches, bone pain, and petechiae. Patients recover in 7 to 10 days without complications. In some instances, patients infected with DV leak plasma into interstitial spaces resulting in hypovolemia and, sometimes, circulatory collapse. This severe and life-threatening syndrome, which is always accompanied by thrombocytopenia and sometimes by frank hemorrhage, is termed dengue hemorrhagic fever (DHF). More severe clinical manifestations of DHF in which plasma leakage is so profound that shock occurs, are referred to as dengue shock syndrome (DSS).

Although the pathogenesis of DHF/DSS is not clearly understood, the association between ADE and the severity of disease has been extensively studied. This association was first described by Halstead and co-workers who observed that the severity of dengue fever was significantly greater in children with maternal antibody specific for DV than in children with no DV-specific maternal antibody. Experimentally, these investigators demonstrated in rhesus monkeys that anti-DV maternal antibody enhances DV infection. The investigators injected monkeys intravenously with small amounts of human cord blood containing anti-DV antibody and immediately challenged them with DV. The monkeys that were injected with DV antibody developed higher levels of viremia for a longer period than
control monkeys. In a clinical setting, sequential infection models demonstrated that a patient who had previously been infected with either one of the four DV serotypes, and was subsequently exposed to DV-2 had a greater risk to develop DSS. The risk for severe form of DV infection was reported to be the highest when the infections were of the DV-1/DV-2 sequence.

It was also found that DV produced a more severe clinical manifestation in older individuals who had subneutralizing levels of antibodies, which were induced by previous DV infections than in individuals who had no previous exposure to the virus. These severe clinical manifestations were more frequently observed in individuals who have antibody against one serotype of DV and were subsequently exposed to a different serotype of DV than in individuals challenged with a homologous serotype. Recent prospective case-control studies conducted by Burke et al. and Kliks et al. demonstrated that presence of DV antibodies is a significant risk factor for increased severity of disease by subsequent DV infection. In these studies, individuals were categorized into the case and control based on the presence and absence of anti-DV antibody. Decay of DV antibody was monitored for the case group and correlated to ADE activity in undiluted sera. Both groups were also monitored for subsequent clinical event with respect to natural DV infection. The investigators observed that the morbidity of DV infection was significantly higher in the case than the control. Mortality due to DHF/DSS was also higher in the case group than the control.

**b) Respiratory syncytial virus**

In general, RSV infections are not always considered serious. However, individuals who develop pneumonia from RSV infection often require hospitalization. Chanock et al. reported that naturally acquired severe RSV infections were almost always seen in the first 6 months of life when children had circulating maternal anti-RSV antibodies. In another study, infants with maternally acquired RSV antibody not only were susceptible to RSV infections, but the rate of severe disease was higher in these infants when compared to infants without maternal antibodies. These observations led to speculation that RSV-
specific antibody may contribute to the severity of clinical manifestations of disease caused by RSV. A recent study demonstrated that infection of a mouse macrophage cell line by RSV is enhanced in the presence of virus-specific antibody. This observation supports the hypothesis that immune-mediated enhancement of disease does occur in human RSV infection and may contribute to the pathogenesis of the disease.

Immune-mediated enhancement of disease has also been described in human infants and children vaccinated against RSV. Several epidemiological and experimental studies found that immunization with an inactivated whole virion RSV vaccine led to development of antibody response, but did not prevent infection with wild-type RSV in children less than 2 years of age. More importantly, subsequent natural infection by wild-type RSV resulted in an extremely high frequency (52-69% of infected children) of severe lower respiratory tract disease (i.e., pneumonia) in the vaccinated group, whereas only 9-10% of infected children became pneumonic in the non-vaccinated group. Furthermore, the duration of illness was longer and the severity of illness was greater in the vaccinated children compared with non-vaccinated children. Results from these studies indicated that children were at increased risk of severe RSV disease following immunization. Similar observations were also made with measles virus. Natural infection with measles after the use of the killed measles virus vaccine produced a severe systemic disease characterized by pneumonia, unusual rash and edema.

c) Feline infectious peritonitis

Feline infectious peritonitis virus is a coronavirus that causes peritonitis and occasionally a fatal pyogranulomatous disease in kittens and cats. Antibody dependent enhancement has been incriminated as a disease-enhancing factor of feline infectious peritonitis. Cats with active or maternal immunity to FIPV often develop an accelerated and more fulminant disease following challenge with FIPV than seronegative cats. The role of antibodies in mediating more severe disease following challenge has been also documented in cats that were injected with FIPV-reactive immune sera or purified immunoglobulin and subsequently challenged with the virus. Furthermore, immune-
mediated disease enhancement has been demonstrated in kittens who had vaccine-derived humoral immunity directed against the spike protein of FIPV. These kittens died earlier than did control animals\textsuperscript{167}. Similarly, kittens immunized with a recombinant vaccinia virus expressing the spike protein of FIPV died earlier than control animals\textsuperscript{167}.

d) Aleutian mink disease

Aleutian disease virus is a parvovirus that circulates in the blood principally as immune complexes, which are fully infectious, both \textit{in vivo} and \textit{in vitro}\textsuperscript{126}. Consequently, viral infection causes a fatal glomerulonephritis in mink due to deposition of soluble immune complexes on renal glomerular membrane or wall of capillary blood vessel, which causes tissue damage by mononuclear cells and complement and consequently results in impairment of renal filtration. Besides formation of soluble immune complexes, ADE of infection has also been suggested as a potential contributing factor to the pathogenesis of ADV\textsuperscript{124,125}. Initially, Porter et al. found that ADV replicated in macrophages and large amounts of non-neutralizing antibody were produced in mink infected with ADV\textsuperscript{124}. They speculated that the early formation of non-neutralizing antibody might lead to virus-antibody complex formation. They further speculated that phagocytosis of these complexes by macrophages could lead to increased infection of the cells by ADV resulting in increased production of progeny virus. In related work, Porter et al. demonstrated that passive transfer of virus-specific antibody at the peak of viral replication resulted in foci of necrosis around virus-infected cells. The investigator concluded that this pathological reaction was due to enhanced complement-mediated cytolysis, suggesting that antibody has the potential to contribute to the severity of disease by ADV\textsuperscript{125}. A recent \textit{in vitro} study by Kano et al. demonstrated that infection of mink peritoneal macrophages by ADE is enhanced by anti-ADV antibody\textsuperscript{77}.

In a trial with an experimental ADV vaccine, the immunization regimen failed to produce any detectable neutralizing antibody to ADV. However, following challenge with a standardized infectious dose of virus by the oral route, higher levels of circulating antibodies were detected in vaccinated mink than in challenged control animals\textsuperscript{125}. Moreover, 8 of 10
vaccinated mink, but none of control animals, developed Aleutian disease. Cumulatively, these observations provide strong evidence that vaccine-induced humoral immunity can lead to a more severe disease course through ADE.

e) Lentiviral diseases

Equine infectious anemia is a lentiviral infection of horses that generally causes a syndrome of fever, anorexia, and anemia with cyclic recurrence during the first year of disease. Subsequently, horses may become asymptomatic or develop a chronic wasting syndrome. Several studies were conducted to evaluate the efficacy of vaccines against equine infectious anemia virus (EIAV) as a model for evaluating AIDS vaccine strategies. These studies clearly illustrated that enhanced severity of disease in vaccinated animals was due to the presence of vaccine-induced antibody. Issel et al. used viremia as a criteria of disease and demonstrated that inactivated whole virus vaccines elicited 100% protection against homologous challenge with avirulent EIAV. In contrast the vaccines failed to prevent viremia following heterologous challenge with a virulent strain of EIAV. However, the vaccine did protect ponies from the subsequent development of clinical symptoms after challenge with the virulent strain. Using viremia as criteria of disease these investigators also evaluated the efficacy of a subunit vaccine composed of lectin affinity-purified viral envelope glycoproteins. This vaccine failed to prevent not only viremia but also the development of subsequent clinical symptoms following challenge with the heterologous virus, while the vaccine provided 100% protection against infection by the homologous virus challenge. In a subsequent study, Wang et al. evaluated a recombinant subunit vaccine consisting of a baculovirus-expressed surface glycoprotein of EIAV in groups of 8 ponies each. Horses immunized with the recombinant vaccine were not protected from challenge with either homologous or heterologous strains of EIAV. Vaccination resulted in significantly higher levels of viremia that persisted for longer period of time. In addition, the severity of disease in vaccinated ponies was greater than in unvaccinated controls following challenge with the virulent heterologous strain. Exacerbation of disease severity in vaccinated animals has also been observed with vaccine...
for other members of the lentiviruses\textsuperscript{101}.

Feline immunodeficiency virus (FIV) has also been used as a model to study lentiviral infection (i.e. HIV-1) for the development of effective vaccines\textsuperscript{9,73}. In some vaccine trials, viremia developed earlier in cats immunized with recombinant envelope (\textit{env}) protein of FIV than unvaccinated animals after challenge with homologous virus. Interestingly, no or low level of FIV-specific antibodies was detected in experimental animals\textsuperscript{135}. Similar studies utilizing various experimental recombinant vaccines have also shown that the animals developed no or poor neutralizing antibodies to the \textit{env} protein. Nonetheless, enhancement of viral infection took place, as the virus load after challenge in vaccinated cats was higher than in unvaccinated cats\textsuperscript{7,67}. In addition high antibody titers to the core protein of FIV have been demonstrated to be associated with enhancement of the disease\textsuperscript{66}.

Currently a similar concern about immune-mediated disease enhancement in HIV vaccine trials is being raised because ADE of HIV infection in human peripheral blood mononuclear cells has been demonstrated \textit{in vitro} with sera from HIV-infected individuals or animals vaccinated with experimental vaccines\textsuperscript{15,60,97}.

\textbf{f) Arteriviruses}

Studies by Christianson et al highlighted the biological significance of ADE related to reproductive problems caused by PRRSV infection. Pregnant sows inoculated \textit{in utero} or intramuscularly with PRRSV that was pre-treated with specific serum prior to inoculation showed higher virus titers in fetal tissues as compared to animals that received the virus alone\textsuperscript{28}. Enhancement of infection in late term fetuses could lead to an increased rate of abortions, stillbirths or weak-born piglets. In addition, the potential risk for disease exacerbation in pigs with antibody of maternal origin was also demonstrated using passive transfer of immunoglobulin specific for PRRSV. Pigs injected immunoglobulin at a low neutralizing antibody developed higher and longer lasting viremia after challenge than pigs received normal swine serum globulin or injected with immunoglobulin containing relatively high level of neutralizing antibody\textsuperscript{175}. 
g) Other viral diseases

Adverse affects of ADE have also been reported in animals vaccinated with experimental rabies or influenza virus vaccines. Sikes et al. evaluated a large number of licensed and experimental rabies vaccines in monkeys. Vaccines were administered either 36 and 73 days prior to challenge or within 6 hours after challenge. Monkeys were injected with $10^{4.5}-10^{5.8}$ mouse lethal doses of rabies virus into cervical muscles. Monkeys vaccinated either before or after challenge, as well as another group of monkeys given with anti-rabies serum, died 6-13 days (mean 11 days) after challenge, while 14 of 17 control animals died 14-63 days (mean 25 days) after challenge. The investigators subsequently coined the term ‘early death’ phenomenon to describe these observations. The same phenomenon has also been demonstrated in mice inoculated intracerebrally with rabies virus 2-4 days after a rabies vaccine was administered intraperitoneally. Later, it was suggested that the ‘early death’ phenomenon was attributed to ADE of rabies virus infection mediated by vaccine-induced humoral immunity.

In work with an experimental influenza virus vaccine, Webster and Askonas found that mice inoculated with one or two doses of inactivated whole virus or subunit vaccines of influenza virus A/USSR/90/77 (H1N1) showed enhanced growth of influenza virus in the lung following intranasal challenge with homologous or heterologous (X-31, H3N2) strains at varying intervals after immunization.

Disease enhancement due to ADE has also been documented in children vaccinated against measles virus. Some children immunized with formalin-inactivated, alum-precipitated measles vaccine developed severe, atypical disease following exposure to wild-type measles virus approximately 2 to 2.5 years and up to 14 years after initial immunization.

2. Contribution to the pathogenesis of virus infection

The presence of non neutralizing antibodies capable of facilitating virus entry into macrophages or cells expressing Fc or complement receptors may contribute to persistence by creating a virus reservoir in macrophages. Latency of HIV infection has been described
to occur via an M phase where the virus escapes by infecting macrophages. However, increase in virus load in the blood of patients infected with HIV-1 has been associated with complement mediated ADE (C-ADE). This C-ADE activity correlates negatively with CD4+ cell counts and usually precedes clinical progression. Szabó and colleagues concluded that there should be a switching from neutralizing towards enhancing antibody production before the onset of clinical presentation. The enhancing antibodies would be expected to be against the immunodominant gp41 of HIV-1 since the level of these are higher in symptomatic patients compared to asymptomatic patients\textsuperscript{131;161}.

Other viruses like PRRSV have been shown to localize in the CNS, particularly in microglial cells\textsuperscript{87} and can persist through a slow replication rate\textsuperscript{5}. Contrary to this the high replicating rate of LDV can establish persistence even when specific cytotoxic T cell responses take place\textsuperscript{166}.

**F. Conclusions**

Antibody-dependent enhancement of virus infection is a phenomenon that is not exclusive to human pathogens but it is also seen involved in the pathogenesis of viruses that affect domestic animals and wild life. Vaccination is the best tool available for the prevention and control of infectious diseases, although good management practices of farm animals and personal or public hygiene are also important measures for this purpose. However, as reviewed above, ADE can be a significant obstacle to the development of effective vaccines. In such cases, the presence of viral epitopes that can enhance virus infection, thus exacerbating disease, should be taken into consideration. Recombinant subunit- or DNA vaccines expressing specific neutralizing epitopes can be one approach for minimizing such potential risk but special care has to be taken in the selection of the genes or peptides used for these to maximize vaccine success in providing protection instead of enhancement. Perhaps the development of novel vaccination strategies or vaccine formulation may also offer new frontiers in the control of diseases enhanced by antibody.
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IDENTIFICATION OF PRRS VIRUS EPITOPES ASSOCIATED WITH ANTIBODY-DEPENDENT ENHANCEMENT AND NEUTRALIZATION OF INFECTION

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A. Abstract

Enhanced infection and replication of porcine reproductive and respiratory syndrome virus (PRRSV) in the presence of specific antibody has been demonstrated *in vitro* and *in vivo*, a phenomenon known as antibody-dependent enhancement (ADE). ADE is considered to be a significant obstacle to developing effective vaccines for many viruses for which ADE has been reported, since virus-specific antibodies of maternal origin or those conferred by vaccination can facilitate the entry of virus into target cells, sometimes resulting in increased severity of disease. In this experiment, the role of specific PRRS viral epitopes in ADE and/or virus neutralization (VN) was assessed *in vitro* using 14 monoclonal antibodies (MAbs) to 4 PRRS viral proteins: nucleocapsid (N), matrix (M), glycoprotein (GP) 5, and GP3. Each MAb represented a distinct epitope on one of these proteins. One-way ADE and VN assays were performed *in vitro* using homologous PRRS virus isolates in the presence or absence of each MAb. ADE activity was determined by detecting a significant increase of progeny virus yield in porcine alveolar macrophage cultures in the presence of individual MAbs. Neutralizing activity was determined by detecting a significant reduction or

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complete blocking of virus replication in MARC-145 cells in the presence of individual MAbs. MAbs could be categorized into 3 groups: enhancing, neutralizing and neither. Neutralizing epitopes appeared to reside on the M, GP3 and GP5 proteins. ADE epitopes were associated with the N and GP5 proteins. Identification of the epitopes responsible for ADE and VN may provide the basis for developing efficacious second-generation vaccines for the control of PRRSV.

B. Introduction

For over a decade porcine reproductive and respiratory syndrome (PRRS) has been a disease of great significance to the swine industry since it first appeared as catastrophic clinical outbreaks in swine herds in North America and Europe in the late 1980's. Despite the efforts to control the syndrome, this disease is still present and responsible for great economic losses for pig producers throughout the world.

Clinically, PRRS induces reproductive disorders in pregnant animals and/or respiratory disease in pigs of all ages. Reproductive disease in pregnant animals is manifested as late-term abortions or premature farrowings. Affected litters have a higher proportion of stillborn and weak born piglets and increased preweaning mortality.

Porcine reproductive and respiratory syndrome is caused by PRRS virus (PRRSV), which is a small, enveloped RNA virus that belongs to the family Arteriviridae with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) of mice, and simian hemorrhagic fever virus (SHFV). Although smaller in size and lacking the surface projections characteristic of coronaviruses, the arteriviruses are classified in order Nidovirales with family Coronaviridae because of common traits in genomic organization and replication strategy.

The PRRSV has a polyadenylated, single-stranded, non-segmented, positive-sense RNA genome of 15.1 kilobases in size. The genome consists of 8 open reading frames (ORFs) that are expressed through the production of a nested set of 6 subgenomic 3' co-terminal mRNAs. ORF 1 encodes for the viral RNA-dependent RNA polymerase. ORFs 2 to 7 are postulated to encode for structural proteins, but only 3 proteins have
been consistently identified in virions and/or lysates of virus-infected cells. These are the 15kD nucleocapsid (N), 19kD matrix (M), and 25kD envelope (E or GP5) proteins that are encoded by ORFs 7, 6, and 5, respectively. Proteins encoded by ORFs 2 to 4 are designated GP2, GP3, and GP4, where ‘GP’ indicates ‘glycoprotein’ and the number designates the ORF from which it is derived. They are postulated to be associated with the viral membrane.

The PRRSV possesses four characteristics that may contribute to difficulties in diagnosis and control of the disease, including production of effective vaccines. These are: 1) tropism for macrophage or macrophage-lineage cells 2) remarkable antigenic variation among PRRSV field isolates 3) enhancement of virus infection by the presence of antibody, known as antibody-dependent enhancement (ADE) and 4) ability to establish persistent infection. Tropism for macrophages is a significant impediment for exposed animals to develop effective local and systemic immunity. The antigenic variability has the potential of rendering useless any preexisting antibody that once was capable of neutralizing the virus and permits the development of new strains that can evade the immune system or revert to virulence. ADE can facilitate the attachment and internalization of the virus into its host cells, such as macrophages and monocytes, through Fc receptor-mediated endocytosis using antibody present at subneutralizing levels. Persistence has significant epidemiological implications related to virus perpetuation in a herd and transmission to naïve animals. ADE of virus infection has been described for various viruses belonging to 12 different families of various taxa and host. Among them are the human immunodeficiency virus type 1 (HIV-1), Ebola virus, West Nile virus, influenza virus, dengue virus (DV), feline infectious peritonitis virus (FIPV), lactate dehydrogenase-elevating virus (LDV), equine infectious anemia virus, Aleutian disease virus and, foot and mouth disease virus, to name a few. ADE plays a role in enhancing the opportunity for viruses to infect target cells, and exacerbation of disease due to ADE has also been well documented for DV and FIPV infections.

ADE has been considered one of the major impediments to the development of
efficacious vaccines for certain viruses such as FIPV, DV, and HIV-1. To minimize the risk associated with ADE in controlling disease by vaccination, efforts have been made to formulate vaccines inducing a balanced immune response and to identify viral component(s) associated with ADE or neutralization of virus infection. In addition, numerous researchers have studied the mechanisms of ADE, particularly cellular events besides simple increased uptake of virus coupled with antibody.

The effectiveness of commercially available vaccines to PRRSV has been questioned because of the persistence of the virus in vaccinated herds. Although many reasons may account for the inefficiency of vaccination in controlling the disease or infection, ADE of PRRSV infection may be one of those reasons. The following study was conducted to characterize the role of PRRS viral proteins in ADE and virus neutralization and identify responsible epitope(s), working toward development of a subunit vaccine that would offer protection against PRRSV.

C. Materials and methods

1. Experimental design

This study attempted to identify epitope(s) associated with neutralization and/or ADE of infection. The role of individual PRRS viral epitopes in ADE and virus neutralization was determined using a panel of 14 murine monoclonal antibodies (MAbs) specific for various PRRSV proteins. Prior to conducting ADE or VN tests, the compatibility between the Fc portion of murine MAb and Fc receptors (FcR) on porcine alveolar macrophages (PAM) was tested by a rosette formation assay. The amount of PRRSV-specific antibody in each ascitic fluid was measured by indirect fluorescent antibody (IFA) test, and adjusted to the same level. Enhancing or neutralizing activity of each MAb was then assessed utilizing an in vitro ADE assay and one-way virus neutralization test, respectively. Differences in virus progeny production between treated and non-treated groups were calculated and statistically compared.
2. PRRSV strains

Two PRRS viruses designated ISU-P and KY-35 were used in ADE and virus neutralization (VN) assays. Both isolates were collected from diseased pigs in swine operations in Iowa and Kentucky, respectively, and were used as antigens for producing the panel of MAbs used in this study (Table 1). These viruses share most of the epitopes studied except those recognized by MAbs ISU19C, ISU45Ad and ISU45B. Both viruses were propagated in MARC-145 cells, a highly permissive clone of the African Monkey kidney cell line MA104, and, after titration, aliquotted and kept frozen in minus 80°C until used.

3. Cells

Porcine alveolar macrophages (PAM) and MARC-145 cell lines were used. The PAM were collected from 5- to 6-week-old piglets free of PRRSV through lung lavage as previously described and used for ADE assay. PAM cultures were prepared by quickly thawing frozen cells, suspending them in RPMI-1640 (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT), 50µg/ml gentamicin (Sigma Chemical Co) and an antibiotic-antimycotic mixture composed of 100 µg/ml streptomycin, 100 IU/ml penicillin and 25µg/ml amphotericin B (Sigma Chemical Co.), and seeding in multi-well plates. The cells were used after 24-hour incubation at 37°C in a humid 5% CO₂ incubator.

MARC 145 cells, were used for virus titrations and VN assays. For use, the cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma Chemical Co.) supplemented with 200mM L-glutamine (GIBCO/BRL Life Science, Grand Island, NY, USA), 10%FCS, 50µg/ml gentamicin (Sigma Chemical Co.), and the antibiotic-antimycotic mixture. The cells were used for the assays after 24 to 48 hour incubation at 37°C in a humidified 5% CO₂ incubator.

4. PRRSV-specific monoclonal antibodies

A total of 14 MAbs specific for PRRSV were obtained as mouse ascites and used in the study. Each MAb was specific for distinct epitopes on N, M, GP5 or GP3 proteins.
Table 1. Characteristics of PRRSV monoclonal antibodies used

<table>
<thead>
<tr>
<th>MAb</th>
<th>Specificity</th>
<th>IFA titer (log10)</th>
<th>Homologous virus</th>
<th>Reactivity to MAb††</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISU15A</td>
<td></td>
<td>7</td>
<td>ISU-P</td>
<td>+</td>
</tr>
<tr>
<td>ISU15B</td>
<td></td>
<td>7</td>
<td>ISU-P</td>
<td>+</td>
</tr>
<tr>
<td>ISU15C</td>
<td>Nucleocapsid</td>
<td>7</td>
<td>ISU-P</td>
<td>+</td>
</tr>
<tr>
<td>ISU15D</td>
<td>(15kD)</td>
<td>6</td>
<td>ISU-P</td>
<td>+</td>
</tr>
<tr>
<td>ISU15E</td>
<td></td>
<td>6</td>
<td>ISU-P</td>
<td>+</td>
</tr>
<tr>
<td>ISU15Hd</td>
<td></td>
<td>6</td>
<td>KY-35</td>
<td>+</td>
</tr>
<tr>
<td>ISU19Ad</td>
<td>Matrix</td>
<td>4</td>
<td>KY-35</td>
<td>+</td>
</tr>
<tr>
<td>ISU19Bd</td>
<td>(19 kD)</td>
<td>6</td>
<td>KY-35</td>
<td>+</td>
</tr>
<tr>
<td>ISU19Cd</td>
<td></td>
<td>4</td>
<td>KY-35</td>
<td>+</td>
</tr>
<tr>
<td>ISU25A</td>
<td>GP5</td>
<td>5</td>
<td>KY-35</td>
<td>+</td>
</tr>
<tr>
<td>ISU25B</td>
<td>(25kD)</td>
<td>5</td>
<td>KY-35</td>
<td>+</td>
</tr>
<tr>
<td>ISU25C</td>
<td></td>
<td>5</td>
<td>KY-35</td>
<td>+</td>
</tr>
<tr>
<td>ISU45Ad</td>
<td>GP3</td>
<td>4</td>
<td>KY-35</td>
<td>+</td>
</tr>
<tr>
<td>ISU45B</td>
<td>(43kD)</td>
<td>4</td>
<td>KY-35</td>
<td>+</td>
</tr>
</tbody>
</table>

† Subfix "d" indicates that given MAb is specific for a discontinuous epitope.

†† Adapted from Yang et al (2000) 72. Both strains are considered homologous regarding all epitopes except ISU19Cd, ISU45Ad and ISU45B MAbs, which are only found in KY-35.

Protein specificity and antibody level of each MAb and PRRSV strains used for production of MAbs are summarized in Table 1. Production, protein specificity and other characteristics of these MAbs have been previously described 71,72. All MAbs were of IgG1 isotype as determined by a commercial mouse MAb isotyping kit (IsoStrip® Mouse Monoclonal Antibody Isotyping Kit, Roche Diagnostic Corporation, Indianapolis, IN, USA). Prior to use the total protein amount in each mouse ascites was determined by spectrophotometry using a commercial protein assay kit (Total Protein Assay Kit, Bio-Rad Laboratories, Hercules, CA, USA) as directed by the manufacturer. The level of PRRSV-
specific antibody in each MAb was determined by a commercial ELISA kit (HerdCheck®
PRRS, IDEXX Laboratory, Westbrook ME, USA) and by an indirect fluorescent antibody
test. The level of antibody in each MAb was then adjusted to the same relative titer
(approximately 1:10,000) according to IFA test results.

5. Indirect fluorescent antibody test

For the preparation of viral antigen 24-day-old MARC145 monolayers were infected
with 200µL of a 10⁴ TCID₅₀ viral suspension of ISU-P strain of PRRSV and incubated at
37°C. Normal cell controls were prepared in the identical manner with cell culture medium
instead of virus. After 48 hours of incubation the cells were washed twice, with 0.01M
phosphate buffered saline (PBS), pH 7.0 and fixed with 80% acetone aqueous solution. A
series of 10-fold dilution (10¹ to 10⁹) was made with each MAb in PBS. Fifty microliters of
each MAb dilution were added in triplicate wells and incubated at 37°C for 30 minutes in a
humidified environment. Unbound MAbs were washed off from wells as above. The
presence of antigen-antibody reaction was visualized by adding 50µL of goat anti-murine
IgG conjugated to fluorescein isothiocyanate (FITC, Kirkegaard & Perry Laboratories, Inc.,
Gaithesburg, MD, USA) to each well and performing immunofluorescence microscopy.
PRRSV-specific antibody titer of each MAb was expressed as reciprocal of the highest
dilution in which specific fluorescence was observed in all 3 wells.

6. Enzyme-linked immunosorbent assay

A commercial ELISA kit (IDEXX Laboratory) was used to determine PRRSV-
specific antibody in each MAb as directed by the manufacturer with two exceptions. Instead
of anti-porcine conjugate provided with the kit, goat anti-mouse IgG labeled with peroxidase
(Kirkegaard and Perry Laboratories, Inc.) was used. The presence or absence of specific
antibody at given dilution was determined using optical density (OD) rather than sample-to-
positive (S/P) ratio. Samples with OD value higher than the upper limit of 95% confidence
interval of mean OD from normal mouse ascites were considered to be positive for antibody
to PRRSV.
7. Rosette assay

The compatibility between murine antibody and Fc receptors on porcine macrophages was determined by a rosette assay utilizing sheep red blood cells (sRBC) coated with murine anti-sRBC polyclonal antibody. Murine anti-sRBC serum was obtained from BALB/c mice following a series of intraperitoneal inoculations of 0.5mL of a 50% suspension of freshly collected, washed sRBC at a two-week interval, and the level of anti-sRBC antibody was endpoint titrated by a hemagglutination assay. Antibody-coated sRBC were prepared by mixing 1% (w/v) sRBC suspension with an equal volume of anti-sRBC serum at a subagglutinating level and incubating the mixtures for 30 minutes at 37°C, then an additional 30 minutes at 4°C.

For the assays, porcine alveolar macrophages (PAM) were prepared in polystyrene 6cm cell culture Petri dishes (Corning Inc., Corning, NY, USA) at a concentration of 10⁶ cells per ml of media and incubated in RPMI 1640 medium- supplemented with 10% FCS, 10mM HEPES (Sigma Chemical Co.) and the antibiotic-antimycotic mixture for 24 hours at 37°C in 5% CO₂ atmosphere. The cells were gently washed once with PBS and 2ml of 1% antibody-coated sRBC suspension were added. The control cells received the same amount of uncoated sRBC. The mixture was incubated for 60 minutes at 37°C and unbound sRBC's were removed by rinsing with PBS. The cells were fixed and stained with Diff-Quick staining (Difeo Laboratories, Detroit, MI, USA) as directed by manufacturer, and observed under 100X magnification. A total of 200 cells in randomly selected microscopic fields were counted and the proportion of rosette forming cells was calculated. A rosette is defined as a macrophage with at least 3 sRBC associated with its membrane. The assay was independently repeated three times, and a mean value between treated and control groups was calculated and analyzed by Student’s t test.

8. In vitro ADE assay

Antibody dependent enhancement of PRRSV infection was assessed by measuring increases in progeny virus yield as previously described. Briefly, PRRSV ISU-P (or KY-35 for MAbs ISU19C, ISU45A and B) was diluted to a 10⁴ TCID₅₀/ml in FCS-free RPMI
1640 medium. For test groups, each virus suspension was mixed with an equal volume of each of the pre-standardized, serially diluted MAbs (10\(^{-1}\) to 10\(^{-7}\)) to be tested. Control groups were prepared by mixing each of the viruses with an equal volume of PRRSV-ADE positive serum or no antibodies at all in the same manner described above. The virus-MAb mixtures were incubated at 37°C for an hour and then 200µL aliquots of each mixture were inoculated in triplicates onto PAM prepared in 96-well plates at a ratio of 1 x 10⁴ PAM/well 24 hours earlier, resulting in 0.1 multiplicity of infection (MOI). After incubation for 1 hour at 37°C, the inoculum was replaced with RPMI 1640 medium with 10% FCS, followed by an incubation period of 2 days at 37°C. At the end of the incubation period, the cells were subjected to 2 cycles of freeze-thawing at minus 80°C and 37°C respectively to disrupt cells that may still contain virus. The amount of PRRSV in each cell lysate was quantitated by virus titration as described below.

9. Virus titration

Each sample containing PRRSV was serially diluted and 100 µl of each diluted sample was inoculated in triplicates onto confluent MARC-145 monolayers prepared in 96-well plates 24 to 48 hours earlier. Plates were incubated for 2 to 3 days at 37°C in a humid 5% CO\(_2\) incubator, and then cells were fixed with 80% acetone aqueous solution. The presence of PRRSV was determined by staining fixed cells with a cocktail of PRRSV-specific MAbs SDOW17 and SR30 (Rural Technologies, Inc., Brookings, SD, USA). Virus titer in each sample was then determined by counting fluorescent foci in wells showing between 50 and 100 foci and expressed as fluorescent foci units (FFU) per milliliter.

10. Virus neutralization assay

Neutralizing activity of individual MAbs was assessed using a fluorescent foci reduction assay with KY-35 PRRSV strain. Briefly, a virus suspension containing approximately 100 FFU/mL based on pre-determined titer was mixed with an equal volume of each standardized MAb. Virus controls were prepared in the identical manner except mixing with PRRSV-positive serum or FCS-free minimal essential medium (MEM, Sigma
Chemical Co.) instead of MAb. Virus-MAb, virus-serum or virus-medium mixtures were incubated for 1 hour at 37°C. After that, 200µL of each mixture were added to triplicate wells containing 24- to 48-hour-old confluent MARC-145 cells monolayers in 96 well plates. After incubation for 1 hour at 37°C, the inoculum was replaced with fresh MEM medium supplemented with 10% FCS and the antibiotic-antimycotic mixture. Then, cells were further incubated for 2 to 3 days at 37°C in a humid 5% CO₂ incubator. At the end of the incubation period, the monolayers were fixed with 80% acetone aqueous solution and stained with the anti-PRRSV MAb cocktail. Virus titers in samples treated with individual MAbs were expressed as FFU/ml as described above and compared to those in virus control.

11. Data analysis

Results of neutralization assays were analyzed by calculating the percent reduction (\( \% R \)) of progeny virus yields (FFUs) in the presence of MAb (\( fcM \)) compared to those in the absence of MAb (\( fcV \)) with the following formula:

\[
\% R = 100 - \left(\frac{fcM}{fcV} \times 100\right).
\]

When reduction of 60% or more in the yield was observed, MAbs were considered to possess neutralizing activity.

Progeny virus yields from ADE assays were determined by calculating FFU/mL for all dilutions of each MAb in the assay as well as virus only controls. Due to the work volume, progeny virus yields (FFU/mL) were assessed for a set of two or three MAb-treated virus groups and one untreated virus control at a time. At a given dilution, geometric means of progeny virus yields between MAb-treated and untreated groups were statistically compared using Wilcoxon-Kruskal Wallis (Rank Sum) tests. Pair-wise comparisons were performed using both Dunnette's method and Student's \( t \) test using the Bonferroni correction to guard against type I error inflation. For all dilutions, data between treated and untreated groups were statistically compared by the general regression model. Significance of
slopes and intercepts was determined at \( p \leq 0.01 \).

### D. Results

In the rosette assay, approximately 80% of the PAM counted formed rosettes when they were incubated with sRBC coated with murine anti-sRBC antibodies, while less than 7% of PAM formed rosettes with sRBC pre-treated with normal mouse serum, demonstrating that the Fc receptors on PAM can react with the Fc portion of murine immunoglobulins (Figure 1).

Each MAb was further characterized for total protein concentration and antibody titer through ELISA and IFA, which were needed for standardization among the MAbs to be used. IFA antibody titers of individual MAbs ranged from \( 10^4 \) to \( 10^7 \) (Table 1). On ELISA, MAbs specific for the N protein (i.e., ISU15A to Hd) showed detectable antigen-specific response; however, no linear relationship could be obtained between optical densities and dilutions (data not shown). Furthermore, antigen-specific reactivity could not be demonstrated on the commercial ELISA with some MAbs, particularly, those specific for M and GP5 proteins. Total protein levels of mouse ascites ranged from 21.84 to 32.36 mg/ml and did not correlate with antibody levels determined by IFA or ELISA. Thus, MAbs were standardized so that all MAbs would have a titer similar to the MAb with the lowest titer according to IFA test results, i.e., \( 10^4 \) IFA titer.

Neutralizing activity of MAbs against PRRSV isolate KY-35 is summarized in Figure 2. Monoclonal antibodies specific for the N protein (ISU15A to Hd) did not neutralize infection of MARC-145 cells by PRRSV. Using 60% reduction in progeny virus production as cut off, MAbs ISU19A, ISU25B and ISU45B were determined to have neutralizing activity to KY-35. Replication of KY-35 in MARC-145 was not inhibited when the viruses were treated with the remaining MAbs.

*In vitro* ADE assays using serially diluted MAbs revealed that MAbs tested at the lowest dilution (approximately \( 10^3 \) IFA units) could be categorized into 3 groups: enhancing, suppressing and neither (Figure 3). Based on progeny virus yield, significant enhancement of PRRSV infection \( (p \leq 0.01) \) was observed only with ISU25C, suggesting that the epitope
Figure 1. Photomicroscopy of H&E stained porcine alveolar macrophages (PAM) exposing to sheep red blood cells (sRBC) pre-treated with murine anti-sRBC serum (A) and untreated sRBC (B). Panel A shows rosette formation indicating the compatibility between porcine Fc receptor and murine immunoglobulin. Observed under light microscope at 20X magnification.
Figure 2. Relative susceptibility of PRRSV (isolate KY-35) to neutralization by monoclonal antibodies (MAbs) specific for distinct epitopes on various PRRSV proteins. Numbers in designation represent molecular masses of PRRS viral protein for which individual MAbs are specific. FFU stands for foci forming units. Each data point represents the mean value of triplicate measurements of each treatment.
Figure 3. Production of infectious progeny PRRSV in porcine alveolar macrophages. Cells infected with 0.1 MOI virus in the presence of monoclonal antibodies specific for distinct epitopes at a rate of approximately $10^3$ IFA antibodies. Each bar represents the geometric mean of net progeny virus yield (foci forming units per milliliter) relative to untreated control group. Error bars are the standard deviation of the mean.
represented by this antibody is associated with ADE of PRRSV infection. Mean virus yield from PAM infected with PRRSV in the presence of MAb ISU25C was of 1.5 log_{10} higher than that from PAM exposed to the virus only. On the other hand, infectivity and/or replication of PRRSV in PAMs was significantly suppressed (p≤0.01) after treatment with MAbs ISU15B, ISU15Hd, and ISU45B. Mean progeny virus yields were reduced by 0.6 log_{10} to 5 log_{10} relative to those of their respective untreated control group. Neither enhancement nor suppression of progeny virus production in PAMs was observed when PRRSV was pre-treated with the remaining MAbs.

Significant enhancing activity (p<0.001) of MAb ISU25C for PRRSV replication continued to be present over a series of dilutions, i.e., lower antibody concentration (Figure 6). In addition, progeny virus yield in PAMs appeared to increase when PRRSV (ISU-P) was pre-treated with MAb ISU15E (y = -0.93795 + 0.0008419 dilution, r^2=0.1445, p = 0.04) at higher dilutions (Figure 4), as was the case with polyclonal anti-PRRSV swine serum used as reference (Figure 8).

Some of the MAbs that significantly suppressed progeny virus production in PAMs at the lowest dilution (i.e., ISU15B and ISU45B) retained the same effect on PRRSV replication even after their concentration was diluted (Figures 4 and 7). In addition to these MAbs, MAbs ISU15A, ISU19B and ISU25B were determined to significantly suppress the production of PRRSV progeny virus in PAMs over a series of dilutions at p≤0.01 (Figures 4 to 6). In comparison, MAb ISU15Hd did not show suppressing activity at any dilution except the lowest. MAb ISU19A was increasingly suppressive on PRRSV replication in PAMs over a series of dilutions (y=0.371859 – 0.0014272 dilution, r^2=0.3652, p=0.0002).

**E. Discussion**

The present study attempted to identify epitopes associated with ADE of PRRSV and those responsible for virus neutralization using a panel of well-characterized MAbs against epitopes on various PRRSV proteins. Monoclonal antibodies have been commonly used for this purpose since each MAb represents the antigenicity of a single epitope. Furthermore, the usefulness of MAbs for this purpose has been proven by other investigators. 

13,19,21,45;50;59.
Figure 4. Production of infectious progeny PRRSV in porcine alveolar macrophages. Cells were infected with ISU-P treated with monoclonal antibodies specific for distinct epitopes of PRRSV nucleocapsid protein. Values represent mean difference in progeny virus yields between treated and untreated groups at each dilution. Error bars are the standard deviation of the mean.
Figure 5. Production of infectious progeny PRRSV in porcine alveolar macrophages. Cells were infected with KY-35 (ISU19C) or ISU-P (ISU19A and B) treated with monoclonal antibodies specific for distinct epitopes of PRRSV matrix protein. Values represent mean difference in progeny virus yields between treated and untreated groups at each dilution. Error bars are the standard deviation of the mean.
Figure 6. Production of infectious progeny PRRSV in porcine alveolar macrophages. Cells were infected with ISU-P treated with monoclonal antibodies specific for distinct epitopes of PRRSV major envelope protein. Values represent mean difference in progeny virus yields between treated and untreated groups at each dilution. Error bars are the standard deviation of the mean.
Figure 7. Production of infectious progeny PRRSV in porcine alveolar macrophages. Cells were infected with KY-35 (ISU45Ad) or ISU-P (SIU45B) treated with monoclonal antibodies specific for distinct epitopes of PRRSV GP3. Values represent mean difference in progeny virus yields between treated and untreated groups at each dilution. Error bars are the standard deviation of the mean.
Figure 8. Production of infectious progeny PRRSV in porcine alveolar macrophages. Cells were infected with ISU-P treated with serum specific for PRRSV. Values represent mean difference in progeny virus yields between treated and untreated groups at each dilution. Error bars are the standard deviation of the mean.
Our study demonstrated that all 14 anti-PRRSV MAbs tested could be categorized into 3 groups: neutralizing/suppressing (e.g., ISU15A, ISU15B, ISU25B, ISU19A, ISU19B, ISU45A, ISU45B), enhancing (e.g., ISU15E and ISU25C), and non-neutralizing/non-enhancing (e.g., ISU15C, ISU15D, ISU15Hd, ISU19C, ISU25A). This observations indicate that individual epitopes of PRRSV may have unique role in ADE and neutralization of virus infection, which is in general agreement with reports of previous similar studies on other viruses.13,21

In general ADE of infection by enveloped viruses is believed to be mediated by epitope(s) associated with envelope protein or membrane-associated protein(s).30,36 Our observations with PRRSV MAbs were in agreement with other investigators' observations. Epitopes associated with ADE are determined to reside on the 15kD nucleocapsid and 25kD envelope (GP5) proteins, whereas epitopes of the 19kD matrix, GP5 and 45kD GP3 proteins were mainly associated with virus neutralization. It is worthwhile to note that MAbs ISU25B and ISU45B suppressed PRRSV replication in both MARC-145 cell line and PAMs. Involvement of GP5 protein in inducing neutralizing or enhancing antibodies have been demonstrated by our laboratory and others.17,31,68,72,75,76 It was, however, unexpected that an antigenic determinant of the GP3 protein, represented by MAb ISU45B, would be associated with inhibition of PRRSV replication. Statistically MAb ISU45B showed much stronger inhibition of PRRSV replication in porcine alveolar macrophages, PRRSV natural target cells, than MAb ISU25B representing an epitope on the GP5 protein (p<0.01). For some North American PRRSV isolates, GP3 protein has been reported as a non-structural protein.37 In contrast, it has been described as a structural protein for some European PRRSV isolates.44 A recent study reported that ORF3 product of a Spanish PRRSV strain from recombinant Baculoviruses provided pigs with protective immunity against PRRSV challenge.54 Further investigation remains to determine the role of GP3 in PRRSV pathogenesis or immunity.

PRRSV infection was also enhanced or suppressed when treated with MAbs specific to epitopes on the nucleocapsid (N) protein (i.e. ISU15E enhanced, ISU15A and B suppressed). These were unexpected observations since no part of the N protein has been
demonstrated to be exposed to the outside of intact virions. Theoretically, such observations are possible if naked or partially stripped viruses containing intact infectious RNA were in the virus preparation used for the assay, which is likely since the virus material used in our study was not purified through zonal centrifugation. However, it also has been demonstrated that the requirement of intact infectious virions for infection in permissive cells can be bypassed through ADE pathway for virus entry 39. Consequently, our observation on ISU15E may have a significant implication in the pathogenesis of PRRSV. The 15kD N protein is known to be highly immunogenic, and abundant expression of ORF 7, the gene encoding for the N protein, in the early stage of infection has been demonstrated in vitro and in vivo 4,34,36,74. Likewise, it has also been reported that high levels of non-neutralizing antibody, most of which is specific for the N protein are produced in pigs following exposure 74. Subsequently, PRRSV could take advantage of ADE for entry to target cells, macrophages or macrophage-lineage cells. Nevertheless, as demonstrated in our results, some of the anti N antibodies may have a suppressive effect, but as stated above this could be related to the presence of naked virus or virions partially stripped from their envelope in the virus suspension used in the assay.

In vitro assessment of ADE activity by antibody can be affected by various factors. These include isotype or subtype of immunoglobulin 22, assay conditions such as virus strain (e.g., homologous versus heterologous), antibody titer, multiplicity of infection 45,46,50, and parameter(s) for measuring enhancement 45,46,50,60,62. All these factors were taken into consideration, including isotype determination. All MAbs used were of the IgG1 subtype, thus, any results from ADE or neutralization assays described in the present study are independent of the immunoglobulin isotype/subtype and a property intrinsic to the epitope bound. However, it should be noted that specific classification of PRRSV MAbs as to their role in VN and ADE may not be conclusive for some MAbs, since antibody concentration of individual MAbs were adjusted to the same or similar antibody level for quality control of the in vitro assays. Previous studies have suggested that PRRSV infection can be inhibited or enhanced in the presence of antibody, depending upon the ratio between immunoglobulins and available specific epitopes or between antibody molecules and virion
The same observation was also made in this study with polyclonal anti-PRRSV swine serum and may be attributed to the fact that an antiserum consists of various subpopulations of antibodies each of which represents different specificity, affinity, and avidity. Since a MAb represents a single epitope, such a phenomenon was not expected to occur. As anticipated, the role of individual MAbs in ADE and VN was distinct as shown by the general regression model, except to ISU19C. This MAb suppressed PRRSV replication when the virus was treated with it at higher concentration \( (p \leq 0.01) \). As antibody concentration was lowered by serial dilution the suppressive effect of this MAb disappeared and became enhancing, however the trend was not considered statistically significant at \( p \leq 0.01 \).

Antibody dependent enhancement of virus infection is a significant obstacle to the development of effective vaccines to control certain viral diseases of public health and veterinary importance. The ADE of PRRSV infection has been suspected as one of the possible reasons for the relative ineffectiveness of vaccination in controlling PRRS. Our present study provides the antigenic basis for developing subunit vaccines that would induce protective immunity with minimal or no risk for ADE. Characterization of enhancing and neutralizing epitopes demonstrated in this study, however, remains to be further studied. Future studies are needed to determine the biological significance of our findings.

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GENERAL CONCLUSIONS

A. Conclusions

In this study, we demonstrated that some of the porcine reproductive and respiratory syndrome (PRRS) virus structural proteins have unique role in immunobiology of PRRS virus. Using a panel of monoclonal antibodies, epitopes that were examined could be categorized into 3 groups: enhancing, neutralizing, and enhancing-neutralizing epitopes. Some of epitopes examined were not classified into any of these groups. Neutralizing epitopes were found to reside on the 19kD matrix (M) and 25kD major envelope glycoprotein (GP5). ADE epitopes were associated with the N and GP5 proteins. Even on the same protein, the role of individual epitopes in ADE and VN was discernible. Identification of the epitopes responsible for ADE and VN may provide the basis for developing efficacious second-generation vaccines for the control of PRRS virus. Yet, genomic sequences corresponding to these epitopes remain to be identified. Animal trials will be required to test the biological relevance of our findings.

B. Future work

Due to the nature of the technique to develop MAbs, it is quite possible that the epitopes recognized are at sites that would not be exposed on the intact virion. The use of immunogold electron microscopy (IGEM) can be used to determine if the epitopes involved in ADE and neutralization are indeed exposed to the outside of the viral envelope or if their recognition is due to damage in the virus envelope. This is especially important in the case of N protein enhancing epitopes since it was reported that it is not exposed to the outside of the virion.

Furthermore, the development of deletion mutant clones of PRRSV proteins would serve to pin point the location of neutralizing epitopes and what changes in their sequences are involved in the generation of ADE epitopes. This knowledge would be used to develop subunit vaccines that would confer protective immunity against the PRRSV and avoid the generation of enhancing antibodies. Animal experiments should be conducted to test the
efficacy of such vaccines as well as longitudinal studies to determine PRRSV persistence after the use of the new vaccine.
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