Antibody dependent enhancement and its potential as a contributing factor to the pathogenesis of porcine reproductive and respiratory syndrome virus infection

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Antibody dependent enhancement and its potential as a contributing factor to the pathogenesis of porcine reproductive and respiratory syndrome virus infection

by

Kyoung-Jin Yoon

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In Charge of Major Work
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For the Major
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For the Graduate College

Iowa State University
Ames, Iowa
1995
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**FIELD ISOLATES OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS VARY IN THEIR SUSCEPTIBILITY TO ANTIBODY DEPENDENT ENHANCEMENT OF INFECTION**

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Antibody dependent enhancement (ADE) of porcine reproductive and respiratory syndrome virus (PRRSV) infection as a contributing factor to viral pathogenesis was studied. Yield of progeny virus and infection rates of porcine alveolar macrophages (PAM) were increased (p<0.01) following treatment of the virus with subneutralizing levels of anti-PRRSV antibody. Maximum increases in virus yields ranged from 1.5 to 2 log_{10}, while maximum increases in infection rates of PAM ranged from 7 to 53 fold. Increased yields and infection rates were directly correlated (r=0.95). Enhancement of virus yield in the presence of antibody was blocked by Protein A, indicating that increased virus yield was due to enhanced uptake of virus by PAM through Fc receptors. However, the yield of progeny virions from individual PAM was also significantly (p<0.01) enhanced in the presence of antibody, suggesting that an additional mechanism may contribute to the increase in virus yield.

The biological significance of ADE was assessed in two experiments. First, ADE of infection was demonstrated in pigs using a completely randomized block design (n=16). The mean level and duration of viremia were greater (p<0.05) in pigs injected with subneutralizing amounts of PRRSV-specific IgG prior to virus challenge than in control pigs injected with normal serum globulin, suggesting that ADE of PRRSV infection has the potential to exacerbate the severity of disease by amplifying virus replication \textit{in vivo}. Conversely, virus replication was significantly (p<0.01) suppressed in pigs with a high level of neutralizing antibody. Second, the period of time that subneutralizing levels of passively supplied antibody can persist and contribute to ADE of PRRSV infection was estimated in 4 pigs injected with PRRSV-specific IgG to yield an initial serum-virus neutralizing (SVN) antibody titer of 3.8 log_{2}. Neutralizing antibody declined to undetectable levels by day 37 post injection (PI), while antibody detected by the immunoperoxidase monolayer assay continued to be detected for an additional 35 days. ADE activity was first detected in undiluted sera on day 20 PI and persisted through day 62 PI, suggesting that passively immunized pigs are susceptible to the potential enhancing effect of ADE for a period of 5 to 6 weeks following the disappearance of neutralizing antibody. A similar period of potential susceptibility to ADE was also demonstrated by regression analysis of
the antibody response of 8 pigs that were infected with PRRSV by the nasal route. The SVN antibodies were estimated to persist for 280 days following infection, while non-neutralizing antibodies were estimated to persist for an additional 6 weeks. These results strongly suggest that ADE has the potential to contribute to the pathogenesis of PRRSV infection in pigs with declining levels of PRRSV-specific antibodies of maternal origin, or induced by vaccination or exposure to wild type virus.

The western immunoblot analysis (WIA) of sera from passively immunized pigs revealed the presence of antibodies specific for the 15kD nucleocapsid and 26kD glycosylated envelope proteins in sera with ADE but no neutralizing activity. Because of internal location of the 15kD protein in the virion, the 26kD protein was concluded to be responsible for inducing antibodies associated with ADE. In addition, the 26kD protein appears to be also involved in the induction of neutralizing activity. In 8 pigs exposed to PRRSV by the nasal route, no SVN activity was detected until after antibodies to the 26kD protein was present as determined by WIA. Consequently, it is likely that the 26kD protein contains antigenic determinants associated with ADE and/or virus neutralization.

Seventeen field isolates of PRRSV, including isolate ISU-P, were evaluated for their susceptibility to ADE of infection mediated by antibodies raised against PRRSV isolate ISU-P and were shown to vary in this respect. This variability was dramatically demonstrated by the neutralization of homologous virus by a concentration of antibody that enhanced the replication of heterologous isolates. These observations imply that antibodies induced by vaccine strains of PRRSV could also do the same in vaccinated animals that become infected with wild type viruses. Therefore, vaccine candidates should be evaluated for their ability to induce ADE activity before their use in the field. Furthermore, the susceptibility of PRRSV isolates to ADE was shown to be inversely proportional (r=0.92) to the ability of the same antibody to neutralize the isolates. Consequently, it may be possible to use the susceptibility of PRRSV isolates to neutralization by vaccine-induced antibody as a surrogate marker for ADE.
INTRODUCTION

Dissertation Organization

This dissertation begins with an abstract and is followed by a review of the literature and a statement of the problem. Three papers present the experimental work performed. The doctoral candidate is the principal investigator and senior author for all three papers. The dissertation concludes with a general discussion, a list of references cited in the literature review and general discussion, and acknowledgments.

Literature Review

History of PRRS

Porcine reproductive and respiratory syndrome (PRRS) is a relatively new viral disease of swine. It was initially known as "mystery swine disease" in the United States (U.S.) because its etiology was unknown at the time. For the same reason, PRRS has been identified by a variety of synonyms since the disease syndrome became recognized in swine populations throughout the world. Some examples of names used for PRRS are summarized in Table 1. The 'porcine reproductive and respiratory syndrome' was designated the official name at the First International Symposium on SIRS/PRRS held in St. Paul, Minnesota in 1992.

The first cases of PRRS were reported in the United States in 1987. In Europe, it was first reported in Germany in 1990 and subsequently in the Netherlands, Spain, Belgium, the United Kingdom, France, and Denmark during 1991 and 1992. The presence of porcine reproductive and respiratory syndrome virus (PRRSV) has recently been reported in Austria, Asia, and South America.

Zimmerman conducted a survey of members of the American Association of Swine Practitioners utilizing clinical manifestations of PRRS to identify the approximate time of appearance of PRRS and spread of the virus in the United States. Criteria for recognizing herds infected with PRRSV included: a) anorexia, b) pyrexia, c) respiratory disease in young pigs, d) increased stillbirths,
Table 1. A list of synonyms for PRRS

<table>
<thead>
<tr>
<th>Names for disease</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>abortus blauw</td>
<td>8</td>
</tr>
<tr>
<td>blue ear disease</td>
<td>9</td>
</tr>
<tr>
<td>blue-eared pig disease</td>
<td>94</td>
</tr>
<tr>
<td>Epidemisch Spätabort der Sauen</td>
<td>332</td>
</tr>
<tr>
<td>Maladie Blue du Porc</td>
<td>332</td>
</tr>
<tr>
<td>Maladie Mystérieuse du Porc</td>
<td>332</td>
</tr>
<tr>
<td>mystery pig disease</td>
<td>216</td>
</tr>
<tr>
<td>new pig disease</td>
<td>216</td>
</tr>
<tr>
<td>pig plague ‘89</td>
<td>163</td>
</tr>
<tr>
<td>plague of 1988-1989</td>
<td>9</td>
</tr>
<tr>
<td>porcine epidemic abortion and respiratory syndrome</td>
<td>314</td>
</tr>
<tr>
<td>porcine viral syndrome</td>
<td>216</td>
</tr>
<tr>
<td>Rätselhafte Schweinekrankheit</td>
<td>332</td>
</tr>
<tr>
<td>SMEDI-like syndrome</td>
<td>163</td>
</tr>
<tr>
<td>Seuchenhafter Spätabort der Schweine</td>
<td>157</td>
</tr>
<tr>
<td>swine infertility and respiratory syndrome</td>
<td>10</td>
</tr>
<tr>
<td>swine plague</td>
<td>332</td>
</tr>
<tr>
<td>swine reproductive failure syndrome</td>
<td>163</td>
</tr>
<tr>
<td>swine reproductive and respiratory syndrome</td>
<td>332</td>
</tr>
<tr>
<td>Syndrome dysgénésique et respiratoire du Porc</td>
<td>332</td>
</tr>
<tr>
<td>Wabash syndrome</td>
<td>332</td>
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</tbody>
</table>
e) increased early farrowing, and f) increased numbers of mummified fetuses. This survey found that 1611 herds were considered to be infected in 19 states and that the first recognized case fitting the criteria was in 1980. Serological survey of banked sera collected from pigs in mid-west swine herds between 1980 and 1989 as part of National Animal Health Monitoring System (NAHMS) revealed that PRRSV was present in Iowa by 1985 and in Minnesota by 1986.228,323

Identification of the etiologic agent

Prior to establishing PRRSV as the cause of PRRS, a number of microorganisms were considered to be as the cause of PRRS. Attention was initially focused on porcine parvovirus, pseudorabies virus, porcine enteroviruses, encephalomyocarditis virus, hog cholera virus, and leptospira since all of these pathogens were associated with reproductive failure.57 Identification of the causative agent was complicated by the isolation of one or more of the previously listed agents as well as mycoplasma, swine influenza virus, a paramyxovirus-type virus, Chlamydia psittaci, and Streptococcus suis from suspected cases of PRRS.1,29,38,73,78,81,144,154,208,216,236,311 Attention was also focused on the role of the mycotoxin, fumonisin in PRRS.12,13

The virus that caused PRRS was first isolated in 1991 in the Netherlands299 and subsequently in 1992 in the United States64 and Canada.79,80 The first virus isolates in the Netherlands and U.S. were designated Lelystad virus (LV) and ATCC VR-2332, respectively. Lelystad virus was isolated from specimens obtained from herds undergoing severe reproductive disorders utilizing porcine alveolar macrophage cultures, while ATCC VR-2332 was isolated from herds undergoing severe respiratory and reproductive problems using the proprietary cell line ATCC CL2621 (Boehringer Ingelheim Animal Health Inc., St. Joseph, Missouri). Both virus isolates were shown to reproduce reproductive and respiratory failures in gnotobiotic pigs that were experimentally infected by the nasal route.66,289

The virus

Classification Porcine reproductive and respiratory syndrome virus is a small, enveloped RNA virus26,310 that has been provisionally classified as a member of the Arterivirus genus of the family Togaviridae.68,197,224,301 Other
members of the genus Arterivirus include lactate dehydrogenase elevating virus (LDV) of mice, equine arteritis virus (EAV) and simian hemorrhagic fever virus. No serological cross reaction has been demonstrated between PRRSV and other Arteriviruses.

Currently, genomic studies of arteriviruses support the establishment of a new family independent from *Togaviridae* family. *Arteriviridae,* *Multiviridae,* and *Mamornaviridae* have been proposed as names for the new family. An even more "coronavirus-like" superfamily that includes coronavirus, torovirus, and arterivirus has been proposed because of the high degree of similarity in their genomic organization and gene expression strategy.

**Physicochemical properties** The PRRSV is spherical in shape. The size of the virus ranges from 48 to 83 nm in diameter. It contains an electron dense icosahedral nucleocapsid that ranges in size from 25 to 30 nm in diameter. The buoyant density of the virus ranges from 1.13 to 1.18 g/ml in CsCl gradients and 1.18 to 1.23 g/ml in sucrose gradients.

Three structural proteins with molecular masses of approximately 15, 19, and 26-30 kilodaltons (kD) have been consistently demonstrated by numerous independent investigators. Endoglycosidase treatment and 3H-glucosamine labeling study revealed that the 26kD protein is a N-glycosylated protein while the 15 and 19 kD proteins are not glycoproteins. Benfield and his associates have recently identified an additional viral protein with a molecular mass of approximately 22-23kD in lysates of cells infected with PRRSV. Canadian investigators have also demonstrated an additional glycosylated protein with a molecular mass of 42kD in virus-infected MA104 cells. However, genomic sequence analysis suggests that PRRSV consists of as many as 6 proteins.

Although the functions of these proteins have not been completely determined, a recent electron microscopic study utilizing colloidal gold labeled with monoclonal antibodies indicated that the 15kD protein is a nucleocapsid protein and the 26kD protein is an envelope protein. The 19kD, 22kD, and 42kD proteins are presumed to be components of the viral envelope. The 15kD protein appears to be highly immunogenic since antibody specific for this protein
can be detected in serum from virus-infected pigs earlier than antibodies to other proteins.\textsuperscript{25,218} Investigators have speculated that the 26kD protein is associated with virus neutralization since antibody specific for this protein was detected by radioimmunoprecipitation (RIP) close to the time at which serum virus neutralizing activity was present.\textsuperscript{55}

The PRRSV is stable for at least 1 month at 4°C and for several months at \(-70°C\). Complete inactivation occurs within 48 hours at 37°C and by 45 minutes at 56°C.\textsuperscript{23,26} Infectivity of the virus is reduced over 90\% at a pH less than 5 or greater than 7.\textsuperscript{26} No chemical inactivatant except chloroform has been tested \textit{in vitro} against PRRSV.\textsuperscript{26} In common with other members of the Arterivirus group, PRRSV does not hemagglutinate red blood cells from mammalian or avian species including sheep, goat, swine, cattle, mouse, rat, rabbit, guinea pig, human type O, duck, and chicken.\textsuperscript{26,314}

\textbf{Genomic organization and gene expression} The genome of the virus is polyadenylated, single-stranded positive sense RNA.\textsuperscript{68,197,215} The genome is approximately 15 kilobases in size and consists of 8 open-reading frames (ORFs).\textsuperscript{68,197} Each of the ORFs 2 through 7 is overlapped with neighboring ORFs in their organization. The ORFs 1a and 1b are predicted to encode the RNA-dependent RNA polymerase, whereas ORFs 2 to 6 are presumed to encode membrane-associated proteins and ORF 7 to encode the nucleocapsid protein. Comparison of the amino acid sequences encoded by each of the ORFs of PRRSV to that of other members of the Arterivirus group indicate that PRRSV is more similar to LDV than to EAV.\textsuperscript{68,197}

Genes are expressed by the production of a 3' co-terminal nested set of 7 subgenomic mRNAs.\textsuperscript{68,197} Each subgenomic RNA contains the same leader sequence at its 5' end. Gene expression of PRRSV is similar to that of Coronaviruses and Toroviruses, rather than that of Togaviruses.\textsuperscript{68,197,301} It is not known how soon PRRSV-encoded mRNA is produced in infected cells, although Bautista et al. reported that viral polypeptide can be detected in infected cells as early as 16 hours postinoculation.\textsuperscript{15} In the case of LDV, viral RNA has been reported to be detected as early as 8 hours after infection.\textsuperscript{239}
**Viral replication** The porcine alveolar macrophage is the only cell to date proven to support virus replication both *in vitro* and *in vivo*. It has not been determined which surface molecules on the cells serve as receptors for the virus and how the virus is internalized into target cells. After internalization, viral replication occurs only in the cytoplasm of infected cells. The virus produces 3' co-terminal nested set of 7 subgenomic mRNAs. Each of subgenomic mRNAs is known to encode for individual viral proteins. Viral RNA-dependent RNA polymerase is the only nonstructural protein identified to date that functions in replication of the viral genome. The nucleocapsid obtains an envelope by budding through the membrane of the smooth endoplasmic reticulum. Enveloped virions accumulate in the endoplasmic reticulum which causes its enlargement. In addition to enlargement of endoplasmic reticulum, severe disruption of mitochondria and formation of double-layered vesicles has also been observed in infected cells. The same changes have been observed in mouse macrophages infected with LDV. An ultrastructural study on PAM inoculated with PRRSV revealed that progeny virus was first observed in the cells at 9 to 12 hours after inoculation with a multiplicity of infection of 1, suggesting that the replication cycle of the virus in the cells is 9 to 12 hours. Progeny viruses are released from the cell initially by exocytosis and eventually by cell lysis. Most PRRSV isolates produce cytolytic infections, but some investigators have reported the existence of noncytopathic PRRSV isolates.

**Virus propagation** The virus is known to replicate preferentially in PAM. Virus-specific cytopathic effects can be detected by light microscopy in the PAM cultures between 48 and 72 hours after inoculation. The virus yield from PAM ranges from $10^5$ to $10^6$ TCID$_{50}$/ml. Peripheral blood monocytes of swine have recently been demonstrated to support PRRSV replication *in vitro*, but progeny virus yield in the monocyte culture was found to be lower ($10^4$ TCID$_{50}$/ml) compared to virus yield obtained with PAM ($10^5$ TCID$_{50}$/ml). More recently, Molitor et al. reported that splenic macrophages and brain microglial cells of swine also supported PRRSV replication *in vitro*. Virus replication has also been demonstrated in established cell lines, such as ATCC CL2621 cells and the African green monkey kidney cell line,
MA104. A comparative study revealed that PAM are more susceptible to PRRSV than the CL2621 cell in terms of the isolation rate of the virus from clinical specimens. However, some PRRSV isolates only replicate in CL2621 or PAM but not both. A more susceptible subpopulation of MA104 cells (MARC-145) has recently been cloned. The virus replication cycle in MARC-145 cells is 48 to 72 hours when the cells are exposed to virus at a multiplicity of infection of 0.01. Maximum progeny virus titers of $10^{8.5}$ TCID$_{50}$/0.1ml have been obtained with this clone.

**Antigenic variation** To date, marked antigenic differences have been demonstrated among PRRSV isolates. Broad antigenic variation is a major concern in the development of effective vaccines and vaccination strategies against PRRS. The existence of antigenic variation among PRRSV isolates was initially demonstrated between European and North American PRRSV using the immunoperoxidase monolayer assay (IPMA). Investigators evaluated the reactivity of polyclonal porcine antibodies raised against LV and ATCC VR-2332 isolate with PRRSV isolates from countries around the world. The investigators were able to distinguish between European and North American isolates based on IPMA antibody titers of reference antisera which were determined using each isolate as antigen. Significantly higher antibody titers were obtained between anti-LV antibody and European isolates or between anti VR-2332 antibody and North American isolates than between other combinations. In another study, 837 swine sera collected from 87 herds in 18 states in the U.S. were tested by fluorescence microscopy for PRRSV-specific antibodies using the Lelystad and ATCC VR-2332 virus isolates. Approximately 58% of the samples tested contained PRRSV-specific antibodies and 36.1% of the samples contained antibodies which reacted with both viruses. Antibodies to Lelystad virus were found in 20.1% of the samples and antibodies to VR-2332 virus were found in 43.8% of the samples.

Field isolates of PRRSV have been shown to vary in their ability to be neutralized by antibody raised against a heterologous virus isolate. McGinley et al. compared 22 North American PRRSV isolates for their antigenic relationship by a one-way serum-virus neutralization (SVN) test using polyclonal porcine antibodies raised against PRRSV isolate ISU-P (ATCC VR-2402). The PRRSV
isolates were placed in one of 3 groups based on their cross neutralization index. The virus isolates in groups 1, 2, and 3 were cross neutralized by the antiserum at a rate of 50%, 25-30%, and ≤15% of the homologous system, respectively. More recently, a similar variability in the susceptibility to neutralization was also observed among European isolates of PRRSV.233

Antigenic diversity among PRRSV isolates has also been demonstrated using monoclonal antibodies (MAbs). Nelson et al. evaluated the reactivities of a panel of 3 MAbs (SDOW17, VO17, EP147) directed against the 15 kD nucleocapsid protein.217 Sixty-three U.S. isolates originating in the midwest and 57 European isolates representing Denmark, France, Germany, Italy, Luxembourg, the Netherlands, Spain, and the United Kingdom were examined using fluorescence microscopy. All 3 MAbs reacted with all 63 U.S. PRRSV isolates, while MAb SDOW17 reacted with the 57 European isolates; neither VO17 or EP147 reacted with any European isolates. More recently, Yoon et al. examined the antigenic relationship of 22 U.S. PRRSV isolates from 8 different states (Arizona, Iowa, Illinois, Kansas, Michigan, Minnesota, North Carolina, Pennsylvania) from 1989 through 1993.329 The antigenic relationship of the isolates was evaluated by fluorescence microscopy using a panel of 5 MAbs (SDOW17, VO17, EP147, M146, M302) specific for the 15kD protein. The 22 virus isolates were categorized into three groups based on their reactivities to the 5 MAbs. Eighteen of 22 isolates were recognized by all MAbs and 3 of the remaining isolates were detected by all MAbs except the MAb EP147. One unique isolate was recognized only with MAb VO17. Similarly, Drew et al. evaluated the antigenic relationship of 18 United Kingdom and 7 continental European field isolates of PRRSV using a panel of 6 monoclonal antibodies specific for the nucleocapsid protein and observed broad antigenic variation among the isolates.92

Genomic sequence analysis of the PRRSV genome has provided genetic evidence for the marked antigenic diversity that was demonstrated by serological tests.96,160,191,192,215 By comparing partial genomic sequences, North American PRRSV isolates were found to have 87 to 95% homology in their nucleotide sequences but were only 64 to 67% similar to European isolates. In these studies, amino acid homology between North American and European isolates was shown to be 60 to 80%.
The antigenic variation of PRRSV with respect to virulence has also been evaluated by a pathological study conducted by Halbur et al.\textsuperscript{119,122} Groups of cesarean-derived colostrum-deprived pigs were infected intranasally with various U.S. PRRSV field isolates collected from swine herds that experienced different types of clinical disease outbreaks. Clinical signs associated with respiratory disease and rectal temperature response were monitored. In addition, pigs from each virus group were periodically killed through 35 days postinoculation, and the blood, lung, lymph nodes, brain, heart, spleen, kidney, and bone marrow were collected from the pigs. Subsequently, gross lung lesions were subjectively scored based on the area of consolidation. Microscopic lesions in tissues of these organs were evaluated. By comparing lesions, the investigators concluded that U.S. PRRSV isolates varied in their pathogenicity. These investigators also observed that virus isolates with lower gross lung lesion scores caused less severe respiratory disease than isolates with higher scores. However, these differences were not able to be correlated with genomic differences among the isolates.\textsuperscript{192}

**Clinical manifestations**

The clinical manifestations of PRRS vary depending upon a number of factors including age of the pig, health status, immune status, reproductive status, and herd management practices.\textsuperscript{111} The PRRSV infection of breeding stock is generally characterized by anorexia, fever, respiratory signs (i.e., coughing and dyspnea), malaise, cyanosis of the extremities in a small number of cases, and reproductive disorders.\textsuperscript{163,179} Reproductive failure associated with PRRSV infection in the breeding age female includes delayed return to estrus or lack of cycling, premature farrowing, and an increase in the number of late term abortions, stillborn pigs, mummified fetuses, and weakborn pigs which usually fail to thrive and die shortly after birth.\textsuperscript{163,179,289} In the breeding age boar, PRRSV infection has shown to cause changes in semen quality.\textsuperscript{76,97,316} These changes were manifested by increases in morphological abnormalities of spermatozoa, and decreases in motility and number of spermatozoa following infection. As many as 25% of boars tested in infected herds experienced a temporary decline in semen quality (volume, motility and morphology of spermatozoa).\textsuperscript{76,97,271}
Clinical infection by PRRSV in neonates and nursery pigs is manifested primarily as respiratory disease. Nursery pigs typically present with coughing and "thumping" and grow poorly. Interstitial pneumonia is the primary cause of lung dysfunction in these pigs. Diarrhea has also been associated with PRRSV infection of neonatal pigs in some herds which commonly results in increased early preweaning death. Infection of PRRSV in nursery pigs has been shown to increase the susceptibility of pigs to secondary bacterial infection. Infections by *Streptococcus suis*, *Salmonella choleraesuis*, *Hemophilus parasuis*, *Pasteurella multocida*, and *Actinobacillus pleuropneumoniae* have been implicated in early preweaning death of nursery pigs which were infected concurrently with PRRSV. Growers and finishers may also present with mild respiratory disease and poor performance. Today, respiratory disease in nursery pigs appears to be the most prevalent manifestation of PRRSV infection (J Zimmerman, Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa, personal communication).

The clinical presentation of herds infected with PRRSV can be categorized into three forms: subclinical, acute (epidemic), or chronic (endemic). Clinical signs of reproductive and respiratory disease are not present in the subclinical form of PRRS. Infection can only be recognized by the detection of PRRSV-specific antibodies. In the acute form, naive herds infected with the virus have all the clinical manifestations of severe reproductive and respiratory disorders. The acute phase has been reported to last anywhere from several weeks to several months. In the endemic form of PRRS, herds become infected and fail to return to the performance level of reproduction and growth that was present prior to infection. Early infertility problems in breeding females and chronic respiratory disease complicated by secondary bacterial infection in young pigs continue to be a problem in these herds. Frequently, poor performance in young pigs results in increased production costs to the producers.

**Epidemiology**

**Prevalence** Porcine reproductive and respiratory syndrome virus is widespread in swine producing regions throughout the world. Seroprevalence studies conducted to assess the spread of PRRSV in the United States revealed
variable infection rates. In a survey of sera collected during the second quarter of 1992 from cull breeding swine in 11 states, 7.3% of the samples were positive by the IFA test. In a study of U.S. herds enrolled in NAHMS during the years 1990 and 1991, 35% of 396 herds in 17 states were seropositive for PRRSV. The seroprevalence in herds ranged from 2% - 82% with a mean seroprevalence of herds within states of 33%. In another study, a total of 2787 sera obtained from 263 pig farms in 13 midwest states during the first 6 months of 1992 were tested by the IFA test for antibodies specific for PRRSV. Approximately 54% of the herds tested were seropositive. Prevalence of PRRS in the U.S. swine herds was directly correlated with swine population density.

In Europe, the number of clinical outbreaks of PRRS has decreased since it reached a peak in early to mid 1991. Although PRRSV infection appears to be widespread in European swine population, there is no thorough report as to prevalence of PRRS in Europe.

**Economic impact** The widespread prevalence of PRRS throughout the world has created an economic hardship for swine producers. The economic losses to the swine producer are due to: a) death loss of pigs, b) increased costs to finish market hogs, c) increased costs of veterinary services and drugs, d) increased labor costs, and d) an increase in nonproductive sow days which creates the need for establishing larger sow and boar inventory to maintain maximum facility usage.

Typical reproductive losses that can be expected in a herd acutely infected with PRRSV were summarized in a study of 4 affected herds in Great Britain. Abortion and premature farrowing rates were up to 3.3% and 20.6% of pregnant sows, respectively. Among pigs born, 26.0% and 18.8% of the pigs were stillborn and mummified, respectively. Neonatal and preweaning death rates reached 88.0%. German investigators evaluated pig losses during and after a PRRS outbreak in 200 sows. The mean number of pigs lost per litter rose from a pre-outbreak level of 22.3% to a final mortality rate of 67.0%. Following the outbreak the mean number of pigs lost per litter returned to the pre-outbreak level. In the United States, Keffaber investigated 11 infected herds in the midwest and reported that the mean number of pigs weaned per litter was 2.9 and the mean nursery death rate was 50%.163
The economic impact of PRRSV infection in a typical U.S. herd undergoing an acute outbreak was evaluated. Assuming that infected herds return to normal following the outbreak, estimated losses were estimated to be $236 per inventoried sow. Potential chronic losses were estimated to be $502 per female in one severely affected herd. Reduced conception rates have also added to the economic impact of PRRS in some herds due to increased nonproductive sow days.

In addition to reproductive losses, productivity of growing pigs is also influenced by infection of PRRSV. Following infection, fattening pigs are often undersized and have retarded growth. Blaha studied the consequence of PRRSV infection in 12 finishing herds in Germany. The average mortality of finishing hogs rose from 2.2% to 4.3% and lung lesions of pigs at slaughter increased from 45% to 70%. Secondary bacterial infections with *Salmonella choleraesuis*, *Streptococcus suis*, or *Hemophilus parasuis* have resulted in mortality rates of 15-25%. Increased rates of bacterial infections following a PRRS outbreak lead to increased vaccine and medication costs, increased labor costs, and decreased average daily gains.

**Host species** Pigs (*Sus scrofa domestica*) are the only known species of domestic animals that are susceptible to PRRSV infection and show clinical manifestations after infection. The presence of antibody in wild boars indicates that feral swine may also be susceptible to PRRSV as well (J. Zimmerman, Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa, personal communication).

Experimental inoculation of various species of birds indicates that some birds may be susceptible to PRRSV infection. Muscovy ducks, guinea fowl, Cornish cross chickens, and mallard ducks were inoculated orally via the drinking water with approximately $10^3$ TCID$_{50}$ of PRRSV per bird. Feces from the birds were collected for 30 days and assayed for virus. No virus was isolated from Muscovy duck feces. Virus was isolated from guinea fowl feces on days 5 and 12, and chicken feces on day 5 post inoculation (PI). PRRSV was isolated from Mallard duck feces at most sampling points between day 5 to 24 PI. No overt clinical signs or seroconversion were observed in any birds at any time during the collection period. Recovery of PRRSV from feces on days ≥3 PI provided
evidence of viral replication in one or more of the systems emptying into the cloaca, rather than passive movement of challenge virus through the gastrointestinal tract. Levels of PRRSV in feces were not determined. Using fecal shedding of virus as the criterion for the susceptibility, Muscovy ducks were found to be completely resistant, guinea fowl and chickens marginally susceptible, and Mallard ducks highly susceptible to PRRSV infection.

Attempts to isolate PRRSV from tissues and sera from trapped wild mice and rats obtained from farms with endemically infected herds were unsuccessful. In addition, mice and rats that were experimentally infected with 10^3 to 10^4 TCID_50's by the nasal, peritoneal and oral routes did not become infected. No virus was isolated from sera or any of the tissues collected. Gross and microscopic examinations did not revealed any abnormalities.

Transmission Direct contact with infected swine has been considered a major route of transmission of PRRSV between pigs, and has been incriminated as a cause of PRRSV outbreaks in naive herds after purchase and movement of infected swine. In Spain, pigs imported from Germany were incriminated as the source of PRRSV infection. Field observations indicate that transmission occurs when weaned pigs are exposed to older pigs. Young pigs which were weaned into an isolation facility did not seroconvert until coming into contact with older pigs. Seroconversion was also demonstrated when uninfected pigs were commingled under experimental conditions with penmates which were infected with PRRSV several weeks earlier. Seronegative finishing pigs seroconverted after they were commingled with experimentally infected sows 14 weeks following inoculation but not when commingled with sows 26 weeks after inoculation. Regarding the exit portal of PRRSV, Yoon et al. reported that the virus was recovered from nasal secretions and feces of experimentally infected pigs for up to 35 days. In contrast, Wills et al. did not detect the virus in the feces of experimentally infected pigs and questioned the role of feces in transmission of the virus. Urine and saliva have been demonstrated to be a source of virus transmission to naive pigs.

It has been proposed that airborne transmission of PRRSV can occur over a distance of 20 kilometers. However, Wills et al.
reported the absence of transmission of PRRSV between infected and uninfected pigs housed in isolation units with a common air source. In subsequent work, these investigators evaluated the possibility of airborne transmission in a more confined area. Three nursery decks were placed parallel to each other and 46 cm apart in the same room. A aluminum partition the size of the deck side walls was suspended halfway between the center deck and a side deck. In 3 of 5 trials, no transmission of virus was demonstrated from infected pigs (n=3) in the center deck to pigs (n=3) in the side deck separated by the barrier, although air flow studies showed that air moved over, under, and around the barrier. In 5 of 5 trials, however, PRRSV-free pigs commingled with infected pigs in the central deck, and pigs in the side deck without a barrier became viremic and seroconverted. The data strongly indicated that airborne transmission does not readily occur and, if it does occur, it does so only over a relatively short distance.

Semen collected from clinically or subclinically infected boars is considered a potential source for transmission of PRRSV. Robertson reported an outbreak of PRRS in swine herds in Great Britain in which the only source of outside contact with pigs was through artificial insemination (AI). Yager et al. also suggested that fresh semen is a possible source of PRRSV in the U.S. swine herds. Experimentally, AI of 2 gilts with freshly collected unextended semen from experimentally infected boars resulted in the development of clinical signs of PRRS and seroconversion by both gilts 3 to 4 days after AI. Swenson et al. demonstrated the presence of viable PRRSV in semen ejaculated from boars that were experimentally infected by the nasal route for as long as 43 days postinoculation. This study was done by a 'swine bioassay' technique in which semen samples were injected into the peritoneal cavity of young pigs. Sera were collected from these pigs weekly for 5 weeks PI and assayed for anti-PRRSV antibody by the IFA test. Seroconversion was an indicative of the presence of infectious virus. These investigators, however, were not able to infect gilts that were artificially inseminated with extended semen collected from a boar experimentally infected with PRRSV. In contrast, the pregnancy rate in gilts receiving virus-contaminated semen was 20% compared to 67% in gilts inseminated with virus-free control semen. Work by Christopher-Hennings et al. also supports prolonged shedding of PRRSV in semen. Using the
polymerase chain reaction (PCR), these investigators detected PRRSV in semen from experimentally infected boars for up to 92 days after initial exposure.

The role of fomites in the transmission of the virus has not been completely evaluated. Under certain conditions, such as a high humidity or in the presence of high concentration of organic materials, PRRSV appears to survive in the environment for extended periods of time. Pigs placed in facilities used for PRRSV studies seroconverted after being placed in non-disinfected rooms 3 to 4 weeks after infected pigs had been removed from the rooms. It was recently found that virus infectivity can be retained in well water for 9 days under experimental conditions.

A study demonstrated that some birds were susceptible to PRRSV infection and shed the virus for extended period of time after exposure. These observations suggest that some species of birds may have the potential to act as biological vectors and transmit the PRRSV over a long distance. To date, no insect vector has been reported to be associated with transmission of PRRSV.

**Diagnosis**

A presumptive diagnosis of PRRSV infection is based on clinical manifestations such as reproductive problems and chronic respiratory disease. The reproductive problems of PRRS are characterized by poor conception rates, late-term abortion, and increases in numbers of stillborn pigs, mummified fetuses, and weakbom pigs. However, because of the similarity of clinical manifestations with those induced by other viral and bacterial pathogens, a differential test is required for a definitive diagnosis. Furthermore, there are no pathognomonic gross or histopathological lesions for the respiratory disease induced by PRRS virus infection, although interstitial pneumonia is a common finding. A definitive diagnosis of PRRSV infection requires the isolation of virus, detection of antibody, detection of viral antigen, or detection of genomic materials.

**Virus isolation** Porcine alveolar macrophages, CL2621 cells, MA104 cells, and MARC-145 cells have been reported to support virus replication in vitro and used for virus isolation from tissues and clinical specimens. PRRSV has been isolated from the spleen.
lungs,24,80,112,153,165,190,223,265,289,296,297 lymph nodes,24,165,265,296,297 heart,24 thymus,112,296,297 tonsil,24,112,265,296,297 nasal turbinate,24 placenta,58 plasma,223,265 serum,14,25,59,91,112,153,165,190,230,265,296,297 the buffy coat layer of citrated blood,165,265 urine,111,265 feces,265,326 semen,280,321 and nasal swabs,231,265,326 Saliva is found to be a source for virus isolation.320 Lung and serum are samples of choice for virus isolation.153 In particular, the presence of a long viremia in young pigs has made serum a good source for virus isolation.296 In cases of late-term abortion and early farrowing, samples should be collected from weakbom pigs rather than mummies, aborted, or stillborn pigs.90,155,296,330

The thermal susceptibility of PRRSV in specimens to different environmental temperature was evaluated.297 The virus isolation rate from positive tissues was 47%, 14%, and 7% when tissues were kept at 25°C for 24, 48, and 72 hours, while virus isolation rates were ≥85% from tissues stored at 4°C and -20°C for the same time periods. In contrast, virus was isolated from all but 1 serum sample kept at 25°C even for 72 hours. This observation indicates that serum may have a protective effect on PRRSV. Current recommendations are that tissues and clinical specimens collected for virus isolation should be kept at refrigerator temperature during shipment to diagnostic facilities in order to enhance the likelihood of isolating the virus.

Although several cells types have been reported to support PRRSV replication, the ability of these cells to grow virus from samples of infected pigs has been complicated by the fact that individual isolates do not replicate in all cell types. In a comparative study, 98 tissues and 73 sera were assayed for the presence of PRRSV utilizing PAM and CL2621 cells.19 Virus was recovered from 7 of 98 (7%) and 4 of 98 (4%) tissue samples in PAM and CL2621 cultures, respectively. Eighteen of 73 serum samples (25%) were found to contain the virus using PAM, but only 2 isolations (3%) were made in CL2621 cell culture. Interestingly, 25 of 82 PRRSV isolates (30%) isolated in CL2621 cells did not grow in PAM and 28 of the isolates (34%) that grew in PAM did not produce cytopathic effect. Five out of 18 isolates (28%) from PAM did not grow in CL2621 cells. These differences demonstrate that at least 2 cell types should be used for virus isolation whenever possible.
Serology  The indirect fluorescent antibody test,\textsuperscript{324} serum virus neutralization (SVN) test,\textsuperscript{26,210} immunoperoxidase monolayer assay,\textsuperscript{312} and enzyme-linked immunosorbent assay (ELISA)\textsuperscript{3} have been used for the detection of PRRSV-specific antibodies. Most North American veterinary diagnostic laboratories have extensively used the IFA test, while European laboratories have relied on the IPMA using PRRSV-infected PAM.\textsuperscript{332} The recent licensure of a commercial ELISA kit (Idexx) is changing this picture.

The IFA and IPMA are thought to be highly specific and sensitive tests. Antibodies to PRRSV are usually detected by these tests between 7 and 15 days after infection.\textsuperscript{112,223,312,324} Both IFA and IPMA reliably detect specific antibodies for 2 to 3 months after infection.\textsuperscript{100,112,223} The ELISA is also reported to be sensitive and specific.\textsuperscript{3,94} One disadvantage of the ELISA was reported to be unacceptable background reaction in some negative pigs.\textsuperscript{94,231} In one study, PRRSV-specific antibodies were reported to be detected by ELISA as early as 10 days after exposure and infected sows remained seropositive for at least 5 months.\textsuperscript{5} The specificity and sensitivity of the commercial ELISA has not been evaluated.

The SVN test is considered to be a specific test, but previous studies have suggested that the SVN test is less sensitive than the IFA and IPMA tests.\textsuperscript{26,210} This low sensitivity of the test is mainly due to the fact that neutralizing antibodies against PRRSV develop as late as 1 to 2 months after infection.\textsuperscript{27,100,112,200,210} The low sensitivity of the test, together with the fact that the test is laborious, has resulted in the SVN test being used primarily as a research tool. A recent report indicated that the sensitivity of the SVN test could be increased by adding 20\% fresh normal swine serum to serum being assayed.\textsuperscript{327} Neutralizing antibodies were detected in the serum of infected pigs at 9-11 days following exposure to PRRSV virus using this modification of the SVN test.

Interpretation of diagnostic information obtained from serological testing has been complicated by several factors. First, antigenic diversity among PRRSV isolates is a major concern in interpreting the serological information especially obtained by the IFA and IPMA tests since false negative results may be due to the strain of virus in use at a diagnostic laboratory.\textsuperscript{61} Secondly, lack of comparative information on the performance of the IFA, IPMA, ELISA and SVN tests in detecting PRRSV-specific antibody over time makes it difficult for diagnosticians
to standardize the tests in use. Thirdly, because of the high prevalence of PRRSV infection in herds, serological information from a single sample is not sufficient for evaluating PRRS status of individual animals. Consequently, it has been suggested that PRRS serology should be used primarily to determine if a herd has been exposed to PRRSV as opposite to virus isolation.

Although serological information is considered to be as a useful tool in tracking the progression of PRRSV infection within a population, the information by itself is not a good predictor of immunity. Viremia is frequently detected in infected pigs in the presence circulating antibody specific for PRRSV, and persistent infection of PRRSV was observed in pigs with relatively high levels of SVN antibodies. In addition, discovery of positive antibody titers against PRRSV in a group of pigs during the outbreak of disease similar to PRRS is not direct evidence of cause in some instances. Consequently, for a definitive diagnosis, virus isolation and/or detection of viral antigen or genomic material are the tests of choice.

Detection of viral antigen Benfield et al. described monoclonal antibodies which have been used to identify PRRSV antigen in lungs of infected pigs. Done et al. have also reported the use of the fluorescent antibody (FA) technique for detection of viral antigen in frozen spleen and lung tissues with monoclonal or polyclonal antibodies. A disadvantage of the FA technique is that the test is not suitable for fixed tissues. Immunohistochemical tests utilizing an immunoperoxidase system or colloidal gold have been developed to detect PRRSV in frozen or fixed tissues. Both tests are reported to be highly sensitive in detecting viral antigens in a variety of tissues including heart, kidney, lung, lymph nodes, spleen, thymus, and tonsil.

Detection of viral genomic material A reverse transcriptase-polymerase chain reaction (RT-PCR) utilizing a nested set of specific primers complementary to the sequences of ORF 7 has been developed to detect viral nucleic acid in infected cells and tissues specimens collected from infected pigs. Size of primers designed for VR-2332 isolate of PRRSV and LV is 22- and 28-mer oligonucleotides, respectively. Target sequences within ORF 7 for amplification are located at between nucleotides 14639 and 14950 for LV, and between
nucleotides 2885 and 3121 for VR-2332. More recently, an in situ hybridization technique that utilizes a nonradiolabeled RNA probe (1000 base pairs in length) that is specific for a partial sequence of ORF 7 of PRRSV has been developed. Although experimental data indicate that the both RT-PCR and in situ hybridization techniques can be used as a sensitive diagnostic tool for detecting the presence of PRRSV, specificity and sensitivity of these techniques have not been thoroughly evaluated.

Prevention and control

Prevention and control of PRRS mainly depends the implementation of good preventive medicine and husbandry. The initial method of prevention was partial or complete restriction of pig movement from herds affected by PRRS. For example, the European Economic Community established control measures for pig movement. These measures mandated that pigs could only be moved to slaughter 8 weeks after clinical signs had disappeared. This restriction applied to herds in which two of three following clinical signs were observed: a) abortion ≥8%; b) stillbirth rate ≥ 20%; and/or c) preweaning mortality ≥25% in a 14 day period. However, this measure was largely ineffective because herds routinely remained infected for longer than 8 weeks. The United Kingdom introduced “draconian” measures to prevent spreading PRRS from affected herds by issuing the “blue-eared pig disease order”. This method also adopted complete restriction of pig movement. It was effective in slowing down the spread of PRRS; however, at the same time, it caused economic hardships to owners of affected herds.

Current prevention of PRRSV infection is dependent on management strategies which maintain high biosecurity standards. This includes limiting traffic on the premise, cleaning and disinfecting transport vehicles, limiting access to buildings, and maintaining rodent control programs. Purchases of breeding stock should be done by matching donor and recipient herds with the same PRRS status. Stock should preferably be purchased from a limited number of sources. It is recommended that all replacement pigs should be quarantined for 3 weeks prior to entry into the herd. In addition, the all-in/all-out production strategies, age segregation, and multisite production have been successfully used to prevent PRRS.
The use of vaccine is being advocated to prevent and/or control PRRS. An experimental killed vaccine has been evaluated in sows and found to provide protection against reproductive disease. In this study, vaccinated sows delivered 23 normal and 2 stillborn pigs, while control sows delivered 36 stillborn pigs and no normal pigs after homologous challenge by the nasal route. Recently, a commercial "modified" live virus vaccine has been made available to the swine industry (NOBL Laboratories). This vaccine has not yet been critically evaluated for efficacy and safety by independent investigators. However, field observations indicate that the efficacy of this vaccine varies from being effective, to occasionally contributing to the disease problem (B Thacker, Iowa State University, Ames, Iowa, personal communication; G Erickson, personal communication).

The control of PRRSV following infection of a herd is primarily based on symptomatic treatment and management practices which reduce stress, minimize exposure to secondary pathogens, and prevent exposure of naive pigs to infected pigs. Use of electrolytes, anti-inflammatory drugs, and antibiotics has been recommended for symptomatic treatment. Management practices designed to reduce stress and minimize secondary infection include: maintaining adequate and clean environmental conditions (temperature, humidity, drafts, and ventilation), feeding good quality feed, preventing exposure to older pigs, widespread use of antibiotics, and vaccination against pathogens found on the farm. Management of the breeding herd is another key area in the control of PRRS. Strategies such as partial depopulation, depopulation/repopulation, test and removal, and modified medicated early weaning have also been evaluated and appear to be useful in eliminating PRRSV from infected herds. However, the long range success of these strategies for eradication of PRRSV is unknown.

Pathogenesis

Introduction Pigs can be experimentally infected with the virus via oral, nasal, intramuscular, intrauterine, intravenous, and intraperitoneal routes. Minimum infectious dose of the virus is estimated to be 10 virions when pigs are exposed to the virus intramuscularly or by the nasal route (M McGinely, Bayer Corporation-Agricultural Division, Shawnee Mission, Kansas, personal
communication). Following nasal challenge with PRRSV, the virus is detected in plasma or serum of the pigs 24 to 36 hours later. One study revealed that pigs that are experimentally infected at 3 days of age became viremic at 12 hours after exposure. Viremia persists approximately for 4 weeks and occasionally for up to 8 weeks. All sites of PRRSV replication have not been identified.

The extended period of transmissibility of PRRSV from infected pigs raised questions as to where the virus localizes in the body and whether pigs become latent/chronic carriers of the virus. Regarding the possibility that PRRSV may induce latency in infected pigs, corticosteroid-induced immunosuppression studies failed to reactivate latent virus in experimentally infected pigs at 12 weeks postinoculation. In contrast, Albina et al. found that movement stress and administration of prednisolone resulted in transmission of PRRS by animals that had seroconverted to PRRSV 15 weeks earlier. More recently, Wills et al. demonstrated the presence of PRRSV in oropharyngeal scraping samples collected from experimentally infected pigs for up to 157 days postinoculation. Overt clinical signs of PRRS were not observed in any of these pigs, indicating chronic infection of PRRSV. These data strongly support field observations that the virus can persist in infected pigs for relatively long periods of time after initial exposure. The investigators suspect that the virus may localize in tonsils of infected pigs.

Initial clinical signs manifested by anorexia, lethargy, and fever appear within 3 to 5 days. In some herds, pigs show transient blue discoloration of ears, vulva, tails, abdomens and snouts. Vascular lesions including swollen endothelial cells and thrombi have been suggested to play a role in the transient blue discoloration of extremities. It is possible that the subcutaneous and periorbital edema which have occasionally been observed in affected pigs may also be attributed to vascular lesions. The initial phase of disease is followed by clinical manifestations associated with respiratory distress and reproductive disorders. Such clinical signs appear within 1 to 2 weeks after infection.

Respiratory disease Clinical PRRSV infection of neonates and nursery pigs is manifested primarily as respiratory disease in the field. Clinical signs are coughing and "thumping". Lungs of affected pigs may appear grossly normal unless infection is complicated with secondary pathogens. Interstitial
Pneumonia is a common observation in infected pigs. Microscopic lesions are characterized by thickening of alveolar septa and accumulation of degenerating cells and proteinaceous debris in alveolar spaces.

Although respiratory disease is a major clinical component of PRRSV infection in the field, it has been difficult to consistently reproduce it experimentally even though histological lesions compatible with those observed in field cases of PRRSV infection were present in experimentally infected pigs. Failure to reproduce respiratory disease in pigs may be due to several factors. First, reproduction of PRRSV-induced respiratory signs may require a synergistic interaction with other respiratory pathogens. For example, Collins et al. were able to demonstrate respiratory signs in 10-day old SPF pigs 7 days after concurrent infection with both PRRSV and *Streptococcus suis* but not with PRRSV alone. Secondly, the genetic constitution of pigs could also influence the response to PRRSV infection. Halbur et al. infected 5-week-old Meishan pigs (n=4) and Yorkshire x Hampshire crossbred pigs (n=4) with two different PRRSV isolates that varied in virulence and evaluated gross and microscopic lesions of lungs and heart collected at 3, 5, 10, and 28 days post infection. They observed that the severity of respiratory disease caused by PRRSV infection was always greater in Meishan pigs than in Yorkshire x Hampshire crossbred pigs, regardless of the virulence of virus isolates used.

The presence of antibody might influence the severity of a PRRSV infection. A preliminary *in vitro* study has shown that it was possible to enhance infection of PAM by PRRSV by treating the virus with optimally diluted anti-PRRSV swine sera prior to inoculating the virus to PAM. Yields of progeny virus were enhanced 10 to 100 times and the proportion of PAM infected was increased 2 to 10 fold, as compared to control group that were not treated with antiserum. An *in vivo* study conducted by Christianson et al. supported antibody dependent enhancement (ADE) of PRRSV infection. Fetuses of sows between 40 and 45 days of gestation were exposed *in utero* to PRRSV mixed with antibody or the virus without antibody. Although no difference in clinical responses, such as fetal death rate, was observed between two groups, higher titers of the virus were demonstrated in fetuses received antibody-virus mixture than fetuses that were injected only with virus.
Antibody-mediated disease enhancement has been demonstrated in several viral infections in other species\textsuperscript{173,185} and may occur in pigs infected with PRRSV. For example, field observations indicate that pigs at 3 to 5 weeks of age are highly susceptible to PRRSV infection and often develop a severe respiratory clinical manifestations of PRRS.\textsuperscript{61,296} This clinical manifestation is referred to as 'post weaning PRRS.' The time period in which post weaning PRRS commonly occurs corresponds to the time period that the level of maternal antibody specific for PRRSV approaches the lower limits of detection. Maternal antibodies specific for PRRSV are generally detected in pigs at 4 to 8 weeks of age.\textsuperscript{6,111,296} Consequently, it is possible that maternal antibodies at subneutralizing levels may contribute to the severity of PRRSV-induced respiratory disease in weaning pigs.

Reproductive disease Effects of PRRSV on female reproductive performance have been extensively studied by inoculating sows and evaluating the effect on the fetus, or by inoculating the fetus directly. It is not known if the reproductive effects seen with PRRSV infection are due to maternal, fetal, or an interaction of maternal and fetal changes. Inflammatory and degenerative changes in the placenta have been reported and virus-like structures has been identified in endothelial cells of fetal and maternal placental capillaries. These changes suggest the potential for placental passage of virus from dam to offspring.\textsuperscript{275} The PRRSV has also been isolated from the placenta of a sow experimentally infected intranasally with the virus.\textsuperscript{58} Moreover, the potential for placental transfer of PRRSV to the fetus was proven when the virus was isolated from piglets born to sows that were infected with PRRSV by the nasal route during 45-50 or 84 days of gestation and anti-PRRSV antibody was detected in precolostral blood samples or ascitic fluids of the piglets.\textsuperscript{58,289}

Studies on the effect of PRRSV infection in different stages of gestation indicate that the age of the fetus determines its susceptibility to PRRSV-induced disease. In one study, sows were inoculated intranasally with PRRSV between 45 and 50 days of gestation.\textsuperscript{58} Fetuses were collected from inoculated sows on days 7, 14 and 21 postinoculation and piglets were obtained at term of pregnancy. No PRRSV was present in the fetuses, while the virus was isolated from live born piglets. These data suggest that PRRSV is able to cross the placenta and to infect
fetuses after mid-gestation. In contrast, Lager et al. evaluated the effect of PRRSV on fetuses by directly injecting the virus to amniotic cavity. These investigators reported that virus was isolated from fetuses in all stages of gestation. The investigators also observed that PRRSV replicated in younger fetuses without gross changes until midgestation and the fetuses began to die thereafter.

Similarly, Mengeling et al. inoculated pregnant gilts at various stages of gestation with PRRSV by the oro-nasal route and monitored the transplacental infection of fetuses. Fetuses at any stages of gestation were shown to be transplacentally infected with PRRSV. However, the incidence of transplacental infection was significantly greater when gilts were exposed to the virus relatively late in gestation, compared with earlier exposure in gestation. Hypotheses as to the cause of age-dependent susceptibility include: a) failure of cells at the maternal-fetal junction to support virus replication until late in gestation, b) development of a susceptible fetal cell population during midgestation, and c) a fetal immune component which enhances viral replication such as antibody.

Little is known about the pathogenesis of PRRSV infection in the boar. Epidemiological and experimental studies have indicated that semen can be a source of PRRSV to naive herds. However, the presence of PRRSV in boar reproductive tract has not been described, even though Wensvoort reported isolation of PRRSV from the genital tract of a boar killed 2 weeks after exposure. The effect of PRRSV infection on boar reproductive function has not been understood completely, although a decrease in semen volume and an increase in morphological abnormality of spermatozoa have been observed in infected boars. Post-infection changes in semen quality and virus shedding in semen have been implicated as a cause of increased returns to estrus seen in the breeding age female following PRRSV infection. It was not clear if this early infertility is due to the inability of spermatozoa to fertilize eggs or due to viral effects resulting in death of the embryo. However, Swenson et al. recently demonstrated that conception occurred in gilts that had been artificially inseminated with PRRSV-contaminated semen. Furthermore, these investigators found the presence of PRRSV in ovaries of these gilts.
Immunology

Pigs develop both humoral and cell-mediated immunity after PRRSV infection. Humoral immunity appears to develop approximately 7 to 14 days after infection, based on initial appearance of detectable circulating antibodies. Relatively high levels of nonneutralizing antibodies to PRRSV are produced in the early stage of infection. Neutralizing antibodies develop approximately 1 to 2 months following exposure, although antibody titers are relatively low. Field observations suggested that PRRSV-specific antibodies may persist for 1 year after initial exposure.

Cell-mediated immunity has been reported to develop following infection of PRRSV. Bautista et al. monitored the T-cell mediated immune response in pigs exposed to PRRSV using lymphocyte blastogenesis assay and a skin test. The study revealed that PRRSV induces an antigen-specific cell-mediated immune response in infected pigs. Antigen-specific lymphocyte proliferation response to the virus was initially detected in virus-infected animals at 28 days following exposure and continued to be detected through 77 days after infection. The skin test revealed that infected animals also develop a virus-specific delayed type hypersensitivity reaction. Induction of virus-specific cytotoxic T lymphocytes by PRRSV infection has not been studied. Although humoral and cell-mediated immunity are known to be induced in infected pigs, the relative importance of humoral and cell-mediated immunity has not been evaluated.

The development of protective immunity to PRRSV has been shown to occur in naturally and in experimentally infected pigs. In an experimental challenge study, 8 gilts were initially exposed intranasally to PRRSV at 86-96 days of gestation and gave birth to an average of 5.8 live pigs, 0.6 stillborn pigs, and 2.1 mummified fetuses. Five months after initial exposure, the same females were bred and subsequently challenged again at 93 days of gestation (7-8 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 infection. Freese and Joo investigated two herds that had a previous history of PRRS outbreak by serological monitoring and virus isolation attempt for 6 months after initial outbreaks. The investigators observed that PRRSV infection spontaneously ceased in one of the two farms investigated. However,
some herds have chronic or cyclic problems of PRRS following initial exposure to PRRSV indicating that virus continues to circulate in the herd.\(^274\) Recent studies by several investigators have indicated that PRRSV can be persistently present in infected pigs in the presence of circulating antibodies.\(^6,28,319,326\) These observations put in question the protective role of humoral immunity against virus.

Frequent outbreaks of secondary infections, particularly by bacterial agents following PRRS outbreak in the field suggest that PRRSV may suppress the immune system.\(^89,165,203,274,289,315\) For example, Keffaber et al. reported that the nursery death rate increased to an additional 25% over the rate normally caused by PRRS only in the presence of secondary infections, particularly with *Salmonella choleraesuis*, *Streptococcus suis*, and *Hemophilus parasuis*.\(^165\) Stevenson et al. also reported a severe systemic salmonellosis in two herds following PRRSV outbreak, suggesting that PRRSV renders pigs more susceptible to salmonella infection.\(^274\) These field observations have been supported by results of controlled experiments. Galina et al. reported that PRRSV predisposes pigs to *Streptococcus suis* meningitis.\(^104\) In the study, specific pathogen free pigs of 4 groups received PRRSV (group 1), PRRSV (group 2), media (group 3), and *S. suis* (group 4), respectively. Four days later, pigs in groups 1, 2, 3, and 4 received media, *S. suis*, *S. suis*, or PRRSV, respectively. Development of clinical central nervous signs typical of *S. suis* infection were evident only in the group 2 pigs which had been previously infected with PRRSV. In contrast, recent studies failed to demonstrate such a synergistic interaction between PRRSV and *Hemophilus, Pasteurella* or *Salmonella* in the pig under experimental conditions.\(^47,69,273\)

The potential for PRRSV to modulate the host immune system is supported by the fact that the virus replicates preferentially in PAM and results in the destruction of these cells.\(^244,312\) A significant decrease in the proportion of lung alveolar macrophages has been observed in pigs following PRRSV infection.\(^203,331\) Under experimental conditions, the proportion of PAM in lung lavage was decreased from >95% of total cells collected to approximately 50% by day 7 post challenge. In addition, alterations in functions of PAM were also observed after PRRSV infection. The production of inflammatory cytokines, such as IL-1 and TNF, in PAM was enhanced and nonspecific bactericidal activity
of the cells was suppressed. Depletion of lymphocytes from lymphoid tissues, such as splenic periarteriolar lymphoid sheaths, tonsillar crypts, mesenteric lymph nodes and the thymic cortex, and decreases in peripheral blood leukocytes after challenge of PRRSV may also lead to the increased susceptibility of infected pigs to secondary infections. 

Experimental studies have been performed to evaluate the interaction of PRRSV with the host immune system. Flow cytometric analysis revealed a down regulation or loss of antigen expression on the surface of infected PAM. This observation may explain why PRRSV is not easily cleared from infected pigs by cell-mediated immune surveillance in the presence of circulating antibodies, resulting in prolonged viremia and persistent infection. In another study, 3 groups of pigs were evaluated at 1, 4, and 10 week of age for their ability to respond to foreign antigen following challenge with PRRSV. The humoral immune responses of pigs that were previously infected with PRRSV to Brucella abortus, Escherichia coli pili antigens, and killed pseudorabies virus were enhanced compared to control pigs. Cell-mediated immune responses to dinitrofluorobenzene, as measured by the delayed type hypersensitivity, were also enhanced in infected pigs compared to control pigs. Consequently, investigators concluded that there was no evidence for systemic immunosuppression by PRRSV infection. Instead, these investigators suggested that PRRSV infection induces polyclonal B- and T-cell activation. A similar observation has been made with respect to infection of mice with another arterivirus, LDV. 

Very little is known about the role of colostrum-derived maternal antibody in PRRSV infection. Specific antibodies have been demonstrated in the colostrum from experimentally infected sows. Albina et al. reported that passive maternal antibody was detected in the serum of piglet as early as 4 days after birth. The investigators also observed some instances in which no maternal antibody was detected in sera of piglets born to infected dams after ingestion of colostrum. Maternal antibody specific for PRRSV has been reported to persist until 4 to 8 weeks of age and occasionally up to 16 weeks of age in pigs nursing immune dams. Although passive immunity is believed to be protective against infection, Molitor reported that pigs from nonimmune dams were not protected following challenge when they were passively given PRRSV
antibodies, while challenged pigs born to immune dams were protected. This observation suggests that antibody alone may not be able to protect pigs from disease and that cell mediated immunity might plays an important role in protecting pigs from PRRS.

**Antibody dependent enhancement**

**Introduction** In general, virus-specific antibodies are considered antiviral and play an important role in the control of virus infections in a number of ways. Antibodies neutralize virus, thereby preventing virus infections of target cells. Antibodies also bind to virus-infected cells and subsequently mediate cell lysis through complement activation or antibody-dependent cell-mediated cytotoxicity mediated by natural killer cells, monocytes/macrophages, and neutrophils. 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.

Antibody dependent enhancement of virus 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. It was first described by Hawkes in 1964 who 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. Subsequently ADE has been described for a variety of viruses representing 12 different families. Some examples are: rabbitpox virus, dengue virus (DV), yellow fever virus, feline infectious peritonitis virus (FIPV), Sindbis virus, LDV of mice, Bunyamwera virus, reovirus, rabies virus, murine cytomegalovirus, influenza virus, human immunodeficiency virus type 1 (HIV-1), respiratory syncytial virus (RSV), and Aleutian disease virus (ADV) of mink.

Common features of the viruses described above are that: a) they replicate, in part or exclusively, in macrophages; b) they induce the production of large amount of antibodies that neutralize even homologous virus poorly 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 antibody raised against heterologous isolates.\textsuperscript{127,185,249}

**Mechanism of ADE** Although the precise mechanism of ADE is not completely understood, it is generally assumed that the increased yields of virus are primarily due to a greater number of susceptible cells being infected.\textsuperscript{70,105,127,158,225} This increase in infection rate of cells is shown to be mediated by receptors, most notably Fc receptor (FcR), which facilitate the uptake of virus-antibody complexes. However, studies also suggested that other mechanism(s) could also account for increased virus yields. Antibody may also increase the efficiency of virus replication, either a) by facilitating the uptake of infectious antibody-virus complexes or b) by increasing the synthesis of viral protein and nucleic acid. For example, Collins et al. demonstrated that antibody increases the numbers of West Nile virus (WNV) attached to mouse macrophage-like cells by comparing radioactivity counts associated with cells infected with radiolabeled WNV in the presence of antibody to that in the absence of antibody.\textsuperscript{106,107} Robinson et al. demonstrated that replication of HIV-1 was initiated sooner in cells when the virus was pretreated with HIV specific antibody, as opposed to when the virus was not treated with antibody.\textsuperscript{259} 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 \textit{in situ} hybridization.\textsuperscript{226} 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 viral replication within the cell 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.\textsuperscript{263} 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 subpopulation 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 culture in which young monocytes are continuously produced. Restriction of virus infection to immunological immature cells was also demonstrated in mice that were persistently infected with LDV.

**Interaction of virus, antibody and receptor in ADE**

**Antibodies mediating ADE**  Enhancement of virus infection has been demonstrated using various sources of antibodies. These sources include polyclonal antisera generated in natural host and other animals, mouse ascitic fluids containing MAbs to the virus of interest, and immunoglobulin 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 antisera 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 MAb 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 may 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.

**Receptors involved in ADE.** Several cell surface molecules, including the FcR, complement receptor (CR), β2-microglobulin, and some CD molecules, have been reported to play a role, or at least to be involved, in mediating ADE of virus infection. 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. 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. These investigators found that ADE of DV infection in monocytes was inhibited by first treating the cells with immunoglobulin prior to exposing cells to virus-antibody mixtures. Peiris et al. conclusively demonstrated that the interaction between virus-antibody and FcR is essential for the ADE of virus infection. 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 Fc portion of antibody.
In humans, there are 3 types of Fc receptors which bind human IgG: FcγRI, FcγRII, and FcγRIII. 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 Fc 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 FcR, changes in any of these three components may modulate the ADE. Of the three components, the FcR can be most easily modulated within relatively short periods of time by treating FcR-bearing cells with certain cytokines or proteolytic enzymes. A quantitative (i.e., number) change or a qualitative (i.e., avidity for Fc portion of IgG) change in FcR 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 Fc 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. More recently, Mady et al. examined the effects of neuraminidase on ADE of DV infection mediated by the low-affinity FcγRII in vitro. 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.

Besides FcR, complement receptors have also been implicated in ADE of virus infection. 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. 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. Subneutralizing levels of HIV-specific antibody enhance virus replication in several human neoplastic cell lines which 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. 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.

Several cell surface molecules are known to enhance virus infections or to be involved in ADE. Takeda et al. demonstrated that ADE of HIV infection in monocytic cells via FcR was blocked by pretreatment of cells with monoclonal antibodies to CD4 molecule, as well as to FcγRI. 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. 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 CD33.180

**Viral proteins/epitopes associated with ADE**  
Viral antigenic determinants associated with envelope protein(s) induce antibodies which mediate ADE.41,70,105,141,225,256,285 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.70,225 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,141 gp120 and gp41 of HIV,257,258,282 HA protein of influenza virus,222,285 G1 and G2 proteins of hantavirus,322 and the F protein of RSV.105 To date, no internal proteins of enveloped 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.141 Reovirus is the only nonenveloped 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.41 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 of swine which, like FIPV, belongs to the family Coronaviridae.

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 neutralized DV-2 infectivity for continuous cell lines very poorly, 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 than in the homotypic serum. Further, 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 difference in epitopic profiles of isolates.
In related work 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. 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 or strongly and weakly associated with ADE. These finding are particularly noteworthy because they suggest that it may be possible to develop vaccines with strong neutralizing and weak ADE inducing characteristics. Similar variability in the susceptibility of HIV-1 isolates to ADE and/or in the ability to induce ADE also believed to exist because a great deal of genomic diversity has been demonstrated among HIV isolates. Currently, variation in the susceptibility of isolates to ADE and in the ability to induce ADE are a great concern in developing vaccines against viruses for which ADE and antigenic diversity have been reported.

**Assays of ADE activity**  Antibody dependent enhancement of virus infection can be studied either in vitro or in vivo. Although in vivo study may have more clinical relevance, in vitro assays utilizing various sources of virus-specific antibody, such as antisera, mouse monoclonal antibodies, fractionated immunoglobulin, are generally performed to determine the ability of antibody to enhance specific virus infections. Two types of ADE assays have been developed for in vitro study to quantitate enhancing activity. One assay is to assess the increase in the production of progeny virus from cells exposed to virus-antibody mixtures. The other assay measures the increase in the proportion of cells being infected after exposure to virus-antibody mixtures. Both ADE assays are conducted by first exposing the virus of interest to antibody at different levels of concentration or to an appropriate antibody-free control serum. Permissive cells are then inoculated with these mixtures. After an appropriate period of incubation, progeny virus yield or numbers of infected cells are determined and compared. The virus yield is usually determined by microtiter infectivity assay or plaque assay using permissive cell lines.
Fluorescence microscopy, in situ hybridization, or infectious foci center assay have also been used to measure the proportion of cells infected with virus.

Morens and Halstead proposed that the occurrence of ADE of virus infections can be recognized by the following observations: a) a significant increase in virus production as measured by quantitative assays at different points on the growth curve; b) 'enhancement profiles' which are characterized by the appearance, peak, decline, and disappearance of infection enhancement produced over at least a 10^3 fold dilution range when the virus output is assayed in cells infected with mixtures of constant amounts of virus and serial dilutions of antibody source; c) the dilution of antibody source at which maximal enhancement is observed is related to other serological measures of binding to virus components; d) infection enhancement is detected with different antibody sources and virus strains tested over a range of multiplicity of infection; and e) other causes of enhanced virus production could be ruled out.

Using an in vitro ADE assay, the ADE activity of antibody source can be expressed in several ways, including endpoint titers, peak enhancement titer and 'enhancement power.' The endpoint titer of enhancing antibody is by definition the highest dilution of serum producing a significant enhancement of infection compared to controls. The peak enhancement titer is a serum dilution at which virus yield or proportion of infected cells is maximal for the antibody source tested. The 'enhancement power or ratio' can be calculated by dividing the virus yield or proportion of infected cells in antibody-supplemented group by the yield or proportion if uninfected cells in control cultures at a selected dilution of serum, or on a selected comparison day if a single dilution of serum is used.

The method of measuring and expressing ADE activity can be of critical importance in attempting to correlate in vitro ADE with an in vivo effect. For instance, in secondary dengue infections, enhancing activity in undiluted sera was a significant risk factor for severe dengue illness when human peripheral blood mononuclear cells were used as indicator cells. In contrast, endpoint ADE titer, such as the highest serum dilution showing enhancing activity was not predictive and paradoxically was higher in children without severe illness.
Role of ADE in disease Antibody dependent enhancement of virus infection has been suggested as a disease enhancing factor for several human and animal viral diseases.\textsuperscript{185} Specific examples include Aleutian mink disease virus, dengue virus, feline infectious peritonitis virus, and respiratory syncytial virus.\textsuperscript{50,51} In addition, ADE has also been implicated as a major obstacle to the development of specific virus vaccines, such as ADV,\textsuperscript{248} bluetongue virus,\textsuperscript{40} DV,\textsuperscript{35} FIPV,\textsuperscript{300} influenza virus,\textsuperscript{306} lentiviruses,\textsuperscript{188,304} measles virus,\textsuperscript{37,251} rabies virus,\textsuperscript{272} and RSV.\textsuperscript{159} 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.

Respiratory syncytial virus In general, RSV infections are not always considered serious. However, individuals who develop pneumonia from RSV infection are required to be hospitalized. 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.\textsuperscript{151} In another study, infants with maternally acquired RSV antibody not only failed to be protected from RSV infections, but the rate of severe disease was higher in these infants when compared to infants without maternal antibodies.\textsuperscript{50} These observations led to the 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 mouse macrophage cell line by RSV is enhanced in the presence of virus-specific antibody.\textsuperscript{105} 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.\textsuperscript{52,103,159,166} 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 nonvaccinated
group. Furthermore, the duration of illness was longer and the severity
of illness was greater in the vaccinated children compared with nonvaccinated
children. Results from these studies indicated that children were at increased
risk to severe RSV disease following immunization.

**Dengue virus infection** Dengue viruses belong to the genus Flavivirus
of the family *Flaviviridae*. There are 4 serotypes: dengue virus types 1, 2, 3, and
4. Dengue virus infections are considered a serious health problems in many
areas of the world. Dengue virus infection can be asymptomatic or cause two
forms of disease. In many cases, DV infection causes a febrile disease referred
to as 'dengue fever' which is characterized by fever, retroorbital 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.

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 an 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.

**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. Similarly, kittens immunized with a recombinant vaccinia virus expressing the spike protein of FIPV died earlier than control animals.

**Aleutian mink disease** Aleutian disease virus is a parvovirus and is known to exist in blood principally as immune complexes which are fully infectious both *in vivo* and *in vitro*. 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{247,248} 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{247} 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{248} 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{158}

In a trial with an experimental ADV vaccine, the immunization regimen even 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{248} 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.

**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.\textsuperscript{152,304} 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.\textsuperscript{152} 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.\textsuperscript{304} 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{188} 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 in vitro with sera from HIV-infected individuals or animals vaccinated with experimental vaccines.\textsuperscript{39,137,185}

\textbf{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.\textsuperscript{272} 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.

**Statement of the Problem**

Porcine reproductive and respiratory syndrome is a relatively new viral disease of swine. It continues to be an economically significant problem in swine producing regions throughout the world. The use of vaccines to control and prevent the disease is being advocated in response to the widespread incidence and economic significance of PRRS. However, antigenic diversity among PRRSV field isolates has posed a significant obstacle to vaccine development. Field and experimental observations to date suggest that the humoral immunity may not provide protection from PRRSV infection and has the potential to contribute to the disease problem. In addition, preliminary studies suggest that a phenomenon known as ADE of virus infection could present obstacles to the development of effective immunization strategies for PRRS. Antibody dependent enhancement has been implicated as a disease enhancing factor in
several viral diseases of humans and animals and recognized as a major obstacle in the development of vaccination strategies against these viruses. Consequently, it was important to know whether or not the same potential exists in PRRSV infection. The work presented here was conducted to determine whether or not PRRSV-specific antibody is a contributing factor to the pathogenesis of PRRS. Specific aims were: a) to determine and characterize ADE of PRRSV infection; b) to evaluate the biological significance of ADE with respect to the pathogenesis of PRRSV; c) to determine if ADE has the potential to interfere with immunization strategies for PRRS; and d) to determine which viral proteins are responsible for the induction of antibodies that mediate ADE.
ANTIBODY DEPENDENT ENHANCEMENT OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS INFECTION IN PIGS

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ABSTRACT

Infection of porcine alveolar macrophages by the porcine reproductive and respiratory syndrome virus (PRRSV) was significantly enhanced in vitro by antibody raised against the PRRSV isolate ISU-P (p<0.01). Increased yields and infection rates were highly correlated (r=0.95) and the ratio of yield to infection rate was greater than 1.4, suggesting that more than one mechanism was responsible for enhanced infection. Antibody dependent enhancement (ADE) of infection was also demonstrated in vivo using a completely randomized block design (n=16). The mean level and duration of viremia was greater (p<0.05) in pigs injected with subneutralizing amounts of PRRSV-specific IgG prior to virus challenge than in control pigs injected with normal IgG. In contrast, virus replication was significantly (p<0.01) inhibited in pigs with neutralizing antibody titers of \(4 \log_2\). The period of time that subneutralizing levels of antibody can persist and contribute to ADE of PRRSV infection was estimated in 4 pigs injected with PRRSV-specific IgG to yield an initial neutralizing antibody titer of \(3.8 \log_2\). Neutralizing activity declined to undetectable levels by day 37 post injection (PI). ADE activity was first detected in undiluted sera on day 20 PI and persisted through day 62 PI. Western immunoblot analysis of sera collected between days 37 and 62 PI detected antibodies specific for the 15kD nucleocapsid and 26kD glycosylated envelope proteins. These results strongly suggest that ADE has the potential to contribute to the pathogenesis of PRRSV infection and is mediated by antibody specific for the 26kD envelope protein.
INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is an economically significant viral disease of swine worldwide. The syndrome was initially observed in the United States in 1987 (15) and subsequently in Europe in 1990 (3). The porcine reproductive and respiratory syndrome virus (PRRSV) was isolated from infected swine in 1991 (36) and has been provisionally placed in the Arterivirus genus of the family Togaviridae (23). Broad antigenic variations among PRRSV isolates have been demonstrated by serological assays using polyclonal (22,37) and monoclonal antibodies (25,39).

The PRRSV causes reproductive failure in sexually mature pigs and respiratory disease characterized by interstitial pneumonia in pigs of all ages (6). Respiratory disease is particularly severe in pigs 3 to 5 weeks of age (15,30,41). This corresponds to the time period when PRRSV-specific maternal antibodies are relatively low. Colostrum-derived antibody specific for the virus is generally detected in young pigs through 4 to 8 weeks of age by the indirect fluorescent antibody test and the enzyme-linked immunosorbent assay (1,6,35). This observation has led to the speculation that antibody dependent enhancement (ADE) of PRRSV infection may play a role in the pathogenesis of the disease. This hypothesis is supported by the work of Choi et al. which demonstrated that virus production could be enhanced in vitro in the presence of subneutralizing levels of PRRSV-specific antiserum (2).

Enhancement of virus infection by antibody has been demonstrated for several different viruses representing 12 different families (18). Specific examples include feline infectious peritonitis virus (FIPV), Aleutian disease virus (ADV) of mink, equine infectious anemia virus (EIAV) and dengue virus (DV) of humans. The role of ADE in viral pathogenesis has been particularly well characterized for DV. It is recognized that DV-induced disease is more severe in individuals who have subneutralizing levels of antibodies induced by previous infection with the same or different DV serotypes than in individuals infected with DV for the first time (7,8,9). In addition, ADE has also interfered with the development of vaccines. Cats, horses, and mink that have been immunized with experimental FIPV, EIAV, and ADV killed vaccines
respectively, developed a more severe disease than unvaccinated control animals following challenge with homologous virulent strains of virus (21).

There is a significant effort currently underway to develop effective immunization strategies to control PRRS. If PRRSV infection is enhanced by antibody, then ADE must be taken into consideration in the development of these strategies. The following studies evaluated the potential of PRRSV-induced antibody to enhance PRRSV infection in pigs.

**MATERIALS AND METHODS**

**Media and reagents.** All growth, maintenance and cell freezing media were supplemented with fetal bovine serum (Sigma Chemical Co., St. Louis, MO) at a rate of 10%, 2%, and 20%, respectively. Each of these media contained 10mM of HEPES and a mixture of antibiotic-antimycotic agents consisting of 100IU/ml penicillin, 10µg/ml streptomycin, 50µg/ml gentamicin, and 0.25µg/ml amphotericin B. In addition, the freezing media contained 10% DMSO (Sigma Chemical Co., St. Louis, MO).

Protein A was prepared by dissolving staphylococcal protein A (Sigma Chemical Co., St Louis, MO) in Hanks’ balanced salt solution (HBSS, Sigma Chemical Co., St. Louis, MO) at a concentration of 0.1% (w/v). The solution was filtered through a 0.22µm membrane filter (Corning Glass Works, Corning, NY) and stored at -70°C until used.

Swine anti-PRRSV sera for use in ADE assays were prepared from blood collected from nine 14- to 28-week-old pigs 55 to 69 days after nasal inoculation with approximately 10³ to 10⁴ TCID₅₀'s of the PRRSV isolate ISU-P. The virus neutralizing and the immunoperoxidase monolayer assay (IPMA) antibody titers of these 9 sera ranged from 1:8 to 1:64 and from 1:256 to 1:1280, respectively.

PRRSV-specific polyclonal immunoglobulin (IgG) for **in vivo** studies was prepared by salt precipitation using saturated ammonium sulfate aqueous solution (16) from a heat inactivated serum pool consisting of equal parts of the above sera. Normal swine serum globulin was prepared in the same manner from 2 control pigs that were not infected with PRRSV. Aliquots of PRRSV-specific and normal globulin were further purified by DEAE cellulose chromatography (16) and concentrated by ultrafiltration for **in vitro** use. Total
protein concentration was estimated using the BCA protein assay kit (Pierce, Rockford, IL).

**Cells and virus.** Porcine alveolar macrophages (PAM) were used for ADE assays and virus isolation. The cells were collected from lungs of 4- to 6-week-old PRRSV-free pigs by lung lavage (38). In brief, lungs were removed from pigs immediately following death and lavaged with cold HBSS containing the antibiotic-antimycotic mixture. Cells were pelleted by centrifugation at 800 x g for 10 minutes, washed 3 times in HBSS, suspended in freezing media at a concentration of 5 x 10^7 cells/ml, and stored at -70°C until used. The total number of viable cells was determined by dye exclusion using a 0.04% trypan blue solution (12). The proportion of PAM among total viable cells was determined by a nonspecific esterase test (14). Frozen cells were reconstituted for use by rapid thawing at 37°C, pelleted by centrifugation, and resuspended in RPMI-1640 growth media to contain 2 x 10^6 viable PAM per ml. The cells were incubated for 24 hours at 37°C in a humidified 5% CO₂ atmosphere prior to use.

The MARC-145 clone (17) of the African monkey kidney cell line, MA 104 was used for virus titration and for the production of PRRSV antigen for use in serological assays and western immunoblotting. The cells were grown in Dulbecco's modified Eagle's media (DMEM, Sigma Chemical Co., St. Louis, MO).

The PRRSV isolate ISU-P (ATCC VR-2402) was used for all assays and in vivo studies (39). This virus was initially isolated in 1992 from an homogenate prepared from a pool of lungs collected from young pigs in a herd that was undergoing an acute outbreak of respiratory disease in the state of Illinois, USA. Porcine alveolar macrophages were used for the initial isolation. The isolate was cloned by 3 rounds of limiting dilutions in PAM and twice by plaquing in MA104 cells. The working stock of virus used in the present study represents the fourth passage in MA104 cells.

**Virus isolation and assay.** Virus was isolated from serum samples using 24-hour cultures of PAM maintained in 48 well cell culture plates (Costar Corp., Cambridge, MA). The cells in individual wells were inoculated by adding 0.2 ml aliquots of undiluted swine serum directly to the media contained in the well. The cell cultures were incubated at 37°C for 7 days or until visible CPE was observed. Two blind passages were made for each sample before the sample was considered negative. Confirmation of PRRSV infection was made by inoculating
media containing suspect virus onto 24 hour-old MARC-145 cell monolayers prepared on 8-chamber glass slides (Nunc Inc., Naperville, IL). The inoculum was removed after a 1 hour adsorption period and replaced with maintenance media. The cell cultures were incubated at 37°C for 48 hours and then examined by the indirect fluorescence antibody technique using a PRRSV-specific monoclonal antibody, SDOW17, specific for the 15kD nucleocapsid protein (25).

Virus was assayed in 96-well microtiter plates (Corning Glass Works, Corning, NY) containing MARC-145 cell monolayers. MARC-145 cells were used in place of PAM because the yield of PAM from individual pigs was relatively small. Serum and cell culture samples to be assayed were serially diluted 10-fold in growth media. Media was removed from individual wells which were inoculated in triplicate with 0.1ml of each serial dilution of sample. One hundred µl of maintenance media were added to each well after a 1 hour adsorption period. The cell cultures were then incubated at 37°C and observed for CPE for 7 days. Virus titers were determined by the method of Reed and Muench (31) and expressed as TCID_{50}/ml.

Serological assays. The serum virus neutralization (SVN) test was performed in 96-well microtiter cell culture plates using MARC-145 cell monolayers as previously described (41). Titers were expressed as the log_{2} of the reciprocal of the highest serum dilution in which no CPE was observed at the end of a 5 day incubation period. The immunoperoxidase monolayer assay was conducted utilizing a biotin-streptavidin horseradish peroxidase system with diaminobenzidine tetrachloride substrate (Kirkgaard and Perry Laboratories Inc., Gaithersburg, MD) as previously described (40). IPMA antibody titers were expressed as the log_{2} of the reciprocal of the highest serum dilution in which a specific color reaction was observed.

Experimental animals. Four- to five-week-old white crossbred pigs were used as sources of PAM, for the production of PRRSV-specific antibody, and for experiments in which the effect and duration of ADE in pigs was evaluated. All pigs were serologically tested for PRRSV-specific antibody by the IPMA at least two times before being used for specific purposes, then again on the day of use. Pigs were obtained from sows that were medicated 9 days prior to farrowing to minimize the possibility of transmitting respiratory pathogens to piglets. Individual piglets were weaned at 14 days of age and maintained on medicated
rations for the duration of experiments. All pigs were serologically tested for pseudorabies virus, swine influenza virus, porcine parvovirus, and transmissible gastroenteritis virus at the end of each experiment.

**Assays for detecting ADE activity.** Antibody dependent enhancement of PRRSV infection was assessed both by measuring increases in virus yield and by determining infection rates of PAM in the presence of subneutralizing levels of PRRSV-specific antibody. Two assays were used to measure increases in progeny virus yield: the standard ADE assay and the modified ADE assay. Increases in the infection rates of PAM were determined by indirect fluorescent antibody microscopy.

The standard ADE assay was a modification of the assay described by Moren and Halstead (24). In brief, test and negative control sera were heat-inactivated at 56°C for 45 minutes. Individual sera were serially diluted 2- and 10-fold in RPMI growth media over a range beyond that in which SVN activity was present. One and a half ml of each dilution of the test serum, the negative control serum, and RPMI growth media alone were separately mixed with an equal volume of RPMI-1640 containing $10^4.3$ TCID$_{50}$s/ml PRRSV. These mixtures were incubated at 37°C for 60 minutes. Subsequently, 1 ml aliquots of each mixture were inoculated in triplicate into individual wells of 48-well cell culture plates that had been inoculated one day earlier with $10^6$ PAM. The plates were again incubated at 37°C for 60 minutes. Inoculums were replaced with 0.5 ml of RPMI growth media and the cultures were incubated for an additional 48 hours at 37°C in a humidified 5% CO$_2$ atmosphere. The virus-infected cell cultures were frozen to -70°C, thawed rapidly in a 37°C water bath, then clarified by centrifugation at 1000 x g for 10 minutes. The supernatant from each well was harvested and assayed for virus activity. The difference in virus yields at each dilution of test and control serum was calculated by the following formula:

$$Yield\ difference = (Y_{ts} - Y_m) \ or \ (Y_{cs} - Y_m);$$

where $Y_{ts}$ = yield of virus in the presence of test serum, $Y_m$ = yield of virus in the presence of RPMI alone, and $Y_{cs}$ = yield of virus in the presence of negative control serum. The yield difference was modeled for each test and control serum by regression on serum dilutions. The general linear test (27) was used to
compare the yield of virus at all dilutions of test serum to the yield of virus generated following treatment with identical dilutions of control serum. Differences significant at p<0.05 were considered indicative of ADE of virus infection.

The modified ADE assay was a variation of the standard ADE assay described above and was conducted in the same manner with the exception that PRRSV was treated with undiluted serum or a specific dilution of PRRSV-specific IgG. Student's t test was used to compare the increase in virus yield following treatment with undiluted serum or IgG preparation to the increase in virus yield following treatment with control serum or a normal serum globulin preparation. Differences significant at p<0.05 were considered indicative of ADE of virus infection.

Antibody dependent enhancing activity as reflected by increases in the infection rates of PAM following treatment with PRRSV-specific antibody was assessed using modification of the protocol described by Olsen et al. (28). The assay was conducted in the same manner as the standard ADE assay through the end of the 48 hour incubation period. At this point, PAM cultures in the 48-well cell culture plates were fixed in cold acetone:methanol (70:30) for 10 minutes. The proportion of cells infected with PRRSV was then determined by indirect fluorescence microscopy using the PRRSV-specific monoclonal antibody SDOW17 and goat anti-mouse IgG conjugated to fluorescein isothiocyanate (Kirkagaard and Perry Laboratories Inc., Gaithersburg, MD). Significant differences (p<0.05) between the infection rates of PAM infected with treated and non-treated PRRSV were considered indicative of ADE activity.

**Western immunoblotting.** Western immunoblot analysis (WIA) was done as previously described (40). In brief, MARC-145 cells were infected at a multiplicity of infection (m.o.i.) of 0.01 and harvested when CPE was present in >95% of the cell monolayer which generally occurred in 3 to 4 days. Virus-infected cell and uninfected control cell preparations were solubilized in a lysis buffer (pH 8.0, 0.05M Tris, 0.15M NaCl, 0.002M EDTA, 0.5% deoxycholate, 0.1% SDS, 1% NP-40, 0.1% sodium azide, 0.1% gelatin, and 0.1% bovine serum albumin) and clarified by centrifugation at 600 x g for 10 minutes. Viral and cellular proteins were then separated by SDS-PAGE using a modified Lammeli procedure (19) on a discontinuous slab gel (70 x 80 x 0.75mm) consisting of a 5% stacking gel and a
14% resolving gel, cross-linked with bis-acrylamide at a ratio of 30:0.8. The separated proteins were electrophoretically transferred from the gel to 0.45μm nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA). The membranes were then immersed overnight in cold (4°C) Tris-buffered saline (TBS, pH 7.5; 20mM Tris, 500mM NaCl) containing 1% gelatin. The membranes were washed for 10 to 15 minutes in gently agitated TBS, then cut into 0.7 cm-wide strips. Duplicate strips containing viral and cellular antigens were reacted with test, control, and reference sera diluted 1:10 in TBS containing 0.05% Tween 20. Antigen-antibody reactions were visualized by immunostaining with peroxidase labeled goat anti-swine IgG (H+L) and subsequent treatment with TMB (3,3′,5,5′-tetramethylbenzidine) membrane peroxidase substrate (Kirkgaard and Perry Laboratories Inc., Gaithersburg, MD). Approximate molecular weights of proteins were determined by comparison with protein standards (GIBCO/BRL Life Technologies, Gaithersburg, MD) using linear regression.

**Demonstration of ADE activity in swine sera.** Sera were collected from 9 pigs 55 to 69 days following nasal inoculation with 10^3 to 10^4 TCID_{50}'s of PRRSV isolate ISU-P. Each of the sera was paired randomly with a PRRSV antibody-free control serum collected from 9 different pigs of similar age. Each pair of test and control sera was assayed in triplicate for ADE activity using the standard ADE assay for detecting differences in progeny virus yield and indirect fluorescence microscopy to detect differences in infection rates of PAM. Both assays were performed concurrently for each pair of test and control sera. The assays were repeated 3 times on each of 3 separate days using the same lot of PAM. The general linear test was used to compare the differences in virus yield and infection rates of PAM for all dilutions of each pair of test and control sera. The relationship between the infection rate of PAM and the yield of progeny virus was then determined by correlation analysis.

**The effect of protein A on ADE of PRRS virus infection.** The Fc receptor of macrophages has been shown to be an essential component in the ADE of several viral infections (10,13,29). Protein A binds to the Fc portion of IgG. Consequently, reacting protein A with PRRSV-specific IgG prior to infection of PAM should significantly reduce virus yield if ADE of PRRSV infection is mediated in whole or in part by Fc receptors. The effect of protein A on ADE of PRRSV infection was assessed as previously described (13) by the modified ADE
assay using a 3 x 2 factorial design in which PRRSV was treated with homologous IgG, normal serum globulin, or RPMI-1640 in the presence and absence of protein A. The concentration of IgG used in the study was selected based on its ability to optimally enhance the yield of PRRSV in the standard ADE assay. Each experiment was done 3 times on 3 different days using 4 different concentrations of protein A, i.e., 25, 50, 100, and 200 μg/ml. Virus yields for all treatments were compared by analysis of variance (ANOVA). A blocking effect of protein A was considered to exist if significant reductions in virus yield (p<0.05) were observed at one or more concentrations of protein A.

The effect of ADE on the duration and level of viremia in pigs. The ability of PRRSV-induced antibody to enhance virus infection in pigs was evaluated in 16 pigs using a completely randomized block design. Pigs were randomly assigned to one of 4 treatment groups of 4 pigs each. One pig from each treatment group were housed in 4 separate rooms. Sera were collected from all 16 pigs prior to treatment and used as controls. Pigs in groups 1, 2, and 3 were injected intraperitoneally with salt precipitated PRRSV-specific IgG at a rate calculated to yield SVN antibody titers of ≤1, 2, and 4 log₂, respectively. Group 4 pigs were injected with an amount of normal serum globulin which was equivalent to the amount of IgG received by pigs in group 1. Seven days later, all pigs were challenged intramuscularly with 10^3 TCID₅₀'s of PRRSV.

Response of treatment groups to virus challenge was assessed by determining the duration and level of viremia. For this purpose, serum was collected from all pigs daily for 7 days beginning on the day of challenge, then every other day through day 28 post challenge (PC). Sera collected on the day of challenge were also assayed for SVN and ADE activity. ADE activity in undiluted serum was determined by the modified ADE assay. In addition, the peripheral blood leukocyte (PBL) profile was monitored in all pigs during this period. Pigs were also observed for signs of respiratory distress and inappetence. All data were analyzed by ANOVA to determine treatment effect, with the exception of data representing changes in virus titers in serum and PBL over time, which were analyzed by the general linear test.

Duration of ADE activity in pigs following the decline of antibodies below neutralizing levels. Six 4- to 5-week-old pigs were housed in the same room. Four of the 6 pigs were injected intraperitoneally with salt precipitated PRRSV-
specific IgG at a rate calculated to yield an SVN antibody titer of $4 \log_2$. The 2 remaining pigs were injected with an amount of salt precipitated normal swine serum globulin that was equivalent to the amount of IgG received by the other 4 pigs. Sera were collected from each pig 2 to 3 times weekly over a 79 day period. All serum samples were assayed for PRRSV-specific antibody by the IPMA and the SVN test. The ADE activity in each undiluted serum was determined by the modified ADE assay. Sera collected on day 0 (the day of injection), day 1, and approximately every 10 days postinjection were also analyzed by western immunoblotting to determine the PRRSV protein specificity of antibody associated with ADE.

**RESULTS**

**Presence of ADE activity in swine sera.** Serum samples were collected from 9 pigs 55 to 69 days following nasal inoculation with PRRSV isolates ISU-P. Each of these sera and a corresponding control serum were assayed for ADE activity by comparing their ability to mediate increases in virus yields and PAM infection rates. Results are summarized in Figure 1.

Antibody dependent enhancement activity induced by PRRSV infection was demonstrated in sera collected from all 9 infected pigs. Significant increases ($p<0.01$) in both progeny virus yield and in the infection rates of PAM occurred when PAM were infected with PRRSV at a m.o.i. of 0.01 following treatment of the virus with all 9 PRRSV-specific antisera at dilutions that did not possess detectable viral neutralizing activity. Maximum increases in virus yields and infection rates of PAM mediated by a specific dilution of each of the 9 sera ranged from 1.43 to 1.97 log$_{10}$, and 0.14% to 1.1% respectively. This increase in infection rates represented a 7 to 53 fold increase. In contrast, no difference in virus yield or infection rates of PAM was observed when PRRSV was treated with control sera or only media.

The mean yield of infectious progeny virus in infected alveolar macrophages was 4.0 virions per infected cell following treatment of PRRSV with control sera or only media. In contrast, the yield of infectious progeny virus per individual PAM was significantly increased following treatment of the virus with the 9 antisera at dilutions in which ADE activity was detected. Under these
Figure 1. Antibody dependent enhancement of PRRSV infection revealed by (A) increases in virus yields as determined by the standard ADE assay and (B) increases in infection rates of porcine alveolar macrophages (PAM)

- = fitted curve

○ = mean of 3 estimates with each of 9 PRRSV-specific antiserums

△ = mean of 3 estimates with 9 control serums
conditions, the mean yield per infected cell ranged from 5.1 to 10.7 virions (Figure 2), suggesting that increased efficiency of virus replication occurred in the cells.

The relationship between increases in virus yields and infection rates of PAM in culture is summarized in Figure 3. The correlation coefficient between virus yield and infection rates was 0.95, which is highly suggestive of a direct relationship between these two parameters of ADE. The relationship between the two parameters is also represented by the equation: \( Y = 1.43X - 0.53 \). The slope of the equation is significantly (\( p < 0.01 \)) greater than 1, which suggests that one or more additional factors besides enhanced infection rate of PAM, such as enhanced efficiency of virus production, may account for the increases in virus yield.

The effect of protein A on ADE of PRRSV infection. An optimum dilution (1:128) of salt precipitated polyclonal IgG specific for PRRSV was treated with varying concentrations of Protein A to determine if ADE of PRRSV infection of PAM could be inhibited as determined by the modified ADE assay. The results of this experiment are summarized in Figure 4. The mean yield of progeny virus following infection of PAM with PRRSV at an m.o.i. of 0.01 after treatment of virus with normal serum globulin and media alone was 2.94 ± 0.16 and 2.93 ± 0.16 log\(_{10}\), respectively. The addition of Protein A to normal serum globulin or media did not affect these virus yields. The yield of progeny virus increased significantly (\( p < 0.01 \)) to 4.97 ± 0.17 log\(_{10}\) when the virus was treated with PRRSV-specific IgG in the absence of Protein A. In contrast, the virus yield was progressively reduced to 4.63 ± 0.09 (\( p < 0.05 \)), 3.36 ± 0.11 (\( p < 0.001 \)), 1.67 ± 0.14 (\( p < 0.001 \)), 1.79 ± 0.08 (\( p < 0.001 \)), and 1.71 ± 0.13 log\(_{10}\) (\( p < 0.001 \)) following the addition of Protein A to PRRSV-specific IgG at concentrations of 25, 50, 100, 150, and 200μg/ml, respectively.

The effect of ADE on the duration and level of viremia in pigs. The potential of PRRSV-induced antibody to enhance virus infection in vivo was evaluated using a completely randomized block design in which 16 pigs were equally and randomly assigned to 4 groups. Three groups were injected with quantities of polyclonal PRRSV-specific IgG to yield SVN antibody titers of \( \leq 1 \) log\(_2\) (Group 1), 2 log\(_2\) (Group 2), and 4 log\(_2\) (Group 3). A fourth group injected with normal serum globulin served as an untreated control.
Figure 2. Mean yield of infectious progeny PRRSV produced in individual porcine alveolar macrophages (PAM) infected with the ISU-P isolate of PRRSV in the presence and absence of homologous antibody.
Figure 3. The relationship between enhanced virus yields and infection rates of porcine alveolar macrophages (PAM) following treatment of PRRSV isolate ISU-P with 9 individual swine serums raised against isolate ISU-P

- = fitted curve

-- = 99% confidence interval
Figure 4. Blocking effect of staphylococcal protein A on the enhancement of PRRSV infection of porcine alveolar macrophages mediated by virus-specific polyclonal immunoglobulin (IgG)
Serum virus neutralizing and IPMA antibody titers together with ADE activity in undiluted serum on the day of virus challenge is summarized for all groups in Table 1. Significant ADE activity was detected in the undiluted sera of all pigs in Group 1 and in one pig in Group 2. No ADE activity was demonstrated in the undiluted sera of Group 3 pigs. The mean SVN titers of Groups 2 and 3 was 1.6 and 3.4 log$_2$, respectively. No SVN activity was detected among Group 1 pigs.

The response of each group with respect to the duration and the level of viremia following intramuscular challenge with isolate ISU-P are summarized in Figure 5. Both the mean duration and the mean level of viremia were significantly lower (p<0.05) for Group 3 than for all other groups, indicating that high SVN antibody titers suppress virus replication in the pig. In contrast, the mean duration and the mean level of viremia were significantly greater (p<0.05) for Group 1 than for the control group or Group 3, indicating that subneutralizing levels of antibody enhance virus replication. In addition, no significant differences were observed between Group 2 and the control group with respect to the duration and the level of viremia.

The response of all 4 groups following PRRSV challenge with respect to PBL profile is summarized in Figure 6. A transient leukopenia occurred in all groups and ranged in duration from 9 to 11 days. The maximum level of PBL depression occurred between 3 and 4 days following challenge. No significant differences were detected between groups (p>0.05). No signs of respiratory distress or inappetence were observed in any pig during the course of the experiment.

**Duration of ADE activity in pigs following the decline of antibodies below neutralizing levels.** The length of time that ADE activity may persist in pigs with declining levels of maternal or vaccine-induced PRRSV-specific antibodies was estimated in 4 pigs which were injected with polyclonal PRRSV-specific IgG. Serum virus neutralizing activity, IPMA antibody titers, and ADE activity in undiluted serum were subsequently monitored for an 80 day period. Results are summarized in Figure 7. The mean SVN antibody titer on day 1 post injection (PI) was 3.8 log$_2$ which subsequently declined to undetectable levels by day 37 PI. The mean IPMA antibody titer was 7.42 log$_2$ on day 1 PI and declined slowly thereafter until becoming undetectable by day 72 PI. Mean ADE activity was first
Table 1. Antibody dependent enhancement (ADE) activity, serum virus neutralization (SVN) antibody titers, and immunoperoxidase monolayer assay (IPMA) antibody titers in 4 groups of pigs on the day of challenge with PRRSV isolate ISU-P

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>ADE activity</th>
<th>SVN activity</th>
<th>Mean SVN titer (log₂)</th>
<th>Mean IPMA titer (log₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>4/4</td>
<td>0/4</td>
<td>N/A^d</td>
<td>5.32</td>
</tr>
<tr>
<td>Group 2</td>
<td>1/4</td>
<td>4/4</td>
<td>1.60</td>
<td>6.13</td>
</tr>
<tr>
<td>Group 3</td>
<td>0/4</td>
<td>4/4</td>
<td>3.32</td>
<td>7.32</td>
</tr>
<tr>
<td>Group 4</td>
<td>0/4</td>
<td>0/4</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

^a Each group contained 4 pigs each. Groups 1, 2, and 3 were injected with PRRSV-specific polyclonal immunoglobulin at a rate calculated to yield SVN antibody titers of ≤1, 2, and 4 log₂, respectively seven days before virus challenge. Group 4 was injected with normal swine serum globulin.

^b Number of pigs in which ADE activity was detected in undiluted serum.

^c Number of pigs in which SVN activity was detected.

^d NA= not applicable
Figure 5. The effect of neutralizing and subneutralizing levels of PRRSV-specific antibody on the (A) duration and (B) levels of viremia in pigs challenged by intramuscular injection of $10^3 \text{TCID}_{50}$ of PRRSV isolate ISU-P. Groups 1, 2, and 3 were injected with salt-precipitated PRRSV-specific polyclonal immunoglobulin at a rate to yield serum virus neutralizing antibody titers of ≤1, 2, and 4 log$_2$. Group 4 was injected with normal swine serum globulin. N = 4 for all groups

* significant difference at p<0.05
Figure 6. The peripheral blood leukocyte response in pigs that were injected with PRRSV-specific polyclonal immunoglobulin (IgG) and challenged 7 days later by intramuscular injection of $10^3$ TCID$_{50}$ of PRRSV isolate ISU-P. Groups 1, 2, and 3 were injected with concentrations of IgG to yield serum virus neutralizing antibody titers of $\leq 1$, 2, and 4 log$_2$. Group 4 was injected with normal swine serum globulin. $N = 4$ for all groups.
Figure 7. Antibody dependent enhancement (ADE) activity, serum virus neutralizing (SVN) antibody titers and immunoperoxidase monolayer assay (IPMA) antibody titers of 4 pigs that were initially injected intraperitoneally with PRRSV-specific polyclonal immunoglobulin at a rate to yield a SVN titer of 4 log₂ and monitored for a 79 day period.
observed in undiluted serum at day 20 PI when the mean SVN antibody titer was less than 1.6 log₂. The mean ADE activity peaked at day 41 PI, then slowly declined at approximately the same rate as the mean IPMA antibody titer until becoming undetectable at day 62 PI.

**Identification of PRRSV protein(s) associated with ADE.** Sera that were collected from the 4 passively immunized pigs described above were analyzed by western immunoblot to determine the specificity of the antibodies associated with ADE. Results of this analysis are summarized in Table 2. Antibodies specific for proteins with molecular masses of 15, 19, 23, and 26kD were detected in all 4 pigs through day 20 PI when ADE activity was first detected. The mean SVN antibody titer on day 20 PI was 1.6 log₂. By day 51 PI, immunoblotting only detected antibodies specific for the 15kD and the 26kD proteins. Antibody specific for the 26kD protein continued to be detected in all 4 treated pigs through day 72 PI, while antibodies specific for the 15kD protein persisted in all 4 pigs until day 51 PI and in 3 pigs until day 62 PI.

**DISCUSSION**

A preliminary study indicated that infection of PAM by PRRSV could be enhanced by treating the virus with subneutralizing levels of antibody prior to infecting PAM (2). Because ADE of virus infection has been shown to contribute to several other viral diseases and to interfere with immunization strategies for viruses such as dengue virus, feline infectious peritonitis virus, Aleutian disease virus of mink, and equine infectious anemia virus (21), it was important to know if ADE has the potential to contribute to the pathogenesis of PRRSV infection and to interfere with immunization strategies for PRRS.

In the work reported here, infection of PAM by the PRRSV was significantly enhanced in vitro by the presence of antibody raised against the PRRSV isolate ISU-P (p<0.01), indicating that PRRSV induces antibodies which are capable of enhancing PRRSV infection (Figure 1). Antibody dependent enhancement of virus infection was also demonstrated in vivo. The mean level and duration of viremia was greater in pigs injected with subneutralizing amounts of PRRSV-specific IgG prior to virus challenge than in control pigs injected with normal serum globulin (Figure 5). In a separate experiment, the time period that pigs
Table 2. Western immunoblot analysis of serums collected from 4 pigs over a 79 day period following injection of PRRSV-specific immunoglobulin (IgG) at a rate to yield a serum virus neutralization antibody titer of 4 log₂

<table>
<thead>
<tr>
<th>PRRSV proteins</th>
<th>Days following injection of IgG</th>
<th>0</th>
<th>1</th>
<th>9</th>
<th>20</th>
<th>30</th>
<th>41</th>
<th>51</th>
<th>62</th>
<th>72</th>
<th>79</th>
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<tbody>
<tr>
<td>26 kD</td>
<td></td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>0</td>
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<tr>
<td>23 kD</td>
<td></td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>4</td>
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<td>0</td>
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<td>0</td>
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</tr>
<tr>
<td>19 kD</td>
<td></td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>2</td>
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<tr>
<td>15 kD</td>
<td></td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>0</td>
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</tr>
</tbody>
</table>

*Number of pigs in which antibody specific for each protein was detected.*
with declining levels of PRRSV-specific antibodies could be expected to be susceptible to ADE was estimated using 4 pigs that were injected with virus specific IgG. Antibody dependent enhancement activity was first detected in undiluted sera when SVN antibody titers dropped below 2 log₂ and persisted in undiluted sera of the pigs for periods that ranged from 5 to 6 weeks, thereafter (Figure 7). Collectively, these observations strongly suggest that ADE of PRRSV infection has the potential to enhance the severity of disease and possibly the susceptibility to PRRSV infection in pigs with declining levels of PRRSV-specific antibodies of maternal origin, or antibodies induced by exposure to wild type or vaccine PRRSV.

In the present study, a direct correlation (r=0.95) was demonstrated between increased virus yields and infection rates of PAM that were mediated by PRRSV-specific antibody. In addition, the ratio between increases in virus yield and infection rate was greater than 1.4 (Figure 3). These observations suggest that more than one mechanism contributed to the enhancement of virus yield in the presence of virus-specific antibody. This conclusion is consistent with the observations that not only was the infection rate of PAM increased but also the yield of progeny virions from individual PAM (Figure 2). The primary mechanism responsible for increased virus yields appears to have been due to an increase in the infection rates of PAM which was facilitated by an interaction between Fc receptors of PAM and virus-antibody complexes. We base this conclusion on the observation that virus yields were significantly reduced when virus/antibody preparations were treated with Protein A prior to exposing PAM to virus (Figure 4). Similar observations have been made by other investigators who studied ADE of DV (10), FIPV (28), West Nile virus (WNV) (29), and ADV infection (13). Increased efficiency of virus production in individual cells in the presence of antibody may have also contributed to increased yields of PRRSV in PAM. This possibility has been previously reported for other viral infections. For example, Robbinson et al. (32) observed higher production of viral components in cells that were infected with human immunodeficiency virus in the presence of virus-specific antibody than in the absence of antibody. The mechanism responsible for the enhanced production of viral components in individual cells was not determined. However, Gollins et al. (5) who studied ADE of WNV presented data that suggest that multiple viral infection of individual cells could
increase virus production. We do not believe that multiple viral infection of individual PAM was responsible for the increased efficiency of virus production in our study because a low m.o.i., i.e., 0.01 was used and infection rates were significantly increased. Nonetheless, the specific mechanism for ADE of PRRSV infection remains to be determined.

Western immunoblot analysis revealed the presence of antibody specific for the 15kD nucleocapsid protein (26) and the envelope-associated 26kD glycoprotein (26) throughout the period that ADE activity was present in undiluted serum of pigs that were injected with PRRSV-specific IgG (Table 2). The location of the 26kD protein in the viral envelope suggests that antibodies induced by this protein are associated with ADE. Other investigators have reported that ADE of other virus infections is also mediated by antibodies induced by envelope-associated proteins (4,11,28,33,34). It is also possible that other envelope-associated proteins of PRRSV that were not detected by our assay, such as the 43kD glycoprotein described by Madassi et al. (20), may induce antibodies that contribute to ADE. The internal location of the 15kD nucleocapsid protein within the virion leads us to believe that antibodies induced by this protein were not associated with ADE even though they were detected throughout the period that ADE activity was detected in undiluted serum. However, it is conceivable that antibody specific for the 15kD protein could contribute to enhancement of PRRSV infection if the integrity of the viral envelope is compromised by host defense mechanisms.

The above study indicates that ADE of PRRSV infection has the potential to contribute to the severity of disease and possibly to increase the susceptibility of pigs to infection by PRRSV. These possibilities are of particular concern with respect to young pigs with declining levels of maternal antibodies since these young pigs are highly susceptible to respiratory disease caused by PRRSV (15,30,41). Results of our study also showed that virus replication was significantly suppressed in pigs with virus neutralizing antibody titers greater than 3.4 log₂ and that ADE activity was not detected in undiluted serum until the SVN titers fell below 2 log₂. Consequently, potential adverse affects mediated by ADE might be avoided by designing immunization strategies that maintain high serum virus neutralizing activity in young pigs.
ACKNOWLEDGMENTS

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REFERENCES


FIELD ISOLATES OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS VARY IN THEIR SUSCEPTIBILITY TO ANTIBODY DEPENDENT ENHANCEMENT OF INFECTION

A paper accepted in Veterinary Microbiology

Kyoung-Jin Yoon, Lie-Ling Wu, Jeffrey J. Zimmerman, Kenneth B. Platt

Abstract

Seventeen porcine reproductive and respiratory syndrome virus (PRRSV) field isolates, including isolate ISU-P, were evaluated for their susceptibility to antibody dependent enhancement (ADE) of infection mediated by antibodies raised against PRRSV isolate ISU-P. Progeny virus yields of ISU-P and 4 of 16 field isolates in porcine alveolar macrophages (PAM) were reduced following treatment with a concentration of antibody that neutralized ISU-P (p<0.01). In contrast, the yields of 12 of 17 field isolates were enhanced (p<0.01). Treatment of all isolates with a 10-fold lower concentration of this antibody significantly (p<0.01) increased virus yields of all isolates in PAM. However, the degree of enhancement varied among the isolates when compared to the enhancement of the yield of ISU-P. While no differences in enhancement were observed among ISU-P and 9 field isolates, yield enhancement of 6 and 1 isolates were less than and more than the yield enhancement of ISU-P, respectively (p<0.05). The degree of enhancement mediated by a high concentration of antibody raised against ISU-P was inversely proportional to the ability of the antibody to neutralize the isolates (r=0.92). In contrast, no direct correlation (r=0.32) was observed between the degree of enhancement mediated by a low concentration of antibody and the ability of the antibody to neutralize the isolates. These data suggest that the variability in the susceptibility of PRRSV isolates to ADE arise from quantitative and/or qualitative differences in the antigenic determinants associated with virus neutralization and/or ADE. The antigenic diversity and the wide range in the susceptibility to ADE that exists among field isolates indicate that ADE should be taken into consideration in the development of effective immunization strategies for PRRS.
1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a highly contagious disease of swine manifested by reproductive failure in breeding age swine and respiratory disease in young pigs (Christianson and Joo, 1994). It is caused by an enveloped, single-stranded RNA virus that has been provisionally classified as a member of the Arterivirus genus of the family Togaviridae (Meulenburg et al., 1993). Currently, the use of vaccines are being advocated to control this disease. However, field observations suggest that the use of a vaccine may exacerbate the clinical manifestations of PRRS in some herds (B. Thacker, personal communication; G. Erickson, personal communication). One possible explanation for these observations is antibody dependent enhancement (ADE) of virus infection in which antibodies of maternal origin or induced by vaccination increase the susceptibility of pigs to vaccine or endemic strains of porcine reproductive and respiratory syndrome virus (PRRSV) as well as contribute to the severity of infection. This possibility has been suggested by results of an earlier study in which that the level and duration of viremia was significantly greater in pigs that were infected with virus following the injection of subneutralizing levels of polyclonal PRRSV-specific IgG than in PRRSV antibody-free control pigs (Yoon et al., 1995c).

Antibody dependent enhancement of virus infection has been reported for several other viruses, most notably dengue virus (DV) of humans (Kurane et al., 1992). More importantly, ADE has been implicated as a significant obstacle to the development of effective vaccines and vaccination strategies for several viruses, including DV, feline infectious peritonitis virus (FIPV), equine infectious anemia virus (EIAV), and Aleutian disease virus of mink (Mascolar et al., 1993). A common characteristic of these viruses is their antigenic variability which has been shown to contribute to not only their susceptibility to ADE but, in some cases, to their ability to induce ADE activity. For example, Halstead and his co-workers (1977 and 1984) have demonstrated that different DV serotypes, as well as different strains within the same serotype, varied in their susceptibility to ADE and in their ability to induce antibodies associated with ADE. Similar observations have been reported by Scott and his associates with respect to FIPV (Corapi et al., 1992; Olsen et al., 1992). Broad antigenic variation among PRRSV isolates has also been demonstrated by several investigators (McGinley et al.,
Consequently, the possibility exists that PRRSV isolates may also vary in their susceptibility to ADE. The following study was conducted to determine if such variations exist among field isolates of PRRSV which could interfere with immunization strategies.

2. Materials and methods

2.1 Media and reagents

All growth, maintenance, and freezing media were supplemented with fetal bovine serum (Sigma Chemical Co., St. Louis, MO, USA) at a rate of 10%, 2%, and 20%, respectively. Each of these media contained 10mM of HEPES and a mixture of antibiotic-antimycotic agents (Sigma Chemical Co., St. Louis, MO, USA) consisting of 100IU/ml penicillin, 10μg/ml streptomycin, 50μg/ml gentamicin, and 0.25μg/ml amphotericin B. In addition, freezing media contained 10% DMSO.

2.2 Anti-PRRSV reference serum

Sera were obtained from 3 pigs at 60 to 63 days following nasal inoculation with 10^4 TCID_{50}/ml of PRRSV isolate ISU-P. The virus neutralizing antibody titers of the 3 sera ranged from 5 to 6 log_{2}. Antibody titers that were determined by the immunoperoxidase monolayer assay ranged from 8.32 to 10.3 log_{2}. Control sera were obtained from PRRSV antibody-free SPF pigs of similar age. Each of the 3 reference antisera was paired randomly with a control serum. Each pair of antibody-positive and control sera constituted one set.

2.3 Cells

Porcine alveolar macrophages (PAM) were used for ADE assays. The cells were collected from lungs of 4- to 6-week-old PRRSV-free pigs by lung lavage using Hanks' balanced salt solution (Sigma Chemical Co., St. Louis, MO, USA) and harvested by centrifugation as previously described (Yoon et al., 1995c). Harvested cells were suspended in freezing media at a concentration of 5 x 10^7 cells/ml and stored at -70°C until used. Immediately prior to use, frozen cells were rapidly thawed in a 37°C water bath and suspended in RPMI-1640 growth media at a concentration of 2 x 10^6 viable PAM per ml. The total number of
viable cells was determined by dye exclusion (Kaltenbach et al., 1958) using a 0.04% trypan blue solution. The proportion of PAM in the viable cell population was determined by a nonspecific esterase test (Kasplow, 1981).

The MARC-145 clone (Kim et al., 1993) of the African green monkey kidney cell line, MA104 was used for virus assays and the serum virus neutralization (SVN) test. These cells were grown in Dulbecco's modified Eagle's media (Sigma Chemical Co., St. Louis, MO, USA).

2.4 Virus

A total of 17 PRRSV isolates were evaluated in the study. The PRRSV isolate ISU-P (ATCC VR-2402) was included among these isolates and used as a reference strain for all comparisons. This virus was initially isolated in 1992 from an homogenate prepared from a pool of lungs collected from young pigs in a herd in which an acute PRRS outbreak was occurring in the state of Illinois, USA. The isolate was cloned by 3 rounds of limiting dilution in PAM and subsequently plaque cloned twice in MA104 cells. Sixteen PRRSV field isolates were obtained from the National Veterinary Services Laboratories, Ames, Iowa. These isolates represented the states of Arizona (AZ), Iowa (IA), Illinois (IL), Kansas (KS), Michigan (MI), Minnesota (MN), and North Carolina (NC) and were isolated from field specimens during the years 1989 through 1993. Field isolates were not cloned. The working stock of all virus isolates used in the study represented the third or fourth passage in MA104 cells.

2.5 Virus assay

Virus titration was performed in 96-well microtiter plates (Corning Costar Corp., Cambridge, MA USA) using MARC-145 cells as previously described (Yoon et al., 1995b). Virus titers were determined by the method of Reed and Muench (1938) and expressed as TCID₅₀/ml.

2.6 ADE assays

A standard and a modified ADE assay were used to determine the relative susceptibility of different PRRSV isolates to ADE. The standard assay was conducted as previously described (Yoon et al., 1995c). In brief, each set of PRRSV-specific antibody-positive and antibody-free sera was heat-inactivated at
56°C for 45 minutes and serially diluted 2- and 10-fold in RPMI growth media over a range beyond detectable SVN activity. One and a half ml of each dilution of the 3 serum sets and RPMI growth media alone were separately mixed with an equal volume of RPMI-1640 media containing $10^{4.3}$ TCID$_{50}$/ml of a specific PRRSV isolate. These mixtures were incubated for 60 minutes at 37°C. Subsequently, 1 ml aliquots of each mixture were inoculated in triplicate into individual wells of 48-well cell culture plates (Corning Costar Corp., Cambridge, MA USA) that had been inoculated with $10^6$ PAM one day earlier. The plates were again incubated at 37°C for 60 minutes. Inoculums were replaced with 0.5 ml RPMI growth media and the cultures were incubated for an additional 48 hours at 37°C. The virus-infected cell cultures were then frozen and thawed once at -70°C and 37°C, respectively, and clarified by centrifugation at 1000 x g for 10 minutes. The supernatant from each well was harvested and assayed for virus activity using MARC 145 cells. The increased yield of virus mediated by PRRSV-specific antibody was determined for each dilution using the formula:

$$\text{Yield difference} = (Y_{ts} - Y_m) - (Y_{cs} - Y_m);$$

where $Y_{ts} =$ yield of virus in the presence of PRRSV specific antibody, $Y_m =$ yield of virus in the presence of RPMI alone, and $Y_{cs} =$ yield of virus in the presence of PRRSV specific antibody-free control serum.

The modified ADE assay was conducted in the same manner as the standard ADE assay with the exception that individual PRRSV isolates were individually treated with a $10^{-1}$ and a $10^{-2}$ dilution of each set of sera. Increased virus yields were determined for each isolate as described above.

2.7 Serum virus neutralization

The SVN test was used in one-way format to determine SVN indices of the 17 PRRSV isolates. The SVN test was conducted as previously described in 96-well microtiter plates using MARC-145 cell monolayers (Yoon et al., 1995b). The titers of all 3 antisera described above in which each isolate was used separately as antigen were determined in duplicate on the same day. This procedure was repeated on 3 separate days and geometric mean (GM) titers were calculated.
2.8 Experimental design

Two experiments were conducted. In the first experiment, 16 PRRSV field isolates were evaluated by the modified ADE assay to determine their relative susceptibility to ADE with respect to that of PRRSV isolate ISU-P. All isolates were coded to avoid test bias and assayed for ADE in triplicate using 2 different dilutions of each set of antibody-positive and negative control sera. The assay was repeated on 3 separate days using the same lot of PAM. Yield differences of each isolate were compared to the yield difference of PRRSV isolate ISU-P by Student's \( t \) test. Subsequently, Duncan's multiple range test was used to determine the relatedness of PRRSV isolates in terms of their relative susceptibility to ADE.

Two PRRSV isolates, AZ-1 and KS-1, were identified by the modified assay as being the most and the least affected by ADE. The susceptibility of these 2 isolates and ISU-P to ADE was further evaluated by the standard ADE assay. The assay was repeated on 3 separate days using the same lot of PAM. Yield differences following treatment with the 3 sets of antibody-positive and control sera were modeled for each isolate by regression on serum dilutions and compared by the general linear test (Neter et al., 1990). Subsequently, the area under the fitted curves which represented ADE of virus yields was determined for each isolate by integration and compared by analysis of variance (ANOVA).

In the second experiment, the relationship between susceptibility of an isolate to both neutralization and ADE was determined. For this purpose, we conducted the one-way SVN test using the same 3 reference antisera that were used in the first experiment and determined the SVN indices as described by McGinley et al. (1993) for 17 isolates including isolate ISU-P. The SVN index of an individual isolate was defined as:

\[
\text{SVN index (\%)} = \frac{\text{GM titer determined with individual isolate} \times 100}{\text{GM titer determined with isolate ISU-P}}
\]

The relative sensitivity of individual isolates to neutralization was determined by comparing SVN indices using ANOVA. Subsequently, the relationship between susceptibility to neutralization and to ADE which was assessed by the
modified ADE assay in the first experiment was determined by correlation analysis.

3. Results

3.1 Variability in the susceptibility of PRRSV field isolates to ADE

Seventeen PRRSV field isolates, including isolate ISU-P, were evaluated to determine their relative susceptibility to ADE mediated by a high and a low concentration of antibody raised against PRRSV isolate ISU-P. Results of this experiment are summarized in Figure 1. Treatment of isolates with a high concentration of antibody raised against PRRSV isolate ISU-P prior to infecting PAM resulted in a reduction of virus yield of ISU-P and 4 additional isolates (IA-7, IA-2, NC-1, IA-3). The virus yields of these isolates were reduced by 0.61 ± 0.08, 0.32 ± 0.08, 0.24 ± 0.14, 0.13 ± 0.07 log_{10}, respectively. In contrast, virus yields of the remaining 12 isolates were increased by treating with the same concentration of the antibody. Increases in virus yields ranged from 0.24 ± 0.00 log_{10} for isolate IL-1, to 0.76 ± 0.14 log_{10} for isolate NC-8 (Figure 1A). No increases or reductions in virus yields were observed among the 16 isolates when they were treated in the same manner with normal control serum.

No reduction in virus yields of any isolate occurred when individual isolates were treated with a 10-fold lower concentration of antibody. Increases in virus yields varied among the isolates (p<0.01) and ranged from 0.16 ± 0.08 log_{10} for isolate KS-1 to 1.83 ± 0.17 log_{10} for isolate AZ-1 (Figure 1B). Yield increase of 1 isolate (AZ-1) was greater than the yield increase of ISU-P (p<0.01), while yield increases of 6 isolates were significantly less than the yield increase of ISU-P (p<0.01). No differences in yield increase were observed among the remaining 9 isolates and ISU-P.

The 2 isolates showing the most (AZ-1) and the least (KS-1) susceptibility to ADE in the modified assay and isolate ISU-P were further evaluated by the standard ADE assay. Results of this experiment are summarized in Figure 2. Treatment of the 3 isolates with PRRSV-specific antiserum prior to infecting PAM significantly (p<0.01) increased the yield of progeny virus of each isolate. The areas described by the fitted curves which are proportional to the virus yields for isolates KS-1, ISU-P, and AZ-1 were 1.56 ± 0.05, 4.17 ± 0.02, and 10.02 ± 0.04, respectively. These results support the finding revealed by the modified ADE
Figure 1. Variability in the susceptibility of 17 North American PRRSV isolates to antibody dependent enhancement mediated by (A) high and (B) low concentrations of porcine antibody raised against PRRSV isolate ISU-P
Figure 2. Comparison of antibody dependent enhancement of infection of 3 PRRSV field isolates AZ-1, ISU-P, and KS-1 in porcine alveolar macrophages following treatment with porcine antibodies raised against the PRRSV isolate ISU-P. Symbols are observations (n=9) and lines are fitted curves for each of the 3 isolates:

AZ-1: \( Y = -0.0085X^3 + 0.0443X^2 + 0.3898X + 2.5671 \)

ISU-P: \( Y = -0.0115X^3 + 0.1126X^2 + 0.0927X + 1.7215 \)

KS-1: \( Y = 0.0056X^3 - 0.1137X^2 + 0.6293X + 2.2277 \)

Control: \( Y = 2.833 \)
assay that PRRSV field isolates vary widely with respect to their susceptibility to ADE.

3.2 The relationship between the relative susceptibility of PRRSV isolates to neutralization and ADE

The 17 PRRSV isolates were compared by a one-way SVN assay for their susceptibility to neutralization by antibodies raised against ISU-P. Results are summarized in Figure 3. The relative difference in the susceptibility of individual isolates to neutralization is reflected by the SVN antibody titers which were determined by the SVN assay using each individual isolate as antigen. The mean SVN antibody titer determined with isolate ISU-P was 5.3 log2. In contrast, the mean SVN antibody titer of the same antiserum that was determined with each of the 16 different isolates ranged from 0.7 (isolate AZ-1) to 4.3 log2 (isolate IA-3). SVN indices of these 16 isolates ranged from 13.2 to 81.1%, indicating that the isolates vary in their susceptibility to neutralization (p<0.01).

The SVN indices of individual isolates were highly correlated (r=0.95), but inversely related to the virus yields of the isolates following treatment with a high concentration of antibody (Figure 4A). This observation indicates that PRRSV isolates with low SVN indices are more susceptible to ADE of infection than isolates with high SVN indices. A similar relationship was observed between SVN indices and the susceptibility to ADE among isolates treated with a 10-fold lower concentration of the same antibody. However, the degree of correlation was markedly lower (r=0.32) because 5 of the 16 isolates fell outside of the 99% confidence interval. The exclusion of these 5 isolates from the analysis also revealed a high degree of correlation (r=0.91) between susceptibility to neutralization and ADE. The lack of correlation between SVN indices and the susceptibility to ADE among 5 isolates could be a reflection of quantitative and qualitative differences in the antigenic determinants of the 5 isolates.

4. Discussion

The primary objective of the above study was to determine if field isolates of PRRSV vary in their susceptibility to ADE. Such a variation could have an adverse effect on immunization strategies for PRRS. Our results clearly indicate that field isolates do vary in this respect. This characteristic of PRRSV was
Figure 3. Antigenic variation of PRRSV field isolates from 7 different states in United States of America as revealed by a one-way serum virus neutralization assay using porcine antibody raised against PRRSV isolate ISU-P.
Figure 4. The relationship between the relative susceptibility of individual PRRSV isolates to neutralization and antibody dependent enhancement in the presence of (A) high and (B) low concentrations of porcine antibody raised against PRRSV isolate ISU-P
- fitted curve
-- 99% confidence interval
dramatically illustrated by the neutralization of ISU-P by a concentration of homologous antibody that significantly (p<0.01) enhanced the replication of 12 of 16 other field isolates tested (Figure 1A). This observation suggests that antibodies induced by vaccine strains of PRRSV could also enhance the replication of PRRSV field isolates in vaccinated animals. In addition, our findings suggest that the replication of vaccine virus in the host could also be enhanced by maternal antibody specific for field isolates of PRRSV. Both of these possibilities could be contributing to the exacerbation of PRRS that is occasionally observed in North American swine herds in which vaccine is being used.

Results of an earlier study in which the potential of ADE of PRRSV infection to contribute to disease was demonstrated (Yoon et al., 1995c) and the preceding study suggests that vaccine candidates should be evaluated for their ability to induce ADE activity prior to their use in the field. In the present study, we demonstrated that isolates with high SVN indices are less susceptible to ADE than isolates with low SVN indices (Figure 4). Consequently, it may be possible to use SVN indices of field isolates, that are determined with antiserum specific for individual vaccine candidates, as surrogate markers for ADE.

The apparent lack of correlation between susceptibility to SVN and ADE mediated by a low concentration of antibody associated with 5 PRRSV isolates (Figure 4B) suggests that ADE can also be mediated by antibody specific for antigenic determinants that are only partially associated with SVN or not associated at all. Studies of FIPV (Corapi et al., 1992) and DV (Henchal et al., 1985; Morens et al., 1987) have revealed the existence of such antigenic determinants. Consequently, the identification of these antigenic determinants in PRRSV and their exclusion from vaccines may be beneficial.

Acknowledgments
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References


CHARACTERIZATION OF THE HUMORAL IMMUNE RESPONSE TO PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS INFECTION

A paper published in the Journal of Veterinary Diagnostic Investigation (Volume 7, pages 305-312, 1995)


ABSTRACT

The development of the humoral immune response against porcine reproductive and respiratory syndrome virus (PRRSV) was monitored by an indirect fluorescent antibody (IFA) test, immunoperoxidase monolayer assay (IPMA), enzyme-linked immunosorbent assay (ELISA), and serum virus neutralization (SVN) test over a 105-day period in 8 pigs experimentally infected with ATCC strain VR-2402. Specific antibodies against PRRSV were first detected by the IFA, IPMA, ELISA, and the SVN test 9-11, 5-9, 9-13, and 9-28 days postinoculation (PI) and reached their maximum values by 4-5, 5-6, 4-6, and 10-11 weeks PI, respectively, thereafter. After reaching maximum value, all assays showed a decline in antibody levels. Assuming a constant rate of antibody decay, it was estimated by regression analysis that the ELISA, IFA, IPMA, and SVN antibody titers would approach the lower limits of detection by approximately days 137, 158, 324, and 356 PI, respectively. In this study, the immunoperoxidase monolayer assay appeared to offer slightly better performance relative to IFA, ELISA, and SVN in terms of earlier detection and slower rate of decline in antibody titers.

Western immunoblot analysis revealed that antibody specific for the 15 kilodaltons (kD) viral protein was present in all pigs by 7 days PI and persisted throughout the 105-day observation period. Initial detection of antibodies to the 19, 23, and 26 kD proteins varied among pigs, ranging from 9- to 35-days PI. Thereafter, the antibody responses to these 3 viral proteins of PRRSV continued
to be detected throughout the 105-day study period. Among these 3 proteins, antibodies specific for the 26kD protein were present immediately before or at the time the SVN activity was detected. These results clearly indicate that the 15 kD protein is the most immunogenic of the 4 viral proteins identified and may provide the antigenic basis for the development of improved diagnostic tests for the detection of PRRSV antibodies. The 26kD protein appears to be highly associated with the induction of neutralizing antibody.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is a relatively new viral disease of swine. In 1987, PRRS was reported as a new, devastating disease of swine in the United States.\textsuperscript{11} It continues to be an economically significant health problem in swine producing regions throughout the world due to losses from respiratory disease in neonates and nursery pigs, and reproductive losses in breeding stock.\textsuperscript{8,13} In response to the economic importance of PRRS, a significant research effort has been mounted to develop reliable diagnostic tests and effective vaccines.

An enveloped RNA virus was identified as the causative agent for PRRS and provisionally assigned to the \textit{Arterivirus} genus of the family \textit{Togaviridae} on the basis of morphological and biological characteristics, genomic organization, and the strategy of gene expression.\textsuperscript{4,9,15,24,25} Although genomic sequence analysis has suggested that the virus may contain as many as 6 structural proteins,\textsuperscript{9,15} only 3 structural proteins with molecular masses of approximately 15, 19, and 26 kilodaltons (kD) have been consistently demonstrated.\textsuperscript{4,25} The functions of these proteins have not been completely determined, but current evidence indicates that the 15kD protein is a nucleocapsid protein, whereas the 19 and 26kD proteins are presumed to be components of the viral envelope.\textsuperscript{3,9,15} The immunobiological roles of the viral proteins have not been characterized, although researchers have speculated that the 26kD protein may be associated with induction of serum neutralizing antibodies against porcine reproductive and respiratory syndrome virus (PRRSV).\textsuperscript{7}

The indirect fluorescent antibody (IFA) test,\textsuperscript{26} serum virus neutralization (SVN) test,\textsuperscript{4,16} immunoperoxidase monolayer assay (IPMA),\textsuperscript{24} and enzyme-
linked immunosorbent assay (ELISA)\(^1\) have been described for the detection of specific antibodies against PRRSV. Currently, most North American veterinary diagnostic laboratories are using the IFA test and/or the SVN test to detect PRRSV-specific antibodies, while European laboratories have relied on the IPMA using PRRSV-infected porcine alveolar macrophages (PAM).\(^8\,^2\) The recent licensure of a commercial ELISA kit (IDEXX) will probably change this picture.

The IFA and IPMA are thought to be highly specific and sensitive tests.\(^2\) Antibodies to PRRSV are usually detected by these tests between 7 and 15 days after infection. Both IFA and IPMA appear to be accurate for 2 to 3 months after infection, but may lose their ability to detect antibodies against PRRS virus as soon as 3 to 6 months after exposure to PRRSV.\(^1\,^1\,^0\,^2\) The ELISA format is also reported to be sensitive and specific.\(^1\) One study found that PRRSV-specific antibodies could be detected by ELISA as early as 10 days after exposure.\(^2\) As yet, the performance of the commercial ELISA has not been reported. The SVN test is also considered to be a specific test, but previous studies have suggested that the SVN test is less sensitive than the IFA and IPMA tests.\(^3\,^5\,^1\,^1\) Neutralizing antibodies against PRRSV may develop as late as 1 to 2 months after infection.\(^1\,^1\,^6\,^2\) However, a recent report indicated that the sensitivity of the SVN test could be increased by adding fresh normal swine serum to serum being assayed.\(^2\) Although difference in the performance is believed to exist among the 4 PRRS serologic tests described above, lack of comparative information on the performance of PRRS serological tests makes it difficult for diagnosticians to standardize the test in use.

Field observations suggest that humoral immunity against PRRSV persists in pigs as long as 1 year after initial outbreak.\(^1\,^6\,^2\) Field observations have also revealed that some herds experience 'cyclic' recurrence of PRRS approximately every 6 to 10 months after initial outbreak.\(^2\) One possible explanation for this observation may be antibody dependent enhancement (ADE) of virus infection. A recent study in our laboratory has demonstrated that PRRSV infection can be enhanced \textit{in vitro} and \textit{in vivo} by the presence of subneutralizing levels of virus-specific antibodies.\(^2\) This study also indicated that ADE potential may persist for 5 to 6 weeks in passively immunized pigs after SVN antibody titers fell below 1:4, suggesting that pigs with declining levels of
PRRSV-specific antibodies may be at increased risk for increased susceptibility to infection and a severe clinical manifestation of PRRS.

The following study was conducted to both characterize the ontogeny of humoral immune response of pigs to PRRSV and to compare the performance of the IFA, IPMA, ELISA and SVN test using sera collected from a homogeneous group of pigs over a 105 day period following nasal inoculation with the ISU-P PRRSV isolate (ATCC VR-2402). Duration of humoral immunity against PRRSV was also estimated to determine if ADE potential exists in pigs with declining level of antibody induced by previous exposure to the virus. In addition, the viral protein specificity of the antibody response of these pigs was determined by western immunoblotting.

MATERIALS AND METHODS

Experimental design  Eight 5- to 6-week-old crossbred pigs were obtained from a PRRSV-free herd. Pigs were numbered and randomly assigned to 2 separately housed groups of 4 pigs each. All pigs were intranasally inoculated with $10^{4.5}$ TCID$_{50}$ of PRRSV. Serum samples were collected from each pig prior to inoculation, every other day during the first 15 days postinoculation (PI), and weekly thereafter through day 105. All sera were aliquoted and stored at $-20^\circ$C until assayed for PRRS virus-specific antibodies by the IFA, IPMA, ELISA, and SVN tests. Aliquots of each serum sample were stored at $-70^\circ$C until assayed for the presence of virus. Prior to performing the serological tests, serum samples were randomized and re-numbered. Results from the serological assays were compared by curvilinear regression.

The viral protein specificity of the antibody response was determined by western immunoblotting in 7 pigs using the same serum samples described above. Pigs were selected for the assay based on the time that neutralizing antibody were first detected, i.e., early responders (3 pigs) in which neutralizing antibodies appeared on days 9 or 11, and late responders (4 pigs) in which neutralizing antibodies were first detected on days 21 or 28 PI. Serum virus neutralizing antibody was not detected in one of the 8 pigs until day 15 PI which prevented its classification as an early or late responder. Consequently sera from this pig were not assayed by western immunoblotting.
**Virus** A cytopathic field isolate of the PRRSV designated ISU-P (ATCC VR-2402) was used in the study. The virus was originally isolated from pigs in a herd undergoing an acute outbreak of PRRS using virus-free porcine alveolar macrophages (PAM). The isolate was purified by limiting dilution in PAM culture and plaque-cloned twice in MA104 cells. The virus reproduced clinical disease compatible with PRRS in experimentally infected pigs and was recognized by PRRSV-specific monoclonal antibody, SDOW17 and polyclonal swine serum raised against PRRSV ATCC VR-2332. In addition, electron microscopy and western immunoblotting revealed a morphological structure and protein composition similar to that reported by other investigators (Pol J and Wagenaar F: 1992, Am Assoc Swine Pract Newsl 4(4):29).

**Virus isolation** Serum samples were diluted 1:5 in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 10mM HEPES, and an antibiotic-antimycotic mixture (100U/ml penicillin, 100μg/ml streptomycin, gentamicin 50μg/ml, 0.25μg/ml amphotericin B). Diluted samples (0.2 ml) were inoculated onto 24-hour-old PAM cultures prepared in 48-well plates. The inoculated cells were incubated at 37°C and observed for the cytopathic effect (CPE) typical of the virus for up to 7 days PI. The presence of PRRS virus in the cultures showing CPE was confirmed by subinoculating onto MA104 cell monolayers prepared on 8-chambered glass slides, incubating for 48 hours, and staining with SDOW17 conjugated with fluorescein isothiocyanate. A sample was considered negative after 2 blind passages in PAM.

**Indirect fluorescent antibody test** The IFA test was performed using a protocol developed at the National Veterinary Services Laboratories, Ames, IA (Frey M, Eernisse K, Landgraf J, et al.: 1992, Am Assoc Swine Pract Newsl 4(4):31). In brief, MA104 cells were placed in 8-chambered glass slides and incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and the antibiotic-antimycotic mixture for 48 to 72 hours at 37°C in a humidified 5% CO₂ atmosphere. PRRSV-infected MA104 cell monolayers were prepared by inoculating culture medium with sufficient PRRS virus to produce 15 to 20 plaque-forming units and incubating for 20 hours at 37°C. The monolayers were then fixed by immersion in 100% acetone for 10 minutes, air dried, and stored at -70°C until used. Viral antigen-free cell controls were prepared in an identical manner. Sera to be tested were serially diluted 2-fold in
0.01M phosphate-buffered saline, pH 7.2 (PBS) beginning with 1:20 dilution. Individual chambers were inoculated with 50μl of each serum dilution and incubated at 37°C for 30 minutes in a humid environment. The preparations were then washed 3 times for 10 minutes each with PBS. Antigen-antibody reactions were visualized by reacting potential antigen-antibody complexes with optimally diluted goat anti-swine IgG conjugated with fluorescein isothiocyanate for 30 minutes at 37°C in a humid environment. Indirect fluorescent antibody titers were recorded as the reciprocal of the highest serum dilution in which typical cytoplasmic fluorescence was observed.

**Immunoperoxidase monolayer assay**  
Porcine reproductive and respiratory syndrome virus-infected MA104 cell monolayers were prepared in 96-well microtitration plates by replacing DMEM supplemented with 10% FBS, 10mM HEPES, antibiotic-antimycotic mixture on confluent 1-day-old monolayers with the media (50μl/well) containing 10^2 TCID₅₀ of virus. After 1 hour incubation at 37°C, 100μl of the media were added to individual wells. Microtitration plates containing both infected and uninfected (control) cell monolayers were maintained at 37°C for 2 days, fixed with cold acetone:methanol (70:30) for 10 minutes, and stored at -20°C until used. Serum samples were serially diluted 2-fold in 0.1M Tris-HCl buffer (pH 7.6) beginning at 1:20. Immediately prior to use, fixed cell monolayers were treated with commercially supplied, pre-diluted, normal goat serum for 30 minutes at ambient temperature to block non-specific binding sites. Subsequently, duplicate 50μl aliquot of each serum dilution were added to individual wells. The preparations were then incubated at 37°C for 30 minutes and washed 3 times with Tris-HCl buffer. Antigen-antibody reactions were visualized with a biotin-streptavidin horseradish peroxidase system using the protocol provided by the manufacturer. In brief, 50μl of biotinylated goat anti-swine IgG conjugate were added to individual wells and permitted to react with potential antibody-antigen complexes for 30 minutes at 37°C in a humidity chamber. The plates were washed as described above and excess buffer removed. Fifty μl of streptavidin conjugated to peroxidase was then added to individual wells and incubated at ambient temperature for 30 minutes. The plates were washed as described above, and antigen-antibody reactions were visualized by adding 100μl of diaminobenzidine tetrachloride substrate to individual wells and incubating for 5 to 10 minutes at ambient temperature. The color reaction
was stopped by washing with distilled, deionized water three times. Immunoperoxidase monolayer assay antibody titers were recorded as the reciprocal of the highest serum dilution in which a specific color reaction was observed.

**Enzyme-linked immunosorbent assay**  The ELISA was performed using a commercial kit (HerdChek®:PRRS)\(^h\) as directed by manufacturer. All reagents necessary for performing the assay were provided with the kit, and the assay was conducted at ambient temperature. In brief, serum samples were diluted 1:40 in a sample diluent. One hundred \(\mu\)l of each diluted sample was added to duplicate wells coated with proprietary PRRS viral antigen or normal cell antigen. Reference positive and negative pig sera, pre-diluted by the manufacturer, were also included in each plate. The plates containing reference and test sera were incubated for 30 minutes, then washed 3 to 5 times with a phosphate buffered wash solution containing Tween (300\(\mu\)l/well). Excess wash solutions were removed and 100\(\mu\)l of anti-porcine IgG conjugated with horseradish peroxidase were added into each well. After a 30 minute incubation, the conjugate was removed and the plates were rinsed as described above. Potential antigen-antibody reactions were visualized by adding 100\(\mu\)l of TMB (3,3',5,5'-tetramethylbenzidine) substrate solution and incubating for 15 minutes. Color reactions were then stopped by adding 100\(\mu\)l of a stop solution containing hydrofluoric acid into each well. Optical density (OD) of each well was measured at 630 nm of wavelength using a computerized microplate reader. The presence or absence of antibody to PRRS was determined by calculating the sample to positive (S/P) ratio as following:

\[
S/P = \frac{\Delta \text{OD of test serum between viral and control antigen}}{\Delta \text{OD of positive reference between viral and control antigen}}
\]

Samples were considered to be positive for PRRSV-specific antibody if the S/P ratio was greater than 0.4.

**Serum virus neutralization test**  The SVN test was performed in 96-well microtitration plates using MA104 cells. Serum samples were heat inactivated at 56°C for 35 minutes prior to performing the test and serially diluted 2-fold in DMEM supplemented with 10% FBS, 10mM HEPES, and the antibiotic-
antimycotic mixture. Each dilution of serum was mixed with an equal volume of PRRS virus containing $10^2 \text{TCID}_{50}$ per 0.1ml and incubated at 37°C for 60 minutes. Two hundred microliters of each mixture were added to a microtitration plate well containing 24-hour-old confluent MA104 cell monolayers and incubated at 37°C. Monolayers were observed at the end of 5 days and antibody titers expressed as the reciprocal of the highest serum dilution in which no CPE was observed. Each sample was run in triplicate.

**Preparation of PRRSV antigens for the western immunoblot assay**

Confluent MA104 cell monolayers were infected with PRRSV at a concentration of $10^4 \text{TCID}_{50}$ per 75-cm² flask and incubated for 3-4 days at 37°C. Virus-infected cells were harvested and pelleted by centrifugation at 1000 x g for 10 minutes. The cell pellet was resuspended and disrupted in a lysis buffer (pH 8.0, 0.05M Tris, 0.15M NaCl, 0.002M EDTA, 0.5% deoxycholate, 0.1% SDS, 1% NP-40, 0.1% sodium azide, 0.1% gelatin, and 0.1% bovine serum albumin) at a rate of 1ml buffer per 0.1ml of cell pellet. The lysis buffer also contained protease inhibitors as described previously. The suspension was stirred overnight at 4°C, then centrifuged at 600 x g for 10 minutes. The supernatant was saved as crude viral antigen, aliquoted, and stored at -70°C. Control antigen was prepared in the same manner using uninfected MA104 cells.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)** A modified Laemmli procedure was used to separate proteins on a discontinuous slab gel (70 x 80 x 0.75mm) consisting of 5% stacking gel and 14% resolving gel, cross-linked with bis-acrylamide at a ratio of 30:0.8. Proteins were solubilized in sample buffer (pH 6.8) containing 0.0625M Tris, 2% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol blue, and 10% glycerol at 100°C for 5 minutes. Ten microliters of each denatured sample (20µg proteins) and 5µl of prestained SDS-PAGE molecular standards were loaded in alternate lanes on the gel. The molecular weight standard contained 6 proteins with molecular masses of approximately 43, 29, 18, 14.3, 6.2, and 3kD. Electrophoresis was carried out using a vertical mini-gel apparatus as directed by the manufacturer. All gels were electrophoresed at 100 volts (Model 1000/500 power supply) until samples reached the stacking/separating gel interface. Sample separation was then completed by electrophoresis at a constant voltage of 200 volts until the dye front
reached 0.5cm from the bottom. This migration limit was imposed on all separations in an attempt to standardize protein migration patterns.

Electrophoretic transfer of proteins  A mini-trans-blot electrophoretic transfer cell was used by following the recommended procedure of the manufacturer. Viral and cellular proteins, along with the standard molecular weight markers separated in gels, were electrophoretically transferred to 0.45μm nitrocellulose membranes immediately following SDS-PAGE. Transfer was carried out at 4°C for 60 minutes at 250 milliamperes in transfer buffer (pH 8.3) consisting of 25mM Tris, 192mM glycine, and 20% (v/v) methanol.

Western immunoblot assay  Nitrocellulose membranes containing viral and control cellular antigens were blocked overnight at 4°C with 1% gelatin dissolved in Tris-buffered saline (TBS, pH 7.5) containing 500mM NaCl and 20mM Tris. The membranes were then washed for 10-15 minutes in gently agitating TBS and cut into strips containing viral and cellular antigens and molecular weight standards. Pig serum samples and reference sera were diluted 1:10 and 1:50 in TBS containing 0.05% Tween 20 (TTBS) and 1% gelatin. The source of the positive reference serum was a serum collected from 3 month-old pig approximately 55 days after it had been experimentally infected with PRRSV isolate ISU-P by nasal inoculation. Normal reference serum was collected from an age-matched pig free of PRRS virus infection. Each diluted serum sample was added to a single membrane strip and incubated for 2 hours at 37°C in a humidified chamber. Following incubation, the membranes were washed 3 times in gently agitating TTBS for 5 minutes. Antigen-antibody reactions were visualized with optimally diluted goat anti-swine IgG (H+L) labeled with horseradish peroxidase and TMB membrane peroxidase substrate. The color reaction was stopped by three brief washes in deionized water. Appearance of virus-specific reactivity was assessed by comparing the antibody responses to viral and cellular antigens. Approximate molecular weights were determined by comparison with the protein standards using linear regression.
RESULTS

All pigs inoculated with PRRSV remained clinically normal over the course of the study. Viremia was first detected 3-5 days PI and persisted through days 9-15 PI. The antibody response over time, as indicated by the IFA, IPMA, ELISA, and SVN tests, is summarized in Table 1 and Figure 1.

Porcine reproductive and respiratory syndrome virus-specific antibodies were first detected by the IFA test on day 9 PI in 4 of 8 pigs. The remaining 4 pigs seroconverted by day 11. The IFA titers rose to ≥640 by 28 days PI in all pigs, then began to decline. Antibody titers in 2 of 8 pigs had fallen below detectable limits by day 105 PI and ranged from 40 to 160 in the remaining 6 pigs.

Specific antibodies were first detected by the IPMA on day 5 PI in 4 of 8 pigs. The remaining 4 pigs seroconverted by day 9 PI. The IPMA antibody titers ranged from 640 to ≥1280 between 28 and 42 days PI. Thereafter, IPMA titers declined slowly, as compared to the IFA titers, ranging from 40 to ≥1280 on day 105 PI.

Antibodies specific for PRRSV were first detected by the ELISA on day 9 PI in 3 of 8 pigs. Four more pigs seroconverted on day 11 PI and the remaining pig seroconverted on day 13 PI. Based on the S/P ratio, ELISA titers rose to maximum value (2.0-3.0 S/P ratio) by 28 to 42 days PI, then began to decline. All 8 pigs remained seropositive through day 105 PI and the S/P ratios ranged between 0.8 to 1.4.

Serum virus neutralizing antibodies were slow to appear relative to antibodies detected by the other three tests. Neutralizing antibodies were first detected in 1 pig on day 9, 2 pigs on day 11, and a fourth pig on day 15 PI. The remaining 4 pigs seroconverted by day 28 PI. Neutralizing antibody titers rose slowly for 63-77 days PI in 6 pigs and then began to decline. However, SVN antibodies in 2 of 8 pigs continued to rise through day 105 PI. Maximum SVN antibody titers ranged from 64 to ≥256 during the 105-day study period. On day 105 PI, neutralizing antibody titers ranged from 8 to ≥256. Assuming a constant rate of antibody decay from peak levels, ELISA, IFA, IPMA, and SVN antibody titers were estimated by regression analysis to drop below the detectable limits of the tests at approximately 137, 158, 324, and 356 days PI, respectively.
Table 1. The antibody response of 8 pigs after intranasal inoculation with PRRSV isolate ISU-P as detected by the indirect fluorescent antibody (IFA) test, the immunoperoxidase monolayer assay (IPMA), the enzyme-linked immunosorbent assay (ELISA) and the serum virus neutralization (SVN) test.

<table>
<thead>
<tr>
<th>Test</th>
<th>Days postinoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>IFA ≥20</td>
<td>0⁬</td>
</tr>
<tr>
<td>IPMA ≥20</td>
<td>0</td>
</tr>
<tr>
<td>ELISA ≥0.4⁬</td>
<td>0</td>
</tr>
<tr>
<td>SVN ≥2</td>
<td>0</td>
</tr>
</tbody>
</table>

*Represents identical responses from samples collected at 7-day intervals 28-98 days PI.

*Number of pigs positive by each test where n = 8.

*Presence or absence of specific antibodies was determined by calculating the sample-to-positive (S/P) ratio of optical densities at 1:40 dilution of samples.
Figure 1. Antibody responses of 8 pigs to PRRS virus infection by the indirect fluorescent antibody test (A), the immunoperoxidase monolayer assay (B), the serum-virus neutralization test (C), and the enzyme-linked immunosorbent assay (D) (The line on each graph represents curve-fitting and dots show distribution of serologic response)
Western immunoblot analysis of early and late neutralizing antibody responders are summarized in Table 2 and Figure 2. Four PRRS viral proteins with molecular masses of approximately 15, 19, 23, and 26kD were consistently identified by western immunoblot (Figure 2). Antibodies specific for the 15 kd protein were first detected in all 3 early responder pigs on day 5 PI. In contrast, antibodies to the 19, 23, and 26kD proteins first appeared 11-15, 15, and 9-11 days PI, respectively. Following their initial detection, antibodies against specific viral proteins were present through the remainder of the study.

No response to viral protein was detected in the 4 late responders until day 7 PI. Antibody to the 15kD protein was detected in all 4 pigs on day 7 PI. Antibody specific for the 26kD protein was first detected 15-28 days PI. Antibodies specific for the 19 and 23kD proteins appeared 21-28 and 28-42 days PI, respectively. As in the early responders, antibodies against specific viral proteins persisted until the end of the study following their initial detection.

Viremia, initial detection of neutralizing antibody, and first appearance of antibodies against each PRRS viral protein are given in Table 3 for individual pigs. Viremia was first detected in all early responders on day 3 and all late responders on day 5 PI. The mean duration of viremia in early responders was 10.3 days PI, in contrast to 14.0 days PI in late responders. The mean onset of neutralizing antibody in early responders was 10.3 days PI versus 24.5 days PI in late responders. The mean onset of antibodies to the 15, 19, 23, and 26 kd viral proteins as determined by western immunoblot was 5.0, 12.3, 15.0, and 10.3 days PI in early responders, and 7.0, 24.5, 33.3, and 19.8 days PI in the late responders.

**DISCUSSION**

The objective of this study was to characterize the ontogeny of the humoral immune response in pigs to PRRS virus infection. The antibody response was monitored using four serological tests routinely used to detect PRRS virus-specific antibodies (IFA, IPMA, ELISA, and SVN). The viral antigen specificity of the antibody response was further characterized by western immunoblot analysis. The period of time for which PRRSV-specific antibodies persist in pigs after infection was estimated by regression analysis.
Figure 2. Representative western immunoblot analysis of the antibody response of pigs to PRRSV infection using pooled sera from 3 early SVN responders in which neutralizing antibodies were first detected between 9 and 11 days PI, and 4 late SVN responders in which neutralizing antibodies were initially detected between 21 and 28 days PI

lane 1: positive reference serum on viral antigen
lane 2: positive reference serum on control antigen
lane 3: early and late responders prior to infection
lane 4: early responders on day 7 PI
lane 5: early responders on day 15 PI
lane 6: late responders on day 7 PI
lane 7: late responders on day 15 PI
lane 8: late responders on day 28 PI
Table 2. A comparison of PRRSV protein specificity of antibodies in pigs (n=7) following nasal inoculation with PRRSV isolate ISU-P as determined by western immunoblot analysis.

<table>
<thead>
<tr>
<th>First detectable SVN response</th>
<th>Viral protein</th>
<th>0</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>9</th>
<th>11</th>
<th>15</th>
<th>21</th>
<th>28</th>
<th>35</th>
<th>42-105a</th>
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</thead>
<tbody>
<tr>
<td>Early respondersb</td>
<td>15 kD</td>
<td>0c</td>
<td></td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
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<td>3</td>
</tr>
<tr>
<td></td>
<td>19 kD</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
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<tr>
<td></td>
<td>23 kD</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>26 kD</td>
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<td>0</td>
<td>1</td>
<td>3</td>
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<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
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</tr>
<tr>
<td>Late respondersd</td>
<td>15 kD</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
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<tr>
<td></td>
<td>19 kD</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
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<tr>
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<tr>
<td></td>
<td>26 kD</td>
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<td>2</td>
<td>3</td>
<td>4</td>
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</tr>
</tbody>
</table>

^Represents identical responses from samples collected at 7-day intervals 42-98 days PI.

bSVN response first detected on day 9 or 11 postinoculation (n = 3).

cNumber of pigs with detectable antibody against each viral protein.

dSVN response first detected on day 21 or 28 postinoculation (n = 4).
Table 3. The chronological appearance of virus, serum virus neutralizing (SVN) activity and antibodies specific for virus proteins in 7 pigs following nasal inoculation with PRRSV isolate ISU-P

<table>
<thead>
<tr>
<th>Pig</th>
<th>Detection of viremia</th>
<th>Appearance of SVN activity</th>
<th>Appearance of antibody against viral proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First day</td>
<td>Last day</td>
<td>Appearance of</td>
</tr>
<tr>
<td>1</td>
<td>3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>11</td>
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<tr>
<td>7</td>
<td>5</td>
<td>15</td>
<td>28</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of days after inoculation with PRRSV.
Porcine reproductive and respiratory syndrome virus-specific antibodies were first detected 5-to-9 (IPMA) 9-to-11 (IFA), 9-to-13 (ELISA), and 9-to-28 (SVN) days PI and, assuming a constant antibody decay rate, were predicted to decline to the lower limits of detection by approximately 137 (ELISA), 158 (IFA), 324 (IPMA), and 356 (SVN) days PI. The initial antibody responses of pigs to PRRS virus infection as revealed by the four tests (IFA, IPMA, ELISA, SVN) evaluated in this study are in general agreement with the findings of other investigators who reported that PRRSV-specific antibodies were initially detected by the IFA test, IPMA and ELISA 7 to 10 days PI in contrast to detection by the SVN test which did not occur until days 28 to 35 PI. The results suggest marked differences in the kinetics involved in the 4 tests. Overall, the IPMA appeared to provide the best overall performance based on its ability to detect antibodies in both early and late infections, as well as the relatively short turn-around time of the test. However, sensitivity and specificity comparisons must be carried out before concluding which is actually the best test. It is also possible that the antibody response of the experimentally infected pigs as detected by the 4 serological tests could have been different if the pigs were infected at an earlier or later age, were of a different genetic background, or were infected with a different PRRSV isolate.

The estimated decay of PRRSV-specific antibodies in pigs after initial infection in our study is compatible with field observations suggesting that anti-PRRSV antibodies persist for approximately 1 year in pigs which had been exposed to the virus. Although recovered pigs are considered to be immune, recurrence of PRRS every 6 to 10 months after initial outbreak has been observed in some swine herds. Recently investigators monitored decay of passive antibody specific for PRRSV in pigs injected with polyclonal immunoglobulin and correlated it to the presence of ADE activity in undiluted serum. Results of the study suggested that pigs with declining levels of virus-specific antibody are susceptible to the potential enhancing effect of ADE for a period of 5 to 6 weeks after the SVN antibody titers in these pigs drop below 1:4. A similar period of potential susceptibility to ADE may also exit in pigs with declining level of anti-PRRSV antibody induced by exposure to wild type virus. In the present study, the SVN antibody titers in infected pigs are predicted to fall to 1:3 or lower by 280 day PI, while nonneutralizing antibody as measure by IPMA are estimated to
persist for additional 44 days. The potential period of increased susceptibility may be an explanation of the cyclic recurrence of PRRS in the field.

The PRRSV consists of at least 3 structural proteins with molecular masses of approximately 15, 19, and 26 kD. In our study we consistently detected 15, 19, 23, and 26 kD viral proteins by western immunoblot (Figure 2). Investigators recently described the detection of 15, 16, 19, 22, and 26 kD proteins by radioimmunoprecipitation. The detection of an additional protein (16kD) is consistent with genomic studies of the PRRSV indicating that as many as 6 structural proteins may be encoded by the viral genome. The failure to detect the 16kD protein in our study may have been due to conformational changes of this protein brought about by the reducing conditions that the viral preparation was subjected to during processing for western immunoblotting. In the current study, antibodies specific for the 15 kD protein were detected in all pigs by day 7 PI and persisted through day 105 PI (Table 2). Antibodies to the remaining 3 proteins (19, 23, and 26kD) were not detected until 15 days PI in any early SVN antibody producing pigs. Antibodies to these 3 proteins were not detected in any late SVN antibody producing pigs until day 42 PI. These results clearly indicate that the 15kD protein is the most immunogenic of the PRRSV structural proteins and may provide the antigenic basis for the development of improved diagnostic tests for the detection of PRRS virus specific antibodies.

The data summarized in Tables 2 and 3 also indicate that the 15kD protein is not associated with neutralizing activity since antibody to this protein was present in 4 of 8 pigs for 14 to 21 days prior to the initial detection of neutralizing antibody. In contrast, The 26kD protein appears to be involved in the induction of neutralizing activity. This conclusion is based on the fact that no SVN activity was detected in any of the 4 late responding pigs until after antibodies to the 26kD protein were present. Further work needs to be done to address the potential role that antibodies specific for the 19 and 23kD proteins may play in viral neutralization. In addition, a previous study has indicated that the 26kD protein is highly associated with the induction of antibody associated with ADE. Consequently, it requires further study to identify antigenic determinants of PRRSV which are responsible for the induction of ADE and/or SVN activity.
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GENERAL DISCUSSION

Introduction

Porcine reproductive and respiratory syndrome is a relatively new viral disease of swine. The disease is manifested by reproductive failure in breeding age swine and respiratory disease in young pigs. Field observations have indicated that pigs at 3 to 5 weeks of age are highly susceptible to PRRSV infection and often develop a severe respiratory clinical manifestation of PRRS. The high incidence of PRRS and the increased severity of disease seen in pigs of this age group has lead to the coining of the term, 'post weaning PRRS.' The time period in which post weaning PRRS commonly occurs corresponds to the time period that the level of maternal antibody specific for PRRSV approaches the lower limits of detection. The apparent correlation between time of occurrence of the post weaning syndrome and low maternal antibody levels has raised the question of whether or not ADE contributes to the pathogenesis of PRRS.

Antibody dependent enhancement of infection has been shown to contribute to disease caused by other viruses, such as dengue virus (DV), feline infectious peritonitis virus (FIPV), Aleutian disease virus (ADV) of mink, and equine infectious anemia virus. Antibody dependent enhancement has also been shown to interfere with the development of effective immunization strategies for these viruses. Recent field observations suggest that ADE may also interfere with immunization for PRRS. Enhanced clinical manifestation of PRRS has frequently been observed in herds after a modified live virus PRRS vaccine was introduced to control the problem. Consequently, the main purpose of this study was to determine if ADE of PRRSV infection is biologically significant and whether or not ADE has the potential to interfere with effective immunization strategies.

Summary of findings

The preceding study demonstrated that infection of PAM by PRRSV is significantly enhanced in vitro in the presence of subneutralizing levels of virus-specific antibody. A similar enhancement of PRRSV infection in the presence of antibody was also observed in vivo. The mean level and duration of viremia
was significantly greater in pigs that were injected with subneutralizing amounts of virus-specific IgG prior to virus challenge than in control pigs which were injected with normal serum globulin. In addition, an experiment in which the rate of decline of injected polyclonal PRRSV-specific antibody was monitored and correlated to ADE suggested that pigs are susceptible to the potential enhancing effect of ADE for a period of 5 to 6 weeks following the disappearance of SVN antibody. A similar period of potential susceptibility to ADE was observed in pigs that were infected with PRRSV by the nasal route. The antibody response of these pigs was monitored by the IFA test, IPMA, ELISA, and the SVN test for 105 days post inoculation. Regression analysis of data indicated that neutralizing antibodies persist for 280 days following infection while non-neutralizing antibodies were estimated to persist for an additional 6 weeks. The study also revealed that field isolates of PRRSV vary in their susceptibility to ADE of infection. This variability was dramatically demonstrated by the neutralization of homologous virus by a concentration of antibody that enhanced the replication of heterologous isolates. Collectively, these observations clearly suggest that ADE of PRRSV infection has the potential to contribute to the pathogenesis of PRRS and to interfere with immunization strategies.

**ADE assays**

Antibody dependent enhancement of PRRSV infection was assessed by measuring increases in virus yield and by determining infection rates of PAM in the presence of PRRSV-specific antibody. All ADE assays used in the present study to measure these two parameters were highly reproducible because no significant variation was observed in results obtained between days on which the assays were performed. In addition, The agreement between two assays in detecting ADE activity in all sera tested was 100%, indicating that the two assays are compatible for detection of virus infection enhancing activity in serum.

Two assays were used to measure increases in progeny virus yield: the standard ADE assay and the modified ADE assay. The standard ADE assay was a modification of the assay described by Moren and Halstead. This assay is considered to be a quantitative assay. The assay was performed by treating a constant amount of virus with a series of dilutions of serum. Subsequently,
PAM were inoculated with the virus-serum mixtures, and changes in virus yield corresponding to each dilution of the serum were monitored. Using this assay, ADE of PRRSV infection was shown to be dependent on antibody concentration of serum (Figure 1, p.55). This characteristic generated an 'enhancement profile' which is characterized by appearance, peak and disappearance of ADE activity based on antibody concentration in each dilution of serum.\textsuperscript{205} The enhancement profile is considered to represent total ADE activity that is present in a serum. In particular, since the enhancement profile is expressed as a polynomial regression curve, the area integrated under the curve may be proportional to enhancing activity of serum for a virus. This method was applied in this study to evaluating the susceptibility of different PRRSV isolates to ADE mediated by antibody raised against a PRRSV isolate. Differences in the susceptibility to ADE among the isolates were clearly demonstrated by this approach (Figure 2, p.82).

The modified ADE assay was a variation of the standard ADE assay. The assay was conducted in the same manner as the standard ADE assay with the exception that PRRSV was treated with undiluted serum or a specific dilution of PRRSV-specific IgG. This assay is considered to be a qualitative test but may be practical as compared to the standard ADE assay. In attempting to correlate in vitro ADE with an in vivo effect, the method of expressing ADE activity is of critical importance. For instance, the presence of enhancing activity in undiluted serum of an individual was a significant risk factor for severe dengue illness by subsequent DV infection.\textsuperscript{169} In the present study, a direct correlation was also demonstrated between the presence of ADE activity in undiluted serum of pigs which was determined by the modified ADE assay prior to challenge and subsequent effect of ADE on the level and the duration of viremia in these pigs following challenge (Table 1 and Figure 5, pp.61-62). Consequently, it is likely that the modified ADE assay can be used in place of the standard ADE assay in assessing ADE activity in sera.

Yield of progeny virions and infection rates of PAM were determined at the end of 48 hour incubation following removal of virus-serum mixtures from cultures. This time period was selected because maximum differences in virus yield or the infection rate of PAM were observed between treatment and control groups in a preliminary study. However, one replication cycle of PRRSV in
PAM was reported to be approximately 20 hours when the cells were inoculated with a multiplicity of infection (m.o.i.) of 1. Consequently, it is not believed that progeny virus yields and cell infection rates observed in the study only represent the first outcome of replication.

The mean yield of infectious progeny virions per macrophage was 4.7 and 11.9 in the absence and presence of virus-specific antibody, respectively. These numbers of progeny virions per cell are considered low, regardless of the treatment. This low yield is believed to be a reflection of the sensitivity of the assay system. Bautista et al. compared the susceptibility of PAM and MA104 cells to PRRSV by comparing the frequency that PRRSV was isolated from clinical specimens using both cell types. These investigators found that higher rates of virus isolation could be achieved using PAM than MA104 cells. In a preliminary study, virus titers determined using PAMs were 2 log$_{10}$ higher than virus titers determined using MA104 cells (unpublished data). Consequently, if virus assays were done using PAM, the virus yield per infected cell would approach approximately 500 to 1000 virions per cell which are in a range of virus yield of arteriviruses. However, MA104 cells were used in place of PAM for virus titration in the present study to avoid the potential inconsistency of results due to the variation in the susceptibility to PRRSV between lots of PAM because the yield of PAM from individual pigs was relatively small.

The infection rate of PAM in the presence of subneutralizing levels of virus-specific antibody approached 1% which is consistent with a m.o.i of 0.01. In contrast, the infection rate of PAM was approximately 0.02% in the presence of only media. The low infection rate in the absence of antibody might be attributed to the presence of high numbers of virus particles which were not capable of attaching to PAM through the interaction with viral receptors on the cells. In addition, the heterogeneity of the PAM population may also have been responsible for the low infection rate. Choi et al. reported that PAM could be divided into several subpopulations based on their density. The cells of these subpopulations varied in their physiological maturity, immunological function, and their susceptibility to PRRSV infection. Yields of PRRSV in macrophage subpopulations ranged from 1 to 5 log$_{10}$. 
Mechanism of ADE of PRRSV infection

The study demonstrated that both total yield of infectious virions and the infection rates of PAM in cultures were increased following treatment of the virus with subneutralizing levels of PRRSV-specific antibodies (Figure 1, p. 55). The primary mechanism responsible for increased virus yields appears to have been due to an increase in the infection rates of PAM which was facilitated by an interaction between Fc receptors of PAM and virus-antibody complexes. This conclusion is based on two observations. First a direct correlation (r=0.95) was observed between increased virus yields and infection rates of PAM (Figure 3, p.58). Secondly virus yields were significantly reduced when virus/antibody preparations were treated with Protein A prior to exposing PAM to virus (Figure 4, p. 59). Protein A binds to Fc portion of antibody, which prevents virus-antibody complexes from attachment to Fc receptor on cells. Similar observations have been made by other investigators who studied ADE of ADV, DV, FIPV, and West Nile virus (WNV) infection.

Additional mechanism(s) may have contributed to the enhancement of virus yield in the presence of virus-specific antibody. This possibility is supported by the fact that the ratio between increases in virus yield and infection rate was greater than 1.4 (Figure 3, p.58). This conclusion is also consistent with the observations that not only was the infection rate of PAM increased but also the yield of progeny virions from individual PAM (Figure 2, p.57). The increased yields of progeny virus in PAM may have been due to increased efficiency of virus production. This possibility has been previously reported for other viral infections. For example, Robinson et al. observed higher production of viral components in cells that were infected with the human immunodeficiency virus in the presence of virus-specific antibody than in the absence of antibody. The mechanism responsible for the enhanced production of viral components in individual cells was not determined. However, Gollins et al. who studied ADE of WNV infection in mouse macrophages presented data which suggested that infection of individual cells by several virions could increase virus production. These investigators found using radiolabeled virus that radioactivity count per cell was significantly increased when the cells were exposed to the labeled virus in the presence of subneutralizing levels of anti-WNV antibody. They also observed using nonradiolabeled virus that virus
yields were enhanced in the presence of the same concentrations of the antibody. The investigators concluded that antibody increased the numbers of WNV attached to individual cells which resulted in increased yields of progeny virus. It is unlikely that multiple viral infection of individual PAM was responsible for the increased efficiency of virus production in the present study because a low m.o.i., i.e., 0.01 was used and infection rates were increased in the presence of antibody.

**Biological significance of ADE in the pathogenesis of PRRSV**

The preceding study demonstrated that pigs with subneutralizing levels of PRRSV-specific antibodies are susceptible to ADE of virus infection, in turn resulting in higher level of viremia for longer period (Figure 5, p.62). This observation indicates that ADE has the potential to increase the severity of disease through amplification of virus replication. The study also revealed that the potential of increased susceptibility to ADE exists for a period of 5 to 6 weeks in pigs with declining levels of PRRSV-specific antibodies after SVN activity drops below detectable levels (Figure 7, p.64). Collectively, these observations strongly suggest that ADE of PRRSV infection has the potential to contribute to post weaning PRRS in young pigs in which levels of maternal antibodies specific for PRRSV are declining.

In the challenge experiment, it was expected that a marked difference in the clinical response would be observed among treatment groups because ADE of other virus infection is often manifested by an exacerbation in the severity of disease. Disease enhancement in the presence of antibody has been most notably demonstrated in humans and cats infected with DV and FIPV, respectively. Although ADE effect was apparent among treatment groups with respect to the duration and the level of viremia, no overt clinical signs of disease were evident in infected pigs in any treatment group. Similar observations have also been made by Christianson et al. who conducted a study in which fetuses of sows were exposed in utero to PRRSV with or without antibody between 40 and 45 days of gestation. The fetuses were subsequently removed from the sows at day 4 and 11 postinoculation and examined. No difference in the fetal death rate was observed between the 2 groups, but significantly higher virus titers were detected in fetuses that were injected with PRRSV-antibody mixtures than fetuses that
were only injected with the virus. The lack of clinical signs of disease especially with respect to respiratory disease was not completely unexpected because the respiratory form of PRRS has not been consistently reproduced under experimental conditions, even though respiratory disease is a major component of PRRS in the field. It is possible that the lack of clinical manifestations in the study may have been due to the challenge route and/or the virus strain used for the study. The intramuscular route was used in the study instead of the nasal route of exposure which is considered to be the natural route of infection. Temperature response and virus shedding pattern were not monitored in the above study. If these parameters had been monitored, it may have been possible to reach a more definitive conclusion with respect to the role of ADE in clinical disease.

The failure to consistently reproduce respiratory disease suggests that this manifestation of PRRS may be multifactorial. The apparent failure of antibody to contribute to the disease process in the study does not rule out ADE as a contributing factor to disease. The contribution of ADE may have been more apparent if a heterologous PRRSV isolate were used as the challenge virus. This possibility is supported by the observation that field isolates of PRRSV varied in their susceptibility to ADE of infection (Figures 1 and 2, pp.81-82). Another factor that has the potential to contribute to the respiratory clinical manifestations of PRRS is concurrent infection with bacteria. The PRRSV replicates preferentially in PAM, which results in the destruction of these cells. The above study demonstrated that infection of PAM by PRRSV is enhanced in the presence of virus-specific antibody. Consequently, ADE of PRRSV infection has the potential to compromise the local immune defense system of the lung through destruction of PAM. It is also conceivable that the function of uninfected PAM could be compromised by soluble viral or virus-induced proteins. In turn, the loss of the first immune defense mechanism in the lung could increases the susceptibility of pigs to infection by other opportunistic respiratory disease pathogens, resulting in exacerbation of respiratory disease. Synergistic effects of PRRSV infection on the severity of disease by subsequent exposure to bacterial pathogens have been demonstrated. For example, Gallina et al. demonstrated that PRRSV infection predisposed pigs to subsequent infection of Streptococcus suis, resulting in increased mortality due to streptococcal
meningitis. Similarly, investigators at the Iowa State University observed that pigs infected concurrently with PRRSV and *Salmonella choleraesuis* showed severe clinical responses, such as fever, depression, inappetence, while no overt clinical signs were observed in pigs infected with either PRRSV or salmonella alone (J. Zimmerman, Iowa State University, personal communication).

The present study indicates that ADE may also be a concern with respect to pigs with declining levels of antibodies that were induced by previous exposure. Experiments in which the neutralizing and non-neutralizing antibody responses of nasally infected pigs were monitored suggests that these antibodies will persist in pigs for approximately 280 days and 324 days, respectively (Figure 1, p.101). Since ADE can only be expressed in vivo in the absence of neutralizing antibody, it is conceivable that pigs in the field are susceptible to the effects of ADE for approximately 44 days. This potential period of increased susceptibility could be an explanation of the cyclic recurrence of PRRS in the field. Field observations have suggested that clinical PRRSV infection reoccurs in herds approximately every 6 to 10 month following the initial PRRS outbreak. Collectively, it appears that ADE has the potential to increase the severity of PRRS in the field among pigs with declining levels of antibody of maternal or vaccine origin, or antibody originally induced by wild type virus infection.

**Significance of the variability in the susceptibility of PRRSV isolates to ADE in the development of immunization strategies**

The above study also demonstrated that field isolates of PRRSV vary in their susceptibility to ADE of infection. This characteristic of PRRSV was dramatically illustrated by the neutralization of isolate ISU-P by a concentration of homologous antibody that significantly (p<0.01) enhanced the replication of 12 of 16 heterologous field isolates tested (Figure 1A, p.81). This observation is particularly significant with respect to the control of disease by vaccination. For example, antibodies induced by vaccine strains of PRRSV could either enhance or suppress the replication of wild type PRRSV in vaccinated animals. In addition, these findings suggest that the replication of vaccine virus in the host could also be enhanced by maternal antibody specific for some field strains of PRRSV. Both of these mechanisms could conceivably contribute to the exacerbation of PRRS that is occasionally observed in North American swine.
herds in which vaccine is being used (B Thacker, Iowa State University, Ames, IA, personal communication; G. Erickson, Rollins Animal Disease Lab, Raleigh, NC).

Variation in the susceptibility to ADE of infection among virus isolates has previously been documented for other viruses, such as DV\textsuperscript{133,141} and FIPV\textsuperscript{70,225}. In the case of DV, differences in the susceptibility of DV isolates to ADE have been suggested to account for different clinical manifestation of DV infection\textsuperscript{128,206}. For example, the severity of DV infection is much greater in individuals with antibodies from previous infection, especially if the previous infection was due to a different serotype\textsuperscript{128}. Isolates of DV and FIPV have also been found to vary in their ability to induce enhancing antibodies\textsuperscript{133,225}. The variability in the susceptibility to ADE and the ability to induce ADE among these viruses have been implicated as important obstacles in the development of vaccines and vaccination strategies\textsuperscript{185}. The present study also demonstrated the potential for ADE of PRRSV infection to interfere with immunization strategies for PRRS. These observations suggest that vaccine candidates should be evaluated for their ability to induce ADE activity before their use in the field. In the present study, PRRSV isolates that were more susceptible to neutralizing activity of antibody were shown to be less susceptible to ADE than isolates with lower susceptibility to neutralization by the same antibody (Figure 4, p.85). Consequently, it may be possible to use the susceptibility of field isolates to neutralization by antibody induced by vaccine candidates, as a surrogate marker for ADE. Field application of this approach in terms of evaluating vaccine candidates needs further work.

Correlation analysis (Figure 4, p.85) indicates that the degree of enhancement mediated by a high concentration of antibody raised against an isolate of PRRSV that was capable of neutralizing infection by homologous virus was inversely proportional to the ability of this antiserum to neutralize the isolates (r=0.92). This observation suggests that the variability in the susceptibility of isolates to ADE may be associated with quantitative and/or qualitative differences in the antigenic determinants associated with virus neutralization. In contrast, a poor correlation (r=0.32) was observed between the susceptibility to SVN and ADE mediated by a low concentration of the antibody. This lower correlation was because 5 of 17 isolates tested fell outside of the 99%
confidence interval. If the 5 isolates were excluded from the analysis, the correlation coefficient was 0.91. The apparent lack of correlation between susceptibility to SVN and ADE mediated by a low concentration of antibody associated with these 5 PRRSV could be a reflection of quantitative and qualitative differences in the antigenic determinants of the isolates. These observations suggest that ADE can also be mediated by antibody specific for antigenic determinants that are only partially associated with SVN or not associated at all.

Role of PRRSV proteins in ADE

Western immunoblot analysis (WIA) revealed the presence of antibody specific for the 15kD nucleocapsid protein and the envelope-associated 26kD glycoprotein throughout the period that ADE activity was present in undiluted serum of pigs that were injected with polyclonal PRRSV-specific IgG (Table 2, p.66). The location of the 26kD protein in the viral envelope suggests that antibodies induced by this protein are associated with ADE. Other investigators have reported that ADE of other virus infections is also mediated by antibodies induced by envelope-associated proteins.\textsuperscript{173,184} It is also possible that other envelop-associated proteins of PRRSV that were not detected by assay in the present study, such as the 43kD glycoprotein described by Madassi et al.\textsuperscript{184} may induce antibodies that contribute to ADE. Because of the internal location of the 15kD nucleocapsid protein within the virion, it is unlikely that antibodies induced by this protein were associated with ADE even through they were detected throughout the period that ADE activity was detected in undiluted serum. However, it is conceivable that antibody specific for the 15kD protein could contribute to \textit{in vivo} enhancement of PRRSV infection if the integrity of the viral envelope is compromised by host defense mechanisms.

The 26kD protein appears to be also involved in the induction of neutralizing activity. This conclusion is based on the observation that no SVN antibody was detected in any pig challenged with PRRSV by nasal route until after antibodies specific for the 26kD protein were present in serum of infected pigs as determined by western immunoblotting (Table 3, p.105). Consequently, it may be possible that the 26kD protein contains antigenic determinants which differ in their function: one is primarily associated with virus neutralization and
the other is not. Studies of FIPV and DV have revealed the existence of antigenic determinants associated with neutralization and ADE on the same protein. In these studies, investigators were able to categorize the monoclonal antibodies that represent the epitopes of envelope protein into 3 groups according to their ability to a) only neutralize, b) both neutralize and enhance, and c) only enhance virus infection. Consequently, the identification of such antigenic determinants associated with ADE on PRRSV proteins and their exclusion from vaccines may be beneficial.

**Immunization strategies to avoid the potential adverse affect of ADE**

Antibody dependent enhancement has the potential to contribute to the severity of disease and possibly to increase the susceptibility of pigs to infection by PRRSV. These possibilities are of particular concern with respect to young pigs with declining levels of maternal antibodies since young pigs are especially susceptible to respiratory disease induced by PRRSV. Results of this study showed that virus replication was significantly suppressed in pigs with virus neutralizing antibody titers greater than 3.4 log₂ and that ADE activity was not detected in undiluted serum until the SVN titers fell below 2 log₂ (Figure 5B, p.62). Consequently, potential adverse affects mediated by ADE might be avoided by designing immunization strategies that maintain high serum virus neutralizing activity in young pigs. In addition, the present study also revealed that strains vary in the susceptibility to ADE and that such a variation may be due to difference in antigenic determinants associated with virus neutralization and/or ADE. Consequently, it may be possible to develop a vaccine with low or no ADE potential. Such an approach could be accomplished by selecting vaccine strains according to their ability to induce enhancing antibody or by genetically engineering a vaccine so that antigenic determinants that are strongly associated with ADE are excluded.

**Future study**

Results of the proceeding study indicate that ADE of virus infection may be a virulence factor in the pathogenesis of PRRSV as well as interfere with immunization strategies. It is highly conceivable that ADE may play a significant role in the induction of disease in young pigs with circulating maternal antibody
specific for PRRSV. This conclusion is based on the following observations. First, ADE activity was demonstrated in undiluted serum of pigs that were injected with virus-specific polyclonal antibody. Secondly, subneutralizing levels of passively supplied antibodies significantly enhanced the level and duration of viremia in pigs following virus challenge. Thirdly, PRRSV isolates were shown to vary in their susceptibility to ADE in vitro.

These observations could be one explanation of the apparent exacerbation of clinical PRRSV infection in swine that has been observed in some herds following vaccination with RespPRRS® marketed by NOBL Laboratories. Therefore, the question of whether or not vaccine-induced antibody can contribute to clinical disease in piglets needs to be resolved. Specifically, can the injection of live vaccine virus into pigs in the presence of maternal antibody specific for a wild type PRRSV increase the level and duration of vaccine virus which could conceivably contribute to the disease process? In addition, can low levels of vaccine-induced antibody can mediate ADE of infection of wild type virus that may be circulating in a herd? For example, the development of vaccine-induced antibody in piglets could be impeded in the presence of maternal antibody but be sufficient to enhance infection of wild type PRRSV.

Since previous studies have been done in pigs with passively supplied PRRSV-specific antibody, another critical question that needs to be answered is whether or not ADE of virus infection can occur in pigs that have been actively immunized with vaccine virus. If ADE of infection occurs in pigs with passive antibody but not in pigs with active immunity, then emphasis should be directed toward developing immunization strategies that induce a strong cell-mediated immunity or persistently maintain high levels of SVN antibody. Furthermore, if ADE does occur in the presence of active immunity, then efforts should be extended to identifying antigenic determinants associated with ADE and excluding them from a vaccine virus.

Since the present study suggests that antibody specific for the 26kD protein of PRRSV is responsible for ADE, initial work for the identification of antigenic determinants associated with ADE should focus on this protein. However, it is possible that other proteins besides the 26kD protein may also be involved in the induction of enhancing antibodies. An experimental approach is to generate a panel of monoclonal antibodies specific for each viral protein and evaluate each
of them for their ability to enhance or neutralize virus infection. This approach has been used to identify determinants associated with neutralization and ADE for other viruses, most notably FIPV.\textsuperscript{70,225}

Differences in the ability of different subclasses of virus-specific IgG to mediate ADE of viral infection has been demonstrated with murine monoclonal IgG specific for DV and FIPV proteins.\textsuperscript{70,141,145,207} It is also known that polysaccharide antigens induce one type of IgG subclass and polypeptide antigens primarily induce the production of a different IgG subclass. There is no reports describing differences in the subclasses of antibodies representing antigenic determinants of polypeptides. this is an area that should be explored to determine if subclass differences are associated with specific antigenic determinants associated with ADE. The identification of such antigenic determinants could lead to their exclusion from vaccines and further reduce ADE activity associated with vaccination.

The respiratory form of PRRS seen in the field has not been reproduced experimentally. Only immunologically naive pigs have been used in these studies to date. The failure to reproduce the respiratory manifestation of PRRS has led investigators to the speculation that additional factor may be involved. One factor that should be investigated is ADE because it increases both the infection rate and virus production in PAM. These cells play a critical role in the pulmonary immune response, and any disruption in their normal function could permit increased virus replication in the lung and increase the lung susceptibility to secondary bacterial infection.
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