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The role of pulmonary intravascular macrophages (PIMs) in porcine reproductive and respiratory syndrome virus (PRRSV) infection in pigs

by

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A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology
Major Professors: Patrick G. Halbur and Mark R. Ackermann

Iowa State University
Ames, Iowa
1998

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This is to certify that the Doctoral dissertation of Roongroje Thanawongnuwech has met the dissertation requirement of Iowa State University.
Dedicated to

Suchan Thanawongnuwech
and
Suchin Thanawongnukul

who have passed from this earth.
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ABSTRACT

Cultured pulmonary intravascular macrophages (PIMs) and pulmonary alveolar macrophages (PAMs) were exposed to porcine reproductive and respiratory syndrome virus (PRRSV) (VR-2385) and infection was confirmed by an indirect immunofluorescence test and transmission electron microscopy. PRRSV did not affect the ability of PIMs or PAMs to internalize or kill Staphylococcus aureus, but significantly decreased the production of superoxide anion (SOA) and myeloperoxidase-H$_2$O$_2$-halide at 24 hours post infection (HPI). PIMs were as permissive as PAMs to infection either with low (ISU-55) or high (VR-2385) virulence PRRSV strains yielding similar progeny titers in vitro. However, PRRSV-infected PIMs from 4-week-old pigs killed fewer bacteria and yielded a higher virus titer than those from 4-month-old pigs at 48 HPI. There was no difference in bactericidal activity between ISU-55- and VR-2385-infected PIMs. Both ISU-55 and VR-2385 infection significantly decreased the production of SOA at 24 and 48 HPI. Pulmonary clearance of copper particles was conducted to measure the effect of PRRSV infection on PIM function in vivo. Pigs were infused intravenously with 3% copper phthalocyanine tetrasulfonic acid (0.2 ml/kg) in saline 30 minutes prior to necropsy after 3, 7, 10, 14, or 28 days post infection (DPI) with uninfected-media, a modified-live virus vaccine (RespPRRS®), or a high virulence strain (VR-2385). Copper concentrations in the lungs of VR-2385-inoculated pigs were significantly lower than levels in the lungs of control and RespPRRS®-inoculated pigs at 7, 10, and 14 DPI. The results suggest: 1) PIMs should be considered as an important replication site of PRRSV; 2) PRRSV had a detrimental effect on bactericidal activity and SOA production of PIMs in vitro; 3) PIMs from younger pigs were more permissive to PRRSV infection; 4) the selected PRRSV strains which differ in their abilities to induce pneumonia in vivo had no significant difference in vitro on virus titer and bactericidal functions; 5) the severity of PRRSV-induced damage to PIMs in vivo differs among PRRSV isolates; and 6) decreased pulmonary clearance of copper particles due to PRRSV infection supports the hypothesis that PRRSV infection may make pigs more susceptible to bacteremic diseases.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure in sexually mature pigs, respiratory disease in pigs of all ages, and increased susceptibility to bacterial and viral diseases. Clinical and experimental studies suggest that PRRS virus (PRRSV) modulates host responses based on the evidence of the role of PRRSV in precipitating secondary infections. Pneumonia, arthritis, meningitis, and infections with pseudorabies virus (PRV) and swine influenza virus (SIV) are common following PRRSV infection (Zeman et al., 1993; Done and Paton, 1995; Zimmerman et al., 1997). An interaction between PRRSV and other agents has been demonstrated under experimental conditions in piglets challenged with *Streptococcus suis* (Galina et al., 1994a) or *Salmonella choleraesuis* (Gray et al., 1996) and with either of two respiratory viruses, porcine respiratory coronavirus (PRCV) or SIV (Van Reeth et al., 1994). The reasons for increased susceptibility to secondary infectious diseases in PRRSV-infected pigs has not been clearly elucidated.

To date, pulmonary alveolar macrophages (PAMs) are the primary cell identified to support replication of PRRSV both *in vitro* and *in vivo*. Choi et al. (1995) found that the activation/maturation stage of macrophages have some effect on the susceptibility to PRRSV infection. Monocytes are inherently resistant to supporting virus progeny production. Monocytes, however, cultured in the presence of monocyte colony stimulating factor (M-CSF) express tissue macrophage markers and become monocyte-derived macrophages (MDMs) which are susceptible to PRRSV replication (Molitor et al., 1996). Increased PRRSV replication is also observed in the mature or activated PAMs based on surface marker expression and PAM functions. Reduction in the numbers of PAMs has been demonstrated in pigs experimentally infected with PRRSV (Done and Paton, 1995). Associated with the altered lung cell dynamics in PRRSV-infected pigs is a decrease in the ability of PAMs to release superoxide anion (SOA) (Molitor et al., 1996).
In addition to PAMs, PIMs are a member of the mononuclear phagocytic system (MPS) which also play an important role in lung defense mechanisms. Because of their intravascular location, PIMs perform the task of blood clearance and are the site of blood cell degradation in pig lungs. Like PAMs, PIMs are from the monocytic origin (Winkler, 1988). The marginated monocytes attach to the endothelium via intercellular adhesion plaques (ICAPs) during differentiation to PIMs (Winkler and Cheville, 1985a). The activation/maturation stage of PIMs may modulate the susceptibility of PIMs to PRRSV infection as well. There is evidence that PRRSV antigen is present in PIMs by immunohistochemistry (IHC), (Halbur et al., 1996a; Rossow et al., 1996). However, the susceptibility of PIMs to PRRSV has not been clearly elucidated. Consequently, a better understanding of the effects of PRRSV infection on PIMs will provide important information on the pathogenesis of PRRSV with respect to the swine respiratory disease complex.

In the past, the information on PIMs was based on the theory that these cells were migrating hepatic Kupffer cells. It was not until the mid-1980 that PIMs were identified as a resident population of lung macrophages. PIMs are tightly adherent to the endothelium, not readily displaced, and capable of specific actions beyond those attributable to activated monocytes. However, removal of macrophages permanently adhered to endothelial cells has not been very successful by vascular perfusion (Morton and Bertram, 1988; Fowler et al., 1991; Chitko-Mckown et al., 1992). These investigators recovered only low numbers of PIMs by vascular perfusion with collagenase. Isolation attempts probably require lung disruption techniques (Rogers et al., 1994). A more successful method of PIM isolation needs to be developed for in vitro study. Eventually, when the nature of the macrophages-endothelial adhesion is understood, vascular perfusion with macrophage washout will be the method of choice.

We hypothesize that PRRSV will infect PIMs in vitro, and that the infection will produce structural damage and functional impairment of the PIMs. To test the hypotheses we will: 1) develop a method to harvest porcine PIMs and grow them in cell cultures; 2) develop the assays to measure phagocytosis and bactericidal functions of PIMs grown in vitro after infection with PRRSV comparing to PAMs; 3) compare low and high virulence strains of
PRRSV isolates in terms of the above assays; and 4) in vivo studies on the effect of PRRSV on pulmonary clearance of copper particles by PIMs.

**Thesis Organization**

This dissertation is presented in an alternate format and includes a general introduction with a literature review, manuscripts prepared and submitted to refereed scientific journals, and a general conclusion. References for each chapter are separate and immediately follow the text of that chapter. The format used in the individual chapters is consistent with scientific journal to which the manuscript was submitted. The first manuscript has been published in *Veterinary Immunology and Immunopathology* (39: 323-335, 1997) and describes the effects of in vitro PRRSV (VR-2385) infection on bactericidal activity of PIMs. The second manuscript has been submitted to *Veterinary Microbiology* and describes the effects of low (ISU-55) and high (VR-2385) virulence strains of PRRSV on bactericidal activity of PIMs. The third manuscript has been accepted for publication in *Veterinary Pathology* and describes the effects of low (RespPRRS®: RespPRRS/Repro™ MLV vaccine) and high (VR-2385) virulence strains of PRRSV on pulmonary clearance of copper particles in pigs. The Ph.D. candidate, Roongroje Thanawongnuwech, is the first author of each manuscript and has been the principal investigator for each experiment.

**Literature Review**

A brief overview of the general structural and functional features of the respiratory system is presented to provide a framework for understanding its responses to injury, and the resulting patterns of disease. Most diseases of the respiratory system are caused by agents arriving either by air borne or blood borne routes, each with its own special pathogenic mechanisms. Pulmonary defense mechanisms are remarkably effective under most circumstances in preventing disease agents from entering or remaining in the lung and in neutralizing these agents if they penetrate initial barriers.
Structure and function of the normal respiratory system

Structure

The respiratory apparatus comprises the nasal cavity, pharynx, larynx, trachea, and lungs with bronchi, bronchioli, and alveoli. The lung parenchyma occupies 80-90% of lung volume; 80% of the parenchyma consists of air in alveolar ducts and alveoli, 9% of capillary lumina, and 8-12% of interalveolar septal tissue (Winkler and Cheville, 1985a, 1987; Warner et al., 1986). The lungs are embedded in the pleural sac. There are two separate blood-conducting systems in the lungs. The arteria pulmonalis systems vascularize the capillary plexus surrounding the alveoli with venous blood from the right ventricle. The close structural and functional parallelism between this bloodstream and the tubular airway system is important as possible infection routes in the lungs. The supporting structures around the trachea, bronchi, bronchioli, and even the wall of the arteria pulmonalis are vascularized with blood from the arteria bronchiaUs tree (Christensen and Mousing, 1992).

In pigs, the lungs are divided with deep fissures into seven lobes. The right lung comprises the apical, cardiac, diaphragmatic, and intermediate lobes. The left lung comprises the apical, cardiac, and diaphragmatic lobes. The left apical and cardiac lobes are not separated by a fissure but only by the cardiac notch. The lobes are subdivided by solid interlobular septa into lobuli. Alveolar parenchyma is divided into structural and functional units called acini. An acinus is the gas-exchange unit of the lung supplied by a single terminal bronchiole. An acinus includes all of the branches of respiratory bronchioles, alveolar ducts, alveolar sacs, alveoli, and associated blood vessels supplied by branching of one terminal bronchiole. The epithelium of primary and secondary bronchioles consist of four cell types: basal cells, intermediate cells, ciliated cells, and non-ciliated (Clara) cells, whereas the epithelium of terminal and respiratory bronchioles consist of two kind of cells: ciliated and Clara cells (Bouljihad and Leipold, 1994). Pathological processes are often retained within lobular structures, typically seen in catarrhal pneumonia with sharp demarcated lines between normal and affected tissues.

The most important cells of the alveolar parenchyma are type I and type II epithelial cells (pneumocytes), alveolar capillary endothelial cells, fibroblasts, and other interstitial cells, and
macrophages. Pneumocyte type I cells are squamous cells which line approximately 93% of the alveolar surface. They have limited capacity to adapt to injury, in part because of their large membrane surface area and minimal enzymatic defense mechanisms. Pneumocyte type II cells are cuboidal cells, line interalveolar septa, and contain characteristic osmiophilic lamellar inclusions in their cytoplasm. The major recognized functions of type II cells are to synthesize pulmonary surfactant, a complex mixture of phospholipids and small amount of protein, and to serve as progenitor cells for the replacement and turnover of alveolar epithelium. The main function of pulmonary surfactant is to decrease surface tension in the alveolar space during expiration. Pneumocyte type II cells synthesize a variety of matrix components including fibronectin, type IV collagen, and proteoglycans. They also metabolize arachidonic acid (AA) to form eicosanoids, such as prostaglandin E2 (PGE2), which may modulate function of other alveolar cells. There is evidence that they can express major histocompatibility complex (MHC) class II molecules and function as antigen-presenting cells (Dungworth, 1993).

**Function**

The vital gaseous exchange between inhaled air and venous blood from the pulmonary artery takes place at the alveolar level. The partial pressures of oxygen and carbon dioxide in the blood are related not only to alveolar ventilation but also to the amount of blood that perfuses the alveoli. The ventilation/perfusion (V\textsubscript{A}/Q) ratio can be calculated when appropriate measurements are obtained. Within the lung at any one time there may exist an uneven distribution of blood flow and ventilation so that areas of low V\textsubscript{A}/Q, normal V\textsubscript{A}/Q, and high V\textsubscript{A}/Q are present in different lung units. For animals at rest and in the standing position, the dorsal aspects have a higher V\textsubscript{A}/Q and the ventral aspects have a lower V\textsubscript{A}/Q. It is likely that with greater activity a resumption of more equal matching of ventilation and perfusion occurs. Mismatches of ventilation and blood flow are probably the most common cause of hypoxemia (Reece, 1993). In the resting pig 10-15% of the alveolar air is exchanged per inspiration. The normal respiratory rate (breaths/minute) varies according to
the age of the animal: piglets and growing pigs, 25-40; finishing pigs, 25-35; sows in gestation, 15-20 (Christensen and Mousing, 1992).

**Defense mechanisms of the respiratory system**

Specific and nonspecific defense mechanisms monitor the continuous challenge of pathogenic agents within the inhaled air in order to maintain favorable working conditions and functional integrity of the lung (Bienenstock, 1984). The mucosal surface of the respiratory tract provides a critical interface between the pig and its environment. While the skin of the pig is well adapted to prevent invasion of potentially harmful agents. The epithelium of the nasal cavity has at least four distinct epithelial types: stratified squamous, transitional, ciliated respiratory, and olfactory epithelium. Ciliated cells are terminally differentiated and have little or no regenerative capacity. Mucous cells and nonciliated cells have the capacity to regenerate themselves as well as to undergo differentiation into ciliated cells and other epithelial types (Dungworth, 1993).

The nasal cavity removes large particles by trapping them with hairs in the nostrils or depositing by gravity in the mucus. Deposition of particles greater than 10 μm, aerodynamic diameter is virtually complete above the larynx (Baskerville, 1981). In addition, a large percentage of inhaled particulates smaller than 10 μm also interact initially with the mucosa of the nasal cavity and nasopharynx. As a result, many viral and bacterial diseases have initial stages of replication in the epithelium and lymphoid tissues of the upper respiratory system before they spread either systemically or are nebulized during inspiration to the lower respiratory tract. The mucociliary blanket consists of cilia bathed in a watery gel on top of which lies mucus with physical properties of a viscoelastic gel in the bronchi and bronchioles giving rise to a continuous flow of about 4-15 mm/minute (Done, 1988). Most of the mucous secretions of the respiratory tract, and the particulate matter they carry are delivered to the pharyngeal cavity and subsequently swallowed. Lung clearance of air-borne microbial agents, as a mechanism of nonspecific defense is attributed to the mucociliary blanket, the coughing mechanism (Green and Kass, 1964; Bienenstock, 1984), and cells of the MPS (Van Furth, 1982). Polymorphonuclear granulocytes are involved in nonspecific defense and
phagocytosis of microbial agents (Bienenstock, 1984; Brain, 1986). Normal mucociliary function depends on structurally and functionally intact ciliated epithelium as well as normal viscous properties and quantity of secretions. Interference with one or more of these predisposes to infection, as will be considered under pathogenesis of bronchopneumonia. Another function of the nasal cavity with its immense venous sinuroids and wet surfaces is humidification and warming of the air before it reaches the lower airways.

The production of specific immunoglobulins is of crucial importance to respiratory immune defense. Trachea, bronchi, and bronchioles contain lymphoid tissue (bronchus-associated lymphoid tissue or BALT) in the lamina propria and submucosa, analogous to gut-associated lymphoid tissue (GALT) in function. In both human and pigs BALT is not present in the normal healthy lung, but rather is a consequence of stimulation with antigen (Walker et al., 1996). Both B cells and T cells have been demonstrated in BALT, with B cells the predominant type. B cells have been demonstrated to be positive for immunoglobulin A (IgA), IgM, IgG, and IgE antibodies. Concentrations of IgA are highest in the airways, but IgG and IgM predominate in the alveoli (Walker et al., 1996). The physical and humoral defenses of the mucociliary blanket are constantly in operation; enhanced by cellular and humoral immune responses recruited from blood at the onset of inflammation, and by sneezing, coughing, and bronchoconstriction provoked by irritation of airway receptors. The cell-mediated immunity (CMI) response is assumed to be of special importance in viral respiratory infections in order to kill the virus-infected cells. In addition, the newborn piglet is capable of absorbing intact lymphocytes from the colostrum. These cells might confer an active cellular immunity from the sow to the piglets (Tuboly et al., 1988). Important nonspecific humoral components of the secretions are interferon, which help limit viral infection in nonimmune hosts, and lysozyme (muramidase) and lactoferrin, which have selective antibacterial activity. The normal bacterial flora of the nose and nasopharynx are important to the epithelial cells. They prevent adherence and colonization by more virulent bacteria (Dungworth, 1993).

Alveolar defense against small-sized particles depends heavily on phagocytosis by PAMs. Nonpathogenic particles and microbes are handled by simple phagocytic activity and
removed in either the mucous flow or the lymphatic system. PAMs move toward the bronchioli and hence eventually onto the mucociliary blanket. As the particle load increases, most particles reach the interstitium by endocytosis across the alveolar type I epithelial cells. Once in the interstitial space, particles move with the flow of lymph and are internalized by interstitial macrophages. Particle-laden macrophages associated with lymphatic vessels occur in peribronchiolar and perivascular clusters, and eventually enter to the local lymph nodes. Pathogenic microorganisms are neutralized with the aid of secretions such as lysozymes, interferon, opsonins, lactoferins, complement factors, and specific immunoglobulins in the mucus. If the invading agents are not neutralized by the PAMs, inflammation will occur. PAMs form the first line of pulmonary defense against infectious agents and particles that are able to penetrate the defense mechanisms of the upper respiratory tract and intrapulmonary airways. In the normal lung, PAMs are derived from peripheral blood monocytes (PBMC) that migrate into the lung after undergoing a maturation step in the interstitial space (Van Furth, 1982). During inflammation, PAMs are derived both from infiltrating PBMC and by local division (Dungworth, 1993; Van Hal et al., 1995).

PAMs appear to consist of heterogeneous populations as measured by discontinuous Percoll density gradient centrifugation (Murphy and Herscowitz, 1984; Shellito and Kaltreider, 1984; Choi et al., 1995). Part of the heterogeneity may be explained by the presence of different maturation stages of PAMs, ranging from small immature, CD14+ RFD9- PBMC-like cells to large, CD14- RFD9+ mature PAMs (Van Hal et al., 1995). Molitor et al. (1996) reported that the larger porcine PAMs appeared to have more cytoplasmic ratios as mature, differentiated macrophages were found in the lower density fraction. PAMs from the higher density fractions showed greater phagocytosis of Pasteurella multocida, SOA production, and TNF-production than PAMs from the lower density fractions. In contrast, binding of opsonized sheep red blood cells through Fc receptors was greater in the lower density cells.

PAMs have a wide array of functions in addition to their capacity to internalize and kill infectious agents and to degrade other internalized particles. They function as regulatory cells controlling inflammatory, immune, and repair processes through release of a wide array of cytokines and other regulatory molecules. Initial particle contact with the plasma
membrane of PAMs is sufficient to induce AA synthesis via the cyclooxygenase and lipoxygenase pathways prior to phagocytosis (Kouzan et al., 1985). Inflammatory and immune functions are promoted by macrophage synthesis and release of cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF), alpha and gamma interferon (α- and γ-IFN), and histamine release factor, as well as by release of inflammatory mediators that include leukotriene (LT) B₄ and LTC₄, platelet-activating factor (PAF), and thromboxane (TX) A₂ (Kirkland and Bockman, 1978; Morrison and Ulevitch, 1978; Scott et al., 1982; Kouzan et al., 1985). Repair processes are generally promoted or otherwise regulated by the release of cytokines including transforming growth factor-β (TGF-β), TGF-α, fibroblast growth factor (FGF), insulin-like growth factor (IGF), and platelet-derived growth factor (PDGF) (Nathan, 1987; Dungworth, 1993). Exposure to agents such as β-interferon or lymphokine-containing supernatants markedly decreases the function of PAMs. These functions include prostaglandin secretion, cytotoxicity, bactericidal activity, and their activity in T cells cultures (Sestini et al., 1984). Influenza virus infection in humans has been reported to transiently suppress bactericidal activity of PAMs (Jakab, 1982), whereas exposure to agents such as Listeria leads to macrophage activation (Johnson et al., 1975). PAMs also play a role in cellular and humoral immune responses through antigen presentation and other accessory cell functions. Under steady-state condition, T cells activation in the lung is tightly controlled by lymphocytostatic signals from resident PAMs via the presence of nitric oxide (NO). Pretreatment of PAMs with granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-α, or TNF-β transiently inhibits both NO production and lymphocytostatic activity of PAMs (Bilyk and Holt, 1995). During the inflammatory process, bronchial epithelial cells can up regulate expression of intercellular adhesion molecule-1 (ICAM-1) following injury and interaction with cytokines. ICAM-1 promotes adhesion and migration of circulation neutrophils and monocytes into airways. Neutrophils from the blood will invade the alveoli and assist macrophages in the phagocytic activity (Dungworth, 1993). The recruited monocytes are initially stimulatory toward T cell activation, and progressively develop both T cell suppressive- and NO synthetic-capacity as they differentiate into mature PAMs in vivo (Bilyk and Holt, 1995). In healthy pigs, the
normal percentages of cell types in the bronchoalveolar mucus is 70-80% PAMs, 11-18% lymphocytes, 8-12% neutrophils, and up to 5% eosinophilic granulocytes (Holt, 1986; Christensen and Mousing, 1992).

In addition to PAMs, macrophage populations in the lungs include PIMs, interstitial macrophages, and dendritic cells. By using flow cytometric analyses, Dethloff and Lehnert (1988) found that the interstitial macrophages exhibited electro-optical characteristics intermediate between those of blood monocytes and PAMs, which is consistent with the concept of the lung's interstitium as a maturation compartment for blood monocytes prior to migration into the alveolar compartment. Dendritic cells are bone marrow-derived motile leukocytes with enhanced antigen-presenting capacity found in the interstitium of alveolar parenchyma and the lamina propria of airways. These cells have numerous, long irregular dendritic processes, an extremely irregular and folded nucleus, and an absence of phagolysosomes. They constitutively express high levels of MHC class I and II molecules and common leukocyte antigen but are incapable of phagocytosing particles efficiently. Macrophages and dendritic cells may be necessary for the presentation of antigens to helper T lymphocytes by inducing both cytokine secretion and clonal proliferation of the T cells. This process is important for primary antibody responses, because in secondary responses B lymphocytes are available for antigen presentation to memory T cells (Dungworth, 1993; Abbas et al., 1994).

**Pulmonary Intravascular Macrophages (PIMs)**

**History of PIMs**

The concept of the mononuclear phagocytic system (MPS) was postulated in 1969 by Van Furth (Van Furth, 1982). Cells of the MPS share similarities in morphology and functions. They originate in bone marrow and are transported by the bloodstream to the tissue, where differentiation occurs. Although it was recognized as early as 1918 that bloodborne particles localized in the lungs of certain species of animals, PIMs were not identified until early 1970s (Schneeberger-Keeley and Burger, 1970; Winker, 1988). Schneeberger-Keeley and Burger (1970) identified phagocytic mononuclear cells adhered to the pulmonary
endothelium of cats. They hypothesized that these cells were hepatic Kupffer cells, which had migrated to the lungs in response to open chest ventilation. The existence of a resident population of PIMs was not considered at the time.

Shortly thereafter, Rybicka et al. (1974) described PIMs in 3- to 4-month-old calves. Because these calves had numerous PIMs and few PAMs, the authors hypothesized that these cells were the precursors of PAMs. It was not until the mid-1980 that bovine PIMs were identified as resident lung macrophages that played a major role in blood clearance (Warner and Brain, 1984; Warner and Brain, 1986).

The presence of PIMs in significant numbers and function have been found only in two orders, with one exception, order carnivore in which there is no theory to explain the existence of PIMs in the cat. Within the order Artiodactyla (even-toed), PIMs have been reported in bovine (Rybicka et al., 1974; Warner and Brain, 1984; Warner and Brain, 1986; Leifsson et al., 1995), sheep (Wheeldon and Hansen-Flaschen, 1986; Warner et al., 1988; Longworth et al., 1992; Rogers et al., 1994), goats (Atwal and Minhas, 1992), pigs (Winkler and Cheville, 1985a; Winkler and Cheville, 1985b; Bertram, 1986; Morton and Bertram, 1988; Sierra, 1990), llama (Staub, 1989), deer (Carrasco et al., 1996), and reindeer (Staub et al., 1992). It was recently shown that PIMs also exist in the order Perissodactyla (odd-toes), horses (Staub, 1989; Frevert et al., 1991; Atwal et al, 1992; Singh et al., 1994).

The existence of PIMs in other mammalian species is controversial. Rabbits (Carrasco et al., 1991), dogs (Niehaus, 1989), rats (DiBattiste, 1988), mice, guinea pigs (Winkler, 1988), baboons (Fracica et al., 1988), and humans (Dehring and Wismar, 1988) have small number of PIMs, but their importance as phagocytes in these species is apparently overshadowed by the macrophages of liver and spleen. There appears to be no absolute criterion that will differentiate between a small population of true PIMs and sequestered or activated monocytes in these animals. However, the existence of liver (Kupffer cells), splenic (reticuloendothelial cells), and bone marrow intravascular macrophages, lining blood vessels remove particulate material from the blood, is well known in these species (Warner and Brain, 1986; Van Furth, 1989). The liver and spleen of rodents contained 80-90% of the injected blood-borne particles of which very small amounts were found in the lungs (Benacerraf et al., 1957). The
lung in these species is not thought to have morphologically or functionally recognizable intravascular macrophages. Recent reports found that PIMs have been detected in rats with chronic biliary cirrhosis and in humans with malignancy and liver diseases (Chang and Ohara, 1996; Warner, 1996).

**Origin and development**

Resident macrophages are generally assumed to begin as bone marrow cells, become circulating blood monocytes, and finally differentiate into mature macrophages at a suitable location. Monocytes constitute approximately 5% of circulating leukocytes in most species and a large fraction of the total accessible monocyte pool is margined or sequestered (Van Furth, 1982). The major site of sequesteration is probably the pulmonary microcirculation. While the majority of the monocytes are only sequestered transiently, a few may adhere more tightly to the capillary endothelium. Interalveolar septa, the habitat of PIMs, are composed of alveolar epithelium, interstitium, and blood capillaries. The air-blood tissue barrier of interalveolar septa is a minimal thickness (0.2 μm) of sites where basal laminae of alveolar epithelium and capillary endothelium are fused. Cells of the continuous endothelial layer of capillaries are joined by tight junctions (Winkler, 1988). Pericytes are closely associated with the capillary endothelium and are probably involved in blood flow regulation in the pulmonary microvasculature (Sims and Westfall, 1983). Pores of Kohn are frequent in carnivores but sparse in ruminants and pigs (Winkler and Cheville, 1984). The amount of capillary volume occupied by PIMs appears to be species-dependent. PIMs occupy 3.65%, 15.3%, 25%, and 6% of the total capillary volume in cats (Schneeberger-Keeley and Burger, 1970), sheep (Warner et al., 1986), 30-day-old and new-born pigs, respectively (Winkler and Cheville, 1987).

Winkler and Cheville (1985a) reported that blood monocytes colonize the porcine lung perinatally, replicate within the capillaries postnatally, and attach to the endothelium by intercellular junctions during differentiation. This data was supported by subsequent work, which described a doubling in the relative volume density of PIMs in piglets from birth to 7 days of age (Winkler and Cheville, 1987) and in lambs from birth to 2 weeks of age.
Differentiated PIMs are rare in newborn pigs, and the majority of cells closely apposed to capillary endothelium consists of monocytes, which are occasionally in mitosis. In 3-day-old and older pigs, most cells apposed to capillary endothelium have characteristics of differentiated PIMs with mitotic figures and fat droplets in the nucleoplasm (Winkler and Cheville, 1985a, 1987). This suggested a monocytic origin of PIMs in pigs (Winkler, 1988). Additionally, PAMs in neonatal pigs have limited phagocytic properties, but functionally competent PAMs appear in 7-day-old pigs (Zeidler and Kim, 1985).

Longworth et al. (1992) reported that within 1 to 3 days after birth the number of ovine PIMs phagocytizing the tracer copper particles or retaining radioactively labeled liposomes was only 10 to 20% of the number at 2-3 weeks of age. In a subsequent study, Longworth et al. (1993) showed that 1-day old lambs had minimal pulmonary hemodynamic or lymph dynamic responses to *E. coli* endotoxin infusion, whereas by 2 to 3 weeks of age, lambs responded in a similar manner to adult sheep. Warner et al. (1986) reported that PIMs population is more differentiated at birth in calves than those in pigs. Ruminant PIMs may comprise a self-replicating population. The relative number of PIMs per mm$^3$ of lung parenchyma is a more specific parameter for the comparison of PIM numbers in different animal species. Estimates assuming a PIM cell volume of 1,500 $\mu$m$^3$, amount to $8 \times 10^3$ PIMs in sheep and $14 \times 10^3$ PIMs in pigs per mm$^3$ of lung parenchyma (Winkler, 1988).

Factors other than age may play a role in the replication, differentiation, and/or maturation of PIMs in the porcine vasculature. Bertram (1986) reported that the porcine PIMs population could change from immature macrophages to mature macrophages or immature epitheloid cells within 24 hours after inhalation of a virulent *Actinobacillus pleuropneumoniae*. Furthermore, PIMs appeared to clear cellular and acellular debris from the blood in pneumonic conditions. After the inciting agent is destroyed, mature macrophages may be capable of returning to a less mature form.

**Morphology**

PIMs are members of the mononuclear phagocytic system (MPS), found preferentially apposed to the underlying endothelium with ICAPs at the thick portion of the air-blood
barrier in the lung of a number of mammalian species (Wheeldon et al., 1986; Winkler and Cheville, 1987). Cell junctions or ICAPs between the closely apposed cell surfaces of PIMs and capillary endothelium are approximately 12-20 nm containing electron-dense material (Winkler and Cheville, 1985a, 1987; Warner, 1986; Morton and Bertram, 1988). These criteria of ICAPs parallel characteristics of epithelial belt desmosomes (zonula adherents, intermediate junction). The formation of ICAPs distinguishes PIMs from PAMs and blood monocytes which do not form ICAPs with endothelial cells (Morton and Bertram, 1988). Similar types of cell junctions, serving the common purpose of anchoring phagocytes to endothelial surface, are found between hepatic Kupffer cells and sinusoidal endothelium, but are not desmosomes or tight junctions (Morton and Bertram, 1988; Winkler, 1988). Mentzer et al. (1987) identified α and β subunits of the LFA-1 membrane molecule in human monocyte-endothelial cell adhesion. DiCorleto and De la Motte (1989) were able to partially block monocyte adherence to cultured endothelial cells by using the lectin wheat germ agglutinin, lactose-1-PO₄, or octylglucoside. However, once the adhesion had occurred, they were unable to detach the monocytes with any of these sugars. While endothelial cell surface glycoproteins may be essential for the initial attachment, they are not involved in the permanent adhesion. Beekhuisen et al. (1991) found partial blockade of adhesion by a monoclonal antibody against the common β-2 subunit of integrins and by MAC-1 in human.

PIMs of bovine, ovine, caprine, porcine, feline, and equine lung capillaries share common ultrastructural characteristics (Schneeberger-Keeley and Burger, 1970; Rybicka et al., 1974; Crocker et al., 1981a; Winkler and Cheville, 1984; Atwal and Saldanha, 1985; Warner et al., 1986; Wheeldon and Hansen-Flaschen, 1986; Atwal et al., 1992). In vivo, PIMs in the porcine lung are large, generally from 20-80 μm in diameter, irregular in shape owing to the fuzzy cell coat (glycocalyx), and have variably sized nuclei and abundant cytoplasm (Winkler, 1988). In vitro comparisons of cells adhered to plastic indicated that porcine PIMs are about half the diameter of PAMs (8.2 μm vs 16.1 μm, respectively) (Morton and Bertam, 1988). However, Fowler et al. (1991) reported a range in diameter of porcine PIMs of 10.4-16.5 μm. Differences in diameter may be due to the isolation procedures used, and to the different external stimulation available to the cells in vitro. The large nucleus is cuboidal to
kidney-shaped. Nuclear features include margination of the chromatin, concentration of chromatin in the centers of nuclei, or interruption of marginated chromatin by nuclear pores (Atwal et al., 1989). Within the cytoplasm, phagosomes, lysosomes, siderosomes, coated pits, rough endoplasmic reticulum (RER), ribosome, Golgi complex, mitochondria, and numerous lamellar structures or micropinocytosis vermiciformis are present (Bertram, 1986; Warner et al., 1986; Wheeldon and Hansen-Flaschen, 1986; Morton and Bertam, 1988; Winkler, 1988). Tubular structures with central, electron-dense lamella, representing intensified forms of receptor-mediated endocytosis, are limited to cells of MPS (Matter et al., 1968). Ligands with multivalent binding capacity to glycocalyx components are associated with the continuous invagination of cell membrane portions that form an intracellular tubular network. Eventually, micropinocytosis cisternae are fused with lysosomal granules (Matter et al., 1968). Degraded blood cells, predominantly erythrocytes located in phagocytic vacuoles, are peculiarities of PIMs (Atwal and Saldanha, 1985). Ultimately, degradation of red blood cells results in disintegration of hemoglobin into hemosiderin and ferritin that are accumulated and stored within siderosomes. Apart from these characteristics that are shared with other cells of the pulmonary alveolar region, three ultrastructural features are unique to PIMs: 1) ICAPs with endothelium; 2) micropinocytosis vermiciformis; 3) phagosomes with cellular debris, particularly, ferritin-containing siderosomes (Winkler, 1988).

In addition to the cytoplasmic organelles listed above, PIMs also contain an extensive cytocavitary network consisting of tubular structures with electron dense central lamellae (Winkler and Cheville, 1985a; Bertram, 1986; Winkler and Cheville, 1987). These structures, which have also designated as microtubules and filaments, give rise to the overall appearance of PIMs of possessing numerous pseudopodia and filopodia (Winkler and Cheville, 1984, 1985a; Atwal and Saldanha, 1985; Bertram, 1986; Wheeldon and Hansen-Flaschen, 1986; Morton and Bertram, 1988; Atwal et al., 1989).

**Function**

The presence of a prominent RER in PIMs is important for protein translation and secretion. Lysosomes within bovine, ovine, and caprine PIMs indicates the synthesis of
hydrolase and other enzymes (Rybička et al., 1974; Winkler and Cheville, 1984; Atwal and Saldanha, 1985; Brain, 1986; Wheeldon and Hansen-Flaschen, 1986). Isolated ovine and caprine PIMs stain positively for α-naphthyl butyrate esterase activity (nonspecific esterase stain), secrete lysozyme, generate oxygen radicals, have receptors for the Fc fragment of immunoglobulin, and release TNF-like activity after endotoxin or PMA stimulation (Warner et al., 1986; Wheeldon and Hansen-Flaschen, 1986; Staub, 1988; Fowler et al., 1991; Staub, 1994).

Endocytosis enables MPS cells to ingest foreign particulates. Endocytosis in PIMs involves both the internalization of large particles (phagocytosis) and the ingestion of macromolecules and fluids (pinocytosis) (Winkler, 1988). Coated pits and vesicles, as well as tubular structures of micropinocytosis vermiformis, are stages of receptor-mediated pinocytosis (Werb, 1983). Singh et al. (1994) demonstrated that the phagocytosis and/or endocytosis by equine PIMs occurred all along the plasma membrane. Atwal and Saldanha (1985) identified the erythrophagocytic role of PIMs in goat lungs. Phagocytosis of erythrocytes is based on the previous recognition of membrane changes and/or immunoglobulin coating (Lasser, 1983). The replacement of fetal hemoglobin-containing erythrocytes is accomplished shortly after birth in calves, sheep, and goats. This parallels observations of numerous siderosomes within PIMs of 7- and 14-day-old pigs (Winkler and Cheville, 1984). Because of their pulmonary location, PIMs are exposed to 100% of the blood circulating in the body (Crocker et al., 1981b). PIMs also are responsible for particulate and bacterial clearance in the species in which they are numerous (Warner et al., 1986; Wheeldon and Hansen-Flaschen, 1986). Singh et al. (1994) reported that phagocytosis or endocytosis by equine PIMs occurred all along the plasma membrane in contrast to other phagocytic cells in which phagocytosis is a local, segmental response of the plasma membrane. In regard to the PIMs, a large fraction of the particles is removed in a single pass through the lung and retained there for at least a month (Warner and Brain, 1986; Longworth et al., 1992; Atwal and Minhas, 1992; Walday et al., 1994). This suggests a resident cell population that does not migrate after particle uptake (Warner and Brain, 1986). While studying the effects of various concentrations of infused Pseudomonas aeruginosa in pigs,
Crocker et al. (1981b) found that as the lung clearance of bacteria decreased, the blood concentrations of bacteria progressively increased. In the common laboratory species (mouse, rat, rabbit, and dog), a variety of the particles including lipopolysaccharide, plastic microspheres, copper particles, colloidal gold, liposomes, or bacteria are rapidly and efficiently cleared by Kupffer cells. Quantification shows that 85-90% of intravenously infused particulate loads, including endotoxin, in these species end up mostly in the liver; the remainder in the spleen or bone marrow (Crocker et al., 1981a; McCuskey et al., 1984). The observations suggest that PIMs are not active in these species.

PIMs may play as large a role in the mechanisms of pulmonary disease as they do in pulmonary surveillance and blood clearance. Fumonisin, a group of naturally occurring mycotoxins produced by *Fusarium moniliforme*, when fed to pigs at sub-lethal concentrations, can inhibit PIMs from removing particulate matter and bacteria from the circulation, thus potentially predisposing swine to infectious diseases (Smith et al., 1996). The uptake of Gram-negative bacteria and endotoxin by PIMs triggers the release of vasoactive and inflammatory mediators *in vitro* resulting in acute pulmonary injury (systemic hypotension, pulmonary hypertension, hypoxemia) in animals with Gram-negative sepsis (Dehring et al., 1983; Bertram, 1986; Chitko-McKown et al., 1992). The accumulation of PIMs seemingly depends on disease processes occurring in the lung, type of bacteria, or route of bacterial exposure. To determine the role of PIMs in the removal of bacteria from the blood stream of sheep, Warner et al. (1987) infused sheep and rats with a single dose of *P. aeruginosa*. Pathological changes were noted in sheep lungs, but not in rat lungs. In the rat, most pathological changes were apparent in the liver, where the majority of bacteria were cleared from the blood. The sheep livers, however, were morphologically normal. However, intravenously injected *Staphylococcus aureus* caused significant pulmonary hypertension in pigs and the bacteria could be found within phagosomes of PIMs, but only minor morphological changes in the lungs have been observed (Dehring et al., 1983). Bertram reported that porcine PIMs had an important role in the clearance of debries and *Actinobacillus pleuropneumoniae (APP)* from the blood as revealed in an experimental model (Bertram, 1986). Similarly, Whiteley et al. (1991) reported that PIMs were highly
phagocytic with internalized neutrophils, platelets, and fibrin in the early intravascular inflammatory events in calves infected with Pasteurella hemolytica. By electron microscopy and immunohistochemistry, Leifsson et al. (1995), reported that bovine PIMs played a role in the uptake of Actinomyces pyogenes from the blood, and in the production of pyemic pulmonary lesions.

Involvement of PIMs in experimental and natural infection of feline cytauxzoonosis has been reported (Glenn and Stair, 1984). Proliferation (schizogony) of the piroplasm Cytauxzoon felis occurs in PIMs and in splenic and lymph-node macrophages of cats. A striking observation is the individual sensitivity of cats to experimental endotoxin administration. Cats that died during the initial, acute phase of endotoxin shock had up to four times higher TX, PGF₂, and PGE₂ concentrations in aortic blood than did surviving cats (Coker et al., 1983).

The susceptibility of PIMs to viral infection has been recently elucidated. Carrasco et al. (1991) found that PIMs from rabbits infected with rabbit hemorrhagic disease, a viral disease characterized by vascular phenomena and pulmonary edema, were more round in appearance and showed fewer ICAPs with endothelial cells comparing to PIMs in control animals. These differences were attributed to the cytopathic effect suffered by these cells. Porcine PIMs were highly susceptible to infection by African swine fever virus (ASFV) and in the acute forms of ASFV, PIMs were the cells of the lung most responsible for the replication (Sierra et al., 1990; Brookes et al., 1996; Carrasco et al., 1996a). The vascular changes consist of the formation of fibrin microthrombi in septal capillaries and the vacuolization of endothelial cells due to the activation of PIMs. Similarly, during the initial phase of disease after experimental infection with swine influenza virus (SIV), neutrophil sequestration, phagocytosis of sequestered neutrophils by PIMs, platelet aggregates, and lung edema are prominent. Clinical signs of respiratory distress during the initial phase of experimental and spontaneous SIV infection are a salient feature (Winkler and Cheville, 1986). However, porcine PIMs were less susceptible than PAMs to infection by pseudorabies virus (Chitko-McKown et al., 1990). Porcine reproductive and respiratory syndrome virus (PRRSV) antigen has been demonstrated in both PIMs and PAMs (Halbur et al, 1996a; Rossow et al.,
however, the differences in susceptibility of PIMs and PAMs to PRRSV have not been clearly elucidated. PRRSV is routinely isolated from PAMs through 7 weeks post infection (PI) and occasionally up to 10 weeks or more (Mengeling et al., 1995). Molitor et al. (1996) found that increased PRRSV replication was observed in both activated monocytes and PAMs during maturation. In contrast, monocytes directly collected and exposed to PRRSV yielded low progeny titers. It appears that PIMs may be a major source of persistent viremia of PRRSV-infected pigs, since the activation/maturation stage of PIMs may modulate the susceptibility of PIMs to PRRSV infection. In addition, B cells, T cells, and neutrophils are refractory to infection as evidenced by absence of progeny virus (Molitor et al., 1996).

A study of PIM function involving the effects of liposomes injected intravenously into sheep has been reported. Miyamoto et al. (1988) found that liposomes affect pulmonary arterial pressure by a mechanism involving the AA cascade, principally TX, which could be blocked completely by indomethacin and 75% by TX synthase inhibitor. It is well known from study of Kupffer cells that TX is not a major product following stimulation by a variety of agents (Kuiper et al., 1988). This may be a significant phenotypic difference between PIMs and Kupffer cells. Bertram et al. (1988) compared the profiles of AA metabolites produced by PAMs and PIMs in vitro. They reported that PIMs appeared to be more active metabolically than PAMs when stimulated by the calcium ionophore, A23187. PAMs formed at least five identified AA metabolites including PGF$_{2\alpha}$, hydroxyheptadecatrienoic acid (HHT), 5-hydroxyeicosatetraenoic acid (HETE), 12-HETE, and 15-HETE. In contrast to PAMs, PIMs formed at least eight AA metabolites including TXB$_2$, PGF$_{2\alpha}$, PGD$_2$, PGE$_2$, HHT, 5-HETE, 12-HETE, and 15-HETE. The major cyclooxygenase metabolite from PIMs was HHT, whereas PAMs produced PGF$_{2\alpha}$. The predominant lipoxygenase metabolites of PIMs were 5-HETE, 12-HETE, and LTB$_4$. Additionally, both macrophage types were capable of producing LTC$_4$, and LTB$_4$ from the leukotriene precursor LTA$_4$. Because of the metabolic capability, PIMs play a significant role in the acute events of pulmonary inflammation and microvascular pressure.
PIMs are activated by uptake of lipopolysaccharide or by uptake of Gram-negative bacteria and exposure to lipopolysaccharide as the organisms are degraded. PIM activation results in the release of such proinflammatory mediators as IL-1, TNF, PAF, and LT and PG metabolites of AA (Warner and Brain, 1990). Thus the secretion of AA metabolites by PIMs, induced by endocytic stimulation, may be one explanation for the pronounced susceptibility of sheep, calved, goats, horses, pigs, and cats to endotoxin-induced lung injury. These mediators can initiate or enhance acute pulmonary inflammation by recruitment and activation of neutrophils, formation of platelet plugs, and activation of the clotting cascade. In addition, activated macrophages are capable of injuring neighboring cells through the generation and release of oxygen radicals and proteolytic enzymes (Nathan, 1987).

Other immune properties of PIMs have been studied. Chitko-McKown et al. (1991, 1992) obtained both PAMs and PIMs from pigs and compared several immune parameters in the two macrophage populations in vitro: bactericidal and phagocytic activity; antibody-dependent cellular cytotoxicity (ADCC); non-MHC restricted cellular cytotoxicity (NMRC); tumoricidal activity; and the production of the cytokines IL-1 and TNF-α. They found that PIMs were as active as PAMs in most parameters measured, but PIMs were more cytotoxic against virally infected target cells, and after endotoxin stimulation PIMs produced more T cell proliferative cytokines. However, PAMs produced more TNF and nitric oxide (NO\textsuperscript{2−}) than PIMs after endotoxin stimulation. Non-stimulated macrophages from both populations produced similar amounts of these substances (Chitko-McKown and Blecha, 1992; Chitko-McKown et al., 1992). The differences in activation of PIMs and PAMs indicate that these macrophage populations may have different roles in lung surveillance.

Porcine reproductive and respiratory syndrome (PRRS)

History of PRRS

Porcine reproductive and respiratory syndrome (PRRS), formerly called mystery swine disease (Wensvoort et al., 1991a), blue-eared pig disease (Robertson, 1992), porcine epidemic abortion and respiratory syndrome (Pol et al., 1991; Terpstra et al., 1991), or swine infertility and respiratory syndrome (Benfield et al., 1992; Collins et al., 1992; Morrison et
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 (Meredith, 1993). The first cases of PRRS were reported in the United States (Hill, 1990) and Canada (Harper, 1991) in 1987. In November of 1990, the first cases of PRRS in Europe were reported in Germany (Egbering, 1991). Subsequently, PRRS spread rapidly through North America, Europe, and Asia (Murakami et al., 1994, Zimmerman et al., 1997b). Serologic evidence suggests that PRRS occurred in Canada as early as 1979 (Carmen, 1995) and in the United States in 1985 (Yoon et al., 1992b); however, the PRRS virus (PRRSV) was first isolated in 1991 (Wensvoort et al., 1991b).

Prior to the isolation of PRRSV, a number of organisms were implicated as the cause of PRRS. The agents associated with SMEDI (stillborn, mummification, embryonic death, infertility) syndrome include porcine parvovirus, pseudorabies virus, leptospira, porcine enteroviruses, encephalomyocarditis virus, and hog cholera virus (Christianson, 1992). Determination of the causative agent of PRRS was complicated by the fact that several of these agents as well as mycoplasma, swine influenza virus, paramyxovirus, chlamydia psittaci, and Streptococcus suis were isolated from suspected PRRS cases (Morin et al., 1990; Bilodeau et al., 1991; Dea et al., 1991; Loula, 1991; Paton et al., 1991b, Wensvoort et al., 1991a; Meredith, 1992).

Prior to the development of a diagnostic method for identifying PRRS-infected herds, a diagnosis of PRRS was based on clinical presentation. The clinical presentation of pigs infected with PRRSV is highly variable, being dependent on a number of factors including age of the pigs, health status, management practices, immune status, and reproductive status. Criteria in a survey of members of the American Association of Swine Practitioners used for recognition of PRRSV-infected herds included: 1) anorexia, 2) pyrexia, 3) respiratory disease in young pigs, 4) increased stillbirths, 5) increased early farrowings, and 6) increased numbers of mummified fetuses (Zimmerman, 1991). Infected nursing pigs may have respiratory signs including dyspnea. Many of the nursing pigs will die within the first week of life and survivors are prone to secondary infection with a variety of bacteria including
Streptococcus suis, Salmonella choleraesuis, Hemophilus parasuis, Pasteurella multocida, and Actinobacillus pleuropneumoniae (Keffaber et al., 1992).

The causative agent of PRRS in the Netherlands was isolated on PAM cultures and designated Lelystad virus (LV) (Wensvoort et al., 1991b). After being passaged three times on PAMs and intranasally inoculated into specific pathogen free (SPF) piglets, the LV caused interstitial pneumonia and was re-isolated from the lungs of infected pigs day 2 after inoculation (Pol et al., 1991). The first report of isolation and propagation of PRRSV (ATCC VR-2332) on a continuous cell line (CL2621) occurred in the United States (Benfield et al., 1992; Collins et al., 1992). Likewise, the ATCC VR-2332 fulfilled Koch's postulates in producing the respiratory form of the disease in gnotobiotic piglets (Collins et al., 1992). The U.S. isolates also replicate in other continuous cell lines; CRL11171 (Meng X-J et al., 1996), MA104 (Kim et al., 1993), or MARC145 (Kwang et al., 1994; Dea et al., 1995; Mardassi et al., 1995). Voicu et al. (1994) successfully infected PBMC with PRRSV. Monocytes, however, are inherently resistant to PRRSV infection and poorly support virus production in vitro, unless activated or induced to mature (Molitor et al., 1996).

The PRRSV is a fastidious, nonhemagglutinating, enveloped RNA virus with 48-83 nm diameter and a nucleocapsid of 20-35 nm (Benfield et al., 1992; Meulenberg et al., 1993; Dea et al., 1995). Nucleocapsids, budding at smooth endoplasmic reticulum (SER), and enveloped viral particles that tend to accumulate in the lumen of ER or Golgi vesicles, are the main features of the viral morphogenesis and the virus is released by exocytosis (Dea et al., 1995). Buoyant density of the virus in CsCl density gradients is estimated to be 1.18-1.20 g/ml (Wensvoort et al., 1991b; Benfield et al., 1992; Mardassi et al., 1994a). The virus is stable at 4 and -70 °C, but is labile at 37 and 56°C (Benfield et al., 1992). The half-life for virus survival is decreased at a pH below 5 or above 7 (Bloemraad et al., 1994). However, the virus could be recovered from well water for up to 9 days and city water for up to 11 days (Pirtle and Beran, 1996). The virus has a single positive stranded, polyadenylated RNA genome of approximately 15 kb containing 8 open reading frames (ORFs), six of which are expressed by a nested set of subgenomic RNAs (Conzelmann et al., 1993; Meulenberg et al., 1993). ORF 1a and 1b are thought to encode the viral polymerase. ORF's 2, 3, and 4 may
encode structural proteins. A single ORF 3 product specific monoclonal antibody (mAb) recognized only a small proportion of PRRSV indicating that the expressed protein is antigenically polymorphic (Drew et al., 1995). ORF 4 has been cloned and expressed (Kwang et al., 1994). A 15 kDa putative nucleocapsid protein (N) and two putative envelope proteins of 18-19 kDa (M) and 24-26 kDa (E) have been identified (Nelson et al., 1993) and shown to be virion proteins (Meulenberg, et al., 1995). ORF 5 encodes a membrane protein (E) which is glycosylated. The gene product of ORF 5, p25, may be a cause of PRRSV-induced apoptosis, as indicated by nucleosome ladder formation, chromatin condensation, and rRNA degradation (Suarez et al., 1996). The M protein is encoded by ORF 6 and is unglycosylated. It is probably a type III integral membrane protein, as found in coronaviruses, and anchored by three successive hydrophobic domains. The N protein, a nucleocapsid protein, is encoded by ORF 7 and elicits a strong immune response.

The entry pathway of PRRSV has been investigated in MARC-145 cells using a variety of drugs that interfere with the pH of intracellular vesicles by different mechanisms (Kreutz and Ackermann, 1996). These investigators concluded that PRRSV entry might occur through a microfilament-dependent endocytic mechanism in which a low pH is necessary for proper virus uncoating.

In regard to morphology, genome organization, replication strategy and protein composition, PRRSV closely resembles lactic dehydrogenase virus (LDV) of mice, equine arteritis virus (EAV) and simian hemorrhagic fever virus, collectively termed Arteriviruses (Plagemann and Moenning, 1992; Conzelmann et al., 1993; Meulenberg et al., 1993) in a new family, Arteriviridae (Pringle, 1996; De Vries et al., 1997). Based on its virion and genome size and icosahedral nucleocapsid symmetry, PRRSV is similar to Togaviridae (Benfield et al., 1992; Ohlinger et al., 1992). However, the genomic organization and translation strategy suggest a closer link to Coronaviruses and Toroviruses (Godeny et al., 1993; Meulenberg et al., 1993) which are in the same newly established order, Nidovirales (De Vries et al., 1997). Based on the sequence homology of the putative M and N genes of the Arterivirus group, PRRSV most closely resembles LDV, but the two do not cross-react serologically (Meng et al., 1995). Indeed no serological cross-reaction has been detected.
between PRRSV and any other virus (Goyal, 1993). Recent serological and biophysical data suggest that the U.S. PRRSV and European LV are the same virus, however, antigenic variation occurs, not only between the U.S. isolates and the European LV, but also among different U.S. isolates (Wensvoort et al., 1992; Nelson et al., 1993). Lesser antigenic differences are also detectable between different European isolates (Suarez et al., 1994; Drew et al., 1995). The multiplex PCR assay has been developed to distinguish between two genotypes of PRRSV directly from the supernatants of virus-infected cell cultures (Gilbert et al., 1997). Genetic comparisons indicate considerable differences between the U.S. and European isolates including deletions as well as point mutations allowed to divide PRRSV isolates into two distinct antigenic subgroups (U.S. and European) (Murtaugh, 1993; Kwang et al., 1994; Mardassi et al., 1994b; Mardassi et al., 1995; Meng et al., 1994, 1995).

Currently, the virulence can only be determined by a comparison following experimental infection in young pigs or pregnant sows. Halbur et al. (1996b) established a method of using cesarean-derived colostrum-deprived (CDCD) pigs for determining the respiratory virulence. Joo and Park (1996) have discussed potential virulence markers for reproductive and respiratory forms of PRRS by using a viral plaque size and by using swine tracheal ring cultures, respectively. Farrowing results from the sows infected with the small plaque PRRSV (MN-Hs) showed lower stillborn pigs and mummified fetuses than did a large plaque virus (MN-HL) (Park et al., 1996).

Pathogenesis

The means of natural transmission has not been clearly defined. Transmission among herds was frequently attributed to airborne spread (Robertson, 1992). Mice, rats, and guinea pigs have been shown to be resistant to infection (Hooper et al., 1994). However, transmission of virus among birds, especially Mallard ducks, has been demonstrated, as well as infectivity and transmissibility of avian-passaged PRRSV in swine (Zimmerman et al., 1997a). Whether birds are active participants in PRRSV epidemiology remains to be determined. Reports of the presence of PRRSV in semen (Yaeger et al., 1993), saliva (Wills et al., 1997), nasal secretion, urine, and feces (Rossow et al., 1994) suggest other routes of
transmission. Pigs can be infected with PRRSV experimentally via oronasal, oral, intranasal, intramuscular, intrauterine, intravenous, and intraperitoneal routes (Collins and Rossow, 1993).

The incubation period of pigs infected with PRRSV is highly variable and dependent on a number of factors including the virulence of virus strain, dose, route, age of the pig, health status, management practices, immune status, reproductive status, and the intensity of observation. The incubation period has been estimated to be 3-24 days post inoculation (DPI) with anorexia and fever being the first clinical signs noticed (Dee, 1992). Most animals become ill 4-5 DPI with PRRSV (Terpstar et al., 1991; Christianson et al., 1992; Rossow et al., 1995; Halbur et al., 1995b, 1996b). Viremia can be detected as early as 1 DPI (Christianson et al., 1993; Rossow et al., 1994), and has been reported to last for as long as 8 weeks (Mengeling et al., 1995) or until the end of 9-week observation period (Loemba, et al., 1996). PRRSV is routinely isolated from PAMs of infected pigs through 49 DPI and occasionally up to 70 DPI (Mengeling et al., 1995). The site of PRRSV replication has not been fully elucidated, although there appears to be a predilection for replication in PAMs or other pulmonary macrophages such as PIMs or other tissue macrophages. However, PRRSV antigen has been detected in resident macrophages in various tissues such as lung, lymph node, tonsil, heart, thymus, spleen, Peyer's patches, liver, kidney, adrenal gland (Halbur et al., 1995a, 1995b, 1996a, 1996b), and brain (Molitor et al., 1996; Rossow et al., 1996; Thanawongnuwech et al., 1997). Other cells in which viral antigen has been detected include pneumocytes, bronchiolar epithelium (Pol et al., 1991; Sur et al., 1996), endothelial cells in the heart, dendritic cells in the lymphoid tissues (Halbur et al., 1995a, 1996a), and muscle tissues (Magar et al., 1995). The lymphoid and respiratory systems have the most severe lesions and are likely the major sites of viral replication (Halbur et al., 1995b; Duan et al., 1997).

The kinetics of the appearance of specific IgM and IgG antibody titers during PRRSV infection have been investigated in pigs naturally- and experimentally-exposed to PRRSV (Loemba, et al., 1996). Specific IgM antibody titers were first detected by indirect fluorescence antibody test (IFA) at the end of the first week of PRRSV infection, peaked by
14-21 DPI, then rapidly decreased to undetectable levels by 35-42 DPI. On the other hand, specific IgG antibody titers peaked by 21-28 DPI and remained unchanged to the end of the 6- or 9-week observation period, concurrently with a persistent viremia. Nelson et al. (1993) demonstrated that peaked IgG antibody titers to PRRSV (IFA titers > 1024) may persist in experimentally-infected pigs for more than 3 months, then decrease progressively to reach very low levels (IFA titers < 20) after more than 300 DPI. However, serum neutralizing (SN) antibody titer > 8 were not detected until 3-4 weeks PI (Loemba, et al., 1996). Blastogenic response in pigs experimentally infected with PRRSV has been reported (Vezina et al., 1996). A transient episode of diminished proliferative response of peripheral blood lymphocytes (PBL) to mitogens phytohemagglutnin (PHA) and concanavalin A (Con A) was observed at 3 DPI, but polyclonal activation of PBL was observed at 7 or 14 DPI by in vitro spontaneous uptake of [3H]-thymidine from the same pigs.

Although the SN test has been reported to be less sensitive than the IFA test, the long term persistence of PRRSV in the experimentally- or naturally-infected animals despite high levels of antibodies to PRRSV, may be due to antibody-dependent enhancement (ADE). Yoon et al. (1996) demonstrated that ADE mediated by antibody specific for the 26-kDa envelope protein had potential to contribute to the pathogenesis of PRRSV infection both in vitro and in vivo. Increases in PRRSV infection rates were mediated by interaction between virus-antibody complexes and Fc receptors on the surface of PAMs. The relative yield of progeny virions from individual cells also increased in the presence of antibody in vitro. ADE may contribute to the susceptibility of pigs to infection by PRRSV.

Gross pathology characterized by multifocal mottled-tan consolidation of noncollapsed lungs and lymphadenopathy variable in distribution and severity (Pol et al., 1991; Christianson et al., 1992; Mardassi et al., 1994a; Rossow et al., 1994, 1995; Halbur et al., 1995b, 1996b). Lung lesions develop subsequent to viremia; are initially most severe in the dorsal caudal lung lobe and are distinctly multifocal without a bronchiolar distribution (Rossow et al., 1995). Microscopy usually reveals interstitial pneumonia, and occasionally, lymphadenopathy, vasculitis, myocarditis, and encephalitis related to the virus strain variation (Collins et al., 1992; Halbur et al., 1995a, 1995b; Rossow et al., 1995). The
multifocal interstitial pneumonia is characterized by three main changes: 1) septal thickening with mononuclear cells, 2) type 2 pneumocyte hypertrophy and hyperplasia, and 3) accumulation of normal and necrotic macrophages in alveolar spaces (Halbur et al., 1995b). Lymphadenopathy with follicular hypertrophy, hyperplasia, and necrosis is consistently seen (Halbur et al., 1995b). Heart lesions are a late feature of experimental infection (Rossow et al., 1994, 1995a). Vasculitis and vasculitis with endothelial budding of virus-like particles has been described in PRRSV-infected neonatal pigs indicated vascular tropism of the virus (Goovaerts and Visser, 1992; Darbes et al., 1996). Necrosis, edema, and hemorrhage in umbilical vessels of fetuses from sows challenged with PRRSV in a research setting has been documented (Lager and Halbur, 1996). Nonsuppurative encephalitis with necrotizing vasculitis has been reported in a field case of PRRSV-infected pigs (Thanawongnuwech et al., 1997). Renal vascular changes characterized by a moderate to marked lymphohistiocytic interstitial nephritis with interstitial edema have also been reported in experimental PRRSV-infected pigs (Cooper et al., 1997). Edema could be the result of vascular damage. PRRSV can be isolated from the lung, brain, tonsil, thymus, spleen, lymph nodes, intestine, heart, nasal turbinates, plasma, serum, and blood cells, co-circulating with antibody which is detectable within 2 weeks (Ohlinger et al., 1992; Rossow et al., 1995; Halbur et al., 1995b, 1996b).

PRRSV has also been isolated from boar semen (Yaeger et al., 1993), bulbourethral gland (Christopher-Hennings et al., 1995) and placenta (Christianson et al., 1993). Viral antigen has been observed in ovarian tissues (Swenson et al., 1995), although the effect of PRRSV on the ovary and the estrus cycle is unknown. PRRSV RNA can be detected by polymerase chain reaction (PCR) in boar semen and sera, and may persist for variable periods of time with responds of 92 DPI and 21-31 DPI, respectively (Christopher-Hennings et al., 1995). Decreases in semen quality have been reported in naturally infected boars (White, 1992). In contrast to the field cases, evaluation of semen quality following experimental inoculation of 2 boars resulted in no changes in concentration, color, progressive motility, or spermatozoa morphology (Yaeger et al., 1993). Post-infection infertility of boars has been implicated as a cause of increased returns to estrus (Gordon, 1992). Transplacental infection was
documented when virus was isolated from piglets born to sows intranasally exposed to PRRSV and PRRSV antibody was identified in precolostral blood (Christianson et al., 1993). Reproductive signs are usually not evident before 25 DPI (Done et al., 1996). The reproductive syndrome is characterized by anorexia, increased late term abortion, increased numbers of stillborn pigs, mummified fetuses, weak live-born piglets, increased preweaning mortality, and delayed return to estrus (Hopper et al., 1992; Zeman et al., 1993). Lager et al. (1994) demonstrated that oronasal exposure of sows at 30 days gestation induced reproductive failure. Several studies report the effects of challenge inoculation on pregnant sows describing various adverse effects on subsequent piglet viability (Terpstra et al., 1991; Christianson et al., 1992; Dea et al., 1992; Yoon et al., 1992a; Christianson et al., 1993). Botner et al. (1994) reported that transplacental virus transmission did not result in increased fetal death. Likewise, the intrauterine infection of pigs with PRRSV at or near the time of conception has little or no effect on their reproductive performance (Lager et al., 1996). This may be due to a greater resistance of younger fetuses to the lethal effects of the virus, or to the inability of the virus to cross the placenta in early gestation. Mengeling et al. (1996) oronasally infected pregnant gilts at day 90 of gestation with various strains of PRRSV and found that all virus strains crossed the placenta to infect fetuses in utero yielded various numbers of late-term dead fetuses depending on their virulences. However, vascular lesions of the umbilical cord have been reported and may be the cause of death in some PRRSV-infected fetuses (Lager and Halbur, 1996).

PRRS-associated respiratory diseases seems to be very age dependent and is usually seen approximately two weeks after weaning (Pijoan, 1996). Galina et al. (1994a) has shown that PRRSV predisposes pigs to *Streptococcus suis* serotype 2 infection and disease resulting in CNS signs and meningitis. However, the interaction is not due to alteration of PAMs functions by PRRSV (Galina et al., 1994b). Coinfection of pigs with PRRSV and *S. cholerae suis* resulted in decreased performance when compared to either control pigs or pathogen alone (Gray et al., 1996). However, Cooper et al. (1995) reported that NEB-1 PRRSV infection did not potentiate common bacterial pathogens: *H. parasuis*, *S. suis*, *S. cholerae suis*, and *P. multocida*. Likewise, Van Alstine et al. (1996) reported that PRRSV
infection did not increase the severity of experimental *M. hyopneumoniae* infection in young pigs. Van Reeth et al. (1994) found that PRRSV by itself was clinically inapparent in feeder pigs, while severe respiratory diseases and production losses occur when respiratory viruses such as porcine respiratory coronavirus (PRCV) or SIV are superimposed on a PRRSV infection. On the other hand Brun et al. (1994) could not show any difference between groups with PRRSV/SIV or SIV alone. Seroconversion, however, to SIV was higher in the PRRSV/SIV group than the SIV-only group. Moreover, these workers were able to show some interaction between PRRSV and unspecified paramixovirus, which resulted in pigs with hypothermia, followed by fever and with inappetence and delay of growth. Other experimental attempts to confirm field observations that PRRSV acts synergistically with a variety of pathogens have not been conclusive (Pijoan, 1996). Variation in the virulence of different isolates in growing pigs has been documented (Halbur et al., 1995, 1996) and may explain the disagreement between research groups in the results of coinfection. Other important factors such as age at time of infection, breed, production style of the unit, environmental conditions, concurrent infections, and the exacerbation of infection by the presence of low levels of PRRSV-specific antibodies may have very important effects on the severity of disease.

**Diagnosis**

Clinical diagnosis is not always straightforward. PRRS diagnosis is based on the combination of clinical manifestations, gross and microscopic lesions, virus isolation results, antigen detection in cells and tissues, and serological tests. The acute reproductive phase is characterized by anorexia, premature farrowings, increased numbers of stillborn pigs, and weak piglets at birth (Dee and Joo, 1994). The acute reproductive phase typically lasts from 2 to 3 months after which reproductive values return to normal. However, decreased farrowing rates and early return to estrus may be persistent features (Done and Paton, 1995). The variable severity of PRRS and the modifying effect of different secondary agents greatly complicate recognition of the disease. Nursery and grower death rate may reach 1-25 percent especially in herds with secondary infections such as *S. choleraesuis, S. suis, H. parasuis,*
encephalomyocarditis virus (EMCV), SIV, or PRCV (Done and Paton, 1995). There are no pathognomonic gross or histopathologic lesions (Terpstra et al., 1991; Collins et al., 1992). However, histopathology has been quite useful to identify the pneumonic process, but is only helpful early on in outbreaks, before bacterial pneumonic pathology obscures the relatively mild viral lesions.

Several cell culture systems used for virus isolation include primary cell culture systems such as PAMs (Dea et al., 1992; Zeman et al., 1993; Molitor et al., 1996), monocyte-derived macrophages (MDMs), and porcine microglia (Molitor et al., 1996) and cell lines such as CL2621 (Collins et al., 1992; Dea et al., 1992), monkey kidney cells (MA104) (Kim et al., 1993), cloned MA104 cells (MARC145), and CRL1171 cells (Meng X-J et al., 1996). A problem that has been recognized with the development of cell lines is that the ability to isolate virus from samples of infected pigs had been complicated. The fact is that isolated do not uniformly grow in all cell types. At this time, only the established cell lines, CL2621, MA104, MARC145, and CRL1171 and PAMs are known to be susceptible to the U.S. isolates. Virus has been isolated from a range of tissues including lung (Dea et al., 1992; Keffaber et al., 1992; Van Alstine et al., 1993a; Halbur et al., 1995b, 1996a, 1996b; Rossow et al., 1995, 1996), spleen (Van Alstine et al., 1993a; Rossow et al., 1995, 1995b; Halbur et al., 1996a), Lymph nodes (Van Alstine et al., 1993; Halbur et al., 1995b; Rossow et al., 1995, 1996), thymus (Van Alstine et al., 1993a; Rossow et al., 1996), tonsil, serum (Van Alstine et al., 1993a; Rossow et al., 1995a, 1995b, 1996a; Rossow et al., 1995, 1996), plasma (Ohlinger et al., 1992; Rossow et al., 1995), buffy coat (Keffaber et al., 1992; Rossow et al., 1995), bone marrow (Rossow et al., 1995), heart, brain (Rossow et al., 1996; Halbur et al., 1995b, 1996a), urine (Goyal, 1993; Rossow et al., 1995), feces (Rossow et al., 1995), placenta (Christianson et al., 1993), nasal swab (Rossow et al., 1995), and nasal turbinates (Rossow et al., 1996). However, serum is a convenient and fairly reliable specimen for sick animals (Van Alstine et al., 1993b), whereas, lung can be readily collected at necropsy. For older pigs, PAMs from freshly killed pigs are more reliable than serum or any other tissues evaluate as diagnostic samples and are often the only samples in which infectious virus and viral antigens are detected more than 3 weeks post exposure (Mengeling et al., 1995). The use of
direct fluorescence antibody (FA) assays for PRRSV antigen detection in frozen tissue sections has been described (Pol et al., 1991; Zeman et al., 1993). Indirect immunostaining methods have been used to detect viral antigens in histological sections of tissues such as lung and lymphoid tissues (Magar et al., 1993; Larochelle et al., 1994; Larochelle and Magar, 1995; Halbur et al., 1994, 1995a, 1995b, 1996a). Reverse transcriptase-polymerase chain reaction (RT-PCR) (Suarez et al., 1994) and in situ hybridization (Larochelle et al., 1996; Sur et al., 1996) have been developed but are not yet routinely applied to clinical specimens.

Serology is the most widely used laboratory diagnostic method and a number of techniques are available. These include immunoperoxidase monolayer assay (IPMA) (Wensvoort et al., 1991a), IFA (Yoon et al., 1992b), SN (Benfield et al., 1992; Morrison et al., 1992a), and enzyme linked immunosorbent assay (ELISA) (Albina et al., 1992). The first serologic test developed for PRRSV antibody detection was the IPMA (Wensvoort et al., 1991a) and has been extensively used in Europe. Most North American veterinary diagnostic laboratories are using the IFA test and/or the SN test to detect PRRSV-specific antibodies. The IFA and IPMA tests are thought to be highly specific and sensitive tests (Wensvoort et al., 1991a; Yoon et al., 1992b). Antibodies to PRRSV are usually detected by these tests between 7 and 15 days and will last until 2 to 3 months after infection (Frey et al., 1992; Van Alstine et al., 1993b; Yoon et al., 1995b). The antibody titers during the decreasing phase could be enhanced by employing a longer incubation time (15 hours) in the IFA test (Joo, 1995). The IFA test for the detection of IgM antibody specific for PRRSV was reported to be useful to identify carrier pigs that are recently infected with PRRSV (Joo, 1995). Specific IgM antibody titers are first detected at the end of the first week, peaked by 14 to 21 DPI, and then rapidly decreased to undetectable levels by 35 to 42 DPI (Loemba et al., 1996).

However, overall performance of the test, including the specificity and sensitivity, has not yet been critically evaluated by other investigators. The SN test is also considered to be a specific test, but previous studies have suggested that the SN test is less sensitive than the IFA and IPMA tests for the detection of an acute infection (Benfield et al., 1992; Morrison et al., 1992a). Recently a modified SN test has been developed to improve its sensitivity by
adding fresh normal swine serum to serum being assayed (Yoon et al., 1994). The modified
test can detect SN antibodies as early as 9-11 days PI. SN titers were high when homologous
PRRSV isolate was used in the test but were markedly lower for heterologous PRRSV
isolates. The ELISA has been also reported to be sensitive and specific that PRRSV-specific
antibodies could be detected as early as 9-10 days PI (Albina et al., 1992; Yoon et al.,
1995b). Automation and high quality control are considered to be strong merits of the test.
A blocking ELISA has been developed and commercial test-kits are now available (Houben
et al., 1995). Yoon et al. (1995b) conducted a study in which the development of the
humoral immune response against PRRSV was monitored by four different serologic tests
over a 105-day period in 8 pigs after intranasal challenge with a PRRSV field isolate.
PRRSV-specific antibodies were first detected by the IFA, IPMA, ELISA, and SN tests 9-11,
5-9, 9-13, and 9-28 DPI and reached their maximum values by 4-5, 5-6, 4-6, and 10-11
weeks PI, respectively. Drew (1995) reported that commercial ELISAs were generally less
sensitive than IPMA when used to assay sera at 10 DPI likely to be due to antigenic variation
among PRRSV. However, Cho et al. (1996) developed an effective blocking ELISA that is
more sensitive than an IFA, particularly with late-infection sera. At the Iowa State
University-Veterinary Diagnostic Laboratory (ISU-VDL), ELISA (Herd Check PRRSV
ELISA, IDEXX Laboratories, Westbrook, Mass.) has proven to be more sensitive and
consistent than the IFA test (Dr. D. Kinker, personal communication). Alternative PRRSV
serology tests used in ISU-VDL include IFA tests for both European and U.S. strains, IgG-
and IgM-specific IFA tests, SN tests, and the IPMA.

Since PRRS is now widespread, single samples will not indicate whether or not infection
has been recent and paired serum samples are to be referred. For a herd diagnosis, a sample
size of 30 will allow 95% confidence of detecting a 10% or higher seroprevalence (Dea et al.,
1992; Joo, 1995). Because finishing pigs have a higher seroprevalence, a sample size of 10
should afford the same confidence interval at the prevalence level of 30% or more (Morrison
et al., 1992b; Joo, 1995). Diagnosis of PRRSV infection as the cause of reproductive failure
or respiratory disease can be achieved by showing seroconversion using paired samples or a
change in antibody titer in paired samples (Van Alstine et al., 1993b). Broad antigenic
variation among PRRSV isolates is a concern in interpreting the serological information because false negative results may be due to the strain of virus in use at a diagnostic laboratory (Bautista et al., 1993; Yoon et al., 1995a). Using the commercial ELISA kit may overcome such potential problems, because the kit contains antigens from several different isolates of PRRSV. Serology is primarily used to determine if a herd has been exposed. For a definitive diagnosis, virus isolation and detection of viral antigen are the tests of choice.

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CHAPTER 2. EFFECTS OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) ISOLATE ATCC VR-2385 INFECTION ON BACTERICIDAL ACTIVITY ON PORCINE PULMONARY INTRAVASCULAR MACROPHAGES (PIMS): IN VITRO COMPARISONS WITH PULMONARY ALVEOLAR MACROPHAGES (PAMS)

A paper published in Veterinary Immunology and Immunopathology 59:323-335, 1997

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Abstract

Porcine pulmonary intravascular macrophages (PIMs) were recovered by in situ pulmonary vascular perfusion with 0.025% collagenase in saline from six 8-week-old, crossbred pigs. Pulmonary alveolar macrophages (PAMs) were recovered by bronchoalveolar lavage from the same pigs for comparisons in each assay. The macrophages were exposed to PRRSV (ATCC VR-2385) in vitro for 24 hours and infection was confirmed by an indirect immunofluorescence test or transmission electron microscopy. Viral particles tended to accumulate in the vesicles of the Golgi apparatus or endoplasmic reticulum. Bactericidal function assays were performed on the recovered macrophages to determine the effects of the virus on macrophage functions. In vitro PRRSV infection reduced the bactericidal ability of PIMs from 68.3% to 56.4% (P < 0.09), and PAMs from 69.3% to 61.0% (P > 0.1) at 24 hours post infection. The mean percentage of bacteria killed by macrophages after PRRSV infection was not significantly different among the treatment groups or between the treatment groups and non-infected controls based on colorimetric MTT bactericidal (Staphylococcus aureus) assay. PRRSV did not affect the ability of PIMs or PAMs to internalize opsonized 125I-iododeoxyuridine-labeled S. aureus (P > 0.05). PRRSV infection significantly decreased the production of superoxide anion (P < 0.01) by 67.0% in PIMs and by 69.4% in PAMs. PRRSV reduced the myeloperoxidase-H2O2-halide product (P < 0.01) by 36.5% for PIMs and by 48.1% for PAMs. The results suggest: 1) PIMs
should be considered as an important replication site of PRRSV; 2) PRRSV may have a
detrimental effect on both PIMs and PAMs; 3) loss of bactericidal function in PIMs may
facilitate hematogenous bacterial infections.

Keywords: Arteriviridae; bactericidal functions; macrophages; phagocytosis; porcine reproductive and respiratory syndrome virus

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) was first isolated on pulmonary alveolar macrophage (PAM) cultures and was designated Lelystad virus (Pol et al., 1991; Terpstra et al., 1991). The first report of isolation and propagation of PRRSV, ATCC VR-2332, on a continuous cell line (CL2621) occurred in the United States in 1992 (Collins et al., 1992). Both Lelystad virus and ATCC VR-2332 induced reproductive failure and interstitial pneumonia in experimentally infected pigs (Collins et al., 1992; Pol et al., 1991; Terpstra et al., 1991). PRRSV is tentatively classified in the proposed family Arteriviridae, which includes equine arteritis virus, lactate dehydrogenase-elevating virus, and simian hemorrhagic fever virus (Meulenberg et al., 1993).

PRRSV appears to replicate extensively in cells of the immune lineage, notably macrophages. PRRSV antigen has been detected in resident macrophages in various tissues such as lung, lymph nodes, tonsil, heart, thymus, spleen, Peyer's patches, liver, kidney, adrenal gland (Halbur et al., 1995a, 1995b, 1996), and brain (Rossow et al., 1996; Thanawongnuwech et al., 1997). Other cells in which PRRSV antigen was detected include pneumocytes, bronchiolar epithelium (Pol et al., 1991; Rossow et al., 1996), and vascular endothelial cells in the lung (Rossow et al., 1996) and heart (Halbur et al., 1995a, 1996).

Pulmonary intravascular macrophages (PIMs) are members of mononuclear phagocytic system, found preferentially apposed to the underlying endothelium with intercellular adhesion plaques (ICAPs) (Winkler and Cheville, 1987). PIMs are numerous in pigs, ruminants, horses, and cats, but appear to be infrequent or absent in species such as dogs, rodents, and human beings (Staub, 1994). Porcine PIMs are derived from monocytes which adhere to the endothelium by ICAPs and replicate within the capillaries postnatally (Winkler
and Cheville, 1987). The lung is the principle site of clearance of blood-borne pathogens and debris in pigs, with PIMs serving as a major component of the systemic host-defense mechanism (Winkler, 1988; Staub, 1994).

To date, PAMs and monocyte-derived macrophages (MDMs), are the primary cells identified to support replication of PRRSV in vitro. By immunohistochemistry, there is evidence that PRRSV antigen is present in PIMs (Halbur et al., 1996; Rossow et al., 1996). However, the susceptibility of PIMs to PRRSV has not been clearly elucidated. Reduction in the numbers of PAMs has been demonstrated in pigs experimentally infected with PRRSV (Done and Paton, 1995). We hypothesize that PRRSV will infect PIMs in vitro and in vivo, and that the infection will produce structural damage and functional impairment of the PIMs. We, therefore, examined the bactericidal (Staphylococcus aureus) function of PIMs in vitro and compared it to PAMs prior to or after PRRSV infection. We also measured phagocytosis and superoxide anion and myeloperoxidase-H$_2$O$_2$-halide production of both macrophage populations. The overall objective of the study reported here was to determine the effect of PRRSV infection on bactericidal activity of PIMs at 24 hours post infection (HPI) before the virus destroyed the macrophages.

2. Materials and methods

2.1 Experimental animals

Six 8-week-old crossbred pigs, from a PRRSV-free herd, were used as donor animals for PAM and PIM collection. All pigs were clinically normal. Sera and lung tissues were collected for serology and PRRSV isolation. PRRSV antigen detection by immunohistochemistry in lungs (Halbur et al., 1995a) was performed. All animals were negative for PRRSV and PRRSV-induced antibodies.

2.2 Initial recovery of macrophages

The method used to recover PIMs by in situ pulmonary vascular perfusion was performed as previously described (Fowler et al., 1991) with minor modifications. Briefly, 100 U/kg sodium heparin and 20 ng papaverine-HCl (Sigma Chemical Co., St. Louis, MO) were administered intravenously to reduce blood clotting and to reduce agonal and post mortem
vasoconstriction. Animals were sacrificed humanely with an intravenous overdose of sodium pentobarbital injection (Anpro Pharmaceutical, Arcadia, CA).

All solutions were prepared using aseptic technique. The pulmonary vasculature was then perfused with 0.01% Na$_2$EDTA in saline (500ml), 0.075% Na$_2$NO$_3$ in saline (500ml), 0.055% CaCl$_2$ in saline (500ml), 0.025% collagenase (type 1A, specific activity 125 U/mg, Sigma Chemical Co., St. Louis, MO) in 0.055% CaCl$_2$ in saline (500ml), and 0.055% CaCl$_2$ in saline (1000 ml), respectively at 24 ml/min using a Harvard apparatus peristaltic pump (Harvard Apparatus, Millis, MA). The outflow from the last two perfusion solutions was centrifuged (400g, 10 min) at 4° C. Cell pellets were resuspended in phosphate buffered saline (PBS), and PIMs were isolated by density gradient centrifugation (400g, 30 min) using Histopaque 1077 gradient (Sigma Chemical Co., St. Louis, MO). PIMs were resuspended in complete media containing RPMI 1640 (GIBCO Laboratories, Grand Island, NY), 10% FBS (HyClone Laboratories Inc, Logan, UT), 3 U polymyxin B/ml, 9 μg gentamycin/ml, and 30 U mycostatin/ml and 10 ml was placed into 100 mm$^2$ tissue culture dishes at 37 °C in a humidified atmosphere with 5% CO$_2$ to allow the cells to adhere.

Soon after vascular perfusion, PAMs were collected from the lungs using the protocol described by Mengeling et al. (1995). Lavage fluid consisting of complete media was gently dispensed and aspirated several times into the lungs to collect the PAMs. Ten ml of the pooled fluid was placed in 100 mm$^2$ plastic tissue culture dishes and kept in the same incubator as the PIMs.

After 1 hour, the culture plates of PIMs and PAMs were washed twice with complete media to remove non-adherent cells. Cells were scraped using a cell scraper (Costar, Cambridge, MA), resuspended in the complete media, and counted manually using a hemocytometer. Viability was determined by tryphan blue dye exclusion. Differential cell counts were obtained by counting at least 200 cells on modified Wright-Giemsa-stained (Diff-Quick, Baxter) cytocentrifuge preparations (Cytospin 2, Shandon Inc.). Nonspecific esterase staining was performed using an α-Naphthyl acetate esterase assay (Sigma chemical Co., St. Louis, MO). Cells exhibiting black granulation were considered to be nonspecific esterase
positive in monocytes, macrophages, and histiocytes. Two hundred cells were graded positive or negative for nonspecific esterase staining.

2.3 Virus

A stock of PRRSV (VR-2385) isolate at the 6th cell-culture passage was prepared in a continuous cell line, ATCC CRL1171, using the procedures described previously (Meng et al., 1996). The titer of this pool was calculated to be $10^{5.56}$ TCID$_{50}$ per ml.

2.4 PRRSV infection of cultured macrophages

Five X $10^5$ macrophages in 100 µl of complete media were placed in flat-bottom microtiter plates, one row (12 wells) for each cell type overnight. The first six wells of PIMs and PAMs were treated with the PRRSV at a multiplicity of infection (m.o.i.) of 1. Negative control cultures containing media alone were used for the next six wells in the same row. Each microplate was subjected to bactericidal or cytochrome C reduction assay after 24 HPI.

Three ml of either PIMs or PAMs was placed into 35 mm$^2$ tissue culture dishes and was treated with PRRSV at a m.o.i. of 1. Negative control cultures containing macrophages with media alone were included in every experiment. After 24 HPI, indirect immunofluorescence test and electron microscopy were performed on each treatment group. PRRSV-infected and negative control macrophages were harvested for phagocytic and iodination assays using a cell scraper.

2.5 Indirect immunofluorescence assay (IFA)

The PRRSV-infected and uninfected macrophages were examined by IFA using the monoclonal antibody SD0W17 (South Dakota State University, Brookings, South Dakota) and a rabbit antimouse IgG-FITC (Sigma chemical Co., St. Louis, MO). Positive staining was determined by bright cytoplasmic fluorescence. Positive or negative staining was counted manually on at least 100 cells.

2.6 Transmission electron microscopy

Cells were washed twice with 0.1 M Na cacodylate buffer (pH 7.2) and fixed 15 min with 3% glutaraldehyde in 0.1 M Na cacodylate buffer at 4°C. Cells were scraped off, centrifuged, and resuspended in fresh fixative at 4°C for several days. Fixed cells were washed 3 times-15 min changes with 0.1 M Na cacodylate buffer before post fixation. Next,
1% cacodylate-buffered osmium tetroxide was used for postfixation at room temperature for 120 min. Then the fixed cells were washed with distilled water, dehydrated through a series of graded acetone (50%, 75%, 95%, and 100%), placed in three changes of 50/50% acetone/plastic, and baked at 60°C for 2 days. The block tip was trimmed with a glass knife, and thin sections were cut with a diamond knife. Thin sections were placed on copper grids, stained with uranyl acetate and lead citrate, and examined in a Hitachi 500 electron microscope.

2.7 Colorimetric MTT bactericidal assay

The bactericidal (S. aureus) activity of the PIMs and PAMs was measured using a modification of the protocol described by Stevens et al. (1991). S. aureus and its antiserum were kindly provided by Drs. J. Roth and D. Frank (Iowa State University, Ames, IA). Macrophages were washed twice with media (RPMI 1640, 5% FBS) containing no antibiotic and 50 μl of 5% FBS media was added resulting in 5 X 10^5 macrophages per well. Fifty μl of 2.5 X 10^7 opsonized bacteria was added to each well resulting in a ratio of 50 bacteria per macrophage. This was done in triplicate. Microtiter plates were then placed on a Micro Shaker II (Dynatech Laboratories Inc, Alexandria, VA) for 1 h at 37°C. The macrophages were lysed by adding 50 μl of 0.2% saponin to each well. Fifty μl of the opsonized bacterial suspension was then added to two control wells (0% killing) and 50 μl of 5% FBS media containing 1% S. aureus antiserum was added in the last control well (100% killing) in each treatment group to ensure that 1) macrophages which had been lysed with saponin did not convert MTT to formazan; 2) saponin-mediated lysis of macrophages did not kill bacteria.

Formazan produced by bacteria was quantitated by measuring optical density (OD) at 550 nm. The OD reading was obtained using an automated 96-channel microtiter plate spectrophotometer (Vmax, Molecular Devices Corporation, Palo Alto, CA) interfaced to a computer. The OD corresponding to 0 and 100% killing of bacteria was established separately by using the OD mean of controls in each treatment. The percentage of bacteria killed by macrophages was determined by the formula:

\[ \frac{[1 - \frac{\text{OD sample}}{\text{OD 100% killing}} - \frac{\text{OD 0% killing}}{\text{OD 100% killing}}] \times 100}{\text{OD 0% killing} - \text{OD 100% killing}} \]
2.8 Cytochrome C reduction assay

This assay measured the amount of superoxide anion (SOA) produced by macrophages during the oxidative metabolic burst by determining the change in OD due to the reduction of cytochrome C (Type III from horse heart, Sigma Chemical Co., St. Louis, MO) comparing to the unstimulated or resting macrophages. The assay was performed using a modification of procedures previously described (Roof and Kramer, 1989; Chiang et al., 1991). The assay was conducted in triplicate. All parts of the assay, unless indicated, used Hank's balanced salt solution (HBSS) without phenol red (GIBCO Laboratories, Grand Island, NY).

Macrophages were washed twice with HBSS and 50 μl of HBSS was added in all wells resulting in 5 X 10^5 macrophages per well. A hundred μl of 0.25% cytochrome C in HBSS was added in all wells. Fifty μl of preopsonized zymosan (7.5 mg/ml in HBSS) was added to stimulate the macrophages. Measurement of SOA production by resting macrophages was performed in a similar manner, except the preopsonized zymosan was replaced with 50 μl of HBSS. The measurement of the standard and background wells was performed similarly to the stimulated and unstimulated wells, except no macrophages were added for zeroing on the microtiter plate spectrophotometer. The microtiter plates were incubated with agitation on a Micro Shaker II at 37°C for 1 h in the dark and the supernatant from each well was transferred into flat bottom microtiter plates after being centrifuged at 1600 g for 5 min. The mean OD at 550 nm (X 1000) of the solution was determined using an automated 96-channel microtiter plate spectrophotometer. Percent SOA production was calculated by the formula:

\[
\frac{\text{OD stimulated wells} - \text{OD unstimulated wells}}{\text{OD unstimulated wells}} \times 100
\]

2.9 Phagocytic assay

A standard phagocytosis assay was conducted using a modification of the procedures previously described (Roof and Kramer, 1989; Yu-Wei Chiang, unpublished). Heat-killed S. aureus labeled with ^125^I-iodo-deoxyuridine (UdR) (Amersham, Arlington Heights, IL) were used to evaluate ingestion by the macrophages. All samples were assayed in duplicate in 12 X 75 mm plastic tubes containing 100 μl of 1.5 X 10^8 cfu of ^125^I-UdR-labeled S. aureus, 50 μl of 2.5 X 10^5 macrophage suspension (bacteria to macrophage ratio = 60:1), 50 μl of 1:6
dilution of porcine anti-\textit{S. aureus} serum, and 300 \mu l of Earle's basic salt solution (EBSS; GIBCO, Grand Island, NY). The tubes were centrifuged at 100 g for 5 min, incubated in a shaking water bath for 20 min, and vortexed to bring the cells back into suspension. After vortexing, extracellular \textit{S. aureus} were lysed with lysostaphin (0.5 ml, 1 IU in PBS) for 30 min at 37°C and the macrophages were washed twice with 2 ml cold PBS. The final pellet was placed in a gamma counter to determine counts per minute (cpm) of radioactivity present. Standard tubes were set up which contained standard amount of \textit{S. aureus} without lysostaphin. Background tubes contained all reactants except macrophages. The percent of the \textit{S. aureus} ingested was calculated using the following equation:

\[
\text{Percent ingestion} = \frac{\text{cpm in reaction tube} - \text{cpm in background tube}}{\text{cpm in standard tube} - \text{cpm in background tube}} \times 100
\]

2.10 Iodination assay

This assay measured the myeloperoxidase (MPO-H_2O_2-halide) activity of macrophages by measuring the amount of ^{125}\text{I} incorporated into the phagolysosomes. This assay was performed in duplicate using a modification of procedures previously described (Roth and Kaeberle, 1981; Roof and Kramer, 1989). The standard reaction mixture contained 50 \mu l of 2.5 \times 10^5 macrophages, 0.1 \mu Ci of ^{125}\text{I} in 50 \mu l of EBSS, 50 \mu l of NaI in EBSS (20 nmole), 50 \mu l of opsonized zymosan, and 300 \mu l of EBSS. The cpm of radioactivity remaining in the trichloroacetic acid (TCA)-precipitate was measured in a gamma counter. The nanomoles of iodide converted to a TCA-precipitate per 10^7 macrophages per hour was calculated using the following equation:

\[
\frac{(\text{cpm unknown} - \text{cpm blank}) \times (40 \text{ nmole NaI}) \times (1 \times 10^7 \text{ cells}) \times (60 \text{ min})}{(\text{cpm standard}) \times (2.5 \times 10^5 \text{ cells}) \times (20 \text{ min})} = \text{nmole NaI/10}^7 \text{ cells/hr}
\]

2.11 Statistical analysis

Data was expressed as mean ± standard deviation (SD), otherwise indicated. The effect of virus inoculation on bactericidal and phagocytic activities, and the production of SOA and MPO-H_2O_2-halide was statistically analyzed at \( P < 0.05 \) level using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). The protected \( t \)-test was performed for further treatment
differentiation when the equal effects assumption in the analysis of variance (ANOVA) was rejected.

3. Results

3.1 Isolation of PIMs and PAMs

The total cell number obtained in this study was between 1 to $4 \times 10^8$ PIMs and greater than $10^9$ PAMs (data not shown). Some pig lungs were susceptible to collagenase pulmonary vascular perfusion which resulted in diminished flow of left atrial effluent. Cell viability was consistently high (> 97%) in both PIMs and PAMs (data not shown). Greater than 85% and 90% of cells isolated were either PIMs and PAMs, respectively, and were macrophages based on morphologic examination and non-specific esterase assay. PIMs appeared to be smaller than PAMs (data not shown). Neutrophils and lymphocytes were the other predominant cell types in both isolations.

3.2 PRRSV antigen detection in macrophages by IFA

Bright cytoplasmic fluorescence (Fig. 1A) was observed in both PIMs and PAMs inoculated with PRRSV for 24 HPI. Approximately 40% of macrophages were positively stained for PRRSV nucleocapsid protein (data not shown). Noninoculated macrophages remained negative.

3.3 PRRSV detection in macrophages by TEM

Electron microscopic examination of ultrathin sections of infected PIMs and PAMs revealed viral particles that tended to accumulate in the vesicles of the Golgi apparatus or endoplasmic reticulum and to be released from the vesicles via exocytosis by fusing of the vesicle wall to the cytoplasmic membrane (Fig. 1B). The intracellular virions appeared as enveloped viral particles 60-65 nm in diameter, with an icosahedric core of about 35-45 nm. Approximately 50% of infected macrophages contained aggregation of viral ribosomes, numerous phagolysosomes, cytoplasmic vacuoles, and residual bodies filled with necrotic debris and damaged cellular components. Swollen mitochondria with cristolysis and flocculation of matrical proteins were also observed in the infected macrophages. The
uninfected cells had intact cytoplasmic organelles and normal nuclear and cytoplasmic membranes.

3.4 Effect of PRRSV infection on the *S. aureus* killing

In the preliminary experiments, a standard curve of bactericidal activity was established by determining the OD of 0% and 90% killing using a protocol previously described (Stevens et al., 1991). The results were not different from the results we obtained (0% and 100% killing) in our experiment. We calculated the percent bacteria killed as described previously. *In vitro* PRRSV infection reduced the bactericidal ability of PIMs from 68.3% to 56.4% (*P* < 0.09), and PAMs from 69.3% to 61.0% (*P* > 0.1) at 24 HPI. Results are shown in Table 1. The mean percentage of bacteria killed by macrophages after PRRSV infection was not significantly different among the treatment groups or between the treatment groups and the control groups.

3.5 Effect of PRRSV infection on the ingestion of *S. aureus*

PRRSV infection caused a 3.6% and 2.6% increase in internalization of opsonized bacteria in PIMs and PAMs at 24 HPI, respectively (Table 1), but these differences were not statistically significant.

3.6 Effect of PRRSV on cytochrome C

The ability of PAMs to produce superoxide anion in response to opsonized zymosan, as measured by the reduction of cytochrome C, was significantly greater than that of PIMs (116.3% vs. 87.3%, respectively; *P* < 0.05). SOA production was significantly (*P* < 0.01) decreased in both PIMs and PAMs after *in vitro* PRRSV infection by 67% and by 69.4% at 24 HPI, respectively (Table 1).

3.7 Effect of PRRSV on iodination

*In vitro* PRRSV infection significantly (*P* < 0.01) inhibited iodination of protein in both PIMs and PAMs by 36.5% and by 48.1% at 24 HPI, respectively (Table 1). Prior to PRRSV infection, the opsonized zymosan activated PAMs iodinated significantly (*P* < 0.05) more protein than the opsonized zymosan activated PIMs (13.5 vs 10.4 nmole NaI/10^7 macrophages/h, respectively).
4. Discussion

*In situ* pulmonary vascular perfusion with 0.025% collagenase in saline was an excellent technique to obtain PIMs. The number of recovered PIMs were less than or equal to those recovered by previous investigators that recovered $3.8 \times 10^8$ PIMs (Fowler et al., 1991). Rogers et al. (1994) recovered 34% of ovine PIMs by proteolytic lung digestion compared to 3% by vascular perfusion technique utilizing iron oxide ($\gamma$Fe$_2$O$_3$) to identify and to isolate PIMs. These results suggest that a large number of PIMs are retained in the lung. We used the vascular perfusion method in this experiment as the effect of iron oxide on PIM function is unknown. Chitko-McKown et al. (1992) reported that collagenase treatment had no effect on macrophage function. We found that some of the 8-week-old pig lungs appeared edematous soon after the collagenase perfusion leading to diminution of the effluent flow because of connective tissue breakdown. Calf lungs (Chitko-McKown et al., 1992) and 4-month-old pig lungs (Thanawongnuwech, unpublished) do not react to the collagenase in this way and collection of perfusate is not limited by lack of flow. Susceptibility to collagenase in individual young pig lungs might be an explanation for the difference in the effluent flow resulting in decreased yield of PIMs. Therefore, more efficient methods of PIM collection may be required to improve the recovery of porcine PIMs for further investigations.

Colorimetric MTT bactericidal (*S. aureus*) assays demonstrated that PRRSV infection reduced the bactericidal ability of both PIMs and PAMs, but not significantly at 24 HPI. However, the production of superoxide anion and MPO-H$_2$O$_2$-halide system was significantly decreased in both PIMs and PAMs. Based on the TEM examination, approximately 50% of the macrophages were intact and able to maintain their bactericidal capabilities at 24 HPI. These macrophages may not have been infected or may be resistant to PRRSV infection. Human influenza virus is thought to impair human monocyte bactericidal (*S. aureus*) activity, but no effect on bactericidal activity by human PAMs was demonstrated *in vitro* (Wilson, 1990). Human influenza virus infection triggers an oxidative burst in monocytes, which leads to reduced oxidative metabolism and depressed intracellular killing, although phagocytosis is not affected (Gardner and Lawton, 1982). Like human influenza virus, PRRSV may mildly stimulate macrophage phagocytosis in early infection, but after 24
hours the virus may have a detrimental effect on the macrophage intracellular killing of bacteria. This may be due to PRRSV reducing oxidative metabolism, inducing apoptosis, or lysis of macrophages. Numerous phagolysosomes and residual bodies have been demonstrated in a number of the infected macrophages in this report. These morphological changes may lead to advanced degeneration and lysis of the macrophages. In addition, both oxidative stress (Li et al., 1996) and PRRSV (Suarez et al., 1996) have been reported to induce apoptosis in PAMs. Further investigation of the effect of PRRSV on PIMs and PAMs should be performed at different periods post infection (that work is in progress in our laboratory).

Chitko-McKown et al. (1991, 1992) found that both porcine or bovine PIMs and PAMs possess similar efficiency in ingesting and killing bacteria but LPS-stimulated PIMs produced more T-cell proliferative cytokines than LPS-stimulated PAMs. In contrast, PAMs produced more TNF-α and nitrite production than PIMs when both populations were stimulated with LPS. We found that PIMs produced significantly less superoxide anion and MPO-H2O2-halide products than PAMs. Destructive products including superoxide anion and MPO-H2O2-halide products secreted by up-regulated macrophages are capable of producing injury to neighboring cells and the interstitium of various tissues by subsequent spilling of toxic materials into the environment (Nathan, 1987). Regulation of these destructive, oxidative products may be more controlled in PIMs to reduce the tissue injury. Obviously, ultimate regulation at the level of release of oxidative products is complex and remains to be investigated. Another possible hypothesis is that PIMs may rely on anaerobic glycolysis like monocytes and peritoneal macrophages, whereas PAMs use both anaerobic glycolysis and aerobic cytochrome oxidation (Wilson, 1990). Destruction of a microorganism requires phagocytosis, formation of a phagolysosome, initiation of a respiratory burst, and release of the products of the respiratory burst plus other macrophage constituents such as enzymes and cationic proteins into the phagosome. PIMs, therefore, may rely primarily on oxygen-independent bactericidal mechanisms.

Voicu et al. (1994) successfully infected peripheral blood monocytes (PBMC) with PRRSV. Monocytes, however, are inherently resistant to PRRSV infection and poorly
support virus production in vitro, unless activated or induced to mature. The differentiation of monocytes to PIMs may modulate the susceptibility of PIMs to PRRSV infection. Demonstration of cytoplasmic PRRSV antigen via IFA and viral particles via TEM in PIMs shows that PRRSV also infects and replicates in PIMs. Since, blood clearance of bacteria is one of the major roles of PIMs (Winkler, 1988; Staub, 1994), the loss of bactericidal function in PIMs may facilitate hematogenous bactericidal infection. An important feature of PRRSV infection is an increased incidence of bacterial disease (Zeman et al., 1993; Galina et al., 1994). However, Cooper et al. (1995) failed to show an interaction between PRRSV and bacterial pathogens. In vivo studies on the effect of PRRSV on PIMs should be done to ascertain, whether the results parallel those observed in vitro.

Acknowledgment

We are grateful to Drs. F. Ahrens, D. Frank, J. Roth, K. Platt, and J. Kinyamu for technical assistance, Drs. D. Frank, M. Ackermann, and J. Zimmerman for editorial assistance, Jean Olsen and Jim Fosse for photography, and Lie-ling Wu and M. Brabec for conducting statistical analysis. This study was supported in part by grants from Healthy Livestock for Iowa Initiative 1996-1997.

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Table 1. Effect of PRRSV on *S. aureus* killing, *S. aureus* ingestion, superoxide anion (SOA) production, and iodination of protein.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Killing (%), mean ± SD</th>
<th>Ingestion (%), mean ± SD</th>
<th>SOA production (OD at 550 nm X 1000), mean ± SD</th>
<th>% production</th>
<th>Iodination [nmole NaI/10^7 cells/h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIMs</td>
<td>68.3 ± 17.4</td>
<td>55.3 ± 6.2</td>
<td>134.9 ± 53.6 unstim 252.7 ± 83.6 stim</td>
<td>+87.3 [a]</td>
<td>10.4 ± 2.9 [a]</td>
</tr>
<tr>
<td>PIMs + PRRSV</td>
<td>56.4 ± 18.6</td>
<td>57.3 ± 5.9</td>
<td>158.2 ± 47.1 unstim 203.8 ± 58.2 stim</td>
<td>+28.8</td>
<td>6.6 ± 2.1</td>
</tr>
<tr>
<td>% Change</td>
<td>-17.4</td>
<td>+3.6</td>
<td>-67.0 [2]</td>
<td></td>
<td>-36.5 [2]</td>
</tr>
<tr>
<td>PAMs</td>
<td>69.3 ± 8.6</td>
<td>50.4 ± 7.1</td>
<td>113.1 ± 59.8 unstim 244.6 ± 100.6 stim</td>
<td>+116.3 [b]</td>
<td>13.5 ± 5.6 [b]</td>
</tr>
<tr>
<td>PAMs + PRRSV</td>
<td>61.0 ± 19.4</td>
<td>51.7 ± 8.8</td>
<td>92.7 ± 48.9 unstim 125.7 ± 54.5 stim</td>
<td>+35.6</td>
<td>7.0 ± 2.1</td>
</tr>
</tbody>
</table>

* Mean ± SD; n = 6

1 [nmole NaI/10^7 cells/h.]

2 % Change of means are significantly different at *P* < 0.01

[ab] Means within the same column followed by dissimilar superscripts are significantly different at *P* < 0.05.
Figure 1. PRRSV propagated in primary cultures of PIMs. A. Infected PIMs with intense cytoplasmic fluorescence observed at 24 HPI. B. Transmission electron micrograph of intracellular viral particles accumulated within the cytoplasmic vesicles of a PIM. Intracellular virion budding from the membrane of a smooth-walled vesicle (arrow). A viral particle expelled from a PIM via exocytosis by fusing of the vesicle wall to the cytoplasmic membrane (arrow head).
CHAPTER 3. INFLUENCE OF PIG AGE AND STRAIN OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) ON VIRUS TITER AND BACTERICIDAL ACTIVITY OF PORCINE PULMONARY INTRAVASCULAR MACROPHAGES (PIMS)

A paper submitted to *Veterinary Microbiology*

Roongroje Thanawongnuwech, Eileen L. Thacker, and Patrick G. Halbur

Abstract

Twelve pigs (six 4-week-old and six 4-month-old cross-bred, specific pathogen free pigs) were used as donors for both pulmonary intravascular macrophages (PIMs) and pulmonary alveolar macrophages (PAMs). The PIMs and PAMs were infected in vitro with low (ISU-55) or high (VR-2385) virulence strains of PRRSV at 1 m.o.i. for comparisons of virus titers at 48 hours post infection (HPI). PIMs were as permissive as PAMs to infection with both PRRSV isolates yielding similar progeny titers ($10^{4.81}$ vs. $10^{5.22}$ TCID$_{50}$/ml, respectively). Both ISU-55 and VR-2385 were able to infect PIMs and no significant difference in virus replication as measured by virus titers between isolates was found ($10^{5.33}$ vs. $10^{4.69}$ TCID$_{50}$/ml, respectively). PIMs from 4-week-old pigs yielded a higher virus titer following PRRSV infection than PIMs from 4-month-old pigs ($10^{5.43}$ vs. $10^{4.56}$ TCID$_{50}$/ml, respectively; $P < 0.02$). Bactericidal activities at 15, 24, and 48 HPI were measured in PIMs. VR-2385-infected PIMs had significantly decreased bactericidal (*Staphylococcus aureus*) activity compared with uninfected PIMs at 48 HPI ($P < 0.05$). There was no difference in bactericidal activity between ISU-55 (low virulence)-infected PIMs and VR-2385 (high virulence)-infected PIMs. Both ISU-55 and VR-2385 infection significantly decreased the production of superoxide anion (SOA) at 24 and 48 HPI ($P < 0.01$). There was no significant difference between virus strains or pig age groups on SOA production. In conclusion, 1)
PRRSV had a detrimental effect on bactericidal activity and SOA production of PIMs, 2) PIMs from younger pigs were more permissive to PRRSV infection, and 3) the selected PRRSV strains, which differ in their abilities to induce pneumonia in vivo were not different when tested in vitro by measuring virus titer and bactericidal functions.

**Keyword**: Porcine reproductive and respiratory syndrome virus; Strain variability; Virus titer; Bactericidal activity; Pulmonary intravascular macrophages; Pigs

### 1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure in sows and respiratory disease in pigs and was first recognized in the United States (US) in 1987 (Hill, 1990) and subsequently in Europe in 1991 (Wensvoort et al., 1991). PRRS virus (PRRSV) is an enveloped single-stranded RNA virus belonging in the order Nidovirales of the family Arteriviridae and genus Arterivirus which also includes lactate dehydrogenase-elevating virus (LDV), simian hemorrhagic fever virus (SHFV), and equine arteritis virus (EAV) (Cavanagh, 1997). Considerable antigenic (Nelson et al., 1993), genetic (Meng et al., 1995; Andreyev et al., 1997), and pathogenic (Halbur et al., 1995; Halbur et al., 1996) variation have been reported between US and European isolates and among US isolates. PRRSV isolate ISU-55 has been demonstrated to be low virulence in contrast to VR-2385 which is high virulence based on the clinical disease in the herds where the isolates originated and severity of experimentally-induced gross and microscopic lesions (Halbur et al., 1996).

PRRSV replicates in resident macrophages of various tissues in which PRRSV antigens were demonstrated (Halbur et al., 1995; Halbur et al., 1996). Molitor et al. (1996) reported that porcine alveolar macrophages (PAMs) and porcine brain microglia were highly permissive to PRRSV in vitro. Pulmonary intravascular macrophages (PIMs), which adhere to the pulmonary capillary endothelium by intercellular adhesion plaques (ICAP) in pigs (Winkler and Cheville, 1987), have been demonstrated to support PRRSV replication in vitro (Thanawongnuwech et al., 1997). We have recently demonstrated significantly decreased pulmonary clearance of copper particles by PIMs in PRRSV-infected pigs.
(Thanawongnuwech et al., 1998). Since PIMs are vital to the clearance of blood-borne pathogens and debris in pigs (Staub, 1994), damage to PIMs would adversely affect pulmonary clearance of bacteria predisposing pigs to infectious diseases (Smith et al., 1996).

Young pigs are more susceptible to PRRSV infection and are more likely to have secondary bacterial infections (Rossow, 1998). Decreased permissiveness of mouse peritoneal macrophages to LDV infection with increasing age of the mouse has been documented (Onyekaba et al., 1989).

An in vitro test to predict the virulence of PRRSV strains would be useful. A marked difference in virulence exists among U.S. PRRSV isolates (Halbur et al., 1995; Halbur et al., 1996). PRRSV plaque variants on MARC-145 cell cultures has been reported to be a potential virulence marker for reproductive form of PRRS (Park et al., 1996). Swine tracheal ring (STR) culture examination for histopathologic and ultrastructural changes has been reported to identify respiratory virulence strains of PRRSV (Joo and Park, 1996). However, the results from experimental challenge of pigs are more conclusive in term of determination of the virulence. We examined the effects of known low and high virulence strains of PRRSV in terms of virus titers and bactericidal (Staphylococcus aureus) activity in PIMs in vitro prior to or after PRRSV infection.

2. Materials and methods

2.1 Animals

The protocol used in this study was approved by the Committee on Animal Care (COAC) of Laboratory Animal Resources at Iowa State University. Six 4-week-old and six 4-month-old cross-bred, specific pathogen free and PRRSV-free pigs were used as donors for PIMs and PAMs.

2.2 Initial recovery of macrophages

Animal were sacrificed humanely with an intravenous overdose of sodium pentobarbital injection (Anpro Pharmaceutical, Arcadia, CA) and euthanized by exsanguination. To reduce blood clotting and to reduce agonal and post mortem vasoconstriction, 100 U/kg sodium heparin and 20 ng papaverine-HCl (Sigma, St. Louis, MO) were administered into the right
ventricle of the heart before the thoracic organs being removed. PAMs and PIMs were harvested from the lungs by bronchoalveolar lavage and by pulmonary vascular perfusion with 0.025% collagenase, respectively as previously described (Thanawongnuwech et al., 1997).

2.3 Viruses

Viruses used in this study were low (ISU-55) and high (VR-2385) virulence strains of PRRSV (Halbur et al., 1996) prepared in a continuous cell line, ATCC CRL11171 (Iowa State University Research Foundation, Ames, IA). Virus titers were $10^{7.46}$ TCID$_{50}$/ml for ISU-55 and $10^{5.56}$ TCID$_{50}$/ml for VR-2385.

2.4 PRRSV infection of PIMs and PAMs

Five $\times 10^5$ of PIMs in 100 $\mu$l of complete media were placed in 3 flat-bottom, microtiter plates, 3 rows (36 wells) overnight. PIMs were inoculated with one of the PRRSV isolates at a multiplicity of infection (m.o.i.) of 1 for 48, 24, or 15 hours (4 wells each) prior to the assays. Negative control cultures containing media alone (4 wells each) were used in the same row. Each microplate was subjected to bactericidal, cytochrome C reduction, or indirect immunofluorescence (IFA) assays on the same day as previously described (Thanawongnuwech et al., 1997).

Two ml of PIMs ($5 \times 10^5$ cells/ml) were placed into 6-well tissue culture plates and were infected with either ISU-55 or VR-2385 (3 wells each) at 1 m.o.i. for 48, 24, or 15 hours. Two ml of PAMs ($5 \times 10^6$ cells/ml) were also placed into 2 wells of 6-well tissue culture plates and were infected with either ISU-55 or VR-2385 at 1 m.o.i. for 48 hours. Negative control cultures containing PIMs or PAMs with media alone were also included. Supernatant from infected wells of either PIMs or PAMs after 48 HPI with one of PRRSV isolates were harvested for virus titration. PRRSV-infected and negative control PIMs were harvested for viability tests and transmission electron microscopy (TEM) examination after 15, 24, and 48 hours post infection (HPI) as previously described (Thanawongnuwech et al., 1997).

2.5 Virus titration

PIMs and PAMs were infected in vitro with either ISU-55 or VR-2385 virulence strains of PRRSV at 1 m.o.i. for comparisons of virus replication at 48 HPI. For virus titration,
growth medium without serum was used as a diluent and serial 10-fold dilutions of the supernatant harvested from infected wells with one of PRRSV isolates were made in 96-well microtiter plates previously seeded with CRL11171. The cell cultures were incubated at 37 °C with 5% CO₂, and were examined for CPE after 48 HPI. Viral CPE was confirmed by indirect immunofluorescence assay using an anti-PRRSV monoclonal antibody SDOW-17 (South Dakota State University, Brookings, SD) (Meng et al., 1996). The titers were calculated by the method of Reed and Muench (1938) and expressed as log₁₀ TCID₅₀/ml.

2.6 Colorimetric MTT bactericidal assay

The bactericidal (S. aureus) activity of the PIMs was measured (Thanawongnuwech et al., 1997). S. aureus and antiserum were provided by Drs. J. Roth and D. Frank (Iowa State University, Ames, IA). Formazan produced by bacteria was quantitated by measuring optical density (OD) at 550 nm. The OD reading was obtained using an automated 96-channel microtiter plate spectrophotometer (Vmax, Molecular Devices Corporation, Palo Alto, CA) interfaced to a computer. The OD corresponding to 0 and 100% killing of bacteria was established separately by using the OD mean of controls in each treatment. The percentage of bacteria killed by PIMs was determined by the formula:

\[
\frac{1 - \text{OD sample} - \text{OD 100% killing}}{\text{OD 0% killing} - \text{OD 100% killing}} \times 100\%
\]

2.7 Superoxide anion (SOA) production

Cytochrome C reduction assay measured the amount of superoxide anion (SOA) produced by PIMs during the oxidative metabolic burst by determining the change in OD due to the reduction of cytochrome C (Type III from horse heart, Sigma, St. Louis, MO) comparing to the unstimulated or resting PIMs (Thanawongnuwech et al., 1997). The assay was conducted in duplicate. The mean OD at 550 nm (X 1000) of the solution was determined using an automated 96-channel microtiter plate spectrophotometer. Percent SOA production was calculated by the formula:

\[
\frac{\text{OD stimulated wells} - \text{OD unstimulated wells}}{\text{OD unstimulated wells}} \times 100\%
\]
2.8 Statistical analysis

Data were statistically analyzed at $P < 0.05$ level using split-split plot analysis of variance (ANOVA) of the GLM procedure of SAS (SAS. 1988. SAS User’s Guild: Statistics. SAS Inst. Inc., Cary, NC) on virus titers at 48 HPI and bactericidal activities and the production of superoxide anion measured at 15, 24, or 48 HPI. The analysis of orthogonal linear contrasts was performed for further treatment differentiation when the equal effects assumption in ANOVA was rejected. Data within the same HPI were evaluated using Scheffe’s test for all pairwise comparisons. The model included main effects (pig age, macrophage type, and virus strain), all two-way interactions, and the three-way interaction. The type 3 mean square of Pig(Age) and Pig*Cell(Age) were used as an error term for pig age and macrophage type, respectively. Most two-way and the three-way interactions were not significant and were dropped from the model.

3. Results

3.1 Virus titration results

PIMs were as permissive as PAMs to infection with both PRRSV isolates yielding similar progeny titers ($10^{4.81} \text{ vs. } 10^{5.22} \text{ TCID}_{50}/\text{ml}$, respectively). Both ISU-55 and VR-2385 were able to infect PIMs and no significant difference in virus titers between isolates was found ($10^{5.33} \text{ vs. } 10^{4.99} \text{ TCID}_{50}/\text{ml}$, respectively). PIMs from 4-week-old pigs yielded a higher virus titer following PRRSV infection than PIMs from 4-month-old pigs ($10^{5.43} \text{ vs. } 10^{4.59} \text{ TCID}_{50}/\text{ml}$, respectively; $P < 0.02$) (Table 1).

3.2 Effect of PRRSV infection on viability of macrophages

PIMs from both age groups had greater than 80% viability at 15 or 24 HPI but less than 70% were viable at 48 HPI in either ISU-55- or VR-2385-infected groups. Greater than 85% of PIMs were viable in the uninfected cell culture. TEM examination of ultrathin sections of PRRSV-infected PIMs from both age groups revealed aberrant ribosomes and tubules, numerous phagolysosomes, cytoplasmic vacuoles, and residual bodies filled with necrotic debris and damaged cellular components (Fig. 1a). Budding characteristics of virions were observed in the smooth-walled vesicles and later apparently free viruses tended to accumulate.
in the vesicles of the Golgi apparatus or endoplasmic reticulum as early as 15 HPI (Fig 1b). Both ISU-55 and VR-2385 were similar in assembly of new virus particles and egress from infected PIMs. The uninfected cells had intact cytoplasmic organelles and normal nuclear and cytoplasmic membranes (data not shown).

3.3 Effect of PRRSV infection on the S. aureus killing

There was no difference in the number of bacteria killed by PIMs from either age group prior to PRRSV infection. VR-2385-infected PIMs from 4-week-old pigs killed significantly fewer bacteria than uninfected PIMs at 48 HPI (51.2% vs. 58.0%, respectively). However, there was no significant difference between ISU-55-infected and VR-2385-infected PIMs in bactericidal activity at any HPI (Table 2). No difference in bactericidal activity between uninfected PIMs and either low or high virulence PRRSV-infected PIMs from 4-month-old pigs was ascertained.

3.4 Effect of PRRSV infection on superoxide anion (SOA) production

Both ISU-55 and VR-2385 infected PIMs had significantly decreased production of SOA at 24 and 48 HPI, but not at 15 HPI. No significant difference was determined between virus strains or pig age on SOA production (Table 3).

4. Discussion

Both PIMs and PAMs possess similar efficiency in ingesting and killing bacteria, but differ in activity and cytokine production (see review article in Chitko-McKown and Blecha, 1992). In this study, we found that both PIMs and PAMs were equally permissive to PRRSV replication. While these pulmonary macrophage populations appear to have different roles in lung surveillance, both PIMs and PAMs serve as important PRRSV replication sites.

Functional and ultrastructural quantification differences between PIMs in newborn and older animals has been demonstrated (Winkler, 1988; Longworth et al., 1992; Longworth et al., 1996). Younger mice possess a higher proportion of LDV-permissive peritoneal macrophages than older mice and the persistent plasma LDV titers are also 10- to 100-fold higher than in older mice (Rowland et al., 1994). Similarly, we demonstrated that PIMs from 4-week-old pigs were more permissive and susceptible to PRRSV than those from the older
pigs in terms of PRRSV titers. The levels of differentiation and activation of monocyte/macrophages have been reported to play an important role in determining their susceptibility to PRRSV (Molitor et al., 1996). Porcine peritoneal macrophages, another population of well-differentiated tissue macrophages, have been demonstrated to be resistant to PRRSV infection while activated monocytes are susceptible (Duan et al., 1997). Macrophage differentiation may reflect altered expressions of surface proteins, which may be viral receptors or transcription factors which are essential for virus replication. Macrophage activation may up- or down-regulate the expression of these factors which is a possible explanation for differences in macrophage susceptibility to PRRSV (Rutherford et al., 1993). However, the reason for the change in PRRSV permissiveness of PIMs with age of pigs is unknown. One explanation for the decreased permissiveness and susceptibility to PRRSV in older pigs is a decrease in the proportion of macrophages that may express a surface protein which acts as the PRRSV receptor. This is why young pigs are more susceptible to PRRSV infection.

Following PRRSV infection, we found that the production of SOA was significantly decreased as early as 24 HPI (Thanawongnuwech et al., 1997). This suggests that PRRSV-infected PIMs may be capable of internalization, but unable to kill bacteria at 24-48 HPI. Likewise, peritoneal macrophages of mice persistently-infected with LDV produce less SOA than uninfected macrophages (Hayashi et al., 1993). PRRSV may induce a similar response in porcine macrophages. Human influenza virus which does not affect phagocytosis, however, triggers an oxidative burst in monocytes leading to reduced oxidative metabolism and depressed intracellular killing (Gardner and Lawton, 1982).

In this study, ultrastructural changes of PRRSV-infected PIMs were demonstrated as early as 15 HPI. Previous studies have shown that after 24 HPI, the infected macrophages underwent cell death by necrosis as a direct consequence of PRRSV replication (Thanawongnuwech et al., 1997) or by virus-induced apoptosis (Suarez et al., 1996). Decreased production of cellular proteins due to the production of viral proteins may lead to advanced degeneration of the macrophages resulting in decreased viability of PIMs by 48
HPI. This could explain why PRRSV infection significantly reduced the bactericidal ability of the PIMs from 4-week-old pigs at 48 HPI, but not at 15 or 24 HPI.

The low and high virulence strains of PRRSV used in this experiment had similar in vitro effects on PIMs in terms of virus titers, bactericidal activity, and SOA production. In contrast, in vivo studies using CDCD pigs found a marked difference in pathogenicity between PRRSV isolates (Halbur et al., 1995; Halbur et al., 1996). The experimental conditions in vivo may provide different microenvironments and macrophage activation factors important for replication of the viruses. In vitro cultures may activate and make most PIMs more susceptible to PRRSV infection than the whole population of PIMs in vivo in which a subpopulation of PRRSV-permissive PIMs may exist. In vitro bactericidal assays of PRRSV-infected PIMs did not correlate with known pathogenic differences of the selected PRRSV strains in vivo. Thus in vitro assays may not be a good indicator of in vivo pathogenicity.

In conclusion, PIMs are as permissive as PAMs to PRRSV infection and the susceptibility to PRRSV infection is age dependent. In vitro bactericidal assays of PRRSV-infected PIMs were unable to differentiate between low and high virulence PRRSV strains as established by in vivo studies. Development of simple techniques to identify virulences of PRRSV isolates would benefit the establishing control measures of endemic PRRS on farms.

Acknowledgement:

This work was supported by Healthy Livestock for Iowa Initiative 1996-1997. The authors thank Dr. D.E. Frank for technical assistance, Drs. F.A. Ahrens, P.S. Paul, and J.A. Roth for use of laboratory equipment, Drs. M.R. Ackermann and J.J. Zimmerman for editorial assistance, and M. Brabec for statistical analysis assistance.
References:


**Table 1.** Virus titers (Log₁₀ TCID₅₀/ml) of different PRRSV isolates from primary macrophage cultures from different pig groups.

<table>
<thead>
<tr>
<th>Source</th>
<th>Virus titers</th>
<th>DF</th>
<th>Mean Squares</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell</td>
<td>PAMs</td>
<td>5.22 ± 1.07*</td>
<td>1</td>
<td>2.038</td>
<td>3.29</td>
</tr>
<tr>
<td></td>
<td>PIMs</td>
<td>4.81 ± 0.83</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pig * Cell (Age)</td>
<td></td>
<td>10</td>
<td>6.197</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>4 wk</td>
<td>5.43 ± 1.05</td>
<td>1</td>
<td>8.459</td>
<td>7.56</td>
</tr>
<tr>
<td></td>
<td>4 mo</td>
<td>4.59 ± 0.68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pig (Age)</td>
<td></td>
<td>10</td>
<td>11.194</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus</td>
<td>ISU-55</td>
<td>5.33 ± 1.16</td>
<td>1</td>
<td>0.966</td>
<td>2.72</td>
</tr>
<tr>
<td></td>
<td>VR-2385</td>
<td>4.69 ± 0.64</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td></td>
<td>20</td>
<td>7.112</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SD, DF = Degree of Freedom, 4 wk = 4-week-old pigs, 4 mo = 4-month-old pigs

**Table 2.** Percent *S. aureus* killed by PIMs at different hours post infection (HPI) with different PRRSV isolates.

<table>
<thead>
<tr>
<th>Virus</th>
<th>HPI</th>
<th>15</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4 wk (4-week-old pigs)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>56.9 ± 12.4</td>
<td>58.6 ± 13.0</td>
<td>58.0 ± 12.7</td>
<td></td>
</tr>
<tr>
<td>ISU-55</td>
<td>62.3 ± 11.4</td>
<td>58.8 ± 12.4</td>
<td>55.9 ± 18.2</td>
<td></td>
</tr>
<tr>
<td>VR-2385</td>
<td>60.7 ± 9.1</td>
<td>55.7 ± 13.9</td>
<td>51.2 ± 19.0b</td>
<td></td>
</tr>
<tr>
<td><strong>4 mo (4-month-old pigs)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>63.9 ± 12.1</td>
<td>65.8 ± 12.7</td>
<td>60.4 ± 18.3</td>
<td></td>
</tr>
<tr>
<td>ISU-55</td>
<td>65.9 ± 11.9</td>
<td>66.8 ± 12.8</td>
<td>61.1 ± 20.6</td>
<td></td>
</tr>
<tr>
<td>VR-2385</td>
<td>67.9 ± 14.1</td>
<td>70.2 ± 15.5</td>
<td>63.0 ± 18.7</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SD (% Bacteria killed), n = 6

b Significantly different from control at $P < 0.05$
Table 3. Percent superoxide anion (SOA) production by PIMs at different hours post infection (HPI) with different PRRSV isolates.

<table>
<thead>
<tr>
<th>Virus</th>
<th>HPI</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td><strong>4 wk (4-week-old pigs)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>155.8 ± 15.3</td>
<td>170.0 ± 18.2</td>
<td>148.9 ± 19.5</td>
</tr>
<tr>
<td>ISU-55</td>
<td>165.1 ± 18.4</td>
<td>110.0 ± 21.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57.5 ± 16.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VR-2385</td>
<td>181.4 ± 17.8</td>
<td>105.1 ± 27.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.7 ± 23.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>4 mo (4-month-old pigs)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>174.2 ± 12.8</td>
<td>160.6 ± 16.6</td>
<td>163.8 ± 14.7</td>
</tr>
<tr>
<td>ISU-55</td>
<td>177.4 ± 12.6</td>
<td>119.9 ± 13.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>96.3 ± 18.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VR-2385</td>
<td>183.2 ± 15.1</td>
<td>125.1 ± 22.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82.7 ± 14.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SD (% SOA production), n = 6

<sup>b</sup> Significantly different from control at P < 0.05
Figure 1. **a.** Transmission electron micrograph of ISU-55-infected PIMs from 4-month-old pig at 15 HPI revealed aberrant ribosomes and tubules (*), numerous phagolysosomes, cytoplasmic vacuoles, and intracellular virus particles accumulated within the cytoplasmic vesicles (arrow head). Bar = 3 μm **b.** Intracellular virion budding from the membrane of a smooth-walled vesicle (arrow head) of ISU-55-infected PIMs. Bar = 0.25 μm L = Lysosomes, M = Mitochondria, N = Nucleus, P = Phagosomes.
CHAPTER 4. EFFECTS OF LOW (MODIFIED-LIVE VIRUS VACCINE) AND HIGH (VR-2385) VIRULENCE STRAINS OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) ON PULMONARY CLEARANCE OF COPPER PARTICLES IN PIGS

A paper accepted by Veterinary Pathology

R Thanawongnuwech, PG Halbur, MR Ackermann, EL Thacker, and RL Royer

Abstract

Seventy-five 3-week-old, cross-bred pigs from a PRRSV-free herd were randomly assigned to 3 groups as uninfected-controls, intranasally inoculated (IN) with RespPRRS/Repro™ modified-live virus vaccine (RespPRRS®), or inoculated IN with a high virulence strain of PRRSV (VR-2385). Pigs were intravenously infused with 3% copper phthalocyanine tetrasulfonic acid (0.2 ml/kg) in saline 30 minutes prior to necropsy at 3, 7, 10, 14, or 28 days post inoculation (DPI) with PRRSV. There were no differences in serum copper concentration in samples collected at 0, 15, or 30 minutes after infusion. Copper concentrations in the lungs of VR-2385-inoculated pigs were significantly lower than levels in the lungs of control and RespPRRS®-inoculated pigs at 7, 10, and 14 DPI (P < 0.05). The greatest difference between the groups was observed at 10 DPI. Liver and spleen copper concentrations were slightly, but not significantly, higher in both PRRSV-infected groups. The percentage of the lung affected by grossly visible pneumonia ranged from 0% to 5.6% in RespPRRS®-inoculated group and 15.2% to 46.4% in VR-2385-inoculated group with lesions peaking at 7 and 10 DPI, respectively. PRRSV antigen was demonstrated in both pulmonary alveolar macrophages (PAMs) and pulmonary intravascular macrophages (PIMs) by immunohistochemistry. Copper particles were demonstrated in the PIMs by light microscopy. PRRSV was isolated from bronchoalveolar lavage fluid of VR-2385-infected pigs from 3-28 DPI and from RespPRRS-inoculated pigs from 7-28 DPI. No PRRSV, PRRSV antibodies, or PRRSV-induced pneumonia was detected in the control group. These results suggest: 1) PRRSV has a detrimental effect on the uptake of copper particles by PIMs,
2) the severity of PRRSV-induced damage to PIMs differs among strains, and 3) demonstration of PRRSV-induced decreased pulmonary clearance supports the hypothesis that PRRSV infection may make pigs more susceptible to bacterial septicemia.

**Keywords:** Copper particles, Pulmonary clearance, Pulmonary intravascular macrophages, Porcine reproductive and respiratory syndrome virus, PRRSV vaccine

**Introduction**

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, positive-stranded RNA virus recently classified in the family *Arteriviridae* which includes equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV). Antigenic, genetic, and pathogenic variation have been reported between US and European isolates and among US isolates. Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure in pregnant sows and respiratory disease in young growing pigs. The prevalence and severity of concurrent disease due to atrophic rhinitis, enzootic pneumonia, *Streptococcus suis, Haemophilus parasuis, Salmonella choleraesuis,* or *Actinobacillus pleuropneumoniae* increases in pigs that survive a PRRS epizootic.

PRRSV has been reported to replicate *in vitro* in porcine pulmonary alveolar macrophages (PAMs) and more recently in porcine pulmonary intravascular macrophages (PIMs). PRRSV has also been demonstrated to replicate in activated monocytes and microglial cells *in vitro.* Pulmonary macrophage populations have important roles in lung surveillance. PIMs adhere to the pulmonary capillary endothelium and serve as a major component of the systemic host-defense mechanism. Copper particles (copper phthalocyanine; Sigma, St. Louis, MO) have been used to study pulmonary clearance and PIM function in swine. We hypothesized that different strains of PRRSV may have different effects on the uptake of copper particles by PIMs. In this study we compared low (RespPRRS/Repro™ modified-live virus vaccine, NOBL Laboratories Inc, Sioux Center, IA) and high (VR-2385) virulence strains of PRRSV in terms of pulmonary clearance of copper...
particles. Such findings could explain the association observed between PRRSV and secondary bacterial diseases in swine herds and help explain why certain PRRSV-infected herds have more problems with secondary bacterial infections.

Materials and Methods

Experimental Design

The protocol used in this study was approved by the Committee on Animal Care (COAC) of Laboratory Animal Resources at Iowa State University. Seventy-five 3-week-old, cross-bred, specific pathogen free, and PRRSV-free pigs were randomly assigned into 3 groups of 25 pigs each. The three treatments included 2 ml intranasal inoculation with uninfected cell culture medium, RespPRRS/Repro™ modified-live virus (MLV) vaccine (RespPRRS® or low virulence group), or VR-2385 isolate (high virulence group). RespPRRS vaccine is approved for intramuscular use and was used extralabel by giving it intranasally. Five pigs from each group were necropsied at 3, 7, 10, 14, or 28 days post inoculation (DPI).

Virus Inocula Preparation

Both RespPRRS and VR-2385 were titrated with serial 10-fold dilutions in 96-well microtiter plates previously seeded with CRL11171 (Iowa State University Research Foundation, Ames, IA).14 The titers of RespPRRS® and VR-2385 were calculated by the method of Reed and Muench18 and were $10^{4.99}$ and $10^{4.47}$ TCID$_{50}$/2 ml, respectively. Challenge inocula consisting of uninfected cell culture medium, RespPRRS®, or VR-2385 were given in 2 ml intranasally.

Clinical Evaluation

Daily clinical respiratory disease scores, ranging from 0 to 6, were given from 0-28 DPI as previously described: 0 = normal; 1 = mild dyspnea and/or tachypnea when stressed; 2 = mild dyspnea and/or tachypnea when at rest; 3 = moderate dyspnea and/or tachypnea when stressed; 4 = moderated dyspnea and/or tachypnea when at rest; 5 = severe dyspnea and/or tachypnea when stressed; 6 = severe dyspnea and/or tachypnea when at rest.13 Other clinical observations such as coughing, diarrhea, inappetence, or lethargy were noted separately and
had no impact on the clinical respiratory score. Rectal temperatures were recorded daily from 0-28 DPI.

**Copper Particle Administration and Necropsy**

Five pigs from each group were necropsied at 3, 7, 10, 14, or 28 DPI. Anesthesia was induced by intramuscularly administration of xylazine (Bayer Corp., Shawnee Mission, KS; 2 mg/kg) and Telazol (Fort Dodge Laboratories Inc., Fort Dodge, IA; 6 mg/kg). Four of the five pigs from each group were given 0.2 ml/kg of 3% copper phthalocyanine tetrasulfonic acid (Sigma, St. Louis, MO) diluted with normal saline (NS) to yield the total infusion volume of 2 ml/kg. The copper particle suspension was slowly infused through an ear vein over a period of 5 minutes. Pigs were in left lateral recumbency during anesthesia prior to euthanasia in order to have the highest perfusion of blood in the left caudal lung lobes. The fifth pig in each group was given only NS so that baseline values of copper in sera and tissues could be established at each DPI. Blood was collected at 0, 15, and 30 minutes after infusion. Pigs were euthanized with an intravenous overdose of sodium pentobarbital injection (Schering-Plough Animal Health Corp., Kenilworth, NJ) 30 minutes after the start of the copper infusion. To assess clearance of copper particles, samples of left caudal lung lobe, the left lobe of liver, spleen, and sera were submitted to the Iowa State University, Veterinary Diagnostic Laboratory (ISU-VDL) for copper analysis.

Complete necropsies were performed on all pigs. An estimated percentage of the lung with grossly visible pneumonia was recorded for each pig based on a previously described scoring system. Other lesions were noted separately and not included in gross lung lesion scores. Combined weight of sternal and medial iliac lymph nodes were recorded at necropsy. Pig body weight was also recorded at necropsy. Sections for histopathologic examination were taken from lung, nasal turbinate, heart, brain, lymph nodes, tonsil, thymus, liver, and spleen. Immunohistochemistry (IHC) was performed on lung, liver and spleen as previously described.

**Copper Analysis**

Copper analysis in serum and in homogenized tissues were treated differently as described previously. Serum samples were diluted with deionized water. The analysis was
performed against standards prepared in glycerol to approximate the viscosity characteristics of the diluted samples. Copper analysis in homogenized tissues was performed by ashing to remove organic materials, dissolving the ash in acid, and aspirating into the atomic absorption spectrometer (Perkin-Elmer 303™, Norwalk, CT). Sera and homogenized tissues were analyzed by atomic absorption spectrometry for absorbance at 324.7 nm. Samples were done in triplicate and calculated in ppm by the formula:

\[
\text{ppm Cu in serum} = \frac{[\mu g \text{ Cu from Standard curve} \times [2 \text{ ml water} + 0.5 \text{ ml serum}]}{\text{ml serum}}
\]

or

\[
\text{ppm Cu in tissues} = \frac{[\mu g \text{ Cu from standard curve} \times [\text{ml dilution}]}}{\text{g sample}}
\]

**Virus Isolation and Serology**

Bronchoalveolar lavage (BAL) was performed aseptically with 50 ml lavage fluid consisting of minimum essential media (GIBCO Laboratories, Grand Island, NY) containing antibiotics (9 µg gentamycin/ml, 100 U/ml penicillin G, and 100 µg/ml streptomycin). Lavage fluid was gently dispensed and aspirated several times into the lungs. The BAL fluid was kept in -70°C until PRRSV isolation was attempted. The CRL1171 cell line was used to isolate PRRSV from BAL fluid. Briefly, 200 µl of BAL fluid was inoculated onto a confluent monolayer of CRL1171 for 1 hour at 37°C in a humidified atmosphere with 5% CO₂. The cultures were then maintained in a complete media with 2% fetal bovine serum (FBS) and monitored daily for cytopathic effect (CPE). Viral CPE was confirmed by indirect immunofluorescence assay. If CPE was not observed within 7 days, the cultures were frozen and thawed and blindly passed 3 times. Monolayers were stained with an anti-PRRSV monoclonal antibody SDOW-17 which recognized a conserved epitope of the PRRSV nucleocapsid protein and FITC-conjugated antimouse IgG (Sigma, St. Louis, MO) and then viewed with a fluorescence microscope for evidence of specific viral antigens. Sera were obtained from all pigs at 0, 7, 14, and 28 DPI and from pigs when necropsied at 3 and 10 DPI. Serum antibodies to PRRSV (S/P ratio ≥ 0.4) were detected using Herd Check PRRSV ELISA (IDEXX Laboratories, Westbrook, Mass.).
**Statistical Analysis**

Data were expressed as mean ± standard deviation (SD) and evaluated using the general linear models procedure of SAS (1988 SAS User’s Guide: Statistics., SAS Inst. Inc., Cary, NC). The analysis of orthogonal linear contrasts was performed for further treatment differentiation when the equal effects assumption in ANOVA was rejected. Data within the same DPI were evaluated using Scheffe’s test for all pairwise comparisons at the $P < 0.05$ level.

**Results**

**Clinical Evaluation**

Respiratory disease scores are summarized in Fig. 1. Respiratory disease in the VR-2385-inoculated group was characterized by mild to moderate dyspnea and/or tachypnea from 3 to 28 DPI. VR-2385-infected pigs were moderately lethargic and anorexic with transient fever (3-16 DPI, peaked at 40.8°C). The RespPRRS®-inoculated pigs experienced no, or very mild, respiratory disease from 3 to 17 DPI. The pigs in this group were also mildly lethargic and anorexic and had mild transient fever (8-16 DPI, peaked at 40.3°C). Control pigs remained normal throughout the 28 days.

**Copper Particle Clearance**

VR-2385-infected pigs exhibited transient blue-purple discoloration of the skin and moderate dyspnea immediately after copper particle infusion. At necropsy, the lungs were blue; however, control (Fig. 2) and RespPRRS® pigs had darker blue lungs than did VR-2385-inoculated pigs (Fig. 3). Blue copper particles were microscopically observed in PIMs of all pigs infused with copper (Fig. 4). Copper particles were commonly found in Kupffer cells in the liver (Fig. 5) and macrophages of the spleen (Fig. 6) in VR-2385-inoculated pigs at 7-14 DPI. Copper particles were uncommonly observed in liver and spleen of RespPRRS®-inoculated or control pigs. Copper concentration in the lungs of VR-2385-inoculated pigs was significantly lower than that in lungs from control or RespPRRS®-inoculated groups at 7-14 DPI ($P < 0.05$) (Table 1). The greatest difference between the
groups was observed at 10 DPI. There was no difference in copper concentration in the lung between control and RespPRRS-inoculated groups.

Although there was an increase in copper concentration in liver and spleen in both PRRSV-infected groups compared with the control group, it was not a statistically significant difference. This may be due to endogenous copper levels in these tissues and the variability between pigs. Endogenous copper was mainly found in liver (9–55 ppm) and significantly decreased with a linear trend as age of pig increased \( (P < 0.05) \) (Table 1). Levels of copper in sera collected at 0, 15, or 30 minutes after copper particle infusion were not statistically different; however, copper levels in serum significantly decreased with a linear trend as the age of pig increased \( (P < 0.05) \).

**Gross Lesions**

Gross lung lesions were scored as an estimated percent of the lung affected by grossly visible pneumonia. VR-2385 isolate induced significantly more severe gross lung lesions (15.2% to 46.4%) than those in RespPRRS-inoculated or control pigs from 3-28 DPI with the peak lesions at 10 DPI \( (P < 0.05) \). The mean percentage of the lung affected by pneumonia ranged from 0% to 5.6% in RespPRRS-inoculated group with the peak lesions at 7 DPI. RespPRRS®-induced gross lung lesions were not significantly different from those of the control group. Mild gross lung lesions attributable to focal bacterial pneumonia were observed in control pigs. The affected areas of lungs in the PRRSV-infected pigs were mottled-tan or diffusely tan in color and failed to collapse (Fig. 3) when compared with the uninfected lungs (Fig. 2). Grossly visible PRRSV-induced lung lesions primarily affected the cranial, middle, and accessory lobes and the ventromedial portion of the caudal lung lobes.

Both VR-2385 and RespPRRS® induced generalized lymphadenopathy as early as 10 DPI. Lymph nodes were enlarged, tan in color, and slightly edematous. Mean lymph node (sternal and medial iliac) to body weight ratios of RespPRRS®- or VR-2385-infected pigs were significantly greater than those of control pigs at 10 and 28 DPI \( (P < 0.05) \). Mean lymph node to body weight ratios of VR-2385-inoculated pigs were significantly greater than those in RespPRRS®-inoculated pigs at 14 DPI \( (P < 0.05) \).
Microscopic Lesions

The number of pigs with pneumonia, rhinitis, myocarditis, encephalitis, and lymphadenopathy in each group is summarized in Table 3. PRRSV-induced pneumonia was characterized by three major changes: 1) type 2 pneumocyte hypertrophy and hyperplasia, 2) septal infiltration by macrophages, and 3) alveolar exudate consisting primarily of foamy macrophages, necrotic macrophages, multinucleated cells, and proteinaceous fluid as previously described. The microscopic lung lesions induced by VR-2385 persisted for 28 DPI and were consistently more severe than those in the RespPRRS® group. There were fewer pigs in RespPRRS®-infected group than in the VR-2385-infected group with evidence of interstitial pneumonia. Blue copper particles were found in the PIMs of all pigs which were given 3% copper particle infusion. The PRRSV-infected PIMs were enlarged and swollen. PRRSV antigen was demonstrated in both PIMs (Fig. 4) and PAMs but not in Kupffer cells or splenic macrophages in RespPRRS® and VR-2385-inoculated groups by IHC.

Mild to moderate multifocal lymphohistiocytic rhinitis was observed after 10 DPI in both the RespPRRS® and VR-2385 -inoculated groups. Rhinitis was more severe in VR-2385 pigs (Table 3). Rhinitis was characterized by mixed leukocytes infiltrating the turbinate epithelium and mild lymphohistiocytic, plasmacytic, and neutrophilic inflammation in the submucosa.

Mild multifocal lymphoplasmacytic and histiocytic myocarditis was observed in both RespPRRS® and VR-2385 groups. The heart lesions were present earlier (14 DPI) in VR-2385-inoculated group. Myocarditis was observed in fewer pigs of the RespPRRS®-inoculated pigs (Table 3). One pig from control group had mild focal lymphohistiocytic myocarditis as well.

Mild encephalitis characterized by multifocal gliosis and lymphohistiocytic perivascular infiltrates was observed only in VR-2385 group in the brain stem and less often in the cerebrum. Brain lesions were observed after 10 DPI (Table 3).

Marked lymphadenopathy was observed by 7 DPI in VR-2385-inoculated group. Similar, but less severe, lymphadenopathy was observed by 10 DPI in RespPRRS®-
inoculated group (Table 3). Lymphadenopathy persisted though 28 DPI in both PRRSV-infected groups. Follicles were hypertrophic and hyperplastic. Necrotic cells were commonly observed within the follicles.

**Virus Isolation**

PRRSV isolation was attempted only from the BAL fluid. PRRSV was recovered from the BAL fluid of 25 of 25 (100%) VR-2385-inoculated pigs, 13 of 25 (52%) RespPRRS®-inoculated pigs, and 0 of 25 (0%) uninoculated controls from 3 to 28 DPI (Table 4).

**Serology**

All pigs were negative for PRRSV serum antibodies (S/P ratio < 0.4) at 0 DPI. Control pigs remained negative through 28 days. PRRSV serum antibodies (S/P ratio ≥ 0.4) were present in 8/20 (40%) of the VR-2385 pigs at 7 DPI, and in all VR-2385-inoculated pigs after 10 DPI. Only 1/5 (20%) RespPRRS®-inoculated pigs had PRRSV serum antibodies at 10 DPI (Table 4). At 28 DPI, all RespPRRS®-inoculated pigs had PRRSV serum antibodies.

**Discussion**

Determination of pulmonary copper clearance in pigs is a relatively simple method to measure PIM function. After intravenous administration of copper particles, the lungs of pigs consistently had the highest copper concentrations when compared to other organs. As in a previous study, the blue copper particles were easily observed in porcine PIMs with light microscopy. Similarly, iron particles given intravenously to sheep were found only in PIMs and not in alveolar and interstitial macrophages, endothelial cells, or circulating monocytes or neutrophils. In horses, it has been demonstrated that PIMs are capable of internalizing copper particles without any manifestation of toxic insult when treated with multiple doses of copper particle suspension over a period of 96 hours. Copper particle internalization is thought to occur principally in coated pits of the PIMs.

The severity of PRRSV-induced damage to PIMs differs among strains. Pigs given a low virulence strain of PRRSV (RespPRRS®) and uninfected control pigs had similar capacity to clear a single intravenous dose of copper particles, whereas the high virulent strain VR-2385 significantly decreased copper clearance. VR-2385-inoculated pigs had significantly
decreased copper concentrations in lungs at 7-14 DPI which is when clinical disease and PRRSV-induced interstitial pneumonia are most severe. This suggests that VR-2385-inoculated pigs had more extensive PRRSV-induced necrosis or apoptosis of PIMs from 7-14 DPI. However, copper levels in lungs were not significantly decreased at 3 or 28 DPI. A sufficient number of PIMs may not be infected by 3 DPI to make a difference in pulmonary clearance of copper particles. By 28 DPI, PRRSV-induced pulmonary lesions are usually resolved and replacement PIMs may repopulate pulmonary capillaries by that time.

African swine fever virus (ASFV) has also been reported to induce damage to PIMs. The intense activation of PIMs and the subsequent release of chemical mediators results in pulmonary edema in ASFV-infected pigs. The ASFV replicates in PIMs at 5-7 DPI and subsequently causes necrosis of PIMs. Like ASFV, PRRSV may have a similar adverse effect on PIMs. This is supported by in vitro evidence that PRRSV replicates in PIMs and infected macrophages undergo cell death by necrosis as a direct consequence of PRRSV replication or by PRRSV-induced apoptosis.

Decreased copper clearance by porcine PIMs has also been documented in fumonisin toxicity. Fumonisin induces ultrastructural alterations in PIMs resulting in an inability to remove blood-borne particles and increased susceptibility to bacteremic diseases. Bertram reported that porcine PIMs had an important role in the clearance of cellular and acellular debris from the blood of pigs infected with Actinobacillus pleuropneumoniae (APP). It is also likely that PRRSV-induced damage to PIMs results in increased susceptibility to bacterial diseases such as APP. Evidence of PRRSV-induced increased susceptibility to Streptococcus suis infection in nursery age pigs has also been experimentally demonstrated. This is consistent with what is reported on farms following a PRRS epizootic and on farms with endemic respiratory diseases associated with PRRS.

A lag between bacterial phagocytosis and killing would be expected to allow for accumulation, replication, and dissemination of viable bacteria in the tissues where clearance normally occurs. Crocker et al. reported that viable bacteria given intravenously were cleared by PIMs and later recovered from the trachea. Since PIM functions are deteriorated by
PRRSV\textsuperscript{26}, despite internalization, viable bacteria may escape intracellular destruction and may eventually cause pneumonia or septicemia.

Hepatic and splenic macrophages are expected to compensate for the decrease in pulmonary uptake by PIMs resulting in increased copper particle uptake\textsuperscript{21} or bacterial clearance in those tissues.\textsuperscript{2} We commonly observed copper particles in Kupffer cells and in splenic macrophages in VR-2385-inoculated pigs from 7-14 DPI but not in other groups or controls. As expected when pulmonary clearance is adequate, there was no significant difference in copper concentration in liver, spleen, or serum between control pigs infused with NS or with copper particles. Measurement and interpretation of the significance of copper level in the livers of young pigs is difficult due to poorly established normal ranges and considerable variability of endogenous copper concentration between pigs.

It is not uncommon for the RespPRRS/Repro\textsuperscript{TM} MLV vaccine to be used intranasally (extralabel) in young pigs in the field.\textsuperscript{22} In our experiment, RespPRRS/Repro\textsuperscript{TM} MLV vaccine produced mild pneumonia but did not adversely affect PIM function. Rhinitis, myocarditis, and encephalitis was observed, but was less severe and less frequent in the RespPRRS\textsuperscript{®} group compared to the VR-2385 group. The RespPRRS/Repro\textsuperscript{TM} MLV vaccines may not provide adequate cross protection in some situations with some PRRSV strains.\textsuperscript{10} The isolation of field virus from vaccinated nursery pigs suggests that vaccination at least does not prevent infection with PRRSV field strains and that there may be limited heterologous cross protection between strains of PRRSV.\textsuperscript{22}

In conclusion, pulmonary clearance of intravenously administered copper particles is a useful and relatively simple method to measure one aspect of PIM function in pigs. PRRSV has an adverse effect on the uptake of copper particles by PIMs and the severity differs among strains. We have documented that the highly virulent VR-2385 isolate of PRRSV decreases the ability of PIMs to clear copper particles. This evidence supports the hypothesis that PRRSV-induced damage to PIMs may result in increased susceptibility to bacteremic disease. These results could explain the increase in the chronic bacterial respiratory diseases, septicemia, and death loss experienced in pigs on farms endemically- or epizootically-infected with high virulent strains of PRRSV.
Acknowledgments

We thank Drs. Ken B. Platt, Jeff J. Zimmerman, and Richard L. Engen for their valuable advice. We also thank Jim Fosse for photography, David L. Cavanaugh for technical assistance, and Julia A. Kinker for copper analysis. This work was supported by the Healthy Livestock for Iowa Initiative 1996-1997.

References


Table 1. Copper analysis in tissues (ppm) after normal saline or 3% copper particle infusion.

<table>
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<tr>
<th>Treatment</th>
<th>3 DPI</th>
<th>7 DPI</th>
<th>10 DPI</th>
<th>14 DPI</th>
<th>28 DPI</th>
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<td>Copper (n = 4)</td>
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<td>25.0 ± 3.4</td>
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<td>24.3 ± 2.5</td>
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<td>21.0 ± 0.8</td>
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<td>12.3 ± 1.9†</td>
<td>15.3 ± 1.5†</td>
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<td>9.1 ± 1.7</td>
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<td>2.1</td>
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<td>3.4 ± 0.5</td>
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<td>3.2 ± 0.3</td>
<td>3.0 ± 0.4</td>
<td>2.6 ± 0.2</td>
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</table>

* Mean ± SD.
† Means within the same column in each tissue when compared to means of the control groups differ significantly at P < 0.05.

DPI = Days post inoculation; NS = Normal saline; Copper = Copper phthalocyanine tetrasulfonic acid (C₁₉H₁₂Cu₂O₂S₄Na₄); RespPRRS® = RespPRRS/Repro™ modified-live virus vaccine (NOBL Laboratories Inc, Sioux Center, IA).
Table 2. Copper analysis in serum (ppm) after normal saline or 3% copper particle infusion at 0, 15, or 30 minutes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>RespPRRS®</th>
<th>VR-2385</th>
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<tbody>
<tr>
<td></td>
<td>NS Copper</td>
<td>NS Copper</td>
<td>NS Copper</td>
</tr>
<tr>
<td>3 DPI</td>
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<tr>
<td>0 min</td>
<td>1.98 2.04 ± 0.21*</td>
<td>1.98 2.30 ± 0.17</td>
<td>2.10 2.13 ± 0.04</td>
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<td>15 min</td>
<td>1.92 1.76 ± 0.17</td>
<td>1.86 1.96 ± 0.13</td>
<td>1.98 1.77 ± 0.08</td>
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<tr>
<td>30 min</td>
<td>1.98 1.76 ± 0.28</td>
<td>1.98 1.98 ± 0.11</td>
<td>1.86 1.73 ± 0.18</td>
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<td>7 DPI</td>
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<tr>
<td>0 min</td>
<td>1.76 1.77 ± 0.34</td>
<td>1.67 2.10 ± 0.25</td>
<td>1.66 1.98 ± 0.18</td>
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<tr>
<td>15 min</td>
<td>1.69 1.45 ± 0.32</td>
<td>1.60 1.75 ± 0.05</td>
<td>1.72 1.76 ± 0.37</td>
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<td>30 min</td>
<td>1.55 1.39 ± 0.32</td>
<td>1.47 1.61 ± 0.13</td>
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<td>10 DPI</td>
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<td>0 min</td>
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<td>15 min</td>
<td>1.33 1.38 ± 0.32</td>
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<td>30 min</td>
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<td>1.80 2.09 ± 0.17</td>
<td>1.56 2.12 ± 0.12</td>
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<tr>
<td>15 min</td>
<td>1.32 1.40 ± 0.16</td>
<td>1.56 1.71 ± 0.18</td>
<td>1.92 1.82 ± 0.10</td>
</tr>
<tr>
<td>30 min</td>
<td>1.32 1.28 ± 0.17</td>
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<td>0 min</td>
<td>1.44 1.74 ± 0.27</td>
<td>1.74 1.79 ± 0.14</td>
<td>1.20 1.52 ± 0.10</td>
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<td>15 min</td>
<td>1.38 1.52 ± 0.25</td>
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<td>30 min</td>
<td>1.32 1.47 ± 0.30</td>
<td>1.74 1.52 ± 0.15</td>
<td>1.40 1.28 ± 0.09</td>
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*min = Minutes after 3% copper particle infusion, See Table 1 for key.
Table 3. Microscopic lesions in control and PRRSV-inoculated (RespPRRS® or VR-2385) pigs.

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<th>Lesions</th>
<th>3 DPI</th>
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ND = No data. See Table 1 for key.

Table 4: The presence of PRRSV serum antibodies and virus isolated from bronchoalveolar lavage fluid.

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AB = Positive for PRRSV serum antibodies (S/P ratio ≥ 0.4), VI = Positive for PRRSV isolation. See Table 1 for key.
Figure 1. Mean respiratory disease scores (0-6) of control and PRRSV-inoculated (RespPRRS® or VR-2385) pigs at 0-28 days post inoculation (DPI).
Figure 2. Lung; pig. 7 DPI with uninfected media. Normal lung after intravenous injection of 3% copper particles in saline (0.2 ml/kg). Lung appeared dark blue due to copper. L = Left.

Figure 3. Lung; pig. 7 DPI with PRRSV (VR-2385). PRRSV-infected lung after intravenous injection of 3% copper particles in saline (0.2 ml/kg). Lung appeared light blue due to copper, and the mottled-tan-red color and failure to collapse was due to PRRSV infection. L = Left.
**Figure 4.** Lung; pig. 7 DPI with PRRSV (VR-2385). Copper particles internalized by pulmonary intravascular macrophages (PIMs). Dark brown staining of PRRSV-antigen (arrow) and the blue copper particles (arrow head) appear in PIMs. Labeled Streptavidin-Biotin (LSAB) immunoperoxidase staining with hematoxylin counterstain. Bar = 10 μm.

**Figure 5.** Liver; pig. 7 DPI with PRRSV (VR-2385). Copper particles (arrow head) appear in Kupffer cells. HE. Bar = 10 μm.

**Figure 6.** Spleen; pig. 7 DPI with PRRSV (VR-2385). Splenic macrophages internalized copper particles (arrow head). HE. Bar = 10 μm.
Figure 7. Estimated mean percent gross lung lesion score of control and PRRSV-inoculated (RespPRRS® or VR-2385) pigs at 3, 7, 10, 14, and 28 DPI. Columns are means ± SD. Means with different superscripts within the same DPI are significantly different ($P < 0.05$).
CHAPTER 5. GENERAL CONCLUSIONS

General Discussion

Although there have been several studies reporting porcine reproductive and respiratory syndrome virus (PRRSV) infection of porcine macrophages, mostly pulmonary alveolar macrophages (PAMs), little attention has been given to describing the role of pulmonary intravascular macrophages (PIMs) in PRRSV infection. The goals of this work were to: 1) develop a method to harvest porcine PIMs and grow them in cell cultures; 2) develop the assays to measure phagocytosis and bactericidal functions of PIMs grown in vitro after infection with PRRSV and comparing those results to PAMs; 3) compare low and high virulence strains of PRRSV isolates in terms of the above assays; and 4) perform in vivo studies on the effect of PRRSV on pulmonary clearance of copper particles by PIMs.

The first major step was to be able to collect PIMs and grow them in vitro. PIMs were successfully recovered by in situ pulmonary vascular perfusion with 0.025% collagenase in saline as previously described (Fowler et al., 1991). However, a large number of PIMs were retained in the lung after the perfusion due to vascular shunt and lack of flow. Within the lung at any one time there may exist an uneven distribution of blood flow as well as the collagenase perfusion and ventilation so that areas of low ventilation/perfusion ratio (V/A/Q), normal V/A/Q, and high V/A/Q are present in different lung units (Reece, 1993). In addition, susceptibility to collagenase in individual young pig lungs might be an explanation for the different in the effluent flow resulting in decreased yield of PIMs (personal observation).

The first manuscript describes the effect of PRRSV (isolate ATCC VR-2385) infection on bactericidal activity of PIMs in vitro. Demonstration of cytoplasmic PRRSV antigen via indirect fluorescence assay and viral particles via transmission electron microscopy (TEM) in PIMs demonstrated that PRRSV infects and replicates in PIMs. Colorimetric MTT bactericidal (Staphylococcus aureus) assays demonstrated that PRRSV infection reduced the bactericidal ability of both PIMs and PAMs, but not significantly at 24 hours post infection (HPI). However, the production of superoxide anion (SOA) and myeloperoxidase (MPO)-H₂O₂-halide were significantly decreased in both PIMs and PAMs. This may be due to
PRRSV reducing oxidative metabolism, inducing apoptosis, or lysis of macrophages. In addition, both oxidative stress (Li et al., 1996) and PRRSV (Suarez et al., 1996) have been reported to induce apoptosis in PAMs. This is the first report to demonstrate PRRSV replication in PIMs *in vitro*.

The initial study provided preliminary evidence that PRRSV infection may have functional impact on PIMs. The second component of this dissertation sought to study the influence of pig age and PRRSV strains on virus titer and bactericidal ability of PIMs at 15, 24, or 48 HPI. We demonstrated that PRRSV replicated in PIMs *in vitro* regardless of pig age. Decreased production of cellular proteins in favor of the production of viral proteins may lead to advanced degeneration of the macrophages resulting in decreased viability and function of PIMs by 48 HPI. This could explain why PRRSV infection significantly reduced the bactericidal ability of the macrophages at 48 HPI, but not at 15 or 24 HPI. Following PRRSV infection, we found that the production of SOA was significantly decreased as early as 24 HPI. This suggests that PRRSV-infected cells may be capable of internalization, but unable to kill bacteria at 24-48 HPI. Likewise, peritoneal macrophages of mice persistently-infected with LDV produce less SOA than uninfected macrophages (Hayashi et al., 1993).

Younger mice possess a higher proportion of LDV-permissive macrophages than older mice and the persistent plasma LDV titers are also 10- to 100-fold higher in younger mice (Rowland et al., 1994). Likewise, we demonstrated that PIMs from 4-week-old pigs were more permissive and susceptible to PRRSV than those from the older pigs. However, there was no significant difference in the effects of pig age on SOA production. Maturation and development of macrophages may have an effect on bactericidal activity and the release products of arachidonic acid metabolism, cytokines, and other mediators of inflammation. Functional and ultrastructural quantification differences of PIMs in newborn and older animals have been demonstrated (Winkler, 1988; Longworth et al., 1992, 1996). This may be an explanation for the decreased permissiveness and susceptibility to PRRSV in older pigs.

Selected low (ISU-55) and high (VR-2385) virulence PRRSV strains in this report had similar effects on macrophages in terms of *in vitro* bactericidal activity and SOA production.
In contrast, *in vivo* studies using CDCD pigs found a marked difference in pathogenicity between PRRSV isolates (Halbur et al., 1995, 1996). The experimental conditions *in vivo* may provide different microenvironments and macrophage activation factors which are important for replication of the viruses.

The third component of the dissertation sought to develop a simple method to further evaluate PIM function *in vivo* in term of pulmonary clearance. After intravenous administration of copper particles, the lungs were the primary organ which consistently had the highest copper concentrations. As in a previous study, the blue copper particles were easily observed in PIMs under light microscopy (Smith et al., 1996). These data suggest that PIMs have potential for the uptake of exogenous copper particles when the particles first pass through the pulmonary capillaries. Equine PIMs are capable of internalizing copper particles without any manifestation of toxic insult when treated with multiple doses of copper particle suspension over a period of 96 hours (Singh et al., 1994). Internalization occurs mostly at coated pits whereas concurrent internalization of nascent globules of the coat takes place at the ruffled surface. Damage to PIMs would likely result in decrease pulmonary copper clearance.

In this study, we found that RespPRRS®-inoculated and uninfected control pigs had similar capacity to clear a single intravenous dose of copper particles, whereas VR-2385-inoculated pigs had significantly decreased copper concentrations in lungs correlated with decreased pulmonary clearance of copper particles by PIMs at 7-14 DPI. Decreased copper uptake by porcine PIMs has also been documented in fumonisins toxicity which induces ultrastructural alterations in PIMs resulting in an inability to remove blood-borne particles, thus increasing the susceptibility to bacteremic diseases (Smith et al., 1996). Bertram (1985) reported that porcine PIMs had an important role in the clearance of debris and *Actinobacillus pleuropneumoniae* (APP) from the blood as revealed in an experimental model. It is likely that PRRSV-induced damage to PIMs results in increased susceptibility to bacterial diseases such as APP.

In conclusion, these studies demonstrated that: 1) PRRSV replicated and had a detrimental effect on PIM function both *in vitro* and *in vivo*; 2) PIMs from younger pigs were
more permissive to PRRSV infection; 3) *in vitro* bactericidal assays of PRRSV-infected macrophages were unable to differentiate between low and high virulence PRRSV strains as established by *in vivo* studies; 4) the severity of PRRSV-induced damage to PIMs *in vivo* differs among PRRSV isolates and correlates with pneumovirulence, and 5) PRRSV effects on PIM function may play a role in the porcine respiratory disease complex (PRDC) of which PRRSV is a major pathogen and secondary bacterial pneumonia and septicemia cause substantial production losses.

**References**


ACKNOWLEDGEMENTS

I have been fortunate to have an opportunity to pursue my higher education here at Iowa State University. It would not have been possible without the help and support of the Royal Thai Government and my colleagues at Chulalongkorn University. The completion of this dissertation and my graduate program also would not have been possible without the support and guidance of many individuals.

My foremost appreciation is to my co-major professors, Drs. Pat Halbur and Mark Ackermann, who have not only guided my research efforts, but provided mentoring and advice in my development as a veterinary pathologist. I am grateful for the moral support, advice, and positive influence of Dr. Eileen Thacker, who later served as a member of my committee. Her generosity and support is greatly appreciated. Dr. John Andrews, who initially served as my major professor, was instrumental in supporting the early parts of the work. I also wish to thank Drs. Ken Platt, Richard Engen, and Jeff Zimmerman as members of my examining committee for their time and effort spent in review of my program of study, preparation of preliminary exams, and review of this dissertation.

I am thankful to Drs. Frank Ahrens, Dagmar Frank, Jim Roth, and Prem Paul for use of laboratory equipment, Dave Cavanaugh for histology service, Jim Fosse for photography, Nancy Upchurch for technical assistance, Ryan Royer and Jeremy Bruna for spending many hours caring for animals and assisting in sample collection, often during unpleasant weather, and Jean Olsen for her friendship and her assistance with electron microscopy.

Richard Hasbrook deserves my boundless gratitude for serving as an inspirational supervisor at sponsored programs. I would also like to thank all of my friends at Iowa State University for their warm friendship, especially, Helmut Haas and Tosak Seelanan for computer assistance. A special thanks is extended to Preecha Prasong, Churee Pramatwinai, Ratree Platt, Sirintorn Yibchok-a-nan, Jackie Kinyamu, Pornitippa Nawagitgul, Peter Akoto, Yanjin Zhang, Susan Schommer, Theresa Young, Barbara Erickson, Theerapol Sirinarumitr, Lani Vincent, Amy Vincent, and Hans Rotto.
Finally, I would not have been able to complete this work without the support of my family and relatives whom I left behind in Thailand. My sincere appreciation is to my mother, Prapaporn Thanawongnuwech for unending support and encouragement for all four of her children, Roongroje, Sirilak, Narong, and Sachee to reach for their highest goals. I also wish to thank my aunt, Petchamanee Siwasangkai, for love and encouragement. Without them nothing would be worth doing.