RESEARCHREPORT
SWINE HEALTH

Title: Macrophage Activation and development of Porcine Circovirus Wasting Disease **NPB #00-014**.

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Date Received: 5/14/2001

I. Abstract: Porcine circovirus (PCV)-2, a newly described single stranded circular DNA virus pathogen of swine is the cause of postweaning multisystemic wasting syndrome (PMWS). In gnotobiotic piglets, PCV-2 infection alone produces asymptomatic infection without evidence of overt PMWS. Gnotobiotic piglets infected with PCV-2 were injected with keyhole limpet hemocyanin in incomplete Freund's adjuvant (KLH/ICFA) and the effects on virus production and development of PMWS were determined. In the first experiment, piglets were injected subcutaneously on the left hip and shoulder and viral burden was assessed in regional lymph nodes draining the injection sites and in contralateral lymph nodes 13/14 days after infection. Immune activation increased the number of virus antigen-positive cells in draining lymph nodes and increased the amount of infectious virus recovered by 1-4 log10. In a second experiment, the effects of injections of KLH/ICFA with or without concurrent stimulation of peritoneal macrophages by intraperitoneal injections of thioglycollate broth on induction of PMWS was assessed. All immunized piglets developed moderate to severe PMWS whereas none of the piglets infected with PCV-2 alone developed PMWS. Greater than 107 infectious virus per gram of tissue was recovered from PMWS-affected piglets. In PMWS-affected piglets, extensive replication of PCV-2 in lymphoid tissues and liver was documented by both immunocytochemistry and quantitative viral titrations.

Lymph node and liver section replicates from experimental PMWS piglets and controls were dually stained for the distribution of PCV-2 structural protein, incorporated bromo-desoxyuridine (BrDU), TUNEL reactivity and cytoplasmic lysozyme. Viral antigen was primarily localized within the cytoplasm and occasional nuclei of lysozyme-positive histiocytes and macrophages. Cellular DNA synthesis was not mandatory for virus production as the majority of virus-positive histiocytes were negative for BrDU incorporation into cell nuclei. In the main, hepatocytes did not contain viral protein and, when detected, antigen was restricted to hepatocyte nuclei only. Apoptosis, as determined by TUNEL, was not a feature of hepatocyte loss in PMWS lesions. These data confirm the tropism of PCV-2 for macrophages and histiocytes and suggest that hepatocyte destruction, characteristic of experimental PMWS, is accomplished by

progressive and widespread hepatocyte necrosis, not by apoptotic mechanisms. Thus, immune activation is a key component of the pathogenesis of PCV-2-associated PMWS in swine and this event is accompanied by upregulation of virus production in macrophages and spread of the virus to many organs and tissues.

II. Introduction: A new disease syndrome of weaned (6-12 week old) piglets was first identified in several "high health" herds in 1991 in western Canada and reported to the swine community in 1997-8 as porcine postweaning multisystemic wasting syndrome (PMWS), a frequently fatal disease characterized by a constellation of clinical signs including progressive weight loss, jaundice and high mortality rates. This is a porcine circovirus-2 (PCV-2) virus infectious disease and has been identified throughout the world. All viral isolates studied to date are essentially identical to each other. Gross expression of PMWS such as generalized lymphadenopathy, hepatitis, nephritis and pneumonia are most severe in weanling pigs. The histologic features of PMWS are systemic angiocentric granulomatous inflammation, syncytial giant cells and intracytoplasmic basophilic viral inclusion bodies in phagocytes. Tissue damage and organ failure results from extensive inflammation and secondary parenchymal cell necrosis.

Even though field studies have implicated PCV-2 as the agent of PMWS, reproduction of PMWS has proven difficult. Inoculation of conventional or gnotobiotic piglets with PMWS tissue extracts or PCV-2 alone frequently resulted in subclinical infection; PMWS, when produced, was less severe than field disease or occurred in some but not all animals within a group. Recently, our laboratory demonstrated that gnotobiotes, co-infected with PCV-2 and PPV, the latter a virus of minimal virulence in neonates, developed disseminated granulomatous inflammation and resultant fatal PMWS. It appears that PCV-2 needs "help" from PPV or other viruses to be expressed as PMWS. What role could PPV have? PPV is known to simulate lymphoreticular hyperplasia through its strong cytotropism for macrophages and actively dividing lymphocytes. The fact that both PPV and PCV-2 appear strongly tropic to macrophages in vivo suggests that potentiation of PCV-2 likely occurs within this cellular compartment. The data suggest the following pathogenesis for PMWS: After co-infection, both PPV and PCV-2 localize in regional histiocytes; PPV spreads rapidly beyond the inoculation site by cell-associated viremia to lymphoid tissues where infection stimulates cellular hyperplasia and induction of anti-PPV immunity and immune clearance of PPV. PCV-2 also replicates in macrophages but effective immunity to PCV-2 is not established. During PPV convalescence, newly arriving and activated macrophages support heightened PCV-2 replication. Increased production of PCV-2 virions facilitates spread to additional phagocytic and lymphocytic cells, PCV-2 escapes lymphoid containment and disseminates to many organs as macrophage-dominant granulomatous inflammation expressed as PMWS. Thus, macrophage activation (mediated by PPV or other factors) is the critical event in the promotion of PMWS. Because cellular stimulation and activation occurs with many different infections and manipulations including prophylactic vaccination. In this grant, we focussed our efforts defining the in vivo effects of localized and systemic manipulations upon quantitative parameters of PCV-2 infection and disease expression as PMWS. The last point (vaccinations) has immediate relevance to the industry as it is now the norm in that very young piglets in SEW programs are vaccinated as early as two weeks of age.

III. Project Objectives: The overall goal of our proposed studies was to simulate the macrophage activating effects of porcine parvovirus (PPV) in gnotobiotic swine in order to test the hypothesis that local or systemic macrophage activation potentiates PCV-2 infection and results in the development of PMWS. This was accomplished in two (3 litter) experiments which tested the hypotheses that:

1. Local activation of macrophages by immunizations potentiates local replication of PCV 2 in lymphoid tissues and,

2. Systemic macrophage activation by immunizations potentiates the systemic replication of PCV-2 and results in PMWS.

IV. Procedures: Tables 1 and 2 below outline the experimental design for each experiment.. In the first experiment, we tested hypothesis one as follows. One-day old piglets were oro-nasally inoculated with PCV-2 containing 4 x 106 infectious virus per ml. Piglets were immunized with keyhole limpet hemocyanin (KLH) in incomplete Freund's adjuvant (ICFA) at 3 and 8 days of age. Actively dividing cells were labeled in situ with BrDU 2 hrs prior to termination. Tissues were collected for histopathologic evaluation and assessment of viral load by titrations and immunohistochemistry.

Table1: Hypothesis 1: Effects of immunization upon virus load in lymphoid tissues

Piglet Groups	Numbers/ Group	Infect with PCV-2	Immunize with KLH in ICFA	Immunize with KLH in saline	Terminate on PID 13/14
A	4	yes	yes	-	yes
B	4	yes	-	yes	yes
C	2-3	no	yes	-	yes
D	2-3	no	-	yes	yes

In the second experiment, we utilized the same basic design and two litters of piglets to determine if immunizations (4 sites instead of the two above), with or without stimulation of peritoneal macrophages with thioglycollate broth, induced PMWS in PCV-2-infected piglets. One day old PCV-2-infected piglets were immunized as in experiment one above. The experiment was terminated when piglets were obviously sick with PMWS or by 35 days of age. As in experiment one, tissues were evaluated for the development of PMWS lesions and the distribution and amount of infectious virus by titrations.

Table 2: Hypothesis 2: Effects of systemic immunostimulation on the development of PMWS in PCV-2 infected piglets.

Piglet Inoculation Group	Piglets per Group	Infected with PCV-2	Local Adjuvant and/or Intraperitoneal injection with: ICFA-KLH only	Glycan only	ICFA-KLH & Glycan	Saline only
A	3-4	yes	yes	-	-	-
B	3-4	yes	-	yes	-	-

C	4-6	yes	-	-	yes	-
D	3-4	yes	-	-	-	-
E	2-3	-	-	-	-	-
F	2-3	-	yes	-	-	-
G	2-3	-	-	yes	-	-
H	2-3	-	-	-	yes	-

As before, piglets were infected at 1 day of age. Groups A, C, F, H given were immunized twice in 4 sites with ICFA-KLH. Groups B, C, G, H were given glycan broth at 3, 15 and 25 days of age to induce sterile peritonitis and influxes of activated macrophages into the peritoneal cavity. As in the first experiment, virus loads were determined by titrations and immunohistochemistry and histologic lesions in lymphoid tissues and liver (the target organs in experimental PMWS) were assessed by histopathology and immunohistochemistry studies using single and dual staining for viral antigen, cytoplasmic lysozyme (a macrophage marker), BrDU for new cell DNA synthesis and for double strand breaks indicative of apoptosis by the TUNEL immunohistochemistry stain.

V. Results and Conclusions: Porcine circovirus (PCV)-2, a newly described single stranded circular DNA virus pathogen of swine is the cause of progressive wasting disease complex known as postweaning multisystemic wasting syndrome (PMWS). PCV-2 infection alone in gnotobiotic pigs does not produce PMWS whereas PCV-2 infection when combined with PPV infection induces PMWS in gnotobiotes. The mechanism(s) whereby PPV potentiates the virulence of PCV 2 has not yet been identified. In these experiments, gnotobiotic piglets, infected with PCV-2, were immunostimulated with an irrelevant protein antigen emulsified in incomplete Freund's adjuvant to mimic one of the proposed mechanisms of potentiation by PPV. Our expectations for these studies were both met and exceeded. A brief summary of our data is presented below.

In experiment one, PCV-2-infected piglets were immunized subcutaneously on the left side and viral burden was assessed in regional lymph nodes draining the immunization sites and in contralateral lymph nodes 13/14 days after infection. Local immunostimulation increased the number of virus antigen-positive cells in draining lymph nodes and increased the amount of infectious virus recovered by 1-4 log10 and this effect was correlated to the increased incorporation of bromo desoxyuridine (BrDU) label into newly synthesized cellular DNA in histiocytes and lymphocytes in the nodes.

In experiment two, the effects of local immunostimulation by subcutaneous immunization with or without concurrent stimulation of peritoneal macrophages by intraperitoneal injections of thioglycollate broth on induction of PMWS in PCV-2-infected gnotobiotic piglets was assessed. All (7/7) systemically immunostimulated piglets developed moderate to severe PMWS whereas none of the piglets infected with PCV-2 and not immunostimulated with KLH/ICFA developed manifestations of disease. Extensive replication of PCV-2, primarily within histiocytic cells was documented by quantitative viral titrations and PCV-2 immunocytochemistry and was correlated to

increased numbers of BrDU-labeled cells in lymphoid tissues and to tissue infiltrations of lysozyme-positive macrophages. Titers in PMWS-affected lymphoid tissues and livers exceeded titers in PCV-2 alone infected piglets by 3-4 log10 infectious units and averaged roughly 1 x 108 infectious units per gram of tissue. In PMWS-affected piglets, liver failure associated with hepatocyte necrosis (not apoptosis or programmed cell death) and granulomatous inflammation was the proximate cause of death. Thus, immunostimulation is a key component of the pathogenesis of PCV-2-associated PMWS in swine.

These experiments are published in Veterinary Pathology 38:31-42, 2000 and the specific details of each experiment can be found there. The manuscript is entitled, "In vivo immunostimulation is the pivotal event in the production of wasting disease in pigs infected with porcine circovirus-2 (PCV-2)" by Steven Krakowka DVM, PhD, John A. Ellis DVM, PhD, Francis McNeilly PhD, Susan Ringler, MS, D. Michael Rings, DVM, MS and Gordon Allan, PhD, Vet Pathol, Five (5) copies of this paper are appended to this report.

In addition a written summary of these experiments was published in the swine educators conference, 2000 sponsored by NPPC for oral presentation in late September of last year. The title of this paper is, "Porcine Circovirus-2 and PMWS: Facts and Speculation on this New Infectious Disease of Swine" by Steven Krakowka, DVM, PhD (The Ohio State University), John A. Ellis, DVM, PhD (University of Saskatchewan) and Gordon Allan, PhD (Queens University, Belfast).

A third paper is "in preparation" and awaits final editing and submission. This paper is entitled, "Hepatic lesions associated with experimental porcine post weaning multisystemic wasting syndrome (PMWS) are not mediated by apoptosis" by Steven Krakowka, DVM, PhD (The Ohio State University), John A. Ellis, DVM, PhD (University of Saskatchewan), Brian Meehan, PhD, Francis McNeilly, PhD and Gordon Allan, PhD (Queens University, Belfast). A draft of this manuscript is enclosed.

The following conclusions were drawn from experiments supported by grant 01-014 from the National Pork Producers Council (NPPC):

1. PCV-2 is the sole cause of wasting disease (PMWS) of swine,

2. PCV-2 infection in gnotobiotic swine is asymptomatic,

3. Histiocytes/macrophages are the initial in vivo cellular target of the virus,

4. Upregulation of macrophage numbers and functions by parenteral immunization potentiates virus replication, facilitates viral spread to nonlymphoid tissues and precipitates PMWS in PCV-2-infected and immunized piglets,

5. Cellular DNA synthesis as determined by BrDU incorporation is not mandatory for virus production in vivo,

6. Liver failure and hepatocyte lesions are mediated by necrosis, not apoptosis, and

7. The implications of these findings for swine production and the practice of prophylactic vaccination for unrelated (to PCV-2) pathogens is profound. These experimental data suggest that the combination of segregated early weaning (SEW) and early (<3 weeks of age) vaccinations with macrophage-targeted adjuvanted vaccine formulations, particularly for Mycoplasma sp may be important co-factors for induction of PMWS in the field.