Short communication

Leukogram abnormalities in gnotobiotic pigs infected with porcine circovirus type 2


1. Introduction

Porcine circovirus type 2 (PCV2) is the causative agent of post-weaning-multisystemic-wasting-syndrome (PMWS), a swine disease first observed in Canada in 1991 (Clark, 1997). PCV2 is ubiquitous in swine herds around the world and most herds are endemically infected. The majority of infected pigs remain clinically unaffected whereas a low percentage can demonstrate severe clinical disease. Studies investigating this enigma have focused on the host immune response and pathogenesis of the disease. In this report we describe a direct relationship between an abnormal leukogram and the onset of clinical disease in a gnotobiotic pig model. These findings are based on data from successive studies using a cell culture propagated PCV2 isolate (Bolin et al., 2001) or viruses derived from infectious DNA clones (Cheung et al., 2007).

2. Materials and methods

2.1. Animals and experimental design

Over the course of 5 animal studies, 64 piglets were derived aseptically from crossbred sows by cesarean
section and housed in sterile isolators as previously described (Gauger et al., in press). Each pig within an isolator (2–4 pigs/isolator – each pig identified by isolator number and a letter) received the same treatment, a sham or PCV2 inoculation (one of three strains – Supplemental Materials and Methods) at about 7–10 days of age, 0 days-post-inoculation (dpi) (Table 1). For inoculation, each pig was restrained and received a 1 ml volume of sham or virus solution divided between each nostril. Pigs in each isolator were randomly pre-selected for euthanasia and necropsy unless they were severely affected at which time they were euthanized prior to scheduled necropsy times.

Experiments 1, 2, and 3 utilized cell culture propagated PCV2a (isolate 688) for PCV2 inoculation. EDTA-treated blood samples and rectal swabs were collected at 0 dpi and at weekly intervals until the time of necropsy. The samples were passed out of the isolator in a sterile fashion. Swabs were cultured for bacteria as previously described (Gauger et al., in press). Bronchoalveolar lavage fluid (BALF), EDTA-treated blood, and whole blood were collected at necropsy. Plasma and BALF were tested for the presence of PCV2 via a gel-based PCR assay as previously described (Nawagitgul et al., 2000).

Experiments 4 and 5 utilized virus derived from infectious clones of PCV2a (isolate DQ629114) and PCV2b (DQ629115) for PCV2 inoculation (Cheung et al., 2007). Rectal swabs were collected at weekly intervals and passed out of the isolator in a sterile fashion to culture for bacteria as previously described (Gauger et al., in press). In Experiment 4, EDTA-treated blood samples were collected at 0, 7, 14, 24, 29, and at necropsy at 35 dpi. In Experiment 5, EDTA-treated blood samples were collected at 0, 14, 19, 22, 26, 29, 33, 36, and at necropsy at 41 dpi. In both experiments, BALF and whole blood were also collected at necropsy. Plasma and BALF were tested for the presence of PCV2 via a quantitative PCR assay as previously described (Opriessnig et al., 2003). Serum harvested at necropsy was tested for PCV2 virus neutralization titers (Supplemental Materials and Methods).

Table 1
Experimental design including the inoculum, the total number of pigs per group and the number of clinically affected pigs in each group.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Controla</th>
<th>PCV2a 688</th>
<th>PCV2ab DQ629114</th>
<th>PCV2b DQ629115</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0/0</td>
<td>2/2d</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>0/4</td>
<td>0/4</td>
<td>–</td>
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<tr>
<td>3</td>
<td>0/6</td>
<td>3/8</td>
<td>–</td>
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</tr>
<tr>
<td>5</td>
<td>0/4</td>
<td>–</td>
<td>7/12</td>
<td>7/12</td>
</tr>
</tbody>
</table>

a Sham-inoculated control pigs.
b Virus inoculum.
c Pigs inoculated with PCV2 and cofactor.
d Numerator – number of clinically affected pigs; denominator – number of pigs in experimental groups.

Plasma samples collected at the time of necropsy were tested by PCR for porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV), swine hepatitis virus (swine HEV), bovine viral diarrhea virus (BVDV), and torque teno virus type 1 and 2 (TTV-1 and TTV-2) as previously described (Gauger et al., in press).

2.2. Leukogram

EDTA-treated blood samples in all experiments were analyzed for white blood cells (WBC) using a multispecies hematology instrument (Hemavet HV950FS, Drew Scientific, Inc., 2003). The WBC count was reported as actual number of neutrophils, lymphocytes, monocytes, basophils, eosinophils, and total WBC per microliter of whole blood. Leukogram values for control pigs determined at the time of necropsy from experiments 2, 4, and 5 (n = 12) were utilized to establish normal values for germ-free pigs. The mean and standard deviation was calculated to establish a normal range for comparative purposes with any values greater than 2 standard deviations from the mean of the control pigs considered as an abnormal value. Control pigs from experiment 3 were not used to derive normal values because isolator 33 became contaminated with bacteria and isolator 35 was colonized with Escherichia coli. The leukogram parameters of the PCV2-infected pigs were reported as being normal or abnormal relative to the control leukogram. In addition to WBC counts, a ratio was determined between the total neutrophil count and total lymphocyte count reported as the N/L ratio.

2.3. Statistical analysis

Differences among clinical-PCV2-infected, subclinical-PCV2-infected, and subclinical-non-infected (control) pigs were analyzed for each experiment and for all experiments combined. Neutrophil to lymphocyte (N/L) ratios and leukogram values were subjected to pair-wise comparisons using the Student’s t-test (JMP, SAS Institute, Cary, NC).

3. Results

3.1. Clinical evaluation

Once the disease process started, the presentation of clinically affected pigs was similar among experiments.
despite infection with different PCV2 strains and co-factors (in experiment 3, pigs were infected with PCV2 in addition to colonization with E. coli or injection with FL-KLH). All PCV2 inoculated groups had a similar clinical presentation and mortality rate, and for the purposes of this paper will be treated as one collective group, i.e., intranasal exposure to PCV2.

3.2. Virus and bacterial detection

All pigs in each of the 5 experiments were negative for PCV2 DNA at 0 dpi. All PCV2-inoculated pigs in each experiment were considered infected since PCV2 was detected in their plasma and BALF at necropsy (Gauger, unpublished observations). PCV2 DNA was not detected in any of the control pigs at the time of necropsy. In experiments 4 and 5, the number of PCV2 genomic copies detected in necropsy plasma or BALF were similar between the PCV2a and PCV2b infected pigs. Among the 46 PCV2-inoculated pigs, 24 were clinically abnormal and PCV2-infected, and 22 were subclinical and PCV2-infected.

No bacterial growth was detected in any of the pigs in experiments 1, 2, 4, and 5. In experiment 3 at 0 dpi the control isolator 33 was found to be contaminated with Chryseomonas luteola and remained so for the duration of the experiment. As expected, E. coli was isolated from isolators 35 and 36 at 0 dpi (4 days post-experimental colonization with E. coli) and remained so for the duration of the experiment.

All pigs tested negative by PCR for PPV, PRRSV, swine HEV, and BVDV nucleic acid. In experiments 4 and 5, TTv-1 and TTv-2 were detected in 3 of the 8 control pigs and in 7 of the 32 PCV2-inoculated pigs. There was no apparent association of positive TTv status with any clinical outcome (Gauger et al., in press). A similar incidence of congenital TTv infection was detected retrospectively in gnotobiotic pigs in experiments 1, 2, and 3 (unpublished results).

3.3. Leukogram

3.3.1. Control pigs

Normal leukogram values from control pigs in experiments 2, 4 and 5 are listed in Supplemental Table 1. Although control pigs in isolators 33 and 35 were subclinically colonized with bacteria, their individual leukogram values did not fall outside of the normal range established by the control pigs (2 standard deviations from the mean) (Supplemental Table 1). However, when the control pigs from experiments 2, 4, and 5 were compared with the experiment 3 control pigs in the student’s t-test, the experiment 3 control pigs had a statistically significant (p ≤ 0.05) increase in mean neutrophil count. No other statistically significant differences were found between these groups.

3.3.2. Subclinical PCV2-infected pigs

At necropsy of 17 of 22 PCV2-infected subclinically affected pigs had abnormal leukograms, i.e., one or more cell populations and/or the N/L ratio were outside the normal range (Table 2). These values were characterized most often by an increased N/L ratio followed by an

Table 2
Neutrophil and lymphocyte values and neutrophil/lymphocyte (N/L) ratios for PCV2-infected pigs at necropsy.

<table>
<thead>
<tr>
<th>Clinically</th>
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<tbody>
<tr>
<td>Experiment</td>
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</tr>
<tr>
<td>1</td>
<td>14A</td>
</tr>
<tr>
<td>1</td>
<td>14D</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
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<tr>
<td>5</td>
<td>51A</td>
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<tr>
<td>5</td>
<td>51B</td>
</tr>
</tbody>
</table>

Actual neutrophil and lymphocyte values (1000 ×) per µL of whole blood and N/L ratios at necropsy for PCV2-infected pigs. Clinically affected pigs are indicated in bold type. Leukogram and N/L ratios 2 standard deviations from control values were considered abnormal.

a Pigs receiving FL-KLH.
b Pigs receiving FL-KLH and E. coli.
c Average values for clinically affected and subclinical pigs from all experiments.
absolute neutrophilia and lymphopenia as determined over several time points (Jain, 1993). Essentially all monocyte, basophil and eosinophil counts at time of necropsy for subclinical, PCV2-infected pigs were within normal limits (data not shown) and no significant differences were found between the sub-clinically affected pigs and control leukogram values.

3.3.3. Clinical PCV2-infected pigs

Abnormal leukograms were identified in all 24 PCV2-infected, clinically abnormal pigs at necropsy (Table 2). Monocyte, basophil and eosinophil counts at time of necropsy for clinical, PCV2-infected pigs were within normal limits (data not shown). Clinically affected pigs had significant ($p \leq 0.05$) increases in neutrophils and N/L ratio, and a significant decrease in lymphocytes compared to the sub-clinical, PCV2-infected pigs and the controls.

3.4. Virus neutralization test

Virus neutralization tests (VNT) were performed on sera collected at the time of necropsy from experiment 5 (Supplemental Table 2). No virus neutralization activity was detected in the control pigs. A 90% reduction (VNT$_{90}$) in virus-infected cells was detected in 8 of 24 PCV2-infected pigs with reciprocal titers ranging from 2 to 128. Virus neutralization tests detecting a 50% reduction (VNT$_{50}$) in virus-infected cells revealed an increase in titer for each of the 8 VNT$_{90}$ positive pigs. Two additional pigs, 51B and 53C, had a VNT$_{50}$ of 8 and 4, respectively. When the 24 PCV2-infected pigs in experiment 5 were divided by clinical status, 2 of the 14 clinically affected pigs had a detectable VNT at time of necropsy, pigs 53D and 54C. They were euthanized 41 and 29 dpi, and had VNT$_{50}$ titers of 16 and 2 and VNT$_{50}$ titers of 128 and 8, respectively. Eight of the 10 subclinical PCV2-infected pigs had VNT titers at time of necropsy. Subclinical pigs 54A and 56B did not develop detectable VNT antibody by the time of necropsy at 29 and 41 dpi, respectively. Of the 24 PCV2-infected pigs, a significant ($p \leq 0.05$) difference was found in the group mean N/L ratio between the 10 pigs that had a detectable VNT$_{50}$ titer and the 14 that did not; group mean N/L ratio of 1.05 (0.36–3.10) vs. a group mean of 4.68 (0.66–12.96).

4. Discussion

As part of a routine procedure, the complete and differential WBCs were evaluated at necropsy for pigs in the experiments 1, 2 and 3. Ten of the 14 PCV2-infected pigs had abnormal leukogram values compared to controls, consisting primarily of neutrophilia and lymphopenia. Upon further examination, clinically affected pigs demonstrated pronounced leukogram abnormalities and significantly elevated N/L ratios compared to both subclinically-infected and control pigs (Table 2). Based on these findings, we hypothesized that the abnormal N/L ratios were predictive of clinical disease, a hypothesis supported by the outcome of experiments 4 and 5.

The goal of experiments 1, 2, and 3 was to characterize the clinical response of germ-free pigs inoculated with a well characterized PCV2 strain, isolate 688. It was anticipated the clinical response would mimic the findings of others in which germ-free pigs given PCV2 alone replicated the challenge virus and remained clinically normal for the duration of the isolator experiment (Ellis et al., 1999; Krakowka et al., 2000). In the previously described studies, pigs developed clinical disease followed by death only in conjunction with a coinfection (Ellis et al., 1999; Krakowka et al., 2000), or an immunostimulating event (Krakowka et al., 2001). In experiments 1, 2, and 3; 2 of 6 pigs inoculated only with PCV2a isolate 688 and 3 of 8 pigs that received PCV2 (PCV2a isolate 688) and a cofactor were considered to be clinically affected (Table 1). All 5 affected pigs from experiments 1 and 3 had similar clinical signs although pig 34B was less affected than the others at the time of its scheduled necropsy at 32 dpi. In addition, all 5 of these pigs had an abnormal leukogram (beyond 2 standard deviations from the control values) for at least one or more of the parameters tested when sampled at necropsy or within the previous 2 days. Except for experiment 3, no known co-factors were given to any of the gnotobiotic pigs. Isolator 33, the control isolator for experiment 3, became contaminated with *Chryseomonas luteola* with no ill effects noted in the contaminated pigs. One of the 4 pigs in isolator 34 inoculated with PCV2 and given an intraperitoneal (ip) injection of FL-KLH was clinically affected at 32 dpi, its scheduled necropsy time. The contributing role of the ip injection is not clear since the remaining three isolator pigs were normal in appearance at the time of necropsy (32 and 46 dpi). Likewise, the role the ip FL-KLH injection and colonization with *E. coli* isolate 68 played in the 2 clinically affected isolator 36 pigs euthanized early (22 and 27 dpi) is not clear since the other 2 pigs receiving the same treatment were normal at 46 dpi, their scheduled necropsy time. The incidence of clinical illness in these “cofactor” groups was similar to the incidence in PCV2-only inoculated gnotobiotic pigs (Lager et al., 2007).

One goal of experiments 4 and 5 was to study more closely the kinetics of PCV2 infection and its effect on the abnormal leukogram through more frequent sampling. The lesions, virus load, and ELISA serology found in these experiments have been reported previously (Gauger et al., in press). WBC counts on serially collected blood samples in experiments 4 and 5 revealed the onset of abnormal N/L ratios beginning by 14 dpi. Most of the pigs that eventually became clinically affected had an abnormal leukogram by 22–24 dpi. The early rise in N/L ratio was attributed to an absolute lymphopenia creating a relative neutrophilia (data not shown). This was followed by an increasing neutrophil count, producing an absolute neutrophilia, more pronounced in clinically affected pigs. The onset of lymphopenia following PCV2 infection has been previously reported and is believed to be directly related to PCV2 infection (Nielsen et al., 2003; Yu et al., 2007a,b). It is not clear how PCV2-infection plays a role in the neutrophilia reported here. Possible explanations include: (1) the neutrophilia may be an artifact of a PCV2 infection in gnotobiotic pigs. However, this phenomenon was also seen in 2 of the 4 isolator 36 pigs that were colonized with the *E. coli* 68 strain, an event that activates the germ-free pig...
immune system more typical of conventional pigs. (2) The neutrophilia was a bystander effect linked to the severe lymphopenia inducing a host response to produce more lymphocytes, and this signal also stimulated abnormal production of neutrophils. Indirect evidence supporting a bystander effect has been reported in the opposite direction where prolonged administration of a cytokine responsible for inducing neutrophil output from the bone marrow (granulocyte colony stimulating factor) in cattle will eventually result in a lymphocytosis and monocytosis (Harp et al., 1991). (3) The production of neutrophils was normal, but the clearance mechanism/emigration of neutrophils was abnormal. However, in immune disorders such as leukocyte adhesion deficiency where neutrophil clearance is impaired, a typical feature would be massive accumulation of neutrophils in the spleen. Such splenic microabscesses were not observed here. (4) The neutrophilia was related to a dysregulated host immune response directed against viral and/or host antigens.

Based on a lack of clinical disease and the presence of a virus neutralization titer, some PCV2-infected pigs in experiment 5 may have developed a protective immune response. An inverse relationship between VNT and clinical disease was found in 12 of the 14 clinically affected pigs. A similar relationship was found when comparing virus load, clinical disease, and ELISA antibody levels in the experiment 4 and 5 PCV2-infected pigs (Gauger et al., in press). When clinical and subclinical pigs were compared, pigs that seroconverted were less likely to have developed clinical disease and had a lower virus load. Although there are a limited number of pigs in experiments 4 and 5, there appears to be a direct relationship between the detection of anti-PCV2IgG and VNT antibody. Six of the 10 pigs that had detectable virus neutralization activity were ELISA positive and pig 53D was suspect ELISA positive (Gauger et al., in press). Pigs 51B, 53C and 54C had low VNT<sub>50</sub> titers and were ELISA negative. The remaining PCV2-infected pigs in experiment 5 were negative by both assays.

Regarding either the development or absence of PCV2 protective immunity, there appears to be 3 responses associated with the category (clinical vs. subclinical) of disease under the conditions of the studies reported here, and previously (Gauger et al., in press). The first outcome is that PCV2-infected pigs that develop ELISA and VNT antibody, have a lower virus load, and are subclinically affected. A second outcome occurs with pigs that do not develop detectable antibody, have a high virus load, and are clinically affected. The third outcome is a mixed response where pigs have become ELISA or VNT positive, may or may not be clinically affected and have a high virus load. Pigs 53C, 53D, and 54C fall into the third outcome.

The environmental variation is minimized among treatment groups and experiments in gnotobiotic pig studies. In essence, the gnotobiotic pig model can evaluate the host-pathogen interaction without environmental influences. However, one limitation of the model is that the germ-free pig does not have a mature or activated immune system. This condition may exacerbate the actions of a pathogen as seen in this pig model in which 52% (24 of 46) of the pigs were clinically affected following challenge with PCV2. This incidence rate is in contrast to conventionally raised pigs experimentally infected with PCV2 (Allan et al., 1999; Balasch et al., 1999; Kennedy et al., 2000; Magar et al., 2000; Rovira et al., 2002; Opriessnig et al., 2004). In addition, 85% of the PCV2-infected pigs in the experiments reported here had an abnormal leukogram; again a very high incidence rate when compared to the limited studies that have been reported for experimentally infected conventional pigs (Segales et al., 2000; Darwich et al., 2003; Nielsen et al., 2003). Another limitation of this experimental model is the capacity of the isolators restricts the size and duration of the experiment to multiples of 4 pigs for approximately 6 weeks. It is not clear how many more pigs might have become clinically affected if allowed to live longer. For example, based on the earliest onset of clinical disease (21 dpi), the 2 pigs euthanized at 11 dpi in experiment 2 (20B, 20C) potentially did not have adequate time to develop an abnormal leukogram. Considering about half of the pigs developed clinical disease, it is likely that one of these pigs would have developed clinical disease had they been allowed to live for another 5 weeks. Collectively, this disease model provides some insight into (1) the span of time between virus challenge and development of an abnormal leukogram and subsequent clinical disease, and (2) the apparent development of a protective immune response in a portion of the pigs following challenge.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2011.06.016.

References


