Effect of granulocyte-macrophage colony-stimulating factor on post-weaning multisystemic wasting syndrome in porcine circovirus type-2-transfected piglets

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Summary

Post-weaning multisystemic wasting syndrome (PMWS) is a complex disease syndrome in swine, affecting nursery and fattening pigs. Although ongoing evidence suggests that porcine circovirus type-2 (PCV2) is the causal agent of PMWS, the host immune system appears to have a crucial role in the PMWS pathogenesis of PCV2-affected pigs. owing to difficulties in producing a biologically pure form of PCV2 devoid of the other viral agents commonly present in swine tissues, we decided to use a tandem-cloned PCV2 DNA providing highly pure grade reagent in order to monitor the virulence of PCV2 alone or with an immunostimulating co-factor, granulocyte-macrophage colony-stimulating factor (GM-CSF). A single intramuscular injection of tandem-cloned PCV2 DNA into 5-week-old piglets produced plasmid to viral genome progeny and infectious particles as early as 8 days post-injection in all the organs tested (the lung, the tonsil and the mesenteric, bronchial and upper-right axial lymph nodes). The initial plasmid load was not detected with the help of primers designed to specifically detect the acceptor plasmid, thus confirming the replication of the viral genome. Despite the presence of a high level of PCV2 genome copies in the lymphoid organs – the tonsil and the lung – and the presence of infectious particles, no detectable clinical manifestations or pathological lesions were observed in the transfected pigs over the period of observation, regardless of whether they had been co-injected with plasmid containing GM-CSF DNA or had received plasmid containing PCV2 DNA alone. GM-CSF encoding DNA injection had no significant effect on viral replication or on the production of viral particles and appearance of the disease.

Keywords
GM-CSF, infectious DNA, intramuscular route, in vivo transfection, PCV2, PMWS
PMWS can be diagnosed from a set of clinical signs correlated with the presence of PCV2 in microscopic lesions. The characteristic clinical symptoms are wasting or in lesser extent failure for the young piglets to gain weight in the normal rate in spite of a normal diet. Other associated signs may include enlarged lymph nodes, paller, diarrhoea, ulceration, icterus, respiratory disorders, coughing, central nervous disorder and sudden death (LeCann et al. 1997; Harding 1997b; Rosell et al. 1999; Quintana et al. 2001). Many attempts have been made in order to reproduce PMWS experimentally either in conventional or in specific pathogen-free (SPF) piglets by using tissue homogenates (Balasch et al. 1999; Albina et al. 2001) or PCV2 grown in cell culture (Ellis et al. 1999; Magar et al. 2000) with success ranging between slight lesions and severe disease. Although severe PMWS can be reproduced with PCV2 alone, severe clinical PMWS has been better reproduced in pigs co-infected with porcine parvovirus (Kennedy et al. 2000; Krakowka et al. 2000), porcine reproductive and respiratory syndrome virus (Rovira et al. 2002) or by using immunostimulating factors, such as keyhole limpet hemocyanin (KLH) emulsified in incomplete Freund’s adjuvant, intramuscular injection of a vaccine against Mycoplasma hyopneumoniae (RespiSure, Pfizer, NY, USA), non-specific immunomodulating drug (Baypamun, Bayer, Leverkusen, Germany) (Allan et al. 2000; Krakowka et al. 2001; Kyriakis et al. 2002; Grasland et al. 2004; Hoogland et al. 2004) which suggests that co-factors are needed in order to reproduce the full spectrum of clinical PMWS.

PCV viruses do not spread easily in cell culture and a high titre of pure virus particles is hard to obtain. Plasmid containing PCV2 DNA was used in PMWS assays with some success by Fenaux et al. (2002). Infectious plasmid containing tandem-cloned PCV2 DNA introduced directly into the lymph node and the liver of piglets gave similar virus distribution and lesions to those observed when piglets were infected with virus stock, suggesting that the plasmid containing PCV2 DNA might replace the virus stock for infection purposes. The main advantage of using infectious DNA is that it leads to cloned viral particles and an endotoxin-free grade DNA preparation is easier to produce than a high viral titre of infectious particles. Recent results demonstrated that clone with a partial duplication of PCV2 genome was shown to be infectious when injected intramuscularly (Roca et al. 2004). We, therefore, explored in this study the possibility of using infectious DNA by intramuscular route for PMWS modelling experiments. The clone used enclosed a full duplication of the genome for delivered PCV2 genome into transfected cells after recombination events. The kinetics of viral replication, virus distribution and PMWS's clinical signs were monitored. The role of immunostimulation in triggering severe PMWS has been demonstrated (Allan et al. 2000; Krakowka et al. 2001; Kyriakis et al. 2002; Grasland et al. 2004), although some workers have found that immunostimulation, such as vaccination with killed parvovirus or commercial vaccine adjuvant, is not essential to initiate severe disease (Ladekjaer-Mikkelsen et al. 2002; Opriessnig et al. 2004; Resendes et al. 2004). Immunostimulants, such as KLH in incomplete Freund’s adjuvant (KLH/ICFA), promote severe PMWS pathogenesis when co-injected with PCV2, jeopardizing the use of immunostimulants in a PCV2 vaccination approach by using attenuated strains or infectious DNA (Krakowka et al. 2001; Grasland et al. 2004). The granulocyte-macrophage colony-stimulating factor (GM-CSF) has been used in a DNA-vaccination protocol with some success (Somasundaram et al. 1999; Flo et al. 2000). GM-CSF is a pleiotropic cytokine produced by both T-cells and B-cells enhancing immune response with little effect on the Th1/Th2 balance (Gerloni et al. 1998; Scheerlinck 2001) especially if the GM-CSF is simultaneously injected with the DNA vaccine (Kusakabe et al. 2000). Its role in the DNA vaccination protocol has been previously investigated (Blanchard et al. 2003a). Alveolar macrophages were shown to be permissive to PCV (McNeilly et al. 1996). Moreover, the addition of the recombinant porcine GM-CSF, a potent macrophage activator, permits virus entry into the nucleus of monocyte-derived macrophages (MDM) in vitro (Gilpin et al. 2001). In the absence of GM-CSF, virus antigen was predominantly located in the cytoplasm, with no nuclear staining observed, suggesting that if cells receive an activation trigger, a PCV2 replication appears to be initiated. Similar observations were made when alveolar macrophages and PBMC were isolated from 4-week-old seropositive piglets (Gilpin et al. 2001). However, further experiments failed to demonstrate in vitro a PCV2 replication in pulmonary macrophages, and MDM when exposed to the virus. However, no stimulation with GM-CSF was used in these later experiments (Gilpin et al. 2003). It was, therefore, interesting to determine more precisely the effects of GM-CSF on viral replication, virus distribution and manifestation of the disease.

Materials and methods

Bacterial strains and DNA

Escherichia coli DH5α were used for plasmid preparation, cloning and subcloning experiments. The plasmid containing the PCV2 DNA was obtained by ligating two copies of the complete genome (AJ623306) in tandem into an acceptor
vector (pBluescript KS+, Stratagene, La Jolla, CA, USA), at the SacI site leading to pTPCV2. PCV2 genome was amplified, as described by de Boisseson et al. (2004), and was digested by SacI restriction enzyme, gel purified and was ligated in tandem in a pBluescript vector. The resulting genetics map has been shown in Figure 1. The porcine GM-CSF DNA cloned in a pcDNA 3.1 vector for expression (designated pGM-CSF) was a gift from F. Lefevre (Somasundaram et al. 1999).

Production of plasmid for in vitro transfection

Tandem-cloned PCV2 DNA (pTPCV2) and pGM-CSF were purified by means of EndoFree plasmid Giga kit columns (Qiagen, Hilden, Germany) and were resuspended in endotoxin-free phosphate-buffered saline (PBS) (Sigma-Aldrich, St Louis, MO, USA). DNA concentration was measured by means of spectrophotometry.

In vitro cell transfection and analysis of plasmids from mammalian expression vector

Pig kidney PCV-free continuous cells (PK15) were transfected with the molecular viral DNA by using Lipofectamine™ 2000 formulation (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s recommendations. Lipofectamine and DNA were diluted inOpti-MEM media (Invitrogen) in 1/2 ratio (w/v) (4 μg of DNA for 500 μl of transfection mix). The mix was then added to 90–95% of confluent cells and was incubated at room temperature for 6 h. The medium was replaced by antibiotic-free minimum essential medium (MEM media) (Gibco, Paisley, UK) supplemented with 5% foetal calf serum. The cells were then incubated at 37°C in 5% CO₂ for 6–90 h until the transgene expressions were assessed. ORF-2 expression and the production of infectious particles of the tandem-cloned PCV2 DNA were confirmed with the help of immunoperoxidase monolayer assay (IPMA) and activity of the pGM-CSF was assessed as described underneath.

In vitro activity test of the pGM-CSF clone

Activity of the pGM-CSF was assessed by means of a TF-1 cell proliferation assay for recombinant GM-CSF. TF-1 cells proliferate only in the presence of GM-CSF or some other cytokines (Kitamura et al. 1989). Cells were grown overnight in the presence or absence of human recombinant GM-CSF (rhGM-CSF), and were thoroughly washed three times with RPMI-1640 medium with 20 min of incubation. After 16 h of culture at 37°C, 20 μl of Alamar blue™ dye (Biosource International Inc., Camarillo, CA, USA) was then added to each well and was returned to the incubator at 37°C for 6 h as previously described (Ahmed et al. 1994; Gogal et al. 1997; Corley & Giambrone 2002). Briefly, the dye when added is in its oxidized form (blue colour, OD 600 nm) that is reduced (pink colour, OD 570 nm), as the cells proliferate. Because of the overlap between the two absorbances, the 600-nm absorbance was subtracted from the 570-nm absorbance in order to obtain the specific absorbance. The specific absorbance of unstimulated cells was subtracted from the specific absorbance of cells incubated with rhGM-CSF. Activity of the pGM-CSF was checked similarly by applying supernatant (100 μl) of 48-h PK15 post-transfected cells onto TF-1 cells. Control was made by means of transfected PK15 cells with pcDNA3.1 DNA and the specific absorbance of pcDNA 3.1-transfected cells was subtracted from the specific absorbance of cells transfected with pGM-CSF.

Infectious DNA preparation for injection

Endotoxin-free grade DNA (≤0.1 EU of LPS/μg DNA) was obtained from PlasmidFactory (Bielefeld, Germany) and was manipulated by using endotoxin-free gloves and materials. The estimated LPS of the preparation was 0.046 EU/μg DNA.

In vivo transfection of 5-week-old piglets

SPF piglets known to be free from PCV1 and PCV2 were obtained as described by Cariolet et al. (1994). Thirty-four 5-week-old SPF piglets were randomly set into six groups and were assigned to three SPF growing rooms in isolated housing with air filtration and controlled air pressure. Two injections of 200 μg of endotoxin-free grade DNA encoding GM-CSF
and 200 μg of endotoxin-free grade pTPCV2 were administered according to the scheme shown in Table 1. Injections were made intramuscularly into the right side of the piglet’s neck. Pigs in group-1 and group-3 were killed by means of intravenous administration of sodium pentobarbital, for gross postmortem examination at one day before the injection and 4, 8, 12, 19 days post-injection, whereas pigs in other group were killed at 35–40 days post-injection. Control pigs in group-5 and group-6 were inoculated either with endotoxin-free PBS (Sigma) or with endotoxin-free grade GM-CSF encoding DNA. Clinical observations and rectal temperatures were recorded daily and pig weights and nutrition were monitored weekly. Blood samples and oronasal swabs were collected weekly. During necropsy, the organs were observed and tissue samples were collected (the lung, the tonsil, the ileum and the bronchial, mesenteric, inguinal and right axillary lymph nodes).

PCV2 ORF-2 protein-based enzyme-linked immunosorbent assay (ELISA)

Serum samples were collected weekly for the detection and titration of PCV2 ORF-2 antibodies by using recombinant PCV2-fused GST/ORF-2 protein, as described by Blanchard et al. (2003b).

PCV2 genome titration by means of real-time polymerase chain reaction (PCR)

DNA was extracted from tissue homogenates (30% w/v) by using the Qiagen Dneasy tissue kit (Qiagen) according to the manufacturer’s recommendations. The number of injected plasmid molecules was quantified by using a TaqMan probe 6-FAM-CCGCTGCGCCTTA-MGB and pBluescript-specific primers 5’-CTGGGCTGTGTCAGGGAAGAAGA-MGB and 5’-GGTGGACT-CAGAGGCA-3’ that were designed in the pUC origin of the pBluescript KS vector (Stratagene) with the help of Primer Express Oligo Design software 2.0 (Applied Biosystems, Foster City, CA, USA). Reactions were performed in duplicate by using a MicroAmp Optical 96-well plate (Applied Biosystems). The thermal conditions were 2 min at 50°C, then 10 min at 95°C followed by 40 cycles with a denaturation step of 95°C for 15 s and annealing/extension at 60°C for 1 min. The PCV2 genome was quantified as described by Blanchard et al. (2004). Basically, the PCV2-specific primers 5’-GGGAG-CAGGGCCAGAATT-3’ (410–427) and 5’-CGCTCTGTGCCC TTG AATAT-3’ (473–452) were designed into the PCV2 ORF-2 region (GenBank accession no. AF201311) and were amplified by means of a 64-bp fragment. The TaqMan probe 5’-ACCTTAACCTTTCTTATTCTG-3’ (430–450) was labelled with the fluorescent reporter dye FAM (6-carboxyfluorescein) at the 5’ end and with the non-fluorescent quencher with the minor groove binder at the 3’ end. A DNA solution of a plasmid carrying a single copy of the PCV2 genome was serial diluted and was used in order to generate a standard curve of quantification. Amplified PCV2 genome copies in sample were inferred from this standard curve. The reactions were performed on an ABI Prism 7000 thermocycler (Applied Biosystems).

Infection particle titration by means of IPMA

Viruses were extracted from tissue with the help of mechanical homogenizer (Bioblock Scientific, Illkirch, France) (30% w/v) in ice-cold PBS. Samples were clarified by means of a 15-min centrifugation at 3000 x g and the supernatants were filtered through 0.22-μm pores, and were stored at -80°C until used. PCV2 titration was performed as described by Ellis et al. (1998). Filtered supernatants of tissue homogenates containing viral particles were serial diluted and were applied to PK15 monolayer cells (free from PCV) at 37°C for 5 h. Cells were washed with sterile buffer (PBS) and were induced with 300 μM D-glucosamine (Sigma) in Earle’s balanced salt (Sigma) in order to enhance viral replication for 30 min at 37°C (Tischer et al. 1987). Cells were then washed with sterile PBS and were incubated for 36 h at 37°C. PK15 adherent cells were then washed with PBS; they were fixed and were permeabilized by using cold acetone at 80% (v/v) for 10 min at -20°C. Endogenous peroxidases were

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Pigs in group-1 and group-3 were serial killed for analyses of genomic load and gross lesions in kinetics. Pigs in group-2 and group-4 were necropsied at the end of the experiment (days 35–40) as pigs in group-5 and group-6 (control).
neutralized for 30 min at room temperature with a mix of H$_2$O$_2$/methanol (1:99 v/v). Cells were washed twice with PBS-Tween-20 0.1% (PBS-T) and were incubated with PBS-T-20 0.1% and PBST-milk 5% (PBS-TM) for 30 min at 37°C; they were then incubated with a 1:300 diluted PCV2-specific pig anti-serum in PBS-TM at 37°C for 1 h and were washed three times with PBST buffer, and were incubated with a 1:150 secondary HRP rabbit anti-swine antibody (DAKO, Glostrup, Denmark). Using AEC substrate buffer and AEC chromogen concentrate (Serotec, Oxford, UK), HRP activity was revealed. The viral titre was expressed in TCID-50 by means of the Kaerber method (Kaerber 1931).

**Sequencing of the viral genome extracted from pig tissue after transfection**

DNA extracted from the inguinal lymph node of one pig from group-2 (35 days post-transfection and immunostimulated) was sequenced, as previously described by de Boisseson et al. (2004), in order to check whether mutations has occurred. The sequencing data were analysed by means of the Vector NTI Contig express suite (informax, Bethesda, MD, USA).

**Statistical analysis**

Non-parametric statistical tests were applied by using the SYSTAT 9 computer software package (SPSS Inc., Richmond, CA, USA). Relative daily weight gains and genomic loads were analysed by comparing the immunostimulated group, the non-stimulated group and the control group with the help of Kruskal–Wallis test. Similarly, the equivalent genome copy numbers of the immunostimulated group and the non-immunostimulated group were analysed by means of the same non-parametric test.

**Results**

**Cloned genomic DNA (PCV2) initiates the production of infectious viral particles by means of in vitro transfection**

Transfection efficiency was determined by appreciation of the green fluorescence ultraviolet radiation of transfected PK15 cells by a GFP encoding plasmid, and was approximately 20–30% of confluent cells (data not shown). Tandem-cloned PCV2 DNA-transfected cells were able to express PCV2 proteins (data not shown). IPMA performed with PCV2-specific antibody confirmed the expression of PCV2 antigen. Ten to fifteen percent of the transfected cells were found positive as early as 10–12 h post-transfection. The presence of infectious particles within these transfected cells or in the supernatant of these cells was then investigated by applying serial dilutions of supernatant or cell lysates to new PK15 monolayer cells (Figure 2). Dynamic in vitro transfection of PK15 with the tandem-cloned PCV2 DNA produced infectious particles both in the cellular fraction and in the growth media of post-transfected cells as early as 6 h post-transfection. The maximum number of infectious particles was found in the host cell fraction at 24 h, the post-transfected cells corresponding to $10^{5.5}$ TCID-50 per $10^6$ cells. A decrease of the infectious load was then observed at 48, 72 and 90 post-transfection. The maximum number of cell-free viruses was found at 24 and 90 h post-transfection, and corresponded to $10^{3.8}$ TCID-50 per $10^6$ cells. The latter peak could be explained by a liberation of virus particles because of increased cell death within the cell population.

**Expression and functionality of the pGM-CSF clone**

The pattern of the proliferative response of TF-1 cells to rhGM-CSF was established (Figure 3). Maximum activity was found between 1 and 5 ng/ml and a saturating effect was observed for concentration above 5 ng/ml. These results confirmed the data obtained by Kitamura et al. (1989). Comparatively, the proliferation observed when the supernatant of pGM-CSF-transfected PK15 cells was applied onto TF-1 cells was similar to the proliferation exerted by 1–5 ng/ml of
rhGM-CSF. Thus, those results confirmed the expression and the functionality of the pGM-CSF clone.

No expression of clinical signs and no gross lesions observed during the assay

None of the tandem-cloned PCV2 DNA-transfected piglets developed any clinical signs during the observation period, not even a day of hyperthermia (Q > 40.5 °C) (data not shown). The average daily weight gain of control pigs (0.78 kg/day) calculated over the 35-day period was similar to the average daily weight gain of the non-immunostimulated transfected pigs (0.84 kg/day) and of the immunostimulated transfected pigs (0.82 kg/day). No significant differences (P > 0.05) were found when immunostimulated and non-immunostimulated transfected pigs were compared with controls, or when all three groups were compared together. At necropsy, no gross lesions were observed in the ileum, the bronchial lymph node, the inguinal lymph node, the mesenteric lymph node, the tonsil or the lung of transfected pigs.

ELISA titration of PCV2 ORF-2 antibodies in blood samples of transfected pigs

Seroconversion was considered effective, when this OD ratio exceeded 1.5. The resulting kinetics of antibody development has been shown in Figure 4. Both immunostimulated and non-immunostimulated transfected pigs exhibited a seroconversion at 2 weeks post-transfection. No obvious effect of GM-CSF was observed either on the date of seroconversion or on the OD ratio value, when the seroconversion profiles of immunostimulated and non-immunostimulated transfected piglets were compared.

Real-time PCR for PCV2 genome quantification in various tissue samples

PCV2 genome was quantified in various tissue samples (the serum, the bronchial lymph node, the inguinal lymph node, the mesenteric lymph node, the axial upper-right lymph node, the tonsil, the lung and the ileum). The axial upper-right lymph node was collected, in particular, because of its location near to the point of injection. A high viral DNA load could be detected after 12 days post-injection in the sera (Figure 5) and in all sampled tissues (Figure 6). Results have been expressed as the mean PCV2 genome equivalent copy number (GECN) per gram of tissue sample or as the mean

![Figure 3](image-url)  
**Figure 3** Proliferation test of the pGM-CSF clone, activity on TF-1 cells, was assessed and was compared to various concentrations of rhGM-CSF. Cells were seeded to 10^4 cells/well and were induced with the presence or absence of 0.1–50 ng/ml of rhGM-CSF (hashed columns); values were blanked against cells with no GM-CSF. Assay of the activity of the pGM-CSF was assessed by adding supernatant of 48 h post-transfected pK15 cells with pGM-CSF (A1); control was made by means of transfected pK15 cells with pcDNA 3.1 (C1) and values were blanked against the transfection control (dotted columns); 1/10th dilution of A1 and C1 was applied to the cells A1/10 and C1/10, respectively. Results shown are the mean ± SD of triplicates. GM-CSF, granulocyte-macrophage colony-stimulating factor; rhGM-CSF, human recombinant GM-CSF.

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**Figure 4** Antibody response of PCV2 DNA-transfected pigs to recombinant PCV2 ORF-2 protein ELISA. Sera of immunostimulated and non-immunostimulated transfected piglets and control piglets were collected during the kinetic analyses. The immunoreactivity of these sera against ORF-2 PCV2 recombinant protein was determined by means of ELISA at dilution 100. The results have been expressed as the mean OD ratio obtained between ORF-2-GST and GST alone for eight piglets in each group. PCV2, porcine circovirus type-2.
PCV2 GECN per millilitre of sera for blood samples. The maximum load for the transfected pigs was found in the mesenteric lymph node at 35 days post-injection and corresponded to $5.54 \times 10^{11}$ GECN per gram for the non-immunostimulated pigs and to $1.24 \times 10^{12}$ GECN per gram for the immunostimulated pigs. The viral genome titre was lower in the sera than that in the other tissues, and was $3.35 \times 10^{7}$ GECN per millilitre of sera for both immunostimulated and non-immunostimulated groups at 14 days post-transfection. Differences between the two groups were not significant ($P > 0.05$). The pBluescript-specific primers were unable to detect injected plasmid, thereby confirming that the quantified viral genomes were initiated after viral replication.

Figure 5 Quantification of PCV2 viral DNA present in blood samples. The graph represents the dynamic titration of PCV2 genome in immunostimulated and non-immunostimulated pigs. Eight pigs were used for each condition and data shown are the mean ± SD. PCV2, porcine circovirus type-2.

Figure 6 Results of the PCV2 genomic load by means of TaqMan PCR assay. The graph represents the mean titration in kinetics of the viral genome present in seven organs (the lung, the tonsil, the ileum and the bronchial, mesenteric, inguinal and right axial lymph nodes) as the mean value ± SD of triplicates observed in these organs from non-immunostimulated pigs and immunostimulated pigs. One pig was tested for each condition at day 4, 8, 12 and 19 and eight pigs for day 35. PCV2, porcine circovirus type-2.

Figure 7 Results of the viral infectious particles in the upper-right axial (a) and bronchial (b) lymph nodes. The infectious viral titre was obtained by means of IPMA and the TCID-50 was calculated per gram of tissue. White histograms represent immunostimulated pigs and hatched ones the non-immunostimulated pigs. IPMA, immunoperoxidase monolayer assay.
Titration of infectious particles by means of IPMA in the bronchial lymph node and the upper-right lymph node of transfected pigs

Viral particles were quantified in the upper-right axial lymph node (Figure 7a) and the bronchial lymph node (Figure 7b) of both immunostimulated and non-immunostimulated pigs. A fairly high number of viruses were present in the organs, corresponding to a maximum titre of $1.10^7.6$ TCID-50 per gram in the bronchial lymph node and to $1.10^5.6$ TCID-50 per gram in the upper-right lymph node for the non-immunostimulated pigs and to $1.10^5.2$ TCID-50 per gram in the bronchial lymph node and to $1.10^4.8$ TCID-50 per gram in the upper-right lymph node for the immunostimulated pigs. However, the titre of viral particles was lower than that of the genome in the organs at the matching time post-transfection.

Molecular characterization of extracted genome from transfected pig organs

DNA extracted from the inguinal lymph node of one immunostimulated pig 35 days post-transfection (group-2) was used as a template to determine the sequence of the viral genome generated by means of the inoculation of the cloned genomic PCV2 plasmid. This sequence was found to be identical to the initial molecular cloned sequence (EMBL sequence AJ3306).

Discussion

It is, generally, accepted that PMWS is a multifactorial disease and that the host immune system plays a major role in disease manifestation. The experimental reproduction of severe PMWS is not easy, nor is the production of a high titre of a biologically pure form of infectious PCV2. Successful in vivo infection of young pigs with a molecular PCV2 DNA has been reported by Fenaux et al. (2002). In this later study, various routes of injection were used; the PCV2 genomic DNA was injected directly into the liver and the superficial iliac lymph node leading to the observation that same PMWS histopathological lesions characteristic of PMWS were observed when infectious DNA or biologically pure PCV2 viral particles were used, but failed to produce the characteristic clinical signs of PMWS. Therefore, endotoxin-free grade DNA and SPF pigs might provide viral cloned progeny and a method of choice for investigation of the factors and co-factors that trigger the clinical symptoms of PMWS. In addition, infectious PCV2 DNA and chimeric PCV2 and PCV1 DNA have successfully been used in order to investigate immunogenicity and pathogenicity of plasmid containing PCV2 and PCV1 DNA (Fenaux et al. 2000; Fenaux et al. 2004).

We have demonstrated that an in vitro transfection of PK15 cells and in vivo transfection of piglets by means of intramuscular route with the full tandem-cloned PCV2 DNA produced rapidly infectious virus. Viral genomes and infectious virus were detected in all the other tissues examined, reaching a peak at 2 weeks and then remaining essentially stable during the observation period of 35 days. Although high levels of PCV2 genome copies and infectious particles were present in all the investigated organs, no clinical signs or gross lesions were detected whether pigs had been co-injected with GM-CSF encoding DNA or had received PCV2 DNA alone. These results slightly differ from those obtained by Fenaux et al. (2002), as they were able to detect gross lesions in in vivo transfected pigs. Similar amount of plasmid DNA was injected and the only differences were the route of inoculation and the PCV2 strain used. These authors used these inoculation routes, as they represent a direct way to reach the lymph nodes and the liver of the pigs where PCV2 is thought to replicate (Choi & Chae 2000; Rosell et al. 2000) and might lead to various levels of viruses within these organs and on apparition of gross lesions. Moreover, various PCV2 strains may vary in replication rate and may have influence on the level of viruses within these organs and on apparition of gross lesions, although variability on the ORF-1 sequence encoding for the replicate between strains were found minimal (de Boisseson et al. 2004).

A lag phase could be expected in virus propagation because of the use of infectious DNA, and representing the aptitude of the DNA to transfect cells, the rate of recombination required to generate the double-stranded intermediate of the viral replication genome and finally the time to obtain a high level of viral particles in the whole organism. However, the seroconversion profile in transfected pigs is similar to that observed after a virus inoculation (Blanchard et al. 2003b) and the time course of DNA propagation suggests that infectious particles are quickly generated, as PCV2 was able to rapidly colonize most organs of the pigs within 2 weeks. Both DNA propagation and the seroconversion profile suggest that the lag phase might be shorter than that initially expected. This short lag phase might be because of the high amount of DNA that was used for transfection leading to dissemination in the body of a high level of transfected cells and rapidly resulting in a high level of viral particle progeny.

It is unlikely that the observed lack of pathogenicity in this assay can be attributed to an attenuation of the PCV2 genome, as no mutation was present. A higher-genome prevalence or a higher titre of virus particles might be required to jeopardize the health status of 5-week-old piglets. However, a correlation
between the PCV2 DNA load and disease development has been demonstrated (Meehan et al. 2001; Sibila et al. 2003; Olvera et al. 2004; Blanchard et al. 2004). A genomic load of $1.10^{11}$–$1.10^{13}$ GECN per gram of lymph node was associated with mild to severe PMWS, when SPF piglets were inoculated with a tissue homogenate obtained from infected pigs (Blanchard et al. 2004; Meehan et al. 2001). The histopathological PMWS lesions observed in tissue samples from conventional pigs with PMWS were significantly correlated with the PCV2 GECN (Blanchard et al. 2004; Sibila et al. 2003; Olvera et al. 2004) and high genomic load was strongly correlated with the appearance of histopathological PMWS lesions. The amount of virus measured in some pigs with clinical signs and mild PMWS corresponded at least to a genomic load of $1.24\times10^{12}$ and $1.10^{9}$ infectious particles (Meehan et al. 2001; Olvera et al. 2004; Blanchard et al. 2004).

For the first time, this study has shown that pigs can host a high viral load and has not shown obvious clinical signs. This might be because of the high purity of the DNA used for transfection, thus emphasizing the importance of co-factors in the development of clinical signs. Surprisingly, high viral replication was observed in non-immunostimulated pigs, suggesting that the viral genome might be able to replicate at a high level without the need for any co-factors. Thus, the host immune system and co-factors might be determinant in developing clinical PMWS but without having a direct influence on viral replication itself.

Unexpectedly, a GM-CSF DNA injection had no effect on the appearance of the disease or on viral replication. Activity of the plasmid encoding GM-CSF in stimulating the pig’s immune response has been checked in our laboratory and in the laboratories of others (Somasundaram et al. 1999; Dufour et al. 2000). The co-delivery of pGM-CSF significantly increases the levels of interferon-γ, interleukin-2 (IL-2) and IL-4 mRNA.

In conclusion, this study proposes a model, eloquent by its simplicity, to assess the impact of viral load and the additional factors required for inducing PMWS. Infectious DNA was shown effective in replicating and colonizing various tissues when directly injected by means of intramuscular route. However, despite the presence of a high level of virus particles and a viral DNA load, the transfected pigs were not ill, implying that co-factors or other pathogenic are crucial in clinical PMWS produced in response to PCV2. The main advantage of using infectious DNA lies in the elimination of problems associated with the presence of other indigenous swine pathogens and/or cytokines or active molecules in a tissue homogenate inoculate. The co-injection of DNA encoding a pleiotropic cytokine (GM-CSF) failed to enhance viral replication and appearance of the disease and suggests that immunostimulation does not seem to play a role in PMWS under those circumstances, also role of macrophages to support viral replication after cellular stimulation is not verified in this model.

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References


