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Immunisation of pigs with a major envelope protein sub-unit vaccine against porcine reproductive and respiratory syndrome virus (PRRSV) results in enhanced clinical disease following experimental challenge

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ABSTRACT

Disease exacerbation was observed in pigs challenged with virulent porcine reproductive and respiratory syndrome virus (PRRSV) following immunisation with a recombinant GP5 sub-unit PRRSV vaccine (rGP5) produced in *E. coli*. Eighteen animals were divided into three experimental groups: group A were immunised twice IM with rGP5, 21 days apart; group B acted as positive controls (challenged but not immunised); and group C were negative controls. Pigs in groups A and B were challenged 21 days after the second immunisation of the group A animals. Following challenge, three pigs given rGP5 exhibited more severe clinical signs than the positive controls, including respiratory distress and progressive weight-loss. Although not statistically significant, the more severe disease exhibited by group A animals may suggest previous immunisation as a contributory factor. The mechanisms of these findings remain unclear and no association could be established between the severity of disease, non-neutralising antibody concentrations and tissue viral loads.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is characterised by reproductive failure in sows and respiratory distress in pigs of all ages (Rossow, 1998). The causative agent, PRRS virus (PRRSV), is a small, enveloped, RNA virus classified within the *Arteriviridae* family (Cavanagh, 1997). The viral genome consists of 15 kb long, linear, polyadenylated RNA that encodes nine open reading frames (ORFs). ORFs 1a and 1b encode proteins with replicase and polymerase activities, while ORFs 2–7 encode virus-associated proteins, of which the glycosylated protein GP5, encoded by ORF 5, is a major component of the virus envelope (Meulenberg et al., 1995). GP5 is associated with the development of neutralising antibodies and host protection (Gonin et al., 1999; Meulenberg, 2000; Ostrowski et al., 2002; Plagemann, 2004a,b) and has been a key target in the design of novel vaccines that overcome safety problems associated with modified live vaccines (Bøtner et al., 1997; Mengeling et al., 1999) and the limited efficacy of inactivated vaccines (Nielsen et al., 1997; Prieto et al., 1997; Scortti et al., 2007).

Experimental vaccines that express native or modified GP5, either alone or in combination with protein M, have been developed, including DNA vaccines (Pirzadeh and Dea, 1998; Kwang et al., 1999; Barfoed et al., 2004; Xue et al., 2004; Fang et al., 2006; Jiang et al., 2006), bacterial vaccines such as *Mycobacterium bovis* BCG or *E. coli* that express recombinant proteins (Pirzadeh and Dea, 1998; Bastos et al., 2004), recombinant viral vaccines such as baculovirus (Plana-Durán et al., 1997), pseudorabies virus (Qiu et al., 2005; Jiang et al., 2007b) and adenovirus (Gagnon et al., 2003; Kheyar et al., 2005; Jiang et al., 2007a), and, more recently, replicon-based vaccines (Mogler et al., 2008; Jiang et al., 2009). However, despite the development of a large number of vaccine candidates, studies in mice or pigs have indicated that their use results in limited protection with, at best, partial reductions in viraemia and tissue viral loads. This lack of protection has been attributed to the induction of weak immune responses, which are usually insufficient to prevent infection after viral challenge, especially where this challenge is heterogeneous in character.

Although the vaccines developed to date have demonstrated little protective efficacy, the only report of an adverse effect was increased lesion severity in infected pigs that had been immunised with *E. coli* GST-ORF5 recombinant fusion protein (Pirzadeh and Dea, 1998). Adverse effects have occasionally been found following the use of other genetically engineered viral vaccines including those against equine infectious anaemia (Wang et al., 1994), herpes simplex-1 (Ghiasia et al., 1999) and influenza (Heinen et al., 2002) viruses. The present study reports disease exacerbation in

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5710 (Spain 2/1991)    ATGAGATGTTCTCACAAATTGGAGCGTTTCTTGACTCCTCACTCTTGCTTCTGGTGGCTT 60
Spain 6/1992           ATGAGATGTTCTCACAAATTGGAGCGTTTCTTGACTCCTCACTCTTGCTTCTGGTGGCCT 60
                       ************************************************************ *

5710 (Spain 2/1991)    TTTTTGCTGTGTACCGGCTTGTCTTGGTCCTTTGTCGATGGCAACGACAGCAGCTCGACA 120
Spain 6/1992           TTTTTGCTGTGTACCGGCTTGTCTTGGTCCTTTGTCGATGGCGACGACAACAGCTCGACA 120
                       ***************************************** ****** **********

5710 (Spain 2/1991)    TACCAATACATATATAATTTGACGATATGCGAGCTGAATGGGACCGAATGGTTGTCCAGC 180
Spain 6/1992           TACCAATACATATATAATTTGACGATATGCGAGCTGAATGGGACCAATTGGTTGTCCAGC 180
                       ********************************************* * ************

5710 (Spain 2/1991)    CATTTTGACTGGGCAGTCGAGACCTTTGTGCTTTACCCGGTTGCCACTCATATCCTTTCA 240
Spain 6/1992           CATTTTGACTGGGCAGTCGAGACCTTTGTGCTTTACCCGGTTGCCACTCATATCCTTTCA 240
                       ************************************************************

5710 (Spain 2/1991)    CTGGGTTTTCTCACAACAAGCCATTTTTTTGATGCGCTCGGTCTCGGCGCTGTGTCCACT 300
Spain 6/1992           CTGGGTTTTCTCACAACAAGCCATTTTTTTGATGCGCTCGGTCTTGGCGCTGTGTCCATT 300
                       ******************************************* ************ *

5710 (Spain 2/1991)    ACAGGATTTGTTGGCGGGCGGTATGTACTCAGCAGCGTGTACGGCGCTTGTGCTTTCGCA 360
Spain 6/1992           ACAGGATTTGTTGGCGGGCGGTATGTACTCAGCAGCATGTACGGCGCTTGTGCTTTCGCA 360
                       ********************************** ***********************

5710 (Spain 2/1991)    GCGCTCGTATGTTTTGTCATCCGCGCTGCTAAAAATTGCATGGCTTGCCGTTATGCCCGT 420
Spain 6/1992           GCGCTCGTATGTTTTGTCATCCGTGCTGCTAAAAATTGCATGGCTTGCCGTTATGCCCGT 420
                       ********************** ***********************************

5710 (Spain 2/1991)    ACCCGGTTTACCAACTTCATTGTGGACGACCGGGGGAGGATCCATCGATGGAAGTCTCCA 480
Spain 6/1992           ACCCGGTTTACTAACTTCATTGTGGACGACCGGGGGAGGATCCATCGATGGAAGTCTCCA 480
                       *********** ************************************************

5710 (Spain 2/1991)    ATAGTGGTAGAGAAATTGGGCAAAGCTGAAGTCGGTGGCGACCTCGTCACCATCAAACAT 540
Spain 6/1992           ATAGTGGTAGAGAAATTGGGCAAANCNGAAGTCGGTGGCGACCTCGTCACCATTAAACAT 540
                       ********************** * ************************** ******

5710 (Spain 2/1991)    GTCGTCCTCGAAGGGGTTAAAGCTCAACCCTTGACGAGGACTTCGGCTGAGCAATGGGAA 600
Spain 6/1992           GTCGTCCTGGAAGGGGTTAAAGCTCAACCCTTGACGAGGACTTCGGCCGAGCAATGGGAA 600
                       ******** ************************************* ************

5710 (Spain 2/1991)    GCCTAG 606
Spain 6/1992           GCCTAG 606
                       ******
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Fig. 1. Alignment of ORF5 sequences of the porcine reproductive and respiratory syndrome virus strains used in the immunisation (Sp-6) and challenge (5710) of the pigs.

pigs, previously immunised with a PRRSV GP5 sub-unit vaccine produced in *E. coli*, following challenge with PRRSV.

Materials and methods

Animal selection

All experimental procedures were approved by The Animal Ethics Committee of The Universidad Complutense de Madrid.

Eighteen 3-week-old cross-bred piglets from a PRRSV-seronegative herd were randomly divided into three groups of six and housed in isolation in pens with concrete floors and an automatic watering system.

Expression and purification of recombinant GP5 protein

The ORF5-encoding region of PRRSV was amplified by RT-PCR (Suárez et al., 1994), prior to cloning in the commercial plasmid pRSET-A (Invitrogen) to generate a pR-PR5 plasmid, which was then used to express the recombinant GP5 protein (rGP5) in *E. coli*.

BL21(DE_3)pLysS competent *E. coli* cells were transfected with pR-PR5 and exponential cultures of transformed bacteria were induced at an optical density ($OD_{600\ nm}$) of 0.6 by adding 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) to the culture media. After 4 h of culture, the cells were lysed and separated by 15% SDS–PAGE. The band corresponding to rGP5 was excised from the gel, electroeluted and the nature of the eluted protein determined by SDS–PAGE analysis followed by Western blotting using hyperimmune PRRSV-specific porcine serum (α-PRRS). The protein concentration in the purified antigen preparation was determined by spectrophotometry.

Virus preparation and culture

The sixth passage in porcine alveolar macrophages (PAM) of strain Spain 6/1992 was used as a template for amplification of the ORF5-encoding region of PRRSV. This strain belongs to the Lelystad-like cluster of type 1 PRRSV. Experimental challenge of immunised pigs was carried out with the seventh passage of field strain 5710 in PAM, which also belongs to the Lelystad-like cluster of European strains (Suárez et al., 1996; Forsberg et al., 2002). The nucleotide sequence of ORF5 from strain Spain 6/1992 (deposited in GenBank with accession number DQ 345733) is 97.50% identical to that of strain 5710 (deposited in GenBank with accession number DQ 345729 under the name Spain 2/1991). The predicted amino acid sequence of the corresponding GP5 protein is 96.52% identical to that of strain 5710. The alignment of the nucleotide sequences of both PRRSV strains is illustrated in Fig. 1. Samples collected after viral challenge were evaluated using PAM cultures (Prieto et al., 1997). Serum neutralisation (SN) assays were performed on MARC-145, a cell clone highly permissive for PRRSV derived from the MA-104 cell line (Kim et al., 1993).

Table 1
Scores assigned to clinical signs during evaluation of pigs following their immunisation with a porcine reproductive and respiratory syndrome virus (PRRSV) GP5 sub-unit vaccine and/or challenge with PRRSV.

Category of clinical sign	Range	Score
Systemic	Normal	0
	Apathy	10
	Anorexia	20
	Lethargy	30
Cutaneous	Normal	0
	Cyanosis	10
Respiratory	Normal	0
	Sneezing	10
	Coughing	10
	Nasal secretion	10
	Laboured breathing	20
	Abdominal breathing	30
Respiratory frequency	Normal	0
	Mild tachypnoea	10
	Severe tachypnoea	20

Experimental design and sample collection

The study had three experimental groups: in group A six pigs (numbered 1–6) were immunised twice IM, once 42 days prior to challenge (day − 42) with 600 μg of rGP5 in 2 mL of incomplete Freund adjuvant and once 21 days prior to the experimental inoculation (day − 21) with 300 μg of rGP5 in 2 mL of incomplete Freund adjuvant. The first immunisation dose was given when the pigs were 28 days old, following an acclimatisation period of 7 days; the six pigs in group B (numbered 7–12) were positive controls and were thus experimentally challenged but not vaccinated; the group C pigs (numbered 13–18) were negative controls and were neither vaccinated nor challenged.

Three weeks following the second immunisation, pigs from groups A and B were inoculated intranasally with 5 mL of PAM culture lysates containing 10^5 tissue culture infectious doses 50 ($TCID_{50}$) of PRRSV strain 5710. Pigs from group C were inoculated intranasally on the same day with 5 mL of an uninfected PAM culture lysate.

Clinical signs and food intake were evaluated daily for each pig following vaccination and experimental inoculation, respectively. Clinical signs were graded using a scoring system adapted from Álvarez et al. (2008). Signs evaluated included lethargy, anorexia, skin discolouration, sneezing, coughing, laboured and abdominal breathing and respiratory rate (Table 1). Serum samples were collected from pigs on the day of each vaccination and at various time-points post-vaccination (p.v.) and post-challenge (p.c.). Serum was stored at −80 °C until used for virus isolation and to determine PRRSV antibody titres. Pigs were euthanased 18 days after virus inoculation and examined post mortem. Samples of lung, tonsil, liver, kidney, spleen and of submandibular, retropharyngeal and superficial inguinal lymph nodes were collected and stored at −80 °C until used for virus isolation.

Virus isolation and titrations

Samples were processed as previously described (Prieto et al., 1997) and were inoculated onto PAM monolayers in duplicate. Cells were incubated for 90 min at 37 °C to facilitate adsorption. The monolayers were then washed twice with Dulbecco's modified Eagle's medium (DMEM) and fresh DMEM supplemented with 10% fetal bovine serum (FBS) was added.

The cells were incubated for 6 days at 37 °C in a humidified atmosphere containing 5% CO_2. Strain 5710 was added to DMEM to final concentrations of 10^4, 10^3 and 10^2 $TCID_{50}$/mL (i.e. 10^3, 10^2 and 10 $TCID_{50}$/well) as positive controls. Only batches of PAM with a minimum sensitivity to infection of >50% of the wells to which 10 $TCID_{50}$ was introduced were used. Virus-free DMEM or FBS were used as negative controls. The presence of a cytopathic effect (CPE) characteristic of PRRSV was determined on days 4–6 post-inoculation. If a CPE was observed, RT-PCR was carried out to confirm the presence of PRRSV (Suárez et al., 1994). Viral titres were determined as described by Scortti et al. (2007), and calculated as described by Reed and Muench (1938) and were expressed as $TCID_{50}$/g (for tissue samples) or $TCID_{50}$/mL (for serum or fluid samples).

Serological examination

Serum samples were examined for PRRSV-specific antibodies using a commercial ELISA (CIVTEST-suis PRRS, Hipra Laboratories). Serum samples collected on days pigs were immunised (days – 42 and – 21), challenged (day 0), and at experiment termination (day 18), were tested using a neutralisation assay performed in MARC-145 cultures (Yoon et al., 1994).

Histopathological examination

Tissue samples collected post mortem were fixed in 10% neutral buffered formalin and dispatched to Dr. J. Segales at The Centre de Recerca en Sanitat Animal (Barcelona, Spain) for histopathological examination and determination of porcine circovirus type 2 (PCV-2) infection status.

Tissues were paraffin-embedded and 4 μm thick sections were cut and stained using haematoxylin and eosin. The potential presence of PCV-2 was evaluated using in situ hybridisation (Rosell et al., 1999), and the possibility of secondary bacterial pulmonary infection assessed using routine bacteriological culture.

Statistical analysis

The occurrence of clinical signs and mean daily weight gains over the entire period p.c. were evaluated for significance using Kruskal–Wallis' non-parametric and Dunn's multiple comparison tests. A Student's *t* test was used to assess significance in differences in rectal temperature prior to and after challenge. Differences in viral and neutralising antibody titres at each sampling point were analysed using a one-way analysis of variance and Duncan's multiple range test. The clinical signs and viral titres recorded for each animal were converted to an approximate area under the curve (AUC) using the trapezoidal rule (Hennen, 2003). AUC was computed from the day of challenge to the end of the experiment (day 18). In the case of clinical signs, the resulting data were compared using Kruskal–Wallis' non-parametric and Dunn's multiple comparison test. AUC values for viraemia were compared using a one-way analysis of variance and Duncan's multiple range tests. All tests were carried out using SPSS software and results were considered statistically significant when $P < 0.05$.

Results

Clinical signs

No adverse reactions to immunisation were observed and pigs from all experimental groups remained clinically normal until virus inoculation, at which point moderate to severe clinical signs were exhibited by animals in groups A and B. Clinical signs were more severe in pigs in group A than in the positive controls (B) (Fig. 2). The highest mean clinical score was recorded for pigs from group A between days 2 and 9 p.c., with three pigs exhibiting severe clinical signs including lethargy, anorexia, mild tachypnoea and laboured breathing. Pig numbers 1, 5 and 6 presented with these signs on days 2–10, 2–7, and 3–7 p.c., respectively.

The most striking sign was severe weight loss, which resulted in significantly lower daily weight gain in group A relative to the other two groups (Fig. 3). The significant decrease in daily weight

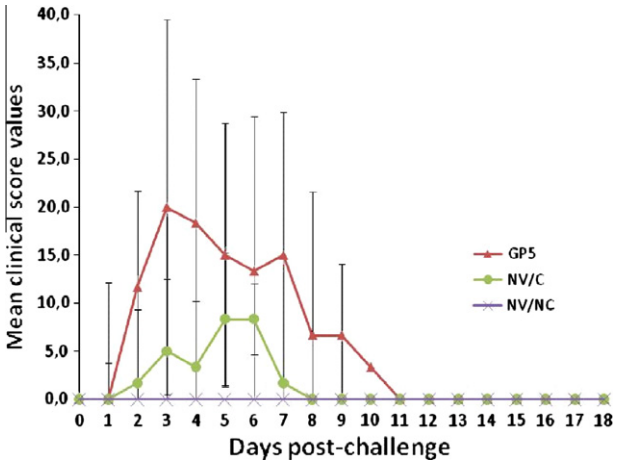

Fig. 2. Graph illustrating the mean clinical scores for each experimental group following experimental challenge with porcine reproductive and respiratory syndrome virus. Red triangles GP5, group A (vaccinated with GP5 protein and challenged); green circles NV/C, group B (challenged only); mauve crosses NV/NC, group C (not vaccinated or challenged).

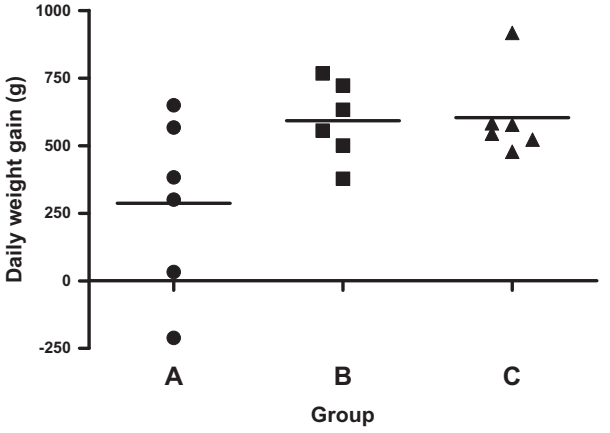

Fig. 3. Graph illustrating daily weight gain of pigs in groups A (vaccinated with GP5 protein and challenged), B (challenged only), and C (not vaccinated or challenged) following experimental challenge with porcine reproductive and respiratory syndrome virus.

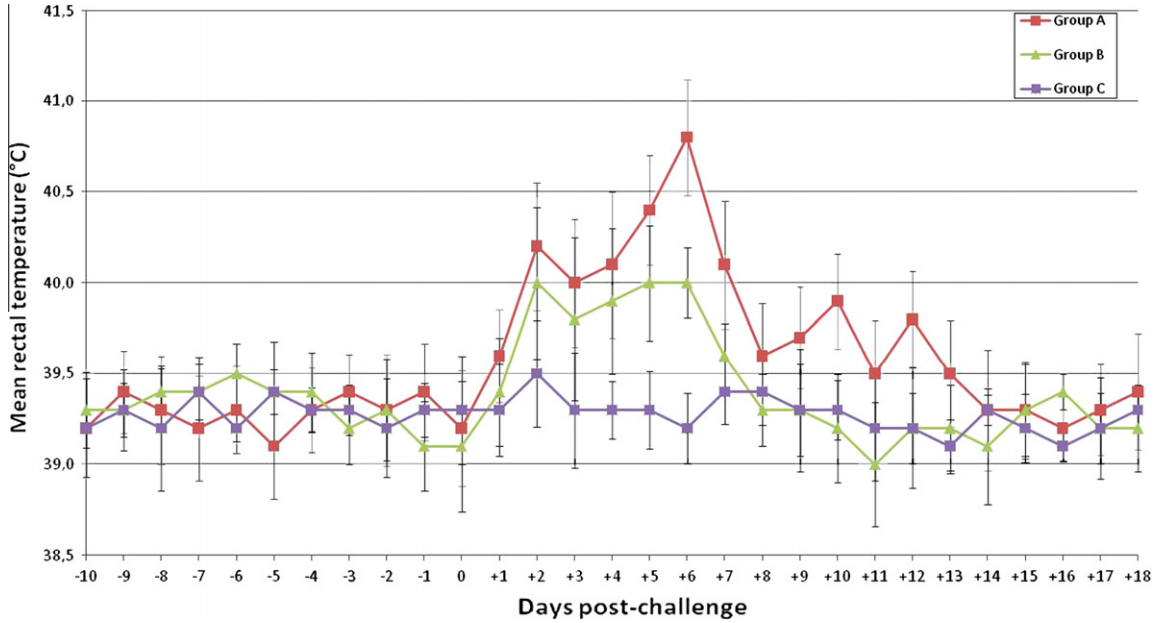

Fig. 4. Graph illustrating the mean rectal temperature (°C) for each experimental group from 10 days before to 18 days post-challenge with porcine reproductive and respiratory syndrome virus. Red squares, group A (vaccinated with GP5 protein and challenged); green triangles, group B (challenged only); mauve squares, group C (not vaccinated or challenged).

gain observed in group A was mostly due to the effect on pig 1, which lost 211 g/day, and pig 5, which gained only 33 g/day p.c. However, due to large individual variations in both clinical scores and daily weight gains within each group, differences were not found to be statistically significant. Pigs in groups A and B developed pyrexia (defined as rectal body temperature $\geqslant$39.7 °C) for at least one day p.c. and mean body temperatures correlated with clinical scores (Fig. 4).

Detection of PRRSV

The results of virus isolation from serum samples collected p.c. are detailed in Table 2. All pigs in groups A and B were viraemic from day 2 p.c. to the end of the experiment, with the exception of pig 1, which was negative on day 18. No virus was found in samples collected from pigs in group C. There was no statistically significant difference in the AUCs between groups A and B and, as would be anticipated, pigs in both challenged groups had statistically significantly higher AUC values than group C pigs ($P \leqslant 0.05$). Differences in mean viral titres in serum samples from groups A and B had statistical significance on day 12 p.c. only. The results of virus isolation from tissue samples are summarised in Table 3. PRRSV was detected in at least one tissue sample from all pigs in groups A and B. No virus was recovered from tissue samples from group C pigs and no statistically significant differences were found between groups A and B in terms of virus tissue distribution or load.

Table 2
Results of virus isolation from porcine serum samples collected post-challenge with porcine reproductive and respiratory syndrome virus in groups A (vaccinated with GP5 protein and challenged), B (challenged only), and C (not vaccinated or challenged). Positive results represent infectivity titres ($\log_{10}$ $TCID_{50}$/mL). – No virus recovered.

Group	Pig number	Days post-challenge				
		2	5	8	12	18
A	1	+(4.0)	+(3.9)	+(3.7)	+(3.8)	–
	2	+(3.8)	+(4.0)	+(3.0)	+(3.5)	+(2.0)
	3	+(3.0)	+(3.9)	+(2.0)	+(3.0)	+(3.0)
	4	+(3.5)	+(4.0)	+(3.0)	+(2.5)	+(2.0)
	5	+(3.8)	+(3.8)	+(3.5)	+(3.6)	+(3.5)
	6	+(3.9)	+(4.0)	+(2.5)	+(4.0)	+(2.5)
B	7	+(3.7)	+(4.0)	+(3.5)	+(3.6)	+(2.5)
	8	+(3.9)	+(3.6)	+(3.8)	+(2.5)	+(3.0)
	9	+(3.8)	+(3.0)	+(3.7)	+(3.0)	+(2.5)
	10	+(3.6)	+(3.5)	+(3.5)	+(2.0)	+(3.0)
	11	+(3.5)	+(3.8)	+(3.0)	+(2.0)	+(3.5)
	12	+(3.0)	+(3.0)	+(3.8)	+(1.0)	+(1.0)
C	13	–	–	–	–	–
	14	–	–	–	–	–
	15	–	–	–	–	–
	16	–	–	–	–	–
	17	–	–	–	–	–
	18	–	–	–	–	–

Serological findings

ELISA and SN tests were negative for all serum samples from all pigs prior to immunisation. On day of challenge, 2/6 pigs in group A had seroconverted as detected by ELISA, although neither had neutralising antibodies. All pigs in groups B and C remained seronegative until challenge. Post-challenge, group B pigs were found to have seroconverted by ELISA and increased antibody titres were noted in pigs from group A. Statistically significantly higher titres were found in group A relative to group B animals at day 18 p.c. ($P < 0.05$). No statistically significant differences were observed between groups A and B in the SN response p.c. (data not shown).

Post mortem findings

Group C pigs did not exhibit macroscopic or microscopic lesions. Group B animals had mild gross lesions including moderate lymph node enlargement and congestion, especially of the mediastinal and mesenteric nodes. No significant microscopic lesions were recorded in these pigs. Similar moderate lymph node enlargement and congestion was observed in three of the pigs from group A. A moderate, sub-acute interstitial pneumonia characterised by alveolar septal thickening and mononuclear cell infiltration was

Table 3
Results of virus isolation from porcine tissue samples collected at necropsy 18 days post-challenge with porcine reproductive and respiratory syndrome virus in groups A (vaccinated with GP5 protein and challenged), B (challenged only), and C (not vaccinated or challenged). Positive results represent infectivity titres (log_{10} $TCID_{50}$/mL). – No virus recovered.

Group	Pig number	RLN[a]	SLN[b]	SILN[c]	Tonsil	Kidney	Spleen	Liver	Lung
A	1	+(2.8)	+(2.5)	+(2.0)	+(3.2)	–	+(2.0)	–	+(3.8)
	2	–	–	–	+(2.8)	–	–	–	–
	3	+(3.2)	+(2.8)	–	+(3.4)	–	–	–	+(3.5)
	4	–	+(2.0)	–	+(2.5)	–	–	–	+(3.5)
	5	+(2.5)	+(2.4)	–	+(2.8)	–	–	–	–
	6	+(3.0)	+(2.6)	–	+(3.5)	–	–	–	+(2.8)
B	7	+(2.6)	+(2.9)	–	+(3.4)	–	–	–	+(3.1)
	8	+(3.1)	+(2.7)	+(2.2)	+(3.1)	–	–	–	+(3.4)
	9	–	–	–	+(2.6)	–	–	–	+(2.9)
	10	+(3.3)	+(2.2)	–	–	–	–	–	–
	11	+(2.5)	+(2.3)	–	+(3.0)	–	–	–	–
	12	+(2.8)	–	–	+(2.8)	–	–	–	–
C	13	–	–	–	–	–	–	–	–
	14	–	–	–	–	–	–	–	–
	15	–	–	–	–	–	–	–	–
	16	–	–	–	–	–	–	–	–
	17	–	–	–	–	–	–	–	–
	18	–	–	–	–	–	–	–	–

[a] RLN, retropharyngeal lymph node.
[b] SLN, submandibular lymph node.
[c] SILN, superficial inguinal lymph node.

observed microscopically in three of the group A pigs (numbers 3, 5 and 6). There was no microscopic evidence of PCV-2 infection and PCV-2 was not detected by in situ hybridisation.

Discussion

The development of safe and effective vaccines against PRRSV infection remains a challenge and a significant priority of the pig production industry. Several promising candidates have failed to deliver effective protection likely due to the fact that even the virus itself, elicits a weak, strain-specific, immune response (Lager et al., 1997a,b; Prieto et al., 2008).

Given that some strains of PRRSV have a greater potential to induce neutralising, cross-reacting antibodies that others (Martínez-Lobo et al., 2008), we set out to assess if a sub-unit vaccine, based on the GP5 protein from one of those PRRSV strains, might induce such protection. However, no protection against a closely related, virulent PRRSV strain was observed in the pigs immunised with this protein, as demonstrated by the induced clinical signs and tissue viral loads p.c.

The lack of protection afforded by vaccines against PRRSV has been attributed to their inability to elicit a robust immune response and to the significant antigenic diversity of the virus (Lager et al., 1997a,b; Prieto et al., 2008). Given that it is possible that neutralising antibodies play a role in the development of protective immunity (Osorio et al., 2002), it was interesting to note that none of the vaccinated animals in the present study developed detectable levels of such antibodies prior to challenge, and only two pigs had developed specific antibodies by day 42 p.v. Somewhat unexpectedly, more severe clinical signs, including respiratory distress and anorexia, were encountered in more of the vaccinated than in the positive control animals. Given this clinical presentation it was important to rule out confounding infection with PCV-2 (Quintana et al., 2001).

Vaccine-enhanced disease has been reported for numerous enveloped viruses, including flaviviruses, alphaviruses, poxviruses, bunyaviruses, rhabdoviruses, coronaviruses, herpesviruses and reoviruses (Porterfield, 1986; Burke, 1992). One of the most commonly proposed mechanisms to explain such an adverse effect is antibody-dependent enhancement (ADE) of virus replication, where non-neutralising antibodies induced by immunisation bind virus and enhance target cell infection, particularly of monocytes and macrophages. Such 'enhancing' antibodies have been demonstrated in the serum of PRRSV-infected pigs (Yoon et al., 1996), as well as in HIV-infected humans (Homsy et al., 1989), SIV-infected macaques (Montefiori et al., 1990), Visna virus-infected sheep (Jolly et al., 1989), and caprine arthritis–encephalitis virus-infected goats (McGuire et al., 1986). Furthermore, non-neutralising, GP5-specific IgG against PRRSV has also been incriminated in increasing the level and duration of PRRSV viraemia (Yoon et al., 1996). Pirzadeh and Dea (1998), found more severe lesions in pigs challenged with a wild-type strain of PRRSV, that had been vaccinated with an *E. coli* GST-ORF5 recombinant fusion protein, and that had developed non-neutralising antibodies against GP5 at the time of challenge, than in non-immunised controls.

In the present study, although non-neutralising antibodies were demonstrated in some animals that had seroconverted before challenge and that did not have detectable levels of neutralising antibodies, a correlation between this antibody level and more severe disease p.c. was not established. Only 1/2 of the immunised pigs that developed non-neutralising antibodies before challenge exhibited enhanced clinical signs, a finding inconsistent with ADE. Although a definitive association between antibody reactivity and enhancement of disease cannot always be established (Raabe et al., 1998), in most cases affected animals have measurable levels of non-neutralising antibodies at the time of infection.

The typical correlation between severity of clinical signs and virus burden observed in ADE of disease was not found in this study. Although the two animals that were most severely affected had persistently high viral titres, other pigs exhibiting mild clinical signs also had a consistently high viraemia during most of the experimental period. However, it must also be considered that while high levels of virus replication are necessary, they are ultimately an insufficient component of ADE (Raabe et al., 1998).

Other immunopathological mechanisms such as T cell responses may be at play in vaccine-induced disease exacerbation, as described for both influenza (Heinen et al., 2002) and respiratory syncytial (Matsuda et al., 1995) viruses. Excess production of pro-inflammatory cytokines, especially interferon-α, tumour

necrosis factor (TNF)-α and interleukin-1, has been correlated with lung pathology in the case of a number of viral infections (van Reeth and Nauwynck, 2000). Interestingly, TNF-α production is linked to weight loss and systemic disease in the case of both respiratory syncytial and influenza virus infection (Hussell et al., 2001). Th-2 cytokine responses have also been implicated in immunopathological processes associated with bovine and human respiratory syncytial virus and Coxsackie virus infection (Boelen et al., 2000; Kishimoto et al., 2001; Kalina et al., 2004). Autoimmunity could also be involved as piglets infected with PRRSV can develop severe hypergammaglobulinaemia and lymph adenopathy (Butler et al., 2008). Further work will be required to assess if any of the above mechanisms are involved in the development of the adverse events described in the current study.

The type of immunogen used in our study may have contributed to disease enhancement as Pirzadeh and Dea (1998), also using a recombinant fusion GP5 protein expressed in *E. coli*, found more severe lesions microscopically in immunised animals. The adverse effects of immunisation we report here have not previously been found in vaccination studies using either whole virus or sub-unit PRRSV proteins (Gagnon et al., 2003; Barfoed et al., 2004; Bastos et al., 2004; Xue et al., 2004; Kheyar et al., 2005; Qiu et al., 2005; Scortti et al., 2006, 2007; Jiang et al., 2007a,b; Prieto et al., 2008).

Conclusions

The results of this study demonstrate that immunisation of growing pigs with a recombinant fusion GP5 PRRSV protein not only failed to provide protection from subsequent viral challenge, but appeared to exacerbate disease. However, the mechanisms of such putative, vaccine-induced disease enhancement remain to be elucidated.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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