Immune response in pigs vaccinated with plasmid DNA encoding ORF5 of porcine reproductive and respiratory syndrome virus

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The ORF5-encoded major envelope glycoprotein (GP5) of porcine reproductive and respiratory syndrome virus (PRRSV) is one of the three major structural proteins of this virus. While some porcine convalescent sera and monoclonal antibodies directed against GP4 and GP5 have the capacity to neutralize the virus in vitro, the protein specificity of porcine neutralizing sera has not yet been established. DNA immunization with a plasmid encoding GP5 of PRRSV, under the control of a human cytomegalovirus promoter, induced anti-GP5-specific neutralizing antibodies in pigs and BALB/c mice. The GP5 protein specificity of neutralizing sera was confirmed by immunoblotting and ELISA. Peripheral blood mononuclear cells obtained from DNA-vaccinated pigs underwent blastogenic transformation in the presence of E. coli-expressed recombinant ORF5-encoded protein, indicating the specificity of the cellular immune response to GP5. Following a massive intratracheal challenge with the virulent IAF-Klop strain of PRRSV, DNA-vaccinated pigs were protected from generalized viraemia and the development of typical macroscopic lung lesions that were observed in unvaccinated, virus-challenged controls, as well as in pigs that were immunized with E. coli-expressed GST–ORF5 recombinant fusion protein. Interstitial pneumonitis and broncho-alveolitis were remarkably milder in DNA-vaccinated animals. These results suggest that the GP5 of PRRSV is a good candidate for a subunit recombinant-type vaccine.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a disease found in swine farms worldwide and it is characterized by reproductive failure such as late-term abortions in sows and by respiratory illness and mortality in young pigs (Dea et al., 1992; Goyal, 1993). The nucleotide sequence, genomic organization and replication strategy of porcine reproductive and respiratory syndrome virus (PRRSV) are related to those of a group of small, enveloped, positive-stranded RNA viruses including murine lactate dehydrogenase elevating virus (LDV), equine arteritis virus (EAV) and simian haemorrhagic fever virus, which are presently classified within the family Arteriviridae, order Nidovirales (Cavanagh, 1997). The genome of PRRSV is about 15 kb in length and contains eight open reading frames (ORFs). ORFs 1a and 1b, situated at the 5’ end of the genome, represent nearly 75% of the viral genome and code for proteins with apparent replicase and polymerase activities (Meulenberg et al., 1993). Six putative structural proteins have been identified and assigned to distinct smaller ORFs, ORFs 2–7, located at the 3’ end of the genome (Mardassi et al., 1995, 1996; Meulenberg et al., 1993). The virion contains three major structural proteins, a 25 kDa envelope glycoprotein (GP3), an 18–19 kDa unglycosylated membrane protein (M), and a 15 kDa nucleocapsid (N) protein, encoded by ORFs 5, 6 and 7, respectively. In addition, the translation products of ORFs 2, 3 and 4, with respective apparent molecular masses of 30, 45 and 31 kDa, also have characteristics of membrane-associated glycoproteins (Mardassi et al., 1995; Meulenberg et al., 1995). Recent findings on the characterization of structural proteins of Lelystad virus, the European prototype strain of PRRSV, indicate that expression products of the above ORFs, designated GPw, GP3 and GPv respectively, are incorporated into virus particles (Meulenberg & Petersen-Den Besten, 1996; Van Nieuwstadt et al., 1996). While pigs develop neutralizing antibodies 4–6 weeks after exposure to virus, the protein specificity of such neutralizing
antibodies is yet to be established. Monoclonal antibodies (MAbs) specific to GP₃ of PRRSV neutralize the virus in vitro (Meulenberg et al., 1997; Van Nieuwstadt et al., 1996) but the reactivity of convalescent pig sera with this viral protein is not constant (Meulenberg et al., 1995, 1997). The ORF4 gene of a North American strain of PRRSV has been also cloned and expressed in E. coli (Kwang et al., 1994). Only 65% of PRRSV-positive sera obtained from affected pig farms reacted positively by immunoblotting with the recombinant ORF4-encoded protein. Therefore, the involvement of ORF4-encoded protein in inducing neutralizing antibodies following PRRSV infection is yet to be determined.

We have recently established that MAbs derived from spleen cells of mice immunized with recombinant ORF5-encoded protein expressed in E. coli neutralized PRRSV in vitro (Pirzadeh & Dea, 1997) and therefore, as for other arteriviruses, notably EAV (Balasuriya et al., 1993) and LDV (Coutelier et al., 1988), the ORF5-encoded glycoprotein of PRRSV is associated with neutralizing epitopes. However, results of our previous studies indicated that immunization with the same antigenic preparation did not induce neutralizing antibodies in pigs (Loemba et al., 1996). The purpose of the present work was to study the immunogenicity of the GP₅ of PRRSV in pigs and to establish if animals exposed to the native form of the protein by means of DNA immunization develop specific neutralizing and protecting antibodies.

Methods

Experimental animals. Twelve crossbred F1 (Landrace × Yorkshire) SPF piglets weaned at 3 weeks of age were obtained from a breeding farm in the province of Quebec, Canada. The breeding stock and piglets were tested and proven to be seronegative for PRRSV, encephalomyocarditis virus, porcine parvovirus, haemagglutinating encephalomyelitis virus, transmissible gastroenteritis virus and Mycoplasma hyopneumoniae. The piglets used in this study were from two different litters and were randomly divided into four experimental groups (Table 1).

Six-week-old female BALB/c and CD-1 mice were purchased from Charles River Laboratories and separated in groups of five mice per cage; cages were equipped with individual filtered air channels.

Virus and challenge. The Quebec cytopathic strain IAF-Klop (Mardassi et al., 1995) used in this study was initially isolated from an acute case of PRRS and propagated in MARC-145 cells, a clone of MA-104 cells highly permissive to PRRSV (Kim et al., 1993), graciously provided by J. Kwang (US Meat Animal Research Centre, USDA, Agricultural Research Service, Clay Centre, NE, USA). Virus titration was provided by J. Kwang (US Meat Animal Research Centre, USDA, Agricultural Research Service, Clay Centre, NE, USA). Virus titration was provided by J. Kwang (US Meat Animal Research Centre, USDA, Agricultural Research Service, Clay Centre, NE, USA). Virus titration was provided by J. Kwang (US Meat Animal Research Centre, USDA, Agricultural Research Service, Clay Centre, NE, USA).

Virus infection with neutralizing and protecting antibodies. Ex vivo expression of the pRc/CMV construct was tested in transient expression experiments in COS7 and 293 cells maintained as confluent monolayers. Cells in 6 cm tissue culture plates were transfected with 15 µg plasmid DNA by calcium phosphate co-precipitation (Graham & Van der Eb, 1973). For indirect immunofluorescence (IF), cells were incubated at 37 °C and fixed with 80% cold acetone for 20 min at 4 °C at various times (18–72 h) post-transfection. The monolayers were then reacted for 30 min with anti-ORF5 rabbit monoclonal hyperimmune serum (Mardassi et al., 1996) and the immune reaction was determined following incubation with fluorescein-conjugated goat anti-rabbit Ig (Boehringer Mannheim), as previously described (Loemba et al., 1996).

Plasmids and recombinant proteins. Viral RNA was extracted from PRRSV-infected MARC-145 cells as previously described (Mardassi et al., 1995). The ORF5-encoding region was amplified by RT–PCR, cloned in the pGEX-4T1 plasmid (Pharmacia); a recombinant fusion protein consisting of glutathione-S-transferase (GST) joined to the N terminus of the ORF5 protein (GST–ORF5) was expressed in E. coli and purified by affinity chromatography on a glutathione–Sepharose column as previously described (Pirzadeh & Dea, 1997). Subsequent SDS–PAGE analysis of the purified protein confirmed that no contaminating bacterial proteins were present in the purified recombinant fusion protein (data not shown). Plasmidic DNA was purified from bacterial lysates by anion exchange chromatography on a hydroxypapatite column (QIAGEN) and then precipitated with isopropanol. This procedure effectively eliminated bacterial protein contaminants. The same amplification product was cloned in the pET21a plasmid (Novagen) to produce a recombinant protein in E. coli consisting of the ORF5-encoded protein fused at the C terminus to six histidine residues (ORF5–6H). The ORF5 coding region was further cloned into the HindIII and Xhol cloning sites of the eukaryotic expression vector pRc/CMV (Invitrogen), downstream of the human cytomegalovirus (HCMV) promoter, to produce pRc/CMV5.

The sequences of the oligonucleotide primers used for the latter amplification were as follows: ETS5 (forward primer), 5′ AACGTTTCCGCCGGCGCATGTTGGGGGAAATGCTTTGACC 3′, which comprises the first ATG codon of the ORF5 gene downstream of a Kozak motif for initiation of translation in vertebrates (Kozak, 1987); and ETR5 (reverse primer), 5′ TCTAGAGGCGAAATGACATCTGGGG 3′, which comprises the C-terminal stop codon of the viral gene. The nucleotide sequence accession number (EMBL/GenBank/DDB) libraries of IAF-Klop strain is U64928. For directional cloning, HindIII and Xhol restriction sites were added at the 5′ ends of the sense and antisense oligonucleotide primers, respectively. Both strands of pRc/CMV5 were sequenced in an Automated Laser Fluorescent DNA sequencer (Pharmacia LKB) in order to confirm that no errors were introduced as a result of PCR amplification.

Transient expression of the GP₅ glycoprotein. Ex vivo expression of the pRc/CMV5 construct was tested in transient expression experiments in COS7 and 293 cells maintained as confluent monolayers. Cells in 6 cm tissue culture plates were transfected with 15 µg plasmid DNA by calcium phosphate co-precipitation (Graham & Van der Eb, 1973). For indirect immunofluorescence (IF), cells were incubated at 37 °C and fixed with 80% cold acetone for 20 min at 4 °C at various times (18–72 h) post-transfection. The monolayers were then reacted for 30 min with anti-ORF5 rabbit monoclonal hyperimmune serum (Mardassi et al., 1996) and the immune reaction was determined following incubation with fluorescein-conjugated goat anti-rabbit Ig (Boehringer Mannheim), as previously described (Loemba et al., 1996).
Table 1. Antibody response of DNA and GST–ORF5 immunized mice and pigs

Antibody titres correspond to the average titres ± SD.

<table>
<thead>
<tr>
<th>Animal group*</th>
<th>Immunogen and dose</th>
<th>Serological tests†</th>
<th>Immunization and sample collection schedule (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0‡</td>
</tr>
<tr>
<td>CD-1 mice</td>
<td>G1: 50 µg pRc/CMV5</td>
<td>ELISA –</td>
<td>140 ± 49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IIF –</td>
<td>35 ± 16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VN –</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>G2: 50 µg GST–ORF5</td>
<td>ELISA –</td>
<td>320 ± 98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IIF –</td>
<td>29 ± 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VN –</td>
<td>–</td>
</tr>
<tr>
<td>BALB/c mice</td>
<td>G1: 50 µg pRc/CMV5</td>
<td>ELISA –</td>
<td>260 ± 120</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IIF –</td>
<td>22 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VN –</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>G2: 50 µg GST–ORF5</td>
<td>ELISA –</td>
<td>640 ± 196</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IIF –</td>
<td>102 ± 31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VN –</td>
<td>–</td>
</tr>
<tr>
<td>Pigs</td>
<td>G1: 100 µg pRc/CMV5</td>
<td>ELISA –</td>
<td>133 ± 47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IIF –</td>
<td>64 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VN –</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>G2: 300 µg GST–ORF5</td>
<td>ELISA –</td>
<td>400 ± 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IIF –</td>
<td>107 ± 30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VN –</td>
<td>–</td>
</tr>
</tbody>
</table>

* Control animals consisted of five BALB/c mice, five CD-1 mice and three F1 piglets. Each control animal was injected with corresponding quantities of parental pRc/CMV plasmid via identical route and frequency. Control animals remained seronegative throughout the observation period.
† ELISA: reciprocal of highest serum dilution reacting with the recombinant ORF5–pH expressed in E. coli. IIF: reciprocal of highest serum dilution at which specific cytoplastic fluorescence was observed in PRRSV-infected MARC-145 cells. VN: reciprocal of highest serum dilution which inhibited 100% of CPE and expression of viral N protein in PRRSV (strain IAF-Klop) infected MARC-145 cells stained by IPMA.
‡ Groups of five mice or three piglets were immunized with pRc/CMV5 plasmid or GST–ORF5 expressed in E. coli on the mentioned days. Blood samples were collected from retro-orbital vein of mice or anterior tibia of pigs prior to each immunization.
§ Pigs were challenged with 5 × 10⁷ TCID₅₀ by intratracheal inoculation.
|| Sample collection only.

Mice immunization schedule. In vivo expression of pRc/CMV5 was verified by immunizing groups of five CD-1 or BALB/c mice with 50 µg pRc/CMV5 diluted in 0.5 ml PBS; the DNA was injected into the tibialis cranialis muscle with a 27 gauge needle. The mice were boosted twice with the same quantities of DNA at 2 week intervals. Control mice received the same amounts of parental pRc/CMV5 vector via an identical route and frequency or three intraperitoneal doses of 50 µg GST–ORF5 in Freund’s complete or incomplete adjuvant.

Pig immunization schedule. Groups of three piglets were injected three times at 2 week intervals with 100 µg pRc/CMV5 diluted in 0.5 ml PBS. Two-thirds of the volume was injected, using a 26 gauge needle, into the tibialis cranialis muscle of the right leg and one-third was intradermally administered into the dorsal surface of the ear. Control piglets received either 100 µg parental vector via an identical route and frequency or 300 µg GST–ORF5.

Virus neutralization and serological tests. Mouse and pig sera were tested for the presence of specific anti-GP₃ antibodies by virus neutralization (VN), IIF, ELISA and Western immunoblotting (WB) tests. The VN test was performed in triplicate with 100 µl serial dilutions of heat-inactivated (56 °C for 45 min) test sera, incubated for 60 min at 37 °C in the presence of 100 TCID₅₀ of the virus in DMEM; the mixtures were put in contact with confluent monolayers of MARC-145 cells that had been seeded in 96-well microtitration plates 48–72 h earlier. Cell monolayers were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ and observed daily for up to 5 days for the appearance of cytopathic effects (CPE). The monolayers were then fixed with a solution of 80% methanol containing 0.05% H₂O₂, and tested for expression of the PRRSV nucleocapsid protein by IPMA (Wensvoort et al., 1991), using the N protein-specific MAb IAF-K8 (Pirzadeh & Dea, 1997). Indirect ELISA was essentially performed as previously described (Pirzadeh & Dea, 1997) with minor modifications. Gel-purified ORF5–pH protein (0.1 µg protein per well) in 0.05 M sodium carbonate buffer, pH 9.0, was used to coat flat-bottomed microtitration plates and peroxidase-labelled goat anti-porcine IgG (Boehringer Mannheim). Neutralizing titres were expressed as the reciprocal of the highest dilution which completely inhibited expression of the viral N protein. IIF was performed on PRRSV-infected and acetone-fixed MARC-145 cells as previously described (Loemba, 1996). Indirect ELISA was essentially performed as previously described (Pirzadeh & Dea, 1997) with minor modifications. Gel-purified ORF5–pH protein (0.1 µg protein per well) in 0.05 M sodium carbonate buffer, pH 9.0, was used to coat flat-bottomed microtitration plates and peroxidase-labelled goat anti-porcine IgG was used to detect the captured antibodies. The substrate solution consisted of 0.1% urea peroxide and 0.02% 3,3’,5,5’-tetramethyl benzidine, in 10 mM citrate buffer, pH 5.0, mixed in equal volumes; absorbance values were determined at 450 nm. WB was also...
performed as previously described (Pirzadeh & Dea, 1997) using either ORF5–pH protein or sucrose gradient-purified PRRSV as antigen.

**Blastogenic transformation test.** At regular post-immunization intervals, pigs were treated with xylazine (Bayer) at a dose of 1 mg/kg and blood samples were collected from the anterior terna carna in vacuum tubes containing 1/10 volume 150 mM sodium citrate in PBS, and then diluted 1:3 in sterile RPMI. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll–Paque (density of 1.077; Pharmacia) centrifugation at 1200 g for 20 min. Mononuclear cells were collected from the buffy coat, pelletted and the residual red blood cells were lysed by incubating cells with 0.53% ammonium chloride for 10 min at 37 °C. After two washes in RPMI, the leukocyte suspension was adjusted to 2 $\times$ 10^9 cells per ml in RPMI containing 20% homologous heat-inactivated PRRSV-negative porcine serum, 50 U/ml penicillin and 50 µg/ml streptomycin. The antigen-specific proliferation was determined by incubating PBMCs in microtitration plates (4 $\times$ 10^5 cells in 200 µl/well in triplicate) for 72 h in the presence of various concentrations (0, 0.1, 10 and 25 µg/ml) of ORF5–pH protein. Blastogenic capacity of the PBMCs under test conditions was confirmed by including controls in triplicate containing 2.5, 5 or 10 µg/ml concanavalin A (ConA; Sigma). After a 72 h stimulation period, the cells were labelled for 18 h with [3H]thymidine (Amersham; 0.1 µCi/well), harvested with a semi-automatic cell harvester (Skatron Instruments) and the incorporated radio-labelled nucleotide was measured by scintillation counting after addition of a fluorescent liquid scintillator (Cytoscan; ICN). The level of proliferation was expressed as the mean of counts per minute (CPM) of the test wells minus the mean of the background CPM in control wells. The control for background levels consisted of PBMC cultures in medium alone.

**Virus isolation.** After collection of blood samples, pigs were euthanized by rapid intravenous injection of sodium pentobarbital (MTC Pharmaceuticals). Specimens were aseptically collected from lungs, spleen, liver, kidneys, and mediastinal and mesenteric lymph nodes. Tissue homogenates were then prepared in DMEM to final concentrations of 0.1, 10 and 25 µg/ml ORF5–pH protein. Blastogenic capacity of the PBMCs under test conditions was confirmed by including controls in triplicate containing 2.5, 5 or 10 µg/ml concanavalin A (ConA; Sigma). After a 72 h stimulation period, the cells were labelled for 18 h with [3H]thymidine (Amersham; 0.1 µCi/well), harvested with a semi-automatic cell harvester (Skatron Instruments) and the incorporated radio-labelled nucleotide was measured by scintillation counting after addition of a fluorescent liquid scintillator (Cytoscan; ICN). The level of proliferation was expressed as the mean of counts per minute (CPM) of the test wells minus the mean of the background CPM in control wells. The control for background levels consisted of PBMC cultures in medium alone.

**RT–PCR.** Total RNA was extracted from tissues collected from challenged animals and from MARC-145 cells inoculated with tissue homogenates. RT–PCR was performed using oligonucleotide primers 1006FS + 1007PR and 1008FS + 1009PR to amplify ORF6 and ORF7 genomic regions of PRRSV, respectively, as previously described (Mardassi et al., 1995).

**Histopathological examination.** Thin sections (5 µm thick) of formalin-fixed, paraffin-embedded tissues from the lungs, spleen, liver, kidneys, thoracic and mesenteric lymph nodes of all pigs were routinely processed for hematoxylin–phloxin–safran (HPS) staining, as described previously (Dea et al., 1991).

**Results**

**Transient expression of the cloned ORF5 gene**

Expression of the ORF5 product was demonstrated in both COS7 and 293 cells lines at 24 and 36 h post-transfection. The identification of GP5 was confirmed by IIF using monospecific anti-ORF5 rabbit antiserum or the porcine anti-PRRSV serum. As shown in Fig. 1, an intense cytoplasmic fluorescence could be observed in approximately 10–15% of the cells and the expressed GP5 tended to accumulate near the perinuclear region.

**Antibody response of mice and pigs**

Sera collected at various times post-immunization (Table 1) were positive for the presence of anti-PRRSV antibodies by IIF. The protein specificity of mouse and pig sera to GP5 was established by immunoblotting with purified whole virus and E. coli-expressed recombinant ORF5–pH fusion protein (Fig. 2) and by ELISA (Table 1). BALB/c mice inoculated with the GST–ORF5 or pRc/CMV5 developed neutralizing antibodies which were first detected 2 weeks after the second booster injection. The VN titres of BALB/c mice sera were estimated between 32 and 64 by week 8 post-immunization and persisted through to the end of the 12 week observation period. In contrast, CD-1 mice did not develop neutralizing antibodies to PRRSV despite a significant anti-ORF5 specific antibody response, detected by ELISA and IIF. Seroconversion was also demonstrated by IIF and ELISA in both groups of vaccinated pigs (Table 1) 15 days after the first injection of either GST–ORF5 or pRc/CMV5. Neutralizing antibodies were detected in sera of the DNA-immunized pigs only 2–3 weeks after the second booster injection (8–9 weeks after first inoculation of plasmidic DNA), and 2 weeks after PRRSV challenge, with estimated titres close to 128. None of the virus-challenged animals in the unvaccinated or GST–ORF5-immunized group developed detectable neutralizing antibodies (VN titres < 8) to PRRSV 2 weeks after infection (Table 1). Control animals were negative for PRRSV and ORF5–pH protein, as determined by IIF and ELISA, throughout the observation period.

**Specific blastogenic response to ORF5–pH**

PBMCs obtained from both groups of immunized pigs underwent specific blastogenic transformation *ex vivo* in a dose-dependent manner in the presence of ORF5–pH protein, whereas [3H]thymidine incorporation by the PBMCs obtained from unvaccinated animals remained at basal level (Fig. 3). Blastogenic transformation indexes of 7–12 and 10–12 were calculated 2 weeks after the second booster injection of GST–ORF5 and pRc/CMV5, respectively. Concentrations higher than 10 µg ORF5–pH protein per ml culture medium did not increase [3H]thymidine incorporation levels in PBMCs from both groups of pigs. No significant variations were observed in the blastogenic response to ConA of vaccinated pigs compared to unvaccinated controls.

**Clinical observations**

Unvaccinated pigs developed clinical signs of respiratory disease, beginning 2–3 days after virus challenge, which
DNA immunization against PRRSV

Fig. 1. Immunofluorescent staining of COS7 cells at 24 h post-transfection with the pRC/CMV5 plasmid. Expression of GP₅ of PRRSV (strain IAF-Klop) was confirmed by IIF following incubation in the presence of the rabbit anti-ORF5 monospecific serum. A similar fluorescent profile was obtained following incubation with the autologous anti-PRRSV porcine hyperimmune serum. Expressed GP₅ protein accumulated mostly in the perinuclear region.

Fig. 2. Reactivity by immunoblotting of the serum of DNA-immunized pigs and mice towards the GP₅ of PRRSV and the recombinant ORF5–pH protein expressed in E. coli. Lane 1: immunoblot showing reactivity of a convalescent pig serum towards three major structural proteins (N, M and GP₅) of PRRSV (strain IAF-Klop). Lanes 2 and 3: reactivity of pig and mouse sera towards GP₅ at day 51 post-immunization with pRC/CMV5. Lanes 4 and 5: reactivity of pRC/CMV5-immunized mouse and pig sera with ORF5–pH recombinant protein expressed in E. coli. Lane 6: reactivity of porcine convalescent serum with ORF5–pH recombinant protein.

Virus isolation

As summarized in Table 2, after a single passage on MARC-145 cells, virus was recovered from tissue homogenates (dilutions of 1/20 and 1/100) of several organs (lungs, spleen, kidneys, liver, lymph nodes) from unvaccinated animals 2 weeks after virus challenge, whereas, apart from lungs and mediastinal lymph nodes, no virus was isolated from other organs of DNA-immunized pigs after two successive passages; this indicates the generalized viraemia of unvaccinated pigs compared to respiratory tract localization of virus in DNA-immunized animals. PRRSV was also recovered from spleen and kidneys of one of the three GST–ORF5-immunized pigs.

Persisted through the end of the 2 week observation period. The principal signs included a marked drop in feed consumption, hyperthermia (40.2–41.7 °C) that persisted for 10–14 days, eyelid oedema, laboured breathing (abdominal respiration) in two pigs accompanied by rasping and crowing sounds heard during inspiration. Apart from a transitory mild fever (39.8–40.4 °C) that lasted not more than 2–3 days, all vaccinated pigs remained clinically healthy during the 2 week observation period following virus challenge. Their average feed consumption and growth rate remained identical to those of unvaccinated, unchallenged controls.

Necropsy findings

Unvaccinated, virus-challenged pigs euthanized at day 14 p.i. had gross lesions that were confined to the respiratory tract and thoracic cavity. Portions of the lungs were tan in colour and partly collapsed, with occasional anteroventral areas of congestion and consolidation. The mediastinal lymph
Fig. 3. *Ex vivo* blastogenic response of porcine PBMCs following incubation in the presence of different concentrations of ConA or ORF5–pH recombinant protein antigen at various times post-immunization. Black bars, DNA immunization; grey bars, GST–ORF5 immunization; open bars, controls. (a) PBMCs obtained from both GST–ORF5- or pRc/CMV5-immunized pigs underwent specific blastogenesis in the presence of ORF5–pH and stimulation indexes of 7–12 were calculated. (b) No significant difference was observed in non-specific mitogen-induced blastogenesis responses of PBMCs obtained from vaccinated and unvaccinated pigs at various times post-immunization.

Table 2. Virus isolation and RT–PCR analysis performed on day 14 post-challenge

<table>
<thead>
<tr>
<th>Pig immunized with:</th>
<th>Organ on MARC-145 cells</th>
<th>Lymph nodes</th>
<th>RT–PCR</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Lung</td>
<td>Spleen</td>
<td>Kidney</td>
</tr>
<tr>
<td>First passage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GST–ORF5</td>
<td>+ (3)</td>
<td>+ (1)</td>
<td>− (0)</td>
</tr>
<tr>
<td>pRc/CMV5</td>
<td>− (0)</td>
<td>− (0)</td>
<td>− (0)</td>
</tr>
<tr>
<td>Unvaccinated controls</td>
<td>+ + (3)</td>
<td>+ + (3)</td>
<td>+ + (3)</td>
</tr>
<tr>
<td>Second passage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GST–ORF5</td>
<td>+ + (3)</td>
<td>+ (1)</td>
<td>+ (1)</td>
</tr>
<tr>
<td>pRc/CMV5</td>
<td>+ (3)</td>
<td>− (0)</td>
<td>− (0)</td>
</tr>
<tr>
<td>Unvaccinated controls</td>
<td>+ + (3)</td>
<td>+ + (3)</td>
<td>+ + (3)</td>
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</table>

+ Virus could be recovered from 1/20 dilution of organ homogenates; + + , virus could be recovered from 1/100 dilution of organ homogenates; − , virus could not be recovered. Of the three pigs studied in each case, the number that tested positive is indicated in parentheses.

Microscopic lesions observed in unvaccinated, virus-challenged pigs were confined to the lungs and consisted of macrophage infiltration, pyknotic cell debris and protein-rich exudate in the lumen of large bronchi and bronchioli, a peribronchiolar and perivascular lymphomononuclear cell infiltration, the presence of lymphomononuclear cells within the alveolar lumen with hyperplasia of type II pneumocytes, mononuclear cell invasion and the presence of pyknotic cells in alveolar septae (Fig. 4 b, c and d). The GST–ORF5-immunized pigs developed intense interstitial pneumonitis, characterized by hyperplasia of bron-
chiolar epithelium and pneumocytes type II of the alveolar endothelium, perivascular cuffing, lymphomononuclear cell infiltration and thickening of alveolar septae (Fig. 5a and b). A remarkably milder interstitial pneumonitis was observed in the DNA-vaccinated pigs. In those pigs, large airways (bronchi, bronchioli), as well as alveolar ducts, were normal in appearance with absence of cells and cellular debris within the lumen (Fig. 5c and d).

Discussion

In vivo expression of foreign proteins via simple injection of plasmidic DNA into mammals was described in the 1980s (Nicolau et al., 1983; Seeger et al., 1984) and a few years later in vivo gene transfer by microprojectiles and particle bombardment was studied (Sanders Williams et al., 1991; Yang et al., 1990). Encouraging results have been also obtained by DNA immunization against viral pathogens and, with few exceptions, laboratory animals such as mice (Davis et al., 1994; Martins et al., 1995; Ulmer et al., 1993; Yokoyama et al., 1995), guinea-pigs (Bourne et al., 1996) and rabbits (Sundaram et al., 1996) have been generally used as the experimental animals. Only a few experiments have been reported in the literature on DNA immunization in farm animals, including cattle (Cox et al., 1993), horses (Lunn et al., 1996), chickens (Fynan et al., 1993) and, more recently, pigs (Gerds et al., 1997). Our poor knowledge of the histocompatibility system of farm animals and the difficulties in handling large animals may be discouraging for some investigators.

In this report, DNA immunization was used to evaluate the immunogenicity of a single gene product of a viral pathogen in its natural host. The data obtained demonstrate that the ORF5-encoded GP₅ of PRRSV elicits neutralizing antibodies in pigs. Furthermore, these experiments suggest that conformational neutralizing epitopes may also be associated with the GP₅ of PRRSV, since immunizing pigs with the recombinant GST–ORF5 protein failed to trigger the immune system to produce neutralizing antibodies. Indeed, fusion proteins expressed in prokaryotic vectors may not effectively mimic the native viral proteins due to the differences in polypeptide folding, disulfide bond formation or post-translational modifications, notably N-glycosylation. It can therefore be assumed that the induction of neutralizing antibodies in DNA-immunized animals is due to correct glycosylation and post-translational modification of the GP₅ expressed in host cells. Recently, recombinant adenoviruses carrying the ORF5 gene of PRRSV strain IAF-Klop downstream of the CMV promoter were constructed and the ORF5 product that was expressed in human 293 cells was found to be glycosylated and processed in the same way as the native viral protein (Gagnon et al., 1997).

In the experiments we describe, the development of neutralizing antibodies cannot be attributed to the virus challenge since they were detected in sera of DNA-immunized pigs only and, at 14 days post-challenge, unvaccinated control pigs had no detectable antibodies. Furthermore, previous studies demonstrated that neutralizing antibodies to PRRSV in naturally or experimentally infected pigs start to be detected at low titres as late as 3–4 weeks after exposure to virus (Loomba et al., 1996; Nelson et al., 1994; Yoon et al., 1995) whereas, in the present study, neutralizing antibodies were detected in sera of DNA-immunized pigs 2–3 weeks after the second booster injection and significant titres of 64–128 were detected 14 days after virus challenge. The appearance of neutralizing antibodies in sera of DNA-immunized, PRRSV-challenged pigs may suggest that virus challenge had a booster effect in developing neutralizing antibodies. This is unlikely since BALB/c mice also developed neutralizing antibodies in the absence of PRRSV challenge.

Neutralizing antibodies in GST–ORF5-immunized BALB/c mice can be attributed to the relatively high quantities of antigen used for immunizing mice compared to that used in pigs (50 µg per mouse versus 300 µg per pig). An alternative interpretation of these results may be that CD-1 mice and pigs are tolerant to the linear epitopes of the E. coli-expressed ORF5-encoded recombinant protein, since CD-1 mice and pigs immunized with GST–ORF5 failed to develop neutralizing antibodies to PRRSV (Table 1). The lower titres of anti-GP₅ antibodies in DNA-immunized mice and pigs compared to the GST–ORF5-immunized animals can be explained by the fact that the injected antigens are available to the B cells and other antigen-presenting cells which can potentially stimulate a strong antibody response. On the contrary, the expressed protein may be retained within the DNA recipient cells and released in smaller amounts into the extracellular space, either by secretion or cell death as a result of cytotoxic protein accumulation. It was previously established that the GP₅ of PRRSV has an apoptotic effect on transfected cells (Suárez et al., 1996). Previous studies indicate that most proteins eliciting high antibody response after DNA immunization are membrane-associated or secreted proteins (Bourne et al., 1996; Davis et al., 1994; Fynan et al., 1993; Ulmer et al., 1993). While GP₅ is a membrane-associated viral glycoprotein, it retains a perinuclear location within the mammalian cells in transient expression experiments (Fig. 1).

Previous studies have established that protection due to DNA immunization is mediated by both CD4⁺ and CD8⁺ T lymphocytes (Doolan et al., 1996; Manickan et al., 1995). According to experimental data on mice, the type of immune response also depends on the route of DNA immunization. It has been previously demonstrated that intradermal immunization can induce very high levels of cytotoxic T lymphocytes mediated by a Th2-like response, whereas intramuscular DNA inoculation favours a Th1-like response (Pertmer et al., 1996; Yokoyama et al., 1997). Since such data are not yet available for large animal models, we used the two inoculation routes simultaneously in order to enhance both types of immune response. In the present study, proliferation assays of PBMCs
Fig. 4. Histological findings in the lungs of control (a) and unvaccinated, PRRSV-challenged (b, c and d) pigs. (a) Spongiform aspect of the lung of a normal pig showing clear airway passages (bronchiole and alveolar duct indicated by arrows) and well-delineated interalveolar septae. (b) General aspect of interstitial pneumonitis with alveolar septae thickened by lymphomononuclear cell infiltration. (c) Free mononuclear cells (arrows), necrotic cell debris and proteinaceous exudate within the bronchiole lumen. (d) Mononuclear cells lining the epithelium of a large bronchi. Note also the focal mild hyperplasia of the respiratory epithelium. HPS staining.

Fig. 5. Histopathological findings in the lungs of GST–ORF5- (a and b) and pRc/CMV5- (c and d) immunized pigs 14 days after challenge with PRRSV (strain IAF-Klop). (a) Localized region of intensive interstitial pneumonitis and accumulation of macrophages and necrotic cell debris within the lumen of a bronchiole with no apparent damage to the epithelium. (b) Normal aspect of the epithelium of a large bronchi with no accumulation of inflammatory exudate within the lumen. Note, on the left, the presence of significant lesions of interstitial pneumonitis (ip) with alveolar septae thickened by lymphomononuclear cell infiltration. (c and d) Moderate interstitial pneumonitis with no apparent damage to the epithelium of the bronchioles and alveolar ducts (arrows). Absence of mononuclear cells and necrotic cell debris within the alveolar lumen. HPS staining.
from pRC/CMV5- and GST–ORF5-immunized pigs showed a specific blastogenesis following stimulation by the recombinant ORF5–pH protein, independent from the production of specific anti-PRRSV neutralizing antibodies. Since the identity of PBMC subpopulation(s) which underwent blastogenesis following antigenic stimulation was not determined, it can be suggested that antigen-specific proliferation may be due to CD4+ and B cell effector cells implicated in eliciting an antibody response.

Our results showed that DNA immunization with a plasmid encoding the GP3 of PRRSV protected pigs from developing the intensive PRRSV-induced lesions observed in unvaccinated virus-challenged controls. Virus dissemination to organs other than the lungs and the accessory lymph nodes was not observed in DNA-vaccinated animals, even after a massive virus challenge, and these animals had a remarkably lower virus burden in their respiratory system compared to the GST–ORF5 vaccinated or unvaccinated controls. Therefore, it appears that ORF5 may be a good candidate for a subunit recombinant-type vaccine against PRRSV. However, the genomic variabilities of the ORF5 genes which have been recently reported amongst North American field isolates (Meng et al., 1995; Pirzadeh et al., 1997) and between North American and European strains (Mardassi et al., 1995; Murtaugh et al., 1995), need further investigation in order to determine their significance in terms of the antigenic determinants involved in protection.

As expected, DNA immunization was not sufficient to inhibit virus persistence and shedding in the respiratory tract during the short 14 day observation period after virus challenge. However, considering the high challenge dose used in our experiments, the protection conferred to the DNA-vaccinated pigs was quite remarkable. Other investigators have reported similar results in connection with post-challenge virus persistence in DNA-immunized animals (Bourne et al., 1996; Cox et al., 1993; Martins et al., 1995; Ulmer et al., 1993). Mucosal immunity is believed to play a role in protection against PRRSV infection, virus persistence and shedding but this aspect of immunity against PRRSV has not been investigated so far.

Finally, the intensive interstitial pneumonia observed in GST–ORF5-immunized animals could be attributed to the antibody-dependent enhancement (ADE) phenomenon which has been reported to occur in PRRSV-infected pigs (Yoon et al., 1997). According to the results obtained by these investigators, subneutralizing levels of anti-PRRSV antibodies have the potential to intensify pathogenesis of PRRSV infection due to ADE, but virus replication is significantly inhibited in the presence of neutralizing antibody titres. The immunoglobulins used in passive immunization studies to demonstrate the occurrence of ADE in PRRSV-infected pigs were extracted from polyclonal pig convalescent sera, but results from immunoblotting studies suggested that the ADE was mediated by antibodies specific to the GP3 (Yoon et al., 1997). The intensity of the lymphomononuclear cell infiltration observed in the lungs of GST–ORF5-immunized pigs which failed to develop neutralizing antibodies to the GP3 may also suggest that subneutralizing anti-GP3 antibodies contribute to ADE of PRRSV infection or, alternatively, the enhanced local secretion of inflammatory cytokines in this group of animals.

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