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Short communication

An adenovirus recombinant expressing the spike glycoprotein of porcine respiratory coronavirus is immunogenic in swine

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The full-length spike (S) gene of porcine respiratory coronavirus (PRCV) was inserted into the genome of human adenovirus type 5 downstream of the early transcription region 3 promoter. The recombinant virus replicated in cultures of the swine testicle ST cell line and directed the synthesis of S antigen with a maximum yield of approximately 26 μg per 10^6 cells. The antigen was cell-associated except in the late phase of the infection, when a small amount (3·5 μg per 10^6 cells) was released. The cell-associated antigen consisted of polypeptides of molecular mass 160 kDa and 175 kDa, comigrating with the authentic precursor S′ and the mature S protein of PRCV, respectively. The extracellular recombinant antigen corresponded to the 175 kDa mature protein. Some recombinant S protein was exposed on the cell surface and was recognized by neutralization-mediating anti-S monoclonal antibodies. Piglets, inoculated oronasally with the recombinant adenovirus vector developed PRCV-neutralizing serum antibodies and were partially protected against PRCV challenge, demonstrating the potential of live adenovirus as vaccine vector.

Porcine respiratory coronavirus (PRCV) (Pensaert *et al.*, 1986) belongs to the *Coronaviridae*, a family of enveloped, positive-stranded RNA viruses. It is antigenically and genetically closely related to enteropathogenic transmissible gastroenteritis virus (TGEV) of swine (Callebaut *et al.*, 1988; Rasschaert *et al.*, 1990; Sanchez *et al.*, 1992) but mainly causes a subclinical respiratory infection. Particularly in industrial finishing pig herds, PRCV infections are highly prevalent and are frequently combined with infections with other respiratory viruses such as swine influenzavirus(es). Experimentally, such dual infections can cause respiratory disease and lung lesions. Therefore, PRCV infection may contribute to the signs of respiratory distress often recorded in pigs in the field (Van Reeth & Pensaert, 1994).

The S glycoprotein of PRCV is associated with the surface projections and is 1209 residues long (Rasschaert *et al.*, 1990). It carries at least two antigenic sites, which are designated A and D (Callebaut *et al.*, 1988; Sanchez *et al.*, 1990) and are the major inducers of neutralizing antibodies (Delmas *et al.*, 1986; Correa *et al.*, 1988). The full-length TGEV S protein expressed by recombinant vaccinia- and baculoviruses is intrinsically capable of being transported to the cell surface, without the cooperation of other viral proteins (Pulford & Britton, 1991; Godet *et al.*, 1991). Since cell surface expression has been reported to enhance the immunogenicity of some antigens (Andrew *et al.*, 1990), all of this suggests that the S glycoprotein may be a useful antigen for the development of a protective anti-PRCV response.

Adenoviruses can cause oral and respiratory infections in various animal species. Therefore, they may be particularly well suited as recombinant live vectors (Tuboly *et al.*, 1993) for antigen delivery at the respiratory mucosal lymphoid tissues. Human adenovirus type 5 (Ad5), the serotype most commonly used in current work, can accept inserts of up to 4 kbp of foreign DNA following a partial deletion of the non-essential early transcription region 3 (E3) from the genome (Graham & Prevec, 1991). A live recombinant Ad5 vector has been demonstrated to be an effective rabies vaccine when administered to skunks and foxes by the oral route (Charlton *et al.*, 1992). Ad5 has been used successfully for expression of the S protein of the coronavirus mouse hepatitis virus (Wesseling *et al.*, 1993).

Previous work in our laboratory has shown that Ad5 causes a subclinical respiratory infection following oronasal administration to pigs (Callebaut *et al.*, 1994). In the present study it was our purpose to examine the

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potential of a recombinant adenovirus-based vector for oronasal vaccination against respiratory viral infection in swine. We report the construction of an Ad5 vector, designated AdgpS, which carries the S gene of PRCV in the E3 region of its genome and directs a high level of glycoprotein S expression in porcine cells in culture. We also describe the biochemical and biological properties of the expressed product and demonstrate that the recombinant vector has immunogenic and protective potential in piglets.

The PRCV isolate TLM 83 (Pensaert *et al.*, 1986) at the third passage in the swine testicle ST cell line was used as the source of viral genomic RNA. A DNA copy of the S gene was obtained by RT–PCR on the viral RNA. The antisense primer 5′ TCTGCTAGCTTAAATTTAATGGACGTGCAC was used for cDNA synthesis. This primer was complementary to nucleotides 3664 to 3684 of the S gene (Rasschaert *et al.*, 1990) and contained an unmatched *Nhe*I site at the 5′ end to facilitate cloning. The sense primer 5′ TAGCTAGCCACACCATGAAAAAATTATTTG, homologous to nucleotides −6 to 16 of the S gene and with a *Nhe*I site added to the 5′ end, was used in combination with the antisense primer for cDNA amplification. Thirty cycles (95 °C for 1 min, 56 °C for 2 min, 72 °C for 15 min) were performed. The PCR product was cloned into Ad5 following described procedures (Graham & Prevec, 1991). Briefly, it was first inserted into the *Xba*I site of the Ad5 transfer vector plasmid pFG144K3, which essentially contains the right-end of the Ad5 genome, but with a deletion of most of the E3 region. Restriction enzyme digestion of the resultant plasmid was performed to verify the integrity of the S gene, inserted at the location of the E3 deletion in an orientation parallel to the direction of transcription of the E3 promoter. Subsequently, the hybrid transcription unit was rescued into the Ad5 genome by homologous overlap recombination following cotransfection of human 293 cells with the recombinant pFG144K3 plasmid along with the overlapping Ad5 subgenomic plasmid pFG173. Plaques developed between 13 and 19 days after cotransfection. Restriction analysis of isolated viral DNA confirmed the presence of the S gene insert. The recombinant virus, inoculated in the porcine ST cells, produced cytopathic effect (CPE) and replicated to a high titre, 10^{10} $TCID_{50}$/ml, which was of the same order of magnitude as that of wild-type Ad5.

The kinetics of S protein expression were determined in cell cultures inoculated with AdgpS at a multiplicity of 10 $TCID_{50}$/cell. Cell lysates (cell-associated antigen) and culture supernatants (extracellular antigen) were assayed by antigen capture sandwich ELISA, performed essentially as described (Callebaut *et al.*, 1982), using pig anti-TGEV capture antibody and pig anti-TGEV horseradish peroxidase conjugate. Serial dilutions of samples

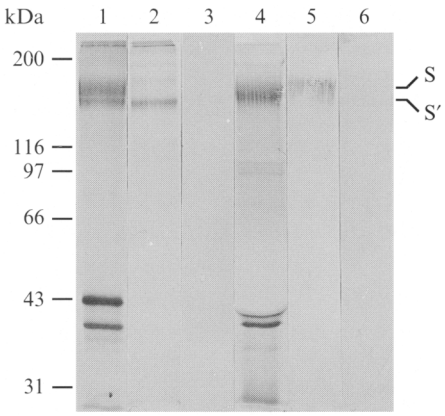

Fig. 1. Immunoblot analysis of recombinant S synthesized in ST cell cultures. Polypeptides revealed by anti-TGEV porcine serum in cell lysates (lanes 1 to 3) and culture supernatants (lanes 4 to 6) from cell cultures infected with AdgpS (lanes 2 and 5), with PRCV (lanes 1 and 4) and with Ad5 (lanes 3 and 6). Precursor S′ and mature S polypeptides are indicated. The positions of molecular mass markers are shown on the left.

were tested and the ELISA titres were converted to S protein concentrations using as a standard a known concentration of highly purified PRCV, prepared as previously described (Callebaut *et al.*, 1988); the S protein content of this virus preparation was one-fifth of the total protein, as estimated by PAGE and densitometry of Coomassie blue-stained gels. Cell-associated S antigen was first detected in the AdgpS-infected cultures at 18 h post-inoculation. The cumulative amount increased at a high rate until 24 h post-inoculation, when 18 μg per 10^6 cells was produced. This was during the early phase of the infection before the appearance of CPE, probably consistent with transcription driven by the E3 promoter. The maximum amount, estimated to be 26 μg per 10^6 cells, was found at 48 h post-inoculation, when 50% of the cells showed CPE. Extracellular S antigen was detectable starting at 48 h post-inoculation. The highest yield of extracellular antigen was 3·5 μg per 10^6 cells; this was reached at 72 h post-inoculation when CPE was complete and cells began lifting off.

Immunoblot analysis was performed to identify the S protein in the cell lysate and culture supernatant at 72 h after AdgpS inoculation. The proteins were resolved on an 8% SDS–polyacrylamide gel under non-reducing conditions and were transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was probed with pig anti-TGEV serum and the reaction was developed using rabbit anti-pig IgG antibodies conjugated to horseradish peroxidase (Nordic) under the conditions described by Correa *et al.* (1988). Samples from cell cultures infected with PRCV and wild-type Ad5 were used as positive and negative controls, respectively. As seen in Fig. 1, in the lysate of AdgpS-infected cells two

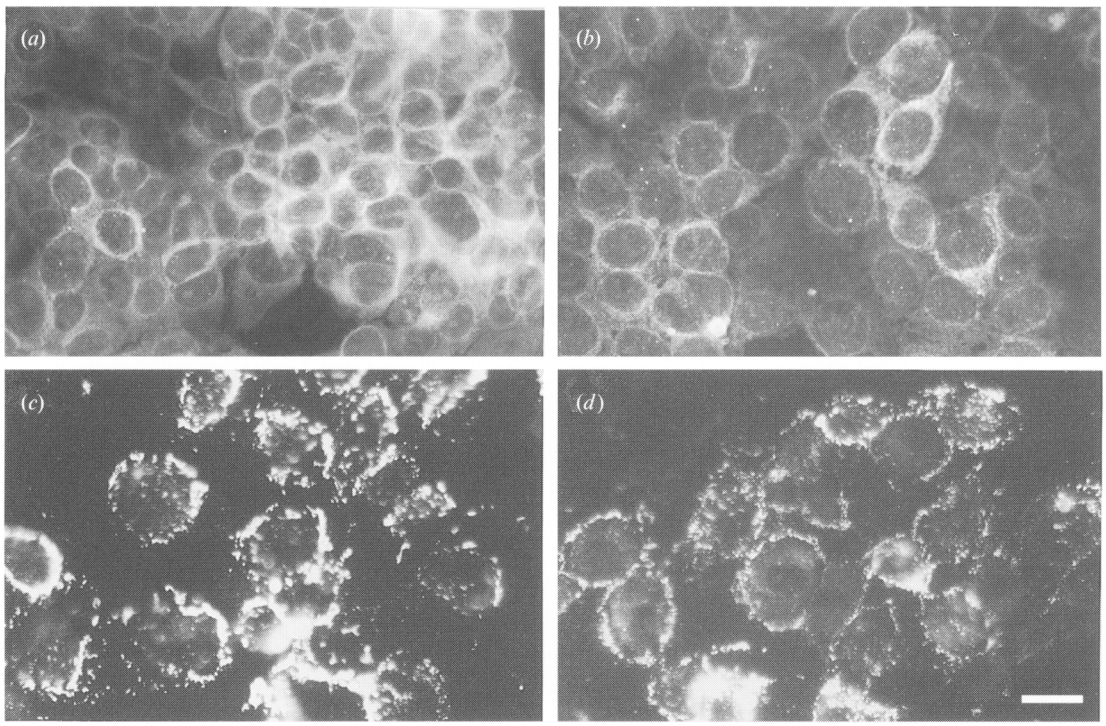

Fig. 2. Cell surface expression of recombinant S antigen in ST cell cultures. The figure shows membrane fluorescence in cells infected with AdgpS (*a*, *b*) and PRCV (*c*, *d*), stained with monoclonal antibodies against antigenic site A (*a*, *c*) and site D (*b*, *d*) of the S protein. Bar represents 20 μm.

major polypeptides of 160 kDa and 175 kDa, respectively, were identified (lane 2). The 160 kDa polypeptide was predominant and had the same mobility as the authentic S′ precursor glycoprotein synthesized in PRCV-infected cells (lane 1) (Rasschaert *et al.*, 1990). The 175 kDa polypeptide comigrated with the authentic mature S glycoprotein, present in PRCV-infected cells as well as in extracellular PRCV (lane 4). The same polypeptides were revealed when the membrane was probed with anti-S monoclonal antibodies (not shown). These findings indicate that the recombinant S protein is properly glycosylated and post-translationally modified. In the supernatant of the AdgpS-infected cells the 175 kDa protein was the single polypeptide species (lane 5). It is not clear whether this finding implies that the mature S protein is secreted, since the protein in the culture supernatant may have been associated with small debris from lysed cells or generated by proteolytic cleavage of cell-associated protein near the membrane anchor.

The distribution of recombinant S antigen on the cell surface was determined by indirect immunofluorescence assay of cells inoculated with AdgpS at a multiplicity of 10 $TCID_{50}$/cell and fixed after 24 h in 0·1 % paraformaldehyde for 30 min at 4 °C as described by To *et al.* (1992). The cells were probed with a 1/100 dilution of two pools of monoclonal antibodies, directed against either the antigenic site A or site D of the S protein (Correa *et al.*, 1988). Following reaction with fluorescein-conjugated goat anti-mouse IgG (Nordic), the cells showed membrane staining (Fig. 2*a*, *b*). Positive and negative controls were performed on cells which had been inoculated with PRCV (Fig. 2*c*, *d*) and with wild-type Ad5 (not shown). This result suggests that some recombinant S protein is transported to the cell surface. However, it cannot be excluded that the appearance of cell surface fluorescence was caused by S protein released from the cells and subsequently bound back to cell surface receptors. In addition, the reactivity with each of the pools of monoclonal antibodies indicates that the recombinant protein has conserved the important antigenic sites.

The potential of AdgpS to induce PRCV-neutralizing antibody and protection against challenge with PRCV was evaluated in conventional piglets, 4 weeks old, obtained from herds without neutralizing serum antibodies to Ad5 and PRCV (titres < 2). Two piglets were inoculated oronasally with $10^{10·4}$ $TCID_{50}$ of AdgpS. Five control piglets were either not inoculated (two piglets) or inoculated oronasally with $10^{9·3}$ $TCID_{50}$ of wild-type Ad5 (three piglets). Upon daily observation for the first 2 weeks, clinical signs were not seen in the inoculated piglets. After 4 weeks the AdgpS-inoculated piglets showed seroconversion against PRCV and against Ad5.

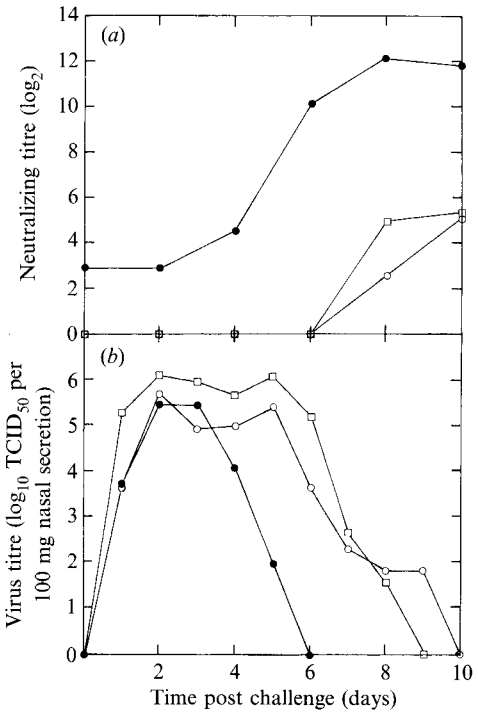

Fig. 3. Geometric mean PRCV-neutralizing antibody titres in serum (*a*) and geometric mean PRCV titres in nasal secretions (*b*) after PRCV challenge of piglets inoculated 4 weeks previously with AdgpS (●) or with wild-type Ad5 (○) or not previously inoculated (□).

PRCV neutralizing activity was detected at titres of 4 and 12; the Ad5-neutralization titres were 8 and 32, respectively. The control piglets, inoculated with wild-type Ad5, had developed Ad5-neutralizing serum antibodies at titres of 48, 64 and 128, but showed no response against PRCV (neutralizing titres < 2). The uninoculated control piglets remained negative for PRCV- and Ad5-neutralizing antibody. These results show that AdgpS is capable of inducing PRCV-neutralizing antibody, but titres are well below the convalescent antibody titres of around 128, previously found in piglets infected with PRCV (Callebaut *et al.*, 1988). In addition, the Ad5 antibody responses induced by AdgpS seem to point to a reduced immunogenic capacity compared with that of wild-type Ad5. It has been demonstrated that E3 gene products may allow Ad5 to escape antiviral defenses in the animal host (reviewed in Wold & Gooding, 1991). Therefore, it cannot be excluded that the deletion of E3 sequences resulted in decreased replication and antigenic stimulation by the AdgpS vector.

At the age of 8 weeks, the individual piglets were exposed to challenge with 10^7 $TCID_{50}$ of PRCV per animal via aerosol, as described (Van Reeth & Pensaert, 1994). The neutralizing antibody response against the challenge virus was determined in serum samples, obtained on the day of challenge and 2, 4, 6, 8 and 10 days later. Nasal swab specimens were collected daily from the day of challenge until post-challenge day 10 and used for the titration of excreted challenge virus. As indicated in Fig. 3(*a*), the AdgpS-inoculated piglets responded within 6 days with a marked increase in their PRCV-neutralization titre, typical of an anamnestic response. Wild-type Ad5 inoculated and uninoculated control piglets showed a response typical of immunologically naive pigs. The titres of challenge virus recovered from the nasal swabs of the three groups of pigs peaked at similar levels, but subsequently titres in AdgpS-inoculated pigs fell more rapidly than the titres in control pigs (Fig. 3*b*). Both AdgpS-inoculated piglets excreted PRCV until 5 days after challenge. The control piglets shed challenge virus until day 8, except one noninoculated and one Ad5-inoculated piglet, which excreted PRCV until post-challenge day 7 and day 9, respectively. The shortened duration of challenge virus shedding indicates that a single oronasal administration of AdgpS is sufficient to provide partial protection against a subsequent respiratory challenge with PRCV. From analysis of the serum antibody response upon challenge, we conclude that the primary anti-S response induced by AdgpS is boosted by the S antigen produced in the initial rounds of replication of the challenge virus; probably the rapid secondary S-specific response causes a concomitant rapid clearing of the infection in the respiratory tract.

On the whole, our data indicate a good prospect for developing recombinant adenovirus vectors for oronasal vaccination against respiratory viral infections in swine. While cloning in the partly deleted E3 region may lower the levels of *in vivo* replication, it may have the benefit of limiting the risks of dissemination of recombinant virus.

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