Construction and characterization of recombinant porcine adenovirus serotype 5 expressing the transmissible gastroenteritis virus spike gene

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Five recombinant porcine adenoviruses of serotype 5 (PAdV-5) carrying the full-length or the 5' 2.2 kb half of the transmissible gastroenteritis virus (TGEV) spike (S) gene were generated by homologous recombination in E. coli strain BJ5183 cells and subsequent transfection of swine testicle cells. The foreign genes were inserted into the E3 region of PAdV-5. One recombinant virus had no deletion in the E3 region, whereas a 1.2 kb fragment was removed from the E3 region in the remainder of the recombinant viruses. One stable construct with a 4.4 kb insertion had a genome size of 109.6% of the wild-type genome, the largest reported for any recombinant adenovirus. Only those viruses that carried the S gene in the left to right orientation expressed the S gene. Three recombinant viruses were tested by oral immunization of pigs and both antibody response and virus shedding were monitored. None of the pigs showed clinical signs and the virus was recovered from rectal swabs until 6–7 days post-infection. Viruses expressing the S gene induced TGEV- and PAdV-5-specific virus-neutralizing antibodies. Moreover, TGEV-specific secretory IgA was detected in the small intestine and in the lungs of the immunized animals.

Introduction

Adenoviruses are potentially effective as vaccine vectors, especially when the development of mucosal immunity is an important consideration, as needed, for example, for the protection against transmissible gastroenteritis. However, adenoviruses suffer from having a limited capacity for foreign gene insertion.

Transmissible gastroenteritis virus (TGEV) is a coronavirus that can cause severe and often fatal diarrhoea in young pigs. The presence of TGEV-specific secretory (s)IgA antibodies in the colostrum and milk of the sows is critical for the survival of the infected piglets. Development of mucosal immunity to TGEV is crucial, not only because it protects the sow from infection but also because sIgA and the sIgA-producing plasma cells, which are transferred into the mammary glands, provide protection for suckling piglets as well.

The protective immunity is directed to the spike (S) protein (Garwes et al., 1978; Jimenez et al., 1986) and four major antigenic sites (A, B, C and D) have been described on the amino-terminal domain (Correa et al., 1990; Delmas et al., 1990). Virus-neutralizing antibodies are directed mainly to the A epitopes but to a lesser extent the D antigenic site is also involved. Earlier studies indicated that the intact globular N-terminal half of the protein is sufficient to achieve a protective immune response equivalent to that induced by the full S protein (Tuboly et al., 1995a).

Although there are several commercially available TGEV vaccines, either inactivated or attenuated, these do not fully protect piglets. Several attempts have been made to develop efficacious recombinant TGEV vaccines. The S gene was expressed in prokaryotic expression vectors (Smerdou et al., 1996), vaccinia virus (Hu et al., 1985), baculovirus (Godet et al., 1991; Tuboly et al., 1994) and most recently in plants (Gomez et al., 1998; Tuboly et al., 2000). The entire S gene and gene fragments of different sizes have also been expressed by human adenovirus (Torres et al., 1996). Hamsters immunized with these recombinant viruses elicited a strong TGEV-specific immune response. Data on the effectiveness of the viruses in swine are not clear and the results suggest that a species-specific porcine adenovirus vector could be more effective than a human adenovirus vector.

Since porcine adenoviruses (PAdVs) do not generally cause disease in swine and since PAdVs are being considered as virus vector vaccines (Tuboly et al., 1993), especially where a...
mucosal immune response is required, the genomes of several PAdVs have been extensively studied. To date, five PAdV serotypes have been described (Haig et al., 1964; Clarke et al., 1967; Kasza, 1966; Hirahara et al., 1990). The E3 region, the site most targeted for foreign gene insertion in adenovirus genomes, has been identified and thoroughly analysed for all five serotypes (PAdV-4, Kleiboeker, 1994; PAdV-3, Reddy et al., 1995; PAdV-1 and -2, Reddy et al., 1996; PAdV-5, Tuboly & Nagy, 2000). Recently PAdV-3 was developed into helper-dependent (Reddy et al., 1999a) and -independent expression vectors (Reddy et al., 1999b; Hammond et al., 2000). Vaccines of helper-independent virus vectors are more practical. To date, two virus genes have been expressed by PAdV-3. The gD gene of Aujeszky’s disease virus was inserted into the E3 region (Reddy et al., 1999b) and the E2 gene of classical swine fever virus was inserted near the right-hand terminus of the virus genome (Hammond et al., 2000). Although both recombinant PAdV-3 viruses expressed the inserted foreign gene, the widespread occurrence of PAdV-3 in swine populations may restrict the use of this serotype as a vaccine vector.

In contrast, the virus involved in the present study (PAdV-5) was originally isolated in Japan (Hirahara et al., 1990) and there are no further reports on the presence of PAdV-5 elsewhere around the world. Our earlier studies indicated that at least 60% of the E3 region was not essential for virus replication (Tuboly & Nagy, 2000), increasing the theoretical vector capacity of PAdV-5 to 2–9 kb, which is much larger than the size given for PAdV-3 (Reddy et al., 1999b).

The objective of this study was to construct stable recombinant PAdV-5 viruses expressing the TGEV S protein responsible for the induction of virus-neutralizing antibodies. Five different types of recombinant viruses were generated carrying either the entire or the amino-terminal half of the S gene. In order to determine the best configuration for the strongest possible expression without including additional promoter sequences, the genes were inserted in both orientations. Viruses with or without a deletion of the E3 region were generated and tested for the expression of the foreign gene. Viruses expressing the S gene were tested for their ability to induce S protein-specific antibodies in pigs, the natural host of both TGEV and PAdV-5.

Methods

**Cells, viruses, virus DNA and cDNA.** The HNF-70 strain of PAdV-5 and the cell culture-adapted Purdai-115 strain of TGEV were propagated in continuous swine testicle (ST) cells (McClurkin & Norman, 1986) as previously described (Tuboly et al., 1995b). Virus titrations, plaque purification and virus neutralization (VN) assays were also performed in ST cells as previously described (Tuboly et al., 1993).

Adenovirus DNA was extracted from PAdV-5-infected ST cells by the method of Hirt (1967) when extensive cytopathic effect (CPE) was seen. TGEV S gene cDNA synthesis and cloning have been described elsewhere (Tuboly et al., 1994).

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**Transfer vector construction.** Full-length genomic PAdV-5 DNA clones were constructed by homologous recombination in *E. coli* strain B5183 cells (Hanahan, 1983) as described (Degryse, 1996). The strategy for the construction of the recombinant transfer vectors is summarized in Fig. 1. Plasmid Rpac was generated by replacing the 1–9 kb SalI–HpaI fragment (spanning part of the pVIII protein and the majority of the E3 coding region) with a unique PacI restriction enzyme (RE) site. The MluI B fragment of PAdV-5 (Tuboly et al., 1995b) was used for the insertion of the S gene. Five different S gene-containing MluI B fragments were generated. One construct contained the entire E3 region while in the remaining four constructs, a 1/2 kb HincII–HpaI fragment was deleted in the E3 region. The five fragments generated were: (i) MluIB-2.2S, which contains the 2–2 kb S' end of the 4/4 kb S gene inserted into the HpaI site of the E3 region in left to right (l–r) orientation; (ii) ΔMluIB-2.2Sc, which contains the 1–2 kb HincII–HpaI fragment of the E3 region replaced by the 2–2 kb S’ fragment in l–r orientation; (iii) ΔMluIB-2.2Sr, which has the same deletion in the E3 as in the ΔMluIB-2.2Sc construct but with the 2–2 kb S gene inserted in the reverse (r–l) orientation; (iv) ΔMluIB-2x2.2S, which contains the 2–2 kb S gene inserted in both the l–r and the opposite r–l orientations as contiguous inverted repeats into the partially...
deleted E3 region; and (v) ΔMluIB-4.4S, which has the entire 4–4 kb S gene inserted into the partially deleted E3 region in 1–r orientation.

The transfer vectors were generated in bacteria by homologous recombination of the modified MluB fragments carrying the S gene and the RPac + genomic clone linearized with PacI. The recombinant clones were analysed and selected by standard miniprep and RE digestion methods (Sambrook et al., 1989). Large-scale DNA preparation of the clones selected for ST cell transformation was performed with the Concert nucleic acid purification system (Life Technologies), according to the instructions of the manufacturer.

DNA transfection and selection of recombinant viruses. Lipofectin-mediated (Life Technologies) ST cell transfections were performed as previously described (Tuboly & Nagy, 2000), following the instructions of the manufacturer. The transfected cells were covered with 0.7% agarose in DMEM supplemented with 10% foetal bovine serum. Plaque formation was monitored daily and ten individual plaques from each transfection were transferred to Eppendorf centrifuge tubes with 1 ml of DMEM on day 7 post-transfection. The tubes were frozen to −70 °C and thawed on ice. The contents were used for the inoculation of duplicate wells of ST cell monolayers in 6-well tissue culture plates.

The cell culture supernatant from each well was collected about 6–7 days after inoculation and stored at −70 °C until the next round of plaque purification. Cells were harvested for RE analysis of the DNA and for Western blotting. Only those viruses that contained the entire expected S gene insert (one from each lineage) were included in further rounds of plaque purification. Viruses selected after three rounds of such plaque purification were designated RPAdV-2.2S, ARPaDV-2.2Sc, ARPaDV-2x2.2S and ARPaDV-4.4S and were used for large-scale virus propagation.

Western blot analysis of recombinant S proteins. Wild-type and recombinant adenovirus-infected cells together with uninfected ST cells were harvested at the peak of CPE formation. The proteins were separated on 10% SDS–polyacrylamide gels as described (Laemmli, 1970) and transferred to nitrocellulose membranes (Sambrook et al., 1989). They were detected with TGEV-specific pig polyclonal antibodies (Tuboly et al., 1994) at a 1:500 dilution. The reaction was developed by the Boehringer Mannheim chemiluminescent detection kit according to the instructions of the manufacturer.

S gene mRNA time-course. ST cells grown in 6-well dishes were infected at an m.o.i. of 10 with the recombinant and wild-type viruses. RNA was extracted with the total RNA extraction kit (RNeasy, QIAGEN) according to the instructions of the manufacturer. The transfected cells were covered with 0.7% agarose in DMEM supplemented with 10% foetal bovine serum. Plaque formation was monitored daily and ten individual plaques from each transfection were transferred to Eppendorf centrifuge tubes with 1 ml of DMEM on day 7 post-transfection. The tubes were frozen to −70 °C and thawed on ice. The contents were used for the inoculation of duplicate wells of ST cell monolayers in 6-well tissue culture plates.

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Construction and selection of recombinant PAdV

Recombinant PAdVs were plaque-purified three times and the presence and orientation of the S gene were confirmed by RE analysis of the virus DNA (data not shown) after each round of plaque purification. The expression of the recombinant S protein was monitored by Western blot analysis. Table 1 summarizes the stability data of each recombinant virus in tissue culture.

Results

Transfer vectors

Five full-length genomic PAdV-5 clones were generated by recombination in E. coli strain BJ5183 cells, each one carrying either the full or a partial S gene. The structure of these vectors, derived from the MluB and ΔMluB clones, is shown in Fig. 1(A). The detailed RE analysis of the transfer vectors indicated that the orientation, location and size of the inserts were as expected.

Animal experiments. Fifteen Yorkshire piglets from a TGEV- and PAdV-5-seronegative herd were weaned 21 days after birth and divided into five groups and housed separately. One group received uninfected ST cell supernatant, one group was immunized with wild-type PAdV-5 and three groups were immunized with the selected recombinant viruses (RPAdV-2.2S, ARPaDV-2.2Sc and ARPaDV-2.2Sc). Each pig received a single oral dose of 1 ml with a virus titre of 5 × 10^8 p.f.u./ml. Blood samples were collected weekly and the clinical signs were monitored daily. The pigs were euthanized after 3 weeks and subjected to post-mortem examination. Contents from the small intestine and parts of the lungs were collected and processed as described (Tuboly et al., 1993) and then tested for the presence of virus and IgA antibodies. For antibody detection, the serum samples and the filtered intestinal and lung contents were heat-inactivated at 56 °C for 1 h. Samples were tested in a TGEV-specific IgG or IgA ELISA as described by Tuboly et al. (1993) and in a TGEV-specific VN microtitre assay (Tuboly et al., 2000). The PAdV-5-specific antibodies were also determined with a VN assay (Tuboly et al., 1993).

Rectal swabs were collected daily to monitor virus shedding. The swabs were processed as described (Tuboly et al., 1995 a) and the virus titres were determined on 96-well plates with ST cells. The viruses isolated at day 5 p.i. were pooled in each group and propagated in ST cells for DNA extraction and RE analysis of the virus DNA.
Table 1. Stability of the recombinant viruses

Results are expressed as the number of S gene (protein)-positive plaques/number of plaques tested. DNA profile (RE) tests and Western blots (Wb) of S protein expression were performed.

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<th>1 Wb</th>
<th>2 RE</th>
<th>2 Wb</th>
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Fig. 2. Northern blot analysis of S gene transcripts probed with a 32P-labelled 2.2 kb DNA fragment representing the 5’ end of the TGEV S gene. Numbers above the gel represent h p.i.; ST, swine testicle cell control; RPAdV-2.2S and ∆RPAdV-2.2Sc, recombinant viruses tested; Wt, wild-type PAadV-5; M, RNA molecular mass markers (kb).

S gene after transfection and the ratio remained low throughout further plaque purifications. In contrast with the rest of the recombinant viruses, the RE digests of the DNA always indicated that only part of the virus population from a single plaque carried the entire S gene, smaller DNA fragments also appeared even after the third plaque purification (data not shown). Similarly, many bands were observed in the Western blots of cells infected with these virus clones.

Expression of the S gene and S protein

S gene expression was monitored by Northern blot analysis of total RNA extracted at 2 h p.i. and every 4 h thereafter from recombinant virus-infected cells and blots were probed with radioactively-labelled 2–2 kb S gene DNA.

RPAdV-2.2S and ∆RPAdV-2.2Sc expressed TGEV S gene-specific mRNA at approximately the same level. The S gene mRNA synthesis in RPAdV-2.2S-infected cells was undetected during early times of virus replication and could be detected only at 18 h p.i., whereas S gene-specific mRNA appeared somewhat earlier in ∆RPAdV-2.2Sc-infected cells, at 14 h p.i. (Fig. 2).

No S gene-specific mRNA was detected in ∆RPAdV-2.2Sr-infected cells (data not shown). The temporal pattern of transcription in ∆RPAdV-2x2.2S-infected cells was similar to that for cells infected with ∆RPAdV-2.2Sc. However, Northern blot analysis of replicates of ∆RPAdV-4.4S-infected ST cell cultures indicated different sizes of transcripts and the ratios of the transcripts were not consistent (data not shown).

For Western blot analysis, cells infected with the different recombinant viruses were collected at 24 h p.i. RPAdV-2.2S and ∆RPAdV-2.2Sc expressed the S protein of the expected 110 kDa size (Fig. 3, lanes 2 and 3) and a similar result was obtained with the ∆RPAdV-2x2.2S recombinant virus (Fig. 3, lane 4). No S protein was detected in cells infected with ∆RPAdV-2.2Sr virus. S protein-specific bands with a wide range of sizes (30–220 kDa) were seen on the blots of samples collected from ∆RPAdV-4.4S-infected cells (Fig. 3, lane 5).

Animal experiments

Pigs orally inoculated with the recombinant viruses RPAdV-2.2S, ∆RPAdV-2.2Sc and ∆RPAdV-2.2Sr remained healthy throughout the experiment with no signs of diarrhoea.
Table 2. Virus shedding based on virus isolation from rectal swabs

Virus titres are expressed as log_{10} dilutions in 0.1 ml.

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Table 3. TGEV- and PAdV-5-specific antibody titres of pigs at 3 weeks post-immunization with recombinant PAdVs or PAdV-5 measured by VN and ELISA

Dilutions for VN start at 1:2 and dilutions for ELISA start at 1:10. Virus titres are expressed as log_{10} dilutions of samples. Mock-infected pigs were negative in all tests.

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<th>VN TGEV Serum IgG</th>
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or respiratory distress. The titre of virus in the rectal swabs collected daily was determined and the results are summarized in Table 2. Virus was detected from day 1 to 7 but was not detected in any of the samples by day 8 p.i. Virus was not recovered from the lungs or from the small intestine of the euthanized pigs at 3 weeks p.i. (data not shown). A sample was considered negative after three blind passages in tissue culture.

All three types of recombinant viruses isolated at day 5 p.i. were tested by RE analysis of the extracted DNA with several of the characteristic REs. The DNA fragment patterns of viruses recovered from inoculated pigs were indistinguishable from those observed for the inocula before the animal passages (data not shown), indicating that the recombinant viruses were stable.
ELISA and VN assays were conducted to detect TGEV- and PAdV-5-specific antibodies in the infected pigs. The results are summarized in Table 3.

VN tests of the sera collected at the end of the experiment showed relatively high TGEV-neutralizing titres (up to 1:64) in groups infected with RPAdV-2.2S and ∆RPAdV-2.2Sc. No TGEV-specific virus-neutralizing antibodies were detected in the samples from pigs infected with ∆RPAdV-2.2Sr or wild-type PAdV-5, or from the mock-infected group. Similar results were obtained in the TGEV-specific ELISA to detect serum IgG. PAdV-5-specific virus-neutralizing antibodies were present in the sera of all animals immunized with recombinant or wild-type PAdV-5 but there was no evidence of such antibodies in the mock-infected group. TGEV-specific antibodies of class A were detected in both the lungs and intestinal contents of the pigs immunized with RPAdV-2.2S and ∆RPAdV-2.2Sc. Intestinal sIgA was present in all of the animals by ELISA. However, the sIgA titres measured in the lungs were lower in all animals and pig no. 2 of the group infected with RPAdV-2.2S was negative.

Discussion

Human adenoviruses provide an efficient vector system for the delivery of porcine coronavirus antigens like those of the TGEV or porcine respiratory coronavirus S protein (Torres et al., 1996; Callebaut et al., 1996). The widespread use of human adenoviruses in domestic animals may be restricted, mainly because of safety concerns. Animal adenoviruses, however, are mostly species-specific, present negligible risk for humans or other animal species and replicate more efficiently than a human adenovirus in the native porcine host, thereby providing a safer and more efficient delivery system in animals.

PAdV-3 carrying the gD gene of Aujeszky’s disease virus (Reddy et al., 1999b) and the E2 gene of classical swine fever virus (Hammond et al., 2000) has already been developed as a recombinant virus vector. However, the widespread prevalence of PAdV-3 may be a limiting factor in their use as recombinant vaccines because of widespread pre-existing PAdV-3-neutralizing antibodies.

In contrast, PAdV-5, to our knowledge, is not present in pig populations and has been reported only once in Japan (Hirahara et al., 1990). The development of PAdV-5 into a recombinant TGEV vaccine is described in this paper. Five helper-independent recombinant porcine adenoviruses have been constructed and tested for their stability and their ability to express the entire or the 5’-2 kb half of the TGEV S gene. Although the genome size of RPAdV-2.2S was, together with the 2-2 kb foreign gene, 106-6% of the original wild-type genome neither the insert nor parts of the E3 region were lost during the plaque purifications or several virus replication cycles in the pig intestine. The insertion and stable maintenance of such a large foreign DNA is in accordance with the findings of Hammond et al. (2000), who increased the genome size of PAdV-3 to 106-8% of the original, despite earlier findings of a maximum of 105% for human adenoviruses (Bett et al., 1993).

One of our goals was to test whether it was necessary to include foreign gene promoter sequences upstream of the insert or if the native PAdV promoters were sufficient to express the gene. In one construct (RPAdV-2.2S) no E3 sequences were removed and the 2-2 kb S gene fragment was inserted in a l–r orientation near the 3’ end of the E3 region, more than 1-8 kb downstream of the putative E3 promoter (Tuboly & Nagy, 2000). S gene-specific transcripts were detected in Northern blots from 18 h p.i., reaching a peak between 18 and 24 h.p.i. The S protein was detected in Western blots, indicating that the native adenovirus promoters were sufficient for foreign gene expression (Torres et al., 1996).

As a result of the 1-2 kb deletion of the E3 region (Tuboly & Nagy, 2000), the rest of the recombinant viruses carried the S gene closer to the E3 promoter than in RPAdV-2.2S. Those recombinant viruses that had the insert in the l–r orientation (∆RPAdV-2.2Sc, ∆RPAdV-2x2.2S and ∆RPAdV-4.4S) started to express the gene at the end of the early replication stages, between 14 and 18 h.p.i., as detected by Northern blot analysis, whereas the virus with the S gene in reverse orientation (∆RPAdV-2.2Sr) showed no signs of S gene expression either in vitro (Northern and Western blots) or in vivo, as judged by the lack of TGEV-specific antibodies in the immunized pigs.

Those ∆RPAdVs carrying a single copy of the 2-2 kb S gene appeared to be stable immediately after the transfection and all plaques tested had the inserted gene of the expected size and at the expected position. ∆RPAdV-2x2.2S, with two sets of the 3’-truncated S gene, produced 7 out of 10 plaques that carried both inserts right after the transfection and became stable during further rounds of plaque purification. The virus did not lose any of the inserts or the PAdV sequences, as detected by RE analysis (data not shown). The size of the genome of this recombinant virus was 106-9% of the original genome size, exceeding the expected maximum of 106-8% (Hammond et al., 2000) described for PAdV-3. The ∆RPAdV-4.4S virus with the full-length S gene did not yield a stable lineage, despite several rounds of plaque purification of the positive viruses. The expected genome size of this virus was also 108-6% of the wild-type genome but unlike the ∆RPAdV-2x2.2S, parts or all of the insert or the PAdV genome were constantly being lost during virus replication. This phenomenon raised questions about current theories of adenovirus genome stability. According to our experiments, the size of the insert may not be the only important factor influencing the stability of the genome, the nature of the foreign transcript and protein may also play a role.

The recombinant viruses were analysed by Western blotting to determine the size of the recombinant proteins. All of the viruses with the gene in a l–r orientation expressed a protein of the predicted size. The estimated size of the S protein in RPAdV-2.2S, ∆RPAdV-2.2Sc and ∆RPAdV-2x2.2S
was 110 kDa. The ARPAvD-4.4S virus preparation also expressed the expected 200 kDa protein but smaller S protein fragments were also detected.

Direct measurement of the amount of recombinant proteins was not carried out, but from comparisons to known amounts of baculovirus- and transgenic plant-expressed S genes (Tuboly et al., 1994, 2000), it was estimated that approximately 5–10 μg S protein/10⁶ cells was obtained at 24 h p.i. This amount is in accordance with that of Torres et al. (1996) for the expression of TGEV S gene and gene fragments in a human adenovirus vector without the help of additional external promoters.

Three recombinant viruses were tested for their ability to induce TGEV-specific immune responses in pigs. Those viruses that carried the 2.2 kb S gene in a 1–r orientation induced a TGEV-specific immune response. It was concluded that a single oral dose of the recombinant virus was sufficient to induce both a systemic and a local humoral immune response. The antibodies induced by the recombinant viruses neutralized both PAdV-5 and TGEV. The presence of TGEV-specific IgA antibodies in the small intestine indicated that a local immune response, particularly important against TGEV, was also induced.

Although challenge experiments were not carried out, we conclude that recombinant PAdV-5 carrying the 2.2 kb S gene fragment could be a useful tool in the protection of swine herds against TGEV. It remains important to test the ability of these viruses to protect pigs from TGEV in field trials involving different age groups of pigs and TGEV strains of differing virulence.

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References


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