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Porcine adenovirus as a delivery system for swine vaccines and immunotherapeutics

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Abstract

Porcine adenovirus (PAdV) has many qualities which make it an ideal choice for use as a delivery vector in swine. It is a low grade pathogen, present almost world-wide in a number of serotypes varying in their virulence and tissue tropism, which may allow for serotype specific vaccine targeting. PAdV is species specific having only been isolated from swine, reducing the possibility of its spread to other animals or man following administration. When engineered to contain a foreign gene, recombinant PAdV (rPAdV) can be grown to high titres in tissue culture cells making it cheap to produce. Knowledge of the complete nucleotide sequence of the PAdV genome has enabled rationally directed insertions of foreign genes which remain stably inserted in the genome and can be expressed at high levels following delivery to the target host. Importantly, recombinant PAdV can be administered by injection or by the oral route in feed or drinking water. We have delivered a range of antigens and immunomodulatory molecules to commercially available pigs using rPAdV and found it to be a very effective delivery system. Significantly, recombinant PAdV serotype 3 is highly effective as a delivery vehicle even when administered in the face of high levels of artificially induced serotype specific neutralising antibody to the vector.

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1. Introduction

Adenoviruses are non-enveloped, icosahedral viruses with a diameter of about 70–100 nm. The genome (Fig. 1) consists of linear double-stranded DNA varying between 26 and 45 kilobase pairs (kbp) depending upon the species (Van Regenmortel et al., 2000, for review see Shenk, 1996). The Adenovirus family has recently been divided into four genera, the Mastadeno, Atadeno, Aviadeno and Siadenoviruses (Pringle, 1998; Van Regenmortel et al., 2000; Büchen-Osmond, 2003). Mastadenoviruses infect only mammals and the Aviadeno- noruses infect only avian species. However, specific members of the third and fourth genera have been shown to infect either mammals, birds, amphibians or reptiles. Analysis of genome arrangements and DNA sequence data has brought about this recent distinction of the Atadeno and Siadenoviruses. Bovine adenovirus (BAV) serotypes 4–8, ovine adenovirus (OAV) isolate 287 and egg drop syndrome (EDS) virus are all included in the Atadeno genus with Frog adenovirus 1 and Tur- key adenovirus A placed in the Siadenovirus genus (Benkő and Harrach, 1998; Benkő et al., 2000; Büchen- Osmond, 2003).

2. Porcine adenovirus

Porcine adenovirus (PAdV) was first isolated from a pig in England in 1964 (Haig et al., 1964). Since then the presence of PAdV in swine has been demonstrated virtually worldwide by virus isolation and serological surveys (Derbyshire, 1989). Depending upon serotype, PAdV can be grown in pig kidney, testis, retina and thyroid cells and in human kidney, canine melanoma and calf kidney cells (Dea and Elazhary, 1984; Kasza,
Porcine adenovirus is regarded as a low grade pathogen in domesticated pigs causing mild diarrhoea of short duration, but with no other clinical signs regularly associated with the infection. Notably, it has also been isolated from apparently healthy pigs with no clinical disease (Derbyshire et al., 1966; Mahnel and Bibrack, 1966; Rasmussen, 1969). Since most infections with PAdV are asymptomatic in pigs, it is not considered to be of economic importance in the pig industry. Porcine adenovirus has been isolated only from swine with no other hosts described, and it is probable that husbandry practices maintaining production pigs in close contact may facilitate the maintenance of PAdV in the herd via faecal–oral transmission.

Porcine adenovirus is classified within the genus *Mastadenovirus* in the *Adenoviridae* family. The non-enveloped icosahedral virus particle contains a double-stranded DNA genome of approximately 34 kbp (Van Regenmortel et al., 2000). Restriction enzyme mapping and sequence analysis of serotypes 1–5 have demonstrated that PAdVs 1–3 are closely related and that PAdVs 4 and 5 are less similar to them and to each other, but that all are distinct from human, murine, canine, bovine and fowl adenoviruses (Garwes and Xuan, 1989; Kleiboeker, 1994; Nagy et al., 2001; Reddy et al., 1996; Reddy et al., 1998).

**3. Recombinant adenoviruses**

Recombinant human adenoviruses have been utilised extensively as viral vectors in human vaccine delivery and in gene therapy (Berkner, 1988; Kovesdi et al., 1997; Miller, 1992). Additionally, a human adenovirus (HAV) serotype 5 recombinant expressing the spike glycoprotein (gpS) of porcine respiratory coronavirus (PRCV) was shown to induce an immune response against this foreign antigen in pigs (Callebaut et al., 1993, 1996). Subsequently, adenoviruses isolated from species other than man have generated increasing interest as delivery systems for use in both animals and man. Recombinant bovine (Mittal et al., 1995), ovine (Xu et al., 1997), canine (Klonjkowski et al., 1997) fowl (Sheppard et al., 1998) and porcine (Hammond et al., 2000) adenoviruses have all been described. Their use has ranged from the species specific delivery of vaccine antigens to cattle, chickens, dogs and pigs, to the delivery of cancer therapeutics in humans (Babiuk and Tikoo, 2000; Fischer et al., 2002; Hammond et al., 2000; Johnson et al., 2003; Voeks et al., 2002).

**4. Recombinant PAdV**

In a comparison of the ability of potential viral vectors to stimulate an immune response in pigs, Tuboly et al. (1993) showed that PAdV 3 could induce both systemic and mucosal antibody responses. Combining this observation with the demonstration that HAV5 could induce an immune response to a foreign antigen in pigs, it was
anticipated that PAdV would prove a more effective species specific vector for delivery of a variety of molecules to swine.

We have constructed and tested a number of recombinant PAdV vectors expressing both antigens and immunomodulatory molecules, such as cytokines, in outbred commercially available pigs and the results of our studies and others are presented in this review.

5. Construction of recombinant PAdVs

In our laboratory, recombinant PAdVs were constructed by insertion of a foreign gene into the right-hand end of the PAdV genome (Fig. 1). Expression of the foreign gene was directed by the viral major late promoter (MLP) which is the strongest adenoviral promoter. The porcine adenovirus MLP and tri-partite leader (TPL) sequences required for directing high levels of foreign gene expression were cloned and inserted into a PAdV expression vector. Foreign genes to be expressed were inserted between these elements and the SV40 PolyA signal sequence. The expression cassette was inserted into the right-hand end of the PAdV genome by homologous recombination and correct orientation confirmed by polymerase chain reaction (PCR) (Hammond et al., 2000). Using this method, no deletions in the PAdV genome are made resulting in the production of a fully replication competent recombinant virus, which may be an important consideration where the recombinant is to be used in outbred swine that may have had prior exposure to PAdV. The insertion made into our first rPAdV which included the E2/gp55 gene from classical swine fever virus (rPAdV-gp55) increased the genome length to 106.8% of wild type and exceeded the expected maximum insert size for a stable Mastadeno virus recombinant by almost 2% (Hammond et al., 2000). An even larger recombinant PAdV has since been constructed with a total genome size of 109.6% (Tuboly et al., 2001), however, prior to insertion of foreign genetic material in that case, a portion of the genome was deleted. Thus, rPAdV-gp55 contains the largest stable insertion that has been made into a non-deleted Mastadeno virus recombinant so far reported.

The Early 3 (E3) region of PAdV has been utilised as an insertion site for the construction of recombinant viruses following prior deletion of E3 viral DNA sequences (Reddy et al., 1999a; Reddy et al., 1999b; Tuboly et al., 2001). It is well known that the open reading frames present in the E3 region of human adenoviruses are not required for viral replication in vitro, but they are thought to play a major role in evasion of the host anti-viral immune response (Russell, 2000). Thus deletion of this region from PAdV recombinants could significantly alter the ability of the vector to replicate in vivo and consequently compromise its ability to successfully express a foreign gene. Also, the foreign genes that have so far been inserted into the PAdV E3 region have not been coupled with a specific PAdV promoter element and have relied solely upon endogenous PAdV promoters for their expression. Use of the MLP may be essential if high levels of gene expression over a broad time period are required, since the E3 promoter functions early and is overwhelmed later in the viral replication cycle (Shenk, 1996).

Reddy et al. (1999a,b) have constructed versions of PAdV3 both as helper dependent and independent vectors. The helper independent (replication competent) rPAdV3 vector contained a gene from Pseudorabies virus (PRV) and was constructed by insertion of the gD coding sequence into the partially deleted E3 region of PAdV3 (Reddy et al., 1999b). A vaccination trial with this recombinant virus in pigs is described below (Hammond et al., 2001a). The helper dependent vector was constructed by deletion of the PAdV3 E1 region, insertion in its place of a reporter gene, Green fluorescent protein (GFP), under the control of the cytomegalovirus immediate early promoter, with subsequent rescue of recombinant virus particles by growth in porcine cell lines expressing HAV-5 E1 proteins. This recombinant was shown to express the GFP gene, but as expected was unable to replicate in swine tissue culture cells without the provision of complementing genes from the human adenovirus E1 region. Such a vector may be of use in vaccination strategies where expression of a gene product is required, but where the spread of the vector within the host is undesirable, such as in specific targeting or gene therapy applications in non-target species rather than in swine. No animal studies have yet been published using this vector. Tuboly et al. (2001) constructed a recombinant PAdV serotype 5 expressing a portion of the S gene from Transmissible gastroenteritis virus (TGEV). Although they were not able to produce a recombinant virus that stably expressed the full length coding sequence of 4.4 kb, a recombinant virus containing a 2.2 kb fragment of the S gene was shown to express the truncated product in tissue culture. When administered orally to pigs, both serum and mucosal antibody responses to the S protein were detected. Sadly however, no TGEV challenge experiment was reported and therefore no protection data are available.

6. Delivery of vaccine antigens by recombinant PAdV

6.1. Classical swine fever virus gp55/E2 gene

Classical swine fever virus (CSFV) infection of pigs results in an acute, fatal disease with mortalities up to 100% in a susceptible population. Classical swine fever is
highly contagious, distributed almost world-wide and is considered the most economically important disease of swine in areas of intensive pig farming (Dahle and Liess, 1992).

We first demonstrated that PAdV was an effective vector for the delivery of vaccine antigens to swine when a single dose of a recombinant PAdV expressing a single gene (gp55/E2) from the CSFV ‘Weybridge’ strain was shown to protect pigs from CSFV challenge (Hammond et al., 2000). In this experiment, outbred commercially available Large White pigs were given a single dose of the recombinant virus (rPAdV-gp55) and monitored daily for any adverse reaction to vaccination. Following vaccination, pigs did not display any clinical disease with their temperatures remaining normal and their ability to thrive unimpeded (Hammond et al., 2000).

More recently, we have also found that vaccine virus cannot be re-isolated at any time after administration by passage of mucosal swab material in tissue culture (unpublished observations). When challenged subcutaneously (s.c.) with a dose of CSFV lethal to susceptible animals, all vaccinated pigs were protected from CSF whereas control pigs all developed severe clinical signs and were euthanased. The unvaccinated control pigs developed fever with temperatures rising above 40 °C by day 3 post challenge and remaining high until they were euthanased with severe disease. Vaccinated pigs, apart from a brief rise in temperature for 2–3 days, remained completely free from disease until termination of the experiment. At post-mortem, no internal signs of CSF lesions could be found and the spleens of vaccinated animals were shown to be free from CSFV antigen by ELISA, whereas those of control pigs all had high levels of CSFV antigen (Hammond et al., 2000). In further experiments, it was demonstrated that no CSF challenge virus could be re-isolated from an rPAdV-gp55 vaccinated group of pigs (Hammond et al., 2001b) suggesting that use of rPAdV-gp55 has the potential to reduce or prevent the common problem of CSFV shedding from vaccinates following challenge.

7. Prime-boost vaccination

Another vaccination strategy that has generated a lot of interest recently is that of a prime/boost regime, where a susceptible target animal is given one dose of a vaccine comprising, for example, a foreign gene expressed from a naked DNA plasmid (DNA vaccination) followed by a second booster dose expressing the same gene but via a different delivery system, such as from a recombinant virus (Ramsay et al., 1997). Such a regime has been shown to prime the immune system to respond to a greater degree following the administration of the booster dose, than when the two doses are delivered via the same route and/or vector.

With a prime/boost vaccination regime, recombinant PAdV-gp55 was shown to be highly effective at boosting the immune response to gp55 following initial priming with a DNA based gp55 vaccine and significantly improved the levels of protection from disease given by a single dose of the DNA vaccine alone (Hammond et al., 2001b). Indeed, the prime/boost regime completely abrogated the transient increase in temperature observed in challenged pigs vaccinated with a single dose of rPAdV-gp55 alone or two doses of the DNA vaccine.

8. Protection from “in contact” challenge

In more recent experiments we have utilised an “in contact” CSF challenge model designed to reproduce an infection more like that seen in the field, with experimentally infected pigs put in close contact with vaccinated and un-vaccinated control swine. One or two doses (21 days apart) of rPAdV-gp55 were administered to commercially available outbred pigs either s.c. or orally and their susceptibility to CSF determined. Control pigs given a lethal dose of CSFV and un-vaccinated “in contact” pigs all developed severe disease and were euthanased. Those pigs vaccinated s.c. with either a single or two doses of rPAdV-gp55 did not develop disease and were protected from the “in contact” challenge. Pigs given two doses s.c. also developed substantial CSFV specific serum neutralising antibodies prior to challenge. In contrast, pigs given a single oral dose of vaccine developed clinical CSF and were euthanased. However, a significant observation from this work was that pigs given two oral doses of rPAdV-gp55 were completely protected from disease even though they did not develop CSFV specific serum neutralising antibodies prior to challenge (Hammond et al., 2003; Johnson et al., 2000b). This result adds further weight to the argument that other immune mechanisms apart from serum neutralising antibodies are operating in protection from CSF, as we have previously suggested (Hammond et al., 2000, 2001c). These results also demonstrate that PAdV based vaccines are effective when delivered either by injection or by the oral route.

9. Pseudorabies virus (Aujeszky disease) gD gene

Pseudorabies virus (PRV) is an alpha herpesvirus which causes the economically important and widespread Aujeszky’s disease (AJD) in pigs (Mettenleiter, 2000). It is highly neurotropic causing nervous and respiratory complications in pigs, the natural host, and in a variety of other animal species (Kluge et al., 1992; Mulder et al., 1997). It is anticipated that the development of a live recombinant rPAdV-PRV vaccine would provide a safe alternative to live PRV vaccines, which,
since they are infectious, maintain the problems associated with live herpes virus vectors of reversion to virulence and/or latency (Kluge et al., 1992; Mulder et al., 1997). The gD gene from PRV was inserted into the E3 region of PAdV-3 (Reddy et al., 1999b), and the resultant recombinant virus (rPAdV-gD) tested in a vaccine trial to determine protection from challenge with PRV (Hammond et al., 2001a).

Five week old, commercially available Large White pigs were given either a single dose or two doses of rPAdV-gD 22 days apart. Pigs were monitored for the development of serum neutralizing antibodies to PRV and challenged three weeks after final vaccination. None of the pigs showed an adverse reaction to vaccination and prior to challenge, pigs given two doses had developed higher levels of PRV specific serum neutralizing antibody than those given a single dose (Hammond et al., 2001a).

Following PRV challenge, pigs were monitored for clinical signs of disease and their ability to thrive and condition recorded. All control animals became sick with elevated temperatures for six days post challenge, whereas vaccinates displayed an increase in temperature for only 2–3 days. Control pigs and those given a single dose all lost condition and their ability to thrive was impaired, but the group given two doses remained healthy (Fig. 2). At post-mortem, gross lesions of pneumonia were found in the lungs of controls and pigs given a single dose of vaccine but the lungs of pigs given two doses were free from disease (Fig. 2). Brain histology demonstrated a marked difference in the severity of infection and frequency of antigen detection, with control and single dose groups being most severely affected and pigs given two doses the least.

Since the gD gene was not specifically linked to a PAdV promoter, it is quite probable that protection against AJD elicited by this recombinant could be improved by coupling the gD gene to the MLP and TPL sequences, similar to the expression system used in the CSFV vaccine experiments with rPAdV-gp55 (Hammond et al., 2000) as this would be expected to increase the level of expression of the protective gD antigen which may increase the magnitude of the immune response.

10. Delivery of therapeutics

Following the successful use of rPAdVs to deliver vaccine antigens to swine, we have since investigated the potential of rPAdV to deliver therapeutic molecules, such as porcine cytokines, to outbred pigs.

11. Delivery of cytokines

11.1. Porcine interferon-γ

Legislated, compulsory withdrawal of the use of in-feed antibiotics in animal feed is becoming more widespread. Therefore, it has become necessary to explore alternative safe and natural means of protecting pigs from diseases associated with intensive farming practices and the re-emergence of disease problems associated with free range piggeries. Cytokines are natural mediators of protective immune responses and as such may be ideal candidate replacements for antibiotics in animal feed (Blecha, 1991).

Interferon gamma (IFN-γ) is an important modulator of the innate and adaptive immune system and is of prime importance in the activation of T-cells and macrophages. It therefore plays a pivotal role in the generation of protective immune responses to many viral and bacterial infections (for review see Samuel, 2001).

Previously, trials in broiler chickens with a recombinant fowl adenovirus expressing the chicken IFN-γ gene demonstrated that treated birds showed increased weight gain compared with untreated control birds (Johnson et al., 2000a; Johnson et al., 2000c). We were interested to determine whether treatment of outbred pigs with IFN-γ would show a similar effect. A recombinant porcine adenovirus expressing the porcine IFN-γ gene was constructed (rPAdV-IFN-γ) and shown to be positive for expression of IFN-γ by bio-assay (data not shown). This rPAdV was then tested in safety trials designed to detect recombinant virus shedding from vaccinated outbred pigs. No recombinant virus could be re-isolated following three serial passages of nasal or rectal swabs in tissue culture and no rPAdV DNA was detected by PAdV specific PCR on the passaged material (data not shown). In the first trial, 10 outbred five week old weaned pigs were inoculated s.c. with a single dose of 2 x 10⁸ TCID₅₀ rPAdV-IFN-γ. Groups of control pigs were either left untreated or

![Fig. 2. Assessment of pig condition following treatment with rPAdV-gD. The overall condition of each pig was assessed and scored on a scale of 1 (poor) to 10 (very good) prior to termination. At post-mortem, lungs were examined for disease pathology and scored for the presence of pneumonia from 0 (none) to 10 (severe). The mean scores for each treatment group are shown as a percent value. ■ = untreated, □ = single dose, □ = two doses.](image-url)
given an equivalent single dose of rPAdV-gp55 as a recombinant PAdV control. Pigs were fed a standard diet of commercially available medicated feed and weights recorded weekly. Mean weights for each group are shown in Table 1. A second trial was carried out using the same vaccination regime and experimental conditions as above, but in which pigs were fed a diet of non-medicated feed which did not contain added antibiotics. Weights were again recorded weekly and the mean weights for each group are shown in Table 1.

In both medicated and non-medicated feed trials it was demonstrated that treatment of pigs with a single dose of rPAdV-IFN-γ resulted in a higher proportion of pigs reaching a defined target weight by day 68, approximately the end of the post-weaning period. Both control groups, either untreated or treated with recombinant PAdV expressing the gp55 antigen, presented with similar, lower proportions of pigs reaching the weight target. Of great interest was the observation that in the non-medicated feed trial there was a 20–30% increase in the number of pigs reaching the target weights than in the control groups. Statistical analysis of the mean weights demonstrated that this increase was significant ($p < 0.05$) compared with both control groups.

These data indicated that treatment of pigs with one dose of rPAdV-IFN-γ at weaning results in significant weight gains to the end of the post-weaning period of the order of 7–8%, both in pigs fed on medicated or non-medicated feed.

A further important observation was that pigs treated with one dose of rPAdV-IFN-γ were more similar in their growth rates throughout the recording period and showed reduced weight variation between individual animals compared with both groups of controls. The implication of this observation is that pigs treated with rPAdV-IFN-γ will be more uniform in their growth rate and reach target weights sooner than untreated groups. Further large scale studies are now required to determine the potential of rPAdV-IFN-γ for use in commercial piggeries as a replacement for in-feed antibiotics.

### 12. Porcine granulocyte colony stimulating factor

Granulocyte colony stimulating factor (G-CSF) is an inducer of polymorphonuclear leukocytes (PMNL) including basophils, eosinophils and predominantly neutrophils, which play a vital role in equipping the innate arm of the immune system to provide the first line of defence against pathogenic bacteria, fungi and parasites (Hubel et al., 2002). G-CSF regulates the normal development of PMNL by directing their maturation and functional capacity and is registered by the Food and Drug Administration in the USA for use in humans to treat patients suffering from neutropenia caused by immunosuppressive therapy or treatment with anti-cancer drugs. G-CSF promotes the production of PMNL thus increasing the patients ability to resolve associated bacterial or fungal infections.

A recombinant porcine adenovirus expressing the porcine G-CSF gene was constructed (rPAdV-G-CSF) (Johnson et al., 2000a). This construct was also tested in safety trials designed to detect recombinant virus shedding from vaccinated pigs. No recombinant virus was re-isolated following three serial passages of nasal or rectal swabs in tissue culture and no rPAdV DNA was detected by PAdV specific PCR on the passaged material (data not shown). Two groups of six outbred 5-week old pigs were housed in adjoining pens. All pigs were bled daily from day 0 (14 days before vaccination) until the end of the experiment (day 37) and their white blood cell counts recorded. One group was vaccinated s.c. with $1 \times 10^5$ TCID$_{50}$ rPAdV-G-CSF on day 14. The second group of six pigs were left untreated. At day 14 post vaccination all pigs were challenged intratracheally with $10^6$ colony forming units/mL of Actinobacillus pleuroneumoniae (HS93 serovar 7) (APP) and monitored daily for clinical signs of APP. Post-mortems were carried out on all pigs on day 12 post-challenge with APP lung lesion scores recorded and general condition noted.

| Group         | Medicated feed | Non-medicated feed |
|--------------|----------------|--------------------|
|              | Day 54 | Day 61 | Day 68 | Day 54 | Day 61 | Day 68 |
| Untreated    | 0%     | 60%    | 40%    | 10%    | 60%    | 60%    |
| rPAdV-gp55   | 0%     | 60%    | 40%    | 10%    | 50%    | 50%    |
| rPAdV-IFN-γ  | 0%     | 60%    | 60%    | 0%     | 90%    | 80%    |

Groups of control pigs were either left untreated or given an equivalent single dose of rPAdV-gp55 as a recombinant PAdV control. Test pigs were given a single dose of rPAdV-IFN-γ. Treatment was given on day 33. Pigs were fed as indicated and weights recorded weekly. Mean weights at 54, 61 and 68 days of age for each group are shown.
13. White blood cell analysis

Up to the day of APP challenge there was no significant difference in overall white blood cell (wbc) counts between the untreated control pigs and those vaccinated with rPAdV-G-CSF. Surprisingly, no increase in peripheral blood neutrophil numbers was observed in the recombinant treated group. However, following challenge with APP, significant differences were observed between the two groups (Fig. 3). Control challenged pigs demonstrated a sharp increase in peripheral blood neutrophils which peaked at 24 h post infection, dropped considerably and then rose to a second peak at eight days post challenge and remained high until termination of the experiment. This significant increase in circulating neutrophils is a typical response observed following experimental infection of pigs with APP (Bosse et al., 2002). In contrast, rPAdV-G-CSF treated pigs showed a delayed and more sustained increase in neutrophil levels that did not peak until 96 h post challenge (Fig. 3). This peak was significantly lower than that of the control pigs and once levels had decreased they did not rise again unlike the untreated group.

Fig. 3. Percent change of white blood cell populations in rPAdV-G-CSF treated pigs. Two groups of six pigs were bled daily for 14 days and their white blood cell counts recorded. On day 14, one group was vaccinated sub-cutaneously with $1 \times 10^5$ TCID$_{50}$ rPAdV-G-CSF. All pigs were then bled daily for a further 14 days. At day 28 post vaccination, all pigs were challenged intratracheally with $1 \times 10^6$ colony forming units/mL of *Actinobacillus pleuromeumoniae* (HS93 serovar 7). The trial was terminated on day 12 post-challenge with overall condition of pigs and APP lung lesion scores at post-mortem recorded. White blood cell types as follows; $\bullet$ = Total white blood cells, $\blacksquare$ = neutrophils, $\triangle$ = lymphocytes, $\chi$ = monocytes, $\star$ = eosinophils. Panels: (a) = rPAdV-G-CSF treated, (b) = untreated.
It is probable that the increase in peripheral blood neutrophil levels was a direct result of the APP challenge and that these neutrophils were being recruited to the lungs in order to combat the infection, although no histology was carried out on lung material. Analysis of the wbc populations of peripheral blood from day 0 when daily bleeding of pigs began, demonstrated generalised daily fluctuations across all cell types from both groups. However, treatment with rPAdV-G-CSF appeared to reduce this fluctuation with far less variation in wbc counts observed from the day of administration when compared with the untreated control group (Fig. 3). This effect was also observed following challenge with APP, where increases in wbc populations including PMNL were less substantial and more tightly constrained in treated pigs than in controls.

Upon termination of the experiment at 12 days post challenge, the condition of all pigs was recorded and scored and at post-mortem all pigs were examined for the presence of APP lesions in the lungs. The condition of all control pigs was very poor whereas all rPAdV-G-CSF treated pigs were in good condition showing no clinical signs of disease. At post-mortem, all control pigs showed large numbers of lesions in the lungs, whereas in contrast, all pigs treated with rPAdV-G-CSF had a significantly reduced lesion load (Table 2). A comparison of the lesion/lobe scores (Moore et al., 2001) for the two groups demonstrated that there was a substantial overall reduction of 94.5% in the clinical score for the rPAdV-G-CSF treated group compared with the untreated controls. A group of control pigs given an equivalent dose of wild type PAdV3 vector did not show any significant reduction in lesion/lobe scores compared with untreated controls (data not shown). This significant result clearly demonstrated that a single dose of rPAdV-G-CSF was able to give considerable protection against a severe experimental challenge with APP, confirming that a recombinant PAdV expressing G-CSF is a potent inhibitor of the bacterial infection APP in pigs.

14. The presence of pre-existing PAdV antibodies in pigs

Porcine adenovirus is thought to be ubiquitous in swine populations around the world (Derbyshire, 1989), with the predominant serotype being PAdV3. Consequently concern has been expressed that PAdV3 would not be a suitable delivery vector due to the potential for pre-existing neutralising antibodies in swine to interfere with recombinant virus “take” in the host (Nagy et al., 2001). PAdV has been isolated from a pig in Queensland, Australia (Kwon and Spradbrow, 1971) and it has previously been reported that the majority of pigs used in the rPAdV-gp55 vaccine trials in Australia were seropositive for neutralising antibody to PAdV3 before vaccination, but that the vaccine was still effective at preventing CSF disease in those animals (Hammond et al., 2000, 2001b,c). Subsequently, in a survey of over 700 pig sera collected from various locations around Australia, we have shown that at a single dilution of 1/8 over 90% of swine tested had specific serum neutralising antibodies to PAdV3 (Table 3). A more detailed analysis

| Pig number | Controls | APP lesion score in the lungs of challenged pigs | rPAdV-G-CSF |
|------------|----------|-------------------------------------------------|-------------|
|            | % Disease (700%) | Lobes affected/7 | % Disease (700%) | Lobes affected/7 |
| Controls   |          |                                                 |             |
| 180        | 70       | 2                                               |             |
| 181        | 70       | 3                                               |             |
| 182        | 15       | 1                                               |             |
| 183        | 40       | 2                                               |             |
| 184        | 140      | 3                                               |             |
| 185        | 20       | 2                                               |             |
| Total      | 355      | 13                                              |             |

Lesion score 1: 355/4200 = 8.5%
Lesion score 2: 355/6 = 59.2
Lobe score: 13/42 × 59.2 = 18.3

rPAdV-G-CSF

| Pig number | % Disease (700%) | Lobes affected/7 |
|------------|-----------------|-----------------|
| 186        | 15              | 1               |
| 187        | 2               | 1               |
| 188        | 5               | 1               |
| 189        | 5               | 1               |
| 190        | 17              | 2               |
| 191        | 0               | 0               |
| Total      | 44              | 6               |

Lesion score 1: 44/4200 = 1.0%
Lesion score 2: 44/6 = 7.3
Lobe score: 6/42 × 7.3 = 1.0%

A group of six pigs was vaccinated subcutaneously with 1 × 10⁷ TCID₉₀ rPAdV-G-CSF on day 14 of the trial. A second group were left untreated. At day 14 post vaccination all pigs were challenged intra-tracheally with 10⁹ colony forming units/mL of APP and monitored daily for clinical signs of disease. On day 12 post infection post-mortems were carried out on all pigs with APP lung lesion scores recorded and general condition noted. Lesion score 1 is given as a percent per group: total %/(700 × N), lesion score 2 is given per pig, per group (total % disease/N) and lobe score is given as total lobes affected/ N(42) × lesion score 2.

Porcine adenovirus is thought to be ubiquitous in swine populations around the world (Derbyshire, 1989), with the predominant serotype being PAdV3. Consequently concern has been expressed that PAdV3 would not be a suitable delivery vector due to the potential for pre-existing neutralising antibodies in swine to interfere with recombinant virus “take” in the host (Nagy et al., 2001). PAdV has been isolated from a pig in Queensland, Australia (Kwon and Spradbrow, 1971) and it has previously been reported that the majority of pigs used in the rPAdV-gp55 vaccine trials in Australia were seropositive for neutralising antibody to PAdV3 before vaccination, but that the vaccine was still effective at
of a smaller sample set of these sera has shown that the titres range from 1/32 to >1/500, with the majority being >1/100 (Fig. 4).

We were interested to determine whether the presence of raised levels of serum neutralising antibodies to PAdV3 could interfere with the efficacy of the CSFV vaccine rPAdV-gp55. In a trial designed to address this question, nine 6-week old Large White pigs were given a s.c. injection of $1 \times 10^6$ wild type PAdV3 in order to induce specific PAdV3 neutralising antibodies. All pigs had low but specific serum neutralising antibody titres against PAdV at the start of the experiment but these increased up to >1/125 by day 35 post administration of PAdV3. Subsequently, three pigs received two doses of rPAdV-gp55 on days 21 and 42 post administration of PAdV3, respectively, three pigs received a single dose of rPAdV-gp55 on day 42 and three pigs were left un-vaccinated. The mean PAdV3 specific neutralising antibody titre of the sera from pigs given two doses of vaccine was >1/37 at the time of first vaccination (day 21) and >1/340 at the time of the second vaccination on day 42. The mean PAdV titre of those pigs given the single dose on day 42 was 1/256. All pigs were challenged with classical swine fever on day 63 post administration of PAdV3 and their response to challenge monitored.

The three un-vaccinated control pigs all developed high fever and related clinical signs of CSF and were euthanased (Fig. 5). The pigs in the single dose group showed a slight increase in temperature for 2–3 days post challenge which then returned to normal and the pigs did not show any other signs of CSF. This was exactly the same response as had been observed previously in CSFV vaccine trials carried out in pigs that had not been given PAdV3 (Hammond et al., 2000, 2001b,c). Pigs given two doses of rPAdV-gp55 did not develop fever nor did they show any signs of CSF. Examination of the spleens from challenged pigs demonstrated the presence of high levels of CSFV antigen in all three controls but none in any of the vaccinates. In addition, those pigs given two doses of rPAdV-gp55 showed a specific boosted level of pre-challenge CSFV neutralising antibody following the second dose of vaccine even in the face of specific neutralising antibody to the vector PAdV3. These results clearly demonstrate that recombinant PAdV serotype 3 is an extremely efficient vector for the delivery of vaccine antigens to swine, and that its efficacy is not inhibited by the presence of serotype specific neutralising antibody in outbred pigs.

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Fig. 4. The presence of neutralising antibodies to PAdV in Australian pig sera. Serum taken from pigs at various state locations around Australia was assayed by serum neutralisation test for the presence of neutralising antibodies to PAdV3. Twelve samples from each state were titrated, except from NT where only five samples were available. Vic = Victoria, NSW = New South Wales, Q’land = Queensland, W.A. = Western Australia, S.A. = South Australia, Tas = Tasmania and NT = Northern Territory.
15. Prophylactic and therapotential

Taken together, the results of our studies strongly suggest that recombinant porcine adenovirus vectors based upon the PAβV3 serotype have the potential to be highly effective delivery vehicles for swine in the field for a wide variety of molecules. Delivery can be successfully achieved parenterally or by the oral route. This appears to be the case even if administered in the presence of considerable levels of specific neutralising antibody to the vector in the potential target host. For use as a delivery vector for therapeutic molecules, large scale trials will determine whether rPAβV-cytokine treatment of production animals can succeed as a natural replacement for in-feed antibiotics. However, our work does strongly suggest that delivery of a cytokine gene by a recombinant porcine adenovirus will prove a highly effective mechanism for introducing such therapotent molecules.

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