Severe acute respiratory syndrome vaccine efficacy in ferrets: whole killed virus and adenovirus-vectored vaccines

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Although the 2003 severe acute respiratory syndrome (SARS) outbreak was controlled, repeated transmission of SARS coronavirus (CoV) over several years makes the development of a SARS vaccine desirable. We performed a comparative evaluation of two SARS vaccines for their ability to protect against live SARS-CoV intranasal challenge in ferrets. Both the whole killed SARS-CoV vaccine (with and without alum) and adenovirus-based vectors encoding the nucleocapsid (N) and spike (S) protein induced neutralizing antibody responses and reduced viral replication and shedding in the upper respiratory tract and progression of virus to the lower respiratory tract. The vaccines also diminished haemorrhage in the thymus and reduced the severity and extent of pneumonia and damage to lung epithelium. However, despite high neutralizing antibody titres, protection was incomplete for all vaccine preparations and administration routes. Our data suggest that a combination of vaccine strategies may be required for effective protection from this pathogen. The ferret may be a good model for SARS-CoV infection because it is the only model that replicates the fever seen in human patients, as well as replicating other SARS disease features including infection by the respiratory route, clinical signs, viral replication in upper and lower respiratory tract and lung damage.

INTRODUCTION

Severe acute respiratory syndrome (SARS) caused 8098 reported cases and 774 deaths in 26 countries (WHO, 2004a) in a single autumn-to-spring period from 2002 to 2003, and had significant effects on the global economy. Serological evidence suggests zoonotic transmission of SARS coronavirus (CoV) into the human population for several years before this outbreak (Zheng et al., 2004); transmission to humans has continued, resulting in at least four independent non-laboratory associated cases in 2004 (Che et al., 2006; Fleck, 2004; Guan et al., 2005; WHO, 2004b). The aetiological agent of SARS has been identified as a novel human CoV by sequencing of its genome (Marra et al., 2003; Rota et al., 2003) and by experimental infection of macaques to fulfil Koch’s postulates (Foucher et al., 2003). It is of particular concern as a zoonosis because it can replicate in a large number of animals including cats, pigs, ferrets, foxes, monkeys and rats (Chen et al., 2005;
Martina et al., 2003; Wang et al., 2005) in addition to Chinese palm civets, raccoon dogs and bats (Guan et al., 2003; Lau et al., 2005). The positive-stranded RNA genome of SARS-CoV is 29.7 kb in length and contains 14 open reading frames (Marra et al., 2003; Rota et al., 2003).

Currently, there are no approved antiviral drugs that effectively target SARS-CoV; hence vaccination is the most likely mode of preventing SARS in people at highest risk. A successful SARS vaccine could be used prophylactically to protect healthcare workers, laboratory personnel and others in high-risk areas. No vaccines are currently licensed for any of the human CoVs, but experimental vaccines have been produced for a number of CoVs for use in chickens, cattle, dogs, cats and swine (Anton et al., 1996; Cavanagh, 2003; Olsen et al., 1993).

Several strategies may be considered for vaccination against SARS-CoV, including a whole killed virus (WKV) vaccine, a live attenuated SARS-CoV vaccine, a viral vector such as adenovirus (Ad) or vaccinia virus expressing SARS-CoV genes, recombinant SARS-CoV proteins or DNA vaccines (See et al., 2005). Live attenuated CoVs, killed CoVs, DNA vaccines and viral-vectorized vaccines have all been used to successfully vaccinate against animal CoVs (Cavanagh, 2003; Holmes, 2003; Navas-Martin & Weiss, 2004). Spike (S) protein has been shown to be involved in CoV pathogenesis and several groups have developed SARS vaccines based on the SARS-CoV S protein as a target (See et al., 2005). A DNA-based vaccine (Yang et al., 2004), a modified Ankara vaccinia virus (Bisht et al., 2004) and a recombinant attenuated parainfluenza virus (Bukreyev et al., 2004) have been shown to induce neutralizing antibodies and reduce pulmonary SARS-CoV replication. Studies from other animal CoV vaccines have shown that the CoV nucleocapsid (N) protein may represent another antigen candidate for vaccine development (Anton et al., 1996; Olsen, 1993). Although antibodies to CoV N proteins have no virus-neutralizing activity, there is evidence that the protein may provide protection in vivo by induction of cell-mediated immunity, although it has also been suggested to induce eosinophilic infiltrates resulting in immunopathology (Deming et al., 2006; Enjuanes et al., 1995; Stohlman et al., 1995; Wesseling et al., 1993). N protein has been shown to generate CoV-specific CD8+ T cells (Boots et al., 1991; Seo et al., 1997; Stohlman et al., 1993, 1995); it also provides protection in animals in response to infection by animal CoV (Collisson et al., 2000; Seo et al., 1997). In addition, vaccination with SARS N protein decreased replication of a vaccinia virus expressing SARS N protein in mice (Kim et al., 2004).

The SARS Accelerated Vaccine Initiative (SAVI) (Finlay et al., 2004) described the first head-to-head comparison of a WKV SARS-CoV vaccine and a combination of attenuated Ad expressing either S or N glycoproteins (Ad S/N) for their ability to protect against live SARS-CoV challenge in a mouse model (See et al., 2006). In the mouse model, the WKV vaccine, in the presence or absence of alum adjuvant, provided protection against live SARS-CoV challenge by the induction of high levels of neutralizing antibodies and reduced SARS-CoV load in the respiratory tract compared with mock-vaccinated mice. Furthermore, the WKV vaccine was more potent than the Ad S/N vaccine in reducing viral replication and in inducing neutralizing antibodies (See et al., 2006). One limitation of mouse models is that they do not reproduce the clinical signs and severe disease of SARS in humans (Glass et al., 2004; Subbarao et al., 2004) unless aged mice are used (Rockx et al., 2007). Ferrets have been used widely for the study of influenza and are susceptible to SARS-CoV infection, exhibiting lung pathology and virus shedding (Martina et al., 2003; ter Meulen et al., 2004). One ferret study indicated that, upon intranasal (i.n.) administration of SARS-CoV Toronto 2 (Tor2) strain, no clinical signs were observed up to 29 days post-challenge, although viral RNA could be detected in pharyngeal swabs (Czub et al., 2005; Weingartl et al., 2004). Our preliminary studies showed clinical signs, viral replication and lung pathology in ferrets infected with SARS-CoV reflecting pathogenesis in humans (To et al., 2004); we therefore decided to evaluate and compare our two SARS vaccine candidates (WKV and Ad expressing S and N protein) in a single trial in the ferret model (Kobinger et al., 2007). This is the first report of two SARS vaccine formats tested directly against each other in both mice (See et al., 2006) and ferrets.

**METHODS**

**Ferrets.** Castrated, descent male fitch ferrets (8–10 months of age) weighing between 800 and 1200 g (Marshall Farms) were pair-housed in stainless steel rabbit cages, with food and water ad libitum. Animals were housed in an AAALAC-accredited facility. All procedures were in accordance with the NRC Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act, and the CDC–NIH Biosafety in Microbiological and Biomedical Laboratories. In addition, all procedures were approved by the Southern Research Institute Institutional Animal Care and Use Committee and the Biosafety Committee.

**Vaccines.** For WKV, the SARS-CoV Tor2 strain was passaged in Vero-E6 cells, purified by ultracentrifugation and subsequently inactivated with β-propiolactone (See et al., 2006). The Ad-based vectors, one expressing the N and the other expressing the S protein, were produced by the McMaster University Vector Facility (See et al., 2007). Expression of the SARS-CoV N or S protein was confirmed by Western blot analysis of lysates from human embryonic kidney (HEK) 293 or HeLa cells infected with the Ad-based vector for 24 h, using convalescent sera from SARS patients as a source of antibodies.

**Ferret vaccination.** Ferrets (groups of eight) were anaesthetized with ketamine (25 mg kg⁻¹), xylazine (2 mg kg⁻¹) and atropine (0.05 mg kg⁻¹). Ferrets received 50 μg WKV vaccine in 0.2 ml, 50 μg WKV plus 500 μg Alhydrogel in 0.2 ml and 0.2 ml PBS subcutaneously. For Ad vectors, 1 × 10⁶ p.f.u. of each virus (Ad-S and Ad-N vaccine) in a total volume of 500 μl PBS was administered i.m. or in 100 μl intramuscularly (i.m.) into the hind leg or 2 × 10⁶ p.f.u. of the AdS virus stock (Ad-Ctrl) i.n.

**Challenge studies.** Ferrets were anaesthetized as above and challenged with 10⁶ p.f.u. SARS-CoV Tor2 in 0.5 ml PBS i.n. at
7 weeks from the initial immunization. Clinical signs such as temperature (measured with a subcutaneous implantable temperature transponder), sneezing, inappetence, dyspnoea and level of activity were assessed daily until sacrifice.

Collection of ferret nasal wash, blood and tissue samples. Samples were collected and processed according to previously described procedures (Zitzow et al., 2002). Nasal washes were collected post-challenge on days 1, 2 and 5. Ferrets were sedated with ketamine (25 mg kg\(^{-1}\)); 0.5 ml sterile PBS containing 1 % BSA, penicillin (100 U ml\(^{-1}\)), streptomycin (100 µg ml\(^{-1}\)) and gentamicin (50 µg ml\(^{-1}\)) was injected into each nostril. Blood was collected from the anterior vena cava in heparanized tubes. Ferrets were euthanized by intracardiac injection of Euthanasia V solution (1 ml per 10 kg of body weight) at an early time point, day 2, and at late time points, day 5 for the Ad vaccine groups and day 6 for PBS and WKV groups. Lungs were collected and lavaged and samples were either frozen on dry ice for virus isolation or placed in formalin for histological analyses. Approximately 2 g lung tissue (collected from each lobe) was homogenized in 2 ml PBS.

Virus titration. Virus titres were determined as previously described (Guo et al., 2004; Schmidt & Emmons, 1989). Duplicate 1/2 log serial dilutions of samples were prepared in 96-well plates and added to confluent Vero E6 monolayers. Cells were incubated at 37 °C, 5 % CO\(_2\) and observed daily for the appearance of virus-specific cytopathic effect (CPE). Virus titre was calculated using the method described by Reed & Muench (1938) and reported as log\(_{10}\) ml\(^{-1}\) TCID\(_{50}\) (g tissue\(^{-1}\)).

Virus neutralization assay. Twofold dilutions of heat-inactivated serum (first dilution of 1 : 10) were tested for the ability to neutralize the infectivity of 100 TCID\(_{50}\) SARS-CoV in Vero cell monolayers as previously described (Zakhartchouk et al., 2005). The cytopathic effect was read on day 3. The dilution of sera that completely inhibited cytopathic effect in 50 % of the wells was calculated as described by Reed & Muench (1938).

Western blot analysis. Membranes were probed first with pooled ferret sera, 1 : 100, in TBS containing 5 % non-fat milk, followed by peroxidase-conjugated anti-ferret immunoglobulin secondary antibodies (1 : 20 000). The blots were processed for detection using the ECL Advanced Western Blotting Detection kit. Recombinant SARS-CoV N protein expressed in Escherichia coli and a truncated S protein (amino acids 318–510) expressed stably in a HEK 293 cell line, as previously described (See et al., 2006), were also used as antigens.

RT-PCR assay for SARS-CoV RNA. Fifty microlitres of lung homogenate was placed in TRizol reagent and processed as previously described (Hogan et al., 2004). RNA was isolated with the RNeasy kit (Qiagen) and tissue RNA was quantified with RiboGreen and treated with DNase. SARS-CoV genome (samples were normalized for quantity of RNA) was detected with LUX primer sets for SARS-CoV N-domain and the Superscript III platinum one-step quantitative RT-PCR system as described by Hogan et al. (2004).

Histology. The cranial and middle lobes of ferret lungs were removed at necropsy and immersed in formalin for 3 days. Tissues were rinsed in tap water and stored in 70 % ethanol. A representative sample of tissue was cut and embedded in paraffin. Tissue sections of 5 µm were prepared on glass slides and baked at 60 °C. Sections were stained with haematoxylin and eosin or the periodic acid–Schiff (PAS) stain.

Statistical analyses. Statistical significance was assessed using the non-parametric Wilcoxon rank-sum test (Hollander & Wolfe, 1973). Differences between mean values for the vaccine groups were considered significant if the P value (two-tailed) was < 0.05. The Fisher exact test was used to determine significance between groups of infected animals for Table 1.

RESULTS

Antibody responses

Ferrets were vaccinated with a combination of recombinant live Ad expressing either the full-length SARS-CoV S or N proteins (collectively called Ad-S/N) or with WKV vaccine on day 0, followed by a boost at 4 weeks. Ferrets (eight per group) were immunized with WKV (± alum) or PBS subcutaneously (s.c.), or with Ad-S/N i.n. or i.m., or control Ad 5 (Ad-Ctrl) i.n. Fig. 1 shows that ferrets vaccinated with WKV vaccine or Ad-S/N i.m. had significant increases in serum neutralizing antibodies (P < 0.05). Neutralizing antibodies were detected within four weeks, and increased by 7 weeks after a boost at week 4. There was not a robust increase in antibody titre after boosting with the Ad vectors, possibly because an anti-adenoavirus immune response prevented boosting efficacy. Addition of alum to the WKV vaccine preparation significantly increased neutralizing antibody titres by an average of 12-fold. Serum antibody responses in ferrets vaccinated with Ad-S/N i.n. were only detectable in 3/4 ferrets and all titres were less than 80. At 7 weeks, the titres were 15-fold higher in ferrets vaccinated with WKV plus alum compared with animals vaccinated with Ad-S/N i.m.

Western blots (data not shown) showed that only SARS-vaccine-immunized ferret sera recognized proteins from SARS-CoV-infected cell lysates corresponding to the molecular masses of N and S proteins (Ying et al., 2004) as well as of purified expressed N and S protein (See et al., 2006).

Clinical signs

All groups of ferrets showed clinical signs after challenge, indicating that ferrets became infected with SARS-CoV and that none of the vaccines tested blocked infection. All ferrets had increased temperatures after challenge, but significant differences between groups were not seen.

Table 1. Percentage (number) of animals in which infectious virus was detected

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Route of immunization</th>
<th>Nasal wash</th>
<th>Lung samples*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-Ctrl</td>
<td>i.n.</td>
<td>100 (7/7)</td>
<td>43 (3/7)</td>
</tr>
<tr>
<td>Ad-S/N</td>
<td>i.n.</td>
<td>88 (7/8)</td>
<td>13 (1/8)</td>
</tr>
<tr>
<td>Ad-S/N</td>
<td>i.m.</td>
<td>100 (8/8)</td>
<td>38 (3/8)</td>
</tr>
<tr>
<td>PBS</td>
<td>s.c.</td>
<td>100 (7/7)</td>
<td>86 (6/7)</td>
</tr>
<tr>
<td>WKV</td>
<td>s.c.</td>
<td>100 (8/8)</td>
<td>63 (5/8)</td>
</tr>
<tr>
<td>WKV + alum</td>
<td>s.c.</td>
<td>100 (8/8)</td>
<td>75 (6/8)</td>
</tr>
</tbody>
</table>

*Combined data from lung homogenate and BAL titres from all days.
Temperatures usually increased by 1–2 °C (range 1–2.5 °C); on average, they peaked at day 2 (occasionally the temperature of an individual ferret peaked on day 3). Serous nasal discharge was apparent by day 1 post-challenge in all groups and continued intermittently until sacrifice, but there was no sustained difference in discharge patterns between vaccinated and control groups. Sneezing was also apparent in challenged ferrets, but again there was no discernible pattern that suggested protection. The two groups that had been previously treated i.n. with Ad vectors (Ad-S/N and Ad-Ctrl) had fewer animals with discharge and lower levels of sneezing; this may reflect changes in nasal mucosa due to adenovirus, as discussed below. Ferret weights did not change significantly over this short challenge period, and there was no diarrhoea. Similarly, activity scores showed no consistent significant difference between groups.

**Evidence of protection**

While infectious virus could be detected in the upper respiratory tract using nasal washes in all groups (45/46 animals), the SARS-CoV antigen-vaccinated groups had fewer animals with detectable live virus in the lower respiratory tract and lungs. Table 1 summarizes the number and percentage of animals from which infectious virus could be detected in the various vaccine groups, on all days, in bronchoalveolar lavages (BAL) and lung homogenate. In the Ad-Ctrl i.n. group, 43 % (3 of 7) of animals had detectable infectious virus in the lungs, compared with only 13 % (1 of 8) of the Ad-S/N i.n.-vaccinated animals. These data suggest that an effective mucosal vaccination may decrease progression of SARS-CoV infections from upper to lower respiratory tract. In addition, the only animal that did not have detectable nasal shedding of live virus was an Ad-S/N i.n.-vaccinated ferret, suggesting that primary mucosal vaccination may be important in controlling shedding and hence viral spread. WKV vaccine also decreased the incidence of detectable virus in lungs from 86 % in the PBS control groups to 63–75 % in WKV-vaccinated animals.

While we expected that Ad-Ctrl delivered i.n. would not protect against SARS challenge, we found that it did provide a measure of protection compared with PBS delivered s.c. (Table 1, Figs 2, 3 and 4). It has been hypothesized that infecting nasal mucosa twice with high doses of adenovirus changes mucosal architecture and its ability to be infected subsequently, a subject for further study. Therefore, we will also compare the Ad-S/N i.m. to PBS controls. When the Ad-S/N i.m. is compared to the PBS s.c., lung infection rates decrease from 86 to 38 %. Viewing the data in this light indicates that both routes of Ad-S/N administration decreased progression of SARS virus into the lungs; however, in the i.m. route it is not possible to say definitively that the adenovirus vectors containing the SARS S/N genes are protective without further controlled studies.

**Fig. 1.** Antibody responses of ferrets to vaccination with SARS vaccines. Ferrets (eight per group) were vaccinated with control adenovirus, Ad-S/N i.n. or Ad-S/N i.m. or WKV in the presence or absence of alum at 0 and 4 weeks and were challenged with SARS-CoV at 7 weeks. Neutralizing antibody titres in serum samples taken at 4 and 7 weeks post-vaccination (reciprocal log₂) are indicated as means±s.d. *, Values with statistical significance (P<0.05) compared with control.

**Fig. 2.** Vaccination reduces SARS-CoV in nasal washes. (a) Groups of eight ferrets were vaccinated and challenged with SARS-CoV. Nasal washes were collected on days 1, 2 and 5 post-challenge for 4 to 8 ferrets. Virus titres (means±s.d.) are expressed as log₁₀ TCID₅₀ ml⁻¹ for each vaccine group. (b) SARS-CoV RNA copy numbers in nasal washes collected as described above. Results are expressed as mean±s.d. P values (two-tailed) were determined by the non-parametric Wilcoxon rank-sum test.
Nasal virus shedding

Ferrets shed virus from the upper respiratory tract in a similar way to human SARS patients (Chan et al., 2004). As shown in Fig. 3(a), WKV-vaccinated ferrets had small but significant reductions in viral titres in nasal washes within the first 2 days post-challenge; alum further reduced viral load (Fig. 2a). The Ad-S/N i.n. vaccine was able to reduce the viral titre by 1000-fold \((P<0.002)\) on day 1 post-challenge and also on day 2. If the Ad-S/N i.m. is compared to the PBS control-vaccinated group, there is also a significant reduction in titre on day 1. SARS-CoV RNA levels were also significantly reduced in Ad-S/N groups on days 1 and 2 as well as the WKV+alum group on day 2 (Fig. 2b).

Virus in the lung

The WKV vaccine and Ad-S/N were also evaluated in ferrets for their ability to reduce viral loads in BAL and lung homogenates. Fig. 3(a) shows that the WKV vaccine, alone or with alum, reduced viral titres approximately 100-fold in BAL. At day 5 post-challenge, virus titres rose in the Ad-vaccinated animals, but both routes of Ad-S/N administration decreased virus titres relative to Ad-Ctrl i.n., although the difference did not reach statistical significance (Fig. 3a). The SARS-CoV RNA levels were also significantly reduced in Ad-S/N groups on days 1 and 2 as well as the WVK+alum group on day 2 (Fig. 2b).

Gross pathology

Analysis of the pathology of lungs and other potential target organs was undertaken to assess viral damage. Only two of the 46 spleens displayed unusual properties; one PBS-vaccinated ferrets and significantly lower in ferrets vaccinated with either WKV alone or in the presence of alum on both days 2 and 6 post-challenge (Fig. 3b). SARS-CoV RNA levels were lower in ferrets vaccinated with Ad-S/N i.n. or Ad-S/N i.m. compared with Ad-Ctrl, although differences were not statistically significant. Compared with PBS-treated animals, the Ad-S/N i.m. provides substantial and highly significant protection. Similar patterns of protection were seen in virus titres and RNA levels in lung homogenates (Fig. 4). Significant reduction of viral RNA was seen in WKV (±alum) on day 2 and day 6 and in Ad-S/N-vaccinated groups on day 2 and day 5 \((P<0.05)\). Data from Table 1 and Figs 2, 3 and 4 show that these vaccines decrease both the levels of virus present in the lungs and the number of animals with detectable virus.
spleen from an Ad-Ctrl i.n.-vaccinated ferret had visible nodules and one spleen was enlarged in the WKV group. SARS-CoV has been detected in human liver (Ding et al., 2004) and can cause liver damage (Guan et al., 2005). There was some change in appearance of the liver in six of the 46 animals in the six groups, namely a pale or tan colour in two animals in each of the PBS, WKV and WKV + alum groups. There was no apparent evidence of enhanced liver pathology in SARS-antigen-vaccinated groups as was reported previously for the modified vaccinia virus (MVA)-vectored SARS vaccines (Czub et al., 2005; Weingartl et al., 2004). Gross pathology indicated frequent significant haemorrhage in lung and mediastinum (the area between the lungs containing the heart, trachea, oesophagus, thymus and lymph nodes), specifically the thymus (Fig. 5), but other organs appeared largely normal. T cell lymphopenia has been reported in SARS-CoV infections of humans (Cui et al., 2003; Li et al., 2003) and in this study, many of the animals, especially in the control groups, had haemorrhage in the thymus (Fig. 5 and Table 2). Fig. 5(a) shows two lobes of the thymus with congestion and haemorrhage, typically beginning at the proximal region (arrow) on day 2 and progressing to the distal portion, as shown in Fig. 5(b), at later time points. These data suggest that SARS-CoV might attack immune organs directly, as this type of pathology is not seen with other viral respiratory infections such as influenza in ferrets (Zitzow et al., 2002). This might contribute to the high virulence of SARS. The mediastina showed thickening and congestion (Fig. 5c) and sometimes severe haemorrhage (Fig. 5d). Lungs showed diffuse haemorrhage (Fig. 5e) and foci of haemorrhage scattered over the lung (Fig. 5f). SARS vaccines reduced both the number of organs affected and the severity, particularly at later times (Table 2).

Lung pathology

Lungs were scored for gross pathology based on severity of damage and proportion of lung area affected on an increasing scale; this went from normal to darkening in colour to areas of haemorrhage (mottling) and finally to multifocal coalescing lesions (most severe disease). All challenge groups showed gross signs of lung damage and all SARS vaccines showed some level of protective efficacy, usually one or two category levels healthier than controls (Table 3). Despite high vaccine-induced antibody titres, none of the vaccines protected lungs completely from apparent gross pathology.

Histopathology

In order to view specific types of lung damage and infiltrating cells, lung samples were observed for histopathological changes. Histopathological analysis (Fig. 5) identified areas of lung damage in all groups; lesions found in the different groups were of a similar nature, but less frequent in SARS antigen-vaccinated groups, in agreement with gross pathology (Table 3). Common pathological features included perivascular and peribronchial inflammation, interstitial pneumonia, supplicative inflammation of the bronchiolos and alveoli (neutrophils) and alveolar oedema and inflammation (focal to diffuse, with lymphocytes and histiocytes and scattered foci of neutrophils). In Ad-Ctrl vector- or PBS-vaccinated animals at day 2 after SARS-CoV challenge, there was marked oedema and haemorrhage throughout the lung tissue and a notable presence of mononuclear cells and neutrophils throughout the parenchyma. There were also areas of dense inflammatory cell infiltrates. The alveolar epithelium was considerably damaged or absent and debris was apparent in the small and intermediate Airways with suggestion of bronchial and goblet cell hyperplasia (Fig. 5g–i), consistent with findings recently reported in ferrets 7 days after infection with a lower dose of SARS-CoV (Chu et al., 2008). The changes to the bronchial epithelium seen here at day 2 are also consistent with the prolonged bronchiolar hyperplasia seen in ferrets at 23 days post-infection, again with a lower dose of virus (Darnell et al., 2007). These data confirm that the ferret model shows significant lung damage with SARS-CoV challenge. By day 5/6, the intensity of inflammatory cell infiltrate had decreased markedly; there was some residual oedema and haemorrhage but there was evidence of lung epithelium recovery.

All types of lung damage were decreased in severity at day 2 by the WKV and the Ad-S/N vaccines, and the lung epithelium was markedly protected. There was a reduction in haemorrhage, oedema and alveolar and bronchiolar epithelial damage (compare controls in Fig. 5j and 5k with vaccine groups in Fig. 5l–o). Moreover, the alum-enhanced WKV vaccine (Fig. 5o) was more protective than the WKV vaccine alone (Fig. 5n) and both were slightly better at protection than the Ad-S/N immunizations, either i.n. (Fig. 5l) or i.m. (Fig. 5m). While there was not much difference in protection seen at day 2 between the two routes of immunization with Ad-S/N, by day 5, the i.n. administration route provided better protection from damage compared to the i.m. route (data not shown). This was not as apparent when scoring lungs on gross pathology (Table 3). At no time in any of the groups was there evidence of eosinophilic infiltration as previously reported in the mouse model of infection (Deming et al., 2006). Together these data confirm that both the WKV and the Ad-S/N vaccines decreased lung damage from SARS-CoV at days 2 and 5.

Interestingly, control-vaccinated animals had the most significant microscopic lung damage at day 2 and were recovering by day 5/6; however, SARS antigen-vaccinated animals showed marked protection on day 2, but with apparent continuing microscopic tissue damage at day 5/6 (although less damage than peak disease in control-vaccinated animals). Therefore, in future SARS-CoV ferret vaccine trials, later time points should also be assessed to explore this delay or possible prolongation in lung damage in order to ensure that there is no vaccine-induced enhancement of disease.
DISCUSSION

Our results show that vaccination of ferrets with either WKV or Ad-vectorized vaccines resulted in the generation of systemic neutralizing antibodies, significantly reduced viral shedding in the ferret upper respiratory tract, decreased viral titres and RNA levels in the lower respiratory tract and modified the progression of organ damage. The WKV + alum vaccine induced 15-fold higher serum neutralizing antibody titres than the other vaccines, but this vaccine did not provide universally better protection. While human antibodies to S protein have shown some protective effect against SARS in ferrets (ter Meulen et al., 2004), our results show that high systemic neutralizing
antibody may not provide adequate protection. The Ad-S/N i.n. was superior in controlling nasal replication and shedding of virus, suggesting that nasal mucosal immunity may have a prominent role in protection. However, protection was incomplete in all vaccine cases. These data suggest that the most efficacious vaccine strategy might be a combination of i.n. primary and systemic vaccination boost, since each delivers different aspects of protection. A heterologous vaccination system might be advantageous, particularly since the boost with Ad did not give a strong increase in antibody (Fig. 1). Perhaps Ad-S/N i.n. priming followed by a WKV systemic boost would be much more effective than either vaccine alone, because this would be expected to provide a good T-cell response, a robust local mucosal response (as shown in Fig. 2) and high neutralizing antibody titre (Fig. 1).

Ferret antibody responses were similar to responses in mice vaccinated with the same vaccines, except that alum showed a greater effect in ferrets than mice and the mouse systemic neutralizing antibody response to the Ad-vectored vaccines was higher than in ferrets (See et al., 2006). Live recombinant vaccines are expected to generate superior cytotoxic T-cell responses, but we were not able to assay T lymphocyte responses in ferrets because of the paucity of ferret reagents. In mice, gamma-interferon-secreting T-cell responses were similar for these vaccines (See et al., 2006).

Our ferret SARS-CoV challenge resulted in ferrets with clinical signs of infection (elevated temperature, nasal discharge and sneezing), in contrast to other reports using the same virus titre (Czub et al., 2005; ter Meulen et al., 2004; Weingartl et al., 2004). Our lab passed the Tor2 virus only once (a total of three passages) prior to challenge to avoid the accumulation of interfering particles or mutations due to tissue culture adaptation. The presence of defective interfering particles common in CoVs might have attenuated the infection in other published challenge studies. Alternatively, the ferrets used in this study were older, and age has been shown to be an important susceptibility factor in the mouse model (Rockx et al., 2007). No other animal, including cynomolgus macaques, has been reported to experience fever, which is the most common sign in human SARS-CoV infection (above 99 %) (Skowronski et al., 2005; Lawler et al., 2006). Thus, ferrets are a good model for SARS-CoV because they are susceptible to infection, support replication in the upper and lower respiratory tracts, develop clinical disease (including fever), shed virus from the upper airway and develop severe lung pathology; this is in agreement with ferret reagents. In mice, gamma-interferon-secreting T-cell responses were similar for these vaccines (See et al., 2006).

Table 2. Gross pathology of the thymus and mediastinum, indicating the percentage (number) of animals displaying the indicated change

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Route of immunization</th>
<th>Bloody thymus*</th>
<th>Mediastinum pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total (days 2–6)</td>
<td>Day 5/6 samples</td>
</tr>
<tr>
<td>Ad-Ctrl</td>
<td>i.n.</td>
<td>57 (4/7)</td>
<td>67 (2/3)</td>
</tr>
<tr>
<td>Ad-S/N</td>
<td>i.n.</td>
<td>13 (1/8)</td>
<td>25 (1/4)</td>
</tr>
<tr>
<td>Ad-S/N</td>
<td>i.m.</td>
<td>25 (2/8)</td>
<td>25 (1/4)</td>
</tr>
<tr>
<td>PBS</td>
<td>s.c.</td>
<td>43 (3/7)</td>
<td>67 (2/3)</td>
</tr>
<tr>
<td>WKV</td>
<td>s.c.</td>
<td>38 (3/8)</td>
<td>50 (2/4)</td>
</tr>
<tr>
<td>WKV + alum</td>
<td>s.c.</td>
<td>38 (3/8)</td>
<td>25 (1/4)</td>
</tr>
</tbody>
</table>

*Area of haemorrhage larger than 3 mm.
†Pathology was more severe in the PBS-vaccinated group.

Table 3. Gross lung pathology

Ferret lungs were analysed on the day of sacrifice and scored for pathology on an increasing scale from normal to multifocal lesions (most severe disease). The number of animals with each lung pathology are shown.

<table>
<thead>
<tr>
<th>Vaccine route:</th>
<th>WKV (s.c.)</th>
<th>WKV + Adj (s.c.)</th>
<th>PBS (s.c.)</th>
<th>Ad-S/N (i.n.)</th>
<th>Ad-S/N (i.m.)</th>
<th>Ad-Ctrl (i.n.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day post-challenge:</td>
<td>2 6</td>
<td>2 6</td>
<td>2 6</td>
<td>2 5</td>
<td>2 5</td>
<td>2 5</td>
</tr>
<tr>
<td>Normal</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Dark pink, dense</td>
<td>3 2</td>
<td>3 1</td>
<td>1 0</td>
<td>0 0</td>
<td>0 1</td>
<td>0 1</td>
</tr>
<tr>
<td>Some dark red</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
<td>0 1</td>
<td>1 0</td>
</tr>
<tr>
<td>Mottled or dark red</td>
<td>1 2</td>
<td>1 2</td>
<td>3 0</td>
<td>1 1</td>
<td>2 2</td>
<td>3 0</td>
</tr>
<tr>
<td>Multifocal lesions</td>
<td>0 0</td>
<td>0 0</td>
<td>0 3</td>
<td>0 3</td>
<td>0 0</td>
<td>0 3</td>
</tr>
</tbody>
</table>
another recent publication (Chu et al., 2008). Ferrets are also outbred, allowing the assessment of a range of individual responses that are documented in human SARS. Finally, the ferret model is a non-rodent model and, compared to non-human primates, they are less expensive, easier to handle and less dangerous. Disadvantages are that the ferret immune system is not well defined, there is a dearth of reagents and because they are outbred, larger numbers are needed to assess statistical significance.

Our results show that i.n. (prime and boost) administration of the control Ad affects the course of the SARS-CoV infection. Even though the boost vaccination and challenge period were separated by 3 weeks, it was noted that animals immunized with control Ad showed decreased viral replication in lungs when compared with that animals immunized with control PBS group. These data suggest that a non-specific response interferes with normal infection rates. We have seen similar results in mouse studies (See et al., 2006), and morphological examination of mouse nasal tissue post prime and boost i.n. with control Ad vector confirmed altered mucosal tissue (data not shown). In the current study, the Ad-Ctrl i.n. is clearly the appropriate control for the Ad-S/N i.n. vaccination; however, PBS injection would be a more appropriate control for the Ad-S/N i.m. vaccination, due to localized mucosal changes produced by repeated i.n. administration of Ad-Ctrl. Compared with PBS s.c., the Ad-S/N i.m. gave significant protection in both the upper and lower respiratory tract and protected these areas from tissue damage. Further experiments, including an Ad-Ctrl administered i.m., will be required to clarify these points.

This is the first report comparing efficacy of two SARS vaccines (whole killed and adenovirus-based) in ferrets, a relevant clinical model. While both vaccines provided some measure of protection, neither blocked the development of significant organ damage, suggesting that other strategies may be necessary to protect humans from SARS. Although all ferrets were sacrificed by day 6 post-challenge, our results did not suggest vaccine-induced immune enhancement of disease in any tissues, such as that observed with feline CoVs (Olsen, 1993; Olsen et al., 1993; Weiss & Scott, 1981). We noted a delay in histopathology in vaccinated groups, but no increase. The lack of vaccine enhancement of disease is further supported by a recent study in WKV-vaccinated and challenged ferrets that were studied for 3 weeks (Darnell et al., 2007). The lack of eosinophilic infiltration is consistent with other reports of ferret infections with SARS-CoV (Chu et al., 2008; Darnell et al., 2007) and differs from the mouse model, in which eosinophil accumulation appears a prominent feature (Czub et al., 2005; Deming et al., 2006). Perhaps this highlights an important intrinsic difference in the species response to virus infection. In future, a heterologous challenge with different SARS-CoV strains is also an important consideration (Rockx et al., 2007). In conclusion, this report adds to others (ter Meulen et al., 2004) that have suggested that a protective SARS vaccine should be possible; however, protection in ferrets (and humans) may be more difficult than the mouse models suggest.

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REFERENCES


