Reverse genetics-generated elastase-dependent swine influenza viruses are attenuated in pigs

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Influenza A virus causes significant morbidity in swine, resulting in a substantial economic burden. Swine influenza virus (SIV) infection also poses important human public health concerns. It has been shown that conversion of the haemagglutinin (HA) cleavage site from a trypsin-sensitive motif to an elastase-sensitive motif resulted in attenuated viruses in mouse models. However, application of this attenuation approach in a natural host has not been achieved yet. Here, we report that using reverse genetics, we generated two mutant SIVs derived from strain A/SW/SK/18789/02 (H1N1). Mutant A/SW/SK-R345V carries a mutation from arginine to valine at aa 345 of HA. Similarly, mutant A/SW/SK-R345A encodes alanine instead of arginine at aa 345 of HA. Our data showed that both mutants are solely dependent on neutrophil elastase cleavage in tissue culture. These tissue culture-grown mutant SIVs showed similar growth properties in terms of plaque size and growth kinetics to the wild-type virus. In addition, SIV mutants were able to maintain their genetic information after multiple passaging on MDCK cells. Furthermore, mutant SIVs were highly attenuated in pigs. Thus, these mutants may have the potential to serve as live attenuated vaccines.

INTRODUCTION

Swine influenza virus (SIV) is a member of the family Orthomyxoviridae, genus Influenza A (Lamb & Krug, 2001). SIV is the causative agent of swine influenza, a highly contagious, acute viral disease of swine; it induces an acute respiratory tract infection and lung lesions. After an incubation period of 24–72 h, the disease begins suddenly, often appearing in many animals in the herd at the same time. Infection is clinically characterized by a high fever, sneezing, rhinitis with nasal discharge, laboured abdominal breathing and bronchial rales at auscultation. In general, morbidity rates may approach 100%, while mortality is usually less than 1%. SIV infections can be associated with secondary bacterial/viral infections and reproductive disorders that can result in abortions. Along with porcine reproductive and respiratory syndrome virus, SIV contributes significantly to post-weaning respiratory disease, causing economic losses due to decreased body condition and an increase in the number of days needed to reach market weight. Currently, H1N1, H3N2 and H1N2 are the dominant subtypes that cause disease in the North American swine population (Olsen, 2002).

SIV infection also poses very important human public health concerns because it naturally infects pigs and can be transmitted to humans (Wells et al., 1991). Since pigs are able to support replication of swine, human and avian influenza viruses, it is very likely that genetic reassortments between these viruses could create novel influenza subtypes. Recently, avian/swine virus reassortant H2N3 influenza A viruses were isolated from diseased swine in the USA. The H2N3 virus has undergone some adaptation to the mammalian host and is able to transmit among pigs and ferrets (Ma et al., 2007). Data from SIV surveillance studies and characterization of influenza virus isolates from pigs are critical for understanding the understanding of long-term evolutionary and epidemiological patterns of human influenza and pandemics (Wells et al., 1991).

The genome of influenza A viruses consists of eight segmented RNAs of negative polarity. The crucial step for infection by influenza A virus is initial virus binding to the cells followed by receptor-mediated endocytosis and fusion of the viral envelope to endosomal membranes (Cross et al., 2001; Skehel & Wiley, 2000). Influenza A virus entry into cells is mediated by the viral surface glycoprotein haemagglutinin (HA). HA has three major roles during virus replication: (i) HA binds to sialic acid receptors on the cell surface; (ii) it allows penetration of the virus into the cytoplasm by mediating fusion between the viral and the endosomal membranes; and (iii) it is the main viral antigen against which neutralizing antibodies are produced (Lamb & Krug, 2001). HA is synthesized as a precursor, HA0, that consists of HA1 and HA2 (Skehel & Wiley, 2000). In order to be infectious, HA0 must be cleaved by host proteases into HA1 and HA2. Therefore, this process
is a crucial determinant of virus pathogenicity (Bosch et al., 1981; Klenc et al., 1975).

Multiple SIV subtypes continue to circulate in swine populations despite available vaccines. Current SIV vaccines are inactivated and their application does not provide the desired immune response and cross-protection against multiple antigenic SIV variants in the field. Application of cold-adapted, live attenuated influenza virus (LAIV) in humans and horses provided a significantly higher and more efficient immune response than killed influenza vaccines (Paillot et al., 2006). Although recent studies by Richt et al. (2006) showed that mutant SIV with a truncated NS1 protein was highly attenuated in pigs and conferred protection against swine influenza (Richt et al., 2006; Solorzano et al., 2005; Vincent et al., 2007), there is no commercially available LAIV for SIV in North America.

It has been shown that conversion of the HA cleavage site from a trypsin-sensitive motif to an elastase-sensitive motif resulted in attenuation of viruses in vivo (Gabriel et al., 2008; Stech et al., 2005). However, these studies were performed with mouse-adapted influenza virus or avian influenza virus in mouse models. Application of this attenuation approach in a natural host has not been achieved yet. Here, we report that using reverse genetics we generated two mutant SIVs derived from strain A/SW/SK/18789/02 (H1N1) (SIV/SK) (Karasin et al., 2004). The mutant SIVs encode modified HA, as such the original trypsin-specific arginine–glycine (Arg–Gly) cleavage site of HA (Garten et al., 1981; Lazarowitz et al., 1973) was replaced with the elastase-sensitive valine–glycine (Val–Gly) or alanine–glycine (Ala–Gly) site (Castillo et al., 1979; Gertler & Hofmann, 1970). These mutations resulted in the generation of HA glycoproteins that are resistant to activation during natural infection by trypsin-like proteases but can be readily activated by elastase in vitro. Furthermore, the mutant viruses are attenuated in pigs, suggesting that these genetically engineered SIVs have great potential to serve as LAIVs for SIV.

**METHODS**

**Cells and viruses.** Madin-Darby canine kidney (MDCK) cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). 293T (human embryonic kidney) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. The influenza A/Swine/Saskatchewan/18789/02 (H1N1) (SIV/SK) virus was obtained from the Prairie Diagnostic Services, Western College of Veterinary Medicine, University of Saskatchewan, Canada. SIV/SK influenza virus was propagated at 37°C in the allantoic cavities of 11-day-old embryonated chicken eggs. Virus titres were determined on MDCK cells by plaque assay as described previously (Shin et al., 2007c).

**Plasmids.** Viral RNA of SIV/SK was isolated from 600 μl allantoic fluid using the RNeasy kit (Qiagen). Viral RNA (0.04 μg) was reverse transcribed into cDNA using Uni12 primer (5’-AGCATAAGGAGG-3’) (Hoffmann et al., 2001) and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. cDNAs were then amplified by PCR using segment-specific primers (Hoffmann et al., 2001). All eight cDNA segments were individually cloned into vector pHW2000 (kindly provided by Drs E. Hoffmann and R. G. Webster; Hoffmann et al., 2000), resulting in constructs pHW-SIV/SK-PB2, pHW-SIV/SK-PB1, pHW-SIV/SK-PA, pHW-SIV/SK-HA, pHW-SIV/SK-NA, pHW-SIV/SK-M and pHW-SIV/SK-NS. Mutations in the HA coding sequence were introduced into pHW-SIV/SK-HA by site-directed mutagenesis as described previously (Shin et al., 2007a). Plasmid pHW-SIV/Ha-R345A, encoding mutant HA with Arg replaced by Val at aa 345, was generated using the primers 5’TGGCCATCCATTCAATCCGGT-AGGCCCTGTTTGGGACATTGCC-3’ and 5’-GGCAATTGCTCCAA-AACAGGCTCACGGATGTGAAGTGGCAC-3’. Similarly, plasmid pHW-SIV/Ha-R345V, encoding mutant HA with Arg replaced by Ala at aa 345, was generated using primers 5’TGGCCATCCATTCAATCCGGT-AGGCCCTGTTTGGGACATTGCC-3’ and 5’-GIATTGCC-TCCAAACAGTCCGGAGGATGTTGGGAC-3’. All of the above plasmids were sequenced to ensure that additional mutations were not introduced during PCR.

**Generation of viruses by reverse genetics.** Wild-type (WT) and mutant viruses were generated using an eight-plasmid reverse genetics system described by Hoffmann et al. (2000). Briefly, 293T and MDCK cells were co-cultured at the same density (2.5 × 10^5 cells per well) in a six-well plate and maintained in DMEM containing 10% FBS at 37°C, 5% CO₂ for 24 h. One hour prior to transfection, medium containing FBS was replaced with fresh Opti-MEM (Invitrogen). To rescue SIV/SK-WT, cells were transfected with eight plasmid constructs (pHW-SIV/SK-PB2, pHW-SIV/SK-PB1, pHW-SIV/SK-PA, pHW-SIV/SK-HA, pHW-SIV/SK-NA, pHW-SIV/SK-NS, pHW-SIV/SK-M and pHW-SIV/SK-NS) by TransIT-LT1 transfection reagent (Mirus). The viruses (rgSIV/SK-R345V and rgSIV/SK-R345A) containing mutations within the HA segment were generated in the same way but substituting pHW-SIV/HA with either pHW-SIV/Ha-R345V or pHW-SIV/Ha-R345A. After 6 h, the transfection mixture was replaced with 1 ml fresh Opti-MEM. Twenty-four hours post-transfection, Opti-MEM (1 ml) containing 0.4% BSA and 2 μg i-(toluene-4-sulphonamido)-2-phenyl ethyl chloromethyl ketone (TPCK)-treated trypsin ml⁻¹ (for WT virus), 1 μg human neutrophil elastase ml⁻¹ (for mutant viruses) or 10 μg porcine pancreatic elastase ml⁻¹ (for mutant viruses) (Serva Electrophoresis GmbH) was added to well. Supernatants were collected 72 h post-transfection.

**Western blot analysis.** Western blotting was performed as described previously (Shin et al., 2007b) with minor modifications. MDCK cells (7 × 10⁴) were plated into 35 mm dishes and were mock-infected or infected with influenza viruses at a determined m.o.i. At 8 h post-infection (p.i.), cell monolayers were lysed; 30 μg total protein was resolved on 10% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were probed with polyclonal antiserum against nucleoprotein (NP) (1:2000) or M1 (1:2000) antibody (raised in our lab) (Shin et al., 2007b) followed by incubation with alkaline phosphatase-conjugated anti-rabbit IgG (1:10 000) (Jackson ImmunoResearch Lab). The immunoblots were visualized by incubating with BCIP/NBT premix solution (Sigma).

**Virus purification.** To prepare virus stocks without any protease residues for animal experiments, we purified tissue culture-grown viruses (SIV/SK-WT, SIV/SK-R345V and SIV/SK-R345A). MDCK cells grown in 10 cm dishes were infected with the viruses. Cells were incubated in the presence of either TPCK-treated trypsin or human neutrophil elastase for 36–48 h in MEM supplemented with 0.2% BSA. Supernatants were harvested and cell debris was removed by centrifugation for 25 min, at 700 g. Viruses were pelleted by ultracentrifugation at 25 000 r.p.m. for 2.5 h at 10°C using a Beckman Coulter Allegra 6R centrifuge, rotor SW28. Pelleted viruses were resuspended in 1 ml TSE buffer (20 mM Tris pH 7.8, 150 mM NaCl pH 7.8, 2 mM EDTA pH 7.6) and were overlaid on a 30–60%
sucrose cushion and further centrifuged at 25000 r.p.m. for 2.5 h at 10 °C using the Beckman rotor SW41. The visible opalescent virus band on the boundary of 30 and 60% sucrose was harvested and stored at −80 °C. Virus titres were determined by plaque assay.

**Infection of pigs with SIV.** Thirty-five 4-week-old SIV-negative pigs were randomly selected and divided into seven groups with five pigs per group. Groups were housed separately in isolation rooms for 1 week prior to infection. At 5 weeks of age, pigs in group 1 were mock-infected intratracheally with 4 ml MEM, while pigs in the remaining groups were infected intratracheally with 4 ml MEM containing 1 × 10^6 p.f.u. ml^-1 or 1 × 10^5 p.f.u. ml^-1 of SIV/SK-WT, SIV/SK/02-R345V or SIV/SK/R345A (Table 1). Pigs in all groups were monitored daily for 5 days and then sacrificed. All animal experiments were conducted at the Vaccine and Infectious Disease Organization, University of Saskatchewan in accordance with the ethical guidelines of the University of Saskatchewan and the Canadian Council of Animal Care.

**Clinical observation and sampling.** Clinical signs including lethargy, apathy, inappetence, reluctance to move, coughing, sneezing, nasal discharge and laboured abdominal breathing were monitored for 5 days. Rectal temperatures were recorded daily and nasal swabs were taken from each pig and placed in 1.5 ml MEM containing antibiotic/antimycotic solution (Invitrogen) and were frozen at −80 °C until the study was completed.

**Necropsy and macroscopic examination of lungs.** Animals in all groups were euthanized 5 days p.i. by intravenous administration of euthanyl (25 mg sodium pentabarbitol ml^-1). At necropsy, lungs were removed in toto and evaluated to determine the percentage of the lung affected with purple–red, firm lesions that are typical of SIV infection. The percentage of areas affected with pneumonia was estimated visually for each lung lobe. Total percentage for the entire lung was calculated based on weight proportions of each lung lobe to the total lung volume (Richt et al., 2003). Tissue samples from the right apical, cardiac and diaphragmatic lobes were taken for virus isolation and histopathology examination.

**Virus titration from nasal swabs and lung tissue.** Lung tissue was processed by mincing with scissors and homogenization. Processing of the tissue was performed in MEM supplemented with antibiotic/antimycotic solution at 10% (v/v) final concentration. Each nasal swab and lung sample was subsequently thawed, vortexed for 15 s and centrifuged at 1600 g for 25 min at 4 °C. Supernatants were collected and 10-fold serial dilutions were prepared in MEM. Each dilution (five replicates) was plated onto confluent MDCK cells in 96-well plates. After 1 h incubation at 37 °C, the diluents were replaced by 200 μl MEM supplemented with 0.2 % BSA and 1 μg TPCK-treated trypsin ml^-1 or 0.5 μg human neutrophil elastase ml^-1. Plates were evaluated for cytopathic effect (CPE) between 24 and 96 h p.i. Virus titres were calculated according to the method described by Reed & Muench (1938).

**Histopathology evaluation.** Tissue sections of lungs were routinely stained with haematoxylin and eosin and examined microscopically for bronchiolar epithelial changes and peribronchiolar inflammation. Lesion severity was scored by the distribution or extent of lesions within the sections, examined as follows: 0, no visible changes; 1, mild focal or multifocal change; 2, moderate multifocal change; 3, moderate diffuse change; 4, severe diffuse change. A single pathologist scored all slides and was blinded for the experimental groups.

**Statistical analysis.** Statistical analysis of body temperatures, macroscopic lesion scores, microscopic lesion scores and virus titres were performed using GraphPad Prism5 statistical software. Differences between the means of each group in each assay were determined by using Mann–Whitney analysis of variance methods. If the mean values of at least one group differed from others with P<0.05, they were considered statistically significant.

### RESULTS

**Generation of elastase-dependent SIV/SK viruses**

To establish a reverse genetics system for SIV/SK, we cloned the eight viral RNA segments from SIV/SK virus into the reverse genetics vector pHW2000 (Hoffmann et al., 2000). Infectious virus was recovered following transfection of these plasmids into co-cultured MDCK and 293T cells. The reverse-genetics-recovered WT SIV/SK (SIV/SK-WT) and the parental WT SIV/SK grew to similar titres in embryonated eggs and caused similar degrees of lesions in pigs (data not shown). To generate a trypsin-resistant virus, we exchanged the nucleotides corresponding to positions 1030 and 1031 in the SIV/SK HA gene with those in SIV/SK/R345A, which would be sensitive to this protease. However, SIV/SK-R345A could not be rescued when human neutrophil elastase was provided, although with slower progression; SIV/SK-R345V virus was rescued at 36 h after transfection, whereas SIV/SK-R345A virus was recovered at 72 h after transfection. The genotype of the mutant viruses was characterized and confirmed by sequencing of the RT-PCR product derived from the HA gene of mutant viruses. Mutant viruses could not be recovered by transfection in the presence of trypsin.

#### Table 1. Assignment of pigs to infection groups

All groups were inoculated intratracheally with 4 ml of virus. NA, Not applicable.

<table>
<thead>
<tr>
<th>Group (n=5)</th>
<th>Inoculum</th>
<th>Concentration (p.f.u ml^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MEM</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>SIV/SK-WT</td>
<td>10^5</td>
</tr>
<tr>
<td>3</td>
<td>SIV/SK-WT</td>
<td>10^6</td>
</tr>
<tr>
<td>4</td>
<td>SIV/SK-R/V</td>
<td>10^5</td>
</tr>
<tr>
<td>5</td>
<td>SIV/SK-R/V</td>
<td>10^6</td>
</tr>
<tr>
<td>6</td>
<td>SIV/SK-R/A</td>
<td>10^5</td>
</tr>
<tr>
<td>7</td>
<td>SIV/SK-R/A</td>
<td>10^6</td>
</tr>
</tbody>
</table>
Fig. 1. Generation, growth properties and genetic stability of mutant viruses. (a) Schematic diagram showing the modifications of the HA cleavage site. (b) Multiple-cycle growth curves of SIV/SK-WT, SIV/SK-R345V and SIV/SK-R345A on MDCK cells. Cells were infected in triplicate with each virus at an m.o.i. of 0.001 in the presence of 1 μg TPCK-treated trypsin ml⁻¹ (for WT) or 0.5 μg human neutrophil elastase ml⁻¹ (for mutant viruses). Supernatants were collected at the indicated time points until 72 h p.i. and titres were determined by plaque assay on MDCK cells. (c) Plaques formed by SIV/SK-WT, SIV/SK-R345V and SIV/SK-R345A viruses on MDCK cells in the presence of trypsin or neutrophil elastase or in the absence of exogenous protease. (d) Mutant viruses were passaged on MDCK cells at a low m.o.i. (0.001) five times in the presence of both trypsin and neutrophil elastase. The supernatants from the fifth passage were serially diluted and plaque assays were performed in the presence of either trypsin or neutrophil elastase. Representatives of the plaque assay results with 10⁶ dilutions are shown here.
**Mutant viruses are strictly elastase-dependent and exhibit the same growth properties as WT SIV in tissue culture**

Multi-cycle growth potential of influenza virus is dependent on proteolytic activation of HA (Klenk et al., 1975). To examine the replication potential of mutant viruses SIV/SK-R345V and SIV/SK-R345A, the plaque size and multiple cycle growth kinetics were compared to that of WT virus. To this end, confluent MDCK cells were infected with SIV/SK-WT, SIV/SK-R345V or SIV/SK-R345A at an m.o.i. of 0.001, supernatants were harvested at the indicated time points until 72 h p.i. and virus titres were determined by plaque assay on MDCK cells. As shown in Fig. 1(b), all of the viruses reached a plateau at 24 h p.i. The growth kinetics and titres of the mutants in MDCK cells were similar to those of the WT virus. These results indicate that a mutation in the HA protein cleavage site did not result in attenuation of virus growth in MDCK cells. To investigate the protease dependence of SIV/SK-WT, SIV/SK-R345V and SIV/SK-R345A, we performed plaque assays in the presence of either trypsin or neutrophil elastase in the plaque overlay or in the absence of an exogenous protease on MDCK cells. As shown in Fig. 1(c), while WT SIV/SK virus was able to form large clear plaques in the presence of trypsin (panel i), SIV/SK-R345V and SIV/SK-R345A viruses formed similar-sized plaques in the presence of neutrophil elastase (panels v and vi). In contrast, SIV/SK-R345V and SIV/SK-R345A viruses were resistant to trypsin (panels ii and iii). Neither the WT nor the mutant viruses were activated without the presence of exogenous proteases (panels vii, viii and ix).

We also tested the growth potential of the two mutant viruses in the presence of porcine pancreatic elastase. SIV/SK-R345V did not grow at all, suggesting that this virus is entirely dependent on human neutrophil elastase activation. Interestingly, although SIV/SK-R345A could not be rescued by porcine pancreatic elastase, it grew in the presence of this protease. After passage of SIV/SK-R345A five times with porcine pancreatic elastase, we sequenced the RT-PCR product of HA derived from SIV/SK-R345A. We found that in front of the cleavage site, Ser344 was replaced by Pro.

**Mutant viruses are genetically stable**

To address the genetic stability of the mutant viruses, they were passaged five times on MDCK cells at an m.o.i. of 0.001 in the presence of both trypsin and neutrophil elastase. Plaque assays were then carried out with 10-fold serial dilutions of the supernatants from the fifth passage, in the presence of either elastase or trypsin (Fig. 1d). Well-defined plaques were seen in the presence of elastase; however, no plaques were detected in the presence of trypsin. At lower dilutions of the supernatants, while cell monolayers were completely disrupted by a higher number of infectious viral particles in the presence of elastase, no infectious particles could be detected in the presence of trypsin (data not shown). After the fifth passage, sequencing results showed that both mutant viruses retained the introduced mutations at the HA cleavage site without any other unwanted mutations, suggesting high levels of genetic stability of the mutant viruses in cell culture.

**Mutant viruses are able to infect cells but their replication is restricted due to the uncleaved HA0**

As a candidate for live attenuated vaccine, a virus should be able to enter cells and complete limited replication cycles. To examine whether this was the case with the mutant viruses SIV/SK-R345V and SIV/SK-R345A, MDCK cells were infected with one of these viruses at an m.o.i. of 10. After virus absorption for 1 h, cells were washed extensively and medium without any extraneous proteases was added. At 8 h p.i., supernatants were harvested and subjected to virus purification, whereas cells were lysed for Western blotting analysis using NP or M1 antibody. NP and M1 expression could be detected in the cells infected with SIV/SK-R345V and SIV/SK-R345A (Fig. 2a, lanes 3 and 4) and expression levels were similar to those in WT virus-infected cells (lane 2). To examine the status of HA present in virus particles, purified virions grown in the presence or absence of corresponding protease were separated by using SDS-PAGE followed by staining with Coomassie blue. HA remained in the form of HA0 in SIV/SK-R345V and SIV/SK-R345A when virus particles were grown without adding elastase (Fig. 2b, lanes 4 and 6). In contrast, the majority of HA0 was cleaved into HA1 and HA2 in SIV/SK-R345V and SIV/SK-R345A virus particles when grown in the presence of neutrophil elastase, although traces of HA0 were visible (Fig. 2b, lanes 3 and 5). As a positive control, HA1 was found in purified WT SIV/SK grown in the presence of trypsin (Fig. 2b, lane 2).

**Elastase-dependent SIV/SK-R345V and SIV/SK-R345A viruses are attenuated in pigs**

Thirty-five 4-week-old, SIV-negative pigs were split randomly into seven groups of five pigs. These were infected intratracheally with 4 ml MEM containing 1 × 10⁵ p.f.u. ml⁻¹ or 1 × 10⁶ p.f.u. ml⁻¹ SIV/SK-WT, SIV/SK-R345V or SIV/SK-R345A. The animals in the control group were mock-infected with medium only (Table 1). Clinical signs and rectal temperature were monitored and nasal swabs were taken daily after virus infection. On day 5 p.i., pigs were euthanized and necropsies were performed. During the 5 day observation period, clinical signs characteristic of SIV infection were observed on days 1, 2 and 3 only in the groups infected with the SIV/SK-WT at both doses. Animals in all other groups did not show any signs of respiratory distress, weight loss or nasal discharge. Mean rectal temperatures in both groups infected with SIV/SK-WT (4 × 10⁵ p.f.u. and 4 × 10⁶ p.f.u.) increased to 40.8 °C on day 1 p.i. (Fig. 3a). This temperature change is significant compared with the control group (P=0.0465). On the following days, the temperature gradually decreased and then slightly rose. By the end of the experiment, the
mean temperatures of animals infected with high and low dose were 40.2 °C \( (P=0.0278) \) and 39.7 °C \( (P=0.0651) \), respectively. Similar temperature kinetics were observed in the group infected with the high dose of SIV/SK-R345A (Fig. 3c), where mean temperature reached 40.6 °C on day 1 p.i. and then remained in the range of 39.5 °C to 40.5 °C. However, pigs in the remaining groups (SIV/SK-R345V infective dose \( 4\times10^5 \) p.f.u. and \( 4\times10^6 \) p.f.u. and SIV/SK-R345A infective dose \( 4\times10^5 \) p.f.u.) did not show significant differences in mean rectal temperatures compared to the control group (Fig. 3b and c).

Nasal swabs were taken daily from all pigs and virus shedding and titres were recorded (Fig. 4). Only pigs infected with SIV/SK-WT shed virus throughout all 5 days. The largest number of animals that shed virus was seen on days 3 and 4 p.i. in the high dose SIV/SK-WT-infected group (Fig. 4a). Virus could be recovered from only one animal on days 3, 4 and 5 p.i. in the low dose SIV/SK-WT-infected group (Fig. 4b). We could not detect any virus in nasal secretion from the pigs infected with SIV/SK-R345V and SIV/SK-R345A.
At necropsy, the percentage of each lung surface with macroscopic lesions was evaluated. The mock-, SIV/SK-R345V- and SIV/SK-R345A- (low or high dose) infected pigs did not show any typical macroscopic lung lesions. In contrast, gross lesions were observed predominantly in the apical and cardiac lobes of SIV/SK-WT-infected pigs, while diaphragmatic lobes were less affected (gross lesions were characterized as purple- to plum-coloured consolidated areas). We also observed hyperemic and enlarged mediastinal lymph nodes in WT virus-infected pigs. The percentage of macroscopic lung lesions in low and high dose SIV/SK-WT-infected pigs was 16.2 and 28.8 %, respectively. These were significantly higher compared with the control group ($P<0.0043$ in both cases) (Fig. 5a). In agreement with these results, SIV/SK-WT could be recovered from lung tissue of all animals infected with the WT virus. The mean virus titres isolated from lungs infected with a high or low dose were $10^{4.5}$ TCID$_{50}$ g$^{-1}$ and $10^{3.5}$ TCID$_{50}$ g$^{-1}$, respectively. No virus could be detected in the lungs of animals infected with SIV/SK-R345V and SIV/SK-R345A viruses (Fig. 5b).

Histopathology results were consistent with previous studies of SIV infection in swine (Straw et al., 1999; Richt et al., 2006). Microscopic lesions were observed most consistently in medium-sized airways. Thus, results obtained from the medium bronchioles were used for comparison of SIV/SK-WT, SIV/SK-R345V and SIV/SK-R345A viruses. The lungs of mock-infected as well as mutant virus-infected pigs showed no or minimal microscopic lesions, whereas moderate to severe lesions were detected in animals infected with the SIV/SK-WT virus (Fig. 6). Virus damage varied from severe necrotizing bronchiolitis and interstitial pneumonia to very mild bronchiointerstitial pneumonia. Epithelial lining was most prominent in SIV/SK-WT-infected pigs due to peribronchiolar lymphocyte and neutrophil infiltration in the airway. Interstitial thickening and lymphocyte infiltration in alveolar walls were minimal and inconsistently present in all groups (Table 2). As seen in the macroscopic lung lesions, microscopic histopathology showed that SIV/SK-R345V- and SIV/SK-R345A-infected pigs showed significantly less lung damage than SIV/SK-WT-infected pigs.

**DISCUSSION**

At present, all SIV vaccines are inactivated and their application does not provide the desired immune response and cross-protection (Brown & McMillen, 1994; Macklin et al., 1998). LAIV are not available for swine, although results of recent studies on NS1 gene-deleted vaccines have been reported (Richt et al., 2006; Solorzano et al., 2005; Vincent et al., 2007). One advantage of live vaccines is that they are delivered intranasally and only replicate to a limited extent, thus they may induce balanced cross-
reactive cell-mediated immunity and humoral antibody responses, providing superior immunity to that induced by conventional inactivated vaccines (Gorse et al., 1995). Data from studies conducted after the application of cold-adapted LAIV in humans and horses showed that live vaccines are capable of inducing a stronger immune response and long-term protection (Cox et al., 2004; Paillot et al., 2006). In contrast, one concern about these LAIV would be possible reassortment between field strains and the vaccine virus, generating new reassortant virus with unpredicted infectivity.

Stech et al. (2005) described an approach to generate a live attenuated virus. Accordingly, a mutant of strain A/WSN/33 with a modified cleavage site within HA was generated which was dependent on proteolytic activation by elastase (Stech et al., 2005). This mutant was strictly dependent on elastase and grew as well as WT in tissue culture, but was entirely attenuated in mice at a virus dose of $10^6$ p.f.u. At a dose of $10^5$ p.f.u., it induced complete protection against lethal infection. These promising results prompted us to investigate whether the strategy was applicable for SIV in its natural host.

Here, we generated two elastase-dependent mutant SIVs. Initially, we constructed plasmid pHW-SIV/HA-R345V, which encodes HA with a modified cleavage site that is susceptible to human neutrophil elastase. We were concerned that upon infection with the virus, neutrophil infiltration could trigger and support virus replication in vivo by releasing elastase, so we designed plasmid pHW-SIV/HA-R345A, which encodes HA with a porcine pancreatic elastase cleavage site at the junction of HA1 and HA2. Both mutant viruses could only be rescued in the

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**Fig. 6.** Histopathological examination of lungs from infected pigs 5 days p.i. (a) Medium-sized bronchioles from the lung of a control pig mock-infected with MEM. (b, c) Normal bronchioles and surrounding blood vessels from the lungs of pigs infected with SIV/SK-R345V mutant virus at a low (b) and high (c) dose. (d, e) Normal bronchioles from the lungs of pigs infected with SIV/SK-R345A mutant virus at a low (d) and high (e) dose. (f, g) The lungs of pigs infected with SIV/SK-WT at a low (f) and high (g) dose showed severe acute necrotizing bronchiolitis and interstitial pneumonia, severe bronchiolar necrosis with basophilic debris and severe neutrophil infiltration in the lumen of bronchioles and bronchi. Neutrophils and macrophages in the alveolar spaces and a mild perivascular lymphoid infiltration were seen. Magnification, ×20; bar, 200 μm.
### Table 2. Histopathology lung scores

Values are the mean ± SD histopathology scores. *Score* = 0.

<table>
<thead>
<tr>
<th>Infection group†</th>
<th>Necrosis</th>
<th>Attenuation of bronchiolar epithelium‡</th>
<th>Hyperplasia</th>
<th>Infiltration of inflammatory cells in bronchiolar lumen</th>
<th>Bronchiolar-associated lymphoid tissue proliferation</th>
<th>Peribronchial lymphocyte and neutrophil infiltration</th>
<th>Perivascular lymphocyte infiltration</th>
<th>Atelectasis</th>
<th>Interstitial thickening and lymphocyte infiltration in alveolar wall</th>
<th>Overall score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>-</td>
<td>-</td>
<td>0.4 ± 0.55</td>
<td>0.8 ± 0.45</td>
<td>0.4 ± 0.55</td>
<td>0.6 ± 0.55</td>
<td>1 ± 0</td>
<td>1 ± 0</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>2</td>
<td>1.6 ± 0.55‡</td>
<td>1.4 ± 0.55‡</td>
<td>1.2 ± 0.45‡</td>
<td>2.2 ± 1.09‡</td>
<td>1 ± 0</td>
<td>2 ± 0.71‡</td>
<td>1 ± 0</td>
<td>2 ± 0.71‡</td>
<td>1.2 ± 0.45</td>
<td>3 ± 0.7‡</td>
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<tr>
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<tr>
<td>4</td>
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<td>0.8 ± 0.45</td>
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<tr>
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</tr>
<tr>
<td>6</td>
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<td>-</td>
<td>-</td>
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<td>0.2 ± 0.45</td>
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<td>0.6 ± 0.55</td>
<td>0.6 ± 0.55</td>
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</tbody>
</table>

*See Table 1 for details.
†Flattening of the epithelial cells lining the bronchioles as part of the repair process following injury.‡Indicates that the group is statistically significantly different compared with other groups (P<0.05).
viruses maintained their growth ability in the presence of appropriate protease in tissue culture, but were highly attenuated in pigs. Currently we are testing the protective immune response of SIV/SK-R345V and SIV/SK-R345A in pigs.

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REFERENCES


