Elastase-Dependent Live Attenuated Swine Influenza A Viruses Are Immunogenic and Confer Protection against Swine Influenza A Virus Infection in Pigs

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Influenza A viruses cause significant morbidity in swine, resulting in a substantial economic burden. Swine influenza virus (SIV) infection also poses important human public health concerns. Vaccination is the primary method for the prevention of influenza virus infection. Previously, we generated two elastase-dependent mutant SIVs derived from A/Sw/Saskatchewan/18789/02(H1N1): A/Sw/Sk-R345V (R345V) and A/Sw/Sk-R345A (R345A). These two viruses are highly attenuated in pigs, making them good candidates for a live-virus vaccine. In this study, the immunogenicity and the ability of these candidates to protect against SIV infection were evaluated in pigs. We report that intratracheally administrated R345V and R345A induced antigen-specific humoral and cell-mediated immunity characterized by increased production of immunoglobulin G (IgG) and IgA antibodies in the serum and in bronchoalveolar lavage fluid, high hemagglutination inhibition titers in serum, an enhanced level of lymphocyte proliferation, and higher numbers of gamma interferon-secreting cells at the site of infection. Based on the immunogenicity results, the R345V virus was further tested in a protection trial in which pigs were vaccinated twice with R345V and then challenged with homologous A/Sw/Saskatchewan/18789/02, H1N1 antigenic variant A/Sw/Indiana/1726/88 or heterologous subtypic H3N2 A/Sw/Texas/4199-2/98. Our data showed that two vaccinations with R345V provided pigs with complete protection from homologous H1N1 SIV infection and partial protection from heterologous subtypic H3N2 SIV infection. This protection was characterized by significantly reduced macroscopic and microscopic lung lesions, lower virus titers from the respiratory tract, and lower levels of proinflammatory cytokines. Thus, elastase-dependent SIV mutants can be used as live-virus vaccines against swine influenza in pigs.

Swine influenza virus (SIV) is the causative pathogen of swine influenza, a highly contagious, acute respiratory viral disease of swine. The mortality of SIV-infected pigs is usually low, although morbidity may approach 100%. Swine influenza is characterized by sudden onset, coughing, respiratory distress, weight loss, fever, nasal discharge, and rapid recovery (38). SIV is a member of the influenza virus A genus in the Orthomyxoviridae family, and the virus has a genome consisting of eight segments of negative-sense single-stranded RNA (29). Epithelial cells in the swine respiratory tract have receptors for both avian and mammalian influenza viruses (13); thus, pigs could potentially serve as “mixing vessels” for the generation of new reassortant strains of influenza A virus that have pandemic capacity. There are a number of reports in which the direct transmission of influenza viruses from pigs to humans has been documented (6, 12, 52), and several of these cases have resulted in human fatalities (19, 35, 40, 53). Consequently, effective control of SIV would be beneficial to both humans and animals.

Until 1998, classical H1N1 SIVs were the predominant isolates from pigs in the United States and Canada (5, 28). In 1997 to 1998, a dramatic change in the epidemiologic pattern of SIV began. Serological studies conducted by Olsen and colleagues in 1997 to 1998 detected a significant increase in H3-seropositive individuals, and H3N2 SIVs were isolated from pigs in both the United States and Canada (17, 54). Furthermore, reassortment between H3N2 viruses and classical H1N1 SIV resulted in the appearance of H1N2 reassortant viruses (14, 15). In addition to the isolation of H4N6 viruses, which are of duck origin, in pigs in Canada (16), wholly avian viruses of the H3N3 and H1N1 subtypes have also been isolated from Canadian pigs (18). In general, three major SIV subtypes exist, i.e., H1N1, H1N2, and H3N2, each of which has multiple genetic and antigenic variants circulating in North American swine populations (18, 28). The increased incidence of avian-like or human-like SIV reassortants raises concerns for public health and requires research devoted to the development of cross-protective SIV vaccines.

Currently available swine influenza vaccines are based on inactivated whole virus of the H1N1 and H3N2 subtypes. Application of these vaccines reduces the severity of disease but does not provide consistent protection from infection (3, 22). In contrast to killed vaccines that are administered intramuscularly, intranasally administered live attenuated influenza vaccines (LAIV) induce an immune response at the site of natural infection. Therefore, an LAIV has the potential to induce broad humoral and cellular immune responses that could provide protection against antigenically different influenza viruses. LAIV based on attenuation of the virus by cold adaptation are available for humans (2) and horses (41). However, to date, no SIV LAIV are commercially available for use in swine in North
A mutant SIV with a truncated NS1 protein was highly attenuated in pigs (36). In addition, this SIV/NS1 LAIV was capable of stimulating a protective immune response against homologous SIVs and a partial protection against heterologous subtype wild-type (WT) SIVs (31, 50). Stech and colleagues demonstrated that the conversion of a conserved cleavage site in the influenza virus hemagglutinin (HA) protein from a trypsin-sensitive to an elastase-sensitive site results in vivo attenuation of the influenza virus in mouse models (9, 37). Furthermore, these elastase-dependent LAIVs were able to induce protective systemic and mucosal immune responses. Recently, we showed that two elastase-dependent SIVs derived from A/Sw/Saskatchewan/1879/02 (SIV/Sk02), R345V and R345A, are attenuated in their natural host, pigs (23). In the current study, we addressed the immunogenic and cross-protective abilities of these mutants.

MATERIALS AND METHODS

Cells and viruses. Madin-Darby canine kidney cells (MDCK) were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). The H1N1 mutant viruses SIV/Sk-R345V (R345V) and SIV/Sk-R345A (R345A) were generated as previously described (23). These two viruses were grown in MDCK cells in the presence of 0.05 µg/mL human neutrophil elastase (Serva Electrophoresis GmbH). Stocks of the R345V and R345A viruses reached titers of 1.7 × 10⁹ and 2.0 × 10⁸ PFU/ml after purification, respectively. The other SIV isolates used in the study were H1N1 A/Sw/Saskatchewan/1879/02 (SIV/Sk02), H1N1 A/Sw/Indiana/1726/88 (SIV/Ind88), and H3N2 A/Sw/Texas/4199-2/98 (SIV/Tx98). These viruses were propagated at 37°C in the bat-like habitats of 11-day-old embryonated chicken eggs. Stock virus titers were 1.4 × 10⁸, 1.33 × 10⁷, and 1.7 × 10⁷ PFU/ml, respectively. Titers for all viruses used in the study were determined on MDCK cells by a plaque assay, as described previously (34).

Experimental design and clinical sampling. For the purpose of this study, we designed two animal trials. In the first trial (Table 1), 35 5-week-old SIV-negative landrace crossbred pigs were obtained from Prairie Swine Center (Floral, Saskatchewan, Canada). Pigs were randomly selected and divided into five groups with seven pigs per group. At 6 weeks of age (day 0), the pigs in group 1 were mock vaccinated with 4 mL of MEM, whereas the pigs in groups 2 and 4 received 4 × 10⁶ PFU of R345V and the pigs in groups 3 and 5 received 4 × 10⁷ PFU of R345A. The viruses or MEM was administered intratracheally (i.t.), ensuring protective abilities of these mutants.

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In the second trial (Table 2), 49 4-week-old SIV-negative pigs were assigned to seven groups with seven animals per group. The animals in groups 1 to 4 were mock vaccinated i.t. with 4 mL of MEM, whereas the pigs in groups 5 to 7 were vaccinated i.t. with 4 × 10⁶ PFU of R345V. Three weeks after the first vaccination, animals in groups 1 to 4 received MEM, whereas the vaccinated groups (5 to 7) received a second dose of 4 × 10⁸ PFU of R345V. Ten days after the second vaccination (day 31), the pigs in all groups were challenged i.t. with 8 × 10⁶ PFU of homologous or heterologous subtype SIVs (Table 2). After the challenge, the pigs were monitored for the presence of clinical signs characteristic of SIV infection and necropsy. Tissue samples from the right aortal, cardiac, and diaphragmatic lung lobes were taken for virus isolation and histopathology examination. Serum samples were collected prior to and after the second challenge and after viral challenge. BALF samples were obtained at necropsy. All animal experiments were conducted under the Veterinary and Infectious Disease Organization at the University of Saskatchewan in accordance with the ethical guidelines of the University of Saskatchewan and the Canadian Council of Animal Care.

Isolation of lymphocytes from tracheo-bronchial lymph nodes. Tracheo-bronchial lymph nodes were dissected in toto at necropsy and stored on ice in AIM-V medium containing 10% fetal bovine serum, 50 mg/mL streptomycin, and 10 mg/mL gentamicin sulfate (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% FBS. Lymph node cells (LNCs) were isolated by finely mincing tissues with a scalpel, filtering the cell suspension through 40-µm nylon cell strainers (Becton Dickinson Labware, Franklin Lakes, NJ), and washing the cells with AIM-V medium supplemented with 2% FBS. Prior to seeding, the cells were resuspended in AIM-V + 2% FBS medium containing 50 µM of β-mercaptoethanol and were counted.

Detection of SIV-specific IFN-γ-secreting cells by enzyme-linked immunoassay (ELISPOT) assay. Nitrocellulose Unifilter 350 microporous plates (Whatman, Florham Park, NJ) were coated with mouse anti-porcine gamma interferon (IFN-γ) monoclonal antibodies (Endogen, Rockford, IL) in coating buffer at a concentration of 5 µg/mL for 16 h at 4°C. The wells were washed, and LNCs were seeded directly at 1 × 10⁶ cells/well in a final volume of 200 µL of AIM-V containing 2% FBS. LNCs were stimulated for 10 h at 37°C with 25 µg/mL of purified UV-inactivated SIV/Sk02 virus, 5 µg/mL of concanavalin A (Sigma-Aldrich), or medium only. After stimulation, the plates were washed five times with PBS containing 0.05% Tween 20 (PBST) and incubated with rabbit anti-porcine immunoglobulin G (IgG) (H+L) (Dianova, South San Francisco, CA) at a dilution of 5 × 10⁵ for 2 h at room temperature. The wells were washed five times and incubated with streptavidin alkaline solution (Jackson ImmunoResearch, West Grove, PA) at a dilution of 5 × 10⁵ for 1.5 h at room temperature. After the wells were washed eight times with double-distilled water, 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium (Sigma-Aldrich)-insoluble alkaline substrate solution was added (100 µL/well), and the plates were incubated for 5 min. The plates were then washed again with double-distilled water and left to dry overnight at room temperature. Spots were counted manually under an inverted light microscope. The number of spots observed in wells stimulated only with medium was counted and subtracted as background. Data were reported as the number of IFN-γ-secreting cells per 10⁶ somatic cells.

LPR assay. LNCs were resuspended in AIM-V culture medium at a concentration of 2.5 × 10⁵ cells/well and stimulated for 72 h at 37°C with 25 µg/mL of SIV/Sk02 antigen, 5 µg/mL of concanavalin A, or medium. Six hours before the 72-h incubation, LNCs were pulsed with 0.4 µCi [5³H]thymidine (Amersham Pharmacia, Piscataway, NJ) as previously described (25). The cells were then harvested using standard liquid scintillation protocols, and the uptake of [³H]thymidine was assessed with a beta counter (Topcounter; Packard Instrument Com-
pany, Meriden, CT). The lymphocyte proliferative response (LPR) was calculated as the mean counts per minute (cpm) of triplicate cultures and expressed as a stimulation index (cpm in the presence of stimulus/cpm in the absence of stimulus).

ELISA for antigen-specific IgG and IgA antibodies and hemagglutination inhibition (HI) assay. For antigen-specific enzyme-linked immunosorbent assays (ELISAs), 2.5 μg/ml of purified UV-activated SIV/Sk02, SIV/Ind88, and SIV/Tx98 antigens were applied to 96-well Immulon-2 plates (Dynex Technology Inc., Chantilly, VA), which were then incubated overnight at 4°C. The plates were blocked for 1 h at room temperature with 100 μl of 1% skim milk in TBST (0.1 M Tris, 0.17 M NaCl, 0.05% Tween 20) and washed four times with PBST. Serum, nasal, and BALF samples were added (100 μl/well) in triplicate at the appropriate dilutions, and the plates were incubated for 1.5 h at room temperature. Samples of previously defined positive control sera and the appropriate negative controls were run on each plate. Subsequently, the plates were incubated with mouse anti-porcine IgA monoclonal antibody (AbD Serotec) or alkaline phosphatase-labeled goat anti-porcine IgG (KPL, Gaithersburg, MD). The IgA ELISAs were developed by the addition of biotinylated goat anti-mouse IgG (H+L) antibodies (DAamed, South San Francisco, CA) and streptavidin alkaline phosphatase solution (Jackson ImmunoResearch, West Grove, PA). After being washed with PBST, the IgG and IgA ELISA plates were developed with the addition of p-nitrophenyl phosphate substrate [10 mg/ml p-nitrophenyl phosphate di(tris) salt crystalline (Sigma-Aldrich), 1% diethanolamine (Sigma-Aldrich), 0.5 mg/ml MgCl₂, pH 9.8]. The optical density (OD) of the reaction product was measured at 405 nm (a 490-nm reference filter was used to detect background, which was subtracted from the measurement reading) on a microplate reader (Molecular Devices SpectraMax Plus 384). The titer of sample was defined as the highest dilution at which the OD of that sample was higher than the defined cutoff (the mean OD of a known negative sample plus two times the standard deviation).

To measure the HI titers, serum samples were treated overnight with receptor-destroying enzyme (cholera filtrate C8772; Sigma Aldrich) at 37°C to eliminate nonspecific HI factors. The viruses used in the HI assays were SIV/Sk02, SIV/Ind88, and SIV/Tx98. HI assays were performed as described elsewhere (27).

Histopathology evaluation. Necropsy, macroscopic examination of lungs, and tissue processing for virus isolation were performed as described previously (23). Tissue sections of lungs were stained with hematoxylin and eosin and examined microscopically for bronchiolar epithelial changes and peribronchiolar inflammation. Lesion severity was scored by the distribution or by the 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. Two independent pathologists blinded for the experimental groups scored all slides.

ELISA for IFN-γ, IL-1, and IL-6 cytokines. In order to detect the IFN-γ, interleukin-1 (IL-1), and IL-6 cytokines, polystyrene microtiter plates (Immulon 2; Dynex Technology Inc., Chantilly, VA) were coated with the capture antibody mouse anti-recombinant porcine IFN-γ clone K9 (R&D no. 27100-1), goat anti-recombinant porcine IL-6 (R&D AF 686), or mouse anti-recombinant porcine IL-1β (R&D MAB 6811) at a concentration of 1 μg/ml in coating buffer. Recombinant porcine IFN-γ (Endogen rPo IFN-γ; 2,000 pg/ml), recombinant porcine IL-6 (R&D 881-PI-025 rPoIL-6; 5,000 pg/ml), and recombinant porcine IL-1β (R&D 881-PI-010 rPo IL-1β; 10,000 pg/ml) were used as standards. Standards and homogenized lung samples were diluted in TBST-0.1% skim milk and added to the coated plates. After overnight incubation at 4°C, the detection antibodies biotinylated mouse anti-recombinant porcine IFN-γ clone F17 (R&D no. 27105-1; 1/1000), biotinylated goat anti-recombinant porcine IL-6 (R&D BAF686; 0.2 μg/ml), and biotinylated goat anti-recombinant porcine IL-1β (R&D BAF681; 0.25 μg/ml) were added to the appropriate wells. Finally, the plates were developed, and the responses were measured as described above. Sample concentrations were calculated using Softmax Pro 5.2 version software (Molecular Devices).

Statistical analysis. Statistical analysis was performed using GraphPad Prism5 (San Diego, CA) and Statistix® (Tallahassee, FL) software. Differences between the means for the two groups (vaccinated versus unvaccinated) in each assay were determined using the Mann-Whitney nonparametric t test. To compare the two vaccines, data from the LPR and ELISPOT assays and for the serum antibodies were transformed and one-way analysis of variance for RANKSUM was applied. If the median values of at least one group differed from the others at a P value of <0.05, the difference between the groups was considered statistically significant.

RESULTS

Live attenuated viruses induce cell-mediated immune responses. To determine whether the mutant viruses were immunogenic, SIV-negative pigs were divided into 5 groups and i.t. vaccinated with MEM, R345V, or R345A (Table 1). Two groups of pigs received one immunization and were euthanized on day 21, whereas two groups of pigs received a second immunization on day 21 and were euthanized on day 31. Animals in the control group received MEM and were euthanized on either day 21 or day 31. At necropsy, tracheo-bronchial lymph nodes and BALF were collected.

To assess the ability of R345V or R345A virus to induce cell-mediated immune responses after vaccination, LNCs were isolated from vaccinated and control pigs, and antigen-specific responses were measured by IFN-γ ELISPOT and LPR assays. As shown in Fig. 1, both R345V and R345A were able to induce a significant number of antigen-specific IFN-γ-secreting cells after only one vaccination. A second vaccination with the same dose of the previously administered vaccines resulted in a further increase in the number of local IFN-γ-secreting cells. This increase was significantly higher than the increase for the groups that received a single vaccination (P = 0.002 for R345V and P = 0.05 for R345A) (Fig. 1A). To further measure cell-mediated responses, we conducted an LPR assay in which we assayed the antigen-specific proliferation of LNCs. Consistent with the IFN-γ ELISPOT results, the LNCs proliferated in response to specific antigens after the first vaccination, with median stimulation indices of 9.57 (R345V) and 38.04 (R345A). Moreover, a second vaccination resulted in a significant increase in the stimulation index (P = 0.02 and P = 0.05 for R345V and R345A, respectively) (Fig. 1B). Statistical analysis showed that there was a significant difference in cell-mediated immune responses between the first and second vaccinations in both groups vaccinated with R345V and R345A. However, there was no statistically significant difference in the cell-mediated immune responses between the two vaccines using these two assays.

Live attenuated viruses induced humoral immune responses. Sera were collected prior to the first vaccination, 21 days after the first vaccination, and 10 days after the second vaccination. SIV-specific antibodies were first examined by an HI assay. All pigs were negative for H1N1 SIV antibodies (HI titer of <1:10) at the start of the experiment, and the seven unvaccinated pigs remained seronegative throughout the experiment. After the first vaccination with R345V or R345A, all pigs had low levels of antibodies against the parental H1N1 strain (HI titer of 20). The second vaccination resulted in a significant increase in the HI titer (HI titers of 160 for R345V and 80 for R345A). Pigs vaccinated with R345V had significantly higher HI titers than the R345A-vaccinated pigs (P = 0.04) (Fig. 2A).

Antigen-specific serum IgG and IgA levels were measured on days 21 and 31 by ELISA using inactivated H1N1 SIV/Sk02 as the capture antigen. As seen in Fig. 2B, both the R345V and the R345A viruses induced a moderate level of IgG after the first vaccination (titers of 321 for R345V and 164 for R345A). The second vaccination led to a considerable increase in the level of IgG (titers of 1,611 for R345V and 955 for R345A). Similarly, the first vaccination was able to induce a detectable
level of IgA (titers of 181.8 for R345V and 84.5 for R345A), and the second vaccination led to an increase in the production of IgA (titers of 746.3 for R345V and 628.7 for R345A) (Fig. 2F). The IgG level in the BALF also increased after the second immunization (median IgG titers of 2.3 versus 0.02 for R345V and 3.0 versus 0.02 for R345A) (Fig. 2G). However, the magnitude of the increase was less than that for IgA.

Similarly, IgA was the dominant antibody subtype in the lower respiratory tract. In the BALF, the IgA titers were significantly higher after the second immunization than after the first vaccination (median IgA titers of 29.0 versus 2,450 [P = 0.0006] for R345V and 52.2 versus 2279 [P = 0.001] for R345A) (Fig. 2F). The IgG level in the BALF also increased after the second immunization (median IgG titers of 2.3 versus 108 [P = 0.02] for R345V and 3.0 versus 112 [P = 0.02] for R345A) (Fig. 2G). However, the magnitude of the increase was less than that for IgA.

**Live attenuated virus induces cross-reactive antibodies.** All pigs were negative for H1N1 and H3N2 antibodies prior to the start of the experiment as determined by an HI assay (HI titer of <10). The pigs vaccinated with R345V seroconverted with respect to SIV/Ind88 H1N1 antigens during the time of study according to the HI assay (Fig. 3A). The median HI titer for SIV/Ind88 was 1:40. However, there were no detectable HI antibodies against SIV/Tx98 H3N2 after two vaccinations with R345V.

The presence of cross-reactive antibodies in the serum and the BALF samples was determined using purified UV-inactivated SIV/Ind88 and SIV/Tx98 as capture antigens in ELISAs. Similarly to HI titers, high levels of serum IgG specific for H1N1 SIV/Ind88 were detected. Although H3N2 cross-reactive serum IgG was detectable, the level was 10 times lower than that of IgG specific for the H1N1 antigen (Fig. 3B). In the lower respiratory mucosa (BALF), R345V-vaccinated pigs had a significantly higher level of IgA antibodies and moderate levels of IgG antibodies that cross-reacted with the homologous antigenic variant H1N1 SIV/Ind88 and the heterologous subtypic H3N2 SIV/Tx98 (Fig. 3C).

**Vaccination reduces macroscopic lung lesions after SIV challenge.** The results described above indicated that (i) both viruses were immunogenic and (ii) two vaccinations were required to induce significantly high levels of immune responses. Considering that R345V exhibited slightly more consistent immune responses and induced stronger immune responses than R345A, in the second protection trial we used the following vaccine strategy. Pigs were immunized with R345V twice within a 3-week interval (Table 2). Ten days after the second immunization, the pigs were challenged i.t. with homologous or heterologous antigenic variant H1N1 SIV/Ind88 and the heterologous subtypic H3N2 SIV/Tx98. No apparent clinical signs with respect to respiratory distress or nasal discharge were observed in H3N2-challenged pigs (Table 2, groups 2 and 3). Unchallenged pigs and pigs vaccinated and challenged with H1N1 SIVs did not show any clinical signs (groups 1, 5, and 6).

No apparent clinical signs with respect to respiratory distress or nasal discharge were observed in the unvaccinated animals challenged with the H1N1 SIV subtypes (Table 2, groups 2 and 3). Unchallenged pigs and pigs vaccinated and challenged with H1N1 SIVs did not show any clinical signs (groups 1, 5, and 6).

The lungs of pigs vaccinated with R345V and challenged with H1N1 viruses (groups 5 and 6) had no gross lung lesions and...
FIG. 2. SIV-specific antibodies induced by the mutant viruses. (A to C) SIV/Sk02-specific HI (A), IgG (B), and IgA (C) levels induced by R345V and R345A were detected in the serum after the first and second vaccinations. (D to G) Mucosal IgG (D and G) and IgA (E and F) antibody titers from nasal swabs (D and E) and from BALF (F and G) were also determined. Each data point represents an individual animal in each treatment group, and median values are indicated by horizontal bars. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. 

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appeared similar to normal lungs. The average lung lesion scores for groups 5 and 6 were significantly lower than those for groups 2 and 3, with \( P < 0.0017 \) (for challenge with Sk02) and \( P < 0.002 \) (for challenge with Ind88) (Fig. 4). Although lung lesions were seen in vaccinated pigs challenged with heterologous subtypic H3N2 virus (group 7), the severity of these lesions was significantly less than the severity of the lesions seen in the corresponding unvaccinated challenged group (group 4); the \( P \) value of the lung lesion score between these two groups was less than 0.05 (Fig. 4).

Vaccination reduces microscopic lung lesions after SIV challenge. SIV-induced histopathological lung lesions are characterized by the detachment of large areas of bronchial/bronchiolar epithelium and by the accumulation of necrotic epithelial cell debris and neutrophils in the airways (46). Consistent with the observed macroscopic lung lesions described above, unvaccinated pigs challenged with H1N1 SIV/Sk02 (group 2) or with H1N1 SIV/Ind88 (group 3) had the most severe histopathological changes, characterized by severe necrotizing bronchiolitis with moderate multifocal necrosis, attenuation of surviving bronchiolar epithelium, and hyperplasia of the bronchial/bronchiolar mucosa (Fig. 5). Severe peribronchiolar and perivascular lymphoid infiltration was accompanied by severe neutrophil infiltration in the lumen of most bronchioles. Furthermore, severe locally extensive interstitial pneumonia with atelectasis, focal alveolar necrosis, and neutrophil infiltration were also observed, as well as infiltration of lymphocytes and macrophages in the alveolar walls and air spaces (Fig. 5B and D). In contrast, as seen in MEM-injected pigs, very mild histopathological changes, such as mild perivascular lymphoid infiltration and occasional mild bronchiolar-associated lymphoid tissue proliferation, were observed in vaccinated H1N1-challenged pigs (groups 5 and 6) (Fig. 5A, C, and E). Focal necrosis of small bronchioles and mild, locally extensive interstitial pneumonia with atelectasis and mixed alveolar inflammatory cell infiltration were found in one vaccinated animal challenged with H1N1 SIV/Ind88 (group 6). Severe peribronchiolar and perivascular lymphoid infiltration
and moderate alveolar atelectasis due to infiltration of mixed inflammatory cell proliferation in the alveolar walls and spaces were observed in all seven unvaccinated H3N2 SIV/Tx98-challenged pigs (group 4) (Fig. 5F). In addition, histopathological changes, such as moderate bronchiolitis with focal necrosis, mild attenuation, and moderate hyperplasia of bronchiolar epithelium, were also found in the animals in this group. In contrast, four of the seven vaccinated pigs challenged with H3N2 SIV/Tx98 (group 7) developed mild to moderate bronchiolitis with rare epithelial necrosis, moderate bronchiolar hyperplasia, and severe peribronchial and perivascular lymphoid infiltration and mild BALT proliferation (Fig. 5G). Lesions and epithelial damage in the remaining three pigs were similar to those in control mock-challenged pigs (groups 1) (Fig. 5A).

**Vaccination reduces virus replication in the lungs.** Tissue sections from the right apical, cardiac, and diaphragmatic lobes were collected and homogenized, and virus titers were determined on MDCK cells. Titers were calculated according to the Reed-Muench method. Each data point represents an individual animal in each treatment group, and median values are indicated by horizontal bars. ***, P < 0.01.

![FIG. 6. Lung virus titers. Lung tissues from the right apical, cardiac, and diaphragmatic lobes were collected and homogenized, and virus titers were determined on MDCK cells. Titers were calculated according to the Reed-Muench method. Each data point represents an individual animal in each treatment group, and median values are indicated by horizontal bars. ***, P < 0.01.](http://jvi.asm.org/)

respectively. The median virus titer for pigs vaccinated and then challenged with heterologous subtypic H3N2 (group 7) was 10^{3.1} TCID_{50/g}. This titer was significantly lower than the virus titers for pigs in group 4 (P = 0.004). No virus could be detected in the lung sections from mock-challenged or vaccinated H1N1-challenged pigs (groups 1, 5, and 6).

**Vaccination reduces the production of proinflammatory cytokines in the lower respiratory tract.** The cytokines tumor necrosis factor alpha (TNF-α), IFN-α, IL-1β, and IL-6 are produced in the lungs during SIV infection and are involved in lung pathology (45, 49). Supernatants from lungs after tissue homogenization were subjected to ELISA to determine the cytokine levels in the lungs. The samples collected at necropsy at 5 days postchallenge that were used for virus isolation were also used for cytokine profiling. TNF-α, IFN-α, IL-1β, and IL-6 were undetectable in the supernatants from lungs of control pigs. TNF-α was undetectable in all groups, probably due to the very narrow window of production (data not shown). In contrast, IFN-α, IL-1β, and IL-6 were detectable on the fifth day after challenge in all vaccinated challenged animals, as well as in unvaccinated challenged pigs. The cytokine levels were significantly higher in all unvaccinated challenged groups than in the corresponding vaccinated challenged animals (Fig. 7). Production of all three detected cytokines correlated with neutrophil infiltration and coincided with the onset of typical SIV clinical signs and lung pathology.

**Antibody titers after virus challenge.** The levels of antibodies in the serum and in the BALF were determined after pigs were vaccinated and challenged with different viruses. As shown in Fig. 8, high levels of HI (titer of 320) and IgG (titer of 2,390) that were reactive to the homologous virus H1N1 SIV/Sk02 were detected in the serum (Fig. 8A and B). Serum IgG antibodies that were cross-reactive with the homologous antigenic variant virus H1N1 SIV/Ind88 were at significantly higher levels after virus challenge (from a median titer of 945 [Fig. 3] to 2,251 [Fig. 8B]; P = 0.002) and reached levels similar to those seen in the H1N1 SIV/Sk02-challenged group. The level of the HI antibody that recognized the homologous antigenic variant virus H1N1 SIV/Ind88 was also significantly increased (from 40 [Fig. 5] to 80 [Fig. 8A]; P = 0.05). Whereas serum IgG antibody cross-reactive with the heterologous virus H3N2 SIV/Tx98 was at a low level before virus challenge (titer of 130) (Fig. 3), after virus challenge the median titer increased to 335.3 (Fig. 8B) (P = 0.03). As in the previous trial, we could not detect any serum HI antibody against H3N2 SIV/Tx98. In the lower respiratory mucosa, IgA antibodies reactive with homologous virus SIV/Sk02 were highly induced after vaccination (titer of 2,750) (Fig. 2F) and virus challenge (titer of 5,874; P = 0.0006), and the levels of IgA antibodies cross-reactive with SIV/Ind88 and SIV/Tx98 also showed significant increases.
(P = 0.007 and P = 0.03, respectively) after the challenge with WT viruses.

DISCUSSION

Attenuation of elastase-dependent LAIV is based on genetically engineering an atypical HA cleavage site that is resistant to activation during natural infection. Replacement of the original trypsin-sensitive (Arg-Gly) cleavage site with the elastase-sensitive (Val-Gly or Ala-Gly) motif resulted in in vivo attenuation in pig (23) and mouse (9, 37) models. Theoretically, the lack of the appropriate protease at the site of infection prevents cleavage of HA, thus disabling the fusion of progeny viruses with the endosome, which in turn blocks virus replication. Previously, we generated two elastase-dependent mutant SIVs (R345V and R345A). The two mutant viruses are genetically stable and are highly attenuated in pigs. Neither the mutant viruses nor any revertant viruses were isolated from the lungs of infected pigs (23); thus, these viruses may have the potential to serve as live attenuated vaccines. In this study, we first assessed the immunogenic properties of these viruses by examining the cell-mediated and humoral immune responses after one and two vaccinations. Afterward, we tested the ability of R345V to induce protective immunity against challenge with homologous and heterologous subtypic SIVs in pigs.

It has long been believed that recovery from influenza virus infection is mediated by cellular immune responses (10, 11, 24), whereas prevention of viral infection correlates with serum and mucosal anti-influenza virus antibody titers (4). In addition, there is a large body of evidence showing that cell-mediated responses are an important contributor to heterologous subtypic immunity in mice and pigs (7, 8, 11, 26). IFN-γ, produced by CD4+ T-helper cell type 1 (Th1) lymphocytes, CD8+ cytotoxic lymphocytes, and NK cells (1), is the major immunomodulator that coordinates the immune responses and establishes an antiviral state of longer duration (32). In our study, one vaccination with R345V or R345A was sufficient to induce significant numbers of local IFN-γ-secreting cells. The twofold increase in the IFN-γ response after the second vaccination suggested that both virus candidates are capable of inducing T-cell activation (Fig. 1A). In addition, results from the LPR assay showed that one vaccination with R345V or R345A induced low proliferation of lymphocytes, whereas a second vaccination resulted in significant LNC proliferation (Fig. 1B). Previous reports on experimental infection with WT SIVs showed that a second exposure to the virus did not result...
in an increase in lymphocyte proliferation or IFN-γ secretion (11, 20), but these results might be attributed to the multiple replication of WT SIVs. In our study, one vaccination was not sufficient to induce the maximum immune response, possibly due to the limited number of replication cycles of R345V in vivo.

Antibody responses to R345V and R345A in the serum and at the respiratory mucosa were measured after the first and second immunizations. Whereas one vaccination induced low levels of antigen-specific IgG, IgA, and HI antibodies in the serum and the BALF, secondary vaccination induced considerably higher titers of antigen-specific IgG, IgA, and HI (Fig. 2). This could be attributed to the restricted replication and short antigen exposure. These data suggest that two vaccinations might be required to generate an adequate immune response and that the two vaccinations could mimic natural immunity after SIV infection and be protective against challenge with WT homologous and heterologous SIVs.

Both R345V and R345A viruses could induce cellular and humoral immunity and showed similar antigenic properties. After comparison of the statistical analyses from all assays performed, SIV mutant R345V showed enhanced serum HI and antigen-specific IgG titers, whereas serum antigen-specific IgA antibody titers, the number of IFN-γ-secreting cells, and the LPR stimulation index were at levels similar to those in R345A-vaccinated pigs. Therefore, we chose R345V as the vaccine candidate in our protection trial.

Two vaccinations via the i.t. route with R345V were sufficient to confer complete protection from challenge with the homologous subtypic H1N1 SIV/Sk02 and the H1N1 variant SIV/Ind88. Vaccinated challenged pigs did not show any of the clinical signs characteristic of SIV infection or elevated rectal temperatures compared with the unvaccinated challenged controls (data not shown). Virus was not detected in the lungs of any of the pigs in these groups, and macroscopic (Fig. 4) and microscopic (Fig. 5) lesions were undetectable or minimal. To demonstrate the ability of the elastase-dependent R345V SIV to induce immunity against an antigenically distinct SIV sub-type, vaccinated pigs were challenged with the heterologous subtypic H3N2 SIV/Tx98 strain. At 5 days postinfection, five of the seven pigs in the R345V-vaccinated H3N2 SIV/Tx98-challenged group had detectable virus in their lungs. However, the median virus titers were significantly lower than those for the unvaccinated H3N2 SIV/Tx98-challenged group (Fig. 6). In addition, macroscopic and microscopic lesions were significantly reduced.

There is growing evidence that the early cytokines are the cause of the clinical signs and the lung epithelial damage as-

![Image](http://jvi.asm.org/)

**FIG. 8.** Antibody titers after vaccination and challenge. The levels of serum HI (A) and IgG (B) titers and mucosa IgA titers from BALF (C) specific for SIV/Sk02, SIV/Ind88, and SIV/Tx98 were determined after pigs were vaccinated twice with R345V and challenged with WT SIVs. Each data point represents an individual animal in each treatment group, and median values are indicated by horizontal bars.
associated with swine influenza (47–49). Early cytokines are produced by nonimmune cells at the site of infection, and these cytokines are responsible for local inflammatory reactions, as well as some systemic effects. IFN-α, TNF-α, and IL-1α and -β are the first cytokines released in the early cytokine cascade (47). These cytokines are rapidly followed by IL-6 and a number of chemokines (44). IFN-α, TNF-α, IL-1, and IL-6 are cytokines with multifunctional activities, and they have been associated with fever, sleepiness, and anorexia. Furthermore, peak cytokine levels directly correlate with virus replication and epithelial lung damage (44, 46). To further strengthen our data for the immunoprotection study, we assessed the production of IFN-α, TNF-α, IL-1α, and IL-6 cytokines in pigs. On day 5 postinfection, we could not detect any released TNF-α (data not shown), probably due to the very narrow window of TNF-α production (first 6 to 8 h postinfection). In all unvaccinated H1N1 SIV-challenged pigs, there was a significant increase in proinflammatory cytokines (IFN-α, IL-1, and IL-6) compared with the R345V-vaccinated challenged animals. However, in pigs challenged with the heterologous subtypic H3N2 SIV/Tx98, the P value between the unvaccinated and vaccinated groups was exactly 0.05 for IFN-α and IL-1β, which is consistent with the partial protection ability of R345V. Taken together, the data that we obtained from macroscopic and microscopic lesions, virus titers, and cytokine release assays clearly showed that two administrations of the R345V virus vaccine conferred full protection against homologous and antigenic variant H1N1 SIVs and partial protection against antigenically distinct H3N2 SIV infection.

Induction of heterologous subtypic immunity after experimental or natural infection with influenza A virus has been described for several species (43, 33, 51). There is mounting evidence showing that the presence of cross-reactive antibodies, especially IgA induced in the respiratory mucosa after natural infection or vaccination with live attenuated vaccines, is strongly correlated with protection from challenge with homologous and heterologous subtypic influenza viruses (21, 50). The importance of cross-reactive IgA is also supported by experiments in which passive transfer of IgA to influenza-naive mice conferred protection (39), whereas mucosal administration of anti-IgA to immune mice abrogated protection from reinfection with the same virus (30). In our study, the R345V mutant virus was capable of inducing a significantly higher level of IgA that reacted with homologous SIV/Sk02 (titer of 2,450) (Fig. 2F) and SIV/Ind88 (titer of 682) (Fig. 3C), and this virus induced moderate levels of IgA that reacted with heterologous subtypic SIV/Tx98 (titer of 251) (Fig. 3C) in the BALF. In addition to the contribution of IgA, cross-reactive IgG antibodies were found in the lungs along with IgA, and these IgG antibodies have been considered a correlate of heterotypic protection (42). Vaccination with R345V also induced the rapid appearance of serum IgG antibodies that reacted not only with homologous SIV/Sk02 (titer of 1,611) (Fig. 2B) but also with the H1N1 antigenic variant SIV/Ind88 (titer of 945) (Fig. 3B) and the heterologous subtypic H3N2 SIV/Tx98 (titer of 130) (Fig. 3B). Cross-reactive IgG was also detected in the BALF, although at a low level (titers of 108 to SIV/Ind88 and 64 to SIV/Tx98) (Fig. 3C). These data suggest that immunization with live R345V-induced influenza virus-specific IgA in the mucosa and IgG in the serum and that the presence of these antibodies may contribute to complete protection against homologous SIV infection and partial protection against heterologous SIV infection.

The presence of cross-reactive IgG in the serum and IgA in the BALF after vaccination and virus challenge suggests that the live attenuated virus could prime immune cells to generate antibodies against common virus epitopes. The significant increase in cross-reactive IgG antibodies in the serum and in IgA in the BALF that was observed after the challenge (Fig. 8) could be due to primed B cells that produce antibodies against common viral antigens following vaccination. This observation is in agreement with the previous findings of Heinen et al., who found that infection of pigs with H1N1 SIV and reinfection with H3N2 significantly increased serum IgG recognizing M2 epitopes and mucosal IgA recognizing NP epitopes (11). In our study, two vaccinations with a modified live virus induced a pattern and kinetics of immune response that had protective characteristics similar to those seen with a past natural infection. The exact mechanism and pathway involved in immune protection against SIV by a live-virus vaccine remain elusive, and further studies are needed to determine to what extent cross talk between the cell-mediated and humoral immune responses plays a role in protection against SIV.

A mutant H3N2 SIV with a truncated NS1 protein has been shown to be highly attenuated and immunogenic in swine. This virus has the potential to be used as a modified live-virus vaccine (31, 36, 50). This vaccine potential is due to the decreased ability of NS1 to antagonize IFN production and IFN’s downstream effectors. Our studies provide an additional attenuating approach that could lead to the development of live-virus vaccines to combat SIV infection. Both NS1-truncated and elastase-dependent LAIV are highly attenuated in swine and are immunogenic. Vaccination with both LAIV was fully protective against infections with homologous SIV and with homologous SIV with an antigenic variant. Vaccination with these LAIV was also partially protective against heterologous subtypic SIV infection. NS1-truncated LAIV can be propagated in embryonated chicken eggs, whereas the elastase-dependent LAIV can be propagated solely in cell culture; in either case, the viruses can grow to high titers. Taking the results together, both LAIV meet the criteria required to be successful live-virus vaccines.

The common objection to the use of live attenuated viruses as vaccines is the possibility of reversion to pathogenicity. The absence of the appropriate protease for cleavage of the R345V HA in vivo allows only a few replication cycles to occur, leading to restricted replication. An important advantage of the short and limited replication is the decreased probability of any reversion or reassortment between the vaccine virus and a WT virus. Although our vaccination requires two administrations and was delivered i.t. (to ensure that all viruses were delivered to the respiratory tracts of the pigs), the advantage of the elastase-dependent live attenuated virus is its ability to induce humoral and cell-mediated immune responses. Most importantly, vaccination with this virus led to reduction in the homologous and heterologous subtypic SIV virus loads and pathogenesis. In addition, the heterologous subtypic immunity induced by the elastase-dependent live attenuated virus could have a significant impact on the epidemiology of novel SIVs emerging in the swine population by reducing viral shedding.
REFERENCES


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