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Protection of weaned pigs by vaccination with human adenovirus 5 recombinant viruses expressing the hemagglutinin and the nucleoprotein of H3N2 swine influenza virus

Ronald D. Wesley*, Min Tang[1], Kelly M. Lager

Virus and Prion Diseases of Livestock Research Unit, National Animal Disease Center, USDA, Agricultural Research Service, P.O. Box 70, Ames, IA, 50010, USA

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Abstract

Swine influenza virus (SIV), subtype H3N2, is a recent reassortant virus that emerged in 1998 in North American swine causing severe respiratory and reproductive disease. In this study, two replication-defective adenovirus recombinants were developed as potential vaccines against H3N2 influenza viruses. Three groups of 3-week-old pigs (10 pigs per group) were vaccinated intramuscularly (IM) with the recombinants; one group was vaccinated with the recombinant adenovirus expressing the influenza virus H3 hemagglutinin (HA) protein, one group was vaccinated with the recombinant adenovirus expressing the nucleoprotein (NP), and one group was vaccinated with both recombinants in a mixture. Two additional control groups (10 pigs per group) were included in the animal trial. One control group was challenged with a virulent H3N2 field strain and one control group remained unchallenged. The results showed that pigs in the groups given the recombinant adenovirus expressing HA alone and HA plus NP developed high levels of virus-specific hemagglutination-inhibition (HI) antibody by 4 weeks post vaccination. Pigs in the group vaccinated with both recombinant viruses in a mixture were completely protected. Complete protection was shown by the lack of nasal shedding of virus following challenge and by the lack of lung lesions at 1 week following the challenge infection. Thus, replication-incompetent adenovirus vaccines given simultaneously to pigs are efficacious for SIV and have the additional advantage over commercial vaccines that suckling piglets have no pre-existing maternally-derived antibody to block early life vaccination.
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Keywords: Recombinant adenovirus vaccine; Swine influenza virus; Subtype H3N2

1. Introduction

Swine influenza virus (SIV) subtype H3N2 emerged in the United States in 1998 as a cause of severe respiratory disease, particularly in finishing pigs and pregnant dams [1,2]. The signs of acute influenza disease were animals with high fevers (40.0–41.5 °C), coughing, labored breathing, abortions and a low percentage of deaths in sows and even in some boar studs [3]. In the US before 1998, influenza disease in swine was caused by classic SIV subtype H1N1 [4]. The inactivated H1N1 vaccine, commercially available since 1994, provided protection to the homologous H1N1 subtype but did not provide significant protection from the emerging disease caused by the H3N2 influenza virus [5,6]. Now the new H3N2 subtype of swine influenza has become well established and widespread in US swine and, more recently, additional reassortant SIVs have been discovered [7,8]. Currently, H1N1 and H3N2 are the dominant subtypes causing disease in North American swine and with some frequency both subtypes can be found cocirculating in the same herd. Bivalent, killed vaccines are now commercially available but new, second generation vaccines that are capable of inducing virus-specific neutralizing antibody plus cell mediated immunity will provide superior protection against the acute influenza diseases.

SIV is an influenza type A virus. The genome of influenza A viruses consists of eight segments of single-stranded, negative-sense RNA encoding 10 viral proteins [9]. RNA segment 4 contains the gene encoding the large hemagglutinin (HA) glycoprotein that projects from the surface envelope of the virion. Segment 5 encodes the nucleoprotein (NP) gene. The viral NP associates with the RNA segments to form a ribonucleoprotein which interacts with

* Corresponding author. Tel.: +1-515-663-7358; fax: +1-515-663-7458.
E-mail address: rwesley@nadc.ars.usda.gov (R.D. Wesley).
[1] Present address: NIH/NIAID/Vaccine Research Center, 40 Convert Drive, MSN 3017, Bethesda, MD 20892, USA.

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the transcriptase complex consisting of PB1, PB2, and PA to form the virus nucleocapsid [9]. The HA immunogen induces predominately a subtype-specific humoral immune response [10,11]. The conserved NP is group-specific stimulating cytotoxic T lymphocytes for cross-reactive immunity to all influenza A subtypes [12–14].

Adenovirus is a double-stranded linear DNA virus with a genome of approximately 36 kb in length. Adenovirus vectors, particularly those constructed from human adenovirus serotype 5 (Ad5), have been used to express genes of interest for use in gene therapy and vaccine development [15–20]. The Ad5 recombinant viruses are often replication-defective due to a large deletion in the early transcription region 1 (E1) of the genome. These replication-defective Ad5 viruses can grow only in cells, like 293 cells, that complement the E1 region of the adenovirus genome [21]. Similarly, many of these vectors contain a deletion in the E3 region of Ad5, which results in a loss of inhibition of the major histocompatibility complex (MHC) class I response leading to an increase in the ability of animals infected by these viruses to develop an immune response to the expressed foreign genes [22]. Moreover, high levels of expression are achieved in the Ad5 vector system when foreign genes are under the control of constitutive promoters like the CMV promoter [23]. Other advantages of the human Ad5 viruses are their broad host range and, in particular for livestock, the lack of pre-existing, maternally-derived antibodies which can interfere with vaccine efficacy in young and growing pigs.

Vaccination strategies using non-replicating virus vectors [24–31] or DNA-based vaccines [11,32] have been used successfully to immunize pigs. In this paper the use of replication-defective human Ad5 as a recombinant vaccine in a non-host species was tested. We show that immunization of weaned pigs with Ad5 recombinants expressing the HA and NP genes of SIV subtype H3N2 is able to elicit a protective immune response. The level of active immunity and the degree of protection for pigs was determined and the potential use of Ad5 as a vaccine vector in pigs is discussed.

2. Materials and methods

2.1. Viruses

The two SIVs used in this study were provided by Dr. Bill Mengeling (Ames, IA) and were from different farms in Iowa. The transgenes expressed by Ad5 were prepared from the RNA of a H3N2 influenza virus isolated in the fall of 1999 from a pig on a farm in northwest Iowa that was experiencing a severe outbreak of respiratory disease. The challenge H3N2 virus was prepared from tissues submitted to the Iowa State University Veterinary Diagnostic Laboratory. The submitted tissues were from a farm with severe respiratory disease and the challenge virus was passed only in pigs as lung homogenates and lung lavage fluids. Sequencing the N-terminal portion of the HA genes indicated that the two viruses were closely related with changes at residues 122 and 357 (99% amino acid identity).

2.2. Construction of the recombinant adenoviruses Ad5-HA-14.2 and Ad5-NP-13.4

The recombinant adenoviruses were constructed using the AdEasy^{TM} vector systems (Qbiogene Inc., Carlsbad, CA) and have been described previously [33]. Viral RNA was extracted from allantoic fluids containing egg passed swine influenza virus (H3N2) using the QIAamp viral RNA kit (Qiagen, Valencia, CA). For RT-PCR the following primers were used. To amplify the HA gene the forward primer was 5′-GTACAGCGGCCGCACCACCATGGGTATGAAGAC-TATCATTGCTT-3′ and the reverse primer was 5′-GTCA-CGATATCTCAAATGCAAATGTTGC-3′. To amplify the NP gene the forward primer was 5′-GTACAGGTAC-CACCATGGCGTCTCAAGGCAC-3′ and the reverse primer was 5′-GTCATGCGGCCGCTCAATTGTCATACT-CCTCTGC-3′. The HA amplicon was digested with Not I and EcoRV and the NP amplicon was digested with Not I and Kpn I. Both amplicons were subcloned at the multiple cloning site of the transfer vector between the human CMV promoter and the SV40 polyadenylation signal, and subsequently transferred into the adenoviral genome by homologous recombination [34]. The recombinant adenoviruses used as vaccines to immunize pigs were designated Ad5-HA-14.2 and Ad5-NP-13.4, respectively. To prepare large stock solutions of Ad5-HA-14.2 and Ad5-NP-13.4, the recombinant viruses were propagated in 293 cells (Qbiogene Inc., Carlsbad, CA) and the upscaling procedure outlined in the manufacturer's manual was followed. From the original transfection of 293 cells each recombinant virus was expanded by three additional passages on 293 cells and then concentrated and purified by discontinuous and continuous CsCl gradients. The recombinant adenoviruses were dialyzed against 10 mM Tris pH 8.0, 2 mM $MgCl_2$, 5% sucrose, titered and stored at −80 °C. The final titers were 10^{11} tissue culture infectious dose 50 ($TCID_{50}$)/ml for the recombinant adenovirus expressing the H3 protein (Ad-HA-14.2) and 2×10^{11} $TCID_{50}$/ml for the recombinant adenovirus expressing NP (Ad-NP-13.4).

2.3. Radiolabeling of cell cultures and immunoprecipitation

Human 293 cells in 60 mm dishes were infected with approximately 5–8 plaque-forming-units (PFU) of Ad5-HA-14.2, Ad5-NP-13.4 or control Ad5 recombinant virus per cell and incubated overnight at 37 °C. The cells were washed twice in methionine-free medium and then radiolabeled in methionine-free medium containing 100 μC/ml [^{35}S]-methionine (Amersham Biosciences Corp., Piscataway, NJ) for 2.5 h. The cells were harvested, lysates were prepared and immunoprecipitation

was carried out as described [35] using H3 or NP specific monoclonal antibodies (MAb 8254 or #5001, Chemicon International Inc., Temecula, CA) or anti-SIV polyclonal serum. [^{35}S]-methionine labeled lysates of SIV-infected Madin-Darby canine kidney (MDCK) cells were prepared similarly. For the SIV lysate, MDCK cells were infected and incubated in McCoys 5A medium containing 0.5 μg/ml of L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin for 7.5 h. The cells were pulse labeled for 2 h.

Before electrophoresis, immune complexes on Protein A Sepharose CL4B beads were denatured by boiling for 5 min in 25 μl of the 2× sample loading buffer. Sample supernatants were layered onto a 12% Tris–glycine precast gel (Invitrogen Corp, Carlsbad, CA) and run in a NOVEX Xcell II mini-cell unit. Following electrophoresis, the gel was washed for 30 min in 1 M Na salicylate in 10% methanol, vacuum dried and exposed to X-ray film for autoradiography.

2.4. Vaccination and challenge of weaned pigs

The experimental design comprised 50 SIV seronegative pigs from a specific-pathogen-free herd that were randomly assigned to five groups (10 pigs per group). These pigs were weaned at 2 weeks of age, delivered to the National Animal Disease Center and allowed to acclimate to their new environment and new feed for 1 week. Each group of pigs was housed separately in an individual animal isolation room. At 3 weeks of age they were vaccinated and 5 weeks later they were challenged as indicated in Table 1.

For vaccination 2×10^{10} $TCID_{50}$ of recombinant virus was given to each pig intramuscularly (IM) in 0.5 ml. For group 4 pigs both viruses were given at 2×10^{10} $TCID_{50}$ in a 0.5 ml mixture. The challenge virus was serially passed only in pigs and the virus titer was determined on MDCK cells as described in Section 2.5. Lavage fluids from the lungs of pigs infected with the challenge virus that showed the most extensive lesions were pooled. The challenge virus titer from the pooled lung lavage fluids was 7×10^5 $TCID_{50}$/ml. For challenge, pigs were anesthetized by IM injection of a mixture of xylazine (22 mg/ml), Telazol® (33 mg/ml, Fort Dodge Animal Health, Fort Dodge, IA), and ketamine (44 mg/ml) at a dose of 1 ml/5.5 kg of body weight. While the anesthetized pigs were breathing deeply the challenge virus was given at 1.5 ml per nostril with a syringe adapted with a tight fitting nasal tip. To reduce the possibility of secondary bacterial infections, oxytetracycline (20 mg/kg) was given IM at the time of challenge and once again at 2 days post challenge. Group 1 pigs were environmental controls. These controls were treated similarly but not vaccinated or challenged. All of the pigs were necropsied 7 days after challenge infection.

Table 1
Experimental design

Group ($n = 10$)	Vaccination	Challenge
1	No	No
2	No	Yes
3	Yes (HA)	Yes
4	Yes (HA + NP)	Yes
5	Yes (NP)	Yes

Clinical signs post challenge were monitored by observing the animals twice per day and daily body temperatures were determined for 5 days post challenge. For virus shedding, nasal swabs from each pig were collected daily from day 0 through day 5. Moistened, dacron polyester tipped applicators (Daigger and Co. Inc., Vernon Hills, IL) were used. After swabbing the applicators were submerged in 1 ml of McCoys transport medium (McCoys 5A medium, Gibco Invitrogen Corp., Carlsbad, CA) supplemented with penicillin (25 U/ml), streptomycin (25 μg/ml), neomycin (25 μg/ml), bacitracin (0.25 U/ml), and gentamycin (50 μg/ml) and promptly frozen and stored at −80 °C. At 7 days post challenge the control pigs and principals were euthanized, lungs were examined for gross lesions and the degree of consolidation on the surface of each of the seven lung lobes was estimated visually. Mean lung scores were calculated as the sum of percent consolidation of each lung lobe divided by 7. McCoys transport medium was also used to collect 20–30 ml of lung lavage fluid per pig.

2.5. SIV isolation and titration from nasal swabs and lung lavages

Madin-Darby canine kidney cells in 24 well plates were washed twice with trypsin-containing medium [McCoys 5A medium supplemented with TPCK-treated trypsin (0.5 μg/ml, Sigma, St. Louis, MO), penicillin (25 U/ml), streptomycin (25 μg/ml), neomycin (25 μg/ml), bacitracin (0.25 U/ml), gentamycin (50 μg/ml), and amphotericin B (2.5 μg/ml)]. The first rinse was quick, followed by a second wash for 30 min at 37C. After the trypsin incubation, the medium was removed and 250 μl of a nasal swab or lung lavage sample (in TPCK-treated trypsin at 0.5 μg/ml) was added to a well and incubated for 2 h at 37 °C. After absorption, samples were aspirated, 1ml of the trypsin-containing medium was added to each well and the plates were incubated at 37 °C in 5% CO_2. Each well was observed daily for viral cytopathic effect (CPE). After 3 days, negative sample wells were passed a second time on trypsin-treated MDCK cells. For this pass, six well plates were used and the entire supernatant (1 ml) of the previous plate's negative well was used to inoculate new wells. Samples were absorbed for 2 h at 37 °C, removed and 4 ml of trypsin-containing medium added to each well. The plates were incubated at 37 °C in 5% CO_2 and observed daily. After 5 days, if no viral CPE was observed, the sample was considered negative.

For SIV titrations of positive nasal swabs, a 10-fold dilution series was prepared from the original sample in the trypsin-containing medium. Confluent MDCK cells in

96 well plates were washed twice with trypsin-containing medium, medium was removed and, in quadruplicate, 50 μl of undiluted or diluted sample was added per well. After 2 h at 37 °C, test samples were removed and replaced with 200 μl of trypsin-containing medium. The plates were incubated at 37 °C in 5% CO_2, observed daily for viral CPE and after 6 days they were fixed with methanol and stained with crystal violet. The positive wells were recorded and the titer calculated using the Karber statistical method [36].

2.6. Serological tests

Hemagglutination-inhibition (HI) tests and HA titrations were performed in microtiter plates [37,38]. Serum samples were pretreated with receptor destroying enzyme (RDE) from *Vibrio cholerae* (BioWhittaker Inc., Walkersville, MD). For pretreatment, 50 μl of serum was mixed with 200 μl of RDE (100 U/ml) and incubated overnight at 37 °C. Next, 150 μl of a 2.5% sodium citrate solution was added and heat inactivated at 56 °C for 30 min. Two hundred μl of treated serum was mixed with 50 μl of a 50% solution of washed turkey red blood cells (RBCs) and incubated for 30 min at room temperature. After this incubation, the samples were centrifuged (800 × *g*, 10 min at 4 °C). The supernatant, a 1 to 10 dilution of the original serum sample, was used in the HI test.

The antigen for the HI test was egg passaged virus that was homologous to the HA transgene used to construct the recombinant vaccine. Four units of HA antigen per well were incubated with a two-fold serum dilution series. Turkey RBCs (0.5%) were added, mixed and allowed to sit undisturbed for 45 min at 4 °C. The final titer was calculated as the reciprocal of the highest serum dilution giving complete inhibition.

2.7. Statistical analysis

A single-factor analysis of variance (ANOVA) was used to analyze mean lung lesion scores, HI titers and virus titers in nasal swabs at each time unit interval. The treatment factor for both titer types and lung lesion analysis was group. The following comparisons were of interest at each time level: group 2 versus group 5 and group 3 versus group 4. A Levene's homogeneity of variance test was used on the data to check if any transformations were necessary. The transformation that stabilized serology variance was $TS = (\text{serology titer} + 0.1)^2$. No transformation of the nasal swab titer data could be found to further reduce the variance (due to group 4 having zero variance), so raw data were used in the analysis. The lung lesion data used the transformation $TL = 1/(\text{lesions} + 0.1)$ to stabilize the variance. A Duncan's multiple range test at the $P = 0.01$ level was used as the multiple comparison procedure for determining pairwise differences if a significant *F*-test resulted from an ANOVA.

3. Results

3.1. Expression of the hemagglutinin and nucleoprotein in Ad5-HA-14.2 and Ad5-NP-13.4 infected 293 cells

Human 293 cells were infected with Ad5-HA-14.2 or Ad5-NP-13.4 recombinant viruses, radiolabeled with [^{35}S]-methionine and expression of the foreign protein was detected by immunoprecipitation with convalescent swine antiserum and with anti-H3 or anti-NP monoclonal antibodies (Fig. 1). Sham-inoculation of 293 cells with recombinant Ad5 that expressed no foreign protein and SIV-infected MDCK cells were used as controls. The HA protein expressed by recombinant Ad5-HA-14.2 previously has been shown to insert into the surface plasma membrane of the infected 293 cells [33]. The immunoprecipitation results demonstrated that the HA from Ad5-HA-14.2 infected 293 cells had a lower apparent molecular weight than the HA from SIV-infected cell lysates. This lower molecular weight was not due to a truncated protein since sequencing of the H3 gene in the shuttle vector demonstrated that the entire gene was inserted. Similarly, the entire NP gene was subcloned but the recombinant Ad5-NP-13.4 expressed NP migrated slightly faster than the NP in SIV-infected cell lysates.

3.2. Hemagglutination-inhibition antibody response to vaccination and to challenge

To determine the immune response to the vaccine expressed HA antigen, serum HI titers were measured prior to vaccination and at 2, 4 and 5 weeks post vaccination and at necropsy which was at 6 weeks post vaccination. Challenge virus inoculation of all pigs except those in group 1 occurred after the 5th week post vaccination bleeding followed 1 week later by necropsy and collection of the final sera at 6 weeks post vaccination. The antibody responses for each group following vaccination and challenge are shown in Fig. 2. Pigs vaccinated with recombinant adenovirus expressing the HA antigen alone or simultaneously with Ad5-NP-13.4 (groups 3 and 4) showed good HI titers (296 and 280, respectively) by 2 weeks post vaccination and very high HI titers by the 4th and 5th week post vaccination. The pigs in these two groups that had already developed high levels of antibody to influenza virus showed an increase but not a marked increase in HI titer 1 week after the challenge infection. The control pigs of group 2 and the pigs vaccinated with recombinant adenovirus expressing NP antigen alone (group 5) developed no detectable HI antibody by 5 weeks post vaccination and both groups developed low HI titers 1 week after challenge. The environmental control group (group 1) remained negative for HI antibody throughout the duration of the experiment.

3.3. Clinical signs

Following challenge infection all vaccinated and control pigs were asymptomatic. During the week following

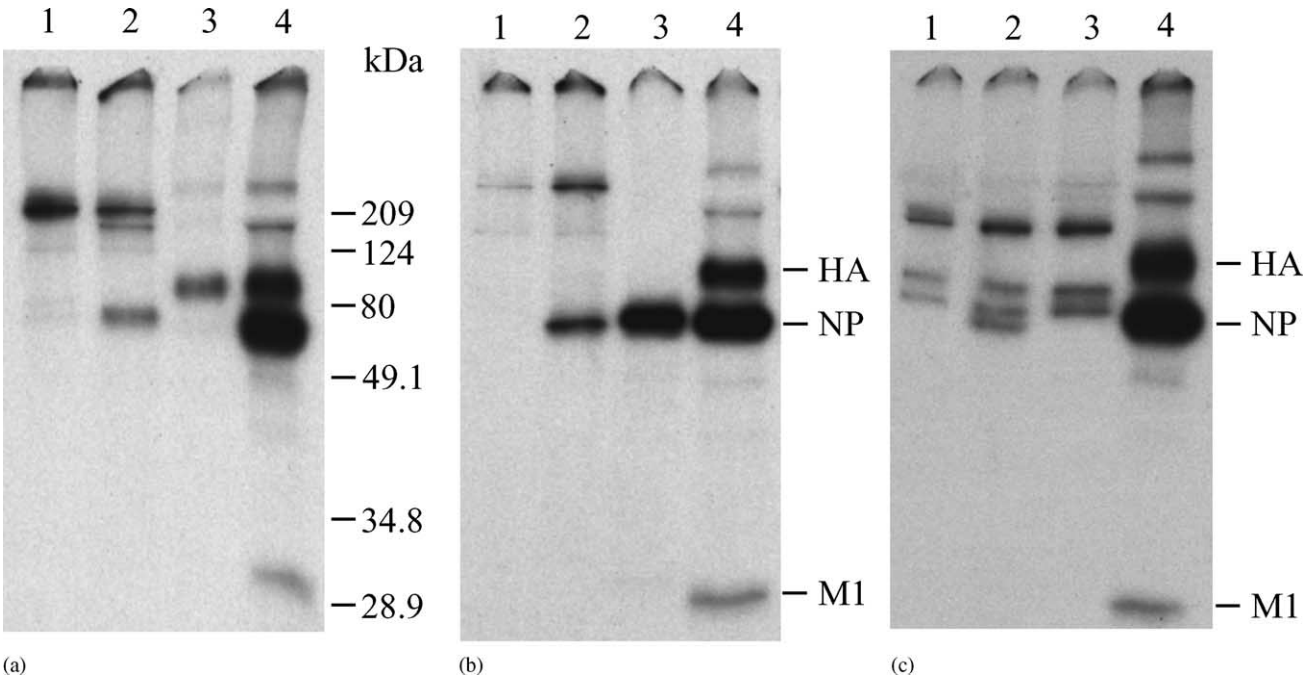

Fig. 1. Immunoprecipitation patterns of HA and NP expressed proteins. (a) Cell lysates immunoprecipated with anti-H3 monoclonal antibody. Lane 1: non-expressing Ad5 control virus infected 293 cells. Lane 2: Ad5-HA-14.2 infected 293 cells. Lane 3: SIV-infected MDCK cells. (b) Cell lysates immunoprecipitated with anti-NP monoclonal antibody. Lane 1: non-expressing Ad5 control virus infected 293 cells. Lane 2: Ad5-NP-13.4 infected 293 cells. Lane 3: SIV-infected MDCK cells. (c) Cell lysates immunoprecipitated with polyclonal anti-SIV pig serum. Lane 1: Non-expressing Ad5 control virus infected 293 cells. Lane 2: Ad5-NP-13.4 infected 293 cells. Lane 3: Ad5-HA-14.2 infected 293 cells. Lane 4 in panels (a), (b) and (c) is SIV-infected MDCK cell lysate immunoprecipitated with polyclonal anti-SIV pig serum.

challenge the pigs were observed twice per day for signs of respiratory disease but no signs occurred under the clean, stress-free conditions of the isolation barn. Body temperatures for all pigs did increase during the 5 days immediately post challenge (Fig. 3). The body temperature profiles also indicated that the challenge infection with H3N2 virus was relatively mild. For the non-vaccinated control pigs that were challenged, the average body temperature for the 10 pigs in the group peaked on day 2 post challenge but remained below 40 °C (the fever threshold). For these group 2 pigs, only 4 of 10 pigs had body temperatures above 40 °C on day 2 post challenge. Conversely, all three groups of vaccinated pigs had group average body temperatures at or just above 40 °C by day 1 post challenge and on day 2,

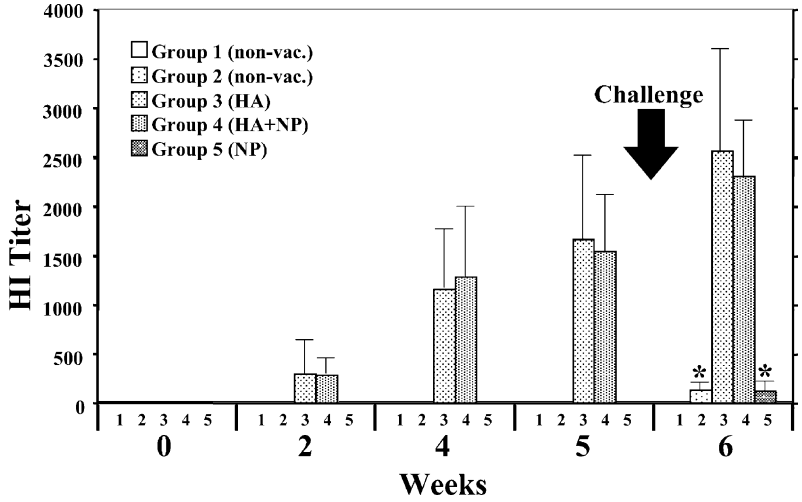

Fig. 2. Geometric mean HI antibody titers and standard deviations for each group post vaccination and post challenge. Mean HI titers for groups 2 and 5 following challenge inoculation (asterisks) are significantly lower than the HI titers of groups 3 and 4 ($P < 0.0001$). Mean HI titers for groups 3 and 4 were not significantly different.

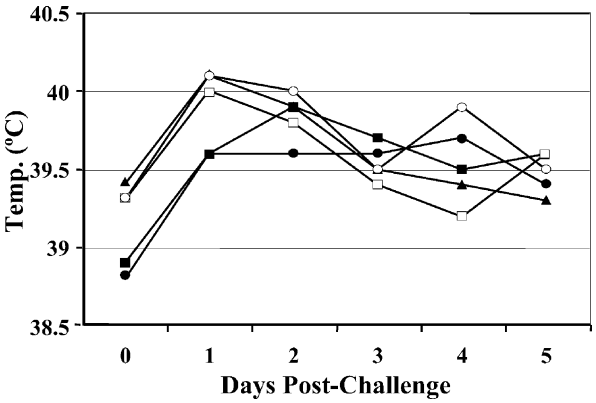

Fig. 3. Post challenge group average body temperatures. Group 1 environmental control pigs (●), group 2 non-vaccinated control pigs (■), group 3 Ad5-HA-14.2 vaccinated pigs (▲), group 4 Ad5-HA-14.2 + Ad5-NP-13.4 vaccinated pigs (□), and group 5 Ad5-NP-13.4 vaccinated pigs (○).

their average body temperatures began to decline and were similar to the challenge control pigs in group 2.

3.4. Nasal shedding of the challenge virus following intranasal inoculation

Nasal shedding for vaccinated and non-vaccinated control pigs are summarized in Fig. 4. The pigs vaccinated with Ad5-HA-14.2 were well protected from challenge infection. No virus was shed from group 4 pigs that were simultaneously vaccinated with both recombinants. Low levels of challenge virus shedding occurred on post challenge days 2, 3 and 4 in group 3 pigs that were vaccinated with Ad5-HA-14.2 alone. Six of the 10 pigs in group 3 did not shed virus during the 5 days post challenge. One group 3 pig shed low levels of virus for three consecutive days. This pig and one other was shedding virus on day 2 post challenge and two additional pigs shed low amounts of virus only on day 4 post challenge. The differences in shedding patterns between group 3 and group 4 pigs were not statistically significant.

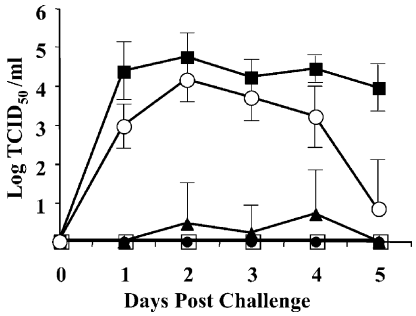

Fig. 4. Nasal shedding patterns post challenge. Geometric mean virus titers ± standard deviations in nasal swabs ($\log_{10}$ $TCID_{50}$/ml) after challenge with 2×10^6 $TCID_{50}$ of H3N2 virus. Group 1 environmental control pigs (●), group 2 non-vaccinated control pigs (■), group 3 Ad5-HA-14.2 vaccinated pigs (▲), group 4 Ad5-HA-14.2 + Ad5-NP-13.4 vaccinated pigs (□), and group 5 Ad5-NP-13.4 vaccinated pigs (○). Pigs in environmental control group 1 and vaccinated group 4 pigs were negative for SIV on the day of inoculation and on each day post challenge. Group 5 pigs shed significantly less challenge virus than group 2 control pigs on days 1 ($P < 0.01$), 4 ($P < 0.01$), and 5 ($P < 0.01$) post challenge. There was no significant difference in shedding between vaccinated pigs in groups 3 and 4.

Some protection occurred in group 5 pigs vaccinated with Ad5-NP-13.4 alone. Compared to the challenged, non-vaccinated control pigs in group 2, the group 5 pigs on average shed less virus on each of the 5 days post challenge (Fig. 4). By day 5 post challenge only low levels of virus were shed by three of the vaccinated pigs while at the same time interval post challenge all non-vaccinated control pigs still shed high levels of virus. These differences in nasal virus shedding between groups 2 and 5 pigs were statistically significant on days 1, 4 and 5 post challenge ($P < 0.01$).

3.5. Gross lung lesions and lavage fluids

Despite a lack of clinical signs in all pigs resulting from the intranasal challenge, all but one of the non-vaccinated, group 2 control pigs showed typical SIV lung lesions (Table 2). The SIV-induced lung lesions at 7 days post challenge were on the apical and cardiac lobes and consisted of reddish areas of consolidation. Group 2 challenged control pigs were the most severely affected with an overall average lung score of 6.4 ± 6.9%. Amongst the vaccinated groups, the levels of protection from the least protected to completely protected were group 5 to group 3 to group 4. Lung scores for the groups 3 and 4 vaccinated pigs were significantly less ($P < 0.0001$) than for either the group 2 non-vaccinated control pigs or for the group 5 pigs vaccinated with the recombinant vaccine expressing NP alone. Only two vaccinated pigs in group 5 had lungs that appeared normal while in group 3 vaccinated pigs, eight of the lungs were normal and for the best protected group of pigs, group 4, vaccinated with both recombinants, all the pigs had normal lungs identical to the lungs of group 1, the environmental control pigs.

Lung lavages were also obtained at necropsy. However, by 7 days post challenge, none of the non-vaccinated control

Table 2
Gross lung lesions at necropsy

Group ($n = 10$)	Normal lungs	Mild to moderate lung scores	Mean lung scores (standard deviation)
1 (non-vaccinated)	10	0	0
2 (non-vaccinated)	1	9	6.4 ± 6.9
3 (HA vaccinated)	8	2	0.04 ± 0.1[a]
4 (HA + NP)	10	0	0[a]
5 (NP vaccinated)	2	8	2.3 ± 3.4

[a] Lung scores for groups 3 and 4 vaccinated pigs were significantly less ($P < 0.0001$) than for group 2 control pigs or for group 5 vaccinated pigs. Differences in lung scores between groups 3 and 4 pigs were not significant.

pigs as well as the vaccinated pigs had detectable virus in their lung lavage fluids (data not shown).

4. Discussion

Overall, the results clearly show that the recombinant adenovirus vaccines for SIV induce significant protective immunity. Protective antibodies, stimulated by Ad5-HA-14.2 expressing the HA protein, had the dominate role for protecting pigs but the recombinant expressing the H3N2 nucleoprotein induced low levels of immunity which was, at least, additive in promoting clearance of the challenge virus. Together the two recombinant vaccines completely prevented nasal shedding of the challenge virus and completely eliminated gross lung lesions. Therefore, the combined vaccination with recombinants expressing both SIV proteins provides the highest degree of protection.

Studies in mice, chickens and pigs have shown that influenza HA alone is enough to provide protection within a subtype via antibody-mediated immunity [10,11,33,39]. Protective immunity is established even at low or undetectable levels of serum antibody to HA. In the present study, HA is shown to be expressed in vitro in Ad5-HA-14.2 infected 293 cells. The molecular weight of the adenovirus expressed HA is smaller than the HA glycoprotein synthesized in SIV-infected MDCK cells. This smaller sized protein is most probably due to differences in post translational modifications of HA in the two different cell cultures, i.e. differences in glycosylation patterns and oligosaccharide side chains. In pigs, the high HI titers show that Ad5-HA-14.2 expressed HA was very immunogenic despite its lower molecular weight. By 2 weeks post vaccination groups of pigs vaccinated with HA singly or with HA simultaneously with NP elicited significant HI titers. By the 4th week post vaccination group average HI titers were above 1000 and by week 5 HI titers continued to rise and were above 1500. These high levels of induced antibody were much better than those induced by DNA-based vaccines, by commercial killed influenza vaccines or by fowl pox vectored HA and NP vaccines in poultry [11,39]. Our results might have been anticipated since recombinant adenoviruses in swine have also stimulated strong antibody-mediated immunity against pseudorabies and foot-and-mouth disease [29,30]. Thus the strong serum HI response to vaccination with Ad5-HA-14.2 was promising and suggested the possibility of solid protective immunity.

The duration and extent of nasal virus shedding was reduced in group 5 pigs vaccinated with Ad5-NP-13.4 alone. This result contrasts with DNA-based NP vaccination in pigs and differs from the immunity induced in chickens vaccinated with fowl pox recombinants expressing the influenza NP gene [11,39]. NP expression in chickens did not boost vaccine protection while in pigs vaccination with recombinant adenovirus expressed NP reduced SIV replication and promoted virus clearance. We have yet to determine the nature of Ad5-NP-13.4 induced immunity in pigs, but generally, NP has been shown to elicit Th-1 type immunity for clearance of influenza A viruses [12–14,40]. This type of cellular immunity is not subtype specific but is broad and cross protective. Incorporating cross protective immunity in a SIV vaccine for pigs is an improvement over commercial inactivated vaccines and an added asset to protect pigs. The type and duration of NP induced immunity in pigs requires further study.

There are a number of advantages for the swine industry to vaccinate with a vectored vaccine like the adenovirus recombinants. (i) The recombinants have a high degree of safety because they are replication-defective. After IM inoculation, the adenovirus recombinants infect muscle cells and other cell types thus presenting the HA and NP antigens to the pig's immune system in a manner that mimics natural infections. But the defective-recombinants do not disseminate further since they do not undergo any additional rounds of replication. (ii) As we show in this study a single dose of recombinant vaccine was sufficient to protect pigs at least from a closely related virulent H3N2 virus. This result contrasts with the time and expense of using inactivated commercial vaccines that require a two dose regime. Moreover, in pigs, replication-defective adenoviruses are better at stimulating humoral immunity than either DNA-based or killed vaccines [11,27]. (iii) Adenoviruses are non-enveloped, hard shelled viruses. The rugged nature of adenoviruses will probably allow for their use in pneumatic guns thus eliminating a swine industry problem of broken vaccination needles in livestock. (iv) And finally, but probably the most important feature, is that there are no pre-existing maternal antibodies [27,29]. Thus, very young pigs as old as 1 day of age can be successfully vaccinated without the worry of vaccine failure due to interference by maternally-derived, suckled antibodies.

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