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Overcoming maternal antibody interference by vaccination with human adenovirus 5 recombinant viruses expressing the hemagglutinin and the nucleoprotein of swine influenza virus

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Abstract

Sows and gilts lack immunity to human adenovirus 5 (Ad-5) vectored vaccines so immunogens of swine pathogens can be expressed with these vaccines in order to immunize suckling piglets that have interfering, maternally derived antibodies. In this study 7-day-old piglets, that had suckled H3N2 infected gilts, were sham-inoculated with a non-expressing Ad-5 vector or given a primary vaccination with replication-defective Ad-5 viruses expressed the H3 hemagglutinin and the nucleoprotein of swine influenza virus (SIV) subtype H3N2. The hemagglutination inhibition (HI) titer of the sham-inoculated group ($n = 12$) showed continued antibody decay whereas piglets vaccinated with Ad-5 SIV ($n = 23$) developed an active immune response by the second week post-vaccination. At 4 weeks-of-age when the HI titer of the sham-inoculated group had decayed to 45, the sham-inoculated group and half of the Ad-5 SIV vaccinated pigs were boosted with a commercial inactivated SIV vaccine. The boosted pigs that had been primed in the presence of maternal interfering antibodies had a strong anamnestic response while sham-inoculated pigs did not respond to the commercial vaccine. Two weeks after the booster vaccination the pigs were challenged with a non-homologous H3N2 virulent SIV. The efficacy of the vaccination protocol was demonstrated by abrogation of clinical signs, by clearance of challenge virus from pulmonary lavage fluids, by markedly reduced virus shedding in nasal secretions, and by the absence of moderate or severe SIV-induced lung lesions. These recombinant Ad-5 SIV vaccines are useful for priming the immune system to override the effects of maternally derived antibodies which interfere with conventional SIV vaccines.
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1. Introduction

Swine influenza virus (SIV) causes a severe respiratory disease, particularly in finishing pigs and

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pregnant sows (Janke, 2000; Olsen, 2002). Clinical signs include pigs with high fever, coughing, labored breathing, abortions and a low percentage of deaths in sows (Easterday and Van Reeth, 1999). The genome of SIV consists of 8 segments of single-stranded, negative-sense RNA encoding 10 viral proteins (Lamb and Krug, 2001). 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 HA immunogen induces predominately subtype-specific humoral immunity (Andrew et al., 1987; Macklin et al., 1998). The conserved NP is group-specific stimulating cytotoxic T lymphocytes for cross-protection (Yewdell et al., 1985; Wraith et al., 1987; Ulmer et al., 1993).

Human adenovirus type 5 (Ad-5) vectors have been used to express foreign genes for use in gene therapy and for vaccine development (Berkner, 1988; Prevec et al., 1989; Graham and Prevec, 1992; Grunhaus and Horwitz, 1992; Eloit, 1995; Hitt and Graham, 2000). The Ad-5 recombinant viruses are rendered replication-defective by introducing a large deletion in the early transcription region 1 (E1) of the genome. These replication-defective Ad-5 viruses grow to very high titers in 293 cells that complement the E1 region of the adenovirus genome (Graham et al., 1977). Similarly, many of these vectors contain an E3 region deletion which results in an enhanced immune response to the expressed foreign genes (Chengalvala et al., 1994). Moreover, high levels of expression are achieved in the Ad-5 vector system when foreign genes are under the control of a constitutive promoter (Ambriovic et al., 1997). Other advantages of the human Ad-5 vectors include their broad host range and the lack of preexisting, maternally derived antibodies which can interfere with vaccine efficacy in young and growing pigs. Sows and gilts generally have some immunity to ubiquitous porcine adenoviruses but they lack immunity to human adenoviruses.

Vaccination strategies using non-replicating virus vectors (Eloit et al., 1990; Konishi et al., 1992; Brockmeier et al., 1993; Le Potier et al., 1997; Mayr et al., 1999, 2001; Monteil et al., 2000; Moraes et al., 2002) or DNA-based vaccines (Macklin et al., 1998; Larsen et al., 2001) have been used experimentally to prime or to immunize pigs. Direct comparisons for efficacy between naked DNA and Ad-5 vectored vaccines using the pseudorabies virus gD glycoprotein have been carried out in pigs with maternal antibodies (Le Potier et al., 1997; Monteil et al., 1997). Theoretically, critical immunogenic proteins of numerous swine pathogens can be expressed with Ad-5 vectored vaccines in order to immunize suckling piglets that have acquired interfering, maternally derived antibodies. These passively acquired antibodies are important for the early protection of piglets but are a common cause of vaccine failure if vaccines are administered to young suckling or nursery-age pigs. Thus, inactivated influenza vaccines for swine are not administered until maternally derived antibodies have decayed to background or to a sufficiently low level. Since this time interval is dependent on the initial amount of passive antibody ingested by each piglet, the actual timing for a successful vaccination is only estimated. Generally, killed influenza vaccines are given twice to weaned pigs as late as 12 weeks and again at 16 weeks-of-age. Consequently, a vaccine that can stimulate immunity in the neonatal pig even in the presence of maternally derived antibodies would offer additional protection and safety.

In this study we used replication-defective, recombinant Ad-5 viruses to prime immunity in 1-week-old piglets that had natural field exposure levels of maternally derived SIV antibodies. Three weeks later, the primed piglets were vaccinated with a commercial SI vaccine yielding an anamnestic humoral immune response and strong protective immunity.

2. Materials and methods

2.1. Experimental design

Six gilts were purchased from a high-health swine herd free of porcine reproductive and respiratory syndrome virus (PRRSV) and delivered to the National Animal Disease Center (NADC) at 70 days of gestation. The gilts were tested and found to be seronegative for PRRSV but had hemagglutination inhibition (HI) titers to SIV (range: 80–320) indicating that the gilts had been naturally infected with SIV subtype H3N2 at some previous time on the farm. The gilts were seronegative for SIV subtype H1N1. All experimental procedures carried out with the gilts and

Table 1
Experimental design

Group	Priming dose with Ad-5 vector	Killed vaccine[a] as booster dose	Challenge
1[b] ($n = 12$)	H3 + NP	No	Yes
2[b] ($n = 11$)	H3 + NP	Yes	Yes
3[b] ($n = 12$)	Sham	Yes	Yes
4[c] ($n = 7$)	No	Yes	Yes
5[c] ($n = 7$)	No	No	Yes

[a] A standard 2 ml dose of End-FLUence® 2 (Intervet, Inc., Millsboro, DE) was given IM.
[b] Pigs in groups 1–3 suckled passive antibodies from naturally infected gilts.
[c] Naïve pigs.

their offspring were performed under the guidelines of the NADC Animal Care and Use Committee.

After farrowing, three of the six litters (35 piglets) suckled their dams for 20–22 days prior to weaning. These pigs were vaccinated or primed while on the dams as outlined in Table 1. Group 1 piglets ($n = 12$) were vaccinated IM at 1 week-of-age with an equal mixture of two replication-defective adenoviruses (2×10^9 $TCID_{50}$ of each virus in a total volume of 0.5 ml); one recombinant adenovirus expressing the H3 hemagglutinin and the other expressing NP (Wesley et al., 2004). At 6 weeks-of-age the group 1 primed pigs were challenged intratracheally with virulent H3N2 virus. Group 2 piglets ($n = 11$) were vaccinated similarly with the recombinant adenovirus mixture at one week-of-age, boosted (IM) at 4 weeks-of-age with commercial vaccine containing inactivated H1N1 and H3N2 subtypes, and challenged at 6 weeks-of-age. The remaining pigs with maternal antibodies (four per litter, $n = 12$) were group 3 pigs that were sham-inoculated IM at 1 week-of-age with 4×10^9 $TCID_{50}$ of replication-defective adenovirus that expressed no foreign protein, boosted with commercial vaccine at 4 weeks, and challenged at 6 weeks. Both the recombinant vaccine mixture and sham control were used within each litter because previously it was shown that vaccine does not spread amongst pigs in close contact (Wesley and Lager, 2005).

Piglets from the three other litters were separated from the gilts at farrowing and prevented from suckling colostrum. The colostrum-deprived piglets (there were 14 by day 14 post-farrowing) were raised on milk replacer (Esbilac, PetAg, Inc., Hampshire, IL) and gradually switched to solid creep feed. Group 4 naïve piglets ($n = 7$), free of anti-SIV antibodies, were vaccinated with commercial vaccine when 4 weeks old and challenged at 6 weeks-of-age. Naïve piglets in group 5, the challenge control pigs ($n = 7$), were challenged at 6 weeks-of-age. Daily body temperatures were determined before challenge and for 5 days post-challenge and animals were observed twice per day for clinical signs. For virus shedding, nasal swabs from each pig were collected on days 0 and 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 5 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. McCoys transport medium was also used to collect 20–30 ml of pulmonary lavage fluid per pig.

2.2. Intratracheal challenge

The challenge virus was prepared from tissues that were submitted to the Iowa State University Veterinary Diagnostic Laboratory and kindly provided by Dr. Pat Halbur. The submitted tissues were from a swine herd with severe respiratory disease and the H3N2 challenge virus was passed only in pigs as lung homogenates and lung lavage fluids. The challenge virus titer from pooled lung lavage fluids was 10^5 $TCID_{50}$/ml. The challenge virus and the SIV that provided the H3 and NP genes for the recombinant vaccines were isolated from different farms but their HA genes were closely related. The N-terminal HA1 portion of the challenge virus H3 gene was 99% identical at the level of deduced amino acids to the HA1 portion of the H3 gene expressed by the recombinant virus.

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. Using a laryngoscope, a small diameter tygon tube was passed over the epiglottis and through the larynx of the anesthesized pig and 2.2 ml of the SIV challenge was deposited in the trachea above its major bifurcation. To reduce the possibility of secondary bacterial infections, oxytetracycline (20 mg/kg, Liquamycin LA-200®, Pfizer Animal Health, Exton, PA) was given IM at the time of challenge and once again at 2 days post-challenge.

2.3. Serological assay

Blood (serum) was collected before priming with the recombinant adenoviruses and at 2–6 weeks-of-age and at 6 weeks plus 5 days. The 4 week serum samples were collected before boosting with the commercial vaccine. The 6 week serum samples were collected before challenge. The 6 weeks plus 5 days sera were collected at necropsy.

Hemagglutination-inhibition (HI) tests and HA titrations were performed in microtiter plates as previously described (Wesley et al., 2004). Serum samples were pretreated with Receptor Destroying Enzyme (RDE) from *Vibrio cholerae* (BioWhittaker, Inc., Walkersville, MD).

2.4. SIV isolation and titration from nasal swabs and pulmonary lavage samples

Madin–Darby canine kidney (MDCK) cells in 24-well plates were washed twice with trypsin-containing medium [McCoys 5A medium supplemented with L-1-Tosylamide-2-phenylethyl chloromethyl ketone (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 37 °C. 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, 1 ml of 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, the 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 scored and the titer calculated using the Karber method for calculating 50% endpoint dilutions (Specter and Lancz, 1986, pp. 193–195).

2.5. Statistical analysis

Single-factor analyses of variance (ANOVAs) were used to determine if there were mean nasal swab and pulmonary lavage titer differences between the five groups of pigs and to analyze group differences in mean body temperature on each day post-challenge. Differences of least squares means at $p \leq 0.05$ were used if a significant F-test statistic was obtained from an ANOVA at $p \leq 0.05$ for determining which groups differed from the others. Levene's homogeneity of variance tests were performed to check for transformation necessity before running analyses. All analyses were run using transformed data where necessary. SAS® Version 9.1.3 was the software used for all ANOVA analyses (SAS Institute, Inc., Cary, NC).

3. Results

3.1. Serological responses

The immune response was measured with the HI test (Fig. 1). The groups 1 and 2 pigs, immunized with

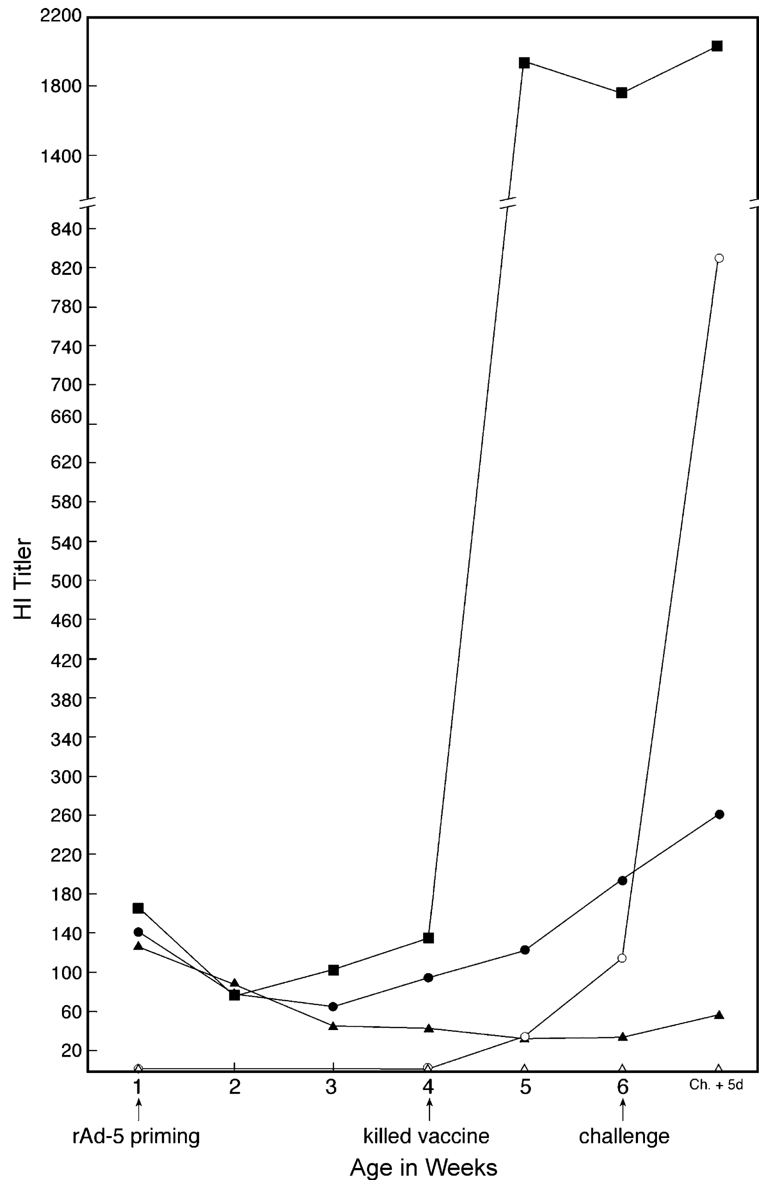

Fig. 1. Mean HI antibody titers for each group of pigs. At 1 week-of-age, groups 1 and 2 were immunized with rAd-5 H3 + NP and group 3 was sham inoculated with a non-expressing rAd-5. At 4 week-of-age, groups 2–4 were given a commercial killed vaccine. At 6 weeks-of-age, all groups were challenged with SIV subtype H3N2. Group 1 (●), group 2 (■), group 3 (▲), group 4 (○) and group 5 (△).

Ad-5 vectored vaccines at 1 week-of-age, developed an active anti-SIV immune response in the presence of maternally derived antibodies by the second week post-vaccination. In contrast, sham-inoculated, group 3 pigs had a steady decay of anti-SIV antibodies. At 4 weeks of age when the HI titer of sham-inoculated group 3 had decayed to 45, the sham-inoculated group and group 2 pigs were boosted with a commercial bivalent vaccine or naïve pigs (group 4) were given the commercial vaccine as a single primary dose. The boosted pigs that had been primed in the presence of maternal interfering antibody had a strong anamnestic response while sham-inoculated pigs did not respond to the commercial vaccine. The naïve pigs started to

develop an immune response by 1 week after vaccination. At 6 weeks-of-age all pigs were challenged with a non-homologous H3N2 virulent SIV. The group 4 pigs showed an anamnestic response to the challenge virus at necropsy 5 days later. All other groups of pigs did not show significant increases in HI titers.

3.2. Clinical signs post-challenge

Intratracheal challenge with SIV was used to increase the severity of infection. Even by this route of infection the clinical signs were mild. Coughing was observed on day 1 post-challenge for a group 4 pig and for two group 5 pigs. Coughing also occurred in two group 3 pigs on day 3 post-challenge. Sneezing occurred in a few pigs (groups 1 and 3) on days 1 and 2 post-challenge. A 1-day febrile spike occurred in all groups of pigs except the group 1 pigs (Fig. 2). The febrile response was the most severe (mean = 41 °C) for group 5 challenge-only pigs. The day 1 post-challenge febrile response was significantly different ($p \leq 0.05$) for all groups of pigs except for groups 3 and 4.

3.3. Gross lung lesions

Severe lung lesions were present in 7 of the 12 pigs in group 3 (Table 2). This demonstrated vaccine failure with the commercial vaccine given at 4 weeks-of-age when the pigs still had inhibiting levels of maternally derived antibodies. Vaccination with a single dose of the recombinant viruses (group 1) in pigs with maternal antibodies eliminated the severity of lung infection and was partially protective. The pigs that were best protected showing the least degree of lung involvement were the group 2 pigs that had been primed with the recombinant viruses at 1 week-of-age and boosted 3 weeks later. The group 2 pigs showed a higher level of protection than even the naïve pigs (group 4) that had been given a primary vaccination at 4 weeks-of-age and challenged 2 weeks later. The challenge-only control group 5 had pigs with only moderate to severe levels of lung involvement consisting of redden areas of sharply demarcated consolidation.

Table 2
Gross lung lesions

	No. of pigs in each group with			
	Normal lung	Mild lesions[a]	Moderate lesions[b]	Severe lesions[c]
Group 1[d]	6 (50%)	3 (25%)	3 (25%)	0
Group 2[d]	9 (82%)	2 (18%)	0	0
Group 3[d]	1 (8%)	2 (17%)	2 (17%)	7 (58%)
Group 4[e]	1 (14%)	4 (57%)	2 (29%)	0
Group 5[e]	0	0	3 (43%)	4 (57%)

[a] <5% in any lobe.
[b] ≥5% to <25% in any lobe.
[c] ≥25% in any lobe.
[d] Pigs in groups 1–3 suckled passive antibodies from naturally infected gilts.
[e] Naïve pigs.

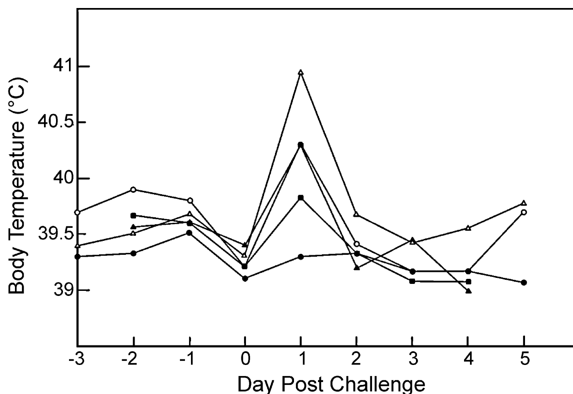

Fig. 2. Arithmetic group mean body temperatures pre- and post-challenge. Group 1 (●), group 2 (■), group 3 (▲), group 4 (○) and group 5 (△).

3.4. Upper and lower respiratory tract virus shedding post-challenge

Naïve pigs vaccinated with commercial vaccine 2 weeks prior to challenge (group 4) had no nasal shedding on day 5 post-challenge nor was virus recovered from pulmonary lavage fluids. For pigs that had acquired maternal antibodies, the priming and boosting strategy offered the best protection. Only 1 of 11 pigs in group 2 shed virus on day 5 post-challenge and no challenge virus was recovered from pulmonary lavage fluids at necropsy. Groups 2 and 4 were not statistically different ($p \leq 0.05$) in terms of either virus recovery from nasal swabs or by recovery from pulmonary lavage fluids (Table 3). The primed-only group 1 pigs did slightly better in terms of reducing virus replication than the vaccinated-only pigs (group

Table 3
Post-challenge virus titer and frequency of recovery from nasal swabs and pulmonary lavage fluids

Group	Nasal shedding			Pulmonary lavage fluids	
	Log $TCID_{50}$/ml (day 0)	Log $TCID_{50}$/ml[a] (day 5)	No. of shedding per total pigs	Log $TCID_{50}$/ml (day 5)	No. of virus positive per total pigs
Suckled maternal Abs					
1	0	3.1 ± 0.4 A[b]	11/12	0.3 ± 0.2 BC[b]	2/12
2	0	0.2 ± 0.2 BC	1/11	0 C	0/11
3	0	3.1 ± 0.4 A	11/12	1.9 ± 0.6 B	7/12
Naïve					
4	0	0 C	0/7	0 C	0/7
5	0	4.1 ± 0.2 A	7/7	4.9 ± 0.2 A	7/7

[a] Arithematic mean ± S.E.M.
[b] Group means followed by the same capital letter are not significantly different based on the differences of least squares means at $p \leq 0.05$.

3) with maternal antibodies. Both groups 1 and 3 pigs had identical mean titers from nasal swabs on day 5 post-challenge but group 1 recombinant adenovirus-primed pigs had fewer pigs from which virus could be recovered from pulmonary lavage fluids and a lower mean virus titer although not significantly lower (Table 3).

4. Discussion

Vaccine failure caused by maternal antibody interference is an important consideration when vaccinating for SI. Consequently inactivated SI vaccines are not administered until maternally derived antibodies are thought to have decayed to low levels. Often pigs can be up to 3 months old before a primary vaccination. Thus, protocols to stimulate active immunity in pigs in the presence of maternally derived antibodies offer substantial improvements for protection of pigs during their critical period of susceptibility for infection and disease while maternal antibodies wane. In this study, vaccine failure in pigs given a killed SI vaccine was demonstrated using a control group that had acquired maternal antibodies (group 3), however, the killed vaccine itself was efficacious in the absence of maternal antibodies (group 4). To overcome the killed vaccine failure, pigs with acquired maternal anti-SIV antibodies were primed with recombinant Ad-5 H3 and NP viruses. Unlike the group 3 pigs, the primed pigs developed an anamnestic HI response to the killed commercial vaccine and demonstrated strong protective immunity. Priming alone without the killed vaccine boost was shown to be partially protective against virulent H3N2 influenza virus.

DNA-based vaccines, in theory, also should elicit immunity in pigs with passive antibodies. However, directed comparisons of a plasmid vaccine and a replication-incompetent Ad-5 vaccine both expressing the same pseudorabies gD glycoprotein replicon have shown that Ad-5 vectored vaccines stimulated or primed immunity in 1-day-old piglets from immune dams while DNA-based vaccines were ineffective (Monteil et al., 1997, 2000). The strong efficacy of Ad-5-based vaccines was attributed to the broad-based transduction of different cell types by the vectored vaccine including the transduction of antigen presenting cells. Ad-5 vectored vaccines stimulated all components of the pig's immune response and are long-lasting because these vaccines mimic a natural viral infection.

A clear memory response as demonstrated by a 14.3-fold increase in the group average HI titer was apparent in recombinant Ad-5 H3 + NP primed pigs following the booster dose of killed vaccine (group 2). As anticipated, this immunization scheme resulted in strong protective immunity demonstrated by limited challenge virus replication because only 2 of the 11 group 2 pigs had lung lesions (mild) and no virus was recovered from pulmonary lavage fluids at necropsy. Group 1 pigs were identical with regard to recombinant Ad-5 H3 + NP priming but showed no anamnestic HI antibody response following challenge which is consistent with limited challenge virus replication. Only 2 of the 12 pigs in group 1 had recoverable virus

at necropsy in pulmonary lavage fluids and the extent of challenge virus replication caused mild and moderate lung lesions in just half of the pigs. In contrast to groups 1 and 2 primed pigs, group 3 pigs had severe lung lesions consistent with challenge virus replication and demonstrating that the killed vaccine given as a primary dose was ineffective in stimulating immunity in pigs with maternally derived antibodies. Moderate amounts of challenge virus were recovered in pulmonary lavage fluids from over half of the group 3 pigs but no strong HI response occurred suggesting that the primary vaccination dose was ineffective because of maternal antibody interference (i.e., no priming). Naïve pigs (group 4) were immunized following primary vaccination with killed vaccine and 2 weeks later gave an anamnestic HI response to challenge infection. This memory response resulted from replication of the challenge virus as also demonstrated by six of the seven group 4 pigs having mild and moderate lung lesions. With the vaccinated naïve pigs, challenge virus replication probably occurred early after infection and resolved because no virus was recovered at necropsy in any of the group 4 pigs. Control group 5 (challenge-only pigs) demonstrated the level of virus recovery on day 5 post-challenge in lavage fluids and nasal secretions and also demonstrated that 5 days were not adequate to detect a HI response to a primary infection with challenge virus.

Often there is uncertainty whether a killed or live virus vaccine will be efficacious in a young pig. In this study we have demonstrated that Ad-5 recombinant viruses expressing the SIV hemagglutinin and the nucleoprotein were capable of stimulating active immunity against SIV in young piglets with interfering maternal antibodies. Although the immunity induced with the recombinant vaccines was good, the Ad-5 recombinants worked even better as a priming dose when followed 3 weeks later with a booster dose of commercial inactivated SIV vaccine.

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