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Influenza vaccine research at the National Animal Disease Center

Amy L. Vincent, DVM, Juergen A. Richt, DVM, Ronald D. Wesley, DVM, Dr. Kelly M. Lager, DVM
Virus and Prion Diseases of Livestock Research Unit, National Animal Disease Center, USDA, Agricultural Research Service, Ames, Iowa 50010, USA

Introduction

Swine influenza virus is a member of the influenza A virus genus that consists of enveloped viruses with a negative sense RNA genome composed of eight gene segments. The eight segments encode the viral structural and non-structural proteins. SIV divergence arises both by random mutations within individual genes (genetic drift) and by reassortment (genetic shift). Reassortment occurs when two different influenza A viruses infect the same cell and a mixing of RNA segments results in a new reassortant virus. Serologic assays differentiate the hemagglutinin (HA) and neuraminidase (NA) influenza virus surface proteins into 16 H and 9 N antigenic groups that can reassort in different combinations so that each subtype can be identified by a specific H and N classification, e.g., H1N1 or H3N2 subtypes. There are some host species-specific subtypes and there are subtypes that can be found in a number of different host species. Most of the subtypes can be found in avian species.

Historically, swine influenza in the United States had a predictable pattern with an epizootic in the fall/winter months similar to that in humans. Prior to 1998, this acute respiratory disease was almost exclusively caused by viruses of the classical-swine H1N1 lineage [1-3]. In 1998, a severe influenza-like illness was observed in pigs on a farm in North Carolina with additional outbreaks in swine herds in Minnesota, Iowa, and Texas. The causative agents for these outbreaks were identified as influenza viruses of the subtype H3N2. Genetic analysis of these H3N2 viruses showed that at least two different genotypes were present. The initial North Carolina isolate (double reassortant) contained gene segments similar to those of the human (HA, NA, PB1) and classical-swine (NS, NP, M, PB2, PA) lineages, whereas the isolates from Minnesota, Iowa, and Texas (triple reassortant) contained genes from the human (HA, NA, PB1), swine (NS, NP, M), and avian (PB2, PA) lineages [4]. By the end of 1999 viruses antigenically and genetically related to the triple reassortant lineage were widespread in the U.S. swine population [5] whereas the double reassortant virus did not spread efficiently among swine. Once established in the swine population, the H3N2 viruses evolved through reassortment with both human and swine viruses. A number of reassortant viruses have been identified, including further H3N2 genotypes [5-7], H1N2 [8, 9], reassortant H1N1 [7], human-like H1N1 and H1N2 [10], and H3N1 viruses [11]. All of these novel reassortants contain the avian PA and PB2 polymerase genes, suggesting that they endow a selective advantage to viruses possessing them. With the acquisition of the avian PA and PB2 genes, the current swine viruses appear to have increased the rate of mutation and reassortment, and thereby, the ability to evade established herd immunity either from natural infection and/or vaccine administration.

When pigs are infected with a virulent influenza virus, complete protective immunity typically develops against re-challenge with homologous virus, i.e., there is little or no detectable virus replication following secondary challenge and there are no lesions associated with challenge [12]. Exposure to a European H1N1 and H3N2 also conferred complete protection against an H1N2 with an unrelated HA protein [13], however vaccination with commercial killed vaccines containing H1N1 and H3N2 did not protect against H1N2 challenge [14]. Complete protection was noted in an earlier study in which pigs immunized with two doses of an inactivated whole virus vaccine were challenged with a different or heterologous SIV strain of the same subtype [15]. However, in other studies using inactivated whole virus vaccines, only partial protection was found following homologous challenge [16-18]. These studies and anecdotal evidence from the field indicate that inactivated vaccines have limited ability to cross-protect against drifted or heterosubtypic viruses and are dependent on the use of relevant cross-reacting parent strains of virus.

Protective immunity against infection with influenza involves both humoral and cellular immunity. Antibodies play a significant role in attenuating/preventing this disease. This is based on the protective quality of colostrum in young pigs [19] and the protection provided by inactivated vaccines [16, 18] that primarily stimulate humoral immune responses. Clinical protection against challenge virus appears to be correlated with the hemagglutination inhibition (HI) titer in the serum of an individual animal [15, 16], i.e., a high HI titer provides better protection against challenge than a low HI titer. This information has led to the suggestion that the presence and magnitude of a HI titer could be a predictor of protection, however this is likely only true when the priming HA inducing the HI titer

is closely related antigenically to the HA of the challenge virus. Other studies have demonstrated the protective qualities of antibodies at the mucosal level. Pigs primed with virulent SIV and then challenged with the same virus 42 days later did not have a detectable anamnestic serum antibody response [12]. However, an anamnestic mucosal immune response (rise in IgA and IgG) was detected in the nasal cavity, the site of challenge, indicating that this compartment of the immune system was stimulated. This data supports the hypothesis that antibody mediated protection at the mucosal level is important for clearing the respiratory tract of SIV and may not be accurately reflected by systemic antibody levels. This is consistent with our own recent findings. When the humoral response fails to prevent infection, cell mediated immunity (CMI) is believed to play a dominant role in clearance of SIV from the lower respiratory tract and subsequent recovery from infection. Cell mediated immunity is also believed to be necessary for heterosubtypic immunity (Het-I) against influenza viruses of different subtypes.

H1 inactivated vaccine studies

We have evaluated and compared the pathogenesis of 10 H1 SIV isolates dating from 1930 to currently active isolates (Vincent et al., submitted). In addition, the HA and NA genes of each isolate were sequenced for genetic comparison and serological cross-reactivity was evaluated using all sera and virus combinations in HI and SN assays. Differences in pathogenicity were detected between isolates, with recent isolates tending to produce more severe disease, increased nasal shedding and higher virus titers in the lung. Serologically, the historical classical viruses tended to have better cross-reaction between historical sera and antigens, with moderate to good cross-reactivity with modern viral antigens. However, the modern sera were less reactive to historical viruses and tended to be less consistent in cross-reactivity within the modern group. There appeared to be an increase in genetic and antigenic diversity coincident with the emergence of the swine triple reassortant H3N2 in 1998. Many of the recent isolates had accumulated amino acid changes in the predicted antigenic and binding sites on the HA protein.

To follow up the *in vitro* cross-reactivity, we evaluated two of the isolates, IA30 (H1N1) and MN03 (H1N2), with substantial genetic variation in the HA gene and failure to cross-react in the HI assay. These isolates were utilized to prepare inactivated vaccines and used to immunize conventional pigs. In addition to the inactivated vaccine primed groups, two groups of pigs were primed with live virus, then challenged with the heterologous virus at the same time interval as the groups vaccinated with killed vaccine. Both inactivated vaccines provided excellent protection against homologous challenge. However, the IA30 vaccine failed to protect against the heterologous MN03 challenge. Surprisingly, 3 of the 9 pigs in this group had substantially greater percentages of lung lesions compared to non-vaccinated MN03 challenge controls. This suggests the IA30 inactivated vaccine may have potentiated the level of pneumonia against the heterologous MN03 challenge. The potentiation may have been immune-mediated due to high levels of presumably non-neutralizing IgG antibodies in the lung of the 3 affected pigs. The vaccines induced an isolate specific HI response against homologous virus, but there was no cross-reactivity with heterologous viruses. Divergent H1 viruses that do not cross-react serologically may not provide complete cross-protection when used as an inactivated vaccine against heterologous challenge. Cross-protection does not appear to be bi-directional as the MN03 inactivated vaccine provided some protection against the IA30 challenge. Although lung lesions consistent with SIV were seen in pigs primed with live IA30 and challenged with MN03, the live challenge and recovery prevented virus shedding from the nose and no virus was isolated from the lungs in our experimental model. This suggests that the use of live virus or a mucosal route of immunization may enhance the efficacy of vaccines to prevent shedding when used in the face of antigenically drifted viruses.

H3N2 modified live vaccine

The development of attenuated MLV or vectored subunit vaccines for swine that induce a balanced immune response including humoral and cell mediated mechanisms are likely to improve homotypic and heterosubtypic protection. A cold adapted live attenuated intranasal influenza vaccine has been approved in the US for use in humans with results from clinical and field trials showing good efficacy [20]. Reverse genetics, or the de novo synthesis of negative sense RNA viruses from cloned cDNA, has become a reliable laboratory method that provides a powerful tool for studying various aspects of the viral life cycle, the role of viral proteins in pathogenicity, and the interplay of viral proteins with components of the host's immune system. A reverse genetics system that allows the generation of influenza A viruses entirely from cloned cDNAs has been described [21, 22] and this technology has been established in our lab at the NADC. This technology permits the generation of viruses with reassortant gene segments and/or gene segments with viable mutations. The use of reverse genetics technology in combination with identification of attenuating mutations could lead to the development of master virus strains that would allow for rapid development of new vaccines with HA and NA genes relevant to newly emerged or drifted viruses in the field.

Mutations in the NS1 gene have been shown to attenuate influenza A viruses in cell culture, in mice, and in embryonated eggs [23]. This effect is believed due to mutations in NS1 gene that cause a loss of function of the viral NS1 protein. A primary function of the NS1 protein is to disrupt

the host's innate immune response to an influenza virus infection and replication. NS1 is an interferon antagonist causing inhibition of the host's interferon mediated antiviral responses, a function that would promote viral replication in the host. Mutations in the N-terminus of the NS1 protein was shown to be important not only for the down-regulation of IFN-β [23], but also TNF-α, IL6, and MIP-1α, other important pro-inflammatory cytokines [24] and these mutant viruses were shown to be attenuated *in vitro* and in mice. We have shown previously that a prototype plasmid derived H3N2 SIV (Sw/A/TX/98) virus with a deletion mutation from amino acid 126 to 153 (Δ126) in the NS1 gene generated *in vitro* could replicate in pigs. It was found that when compared to the wild type parental H3N2 SIV, the *in vitro* generated virus was not shed from the nose, did not replicate to the same extent as the parental virus, and was highly attenuated compared to the wild type virus [25].

In a more recent study, four-week old pigs were vaccinated and boosted with the TX98 NS1Δ126 modified live virus (MLV) via the intratracheal route (Richt et al., submitted). Pigs were challenged with wild type homologous H3N2 or heterosubtypic classical H1N1 SIVs and necropsied five days later. The MLV was highly attenuated and completely protected against challenge with the homologous virus. Vaccinated pigs challenged with the heterosubtypic H1N1 virus demonstrated pathologic lung changes similar to the non-vaccinated H1N1 control pigs. However, vaccinated pigs challenged with H1N1 had significantly reduced virus shedding from the respiratory tract when compared to non-vaccinated, H1N1 challenged pigs. All vaccinated pigs developed a significant level of HI titer, serum IgG, and mucosal IgG and IgA antibodies against parental H3N2 SIV antigens.

A separate study evaluated the efficacy of the MLV when used via the intranasal or intramuscular route when challenged with homologous virus. Furthermore, pigs were vaccinated via the intranasal route and challenged with wild type homologous H3N2, a genetic and antigenic variant H3N2 (CO99) and a reassortant H1N1 that contained internal genes similar to the triple reassortant H3N2 viruses (Vincent et al., in preparation). Results from recent studies will be summarized in the oral presentation. It is apparent that a complex host response involving CMI and humoral mechanisms contribute to the immunity established from MLV SIV vaccines and the response to MLV may be superior to that induced by inactivated influenza vaccines. Future work is planned to address these areas.

H3N2 adenovirus vectored vaccines

Recombinant human adenoviruses have been demonstrated to be effective vectors for insertion of antigens from infectious agents for use as vaccine candidates in many species, including those of veterinary importance. Several of these vaccine candidates, specifically those created from human adenovirus serotype 5 (HAd5), have been shown to provide excellent protection from challenge with foot-and-mouth disease virus [26-28] and swine influenza virus [29]. Vaccination with HAd vectors have been shown to induce both humoral and cell-mediated immunity, and those given by a mucosal route have been shown to provide superior, long lasting mucosal immunity [30-34]. In studies at the NADC, 2 replication-defective adenovirus recombinants were developed as potential vaccines against H3N2 influenza viruses [29]. Pigs were vaccinated intramuscularly with the recombinants only; vaccinated with the recombinant adenovirus expressing the influenza virus H3 HA protein, vaccinated with the recombinant adenovirus expressing the nucleoprotein (NP), or vaccinated with both recombinants in a mixture. The results showed that pigs in the groups given the recombinant adenovirus expressing the HA protein developed high levels of virus-specific HI antibody by 4 weeks post vaccination. Pigs in the group vaccinated with recombinant viruses expressing both the HA and NP in a mixture were completely protected against homologous challenge, shown by the lack of nasal shedding of virus following challenge and by the lack of lung lesions at one week following the challenge infection.

In addition, the safety and efficacy of a human adenovirus-5 vaccine for protecting weaned pigs against swine influenza virus subtype H3N2 infection were evaluated when administered via 2 injection methods [35]. Weaned pigs received a 10-fold serial dilution of recombinant adenovirus expressing H3 HA and a constant amount of recombinant adenovirus expressing NP, either with a needle-free injection device or by traditional IM injection. Vaccinated pigs and non-vaccinated controls were challenged 5 weeks later. After challenge, pigs were observed for clinical signs and nasal secretions were tested for virus. On day 5 after challenge, pigs were euthanatized; lungs were examined for gross lesions and bronchoalveolar lavage samples were tested for virus replication. The HI antibody response was elicited in a dose-dependent manner. Traditional IM-administered vaccination induced consistently higher HI responses than vaccination via needle-free injection, but the differences were insignificant. Likewise, traditional IM administration was superior at reducing nasal virus shedding except at the highest dose, at which both methods blocked virus replication. The severity of lung lesions was reduced in a dose-dependent manner by both vaccination methods. The replication-defective vaccine virus was not transmitted to sentinel pigs.

Sows and gilts lack immunity to Ad-5 vectored vaccines so immunogens of swine pathogens can be expressed by these vaccines in order to immunize suckling piglets that have interfering, maternally-derived antibodies against the swine pathogen. 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 HA and the NP of SIV subtype H3N2 (Wesley et al., submitted). The HI titer of the sham-inoculated group showed continued antibody decay whereas piglets vaccinated with Ad-5 SIV 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.

Conclusions

It is evident from the increasing number of novel subtypes and genetic variants identified in cases of influenza pneumonia in pigs that controlling swine flu will only continue to be more dynamic and difficult. New strategies of vaccine development must be considered to keep up with the ever-evolving influenza virus as well as the hurdle of maternal antibody interference with inactivated vaccines. The demonstrated safety and efficacy of the cold-adapted modified live virus vaccine in human medicine has paved the way for investigating modified live vaccines in swine medicine. In addition, it has been shown that strain, route of administration, and use of vaccine additives can play a role in enhancing heterologous protection. Future studies at the NADC will address each of these areas as well as the further use of reverse genetics to genetically engineer viruses with vaccine potential (live or inactivated); the use of reverse genetics to investigate additional genes that may play a role in virulence with the potential for attenuating mutations; and the use of Ad-5 vectored vaccines. Continued efforts will be made to collaborate with diagnosticians and practitioners to keep current with the circulating viruses in the field and evaluate the relevance of genetic phylogeny and *in vitro* cross-reaction to cross-protection. In addition, to more fully understand experimental vaccine efficacy, the CMI and humoral immune responses at the systemic and mucosal levels will be evaluated in future animal studies.

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