Title: Improvement of vaccine protection against swine influenza: Proof-of-concept - **NPB #08-261**

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Industry Summary

Swine influenza is a growing problem in the U.S. swine industry although the disease and etiology have been known for a long time. Historically, swine influenza in the U.S. had been only due to classical H1N1 (i.e., α clade) swine influenza virus (SIV) and can be relatively well controlled through vaccination using inactivated viruses. However, after introduction of H3N2 SIV, emergence of numerous reassortants between H1N1 and H3N2 and increased antigenic changes within each subtype have become a nightmare for swine veterinarians and producers from a disease control standpoint and have raised the need for better vaccination strategies. In order to develop an effective vaccine, better understanding of the immune ontogeny of pigs to SIV infection was necessary as natural exposure induces a strong and protective immune response. The proposed study was intended to combine findings on the role of each SIV protein in immunity with an advanced vector technology to formulate a novel way for delivering specific viral antigens to pigs in a targeted manner so that enhanced yet balanced immune response to SIV can be obtained in the animals.

The main objective of the study was to generate an immunization vector capable of providing the source of both immunogenic endogenous and exogenous antigens for the balanced stimulation of the immune system (i.e., both virus-specific antibody and cell-mediated immunity). Baculovirus was chosen as antigen exchange vector to prove the concept of surface display-endogenous expression of targeted antigens for better immunization against SIV. The target antigens were hemagglutinin (HA) for surface display and matrix (M) protein for endogenous expression. The resulting recombinant baculovirus was then used as immunogen (i.e., vaccine) and evaluated in animal trials measuring parameters associated with humoral and cell-mediated immune responses. A viable recombinant baculovirus displaying immunologically recognizable HA protein on the surface and harboring functional M gene was successfully constructed. After immunized with the recombinant baculovirus twice, pigs developed antibodies against the HA protein which were measurable by HI, ELISA and SN tests. The kinetic of antibody response was comparable with that in pigs infected with the donor SIV. However, antigen-specific CMI response was much weaker in immunized pigs as compared to that in challenged pigs, suggesting that replication of the recombinant baculovirus in pigs was not optimal or matrix protein did not contain T-cell epitopes.

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Although optimal CMI response could not be obtained in pigs and further work remains to address this issue, the proposed work was a new approach combining the capability of surface display, mammalian cell transduction and the sequential endogenous antigen expression in the same baculovirus vector construct, mimicking the natural infection pathway of the virus on host cells. Therefore, it should provide a new tool for safe antigen delivery, which in turn enhances protective immunity by inducing the balanced immune responses against the target pathogen. A similar platform would allow biologic firms to rapidly formulate a vaccine using contemporary SIV strains once a cloning cassette is formulated.

Keywords: swine influenza virus; vaccine; recombinant baculovirus; surface display; endogenous expression; antigen exchange vector

Scientific Abstract

Swine influenza is a growing and re-emerging concern in the U.S. swine industry. Historically, swine influenza in the U.S. had been only due to classical H1N1 (i.e., α clade) swine influenza virus (SIV) and can be relatively well controlled through vaccination using an inactivated virus. However, such stability was disturbed after introduction of H3N2 SIV, which resulted in the emergence of numerous reassortants between H1N1 and H3N2 and increased antigenic changes within each subtype. All these occurrences have negatively impacted the efficacy of current commercial vaccines. Such an inferior effectiveness of current inactivated virus vaccines raises the need for better vaccination strategies for SIV. It was then our hypothesis that the delivery of multiple exogenous and endogenous SIV antigens/genes in an organized manner (i.e., mimicking natural infection) induces effective and balanced humoral and cell-mediated immune responses in immunized animals. The main objective of the study was to generate an immunization vector capable of providing the source of both immunogenic endogenous and exogenous antigens for the balanced stimulation of the immune system.

The proposed work started with constructing a recombinant baculovirus (*Autographa californica* nucleopolyhedrovirus, AcMNPV) that can serve as multiple SIV antigen exchange vector for surface display (i.e., pseudotyping antigen) as well as endogenous expression of targeted antigens using gene cloning and baculovirus recombinant techniques. The target antigens were hemagglutinin (HA) for surface display and matrix (M) protein for endogenous expression based on our recent assessment of their role in the SIV immunity. A classical H1N1 SIV isolate (A/Sw/IA/1992) was used as the donor of the target genes. The resulting recombinant baculovirus was then used as immunogen (i.e., vaccine) and evaluated in animal trials measuring parameters associated with humoral and cell-mediated immune responses in comparison to pigs inoculated with SIV (A/Sw/IA/1992) and wild type baculovirus.

A viable recombinant baculovirus which displayed the immunologically recognizable HA protein on the surface and harbored functional M gene was successfully constructed. When injected with the recombinant baculovirus twice, pigs developed antibodies against the HA protein which were measurable by HI, ELISA and SN tests. The antibody kinetic was comparable with that in pigs inoculated with SIV itself. Pigs inoculated with wild type baculovirus did not produce antibody specific for the HA protein. However, antigen-specific CMI response (blastogenesis and interferon-γ production) was much weaker or minimal in the immunized pigs as compared to that in challenged pigs. Although optimal CMI response could not be obtained in pigs and further work remains to address that issue, the proposed work demonstrated a new approach combining the capability of surface display, mammalian cell transduction and the sequential endogenous antigen expression in the same baculovirus vector, mimicking the natural infection pathway of the virus on host cells. Therefore, a similar approach might provide a new tool for safe antigen delivery, which in turn enhances protective immunity by inducing both humoral and CMI responses against SIV.

Introduction

Swine influenza has been one of the major respiratory diseases in pigs throughout all stages of production since the disease was identified in 1918.[14] Swine influenza virus (SIV), a member of Influenza A virus, has been mainly responsible for the disease. The virus has 8 segmented RNA molecules, each of which encodes distinct structural and non-structural proteins of the virus.[9] Being an RNA virus, point mutations of the genome during *in vivo* replication are common, leading to antigenic drift. Since segmented genomes are interchangeable between 2 strains of SIV (i.e., reassortment), major phenotypic changes known as antigenic shift occasionally occur, leading to an influenza epizootic in animals or epidemic or pandemic in humans.

Although SIV is known for its high degree of genetic and antigenic variations, only one subtype (i.e., H1N1) of SIV had been responsible for "swine flu" in the United States and the virus had been antigenically stable since it was first discovered in 1930.[14] Hence, vaccination was very effective for controlling and preventing "swine flu". This picture was, however, dramatically changed in late 1998 by the emergence of a new subtype (H3N2) in U.S. swine.[17] The emergence of the new subtype not only caused an epidemic (epizootic) in naïve populations but also affected the stability of the virus. Since H3N2 was introduced, several reassortants between H1N1 and H3N2 emerged.[12,16] In addition, the rate of antigenic drift has significantly accelerated, resulting in antigenic variants within the same subtype which minimally cross react to each other.[2,8,15] A high degree of genetic and antigenic changes became a significant impediment to the efficacy of current commercial SIV vaccines since the current vaccines (inactivated form) heavily focus on immune protection by antibody against hemagglutinin. Such an inferior effectiveness of the current vaccines in controlling or preventing swine influenza raises the need for new vaccination strategies.

Protective immunity against viral pathogens relies on the effective induction of both humoral and cell-mediated immune (CMI) responses. Exposure of T and B lymphocytes of the immune system to the proper antigen(s) is pivotal for the induction of protective immunity. In general, T-cell priming against extracellular and intracellular antigens is known to be mediated by the processing and presentation of an antigen in combination with class II and I MHC molecules, respectively. In contrast, cross-linking of regular, rigid and organized antigens in a distance of 5-10 nm by B lymphocytes is a critical signal for B-cell activation leading to antibody production.[1,3] Therefore, we hypothesized that the delivery of multiple exogenous and endogenous SIV antigens in an organized (i.e., physically structured) manner induces effective and balanced humoral and CMI responses to SIV in immunized pigs and overcomes the ineffectiveness of antibody due to antigenic difference of incoming SIV, providing overall better protection against SIV. The following study was conducted to construct such an immunization vector.

Objectives

The objective of the study is to generate an immunization vector platform capable of providing the source of both endogenous and exogenous SIV antigens for the balanced stimulation of the immune system. We chose a baculovirus as the antigen exchange vector to prove the concept of surface display-endogenous expression of target antigens for better immunization against SIV.

Materials and Methods

Several recombinant baculoviruses (*Autographa californica* nucleopolyhedrovirus AcMNPV) were constructed to: a) either display the ectodomain of the target antigens on its surface by fusing the target gene with the signal peptide and transmembrane domain of AcMNPV gp64, or b) carry foreign viral genes under the control of mammalian-active promoters.[4,6] Based on results of a recent study in our and other laboratories,[5,10,11] HA gene were selected for surface display (i.e., neutralizing epitopes) while M gene was selected for

endogenous one (i.e., T-cell epitopes) for proof-of-concept. Green fluorescent protein (GFP) gene will be inserted into all recombinants as a transient tracking device. A classical H1N1 (α cluster) SIV isolate (A/Sw/IA/1992)[5] was the donor of necessary viral RNA molecules.

Each recombinant baculovirus, particularly the insert of foreign gene, was sequenced if the recombinants were constructed correctly in relation to orientation of insertion using primers corresponding to flank ends of AcMNPV. Surface display of SIV target antigen (peptide) was confirmed by immune gold electron microscopy using SIV-specific polyclonal antibody of swine origin. The presence of neutralizing/blocking epitope(s) in peptides displayed on the surface of AcMNPV was determined by serum neutralization (SN) and hemagglutination-inhibition (HI) tests in a competitive format. That is, capture of SN or HI antibody from the serum by epitopes, if present, that displayed on the surface of the recombinant baculovirus reduces SN antibody titer against SIV infection to MDCK cells or HI titer against viral hemagglutination activity with rooster erythrocytes; otherwise, SIV infection to the cells or HA activity will be blocked. Expression of endogenous foreign antigen and efficiency of gene delivery to mammalian cells were determined by Western immunoblotting and immunocytochemical staining of cells transduced with recombinant baculoviruses.

The immunogenicity of the successfully constructed recombinant baculovirus was assessed in pigs. All animals were purchased from a SPF herd free of SIV, PRRSV and *Mycoplasma hyopneumoniae* and housed in the Large Animal Infectious Disease Facility. Pigs (n=6 per treatment) were injected with the recombinant baculovirus or H1N1 donor SIV at a rate of 10^9 plaque-forming unit (PFU) and 10^7 median egg infectious dose (EID_{50}) per ml, respectively. The recombinant was given twice at 2 week intervals. The SIV-infected group served as the positive control of natural infection. Another group of 6 pigs were injected with wild –type baculovirus without any SIV antigen and served as sham-inoculated control. Humoral (serum antibody) and antigen-specific T-cell responses (PBMC) of each pig were assessed on day 0 and thereafter every 7 days using HI test[5], SN test, H1N1 ELISA (IDEXX), blastogenesis and ELISPOT assay[13] for 6 weeks total. Each parameter was analyzed by treatment and time to characterize the immunogenicity of the recombinant baculovirus that carry SIV antigens in comparison to those in pigs exposed to SIV.

Results

After numerous attempts, a viable recombinant baculovirus which displayed the HA protein on the surface and harbored M gene was successfully constructed. The HA protein was recognized by anti-SIV pig serum on immune-electron microscopy, indicating that the protein was immunologically reactive. Possession of neutralizing/blocking epitope(s) in the HA protein displayed on the surface of the recombinant was confirmed as HI titer and SN titer of the antisera against SIV was reduced. The reducing effect had a positive correlation with the amount of recombinant baculovirus added to the sera. Expression of the M gene cloned into the baculovirus in Sf-9 cells and transfected MDCK cells was detected by an immunofluorescence microscopy using M protein-specific monoclonal and polyclonal antibodies. The intensity of antigen production was much greater in Sf-9 cells than MDCK cells. The presence of M protein in the lysate of each cell type was also visualized on Western immunoblotting using the same antibodies.

When the resulting recombinant baculovirus was given to pigs, pigs developed antibodies against the HA protein which were measurable by HI (≥1:20), ELISA (≥0.4 S/P ratio) and SN (≥1:10) tests on 7, 14 and 21 days after 2 does injection at 2 weeks internal. Levels of ELISA and SN antibodies continued to rise until the end of sampling (6 weeks total/4 weeks after 2nd injection), where as HI titer started to decline after 2 weeks post 2-dose injection. Overall kinetic of antibody response in pigs immunized with the recombinant baculovirus

was similar to that in pigs infected with SIV although challenged pigs had higher HI and SN antibody titers than did the immunized pigs. Antibody response to the HA protein in the immunized pigs was confirmed by Western immunoblotting using lysate of SIV-infected MDCK cells. Weak antibody response to M protein was noted on 3 weeks after the 2nd injection. However, antibody to other SIV proteins such as nucleoprotein, neuraminidase and NS1 was not detectable in any of serum samples collected from the immunized pigs. Pigs inoculated with the wild type baculovirus did not produce antibody specific for the HA protein which was measurable by any of the serologic assays.

In contrast to promising antibody response by the recombinant baculovirus, antigen-specific CMI responses were much weaker in the immunized pigs as compared to that in pigs infected with SIV. In the challenged pigs, lymphocyte proliferation response of PBMC was detectable on day 7 after infection when stimulated by inactivated virus (10 HAU/ml), HA protein (10μg/ml or M protein (10μg/ml) and last until the end of the study. IFN-γ production in PMBC was also detectable by ELISPOT assay on day 7, started to decline after day 14 but was still above the detectable level by the end of the study. While a similar lymphocyte proliferation response was observed in the immunized pigs, IFN-γ production in PMBC was transient (day 7 only) and at a much lower level in the immunized mice as compared to that in the infected pigs.

Discussion

Swine influenza has become a re-emerging issue in the U.S. swine industry since introduction of H3N2. Due to frequent emergence of reassortants and increased antigenic drift within the same subtype of SIV, the efficacy of conventional killed vaccines for SIV is suboptimal unless the antigenic property of vaccine strain and the virus affecting pigs matches, raising the need for better vaccination strategies including consistent update of vaccine strains.

In order to develop an effective vaccine, better understanding of the immune ontogeny of pigs to SIV infection is necessary since natural exposure induces a strong and protective immune response. In human medicine, effort has been made to find a way to induce immunity to conserved influenza virus antigens. Much of previous work has focused on cell-mediated immunity, particularly induction of cytotoxic T lymphocyte (CTL) response. Unfortunately traditional killed vaccines cannot induce CTL response even though certain adjuvants or addition of an immune modulator to a vaccine have been reported to be effective in inducing CMI. The proposed study was intended to combine findings on the role of each SIV protein in immunity with an advanced vector technology to formulate a novel way for delivering specific viral antigens to pigs in a targeted manner so that enhanced yet balanced immune response to SIV (i.e, both humoral and CMI responses) can be obtained in the animals. In this sense, the proposed work was successful in construct an antigen exchange vector containing target antigens of SIV. As expected, the HA protein which was expressed on the surface of the vector induced both antibody and CD4+ T-cell response (as evident with lymphocyte proliferation). Knowing that conventional killed virus vaccines are able to confer protective immunity against SIV, the vaccine made of the recombinant baculovirus should be able to do the same.

Concerning heterologous protection, it was hoped that endogenous expression of M gene via the recombinant baculovirus is going to induce strong CMI response particularly in conjunction with IFN-γ as surrogate of CTL response. Unfortunately that was not the case under conditions presented in the proposed study. Although further work remains to be conducted to identify reasons for weak or suboptimal CMI response, one can speculate a few things. One is that matrix protein may not contain appropriate T-cell epitopes. In a study using mice, nucleoprotein was shown to be involved in CMI. Yet, such an immunological event was not reproducible in other mammalian hosts including humans. Second, T cell epitopes on the M protein may

require a specific configuration or physical presentation to be recognized by T cell receptors. Such a configuration may be present in its nature form (i.e., virion) and might have been altered during cloning into baculovirus as foreign gene. Third, it is possible that the recombinant baculovirus in pigs was not optimal. If this was the case, other antigen exchange vectors can be considered since vectored vaccines have been successfully used for equine influenza virus or avian influenza viruses.

Although optimal cell-mediated immunity could not be obtained in pigs and further work remains to address that issue, the proposed work demonstrated a new approach combining the capability of surface display, mammalian cell transduction and the sequential endogenous antigen expression in the same baculovirus vector which could mimic the natural infection pathway of the virus on host cells. Therefore, it might provide a new tool for safe antigen delivery, which in turn enhances protective immunity by inducing both humoral and CMI responses against SIV. This type of platform would allow biologic firms to rapidly formulate a vaccine using contemporary SIV strains once such a vector is generated.

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