Elastase-Dependant Live Attenuated Swine Influenza Virus Vaccine in Pigs

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Abstract: Swine influenza (SI) is an acute, highly contagious, respiratory disease of swine caused by influenza A viruses. In addition, SI infections possess significant human public health concerns as they may serve as intermediate host for the generation of new pandemic viruses. Vaccination is still the primary method for the prevention and control of SI. Currently, commercially available vaccines against SI are a combination of inactivated swine influenza viruses (SIVs) with oil adjuvant. Their application induces mainly humoral immune response, which may not be protective against virus variation in the field. In contrast, application of live attenuated influenza vaccines (LAIV) mimics natural infection and induces strong, cell-mediated and humoral immunity. Furthermore, LAIV induces cross-protective immunity against different subtypes of influenza A viruses and are currently unavailable for SI. Using reverse genetics technology we generated mutant SIVs with the modified cleavage site within hemagglutinin (HA) segment. These viruses are fully dependent on the presence of human neutrophil elastase for their growth in tissue culture and are completely attenuated when administered to pigs. Furthermore, application of these live attenuated, elastase-dependent swine influenza viruses as a live vaccines resulted in the significant protective humoral (systemic and mucosal), cell-mediated and cross-reactive immunity in pigs. The purpose of this review is to provide recent advances in SIV live vaccine development by modifying the hemagglutinin cleavage site. Here, we review the design and generation of elastase-dependent mutant SIV by reverse genetics; evaluation of its genetic stability and pathogenicity in pigs; evaluation of its immunogenicity and protection efficacy after intratracheal and intranasal application to diverse SIV challenges including 2009 pandemic H1N1 virus.

Keywords: Live attenuated vaccine, swine influenza.

GENETIC ENGINEERING AND CHARACTERIZATION OF ELASTASE-DEPENDENT SIVS

The crucial steps for infection by influenza A viruses are initial virus binding to the cellular receptors followed by receptor-mediated endocytosis and fusion of the viral envelope to endosomal membranes [1]. The exposure of the fusion peptide within HA requires prior cleavage of the HA by host proteases at a specific motif [2-4]. These specific motifs are monobasic in the case of all mammalian and non-pathogenic avian strains and polybasic in the case of all highly pathogenic avian influenza viruses [1]. Dependence of influenza virus replication on the host factors resulted in the idea of replacing the natural cleavage motif of the HA by another motif susceptible to a protease which is not accessible to the virus during the natural infection [5]. By using reverse genetics techniques [6] we generated two elastase-dependent mutant SIVs (SIV/R345V and SIV/R345A) from parental virus A/SW/SK/18789/02 H1N1 SIV (SIV/Sk02) [7]. SIV/R345V virus contained mutation at position 345 where amino acid Arginine is replaced with Valine, while SIV/R345A SIV had replaced Arginine with Alanine at the same position (Fig. **1**). These amino acid substitutions created new cleavage sites that are susceptible to human neutrophil elastase (SIV/R345V) and porcine pancreatic elastase (SIV/R345A) respectively. The rational for genetically engineering two viruses with similar but different cleavage sites was the concern that activation of neutrophils during the immune response after vaccination with LAIV could trigger release of elastase and thus facilitate viral replication. Therefore we designed construct with the cleavage site that is sensitive to porcine pancreatic elastase which is chemically different from human neutrophil elastase and not naturally present in the respiratory tract. Surprisingly, both mutant viruses could be rescued only in the presence of human neutrophil elastase. Although SIV/R345A was able to grow in the presence of pancreatic elastase, it contained the additional mutation in the HA segment at amino acid position 344 (Pro344-Ala-Gly instead of Ser344-Ala-Gly), which was most likely the result of adaptation to selection pressure of pancreatic elastase during the multiple passages in tissue culture [7].

In order to be considered as a live vaccine candidate, genetically modified live virus should be able to maintain wild type phenotype (genetic stability, growth properties, infectivity, etc.) in the presence of the proper protease *in vitro* and be able to induce immune response similar to that observed after natural infection *in vivo*. We thus characterized two mutant viruses by comparing the multi-cycle growth properties, protease dependence and genetic stability to the wild type parental SIV/SK02 in *in vitro* conditions. Both R345V and R345A viruses were solely dependent on human

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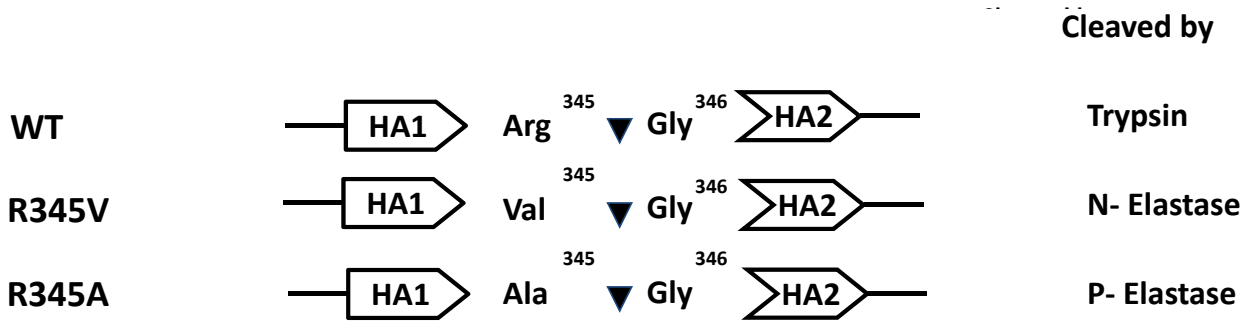

Fig. (1). Schematic diagram showing the modifications of the HA cleavage site. N- elastase: neutrophil elastase. P-elastase: porcine pancreatic elastase.

neutrophil elastase activation and grew to equivalent titers in the presence of human neutrophil elastase as the parental SIV/SK02 wild type (WT) virus in the presence of trypsin. Furthermore, tissue culture grown R345V and R345A SIVs were able to infect cells, synthesizing similar quantities of viral proteins as the parental virus [7].

The major concerns for use of LAIV are the potential reversion to wild type phenotype and reassortment of LAIV with other wild type SIVs in case of persisting infection, which could lead to generation of new pathogenic viruses. Understanding the importance of this safety concern, we assesed the genetic stability and reversion ratio for both R345V and R345A SIVs in tissue culture. Both R345V and R345A SIVs were grown in the presence of trypsin and elastase for five consecutive passages and supernatants were then serially diluted and subjected to plaque assays in the presence of either trypsin or elastase. Well defined plaques were detected only in the presence of human neutrophil elastase. In addition, sequencing results of RNA genetic material showed that both mutant viruses retained the introduced mutations at the HA cleavage site without any additional mutations. These results suggested that the mutant viruses possess high level of genetic stability in cell culture.

The pathogenicity of the mutant viruses was then tested. Pigs were intratracheally inoculated 4×10^5 PFU of R345V or R345A; or 4×10^6 PFU of R345V or R345A, respectively. During the five-day observation period after virus inoculation, clinical signs characteristic for SIV infection were observed only in the groups infected with the WT SIV at both doses. Animals in all other groups inoculated with R345V and R345A did not show any signs of respiratory distress, weight loss or nasal discharge. Most importantly, at necropsy we could not detect significant presence of SI characteristic clinical signs, macroscopic, microscopic lesions or virus particles in the lungs of challenged animals. These data demonstrated that both R345V and R345A were highly attenuated in pigs and that they have a potential as LAIV for SIV in pigs [7].

IMMUNE RESPONSES TO ELASTASE-DEPENDENT SIVs

Natural immunity to SIV depends on the activation of both cell-mediated immunity (CMI) and humoral responses triggered by the virus replication. Multiple factors such as virus pathogenicity, virus load, length of disease acute phase and innate immunity contribute to the strength and duration of natural immunity [8]. Since both of our vaccine candidates were capable of completing only a limited number of replication cycles *in vivo*, we assessed their immunogenic properties in pigs after one and two intratracheal (IT) administrations [9]. Immunogenicity of R345V and R345A was determined based on the capability of mutant viruses to induce both cell-mediated and humoral immune responses at the site of infection/inoculation. As the readout for induction of CMI we used IFN-γ ELISPOT and lymphocyte proliferative response (LPR) assay. Our data showed that both viruses were able to induce considerable numbers of IFN-γ secreting cells and lymphocyte proliferation in response to SIV specific antigen. Moreover, a second vaccination resulted in a significantly enhanced CMI than only one IT administration of both R345V and R345A [9], (Table **1**). It is most likely that one vaccination was not sufficient to induce maximum immune responses, possibly due to limited replication cycles of elastase-dependant mutant SIVs *in vivo*.

Consistent with the CMI, the induction of a humoral immune response in the serum and in upper and lower respiratory mucosa was low or undetectable after only one vaccination in the majority of tested pigs. However, the levels of serum hemagglutination inhibition (HI), IgG and IgA as well as mucosal IgA and IgG (from bronchoalveolar lung lavage and nasal passages) were significantly increased after a second vaccination in both R345V and R345A vaccinated pigs (Table **1**). Although both R345V and R345A induced immunity to SIV to similar levels, comparison of collected data on immunogenicity from two vaccine candidates showed that R345V induced more consistent and slightly better immune responses. In addition, R345V contained cleavage site that was solely dependent on the presence of human neutrophil elastase. Furthermore, R345V was able to induce higher levels of serum IgG and mucosal IgA antibodies that are cross-reactive to antigenically distinct H1N1 and H3N2 SIV subtypes [9]. Therefore, R345V was selected as a candidate for LAIV against SIV challenge, and its immuno-protection ability was further assessed [9].

The protection capability of R345V was assessed by determining the severity of clinical signs, percentage of gross and microscopic lung lesions, virus titers in lungs (Table **2**) and levels of pro-inflammatroy cytokines after the challenge with wild type SIVs (Table **3**). Two vaccinations with three weeks apart with R345V LAIV via IT route were sufficient to provide complete protection of pigs against

Table 1. Summary of Immunogenicity Study (IT)

Immune Responses	R345V	R345V	R345A	R345A
	1st Vaccination	2nd Vaccination	1st Vaccination	2nd Vaccination
*IFN-γ	100	231	102	233
**LPR	9.57	49.24	38.04	82.07
IgG serum	321	1611	164	955
IgA serum	181.88	746.26	84.50	628.65
IgG BALF	2.33	108	3.41	112
IgA BALF	29.03	2450	52.16	2279
IgG nasal	1	11	0	10
IgA nasal	0	77	0	59
HI	10	160	10	40

* Median number of IFN-γ secreting cells after 1st and 2nd vaccination with R345V or R345A measured by ELISPOT.
** Median values for stimulation index of lymphocytes proliferative response assay.
Antibody titers were shown as median values.

Table 2. Summary of Vaccination and Challenge Study (IT & IN)

Route	Factor	Vaccination/Challenge					
		MEM/SK02	R345V/SK02	MEM/IND88	R345V/IND88	MEM/TX98	R345V/TX98
IT	Lung lesion score	16.14	0.32	23	0.36	12.5	4.93
IT	Histopathology score	3	1	2	1	2	2
IT	Virus titer log10	4.16	0	3.83	0	4.995	3.16
IN	Lung lesion score	25.11	0.6	10.33	1.21	12.92	4.29
IN	Histopathology Score	4	1	3	1	3	1.5
IN	Virus titer log10	3.7	0	5.29	0	5.12	1.36

Lung lesion score: The percentage of the areas of the lung affected with purple-red, firm lesions typical for SIV infection.
Histopathology score: Lesion severity was scored by the distribution or extent of lesions within the sections examined as follows: 0: no visible changes; 1: mild focal or multifocal change; 2: moderate multifocal change; 3: moderate diffuse change; 4: severe diffuse change.
SK02: /SW/SK/18789/02 (H1N1)
IND88: A/Sw/Indiana/1726/88 (H1N1)
TX98: A/Sw/Texas/4199-2/9/98 (H3N2)

homologous subtypic H1N1 SIV/Sk02 and H1N1 variant A/Sw/Indiana/1726/88 infection. However, R345V LAIV vaccinated pigs were only partially protected against heterologous subtypic H3N2 A/Sw/Texas/4199-2/9/98 infection [9]. All vaccinated animals showed significant reduction in all tested disease parameters compared to unvaccinated and challenged animals. In addition, serum IgG and mucosal IgA antibody responses in vaccinated pigs were high and correlated with previous reports that evaluated immune responses and protection ability to infection with SIV in pigs [10,11].

The results using R345V as LAIV were encouraging for testing its immunogenicity and protection ability via the more feasible intranasal (IN) route. IN vaccination could be a choice for mass herd vaccination since it enables use of smaller volumes and antigen delivery is similar to the natural infection [12,13].

Our IT results showed that two vaccinations were required for optimal immunity to SIV in pigs, thus we used the same regimen for IN vaccine administration. As expected, IN administered R345V was capable of inducing CMI measured by the IFN-γ ELISPOT. Moreover, 10^7 PFU of vaccine dose of R345V given IN was sufficient to induce cross-reactive CMI to antigenically distinct H1N1 and H3N2 SIV antigens [14]. Humoral immune responses measured in serum (HI, IgG) and mucosal surfaces (IgA) were undetectable to low after only one IN administration of R345V, while the second vaccination via the same route resulted in a significant increase of SIV specific antibodies [14]. In addition, R345V administered IN induced mucosal IgA and serum IgG that are cross-reactive to antigenically distinct H1N1 and H3N2 SIV antigens (Table **4**). Furthermore, microscopic results showed that IN vaccinated and challenged group exihibited mild microscopic lesions

Table 3. Summary of Cytokine Production after Vaccination and Challenge (IT & IN)

Route	Cytokines	Vaccination/Challenge					
		MEM/SK02	R345V/SK02	MEM/IND88	R345V/IND88	MEM/TX98	R345V/TX98
IT	IFN-α	265.85	45.29	828.74	13.78	386.91	80.26
IT	IL-1	605.31	209.05	751.62	332.09	650.51	418.73
IT	IL-6	56.72	9.31	87.64	25.00	62.70	16.61
IN	IFN-α	147.41	8.60	634.33	13.64	400.00	47.23
IN	IL-1	1800.67	103.97	988.71	249.16	1036.09	397.42
IN	IL-6	254.56	1.90	105.60	15.11	168.35	50.21

IFN-α, IL-1 and IL-6 production in pigs after two vaccinations with R345V via IT (10^6 PFU) or IN (10^7 PFU) route and challenged with H1N1 Sk02, H1N1 IND88 or H3N2 Tx98 viruses.
SK02: /SW/SK/18789/02 (H1N1)
IND88: A/Sw/Indiana/1726/88 (H1N1)
TX98: A/Sw/Texas/4199-2/9/98 (H3N2)

characterized as mild peribronchial infiltration, which is similar to the pathological results observed in the mock challenged control group [14], (Table **2**).

In agreement with the IT study, the IN administered R345V conferred total protection against H1N1 antigenic subtype and partial protection against heterologous H3N2 SIVs' challenge (Table **2**). In addition, the antibody responses in serum (HI, IgG) and respiratory mucosa (IgA) were at considerable high levels. Production of pro-inflammatory cytokines (IFN-α, IL-1 and IL-6) was significantly reduced in all vaccinated and challenged animals compared to unvaccinated and challenged controls (Table **3**). Although the number of virus positive animals after challenge was higher than in the IT study, there was still a significant reduction in virus titers in vaccinated and challenged group than in unvaccinated and challenged group [14], (Table **2**).

Recently, we evaluated the ability of the elastase-dependent live attenuated influenza A virus R345V to protect pigs against the pandemic H1N1 2009 influenza virus [15]. Pigs vaccinated IN or IT with the R345V had significantly reduced macroscopic and microscopic lung lesions and lower viral loads in the lung and in nasal swabs. Thus, elastase-dependent SIV mutant can be used as a live-virus vaccine against swine influenza in pigs. In addition, low levels of cross-neutralizing antibodies to H1N1 2009 were elicited prior to challenge after vaccination with R345V, whose parental virus is a swine adapted avian strain [15].

Table 4. Summary of Cross-reactive Antibodies after Vaccination with R345V (IT & IN)

Cross-reactive Ab	IT		IN	
	Antigen			
	IND88	TX98	IND88	TX98
HI serum	40	<10	<10	<10
IgG serum	945	130	483	320
IgA BALF	682	251.01	391	303

Cross-reactive HI, IgA and IgG antibody titers are shown as medians of each group.
IND88: A/Sw/Indiana/1726/88 (H1N1)
TX98: A/Sw/Texas/4199-2/9/98 (H3N2)

All together, our data showed that two administrations of R345V via IT or IN route are required to induce immune response and homologous and heterologous protection similar to one observed after natural SIV infection. Other LAIV vaccines can induce strong immune response after only one administration, which is attributed to the prolonged virus replication and antigen exposure [16,17]. However, longer replication of vaccine strain extends the possibility for the influenza virus reassortment between vaccine and field strains. In our model, virus attenuation occurs most likely within the first 24 h.p.i., which significantly reduces the timeframe for the possible reassortment with wild type field SIVs.

CONCLUSION REMARKS

The main advantage of LAIV compared to their inactivated counterparts is the induction of both CMI and humoral immunity at the site of infection that resemble the natural infection [13]. The activation of CMI and strong mucosal antibody response are crucial in developing long-lived cross-protective immunity against influenza A virus infections. However, the major concern for using LAIV is possible reassortment between vaccine and wild type virus, which will lead to the generation of new circulating viruses. The general fear that the HA gene (as the most dominant antigenic component) of LAIV would reassort into circulating viruses could be prevented by an HA with modified cleavage site. Using the well established molecular techniques, influenza viruses with modified cleavage site can be easily engineered and rapidly propagated *in vitro* in the presence of the appropriate proteases [5,7]. The mutation within HA cleavage site would serve as an attenuation phenotype which could be inherited by the wild type virus in the case of reassortant. In addition, these viruses will have limited replication ability *in vivo* which narrows the window for possible reversion or reassortment with wild type SIVs,

making this LAIV a safer candidate. Despite the restricted replication and short antigen exposure, SIV cleavage mutants showed significantly better immunogenicity than their currently available inactivated counterparts. Complete protection to divergent H1N1 SIV challenge and partial protection to H3N2 SIV challenge demonstrated that the elastase-dependent swine influenza virus is a promising potential LAIV against swine influenza in pigs.

CONFLICT OF INTEREST

Declared none.

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