Intranasal Immunization of Pigs against Influenza with Synthetic Peptides Based on the Cassette Theory

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ABSTRACTS

Synthetic petide vaccines prepared according to the cassette theory(Naruse et al., 1994), formalin-inactivated virus and ether-split vaccine of influenza virus A /Aichi/68(H3N2) were administered intranasally to the pigs. The peptide vaccines contained single or double sets of the peptide sequence of hemagglutinin 127-133 of influenza virus A/Aichi/2/68(H3N2) inserted into the H-2 class allele specific amino acid motif of the agretope composed of residues 43-58 of pigeon cytochrome c in the mouse bearing A^k or A^b. After 4 times immunization, pigs were challenged with 10^{4-5} PFU of virus intranasally. Intranasal immunization of the pigs with inactivated virus vaccine and split virus vaccine induced antibody production against A/Aichi/2/68(H3N2) virus, protecting them from infection. Synthetic peptide vaccine which contains 2 sets of epitope amino acid sequence evoked protective immune response although antibody response was not detected in the pigs. The present results indicated that intranasal route can be applied for inducing not only mucosal immunity but also systemic immunity against influenza virus infection. Based on the present finding we assume that synthetic peptide vaccine prepared according to Cassette theory may be applicable to the heterogenous species including human.

Keywords : Influenza virus; pig; synthetic peptide vaccine; intranasal immunization; cassette theory

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INTRODUCTION

Swine influenza caused by influenza A viruses is a highly contagious, acute viral disease of the respiratory tract affecting pigs. Swine influenza has economic consequences in that sick pigs lose weight or their weight gains are reduced, and that deaths occasionally occur after secondary infections. Besides, it is believed that human Influenza pandemics are originated from pig source (1). There is a compelling evidence of the pig roled as a 'mixing vessel' in genetic reassortment events(2).

The interception of viral transmission to human from intermediate animal is a pivotal conception in the prevention of zoonoses. Therefore, pig's vaccine against influenza virus, especially in the regions where ducks and pigs are raising together(3), can reduce not only the loss of productivity in stock raising but also human influenza epidemics.

Use of inactivated influenza vaccine is the single most important measure in preventing or attenuating influenza infection(4). But the use of intact influenza viruses for the vaccination of humans by parenteral inoculation suffers from certain disadvantages. Since intact virus vaccines often cause undesirable febric reactions, Webster and Laver(1966) determined the efficacy of influenza virus subunit vaccines using ether-disrupted virus(5). And they ascertained that the ether-treated vaccines were not pyrogenic and induced anti-hemagglutinin levels that were equal to those induced by the intact viruses.

Recently, based on hapten-carrier concept, synthetic vaccines have been prepared by combining a fragment of a pathogen with a foreign helper T-cell determinant. Peptide vaccine design consideration includes the need to raise protective antibody against the pathogen resulting from activation of helper T cells specific for the pathogen. In general, agetopes and epitopes function independently. Ogasawara et al.(6, 7) determined the H-2 class II(A^k and A^b) allele specific motif of a peptide fragment derived from residues 43-58 of pigeon cytochrome c(p43-58). Residues 46 and 54 of p43-58 and its related peptide 50V worked as agretopes. Based on this finding, they reported potential peptide vaccine according to the 'cassette theory' (8, 9, 10). That synthetic peptide vaccines against influenza Aichi(H3N2) virus were prepared by introducing HA 127-133 residues of the A/Aichi/68 influenza virus hemagglutinin, the highly conserved amino acid among H3 subtype influenza viruses, into the frame component residues 43-46 and 54-58 of p43-58 analogues. And that vaccine was

shown to be effective in preventing infection with a naturally occurring antigenic variant, A/Texas/1/77(H3N2) carring the same sequence at 127-133 of the HA as Aichi/68 virus in I-A^b mice(11), suggesting that this synthetic peptide vaccine prepared according to the cassette theory can overcome the ineffectiveness of classical vaccines attributable to antigenic drift of influenza viruses and at the same time can avoid eliciting undesirable extraimmune response.

Since influenza virus infects via respiratory tract, the local immunity should be important for the prevention from influenza virus infection(12, 13). Besides, local administration may have a lot of advantages in safety consideration of vaccine. The purpose of the present investigation was to confirm the possibility of extension in applying these noble synthetic peptide vaccines prepared according to the 'cassette theory' for mouse MHC to another species of animals and to develope new delivery method of vaccination which have the advantages of safety and convenience for eliciting local immunity on the respiratory mucosa.

MATERIALS AND METHODS

Animal

Specific pathogen free(SPF) pigs(F1 between Landrace & Durock) obtained from Hokuren Takikawa Swine Station, Hokkaido, Japan were used at the age of 4-5weeks.

Virus

Influenza virus strain A/Aichi/2/68(H3N2) was grown in the allantoic cavity of 11-day-old embryonated hen egg. For the preparation of inactivated viral vaccine, formalin was added to make 0.01% of the allantoic fluid(AF) and clarified by centrifugation(about 8,000g, 1hr, 5℃), followed by ultracentrifugation(140,000g, 1hr, 5℃ with cusion of 50% sucrose). The Pellet was ultracentrifuged with sucrose density gradient(140,000g, 2hr, 5℃ with 20, 30, 40 and of 50% sucrose). For preparing hemagglutination inhibition(HI) test material, glycerol and AF of virus infected 13-day-old embryonated hen egg was mixed to the 1:1 ratio(v/v) and stored in the refrigerator. Virus to be used as antigen in ELISA was purified from allantoic fluid using sucrose gradient. Titration of virus in the AF was performed by plaque forming assay using MDCK monolayer cells.

Peptides

Peptides were prepared using an automatic peptide synthesizer(430A, Applied Biosystems Inc., Foster City. CA) as previously described(7) and separated from PAM resin with trifluoromethane sulfonic acid(TFMSA) and then purified to more than 95% by reverse-phase high-performance liquid chromatography on a Vydac C18 column(Waters Japan, Tokyo, Japan). To confirm the amino acid compositions of the sysnthetic peptides, the peptides were subjected to fast atom bombardment(FAB) mass spectroscopic analysis using JMS-HX110(JEOL, Tokyo, Japan).

Peptide vaccines were applied as the two suspension forms, i.e., YEGFSWTGVTQNKAKGIT(18-mer), and YEGFSWTGVTQNKAKGITWTGVTQN(25-mer) anchored to liposome. The 25-mer peptide has additional one set of epitope sequence subjoined at the end of 18-mer peptide prepared according to the cassette theory.

Immunization

Pigs were immunized intranasally with various preparations including formalin-inactivated virus, ether-treated split virus and two kinds of synthetic peptide vaccine, 18-mer and 25-mer. Thereafter, pigs were boostered thrice at weekly interval.

In Vivo Challenge

The viral challenge was performed by placing 1ml PBS containing 10^4-10^5 PFU(plaque-forming unit) of influenza virus A/Aichi/2/68 strain in the nostrils of the pigs 1 week after the last immunization. Nasal swabs were taken from those imm-unized pigs daily after challenge. Fifty microliters of serially diluted nasal swabs with phosphate-buffered saline containing 0.1% bovine serum albumin(pH 7.2) were inoculated on the MDCK monolayer

cells and incubated for 1 hr at room temperature for absorption. After washing, each culture received agar overlay medium consisting of Eagle's minimum essential medium, 1% bacto agar(Difco), and approximately 5g of trypsin (Difco) per ml of agar medium. After a 48-hr incubation at 35℃ in 5% CO2, the plaques those were formed in MDCK monolayer cells inoculated with the nasal swabs diluted optimally were counted.

Antibody detection

Antibody levels of sera and nasal swabs were measured by HI test and ELISA. HI test was done by using 4HA unit of virus in AF and 0.5% chick red blood cells in saline. For ELISA HRP conjugated goat anti-swine IgG, IgM and IgA were used as the tracer of anti-influenza virus antibody. ABTS was used as the chromogen. Absorbance was measured at 405nm.

RESULTS

The synthetic petide(18-mer), first, was examined for its ability to induce antibodies against A/Aichi/68 virus. Three times immunization of each 1mg synthetic peptide anchored to liposome were done at interval of 2 weeks. Formalin-inactivated virus was dosed at 1.0 mg to pigs and weekly booster immunization was done three times after 2 weeks of first immunization. ELISA and HI test were performed to detect anti-influenza antibodies. ELISA was used to assay for specific IgG, IgM and IgA antibodies against influenza virus, respectively, in nasal swabs and sera.

The pigs immunized intranasally with inactivated virus showed antibody responses against A/Aichi/2/68 virus in the sera and nasal secretions at the time of challenge when determined by ELISA. And IgG, IgM and IgA type of anti-Aichi antibodies were detected in the serum samples. IgA antibodies were outstanding especially in the nasal swab samples. But those of the other groups did not show detectable antibody response both in the sera and nasal swabs (Figure 1).

There was no remarkable change of antibody response in the sera and nasal swabs of the pigs immunized with inactivated virus two weeks after challenge. But there were increase of antibody responses in the sera and nasal swabs of the pigs immunized with synthetic peptide and vehicle control(liposome), indicating that virus replication were occured in the respiratory mucosa and stimulated immune system of the animals(Table 1).

Nasal swab samples were taken and inoculated to MDCK monolayers daily after intranasal challenege of 10^5PFU A/Aichi/2/68 virus. Viruses were not recovered from the nasal swabs of pigs immunized with inactivated virus, while recovered from $2x10^1$ to $4.5x10^3$ PFU/ml in the nasal swab samples of the other pigs including negative control group, indicating that intranasal immuniza-

tion of inactivated virus can induce antibodies against influenza virus and prevents viral replication to protect pigs from infection of challenge of virus(Table 2).

Once it was established that intranasal immunization of inactivated virus only was capable of eliciting anti-influenza specific humoral immune responses, the efficacy of the various vaccine preparations to protect pigs from viral challenge was determined. From the virus materials, ether-treated split virus and formalin-inactivated virus were prepared. For synthetic peptide vaccine, additional one set of epitope sequence was subjoined at the end of 18-mer peptide(25-mer peptide).

Dose of vaccine preparations originated from virus material were adjusted to 100μg equivalent of protein per ml. Synthetic peptide was adjusted to 1.0mg per ml. Weekly immunization was done four times intranasally in all experimental groups.

Antibody titers in serum and Nasal swab of the pigs immunized with various vaccine preparations 1 week after 4th immunization were shown in Table 3. Those of originated from virus material induced remarkable antibody response in the pigs showing HI titers of 1:64 to 1:256. Synthetic peptide vaccine(25-mer) did not induce detectable antibody response in the sera and nasal swabs.

After challenge of Aich/2/68, nasal swab samples were taken daily for 10 days. Virus shedding in the nasal secretions were quantitated by plaque formation assay. Table 4 shows the results of virus recovery from the nasal swab samples. Nasal swabs from the pigs immunized with virus originated vaccine preparations did not show plaque formation within the whole period.

Interestingly, nasal swabs from one pig immunized with 25-mer peptide vaccines did not show plaque formation, and even those of other showed only 101PFU/ml at day 3, 5 and 6 of challenge although they did not show detectable antibody response in the sera and nasal secretions at the time of challenge. But, sera of the pigs immunized with peptide vaccines showed an increasing HI titers 1:128 to 1:256 two weeks after challenge(Table 5), suggesting that the virus replication was progressed at the respiratory mucosa and initiated immune response even though virus shedding was inhibited in the pigs immunized with 25-mer synthetic peptide vaccine.

DISCUSSION

Since there is no specific treatment for swine influenza, immunoprophylaxis with vaccines may be the most useful method for controlling swine influenza. For active immunization, inactivated viral vaccines containing oil adjuvant have been developed by Gourreau et al.(15).

The most important matters to consider in applicating vaccine are the efficacy and safety of the preparations. The synthetic peptide holds several advantages over conventional vaccines, including safety considerations, the ability to target the immune response, the possibility of preparing multipathogen vaccines and relatively long shelf-life.

In the present study, the pigs vaccinated intranasally with formalin-inactivated virus and ether-treated split virus preparations showed impressive antibody response against influenza virus A/Aichi/68, and they did not shed virus after challenge of live virus. These vaccines induced IgG, IgM and IgA antibodies in the sera and nasal fluid. The IgA antibody that is crucial to inhibit expansion of influenza virus in vivo was the dominant antibody class both in the sera and nasal swabs.

In contrast, the pigs vaccinated with synthetic peptide 18-mer and 25-mer did not show any detectable antibody response in HI test and ELISA. But out of two pigs vaccinated with synthetic peptide 25-mer, one pig did not shed virus in nasal fluid and also the other pig shed relatively little amount of virus compared to liposome control group and vaccinated group with 18-mer synthetic peptide, suggesting that 25-mer peptide may induced some protective immune response to the challenge virus by unknown priming with local immune system.

Ogasawara et al.(8) reported on synthetic peptide vaccine against influenza virus A/Aichi/68 based on the cassette theory, which contain agretopic motif and epitopic motif of I-A^b mice all together, the peptide vaccines induced both helper T-cell responses and production of antibodies that were specific for influenza virus A/Aichi/68 but not for the major histocompatibility complex binding frame in mice bearing A^k or A^b. In this study, all the pigs vaccinated with synthetic peptides did not show antibody response even against the synthetic peptide 18-mer or 25-mer in ELISA(data not shown).

Although, the synthetic peptide(25-mer) did not induce any antibody produc-

tion in the immunized pigs but the mechanisms which prevent viral replication in the respiratory mucosa of the challenged pigs should be investigated further. Based on the present finding we assume that synthetic peptide vaccine prepared according to Cassette theory may be applicable to the heterogenous species including human.

ACKNOWLEDGEMENT

This research was supported by KOSEF of Post-Doctral supporting program.

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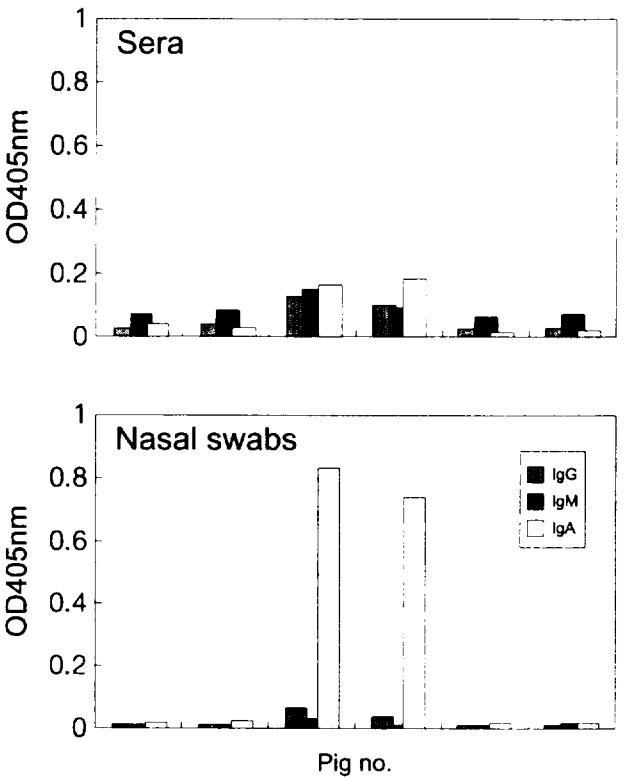

Figure 1. ELISA of sera and nasal swabs from pigs immunized with formalin-inactivated virus or sythetic petide(18-mer) at the time of challenge.(p1, p2 : dosed with liposome vehicle; p3, p4 : dosed with formalin-inactivated virus; p5, p6 : dosed with synthetic petide)

Table 1. HI antibody titers of sera and nasal swabs at the time and 2 weeks after challege

Vaccine	Pig no.	Serum*		Nasal Swab	
		0wk	2wk	0wk	2wk
Liposome	1	<8	512	<8	16
Control	2	<8	256	<8	32
Synthetic	3	<8	512	<8	32
Peptide(18-mer)	4	<8	512	<8	32
Inactivated	5	128	128	256	<8
Virus	6	128	128	128	<8

* Sera were treated with same volume of receptor destroying enzyme at 37°C, 16hr followed 56°C, 1hr.

Table 2. Virus recovery from the nasal swabs of pigs

Group	Pig no.	days after Challenge							
		1	2	3	4	5	6	7	8-14
Liposome	1	-	++	++	++	+++	+++	+++	-
Control	2	-	-	-	+++	+++	+++	+	-
Synthetic	3	+	+++	++++	+++	+++	+++	-	-
Peptide(18-mer)	4		- ++	+++	++	+++	+++	-	-
Inactivated	5	-	-	-	-	-	-	-	-
Virus	6	-	-	-	-	-	-	-	-

Challenge dose: 10^5 PFU
- : less then 10^1 PFU/ml
+ : ≤10 PFU/ml swab
++ : 11 - 100 PFU/ml swab
+++ : 101 - 1000 PFU/ml swab
++++ : 1001 - 10000 PFU/ml swab

Table 3. Antibody titers of pig sera and nasal swab 1 week after 4th immunization

Group	pig no	Serum				Nasal Swab			
		HI	ELISA			HI	ELISA		
			IgG	IgM	IgA		IgG	IgM	IgA
Liposome	1	<8	<100	100	<100	(16)*	<10	10	<10
Control	2	<8	<100	100	<100	(16)	<10	10	<10
Synthetic	3	<8	100	<100	<100	(16)	<10	<10	<10
Peptide(25-mer)	4	<8	<100	<100	<100	(16)	<10	<10	<10
Split Vaccine	5	64	800	400	200	64	160	160	160
	6	128	1600	200	400	128	160	80	640
Inactivated	7	64	800	200	400	256	320	320	640
Virus	8	64	800	400	400	128	160	160	160

* (16) indicates incomplete HI

Table 4. Virus recovery from the nasal swabs of pigs

Vaccine	Pig no.	Days after challenge						
		1	2	3	4	5	6	7-10
Liposome	1	-	++	+++	+++	+++	+++	-
Control	2	-	+++	+++	+++	+++	++	-
Synthetic	3	-	-	+	-	+	+	-
Peptide(25-mer)	4	-	-	-	-	-	-	-
Split	5	-	-	-	-	-	-	-
Vaccine	6	-	-	-	-	-	-	-
Inactivated	7	-	-	-	-	-	-	-
Virus	8	-	-	-	-	-	-	-

Challenge dose : 10^4 PFU
- : less then 10^1 PFU/ml
+ : 10 PFU/ml swab
++ : 11 - 100 PFU/ml swab
+++ : 101 - 1000 PFU/ml swab

Table 5. HI antibody titers change of sera and nasal swabs at the time and 2 weeks after challege

Vaccine	Pig no.	Serum*		Nasal Swab	
		0wk	2wk	0wk	2wk
Liposome	1	<8	128	8	16
Control	2	<8	256	8	8
Split vaccine	3	64	64	64	32
	4	128	64	128	16
Inactivated	5	64	64	256	<8
Virus	6	64	64	128	<8

* Sera were treated with same volume of RDE at 37℃, 16hr followed 56℃, 1hr.