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FULL PAPER

Mucosal vaccination against influenza: Protection of pigs immunized with inactivated virus and ether-split vaccine

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Abstract

Effective vaccinations against swine influenza reduce the economic loss of pig industries, and also may minimize the possibility of emergence of new pandemic viruses, since pigs are intermediate hosts to generate reassortant viruses among avian and mammalian influenza viruses. In this study, we showed that intranasal immunization of pigs with formalin-inactivated or ether-split influenza vaccine (A/Aichi/2/68) induced virus-specific IgG, IgM, and IgA antibodies in their nasal secretions and sera, resulting in complete protection from virus challenge. Antibody response to the challenge virus was not observed in the immunized pigs, suggesting that the replication of the virus in the primary targets, respiratory epithelial cells, was inhibited. The present results indicate that intranasal immunization of pigs with inactivated vaccines is effective to control swine influenza, and also provide a good model, as well as a mouse model, to evaluate an intranasal application of influenza vaccine for humans.

Introduction

Swine influenza is a highly contagious acute viral disease of the respiratory tract, and has economic consequences in that infected pigs lose weight and occasionally die by secondary infections[3]. In addition to the significance of this disease with economic loss of pig industries, there is, in fact, an another issue that should be considered with control of swine influenza.

Pigs are shown to be susceptible to both

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human and avian influenza A viruses[2,5,8], which may be explained by the existence of the receptors for both viruses on the epithelial cells of their respiratory tracts[6]. The human pandemic strain, A/Hong Kong/2/68 (H3N2), has been shown to be a genetic reassortant between human and avian viruses, and such an event might occur in pigs coinfected with the viruses[22]. Thus, pigs are thought to be intermediate hosts playing an important role to generate human pandemic influenza viruses[14,22].

The influenza vaccines that are commercially available for animals, including humans, horses, and pigs do not provide complete protection from virus infection while they may decrease the incidence and severity of clinical disease[12]. These vaccines are inactivated intact virus or subunit vaccines, and injected by parenteral route to induce serum antibody response. On the other hand, there is a large number of works showing that the mucosal immunity represented by local secretory antibodies such as IgA, is rather more essential for protection of animals from infections of mucosal tissues than systemic immunity[10,11]. Since the secretory antibodies play an important role to exclude viruses from the mucosal surfaces of animals, induction of the secretory antibodies may provide complete protection from virus infections through the mucosal routes by prohibiting their initial replication in the mucosal epithelial cells[17,18,20].

Influenza virus initially infects the epithelial cells of the respiratory tracts of the animals. Accordingly, it has been shown that the local secretory antibodies play an important role for the protection of mice from the virus infection[4,19,21]. The aim of this study is to evaluate intranasal vaccination of pigs with inactivated influenza vaccines, which have a potential to induce secretory antibodies and hence to elicit protective immunity in the animals.

Materials and Methods

Animals

Three-week-old specific pathogen free pigs (F1 between Landrace and Durock) were purchased from Hokuren (Sapporo, Japan).

Virus and vaccines

Influenza virus, A/Aichi/2/68 (H3N2) (Aichi/2/68), a prototype strain of human H3N2 viruses, was propagated in the allantoic cavities of 11-day-old embryonated chicken eggs at 35℃ for two days. The virus was purified by high speed centrifugation of infected allantoic fluid, followed by differential centrifugation through a 10 to 50% sucrose density gradient and pelleted[9]. For ether-split vaccine, the purified virus was mixed with equal volume of ether, followed by incubation for 30 min at room temperature with stirring. The mixture was centrifuged (3,000rpm, 15 min), and the aqueous phase was collected and evaporated. Formalin was added to the aqueous phase at the final concentration of 0.01%. For inactivated intact virus vaccine, formalin was added to the allantoic fluid and then the virus was purified as described above.

Immunization and protection tests

Pigs were immunized intranasally with 100μg viral protein of each vaccine diluted with 1 ml of PBS (0.5ml for each nostril) 4 times weekly. Control pigs were given PBS. One week after the final immunization, the sera and nasal swabs were collected for antibody detection, and the pigs were challenged intranasally with 1 ml of 10^4 plaque forming units (PFU) of the virus. To examine virus recovery, the nasal swabs were collected from the pigs daily after the challenge. The swabs were soaked into 1 ml of PBS containing antibiotics. Virus titers were determined by

plaque assays using MDCK cells as described previously[7].

Antibody assays

Sera and nasal swabs were examined for their antibody titers by hemagglutination-inhibition (HI) test[15] and enzyme-linked immunosorbent assay (ELISA)[7]. For HI tests, the sera were treated with receptor destroying enzyme (Takeda Chemical Industries) to reduce nonspecific HI activity by serum inhibitors. In the ELISA, the wells were coated with disrupted Aichi/2/68 obtained by treating purified virions with 0.05M Tris-HCl (pH 7.8) containing 0.5% Triton X-100 and0.6M KCl at room temperature and diluted in PBS. The reactions were detected using goat anti-pig IgA, IgM, or IgG antibodies conjugated to horseradish peroxidase (Bethyl Laboratories).

Results

Antibody response of pigs immunized intranasally with formalin-inactivated virus or ether-split vaccine

Four and two pigs were immunized intranasally with formalin-inactivated virus and ether-split vaccines, respectively. One week after the final immunization, sera and nasal swab samples were collected from these immunized pigs and 4 control pigs, and examined for their antibody responses (Table 1). We found that both formalin-inactivated virus and ether-split vaccines induced systemic and mucosal antibody responses. Remarkable levels of IgG, IgM, and IgA antibodies specific to the virus were detected both in the sera and nasal secretions of the immunized pigs. No significant difference in the ability to induce systemic and mucosal antibody responses was observed between formalin-inactivated virus and ether-split vaccines.

To monitor the antibody levels in the immunized pigs during the experiments, serum and nasal swab samples were collected from each two of four pigs in the groups I (formalin-inactivated vaccine) and III (control) weekly after the first immunization, and IgG and IgA antibody titers in the sera and the nasal secretions, respectively, were determined

Table 1. Virus-specific antibody titers[a] of pigs immunized intranasally with inactivated vaccines.

group	pig no.	serum			nasal swab		
		IgG	IgM	IgA	IgG	IgM	IgA
I. formalin-	1	800	200	400	320	320	640
inactivated	2	800	200	400	160	160	160
vaccine	3	640	NT[b]	NT	160	NT	160
	4	640	NT	NT	160	NT	640
II. enter-split	5	800	400	200	160	160	160
vaccine	6	1600	200	400	160	80	640
III. control(PBS)	7	<10	<10	<10	<10	<10	<10
	8	<10	<10	<10	<10	<10	<10
	9	<10	<10	<10	<10	<10	<10
	10	<10	<10	<10	<10	<10	<10

[a] IgG, IgM, and IgA antibodies in the samples of individual pigs were detected by ELISA as described in the Materials and Methods. Antibody titers are expressed as the reciprocals of endopoint dilution of the samples.

[b] Not tested.

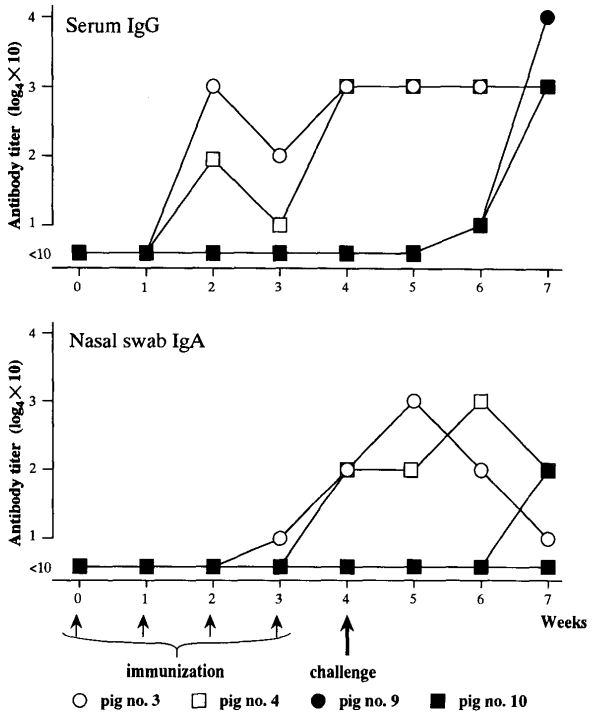

Fig. 1. Serum IgG and nasal IgA antibody responses in pigs after immunization and virus challenge. Samples were collected from pigs nos. 3 and 4 that were immunized with formalin-inactivated virus vaccine, and control pigs no. 9 and10 every week. Antibody titers were determined by ELISA.

(Fig. 1). Both of the immunized pigs (nos. 3 and 4) produced detectable serum IgG antibodies after the second immunization, while nasal IgA antibodies became positive after the third or forth immunization. One week after the final immunization, antibody titers of serum IgG and nasal IgA reached up to 640 and 160, respectively.

Protection of pigs from intranasal virus challenge

To evaluate the contribution of the antibodies to protective effects, pigs were challenged with Aichi/2/68 at one week after the final immunization. Nasal swab samples were collected daily for 10 days and examined for virus recovery (Table 2). On 2 - 7 days after the virus challenge, control pigs (group III) shed the virus at the titers of $10^{1.3}$-$10^{2.9}$ PFU/ml of samples. In contrast, no virus was detected in the samples of two pigs in group II (pigs nos. 5 and 6) and two out of four pigs in group I (pigs nos. 1 and 2) throughout the periods. Only a low level of the virus was recov-

Table 2. Virus recovery from the nasal swabs of pigs immunized inranasally.

group	pig no.	days post infection[a]									
		0	2	3	4	5	6	7	8	9	10
I	1	—[b]	—	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—	—	—
	3	—	—	1.5[c]	—	—	—	—	—	—	—
	4	—	—	1.5	—	—	—	—	—	—	—
II	5	—	—	—	—	—	—	—	—	—	—
	6	—	—	—	—	—	—	—	—	—	—
III	7	—	1.8	2.9	2.5	2.8	2.3	—	—	—	—
	8	—	2.0	2.8	2.2	2.7	1.3	—	—	—	—
	9	—	1.9	2.0	1.8	2.9	2.9	2.1	—	—	—
	10	—	—	2.6	2.5	2.6	1.6	—	—	—	—

[a] Nasal swabs were collected from each pigs daily after the virus challenge.

[b] — ; not detected.

[c] Titers are expressed as log_{10}PFU/ml of swab sample.

Table 3. Serum HI antibody response of pigs immunized intranasally with inactivated vaccines.

group	pig no.	week post infection[a]	
		0	3
I	1	256[b]	256
	2	256	256
	3	64	64
	4	64	64
II	5	64	64
	6	128	64
III	7	< 8	128
	8	< 8	256
	9	< 8	1024
	10	< 8	512

[a] The sera were collected before and 3 weeks after virus challenge.

[b] Titers are expressed as the reciprocals of serum dilution.

ered from two pigs (pigs no. 3and 4) in group I at three days after the challenge.

Antibody response of pigs after virus challenge

In order to know whether the immune response against the challenge virus was induced, serum samples were collected from all the pigs on 0 and 21 days after the challenge and examined for HI antibody titers. As shown in Table 3, no increase in the titers was found in pigs in group I, although pigs nos. 3 and 4 shed the virus, or in those in group II. The control pigs seroconverted after the challenge. In order to examine a effect of protection by inactivated intact virus in detail, serum and nasal swab samples collected from each two of four pigs in groups I (pigs no. 3 and 4) and III (pigs no. 9 and 10) after the virus challenge were tested to compare their antibody titers with those of the samples collected from them before challenge (Fig. 1). In contrast to the control pigs in which significant IgG antibody raise were found, indicating that the replication of the challenge virus occurred, both of the immunized pigs did not show the increase in antibody titers after the virus challenge. Similarly, significant increase in nasal IgA antibody titers was not observed with the immunized pigs.

Discussion

Influenza A viruses infect a large variety of animal species[22]. Among them, pigs are an important host in influenza ecology since they are susceptible to infection with both avian and mammalian influenza A viruses. Although only viruses of subtypes H 1 N 1 and H 3 N 2 have been spread widely in pigs thus far[1], there are many epidemiological evidence that human-to-pig and avian-to-pig transmission could occur in nature[1,23]. It is also noted that avian H 9 N 2 viruses which were isolated from avian species in Southern China were recently isolated from humans and pigs in that area[13,16]. Maintenance of current influenza A viruses in the pig population or the frequent introduction of new viruses from avian species could be a threat of the generation of reassortant viruses which may emerge as pandemic strains in humans. Besides their roles in genetic reassortment, pigs are sometimes sources for zoonotic trasmission of swine viruses to humans[12]. Thus, much attention should be paid to the control of swine influenza, not only to reduce the economic loss of pig industries but also to minimize the possible transmission of the viruses, including reassortant viruses, to humans.

Vaccines currently used for animals and humans are injected through parenteral rourtes (eg. subcutaneous) to induce serum antibodies. However, systemic immunity including serum antibody and cytotoxic T-cell responses does not prevent initial infection on the mucosal epithelial cells[17,18], although these responses may reduce the severity of

the disease and eliminate the virus from the infected animals[12]. In contrast, since the mucosal surfaces are the portals of the entry for many pathogens including influenza virus, mucosal immunity protects animals from virus infections by inhibiting initial replication of the virus at the mucosal tissues. In this study, the intranasal immunization of pigs with formalin-inactivated or ether-split influenza vaccine induced virus-specific IgG, IgM, and IgA antibodies in their nasal secretions, resulting in complete protection from the virus challenge. The present results are consistent with our previous studies on intranasal immunization of animals against viral infections[17-20].

The present study showed that a complete protection of pigs from influenza A virus infection could be attained by the induction of a specific immune defense at the site of the entry for the virus. This strategy should eliminate any opportunity to generate reassortant viruses in pigs, while systemic immunization does not appear appropriate for preventing an initial repication of the virus in the respiratory tissues. In addition, there is a number of practical advantages for mucosal immunization with non-invasive viral antigens with respect to the simplicity, easy storage and delivery, and cost effectiveness, which should abolish the risks inherent to the use of live virus vaccines.

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