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Oral vaccination with inactivated influenza vaccine induces cross-protective immunity

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Abstract

Oral vaccination would provide an easy and safe measure to prevent infectious diseases by facilitating mass immunization. We investigated the feasibility of oral vaccination with inactivated whole influenza virus (A/PR8/34). Oral vaccination of mice induced high levels of serum IgG and IgA antibodies specific to the homologous virus (A/PR8) as well as cross reactive to heterologous (A/California/04/09) and heterosubtypic viruses (A/*Philippines*/2/82). IgG1 isotype antibodies were found to be induced at significantly higher levels than IgG2a antibodies. These antibodies induced by oral vaccination exhibited hemagglutination inhibition activities. High levels of both IgG and IgA antibodies were induced in vagina and lungs. Mucosal IgA antibodies were also elicited in other sites including saliva, urine, and fecal samples. Orally vaccinated mice were completely protected against challenge with homologous or heterologous viruses, and partially protected against heterosubtypic virus. Importantly, high recall antibody secreting cell (ASC) responses were induced in spleen, indicating the generation of memory B cells by oral vaccination. The present study therefore presents new findings of cross-reactive antibodies at systemic and diverse mucosal sites, recall antibody responses, and cross-protective efficacies by oral vaccination, thus supporting a proof-of-concept that oral delivery of vaccines can be developed as an effective vaccination route.

Keywords

Influenza; Oral vaccination; Cross protection

Introduction

Influenza epidemics and the threat of novel pandemic strains remain major health concerns since influenza can cause high morbidity and mortality rates. Delivery of vaccines via the

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oral route is the most convenient and safe way of vaccination as demonstrated for the case of live attenuated polio vaccine. Oral vaccination would significantly reduce opportunistic and iatrogenic infections due to the use of unsterile needles as well as needle-stick injuries, which are especially a high risk in developing countries [1,2]. In contrast to parenteral vaccinations, oral vaccination can induce immune responses in mucosal sites [3–5], which might give protection against influenza infection at the port of entry [6].

Previous clinical studies demonstrated that oral vaccination with influenza vaccines in water-in-oil emulsion or enterically coated capsules induced significant levels of IgA antibodies in saliva and nasal wash samples, but IgG antibody responses were at low or below detection levels [3,4,7]. Despite the possibility to induce effective mucosal immune responses, influenza vaccines are required to induce a sufficient level of virus-specific antibodies in the serum to meet current regulatory requirements for immunogenicity. To achieve sufficient serum antibody levels, preclinical studies on oral immunization with influenza vaccines have focused on the use of potent adjuvants such as *Escherichia coli* heat-labile enterotoxins [8–10] or complex vaccine formulations including bile salts and lipid vesicles or biodegradable and biocompatible microspheres [5,11–14].

Cross reactivity and protection after oral vaccination with influenza vaccines in the absence of adjuvant has not been well investigated. In this study, we tested the feasibility of oral vaccination with inactivated whole virus vaccine without using an adjuvant. Mice orally immunized with inactivated virus induced high levels of IgG and IgA antibody responses at systemic and mucosal sites, which were found to be cross-reactive. This study also provides a proof-of-concept that oral vaccination can induce cross-protective immune responses.

Methods

Virus and cells

Influenza virus A/PR8/34 (H1N1, A/PR8), A/California/04/09 (H1N1) and A/Philippines/2/82 (H3N2) were grown in 10-day-old embryonated hen's eggs and purified from allantoic fluid by using a discontinuous sucrose gradient (15%, 30%, and 60%). Inactivation of the purified virus was performed by mixing the virus with formalin at a final concentration of 1:4000 (v/v) as described previously [15]. Inactivation of the virus was confirmed by a plaque assay on confluent monolayers of Madin–Darby canine kidney (MDCK) cells and by inoculation of the virus into 10-day-old embryonated hen's eggs. For challenge experiments, mouse-adapted influenza viruses A/PR8/34 and A/Philippines/2/82 or a human pandemic virus isolate, A/California/04/09 were prepared as lung homogenates from intranasally infected mice and used for challenge.

Immunization and challenge

Female inbred BALB/c mice (Charles River) aged 6–8 weeks were used. Twelve mice in each group were orally administered 100 µl phosphate-buffered saline (PBS) containing 25 µg of inactivated A/PR8 virus on days 0 and 30. Oral administration was carried out using a stainless steel feeding needle with a silicone tip. For challenge infections, isoflurane-anesthetized mice were intranasally infected with the following doses of virus (A/PR8/34:

25 × LD₅₀, A/California/04/09: 5 × LD₅₀, A/Philippines/2/82: 5 × LD₅₀) at week 4 after boost. Mice were observed daily to monitor changes in body weight and to record death (25% loss in body weight as the Institutional Animal Care and Use Committee (IACUC) endpoint). All animal studies were approved by the Emory University Institutional Animal Care and Use Committee (IACUC).

Sample collections

Blood samples were collected by retro-orbital plexus puncture before immunization and 2 weeks after priming and booster immunizations. Vaginal lavage fluids were collected by washing the vagina with 200 µl of PBS. Ten pieces of freshly voided feces were collected at 2 weeks after boost, weighed, and resuspended in PBS with NaN₃ (0.01%) in a ratio of 1 mg of feces in 1 ml PBS solution. The feces were re-suspended in PBS by vigorous vortex until solutions were homogenous. Samples were then spun in a microcentrifuge for 10 min and supernatants were collected. Saliva and urine samples were collected at week 2 after boost. Carbamoylcholine chloride (2 µg, 100 µl PBS) was intraperitoneally injected to stimulate secretion of saliva during sample collection. Lung homogenates in 1 ml Dulbecco's modified Eagle's medium were centrifuged at 1000 rpm for 10 min to collect supernatants, which were frozen and kept at -80 °C until used for determination of virus titers and IgG and IgA antibody levels. Lymphocytes from spleen samples were collected from sacrificed mice to detect influenza virus-specific antibody-secreting plasma cells (ASC).

Antibody responses and hemagglutination inhibition (HAI) titers

IgG, IgG1, IgG2a and IgA antibody levels specific to influenza viruses in the serum or mucosal samples or lung were determined by enzyme-linked immunosorbent assay (ELISA) as previously described [16]. Briefly, ninety-six-well microtiter plates (MaxiSorp immunoplate; Nunc Life Technologies, Basel, Switzerland) were coated with 100 µl of inactivated viruses (4 µg/ml) or purified soluble HA proteins (Immune Technology Corp, NY) in coating buffer (0.1 M sodium carbonate, pH 9.5) at 4 °C overnight. Serially diluted sera or lung samples were added to wells to determine influenza virus-specific binding antibody levels. HAI titers were determined using 0.5% chicken red blood cells and 4 HA units of A/PR8, A/California/04/09, or A/Philippines/2/82 as described [17].

Lung viral titers

Lung viral titer assay was performed using MDCK cells following a previously described procedure [16,17]. Briefly, serially diluted lung homogenates were added to the cell monolayers. After incubation for 2–3 days, the cells were fixed with 0.25% glutaraldehyde and stained with 1% crystal violet to visualize plaques.

Detection of antibody-secreting cells in the spleen

To detect influenza virus-specific antibody-secreting cells, inactivated viral antigens (A/PR8, A/California/04/09, A/Philippines/2/82) were used to coat 96-well culture plates (Costar). Freshly isolated cells from spleen (1 × 10⁶ cells) were added to each well and incubated for 2 or 6 days at 37 °C with 5% CO₂. After removing cells from the culture plates, HRP-conjugated secondary goat-anti-mouse antibodies were incubated. As a measure of HRP

activity, the substrate *o*-phenylenediamine (Zymed, San Francisco, CA) was used and the optical density was read at 450 nm.

Statistics

All parameters were recorded for individuals within groups. Statistical comparisons of data were carried out using the analysis of variance and Npar one-way Kruskal–Wallis tests of the PC-SAS system. *P* values of <0.05 were considered significant.

Results

Oral vaccination induces systemic and mucosal immune responses

We have investigated the immunogenicity of inactivated influenza vaccine in a mouse model after oral administration. Mice were orally immunized with inactivated influenza vaccine (A/PR8) at weeks 0 and 4. Substantial levels of serum IgG antibodies specific to A/PR8 virus were observed after priming compared to the control (Fig. 1A). Oral boost vaccination further increased the levels of IgG antibodies specific to A/PR8 by 5 fold. Also, serum IgA antibodies specific to A/PR8 virus were induced by prime boost oral immunization (Fig. 1B). When antibody isotypes were determined, IgG1 isotype antibodies were induced at higher levels (Fig. 1C), indicating T helper type 2 (Th2) antibody responses. The results indicate that oral vaccination with inactivated influenza vaccine induces virus specific serum IgG and IgA antibodies.

Since oral delivery is considered to be a mucosal vaccination route, we determined virus specific IgG and IgA antibody responses in various mucosal sites. High levels of both IgG (Fig. 1D) and IgA (Fig. 1E) antibodies were induced in vaginal washes after oral vaccination with inactivated A/PR8 vaccine. It is interesting to note that high levels of IgA antibodies were detected in saliva samples. Significant levels of IgG and IgA antibodies were also observed in urinary samples after oral vaccination. Finally, low but substantial levels of IgG and IgA antibodies were detected in fecal samples. These results indicate that oral vaccination can provide an effective vaccine delivery route for inducing IgG and IgA antibodies in both systemic and mucosal sites. Also, determining antibody levels in the saliva and urinary samples might provide a convenient and easy diagnostic assay for evaluating vaccine efficacy.

IgG antibodies induced by oral vaccination are broadly cross reactive

Induction of cross reactive antibodies is an important goal for vaccination, and mucosal delivery of vaccines can be effective in inducing cross reactive responses. The 2009 H1N1 virus (A/California/04/09) is antigenically different from previous seasonal influenza H1N1 viruses and we therefore determined antibody reactivity with that virus. High levels of IgG antibodies cross-reactive to the 2009 H1N1 virus were induced in boost immune sera after oral vaccination with inactivated A/PR8 virus (Fig. 2A). Importantly, a similar level of IgG antibodies cross-reactive to the purified HA protein of the 2009 H1N1 virus was observed (Fig. 2A), indicating that cross reactive antibodies are binding to the HA protein. To further extend the breadth of cross-reactivity, we also determined the cross reactivity to heterosubtypic 1982 H3N2 virus (A/Philippines/2/82). Substantial amounts of IgG

antibodies that were induced by oral vaccination were cross reactive to the heterosubtypic H3N2 virus (Fig. 2B) although their reactivity was lower compared to heterologous cross reactivity within the H1N1 subtype viruses (Fig. 2A). These IgG antibodies were also found to be cross-reactive to the soluble H3 HA protein at lower levels (Fig. 2B). Therefore, these results indicate that oral delivery can be an effective vaccination approach for inducing cross reactive immune responses.

Cross-reactive antibody subclasses and hemagglutination inhibition activities by oral vaccination

It is informative to analyze subclasses of cross-reactive antibodies. The analysis of cross-reactive IgG antibody isotypes revealed that IgG1 was induced as a dominant isotype after oral vaccination, which was cross-reactive to the 2009 H1N1 (A/California/04/09) and the 1982 H3N2 (A/Philippines/2/82) viruses (Fig. 2C and D). We also found that IgA antibodies induced by oral vaccination were cross reactive to the heterologous 2009 H1N1 virus (Fig. 2E) and the heterosubtypic H3N2 virus (Fig. 2F) where effective IgA concentrations were approximately 25 ng/ml. Serum IgA antibodies cross reactive to soluble HA proteins were very low, near to background levels (data not shown). Hemagglutination inhibition (HAI) activity is considered as a functional antibody response providing a better protective immune correlate. We determined homologous and heterologous HAI titers in serum samples. HAI titers of 1/80 were observed with the homologous A/PR8 virus (Fig. 3). Importantly, HAI titers of 30 were found to be cross-reactive to the heterologous 2009 H1N1 and heterosubtypic H3N2 viruses, which is significantly higher than those in naïve serum controls (Fig. 3). Thus, oral vaccination can induce cross-reactive IgA and IgG1 antibodies as well as functional cross reactive antibody responses.

Oral administration of vaccines induces heterologous and heterosubtypic immunity

To determine the protective efficacy of oral vaccination, we challenged the vaccinated and naïve mice with a high dose (50 LD50) of homologous virus A/PR8 (Fig. 4A and B). All naïve mice showed rapidly declining body weight changes and died by day 4 post infection, which is an early time point due to the high lethal dose. The vaccinated mice showed only 5–8% decreases in body weight (Fig. 4A) and 100% survival (Fig. 4B). Therefore, oral vaccination can provide an effective method for inducing protective immunity against respiratory influenza infection.

To investigate the cross protective efficacy of oral vaccination, we tested the cross protection against the 2009 H1N1 virus. Naïve mice showed progressive loss in body weight (Fig. 4C) and all died by day 8 post infection with A/California/2009 (10 LD50) (Fig. 4D). In contrast, 100% of vaccinated mice survived although these mice also experienced significant losses in body weight. Last, we tested the heterosubtypic cross protection against the H3N2 influenza virus A/Philippines/82 (10 LD50). Vaccinated mice showed partial survival of 30% although they suffered loss in body weight similar to the naïve infected mice (Fig. 4E and F). These results support the conclusion that oral vaccination can induce cross protective immunity against heterologous and heterosubtypic viruses.

Lung viral loads are significantly reduced in orally vaccinated mice

To better assess the protective efficacy of oral vaccination, we determined viral loads at day 4 post challenge. After homologous virus (A/PR8) challenge, the orally vaccinated group showed 200 fold lower lung viral titers compared to those in naïve infected mice (Fig. 5). For heterologous challenge with the 2009 H1N1 virus, there was a 3-fold reduction in viral load in the oral vaccination group compared to the naïve infection control. In the case of heterosubtypic challenge with H3N2 A/Philippines/82 virus, the orally vaccinated group showed 6 fold lower viral lung titers compared to the naïve control group. These results indicate that oral vaccination can induce protective immune responses which effectively control viral replication of homologous virus and confer partial inhibition of heterologous viruses.

Vaccines delivered orally induce cross reactive antibody responses in lungs

We determined the levels of antibodies in lungs at day 4 post challenge (Fig. 6). Antibody levels were not observed in infected unvaccinated mice at a detectable level (Fig. 6A). Higher levels of IgG and IgA antibodies specific to the homologous viral antigen A/PR8 virus were induced in the orally vaccinated group that was challenged with A/PR8 virus (Fig. 6A and D). More importantly, it is worth noting that IgG and IgA antibodies cross reactive to the 2009 H1N1 virus were found to be induced in the orally A/PR8 vaccinated group at day 4 post challenge with the 2009 H1N1 virus (Fig. 6B and E). Levels of cross-reactive antibodies were lower than those to the homologous A/PR8 virus. Similarly, lung IgG and IgA antibodies that were cross reactive with the heterosubtypic H3N2 A/Philippines/82 virus were induced at significantly higher levels in orally vaccinated mice at day 4 post challenge compared to the naïve infected control (Fig. 6C and F). Therefore, oral vaccination can induce cross-reactive IgG and IgA antibody responses locally in lungs, at an early time post challenge.

Oral vaccination induces antibody secreting cell responses systemically

One of the main goals of vaccination is to generate long-lived memory B cells that can rapidly differentiate into antibody secreting plasma cells upon exposure to antigens [18]. To determine antibody secreting cell responses after oral vaccination, spleen cells were collected from orally vaccinated mice at day 4 post challenge and subjected to *in vitro* cultures (Fig. 7). We found that significant levels of IgG antibodies specific to the homologous A/PR8 virus were secreted into culture supernatants by spleen cells from the group of orally vaccinated and A/PR8 challenged mice. Also, substantial amounts of IgG antibodies cross reactive to the 2009 H1N1 virus were observed from splenocytes of orally vaccinated and challenged mice with the 2009 H1N1 virus after 2 or 6 days of culture (Fig. 7). The levels of cross reactive IgG antibodies to A/Philippines/82 virus were also significantly higher than those from the naïve infected control (Fig. 7A). We also determined the IgA antibodies in the same culture supernatants (Fig. 7B). IgA antibodies specific to A/PR8 were detected after 2 days of culture and further increased after 6 days of culture. Although IgA antibodies cross reactive with the 2009 H1N1 virus or H3N2 A/Philippines/82 virus were not detected after 2 days of culture, significant levels of cross reactive IgA antibodies were found to be secreted after 6 days of culture. Therefore, oral vaccination can

generate memory B cells systemically which have the capacity to rapidly differentiate into antibody secreting cells upon influenza virus infection.

Discussion

In order to improve the coverage of vaccination, the oral delivery of influenza antigens would be an attractive approach. In the present study, we investigated immune responses and cross protective efficacies in mice after oral vaccination with the inactivated whole virus. This study presents new findings that the antibodies induced by oral vaccination were found to be highly cross reactive to heterologous H1N1 and heterosubtypic H3N2 viruses, that oral vaccination could confer homotypic and heterotypic protective immunity, and that oral vaccination with whole inactivated virus can induce memory B cells with capacity to rapidly respond upon a virus challenge. Therefore, the present study provides proof-of-concept of the feasibility of oral vaccination for inducing broadly cross protective immunity.

We observed that oral vaccination with the inactivated whole virus induced high levels of serum IgG and IgA antibodies. In contrast, in previous comparative studies, intramuscular immunization induced higher levels of serum IgG antibody responses but did not induce serum IgA antibody responses compared to oral vaccination [8,19]. In a preliminary comparative study consistent with these previous reports [8,19], we found that oral vaccination showed higher levels of mucosal IgA antibodies cross-reactive to heterologous virus (data not shown), whereas intramuscular immunization showed higher levels of serum IgG antibodies. Regarding cross reactivity to heterosubtypic virus, oral vaccination was found to be more effective in inducing heterosubtypic cross reactive antibodies as determined by HAI and mucosal IgA antibodies although intramuscular immunization induced higher levels of IgG binding antibodies cross-reactive to heterosubtypic virus. However, more comprehensive studies including the effects of dosage are needed since the doses of oral vaccines required for protective immunity are much higher than intramuscular immunization doses. Also, the levels of IgG1 isotype antibody were induced at higher levels than IgG2a antibody after oral vaccination. Langerhans dendritic cells in the mucosal layers might be involved in uptake of antigens delivered via the oral route, which is more likely responsible for inducing Th2 type immune responses as suggested previously [20,21]. Intramuscular immunization with whole inactivated virus or virus-like particles was shown to induce IgG2a as a dominant isotype antibody [22–24]. The size of antigens (soluble versus particles, small versus large particles) was also demonstrated to influence the types of immune responses either after intramuscular immunization [24] or oral delivery [13]. The immune mechanisms behind the different patterns of immune responses depending on routes of vaccination remain to be determined.

Previous clinical studies demonstrated that oral immunization with influenza vaccines is safe. These oral immunizations induced IgA antibodies at mucosal sites, but induced low levels of serum IgG responses with low protective efficacies in humans [3–5,7]. Our study used a high dose of vaccine with two immunizations in mice, showing that significant levels of both IgG and IgA antibodies were induced in different mucosal samples and serum. Vaccinated mice were 100% protected against homologous and heterologous viruses, and partially protected against heterosubtypic virus. These are encouraging results compared to

human clinical trials where a single lower dose of vaccine was used. Challenges in developing oral vaccination in humans include the requirement for higher doses of vaccines and lower efficacy in inducing systemic antibody responses. Influenza antigen may need to be protected from gastric fluids to maintain stability of antigen activity. Low stability of vaccines in gastric fluids might be the reason that higher oral doses and multiple immunizations are required. These challenges are difficult to resolve in developing effective oral vaccines and might be reasons preventing the general application of influenza oral vaccination to humans. It is therefore important to choose the optimal dose and develop methods for stabilization of vaccine antigens and repeated vaccinations to ensure an effective immune response in humans, which may avoid immune tolerance and result in induction of protective immunity.

An advantage of mucosal vaccine delivery is to induce protective immune responses at the various mucosal sites where pathogens enter the body. By virtue of the common mucosal immune system, it is possible to achieve immune responses at remote mucosal surfaces by administering a vaccine orally. This phenomenon involves gut-associated lymphoid tissues such as the Peyer's patches and mesenteric lymph nodes, which leads to the dissemination of antigen-sensitized immune cells to distant mucosal tissues via the efferent lymphatics [26]. In particular, mucosal IgA antibodies are known to be produced after oral vaccination [7]. Clinical studies demonstrated that the majority of persons orally immunized with enterically encapsulated influenza vaccines responded with fourfold or higher IgA antibody responses in nasal secretions without significant increases in serum IgG antibody titers [7,27]. Also, over 90% of individuals who were vaccinated by the oral route with live attenuated influenza virus vaccine responded by showing enhanced hemagglutination inhibiting activity without clinical manifestations [28]. Consistent with previous findings, the present study demonstrates that high levels of both influenza antigen-specific IgG and IgA antibodies were detected in vaginal wash and lung extract samples. In addition, high levels of antigen-specific IgA antibodies were present in saliva and urine samples. Therefore, oral delivery provides a promising vaccination method for inducing immune responses in remote mucosal tissues.

More importantly, mucosal immune responses induced by intranasal or oral vaccination might offer a broader protection against antigenically drifted strains. Previous studies demonstrated that intranasal immunization of mice with high doses of an inactivated virus, influenza virus-like particles or co-immunization with an enterotoxin adjuvant induced IgG and IgA antibodies cross-reactive to different subtypes of influenza viruses [29–31]. Cross-reactive antibody responses induced by intranasal immunization but not by parenteral immunization were considered to contribute to providing cross protection [29,31]. Also, mice that were intranasally immunized with a high dose of whole inactivated virus in the presence of cholera toxin adjuvant induced cross reactive antibodies at systemic and mucosal sites, which were shown to have cross reactive neutralizing and HAI activities against a heterosubtypic virus as a recall response [15]. However, it was reported that side effects including facial paralysis were associated with intranasal influenza vaccination with an inactivated virosomal vaccine in the presence of an enterotoxin adjuvant [32,33]. These adverse effects resulted in the withdrawal of the intranasal vaccination from the market. No side effects (such as local or systemic reactogenicity often observed in systemic

immunization) are expected in oral vaccination. Also, in contrast to intranasal immunization, oral delivery is less likely to affect the neuronal system. Thus, oral vaccination is considered safer and less complicated than intranasal immunization [34,35]. However, the efficacy of oral vaccination needs to be improved.

In a previous study, oral immunization with inactivated influenza virus absorbed onto chicken erythrocytes but not the same vaccine given subcutaneously was shown to induce broader cross protection [14]. Also, gastrointestinal delivery of live baculovirus expressing influenza hemagglutinin was shown to protect mice against a heterologous virus strain [36]. In the present study, oral vaccination of mice without any adjuvant induced serum and lung IgG and IgA antibodies that were cross reactive to variant viruses. In addition, these antibodies were found to have cross reactive HAI activities, a better correlate for cross protection. The orally vaccinated mice were protected against homologous as well as heterologous virus challenge. This study and others therefore suggest that oral delivery can be an effective vaccination method for inducing broader protective immunity.

An important goal for vaccination is to induce long-lived memory B cell responses. The present study demonstrated that oral vaccination with inactivated influenza vaccine can induce the generation of cross-reactive B cells that are capable of rapidly differentiating into antibody secreting plasma cells. Antibodies secreted by B cells were found to be highly cross-reactive to heterologous and heterosubtypic viruses. Overall, we have shown that oral vaccination with inactivated whole virus vaccines can induce cross-reactive antibodies as well as cross reactive memory B cell responses. Further studies are needed to develop effective oral delivery vehicles so that reduced doses of vaccines will provide good protection.

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Fig. 1.

Antibody responses in serum and mucosal secretions elicited by oral vaccination. (A) Serum IgG antibodies specific to A/PR8/34 virus. (B) Serum IgA antibodies specific to A/PR8/34 virus. (C) Serum isotypes of antibodies specific to A/PR8/34 virus. IgG, IgA, IgG1 and IgG2a serum antibodies specific to the homologous virus A/PR8/34 were determined at week 2 after prime and boost. Significantly higher IgG or IgA titers against A/PR8 viruses were detected at boost compared to prime ($*P < 0.001$). IgG2a and IgG1 responses were determined in the serum after boost. Serum was serially diluted and optical density readings at 450 nm (OD_{450}) are shown in C. (D) Mucosal IgG antibodies specific to A/PR8/34 virus. Naïve indicates the highest background IgG level from naïve mouse feces compared to other mucosal samples. (E) Mucosal IgA antibodies specific to A/PR8/34 virus. A/PR8/34 specific IgG and IgA in mucosal samples were determined at week 2 after boost. Naïve indicates the highest background IgA level from naïve mouse feces compared to other mucosal samples. Samples (100 μ l) were diluted as 5 times and used for per well. Significantly higher IgG (A) or IgA (B) responses were detected except saliva IgG compared to naïve. For IgG, $*P < 0.001$ to vaginal wash, $^{\#}P < 0.01$ to urine and feces. For IgA, $*P < 0.001$ to vaginal wash, saliva and urine, $^{\#}P < 0.01$ to feces.

Fig. 2.

Cross-reactive antibody responses induced by oral vaccination. (A) IgG antibodies cross-reactive to the 2009 H1N1 virus (A/California/2009) and to the soluble H1 HA protein derived from the A/California/2009 virus. (B) IgG antibodies cross-reactive to the 1982 H3N2 virus (A/Philippines/2/82) and to the soluble H3 HA protein derived from the A/Philippines/2/82 virus. At week 4 after oral boost vaccination, cross-reactive IgG antibody responses were determined by ELISA using whole virus or purified soluble HA protein as a coating antigen. (C) Serum IgG1 and IgG2a antibody responses cross-reactive to the 2009 H1N1 pandemic virus (A/California/04/09). (D) Serum IgG1 and IgG2a antibody responses

cross-reactive to the 1982 H3N2 virus (A/Philippine/2/82). Cross-reactive IgG1 and IgG2a antibody responses were determined after boost. Serum samples were serially diluted and ELISA was performed to determine serum IgG1 and IgG2a antibodies. (E) Serum IgA antibody titers to the A/California/04/09 (H1N1) (* $P < 0.05$). (F) Serum IgA antibody titers to the A/Philippines/82 (H3N2) (# $P < 0.01$).

Fig. 3.

Hemagglutination inhibition titers against homologous and heterologous viruses. HAI titers against the homologous (A/PR8/34), heterologous (A/California/04/09), and heterosubtypic (A/Philippine/2/82) viruses were determined using immune sera collected from mice at week 2 after boost. Viruses grown in eggs (4 HA units) were used for HAI titers. Naïve sera were used as control. Significant higher HAI titers were observed in immune sera compared to those in naïve sera (* $P < 0.01$).

Fig. 4.

Oral vaccination induces cross-protection. (A and B) Protection against the homologous virus. Orally immunized mice ($n = 6$ per group) were challenged with homologous A/PR8/34 virus (50 LD_{50}) and monitored daily for body weight changes (A) and survival rates (B). (C and D) Cross protection against a heterologous H1N1 virus. Orally immunized mice ($n = 6$ per group) were challenged with the heterologous virus (A/California/04/09) (10 LD_{50}). Mice were monitored daily for 14 days for body weight changes (C) and survival rates (D). (E and F) Heterosubtypic cross protection. Orally immunized mice ($n = 6$ per

group) were challenged with a heterosubtypic virus (A/Philippine/2/82) (10 LD₅₀). Body weight changes (E) and survival rates (F) are shown.

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Fig. 5.

Oral vaccination reduces lung virus titers Lung samples from mice immunized with inactivated PR8 viruses ($n = 6$ per group) were individually collected at day 4 post-challenge with a lethal dose of A/PR8/34 (A), or A/California/04/09 (B) or A/Philippines/2/82 (C) viruses. Each lung sample from a mouse was suspended in 1 ml with Dulbecco's modified Eagle's medium. Significantly lower lung virus titers were detected in immunized mice compared to naïve (* $P < 0.0001$ to #PR8 Cha, ** $P < 0.01$ to 2009 H1N1 Cha, $P < 0.001$ to Phil Cha).

Fig. 6.

Lung IgG and IgA responses. Lung IgG and IgA antibody responses to A/PR8/34 (A and D), A/California/04/09 (B and E) or A/Philippines/2/82 (C and F) virus were determined at day 4 post challenge. Significantly higher levels of IgG and IgA responses were detected in orally vaccinated mice compared to naive mice after challenge infection ($P < 0.01$).

Fig. 7.

Oral vaccination induces antibody secreting cell responses. Mouse spleen cells were prepared at day 4 post challenge, added to the culture plates coated with whole viral antigens (A/PR8/34, or A/California/04/09, or A/Philippines/2/82), and then incubated for 2 days (D2) or 6 days (D6). Antibodies secreted and bound to the culture plates were determined by ELISA. (A) IgG antibodies to homologous (A/PR8), heterologous (A/California/04/09), heterosubtypic (A/Philippines/2/82) antigens. (B) IgA antibodies to homologous (A/PR8), heterologous (A/California/04/09), heterosubtypic (A/Philippines/2/82) antigens. Higher levels of IgG and IgA antibodies binding to the homologous or heterologous viruses were observed after 6 days of culture compared to those at day 2 ($P < 0.01$). Significant differences were found between IgG antibodies against PR8 Ag and 2009 H1N1 Ag or 1982 H3N2 Ag at day 2 and 6 after culture ($*P < 0.05$), between IgG antibodies against 1982 H3N2 virus antigen and non-antigen coated control ($P < 0.01$) at day 2 and 6, between IgA antibodies against PR8 Ag and 2009 H1N1 Ag (A/California/04/09) or 1982 H3N2 Ag (A/Philippines/2/82) ($\#P < 0.01$ at day 2, $**P < 0.05$ at day 6), between IgA antibodies against 2009 H1N1 and 1982 H3N2 at day 6 ($**P < 0.05$).