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# Neuraminidase gene homology contributes to the protective activity of influenza vaccines prepared from the influenza virus library

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Whole-virus (WV) vaccines from influenza A/duck/Hokkaido/77 (H3N2), and its reassortant strains H3N4, H3N5 and H3N7, which have the same haemagglutinin (HA) gene but different neuraminidase (NA) genes, were prepared from our influenza virus library. Mice were intranasally immunized with equivalent doses of each vaccine (1–0.01 μg per mouse). All of the mice that received the highest dose of each vaccine (1 μg per mouse) showed equivalent high HA-inhibiting (HI) antibody titres and survived the H3N2 challenge viruses. However, mice that received lower doses of vaccine (0.1 or 0.01 μg per mouse) containing a heterologous NA had lower survival rates than those given the H3N2-based vaccine. The lungs of mice challenged with H3N2 virus showed a significantly higher virus clearance rate when the vaccine contained the homologous NA (N2) versus a heterologous NA, suggesting that NA contributed to the protection, especially when the HI antibody level was low. These results suggested that, even if vaccines prepared for a possible upcoming pandemic do not induce sufficient HI antibodies, WV vaccines can still be effective through other matched proteins such as NA.

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## INTRODUCTION

Influenza virus is the causative agent of influenza, which can pose a serious public threat. Vaccination against influenza is the most effective method for preventing this disease and its potential complications (Cox & Subbarao, 1999). Influenza virus has two main immunogenic surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA), which are antigenically classified into 16 and nine subtypes, respectively, in the avian reservoir (Kida & Sakoda, 2006; Kilbourne, 2006b).

The currently available and licensed vaccines for humans include formalin-inactivated whole-virus (WV), split, subunit and live-attenuated vaccines. An influenza virus library containing 144 antigenically different types of influenza virus with all the combinations of HA and NA types (Isoda *et al.*, 2008; Itoh *et al.*, 2008; Kida & Sakoda, 2006; Samad *et al.*, 2011; Soda *et al.*, 2008a, b; Tamura *et al.*, 2010) has been prepared and stored at the Office International des Epizooties (OIE) Reference Laboratory for Avian Influenza at Hokkaido University in anticipation of the possible appearance of a new influenza pandemic

(Kida & Sakoda, 2006). We previously introduced a system for efficient influenza vaccine production during an influenza pandemic in which vaccine was prepared from the influenza virus library by cultivation in Madin–Darby canine kidney (MDCK) cells and administered through the nasal route to prove efficiency in mice (Haredy *et al.*, 2013).

Meanwhile, split-type vaccines have been mainly used throughout the world and the main goal of the current seasonal influenza vaccines is to induce antibodies against HA. Influenza vaccination is thought to elicit neutralizing antibodies that block virus attachment to host cell receptors or interfere with HA-mediated viral fusion (Kida *et al.*, 1983; Sandbulte *et al.*, 2007; Yoden *et al.*, 1986). Thus, the serum HA-inhibiting (HI) antibody titre is used as an immunological correlate of the protection elicited by currently licensed vaccines. An HI titre $>1:40$ appears to be correlated with vaccine efficacy in humans (Black *et al.*, 2011; Coudeville *et al.*, 2010; Luytjes *et al.*, 2012). Recently, the Flublok vaccine, which contains only the HA protein, was approved for human use in the USA. This vaccine assumed to induce HA inhibition antibodies to prevent influenza infection (Cox *et al.*, 2008). Thus, the current trend in influenza vaccine development is to give less importance to or ignore the immune response against NA or other influenza proteins.

Although the currently available inactivated vaccines are thought to also induce a NA-specific antibody response, the NA antibody response is not always measured (Johansson & Cox, 2011; Johansson *et al.*, 1987; Kilbourne *et al.*, 1995; Valette & Aymard, 2002). Furthermore, the impact of NA heterogeneity on the immune response of influenza vaccines has not been investigated in a systematic way, so it is still unclear how a change in NA might affect the immune response or virus clearance. Thus, in this study we examined the effect of NA heterogeneity on vaccine efficacy using formalin-inactivated WV vaccines prepared from the influenza virus library and administered through the nasal route in mice.

## RESULTS

### HI antibody responses following immunization

To examine the induction of HI antibodies by the prepared vaccines containing different NA genes (N2, N4, N5 and N7), HI antibody titres in the sera of the immunized mice were determined. Mice immunized with vaccine doses of 1, 0.1 and 0.01 μg showed decreasing HI antibody titres for all the vaccine preparations. Moreover, the same dose of vaccines with different NAs induced equivalent HI antibody titres against either the HA parent A/duck/Hokkaido/77 (H3N2) or the different homosubtype A/Aichi/2/68 (H3N2) strain. Vaccines administered at a dose of 1, 0.1 and 0.01 μg induced HI antibody titres of ~1 : 50, 1 : 10 and $<1:4$ against the HA parent H3N2 strain (Fig. 1a), and ~1 : 40, 1 : 8 and $<1:4$ against the other H3N2 strain, respectively (Fig. 1b).

### Protection of immunized mice against H3N2 viruses challenge

We next observed the survival of immunized mice after being infected with 100 $LD_{50}$ of mouse-adapted A/duck/Hokkaido/77(H3N2) or A/Aichi/2/68 (H3N2). All of the PBS-treated mice lost >20 % of their body weight (data not shown) and died by 5 days after infection (Fig. 2). All of the mice immunized with the 1 μg dose of vaccine lost <10 % of their body weight after infection (data not shown), and showed 100 % survival against both the HA parent strain (Fig. 2a) and the other H3N2 strain A/Aichi/2/68 (Fig. 2c). Mice immunized with the 0.1 μg dose of H3N2 also showed 100 % survival and a body weight loss

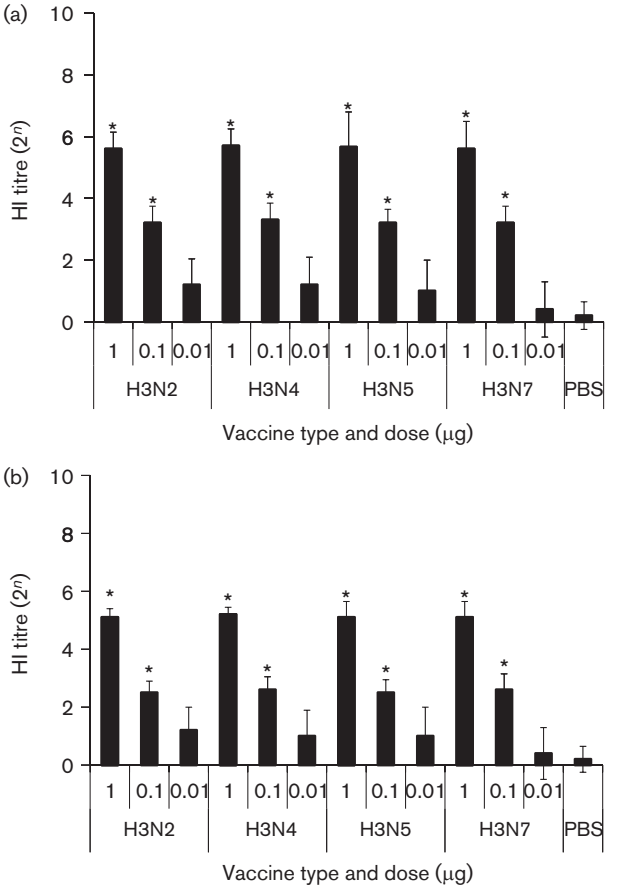


**Fig. 1.** HI antibody responses after vaccination. Mice sera were tested 2 weeks after the final immunization with H3N2, H3N4, H3N5 or H3N7 vaccine prepared at three different doses (1, 0.1 or 0.01 μg per mouse). Columns represent the mean ± SD of five mice. (a) HI response against the mouse non-adapted parent strain A/duck/Hokkaido/77 (H3N2). (b) HI response against the different homosubtype A/Aichi/2/68 (H3N2) strain. *$P<0.01$ versus the PBS immunized group.

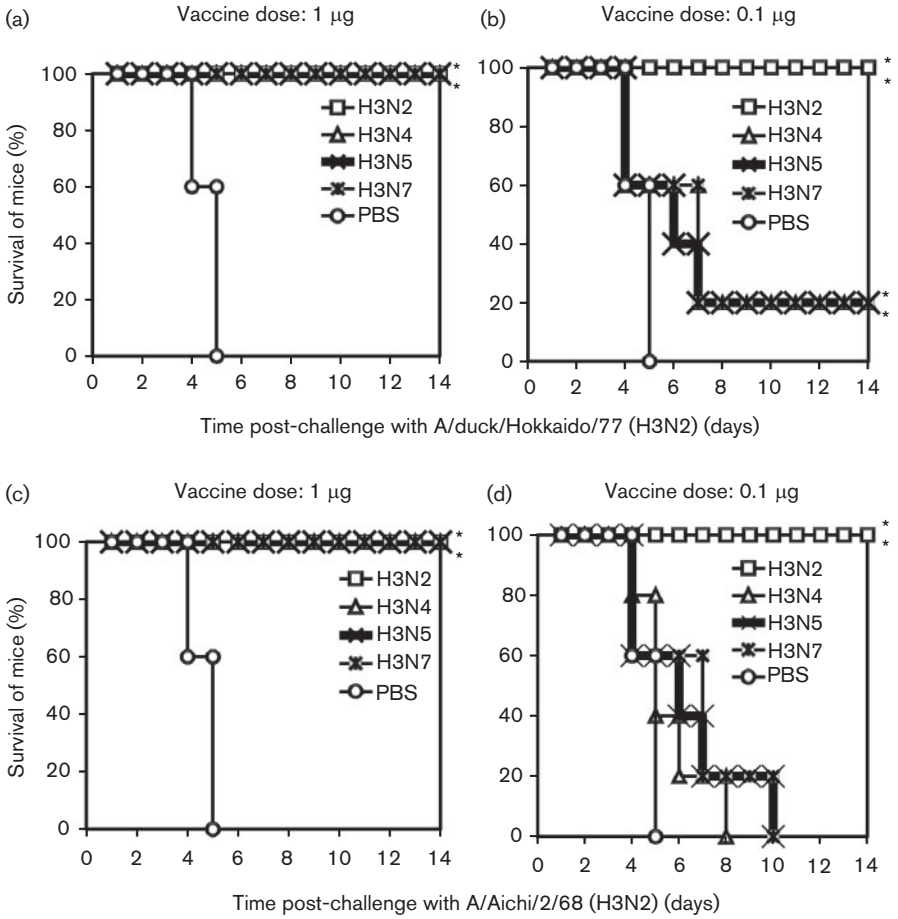


**Fig. 2.** Protection of mice by intranasal immunization. Six-week-old female BALB/c mice were divided into immunized groups ($n$=5 per group) and a control PBS group ($n$=5). All the members of each group were immunized twice with the same vaccine dose (1 or 0.1 μg) or PBS. Fourteen days after the second immunization, the mice were infected with 100 $LD_{50}$ of either (a, b) the HA parent strain A/duck/Hokkaido/77 (H3N2) or (c, d) the different homosubtype strain A/Aichi/2/68 (H3N2) and the mortality was assessed. **$P$<0.01 versus the PBS-treated group.

of <10 % (data not shown) against A/duck /Hokkaido/77 (H3N2) (Fig. 2b), and against the different homosubtype strain A/Aichi/2/68 (H3N2) (Fig. 2d). However, vaccines containing the heterologous NA at the 0.1 μg dose resulted in 80 % mortality from the A/duck /Hokkaido/77 (H3N2) strain (Fig. 2b) and 100 % mortality from A/Aichi/2/68 (H3N2) (Fig. 2d). The vaccine dose of 0.01 μg was unable to protect the mice against either H3N2 challenge viruses (data not shown).

## Virus clearance in the lungs of immunized mice

We next determined the virus clearance in the lungs of the infected mice immunized with different vaccines. Immunization with a 1 μg dose of any of the vaccines completely cleared the challenge virus from the lungs of all the immunized mice (Fig. 3a). The lungs of mice immunized with a 0.1 μg dose of vaccines with a heterologous NA showed no significant decrease in the challenge virus titre compared with those of PBS-treated mice, whilst the lungs of mice immunized with a 0.1 μg of dose of the H3N2 vaccine showed significantly decreased titres of the H3N2 challenge viruses (Fig. 3b, c). As expected, the extremely low dose of vaccine failed to clear the virus 3 days after infection (data not shown).

## NA-inhibiting (NAI) antibody detection in mouse serum

As the equivalent vaccine doses resulted in equivalent HI antibody responses, we next tested the immunized mice for NAI antibodies. Significant NAI activity was detected in the serum samples from mice immunized with a 1 μg dose of each vaccine against its parent strain (Fig. 4a, b). However, NAI activity against the H3N2 virus could not be detected in the sera of mice immunized with the H3N4, H3N5 or H3N7 vaccine (Fig. 4a). The NAI activity was below the detection limit for the infected mice immunized with a 0.1 μg dose of any vaccine preparation.

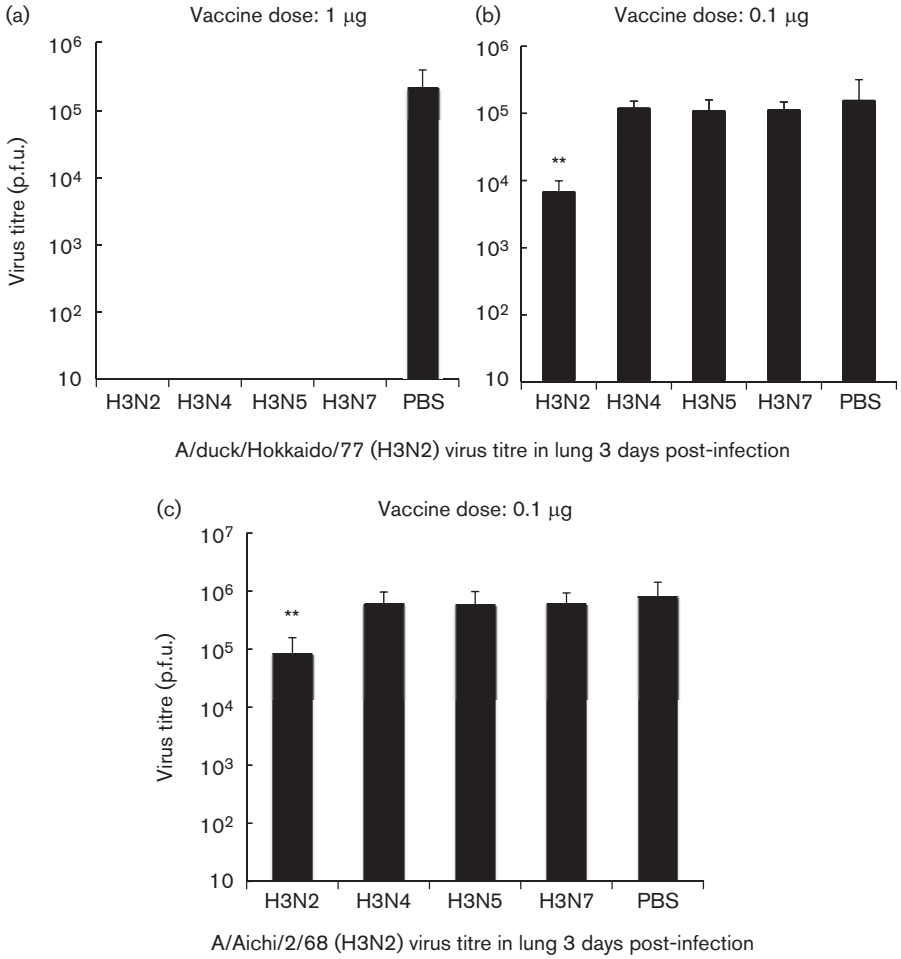


**Fig. 3.** Virus clearance in the lungs of immunized mice. Mice were immunized with 1 or 0.1 μg H3N2, H3N4, H3N5 or H3N7 vaccine, or PBS, and challenged by H3N2 mouse-adapted strains 2 weeks after the second immunization. Three days after infection, the lungs were harvested and the virus titre of (a, b) A/duck/Hokkaido/77 (H3N2) or (c) A/Aichi/2/68 (H3N2) in the lung homogenates was determined. Values represent the mean ± SD of 10 mice from two independent experiments. $^{**}P<0.001$ versus the PBS-treated group.

## DISCUSSION

In the present study, we examined the importance of the NA gene in seed virus strains in the influenza virus library (Isoda *et al.*, 2008; Itoh *et al.*, 2008; Kida & Sakoda, 2006; Samad *et al.*, 2011; Soda *et al.*, 2008a, b) for vaccine preparations.

The H3N2, H3N4, H3N5 and H3N7 virus strains chosen from the library had an identical HA and a different NA gene. These strains were used to prepare formalin-inactivated WV vaccines by cultivation in MDCK cells (Haredy *et al.*, 2013). HA and NA are located on the surface of influenza viruses, and are known to be the primary antigens involved in eliciting the humoral immune response to influenza viruses (Gamblin & Skehel, 2010; Kilbourne, 2006a). Supporting this notion, vaccination using inactivated influenza virus induces antibodies mainly to HA and to a lesser extent to NA (Kendal *et al.*, 1980). An HI antibody titre $\geqslant 1:40$ is considered to be the immunological correlate to influenza vaccine effectiveness in adult humans (The European Agency for the Evaluation of Medicinal Products Committee for Proprietary Medicinal Products, 1997; FDA CBER, 2007; Wood & Levandowski, 2003). Accordingly, the presence of HA antigen and the induction of HI antibody are the main properties used to determine the quality of a licensed vaccine.

In this study, we demonstrated that high doses of WV vaccines induced high HI antibody titres and provided similar protection against H3N2 viruses, regardless of whether the NA was heterologous. This result is consistent with the current knowledge about HI antibodies, which are known to directly neutralize virus infectivity (Johansson *et al.*, 1987; Kilbourne *et al.*, 1987). This finding was supported by the observation of complete virus clearance in the lungs of immunized mice 3 days after infection with the challenge virus (Fig. 3a).

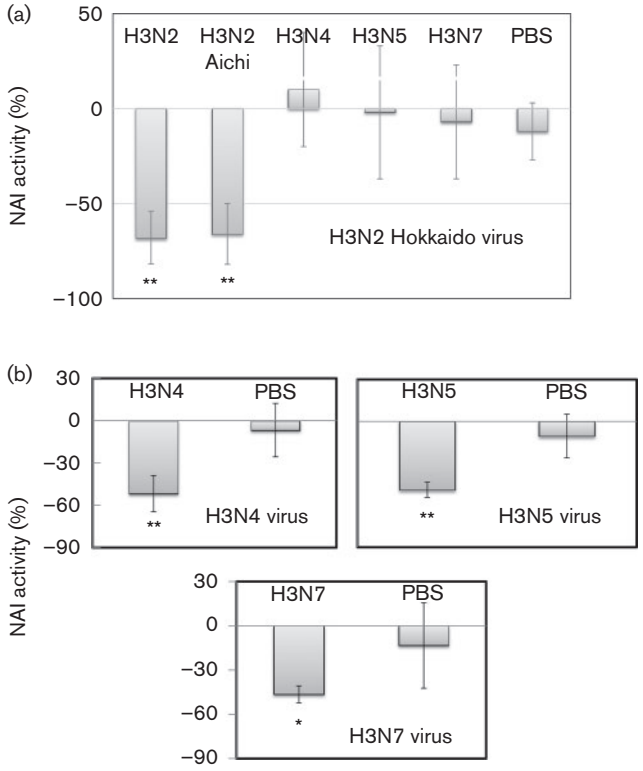


**Fig. 4.** Inhibition of NA by serum from mice intranasally immunized with 1 μg H3N2, H3N4, H3N5 or H3N7 formalin-inactivated WV vaccine. Mice ($n$=5 per group) were immunized twice with PBS or with 1 μg formalin-inactivated H3N2, H3N4, H3N5 or H3N7 WV vaccine. Fourteen days after the final immunization, the sera were harvested and examined for NAI activity. The NAI activity was then assessed for all the samples against (a) A/duck/Hokkaido/77 (H3N2) and (b) the homo-virus of each immunized group. Values represent the mean ± SD of the percentage of NA inhibition. $^{**}P<0.01$ and $^{*}P<0.05$ versus the PBS-treated group.

Interestingly, at a lower dose of vaccine (0.1 μg), which elicited lower HI antibody titres, only the H3N2 vaccine was effective against H3N2 viruses, whilst the mortality rate in mice immunized with vaccines containing a different type of NA ranged from 80 to 100 %. Similarly, at this dose (0.1 μg) of vaccine, significant viral clearance was observed in the lungs of H3N2-immunized mice compared with the groups immunized with the other vaccines. These results suggested that in mice in which insufficient HI antibody was induced, other viral antigens such as NA become important for protection.

We performed NAI assays using the serum of immunized mice, and confirmed that H3N2, H3N4, H3N5 and H3N7 had significant NAI activity against their own homologous viruses. Moreover, the NAI activity from the serum of H3N4-, H3N5- or H3N7-immunized mice showed no cross-inhibition against the H3N2 virus. This result was in agreement with several reports on the effect of NA on immune responses, in which no inter-NA subtype cross-reactive antibodies were detected (Avellaneda *et al.*, 2010; Beato *et al.*, 2007; Capua *et al.*, 2003; Peeters *et al.*, 2012; Wang *et al.*, 2011). These results could be attributed to the low homology between NA protein subtypes.

Recently, the importance of the immune response to NA has become more apparent. For example, it was shown that formalin-inactivated WV vaccine prepared in Vero cells induces an anti-NA immune response (Fritz *et al.*, 2012). In addition, it was reported that individuals with a sufficient immune response to influenza N1 in seasonal vaccine preparations show a cross-reactive immunity to the N1 associated with avian influenza H5N1 (Sandbulte *et al.*, 2007). Moreover, it was observed that the cross-protection against H5N1 obtained with trivalent seasonal or H1N1-only preparations formulated in adjuvant can be attributed to both HA and NA, but that the production of NAI antibodies is strongly correlated with protection in the ferret model (Rockman *et al.*, 2013). Our present results also indicate that the immunity to influenza NA is important in mice. Finally, it is known that NA is a cleavage enzyme releasing the virion from the infected cell surface. Unlike the HA neutralization that prevents infections, it would be expected that NA antibodies inhibit the influenza virus release and limit the infection spread. (Seto & Rott, 1966; Webster & Laver, 1967). Thus, both anti-HA and anti-NA antibodies are correlated with the reduction in influenza virus infections, and ensuring the induction of both antibodies by vaccination is desirable (Couch *et al.*, 2013). We conclude that for the influenza virus library to produce an effective vaccine, both HA and NA should be considered.

## METHODS

**Cells.** MDCK cells, which were kindly provided by the Kanonji Institute (The Research Foundation for Microbial Diseases of Osaka University, Kagawa, Japan) were maintained in a serum-free medium, Opti-Pro SFM (Invitrogen), supplemented with 0.3 mg L-glutamine $ml^{-1}$ and 8 μg gentamicin $ml^{-1}$. The cells were maintained at 37 °C in 5 % $CO_2$ (Yamada *et al.*, 2012).

**Viruses.** Non-pathogenic H3N2 A/duck/Hokkaido/77 in mice, and its reassortant viruses H3N4, H3N5 and H3N7, from the influenza virus library stored at the OIE Reference Laboratory for Avian Influenza at Hokkaido University were used. The reassortant viruses were prepared by mixing parental A/duck/Hokkaido/77 H3N2 virus with A/duck/Hokkaido/18/00 (H10N4), A/shearwater/Australia/1/72 (H6N5) or A/seal/Massachusetts/1/80 (H7N7), as described previously (Soda *et al.*, 2008b). The obtained viruses contained the same HA as the parental strain A/duck/Hokkaido/77 (H3N2) and a different NA from the other strains. In addition, we used the homosubtype strain A/Aichi/2/68 (H3N2), which is also available from the OIE Reference Laboratory for Avian Influenza at Hokkaido University, and has a deduced amino acid sequence with 97 % HA identity and 92 % NA identity to A/duck/Hokkaido/77 (H3N2).

**Virus titration.** Ten-fold serially diluted viruses in 200 μl minimal essential medium (MEM) with 0.1 % BSA were applied to confluent monolayers of MDCK cells in six-well plates and incubated at 35 °C for 1 h. Unbound viruses were removed and the cells were washed with MEM. The cells were then overlaid with 2 ml virus growth

medium containing 0.8 % agarose (Sigma) and 0.8 μg crystal trypsin $ml^{-1}$ (Sigma). After 72 h incubation at 35 °C, the cells were fixed with 10 % formaldehyde, and stained with 0.1 % crystal violet solution. Plaques were counted and expressed as p.f.u.

**Mouse adaptation of viruses.** To generate the challenge strains for protection experiments, A/duck/Hokkaido/77 (H3N2) and A/Aichi/2/68 (H3N2) strains that were pathogenic in mice were generated as described in our previous report (Haredy *et al.*, 2013). Briefly, mice were intranasally infected with virus ($10^8$ p.f.u. per mouse) and their lungs were harvested 3 days later. The lung tissue was homogenized in 1 ml PBS using a Multi-beads Shocker (Yasui Kikai), spun at 10 000 ***g*** for 10 min and the supernatant was used for a second infection. These steps were repeated for a total of 10 times. The mouse-adapted viruses were then grown once in MDCK cells and the $LD_{50}$ for the viruses in mice was assessed.

**Influenza vaccines.** The seed viruses H3N2, H3N4, H3N5 and H3N7 were grown for vaccine production using the BelloCell cell culture system (CESCO Bioengineering), and the WV vaccines after formalin inactivation were prepared as described previously (Haredy *et al.*, 2013). The vaccine concentrations were adjusted to 1, 0.1 or 0.01 μg per 20 μl dose.

**HI antibody test.** Six-week-old female BALB/c mice (Japan SLC) were divided into 12 groups ($n$=5 per group) for treatment with the four types of vaccine, each at three concentrations. Each mouse was immunized intranasally with 20 μl vaccine (1, 0.1 or 0.01 μg per dose). In addition, a control group of five mice was given PBS intranasally (20 μl per mouse). Immunization was performed twice with a 3 week interval between each dose. Two weeks after the second immunization, the serum was collected from each mouse. Each serum sample was treated with receptor-destroying enzyme from *Vibrio cholera* (Denka-Seiken) according to the supplier's protocol, and the HI antibody titre against both A/duck/Hokkaido/77 (H3N2) and A/Aichi/2/68 (H3N2) was determined using 0.75 % guinea pig erythrocytes by a standard method (Cottey *et al.*, 2001).

**Virus challenge.** Six-week-old female BALB/c mice (Japan SLC) were divided into 12 groups ($n$=5 per group) and immunized intranasally with 20 μl per mouse of vaccine (1, 0.1 or 0.01 μg per dose) as described above. In addition, a control group of five mice was given PBS intranasally (20 μl per mouse). Immunization was performed twice as described above. Two weeks after the final immunization, the vaccine-immunized and PBS-administered mice were challenged via the intranasal route with 100 $LD_{50}$ of mouse-adapted A/duck/Hokkaido/77 (H3N2) ($10^6$ p.f.u. per mouse). The mortality and weight loss of the mice were assessed daily for up to 14 days thereafter. To confirm the results of the challenge by the parent strain, the challenge experiment was repeated for the all vaccine groups against 100 $LD_{50}$ of the mouse-adapted homosubtype strain A/Aichi/2/68 (H3N2) ($10^6$ p.f.u. per mouse). All of the challenge experiments for either H3N2 strain were performed twice independently.

**Virus clearance.** Six-week-old female BALB/c mice were grouped and immunized as described for the HI antibody experiment. Two weeks after the final immunization, the mice were infected intranasally with 100 $LD_{50}$ influenza virus A/duck/Hokkaido/77 (H3N2) or A/Aichi/2/68 (H3N2). Three days later, the lungs from each mouse were excised and homogenized using a Multi-beads Shocker as described above for the mouse adaptation of viruses. The viral titre in the lung homogenate of each mouse was estimated using a plaque assay as described above. All the experiments assessing protection against either H3N2 strain were performed twice independently.

The Institutional Animal Care and Use Committee of the National Institute of Biomedical Innovation (approval number DS21-21) authorized this animal experiment and all the experiments were performed according to the guidelines of this committee.

**NAI activity assay.** The NAI activity was measured in the sera of 0.1 and 1 μg vaccine-immunized mice, and of PBS-treated mice. The NAI was also measured in the sera we had in stock from 1 μg A/Aichi/2/68 (H3N2) intranasally immunized mice. The NA activity was assessed using the NA-Star assay kit (Applied Biosystems), according to the manufacturer's instructions. Suitable amounts of virus were pre-incubated with serum samples diluted in the NA assay buffer at 37 °C for 2 h and then incubated with the NA-Star substrate for 30 min. After the addition of NA-Star accelerator, the chemiluminescent signals were measured at a rate of 1 s per well using a Wallac 1420 ARVOMX (PerkinElmer Japan). The relative NAI activity was determined as the mean per cent inhibition for five wells.

**Statistical evaluations.** Statistical analyses were performed using Microsoft Excel 2013 software. Fisher's exact test was used to evaluate the differences between groups in the mortality experiments. To analyse the data in other experiments, a standard non-paired Student's $t$-test was used. A $P$ value of $<0.01$ was considered significant.

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