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.

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...
et al. (Black 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 1:8 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 LD50 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...
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.

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**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 LD₅₀ 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.
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 $\geq 1:40$ 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. 3.** Virus clearance in the lungs of immunized mice. Mice were immunized with 1 or 0.1 $\mu$g 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.
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 VW 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 mgL-glutamine ml⁻¹ and 8 µg gentamicin ml⁻¹. The cells were maintained at 37 °C in 5% CO₂ (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 (10⁶ 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 LD₅₀ 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 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 NA 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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NA gene homology impact on influenza vaccine


