The Journal of Microbiology (2012) Vol. 50, No. 3, pp. 478–488
DOI 10.1007/s12275-012-1573-z

Evaluation of the Efficacy of a Pre-pandemic H5N1 Vaccine (MG1109) in Mouse and Ferret Models

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(Received November 15, 2011 / Accepted March 9, 2012)

The threat of a highly pathogenic avian influenza (HPAI) H5N1 virus causing the next pandemic remains a major concern. In this study, we evaluated the immunogenicity and efficacy of an inactivated whole-virus H5N1 pre-pandemic vaccine (MG1109) formulated by Green Cross Co., Ltd containing the hemagglutinin (HA) and neuraminidase (NA) genes of the clade 1 A/Vietnam/1194/04 virus in the backbone of A/Puerto Rico/8/34 (RgVietNam/04xPR8/34). Administration of the MG1109 vaccine (2-doses) in mice and ferrets elicited high HI and SN titers in a dose-dependent manner against the homologous (RgVietNam/04xPR8/34) and various heterologous H5N1 strains, (RgKor/W149/06xPR8/34, RgCambodia/04xPR8/34, RgGuangxi/05xPR8/34), including a heterosubtypic H5N2 (A/Aquatic bird/orea/W81/05) virus. However, efficient cross-reactivity was not observed against heterosubtypic H9N2 (A/Ck/Korea/H0802/08) and H1N1 (PR/8/34) viruses. Mice immunized with 1.9 μg HA/dose of MG1109 were completely protected from lethal challenge with heterologous wild-type HPAI H5N1 A/EM/Korea/W149/06 (clade 2.2) and mouse-adapted H5N2 viruses. Furthermore, ferrets administered at least 3.8 μg HA/dose efficiently suppressed virus growth in the upper respiratory tract and lungs. Vaccinated mice and ferrets also demonstrated attenuation of clinical disease signs and limited virus spread to other organs. Thus, this vaccine provided immunogenic responses in mouse and ferret models even against challenge with heterologous HPAI H5N1 and H5N2 viruses. Since the specific strain of HPAI H5N1 virus that would potentially cause the next outbreak is unknown, pre-pandemic vaccine preparation that could provide cross-protection against various H5 strains could be a useful approach in the selection of promising candidate vaccines in the future.

Keywords: HPAI H5N1, pre-pandemic vaccine, cross-reactivity, ferrets

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Introduction

In recent years, the next pandemic threat has been posed by highly pathogenic avian influenza (HPAI) H5N1 viruses continuously isolated in wild birds and domestic poultry since 1996 (Sims *et al*., 2005). The HPAI H5N1 virus strains cause high mortality, resulting in huge economic losses to the poultry industry (Shortridge, 1999; World Health Organization, 2008). After being first identified in Southeast Asia, the H5N1 virus spread rapidly across Eurasia and Africa (Sabirovic *et al*., 2006; De *et al*., 2007). Notably in Asia, these viruses have also been found infecting pig populations in China, Indonesia, and Vietnam (Choi *et al*., 2005; Shi *et al*., 2008; Nidom *et al*., 2010). The spread of H5N1 was not only confined to animals, but was also found among humans, causing more than 320 human deaths worldwide. Infections were reported from Azerbaijan, Cambodia, China, Egypt, Indonesia, Iraq, Laos People's Democratic Republic, Nigeria, Pakistan, Thailand, Turkey, and Vietnam, with a case fatality rate of approximately 59% (World Health Organization, 2011). While the human fatality rate is quite high, human-to-human transmission of the H5N1 virus may not happen efficiently unless the virus mutates, or exchanges genes with a circulating human influenza virus (Subbarao and Kuke, 2007). Even so, the high mortality rate of H5N1 is an important public health concern, prompting the need to develop various approaches for dealing with H5N1 infections in humans.

Eradication of HPAI H5N1 virus-infected poultry is a primary but temporary measure for curbing human exposure. However, ducks and geese in nature are asymptomatically infected with these avian influenza viruses, making it impossible to completely avoid contact (Chin *et al*., 2002; Webster *et al*., 2002; Subbarao *et al*., 2003). Thus, active surveillance and monitoring of HPAI viruses and the development of HPAI vaccines are considered to be the optimal strategies for the prevention and control of influenza pandemics. In the case of a possible influenza pandemic in humans, stockpiling of an effective influenza vaccine is urgently needed. Because pandemics have the potential to spread rapidly through human populations across a large region,

it is crucial for a candidate pandemic vaccine to be made available to the public on short notice. In addition, cross-protectiveness of the pandemic vaccine is equally important since influenza viruses readily undergo antigenic drift and shift due to an error-prone RNA polymerase activity, or re-assortment, producing escape mutants that may be resistant to subtype specific vaccines. Dating back to 2000, more than 10 phylogentic clades of H5N1 viruses had already evolved based on their H5 HA genes (Smith *et al*., 2006; Yamada *et al*., 2006; Salzberg *et al*., 2007; World Health Organization, 2011). Thus, the immunity conferred by a vaccine should be effective against heterologous viruses that are antigenically different from the original vaccine strain (Govorkova *et al*., 2006). Through the years, the use of reverse-genetics technology has permitted the production of vaccine strains that have the advantage of rapid vaccine preparation (Schickli *et al*., 2001; Hoffmann *et al*., 2002) and have shown the ability to provide cross-protection by inducing high anti-HA antibodies in ferrets immunized by reverse-genetics vaccines (Govorkova *et al*., 2006; Lipatov *et al*., 2006). Attempts to use various virus strains, dosages, different types and amounts of adjuvant in reverse-genetics vaccines are done continuously. In this study, a reverse-genetics vaccine (MG1109), which contains the HA and NA genes of a clade 1 (A/VietNam/1194/04) highly pathogenic H5N1 virus in the internal backbone of A/PR/8/34 (PR8) (H1N1), was investigated for its efficacy. The vaccine was prepared with a WHO recommended pre-pandemic vaccine strain (A/VietNam/1194/04, clade 1) (Murakami *et al*., 2008) intended for human use in a future unprecedented event. We previously demonstrated that the high HI titer could afford cross-clade protectivity of H5N1 (lethality, viral titers, and virus shedding) in vaccinated hosts using lab-generated H5N1 (RgKoreaW149/06xPR8, clade 2.2 and RgKoreaES223N/03XPR8, clade 2.5) vaccine strains (Song *et al*., 2009a). The use of mammalian models has been essential in the pre-clinical screening of such pre-pandemic vaccines to examine initially vaccine efficacy and safety, prior to conducting clinical trials in human subjects. Therefore, mice and ferret models were used to evaluate the potency of the MG1109 vaccine for protecting against a challenge of a heterologous clade 2.2 HPAI H5N1 isolated from fecal samples of wild birds in South Korea and a heterosubtypic H5N2 virus that became virulent in mice after serial lung-to-lung passage (Song *et al*., 2009b). Results indicate that high HI titers due to pre-pandemic vaccination in mice and ferrets not only provided immunity in the animal models, but at the same time inhibited virus shedding through the nasal route and viral replication in major target organs, suggesting a positive correlation between induced HI titers from the vaccine and adequate immunization methods.

Materials and Methods

Viruses

The HPAI H5N1 (A/Environment/Korea/W149/06) and mouse-adapted H5N2 (A/Aquatic bird/Korea/ma81/07) viruses used in this study for an animal challenge experiment were isolated from fecal specimens of wild birds (Lee *et al*., 2008; Song *et al*., 2009b) (Table 1). Viruses were serially diluted 10-fold and inoculated in 10-day-old embryonated chicken eggs and the titers were calculated as $\log_{10}$ EID_{50} per milliliter ($\log_{10}$ EID_{50}/ml) (Reed and Muench, 1938). Stock viruses were kept at -80°C and thawed right before use. Mouse 50% lethal dose (MLD_{50}) was defined as the EID_{50} resulting in 50% mortality, calculated by the method of Reed and Muench (1938). All usage of the H5N1 and H5N2 viruses, both *in vitro* and *in vivo*, was conducted using approved biosafety conditions within a level 3 (BSL-3+) facility. To confirm cross-reactivities, A/Ck/Korea/SH0802/08 (SH0802) (H9N2) virus collected from slaughterhouses (Park *et al*., 2011) and A/Aquatic bird/Korea/W81/05 (W81) (H5N2) virus from a wild aquatic bird (Song *et al*., 2009b) were also included in the serologic assays.

Construction of plasmids and reverse genetics

The reverse-genetics RNA transcription vector, pHW2000, and eight plasmid constructs containing the genomic cDNAs of influenza PR8 (H1N1) were kindly provided by Dr. Robert G. Webster. The RgVietNam/04xPR8/34 (MG1109) reassortant virus antigen was obtained from the National Institute for Biological Standards and Control (NIBSC, UK). The RgKor/W149/06xPR8/34, RgGuangxi/05xPR8/34, and RgCambodia/04xPR8/34 reassortant viruses containing the HA and NA genes of A/EM/Korea/W149/06 (H5N1), A/Guangxi/1/05 (H5N1) or A/Ck/Cambodia/7/04 (H5N1) were generated, respectively, in the background of PR8 (H1N1) by plasmid-based reverse genetics as described by Song *et al*. (2009a). The HA and NA genes of A/Guangxi/1/05 and A/Ck/Cambodia/7/04 were synthesized based on GenBank

Table 1. Virus strains to confirm cross reactivity against MG1109[a]

Original Virus	Subtype	Clade	Abbreviation	Polybasic cleavage site	Genotype
A/Puerto Rico/8/34	H1N1		PR8	none	Wild-type
A/Ab/Korea/W81/05	H5N2		W81	none	Wild-type
A/Ck/Korea/SH0802/08	H9N2		SH0802	none	Wild-type
A/Viet Nam/1194/04	H5N1	1	RgVietNam/04xPR8/34	Deleted	VietNam/04 + PR8/34[b]
A/Ck/Cambodia/7/04	H5N1	1	RgCambodia/04xPR8/34	Deleted	Cambodia/04 + PR8/34[b]
A/Guangxi/1/05	H5N1	2.3.4	RgGuangxi/05xPR8/34	Deleted	Guangxi/05 + PR8/34[b]
A/Em/Korea/W149/06	H5N1	2.2	RgKor/W149/06xPR8/34	Deleted	Kor/W149/06 + PR8/34[b]

[a] MG1109 is the vaccine derived from inactivated and purified RgVietNam/04xPR8/34 viral antigen.
[b] Created by reverse-genetics method recombining the surface genes (HA and NA) of the virus and the internal genes from PR8/34 as its genetic backbone. Ab, aquatic bird; Ck, chicken; Em, environment.

data sequences and were confirmed through re-sequencing at Cosmo Genetech (Korea). The HA protein sequences were modified so as to remove the polybasic amino acid motif at the proteolytic cleavage site of the H5 viruses, creating the avirulent-type as described elsewhere (Horimoto *et al.*, 2006; Murakami *et al.*, 2008). The 3 reverse-genetic (Rg) H5N1 reassortant viruses (RgKor/W149/06xPR8/34, RgGuangxi/05xPR8/34, and RgCambodia/04xPR8/34) were used for the serological assays (Table 1).

Vaccine generation

The mock-up influenza vaccine virus (MG1109) was propagated in 11 day-old-embryonic eggs at 37°C for 72 h. The allantoic fluid containing viruses was harvested and concentrated to 1/10 of the original volume using a molecular cut-off concentrator apparatus. The concentrated viruses were purified by sucrose density gradient centrifugation. The purified vaccine viruses were inactivated with formalin. The inactivation of vaccine viruses was confirmed by absence of virus growth in 2 consecutive passages in eggs.

The HA protein content in the purified vaccine bulk was determined by the standard single radial immunodiffusion (SRID) technique, using standard sera and antigens obtained from the National Institute for Biological Standards and Control (Hertfordshire, UK). After dilution of the vaccine bulk to appropriate antigen concentration, antigen was adsorbed to aluminum hydroxide as adjuvant at 1 mg/ml Alum.

Vaccination and virus challenge

Four-week-old BALB/c mice were purchased from Samtako (Korea) and 15–16-week-old ferrets (*Mustela putorius furo*) were purchased from Marshall Co. Ltd. (USA). All animals had tested seronegative against H1N1-like, H9N2-like, and H5-like viruses by hemagglutination inhibition (HI) assays prior to this study (Palmer *et al.*, 1975).

Groups of mice (12 per group) were vaccinated intramuscularly (IM) with 2 doses each of inactivated MG1109 vaccine containing 0.9, 1.9, 3.8, or 7.5 μg/dose of HA containing 250 μg of aluminum hydroxide adjuvant in 0.25 ml of sterile phosphate-buffered saline (PBS), administered 3 weeks apart. Two weeks after the last immunization, mice were intranasally (i.n.) challenged with 100 MLD_{50} of the wild-type A/EM/Korea/W149/06 (H5N1) and A/Ab/Korea/ma81/07 (H5N2) viruses. The control group received 250 μg of aluminum hydroxide adjuvant only in 0.25 ml of sterile PBS (Song *et al.*, 2009a). The kinetics of survival was analyzed by Kaplan-Meier curves using the Prism 5.0 program.

Groups of ferrets (6 per group) were vaccinated intramuscularly with 2 doses each of the inactivated vaccine containing 1.9, 3.8, 7.5, or 15 μg/dose of HA with aluminum hydroxide adjuvant, administered 3 weeks apart. Virus challenge with 6.5 $\log_{10}$ EID_{50} of A/EM/Korea/W149/06 was performed by administering the virus i.n. 2 weeks after the last immunization. The control group received 500 μg/0.5 ml of aluminum hydroxide adjuvant (Song *et al.*, 2009a).

Sera, nasal wash sample, and tissue collection

Sera from mice and ferrets were collected 2 weeks after each vaccine administration and stored at -80°C until use. Lung tissues of mice [at 1, 3, 5, and 7 days post-infection (dpi)], and lung, kidney, and spleen tissues of ferrets (at 2 and 5 dpi) were harvested and homogenized with equal

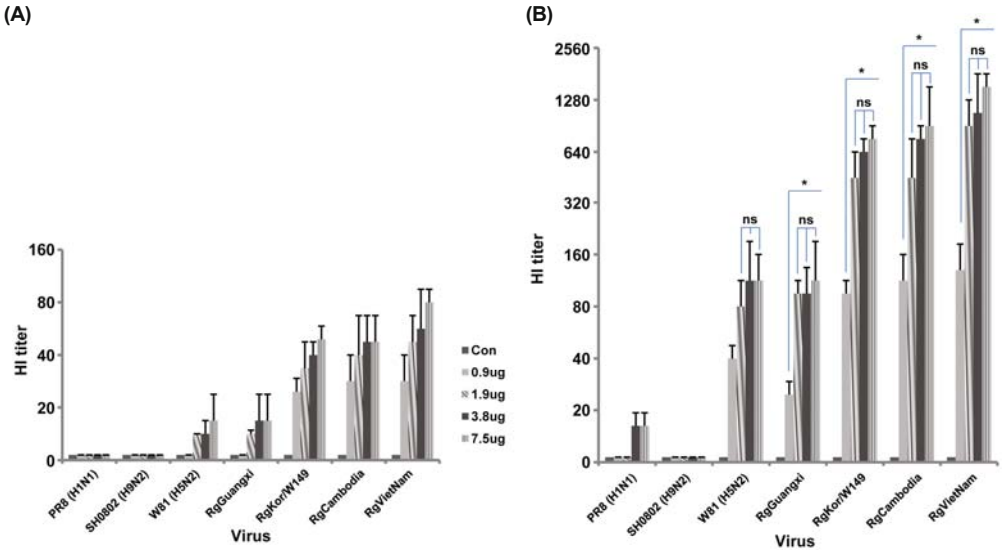

Fig. 1. Serum antibody response in mice administered different doses of the MG1109 pre-pandemic H5N1 vaccine. Differences of mean hemagglutinin inhibition (HI) titers induced in mice immunized once (A) or twice (B) with various doses (0.9 μg, 1.9 μg, 3.8 μg, or 7.5 μg HA antigen) and frequency of MG1109. Two weeks after each vaccination, the collected sera from the vaccinated mice were used to perform HI assays against wild-type SH0802 (H9N2), W81 (H5N2), PR8 (H1N1) or reverse-genetics recombinant H5N1 viruses [RgVietNam/04xPR8/34 (RgVietNam), RgCambodia/04xPR8/34 (RgCambodia), RgGuangxi/05xPR8/34 (RgGuangxi), RgKor/W149/06xPR8/34 (RgKor/W149)]. Data are mean titers±standard deviation. *p<0.05 for comparison between 0.9 μg group and bar-indicated groups. Abbreviation, ns; not significant.

volumes (1 ml/g tissue) of PBS containing antibiotics. Tissue homogenates were clarified by centrifugation at 12,000×*g* for 10 m at 4°C and then supernatants were transferred to new tubes. Ferret nasal washes were collected with 1 ml PBS containing antibiotics at 2 and 5 days after virus challenge. Samples were 10-fold serially diluted immediately after collection and then inoculated into 11-day-old embryonated chicken eggs for virus titration as computed by the Reed and Muench method, with the results expressed as $\log_{10}$ EID_{50}/ml or per g of tissue ($\log_{10}$ EID_{50}/g) collected (Reed and Muench, 1938). The limit of virus detection was set at <0.7 $\log_{10}EID_{50}$/ml or $\log_{10}$ EID_{50}/g and the virus titers were compared by standard student *t*-test. Hematoxylin and eosin (HE) staining of lung samples of ferrets was done as described elsewhere (Song *et al*., 2009b).

Serologic assays

HI assays were done as described elsewhere (Lu *et al*., 1999). Briefly, serum samples were treated with receptor-destroying enzyme (RDE, Denka Seiken, Japan) to inactivate non-specific inhibitors with a final serum dilution of 1:10. RDE-treated sera were 2-fold serially diluted and equal volumes of virus (8 HA units/50 µl) were added to each well. The microplates were incubated at room temperature for 30 min followed by the addition of 0.5% (v/v) chicken red blood cells (RBCs). The plates were gently mixed and incubated at 37°C for 30 min. The HI titer was determined by the reciprocal of the last dilution that did not exhibit agglutination of the chicken RBC. The detection limit for the HI assay was set to <20 HI units.

The serum neutralizing (SN) assay was performed as previously described, with modifications, to determine cross reactivity of the collected sera from mice and ferrets (Palmer *et al*., 1975; Benne *et al*., 1994). Viruses used for the SN assay were diluted from virus stock solutions at a titer between 100–300 $TCID_{50}$/0.1 ml. Initial serum dilutions of 1:10 were made using PBS. Two-fold serial dilutions of all samples were made to a final serum dilution of 1:10,240. To each serum dilution, 50 µl of 100–300 $TCID_{50}$/0.1 ml of virus was added and incubated for 1 h at 37°C in 5% CO_2. Following incubation, the virus and serum mixtures were added to 96-well tissue culture plates containing confluent MDCK cell monolayers (~1.5×10^4 cells/well) and incubated for 48 h at 37°C in 5% CO_2. After infection, the plates were monitored for the appearance of any cytopathic effect (CPE). Viral replication in the supernatant of each well was confirmed by a hemagglutination test.

Statistical analysis

The Student's *t* test (two-tailed, unpaired) was used to determine the significance of differences between two sets of values and the log rank (Mantel-Cox) test was used to determine the *p*-value of survival calculated by the Prism 5.0 program (GraphPad).

Results

Immunogenicity of MG1109 in mice and ferrets

To test the immunogenicity of the MG1109 pre-pandemic H5N1 vaccine, groups of 4-week-old mice were IM vaccinated with 0.9, 1.9, 3.8, and 7.5 µg/0.25 ml dose of the HA antigen containing aluminum hydroxide-adjuvant or mock-vaccinated with the adjuvant alone, twice, 3 weeks apart. Two weeks after each vaccine administration, mouse sera were obtained to determine mean basal HI titers against viruses tested, as listed in Table 1. Single administration of the different dose preparations of the MG1109 vaccine in-

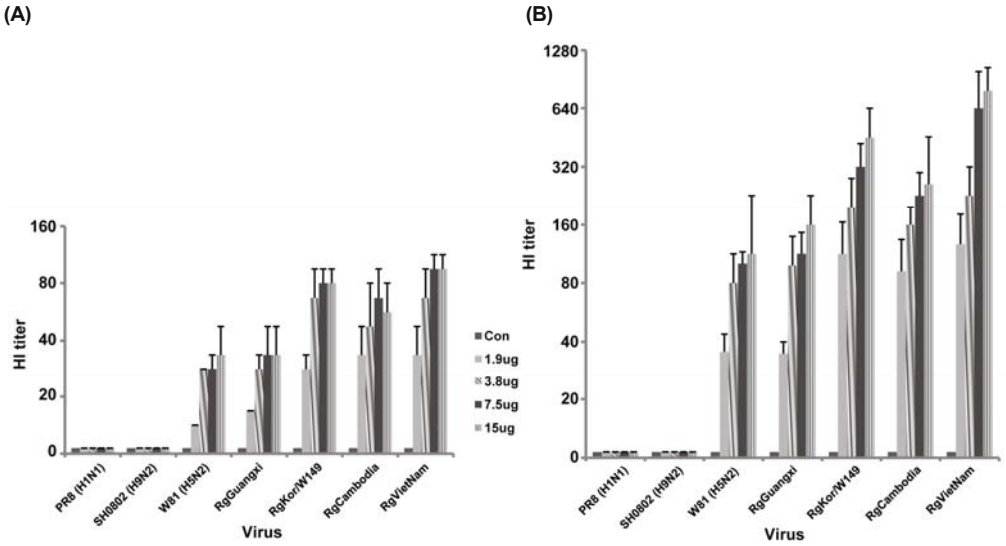

Fig. 2. Serum antibody response in ferrets administered different doses of the MG1109 pre-pandemic H5N1 vaccine. Differences in mean HI titers induced in ferrets immunized with various dosages of MG1109. Two weeks after each immunization (A, Single; B, Double), the collected sera from the vaccinated ferrets were used to perform the HI test against wild-type SH0802 (H9N2), W81 (H5N2), PR8 (H1N1) or reverse-genetics recombinant H5N1 viruses [RgVietNam/04xPR8/34 (RgVietNam), RgCambodia/04xPR8/34 (RgCambodia), RgGuangxi/05xPR8/34 (RgGuangxi), RgKor/W149/06xPR8/34 (RgKor/W149)]. Data are mean titers±standard deviation.

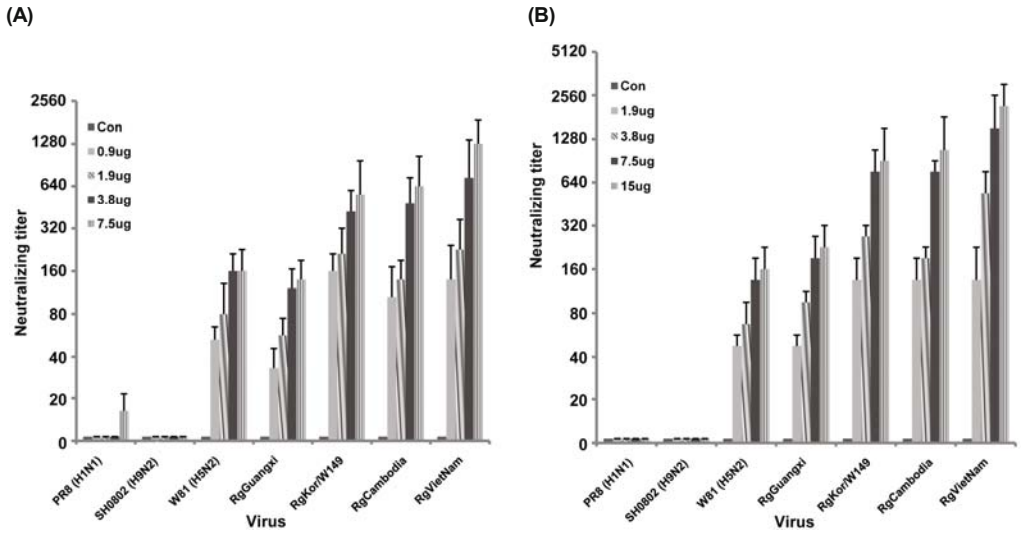

Fig. 3. Neutralization assay for serum collected from MG1109-vaccinated hosts. Differences of mean serum neutralizing titers induced in mice (A) and ferrets (B) immunized twice with the various doses of MG1109. Two weeks after the last vaccination, the collected sera from the vaccinated hosts were used to determine *in vitro* micro neutralization of the pre-selected viruses in MDCK cells. Data are mean titers±standard deviation.

duced modest antibody titers against most of the H5 viruses used but not against the heterosubtypic PR8 (H1N1) or SH0802 (H9N2) virus (Fig. 1A). Homologous virus reactivity (RgVietNam/04xPR8/34) demonstrated the highest antibody titers, reaching up to 80 HI units at 7.5 μg/dose but was only about 30 HI titers at the lowest concentration (0.9 μg/dose of HA). Comparable cross-reactivities were observed against a clade 2.2 virus (RgKor/W149/06xPR8/34) and a clade 1 virus (RgCambodia/04xPR8/34) H5N1, with slightly over 40 HI titers at the maximum antigen dose. In contrast, none of the vaccine doses could induce HI titers beyond the positive limit of detection (≤20 HI titers) against RgGuangxi/05xPR8/34 (H5N1, clade 2.3.4) and heterosubtypic W81 (H5N2). Interestingly, serum samples collected after the second vaccination efficiently enhanced antibody titers against the clade 1 (RgVietNam/04xPR8/34 and RgCambodia/04xPR8/34) and clade 2.2 (RgKor/W149/06xPR8/34) H5N1 viruses, except for the clade 2.3.4 (RgGuangxi/05xPR8/34) virus, most notably among groups that received 1.9, 3.8, and 7.5 μg/dose of HA (Fig. 1B). Induction of serologic responses in these vaccine groups ranged from 320–1280 HI units. Moderate increases in antibody response were also noted against RgGuangxi/05xPR8/34 and the heterosubtypic W81 (H5N2) (up to 80 HI units). Similar to sera collected after a single vaccination, cross-reactivity against PR8 (H1N1) or SH0802 (H9N2) did not exceed the pre-set positive cut-off titer (<20 HI units) in any of the double-vaccinated groups. Mock-vaccinated mice had no detectable HI titers in all groups tested (data not shown). Although the HI titers appeared to have a dose-dependent correlation with respect to the antigen administered, particularly in immunized groups from 0.9 to 1.9 μg of HA, no substantial advantage for cross-reactivity was observed in higher vaccine doses (>1.9 μg HA) in mice.

Serologic antibody response elicited by the MG1109 vaccine was also measured in ferrets receiving one and two doses of 1.9, 3.8, 7.5, or 15 μg of HA. Ferret sera were collected from vaccinated hosts two weeks after each administration to determine the mean HI titers elicited against the pre-selected viruses as used above. Similar to results obtained for mouse vaccinations, increased quantities of antigen induced elevated HI titers against homologous or heterologous H5N1 viruses as well as the heterosubtypic W81 (H5N2) virus, but not against the PR8 (H1N1) or the SH0802 (H9N2) virus (Fig. 2). At single doses, about 40 to 80 HI units were induced against the RgKor/W149/06xPR8/34,

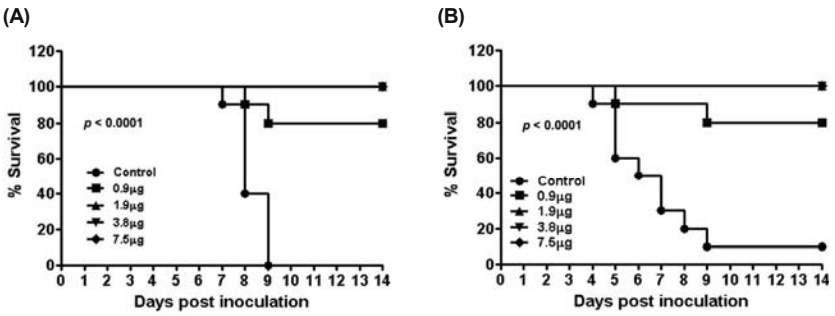

Fig. 4. Survival test of MG1109-vaccinated mice. Efficacy of the vaccination was verified by survival rate. Groups of 10, 5-week-old, Balb/c mice were inoculated intramuscularly and challenged with a wild-type HPAI H5N1 A/EM/Korea/W149/06 (100 MLD_{50}) (A) and mouse adapted A/Ab/Korea/ma81/07 (100 MLD_{50}) (B) influenza viruses.

Table 2. Clinical signs of influenza infection observed in MG1109–vaccinated mice[a]

Group No. (Antigen content)	Clinical signs	Mobility
1. Alum only (250 μg)	5–8 DPI : Ruffled fur,	5 DPI : Low
	Labored breathing	6–8 DPI : Very low
	9 DPI : All died	9 DPI : All died
2. 0.9 μg HA/dose + 250 μg Alum	3–9 DPI : Ruffled fur	3–9 DPI : Low
3. 1.9 μg HA/dose + 250 μg Alum	3–5 DPI : Ruffled fur	3–5 DPI : Low
4. 3.8 μg HA/dose + 250 μg Alum	3–5 DPI : Ruffled fur	3–5 DPI : Low
5. 7.5 μg HA/dose + 250 μg Alum	3–4 DPI : Ruffled fur	3–4 DPI : Low

[a] Groups of mice were intranasally challenged with 100 MLD_{50} of the wild-type A/EM/Korea/W149/06 HPAI H5N1 virus two weeks after the last immunization. Clinical signs of mice were monitored daily for 14 days after challenge.

RgCambodia/04xPR8/34 and RgVietNam/04xPR8/34 viruses starting from 3.8 μg HA, whereas none of the antigens administered could elicit higher than 40 HI titers against the RgGuangxi/05xPR8/34 (H5N1) or W81 (H5N2) virus (Fig. 2A). After the second immunization, all vaccine groups had substantially elevated cross-reactivity against the RgKor/W149/06xPR8/34, RgCambodia/04xPR8/34 and RgVietNam/04xPR8/34 viruses (> 80 HI titers) (Fig. 2B). Accordingly, at least 3.8 μg of HA was sufficient to achieve about 80-160 mean HI titers against the clade 2.3.4 H5N1 (RgGuangxi/05xPR8/34) virus and heterosubtypic W81 (H5N2) virus but the lowest dose of the antigen (1.9 μg) could not elicit more than 40 HI units. Mock-vaccinated ferrets had no detectable HI titers in all groups (data not shown). These data illustrate that two doses of the adjuvanted MG1109 vaccine containing 3.8 μg HA might be needed to induce cross-reactive HI titers in ferrets. Taken together with the mouse experiments, MG1109 is cross-reactive to the panel of H5N1 viruses belonging to different clades that also extended to the heterosubtypic H5N2 (W81) avian influenza virus used

Table 3. Clinical signs observed in MG1109-vaccinated ferrets[a]

Group	Antigen	Days post infection	Clinical sign[b]	Mobility[c]
1	500 μg Alum	0	0	0
		1	1,2	0
		2	1,2	1
		3	1,2	1
		4	1,2,3,4	1
		5	1,2,3,4	1
2	1.9 μg HA/0.5 ml + 500 μg Alum	0	0	0
		1	1	0
		2	1,2	1
		3	1,2	1
		4	1	1
		5	0	0
3	3.8 μg HA/0.5 ml + 500 μg Alum	0	0	0
		1	1	0
		2	1,2	1
		3	1	1
		4	0	0
		5	0	0
4	7.5 μg HA/0.5 ml + 500 μg Alum	0	0	0
		1	1	0
		2	1,2	1
		3	1	0
		4	0	0
		5	0	0
5	15 μg HA/0.5 ml + 500 μg Alum	0	0	0
		1	1	0
		2	1,2	1
		3	1,2	0
		4	0	0
		5	0	0

[a] Two weeks after the last immunization, vaccinated ferrets were intranasally challenged with 6.5 $\log_{10}EID_{50}$/0.5 ml of A/EM/Korea/W149/06 HPAI H5N1 virus. Signs of the disease were scored as follows:
[b] Clinical signs: 0, Normal; 1, Increased body temperature; 2, Decreased food intake; 3, Frequent coughing; 4, Nasal congestion.
[c] Mobility: 0, Normal; 1, Decreased mobility.

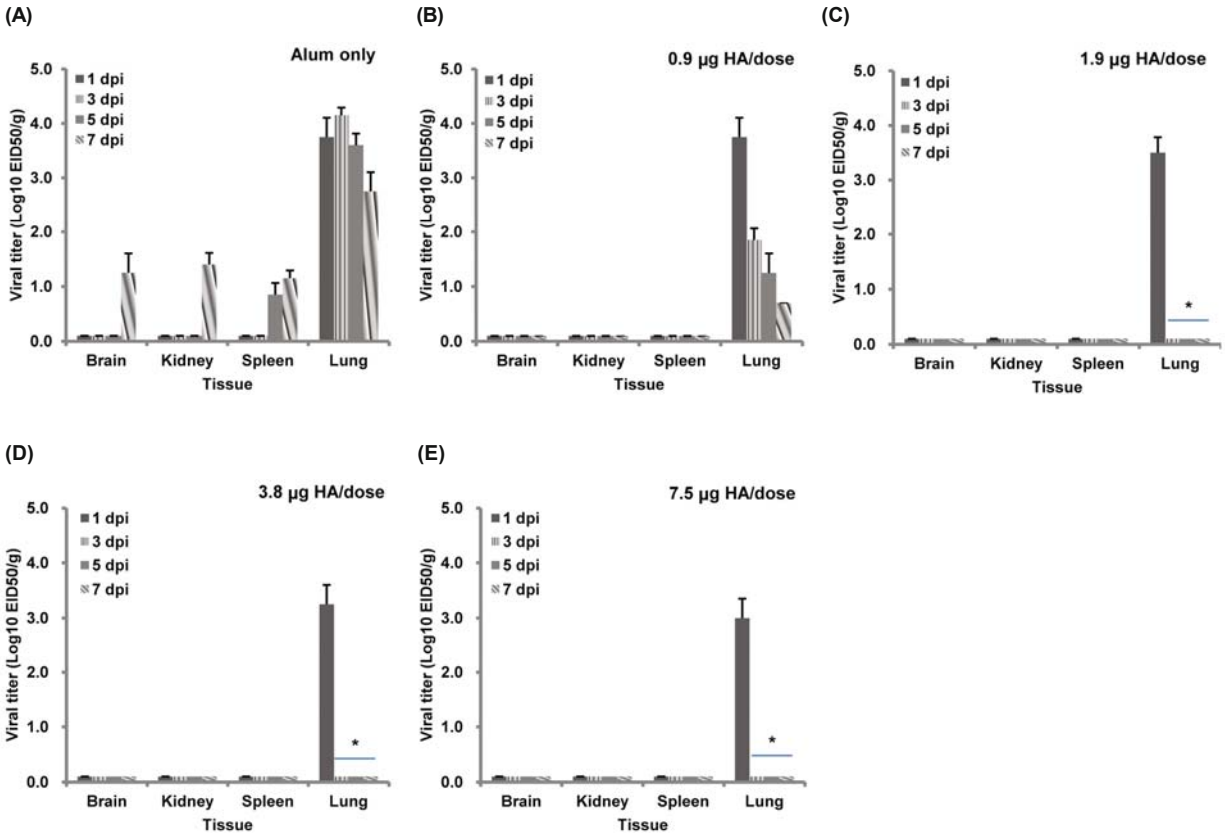

Fig. 5. Virus titration in MG1109-vaccinated mice. After i.n. infection with a lethal dose of HPAI H5N1 (A/EM/Korea/W149/06), brain, kidney, spleen and lung tissues were harvested on 1, 3, 5, and 7 dpi for virus titration by inoculation of 10-day-old embryonated chicken eggs. Viral titers are expressed as $\log_{10}EID_{50}$ per gram of tissue collected according to the method of Reed and Muench (1938). Data are mean titers ± standard deviation. *$p<0.05$ for comparison between lung viral titer of alum only group and various dose groups, respectively.

in this study, after receipt of two vaccine doses of at least 1.9 μg and 3.8 μg/dose of HA in mice and ferrets, respectively.

In vitro virus neutralization of immune sera obtained from MG1109-vaccinated mice and ferrets

Two weeks after two administrations of the different doses of the MG1109 vaccine, the sera obtained from mice or ferrets were tested by serum neutralizing (SN) assays to examine whether the sera can cross-neutralize *in vitro* the various influenza viruses used for testing serologic cross-reactivities above. Notably, marked increases in virus-neutralizing antibody titers (≥320 neutralizing units) were detected in sera obtained from mice and ferrets that received at least 3.8 μg and 7.5 μg HA, respectively, particularly against the clade 1 (RgVietNam/04xPR8/34 and RgCambodia/04xPR8/34), and clade 2.2 (RgKor/W149/06xPR8/34) H5N1 viruses (Fig. 3). It is also interesting to note that the sera from double-vaccinated animal models were able to cross-neutralize the clade 2.3.4 H5N1 RgGuangxi/05xPR8/34 and heterosubtypic W81 (H5N2) (≥ 40 neutralizing units) viruses, albeit to a lower extent relative to the other H5N1 viruses. However, none of the immunized sera could successfully neutralize representative viruses of the H1N1 (PR8) and H9N2 (SH0802) subtypes (Fig. 3). Therefore, cross-neutralization of test viruses appeared to correlate with vaccine-induced cross-reactivity observed in the HI assays (Figs. 1 and 2).

Clinical signs and survival rates of mice and ferrets

Two weeks after booster vaccination, all groups of mice were challenged i.n. with 100 times 50% lethal dose (100 LD_{50}) of a wild-type field isolate A/EM/Korea/W149/06 (H5N1) or a mouse-adapted A/Ab/Korea/ma81/07 (H5N2) virus to evaluate the protective efficacy of the H5N1 prepandemic vaccine in mice. After challenge, MG1109-vaccinated and mock-vaccinated (administered only with the aluminum hydroxide adjuvant in PBS) mice were monitored for morbidity and mortality due to influenza virus infection for 14 days. All mock-vaccinated mice (Group 1) died within 10 dpi of the HPAI H5N1 (Fig. 4A) or virulent mouse-adapted H5N2 (Song *et al.*, 2009b) (Fig. 4B) virus challenge. On the other hand, about 20% of the vaccine group, which received the lowest antigen (Group 2, 0.9 μg HA) succumbed due to infection of either virus. Interestingly, mortality was not observed among vaccine groups administered with at least 1.9 μg HA antigen (Groups 3 to 5). While most of the infected mice exhibited ruffled fur indicative of influenza-like illness, labored breathing was only

evident in the mock-vaccinated group, particularly at 3 days prior to death (Table 2). In addition, groups administered with the lowest dosage of the antigen exhibited less mobility after virus challenge with Group 2, extending to 9 dpi, whereas Groups 3, 4, and 5 appeared to recover more rapidly (Table 2 and Fig. 4).

A similar pattern of influenza-like disease was also observed in vaccinated ferrets during virus challenge with 6.5 $\log_{10}$ EID_{50} of the A/EM/Korea/W149/06 (H5N1) virus. Clinical signs were generally noted in a dose-responsive manner, with the mock-vaccinated group showing more severe disease signs, which included frequent coughing, nasal congestion, and inactivity up to 7 dpi (Table 3). Close monitoring of the body temperatures revealed that ferrets belonging to Groups 3–5 were within normal variation range while those belonging to Groups 1 and 2 showed a 1.5–2°C degree increase coinciding with the aforementioned clinical signs at 2 and 4 dpi. Soon after, however, the body temperature of the ferrets in Group 2 started to normalize and the animals began showing signs of recovery. In contrast, ferrets in Group 1 (mock-vaccinated group) continued to exhibit elevated body temperatures as compared to the other groups, but none died (data not shown)

Viral replication in mice and ferrets after challenge

After evaluating the ability of a pre-pandemic MG1109 vaccine to protect immunized mice and ferrets from challenge with an HPAI H5N1 virus, we additionally examined the ability of the pre-pandemic H5N1 vaccine to suppress virus growth in these hosts. Three mice from each vaccine group were sacrificed at 1, 3, 5, and 7 dpi and their respective lung, kidney, spleen, and brain tissues were harvested for virus titration by inoculation in embryonated chicken eggs. The virus (A/EM/Korea/W149/06) was isolated from mouse lungs, where they persisted up to 7 dpi in mock-vaccinated and the 0.9 μg HA-vaccinated groups. Modest titers were also detected in the brain, kidney and spleen of the alum only (mock) vaccine group (Group 1) (Fig. 5). Although A/EM/Korea/W149/06(H5N1) was detected in the lungs at day 1 pi, in the other vaccine groups, which received higher vaccine doses (≥ 1.9 μg HA), the challenge virus did not show any extra-pulmonary virus replication, indicating suppression of viral spread in mice.

Vaccinated and subsequently infected ferrets were also sacrificed at day 2 and 5 pi (3 animals/day). Nasal washes were obtained prior to euthanizing, and tissue samples (spleen, kidney, and lung) were collected to monitor virus growth in homogenates. Except for the 1.9 μg HA vaccine recipients, relatively low virus titers (≥ 2 $\log_{10}$ EID_{50}/ml reduction) were detected in the upper respiratory tract of immunized ferrets (≥ 3.8 μg HA) at 2 dpi compared to the mock vaccine group, where infection persisted up to 5 dpi (with titers up to 2.6 $\log_{10}$ EID_{50}/ml) as determined in nasal washes (Fig. 6A). Two doses of 1.9 μg HA antigen could not efficiently inhibit virus growth in the upper respiratory tract of ferrets at both the time points tested (2.3 $\log_{10}$ EID_{50}/ml peak titers). Interestingly, animals receiving two doses of the antigen containing 3.8 μg HA or higher did not demonstrate detectable virus titers in nasal washes at 5 dpi. For the collected and processed tissue samples, the control group displayed viral replication at 2 and 5 dpi in the lungs with titers up to 3.2 $\log_{10}$ EID_{50}/g of tissue (Fig. 6B). Although the challenge virus still managed to replicate in lungs of the 1.9 μg HA antigen dose group at 2 dpi, it was already barely detected at 5 dpi (<0.7 $\log_{10}EID_{50}$/g). Infectious virus was also retrieved from spleen and kidney tissue samples of mock-vaccinated group at 2 dpi (titers up to 1.3 $\log_{10}$ EID_{50}/g) but none were recovered from the MG1109-immunized groups in any of the days sampled (Fig. 6B).

Histopathological examination (HE staining) of ferret lung tissue samples prepared at 5 dpi from each group demonstrated severe signs of inflammation and pneumonia in

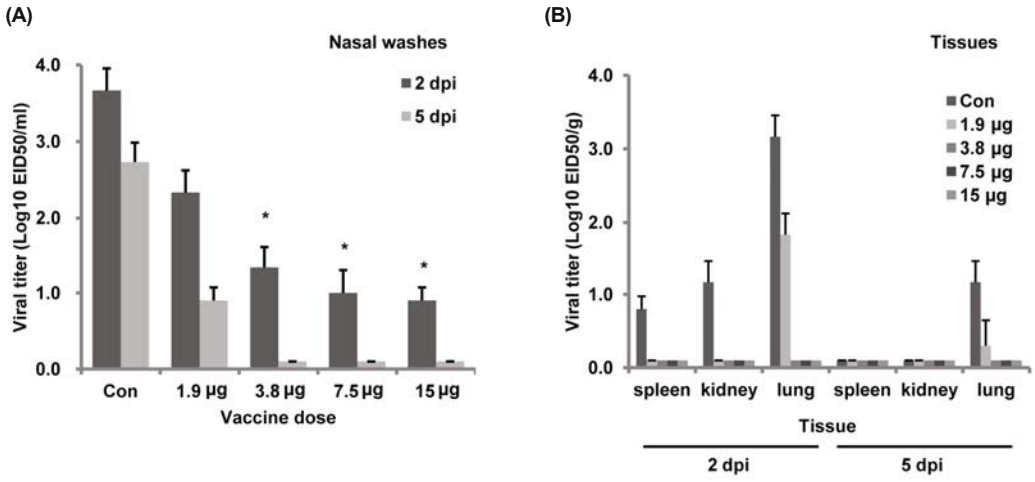

Fig. 6. Virus titration in MG1109-vaccinated ferrets. Nasal washes (A) and various tissue organs (B) were collected day 2 and 5 pi from ferrets infected with the HPAI H5N1 (A/EM/Korea/W149/06) virus. Samples were processed to determine viral titers at the specified time points by egg titration expressed as $\log_{10}EID_{50}$ per ml or per g of tissue collected (Reed and Muench, 1938). Data are mean titers ± standard deviation. *p<0.05 for comparison between viral titer of alum only group and various dose groups at 2 days pi, respectively.

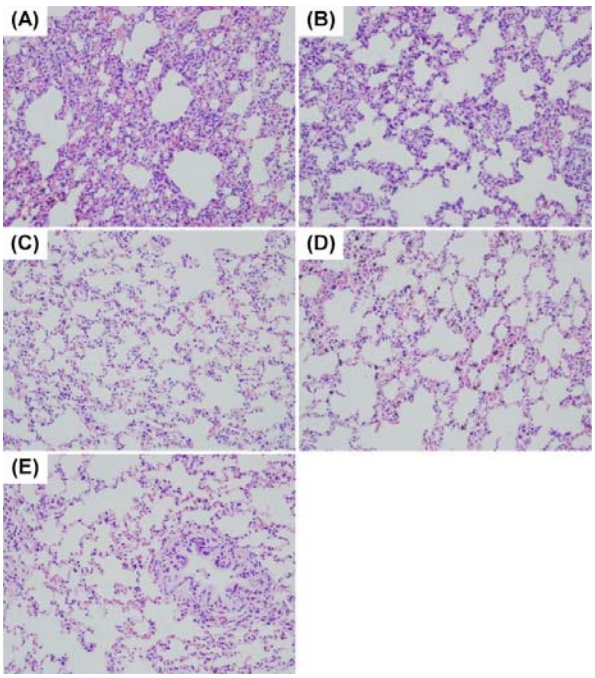

Fig. 7. Histopathological examination of lungs from MG1109-vaccinated ferrets. Lungs from vaccinated and subsequently infected ferrets were collected at 5 dpi and processed for hematoxylin and eosin staining: (A) mock-vaccinated group; (B) 1.9 μg HA antigen group; (C) 3.8 μg HA antigen group; (D) 7.5 μg HA antigen group, and; (E) 15 μg HA antigen group. Magnification 200×.

mock-vaccinated and 1.9 μg HA-vaccinated ferrets. In contrast, groups that received two doses of the antigen, containing 3.8 μg HA or higher, exhibited moderate lung pathology. These observations appear to correlate with the viral replication kinetics and morbidity induced by infection with the A/EM/Korea/W149/06 (H5N1) virus in this host (Fig. 7).

Discussion

The significance of cross-protective pre-pandemic or pandemic vaccines has always been emphasized because pandemic virus strains are difficult to predict and vaccine production is time consuming. Vaccines that can cross-protect against antigenically different H5N1 viruses are desirable, especially when current stockpiling of influenza virus vaccines are still inadequate. Moreover, if a vaccine can also inhibit virus shedding, the proliferation of the virus will be hindered and better controlled among the affected population. With this ideal vaccine in mind, we tried to evaluate the efficacy of MG1109 to provide protection and inhibit virus shedding and spread, even against a heterologous HPAI H5N1 virus challenge in mice and ferrets. These animal models have been very useful in the pre-clinical assessment of the cross-reactivity and protective efficacy of vaccines formulated against HPAI H5N1 viruses. Mice and ferrets were vaccinated with the MG1109 vaccine, derived from RgVietNam/04xPR8/34, at different doses to determine the appropriate dosage sufficient for effective immunization. Mice vaccinated with MG1109 showed elevated levels of mean HI titers in a dose-dependent manner against the selected Rg H5N1 viruses [RgVietNam/04xPR8/34 (clade 1), RgCambodia/04xPR8/34 (clade 1), RgGuangxi/05xPR8/34 (clade 2.3.4), and RgKor/W149/06xPR8/34 (clade 2.2)], including the H5N2 virus (A/AB/Korea/W81/05); appreciable immunogenicity was not observed against the heterosubtypic H9N2 (SH0802) and H1N1 (PR8) viruses indicating specificity of MG1109 to variant H5 viruses. We also noted that HI titer of the vaccinated groups receiving more than 1.9 μg/HA, particularly after the second doses, were not substantially elevated which suggest that at least 1.9 μg/HA doses might be sufficient to elicit cross-reactive antibody titers in mice. Similarly in ferrets, recognized as the most relevant and suitable hosts for influenza virus studies (Govorkova *et al.*, 2006; Hampson, 2006), immunization with various defined doses of HA antigen to raise HI titers, elicited dose-dependent immune responses even against genetically divergent H5 strains used in the present study. SN assays also confirmed the presence of neutralizing antibodies in immune sera from mice and ferrets, corroborating the results obtained with the HI tests. These results suggest the presence of common antigenic epitopes between the LPAI H5 and genetically divergent HPAI H5N1 viruses. In particular, we noted closely related antigenicity between the clade 1 and clade 2.2 viruses. Although there was relatively lower antigenicity against the clade 2.3.4 H5N1 (RgGuangxi/05xPR8/34) and heterosubtypic H5N2 (W81) compared to other H5N1 viruses (40-160 versus 160-1280 HI titers), the observed cross-reactivity of MG1109 still indicates its suitability as a candidate pre-pandemic H5N1 vaccine in the future, considering its potential to provide broad immunity.

The absence of other wild-type H5N1 viruses available for challenge limited our findings to confirm broad cross-protectivity. Remarkably though, we have shown that the MG1109-induced cross-reactivity could protect against lethal challenges with a field clade 2.2 A/EM/Korea/W149/06 H5N1 isolate and a virulent mouse-adapted A/AB/Korea/ma81/07 (H5N2) virus in mice. Two vaccine doses of at least 1.9 μg HA protected mice from dying, minimized morbidity, and suppressed virus replication in the collected tissue samples, particularly in the lungs (Figs. 4 and 5). Thus, these results show the efficacy of the pre-pandemic MG1109 vaccine in mice (at least at 1.9 μg HA antigen dosage) under stringent conditions of lethal infection (100 MLD_{50}), which would otherwise cause disease and certain death. MG1109-vaccinated ferrets were also protected from the HPAI H5N1 virus challenge allowing immunized hosts to recover quickly, with suppression of severe disease signs and reduction of virus replication in the upper respiratory tract. After two vaccine doses containing at least 3.8 μg HA, the HPAI A/EM/Korea/W149/06 (H5N1) virus was not recovered from nasal washes at 5 dpi. We have shown here and previously (Song *et al.*, 2009a) that A/EM/Korea/W149/06 (H5N1) has expanded tissue tropism in naïve ferrets. Although the HPAI H5N1 virus challenge was initially recovered in the lungs of the 1.9 μg HA-immunized group at 2 dpi, it was already barely detected at 5 dpi.

Furthermore, in ferret groups immunized with 3.8 μg HA antigen doses, or more, virus replication was prevented in all the various organs tested, apart from some suppressed viral growth in the upper respiratory tract, which further supports the potential ability of MG1109 to protect against the heterologous HPAI H5N1 virus. The observation that high immune titers elicited by doublevaccination of at least 3.8 μg HA, are consistent with efficient inhibition of virus shedding and systemic viral spread seen previously after experimental inoculation in animal models (Govorkova *et al*., 2006; Suguitan *et al*., 2006; Isoda *et al*., 2008; Mahmood *et al*., 2008; Song *et al*., 2009a). Altogether, the observed vaccine-induced immunogenic titers were supported by the protective effects against the heterologous virus seen in vaccinated, then subsequently infected, mice and ferrets.

Our use of varying doses and frequency of vaccine administration allowed us to determine an appropriate vaccination regimen. We were able to show that 2-dose vaccinations may be an optimal strategy for inducing cross-reactive immune responses against the antigenically drifted H5N1 and perhaps, genetically divergent H5N2 viruses within immunologically naïve populations. We were also able to determine that alum-adjuvanted low-antigen compositions were sufficient to induce cross-reactive antibody titers in mice and ferrets. A previous vaccine efficacy study (Miyaki *et al*., 2010) was reported for the A/VietNam/1194/04 strain. In mice, the H5N1 inactivated whole virus vaccine elicited higher hemagglutination-inhibition (HAI) titers with aluminum hydroxide ($Al(OH)_3$) and monophosphoryl lipid A (MPLA) adjuvants as compared to the same vaccine dose without any adjuvant. Aluminum hydroxide has been most widely used as adjuvant for human vaccines due to its cost-effectiveness and ease of application (Clements and Griffiths, 2002). However, since its usefulness in the context of influenza vaccines for humans are still equivocal (Gherardi *et al*., 2001; Lindblad, 2004), evaluation of other potential adjuvants might be required during vaccine preparation.

There have been various reports on H5N1 pre-pandemic vaccine efficacy from studies using various clades of HPAI H5N1 viruses. For example, Harada *et al*. (2011) reported immunogenicity and protective efficacy of adjuvanted clade 2.3.4 H5N1 pre-pandemic influenza vaccine against clade 1 and 2.2 viruses. In addition, the A/VietNam/1194/04 strain-based adjuvanted vaccine protects against clade 2.1 (A/Indonesia/5/05) of H5N1 influenza viruses in animal models (Baras *et al*., 2008; Bodewes *et al*., 2010). Further, an adjuvanted clade 1 (A/VietNam/1194/04) pre-pandemic vaccine elicited broad clade 2 (2.1, 2.2, and 2.3) cross-reactivity in humans (Leroux-Roels *et al*., 2008). In this regard, the A/VietNam/1194/04-based vaccine (MG1109) has potential as a pre-pandemic vaccine for national stockpiling.

In summary, the MG1109 vaccine in the present study demonstrated cross-reactivity and cross-neutralization against several clades of H5N1 viruses, including a heterosubtypic avian H5N2 isolate. Vaccine-induced sero-reactivity also correlated with protection against virulent heterosubtypic H5N2 (mice) and heterologous HPAI H5N1 (mice and ferrets) virus challenge that limited viral growth in target organs, results which are desirable features of a pre-pandemic vaccine. Although developing technologies and methods that will enable the reduction of administration dose/frequency are still needed, these results may play a role in the assessment and selection of promising vaccine candidates against H5 viruses, with their growing threat to cause the next influenza pandemic.

Acknowledgements

This work was supported by grant 2008-E00311-00 of the KCDC (Korea Center for Disease Control & Prevention), KGCC (Korea Green Cross Co.) and partially by a National Agenda Project grant from the Korea Research Council of Fundamental Science and Technology and the KRIBB Initiative program (KGM0821113). We thank Su-jin Park and Eun-hae Choi, for technical assistance.

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