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Intranasal Adenovirus-Vectored Vaccine for Induction of Long-Lasting Humoral Immunity-Mediated Broad Protection against Influenza in Mice

Eun Hye Kim,[a] Hae-Jung Park,[a] Gye-Yeong Han,[a] Man-Ki Song,[a] Alexander Pereboev,[b] Jeong S. Hong,[c] Jun Chang,[d] Young-Ho Byun,[e] Baik Lin Seong,[e] Huan H. Nguyen[a]

Viral Immunology Laboratory, International Vaccine Institute, Seoul, South Korea[a]; Gene Therapy Center[b] and Department of Cell Biology,[c] University of Alabama at Birmingham, Birmingham, Alabama, USA; Graduate School of Pharmaceutical Sciences, Ewha Womans University, Seoul, South Korea[d]; Department of Biotechnology and Vaccine Translational Research Center, Yonsei University, Seoul, South Korea[e]

ABSTRACT

Influenza vaccines aimed at inducing antibody (Ab) responses against viral surface hemagglutinin (HA) and neuraminidase (NA) provide sterile immunity to infection with the same subtypes. Vaccines targeting viral conserved determinants shared by the influenza A viruses (IAV) offer heterosubtypic immunity (HSI), a broad protection against different subtypes. We proposed that vaccines targeting both HA and the conserved ectodomain of matrix protein 2 (M2e) would provide protection against infection with the same subtype and also HSI against other subtypes. We report here that single intranasal immunization with a recombinant adenovirus (rAd) vector encoding both HA of H5 virus and M2e (rAdH5/M2e) induced significant HA- and M2e-specific Ab responses, along with protection against heterosubtypic challenge in mice. The protection is superior compared to that induced by rAd vector encoding either HA (rAdH5), or M2e (rAdM2e). While protection against homotypic H5 virus is primarily mediated by virus-neutralizing Abs, the cross-protection is associated with Abs directed to conserved stalk HA and M2e that seem to have an additive effect. Consistently, adoptive transfer of antisera induced by rAdH5/M2e provided the best protection against heterosubtypic challenge compared to that provided by antisera derived from mice immunized with rAdH5 or rAdM2e. These results support the development of rAd-vectored vaccines encoding both H5 and M2e as universal vaccines against different IAV subtypes.

IMPORTANCE

Current licensed influenza vaccines provide protection limited to the infection with same virus strains; therefore, the composition of influenza vaccines has to be revised every year. We have developed a new universal influenza vaccine that is highly efficient in induction of long-lasting cross-protection against different influenza virus strains. The cross-protection is associated with a high level of vaccine-induced antibodies against the conserved stalk domain of influenza virus hemagglutinin and the ectodomain of matrix protein. The vaccine could be used to stimulate cross-protective antibodies for the prevention and treatment of influenza with immediate effect for individuals who fail to respond to or receive the vaccine in due time. The vaccine offers a new tool to control influenza outbreaks, including pandemics.

Influenza A virus (IAV) is responsible for seasonal influenza epidemics and infrequent, unpredictable pandemics that cost millions of human lives (1). The infection is largely uncontrolled due to variation in the virus' two outer membrane glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Current licensed influenza vaccines aimed to induce antibodies (Abs) directed against HA and NA (2–4) provide protection against infection with same (antigenically matched) virus strains that are included in the vaccines. Therefore, the composition of influenza vaccines has to be revised every year due to antigenic drift of seasonal influenza viruses. Vaccines designed to elicit cross-protection against infection with different subtypes would not need to be updated annually and could be stockpiled to combat influenza pandemics. A number of approaches targeting conserved IAV proteins to induce cross-protection have been developed and preclinically evaluated.

Since the Abs against the highly conserved ectodomain of matrix protein 2 (M2e), the viral ion channel protein, were shown to restrict influenza virus replication and reduce morbidity and mortality (5–9), M2e is an attractive target for the development of vaccines inducing broad protection (8, 10–13). Although the M2e-based vaccine candidates induced significant immune responses to the protein, the levels of cross-protection were modest. M2e-based vaccine strategies included expressing M2e as a fusion protein with the hepatitis B core antigen (14), or the delivery of M2e peptide conjugates (15). Another vaccine approach involved priming with M2-DNA and boosting with recombinant adenovirus (rAd) encoding M2, which resulted in protection against challenges with virulent IAV, including an H5N1 strain (16). More recently, mucosal immunization with recombinant M2 (17) or influenza virus-like particles induced cross-protection in mice (18).

HA consists of a variable head (HA1) and conserved stalk (HA2) domains (19) that are involved in attachment of virus to

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Address correspondence to Huan H. Nguyen, hhnguyen@ivi.int.

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sialic acids on cellular receptors and fusion of the viral and cell membranes, respectively. Conserved epitopes in the stalk HA (HA2 domain) were identified (20), and the vaccines targeting the conserved stalk HA for induction of cross-protective immunity recently yielded promising results in preclinical animal models (21, 22). Stalk-specific Abs contribute to cross-protection (23, 24), and vaccines based on chimeric HA constructs that express unique head and stalk combinations elicit HA stalk-specific Abs, along with cross-protection (25). Repeated immunization of mice with the constructs that expressed the same HA stalk linked to HA head domains of different subtypes elicited significant levels of HA stalk-specific Abs, along with cross-protection (25).

Replication-deficient rAd has been used extensively for genetic vaccination (26–30) or boosting of primed immune responses (31). We have recently shown that intranasal (i.n.) immunization with of rAd encoding conserved nucleoprotein elicited mucosal immunoglobulin A (IgA) and CD8 T-cell responses in mice and provided potent protection against influenza virus infection (32). Vaccination with rAd encoding IAV HA has been proven efficient in generating protective immunity against homologous or heterologous challenge (33–36). More recently, i.n. immunization with rAd encoding HA conferred cross-protection against other subtype of the same group of IAV (37). In humans, i.n. administration of rAd encoding HA was demonstrated to be safe and immunogenic (38), which supports the development of rAd-vectored vaccines for use in humans.

It is well established that mucosal immunization leads to the generation of superior mucosal immunity by inducing secreted IgA and T-cell responses both at the site of immunization and at distal mucosal sites (39, 40). Since i.n. immunization is the most effective method for the induction of cross-protection against influenza in mice (41) and the respiratory tract is the natural site of influenza virus and adenovirus replication, rAd vectors are an ideal platform for i.n. vaccines against influenza. In the present study, we designed, constructed, and evaluated the efficacy of a novel rAd-vectored vaccine encoding H5 HA and M2e to confer protection against H5 virus and cross-protection against other subtypes upon i.n. immunization in mice.

MATERIALS AND METHODS

Generation of rAd vectors. Replication-defective human Ad serotype 5-derived vector encoding humanized full-length H5 HA (rAdH5), four tandem copies of the ectodomain (M2e) of the M2 (rAdM2e), or fusion of HA and four tandem copies of M2e (rAdH5/M2e) were generated (Fig. 1A). Influenza M2 and HA antigen amino acid sequences were from A/Vietnam/1203/2004 (H5N1; GenBank accession numbers AY651388 and AY651334, respectively). The rAd vector encoding H5 HA was selected for immediate evaluation as a vaccine candidate against a potential H5N1 pandemic. The codon-optimized H5 HA sequence (synthesized by GenScript, Piscataway, NJ) was cloned into the pShuttle/CMV plasmid, which allowed for homologous recombination with a plasmid encoding an Ad backbone in *Escherichia coli* BJ5183. rAd vectors were subsequently generated by transfecting recombinant plasmids containing the bioengineered Ad genomes encoding the transgenes into 293 cells. The vectors were mass produced, purified, and titrated according to the AdEasy manual (Strategene, La Jolla, CA). The genomes were sequenced to confirm the presence of M2e and HA genes and their flanking Ad sequences. M2e and HA of H5N1 origin were validated in lysates prepared from transduced HeLa cells by Western blot analysis using Abs to M2 (kindly provided by W. Gerhard, The Wistar Institute, Philadelphia, PA) and H5-specific IgY (42), respectively (data not shown).

Viruses. Mouse-adapted A/PR/8/34 (PR8) (H1N1) virus was prepared as lung homogenates of intranasally infected mice were used for challenge as previously described (43). A/Aquatic bird/Korea/W81/2005 (H5N2), isolated from a wild bird in Korea in 2006 (kindly provided by Young-Ki Choi, Chungbuk University, Chungbuk, South Korea), was adapted by multiple passages (15 times) in BALB/c mice. After the final passage, a single plaque isolated by three consecutive plaque purification on MDCK cells was amplified in embryonated chicken eggs, and the mouse 50% lethal dose (LD_{50}) of the H5N2 virus was determined for the challenge experiment.

Animals. All animal experiments and animal procedures conformed to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the International Vaccine Institute. Female BALB/c mice aged 6 to 8 weeks were purchased from Orient Bio, Inc. (Orient, Inc., South Korea). Animals were housed in specific-pathogen-free barrier facilities with a 12-h dark and light cycle and free access to water and food.

Cell lines. Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34; American Type Culture Collection [ATCC], Manassas, VA) were maintained in ATCC-formulated Eagle minimum essential medium containing 10% fetal bovine serum (HyClone) and penicillin-streptomycin (Gibco) at 100 U/ml and 100 μg/ml, respectively.

Recombinant chimeric protein. The chimeric cH9/1 protein containing the stalk domain of PR8 and the globular head domain of A/guinea fowl/Hong Kong/WF10/99 (cH9/1) was kindly provided by Peter Palese, Icahn School of Medicine, Mount Sinai, New York, NY (44).

Generation of Abs using vaccine candidates. Mice were i.n. immunized with 10^7 PFU in 50 μl of rAd encoding HA of H5 virus fused with M2e (rAdH5/M2e) or a sublethal dose of live mouse-adapted PR8 in 50 μl of phosphate-buffered saline (PBS). Four weeks after immunization, sera were collected and pooled for passive immunization of naive mice. Formaldehyde-inactivated PR8 was prepared by treatment of egg-grown PR8 with 0.02% formaldehyde overnight followed by dialysis to remove formaldehyde. Each mouse was immunized i.n. with 50 μl of 2×10^7 PFU of inactivated PR8 mixed with 2 μg of cholera toxin (List Biological Laboratories, Inc., Campbell, CA). The PR8-primed mice were then boosted by a second immunization using the same procedure. Four weeks after the second immunization, sera were collected and pooled for passive immunization of naive mice.

Immunization. For i.n. immunization, mice were anesthetized by an intraperitoneal injection of ketamine-xylazine (0.15 and 0.03 mg/kg, respectively) and i.n. inoculated with rAd encoding green fluorescent protein (GFP), rAd encoding HA of H5 virus and M2e (rAdH5/M2e), and rAd encoding either HA (rAdH5) or M2e (rAdM2e) in total of 50 μl (25 μl per each nostril). The rAd vaccine doses are specified in the figure legends.

Infection with influenza viruses. The mouse LD_{50} was determined by inoculating groups of eight mice i.n. with serial 10-fold dilutions of virus as previously described (45). For lethal infection, ketamine-anesthetized mice were i.n. inoculated with 5× the LD_{50} of mouse-adapted PR8 or A/Aquatic bird/Korea/W81/2005 (H5N2) viruses resuspended in 50 μl of PBS per animal.

Passive immunization. Anesthetized naive mice were passively immunized by i.n. administration of 50 μl of 1:5 diluted sera 50 μl of 1:5-diluted sera. Six hours later, the recipient mice were infected with 5× the LD_{50} of mouse-adapted PR8 or A/Aquatic bird/Korea/W81/2005 (H5N2) in 50 μl of PBS per animal.

ELISA. A standard enzyme-linked immunosorbent assay (ELISA) was performed to detect antigen-specific Abs in the sera. MaxiSorp Nunc Immuno plates (96 well; Nalgene Nunc International, Naperville, IL) were coated with whole virus particles, M2e, or chimeric protein (cH9/1) at a concentration of 5 μg/ml (100 μl per well). The plates were then blocked for 1 h with PBS containing 0.1% (vol/vol) Tween 20 (TPBS) with 3% bovine serum albumin (BSA). Serum was prediluted to 1:100, serially diluted to 1:5 in blocking buffer, and then adsorbed onto plates for 2 h. After extensive washing with TPBS, the bound immunoglobulins were detected with goat anti-mouse Ig(H+L) horseradish peroxidase-conju-

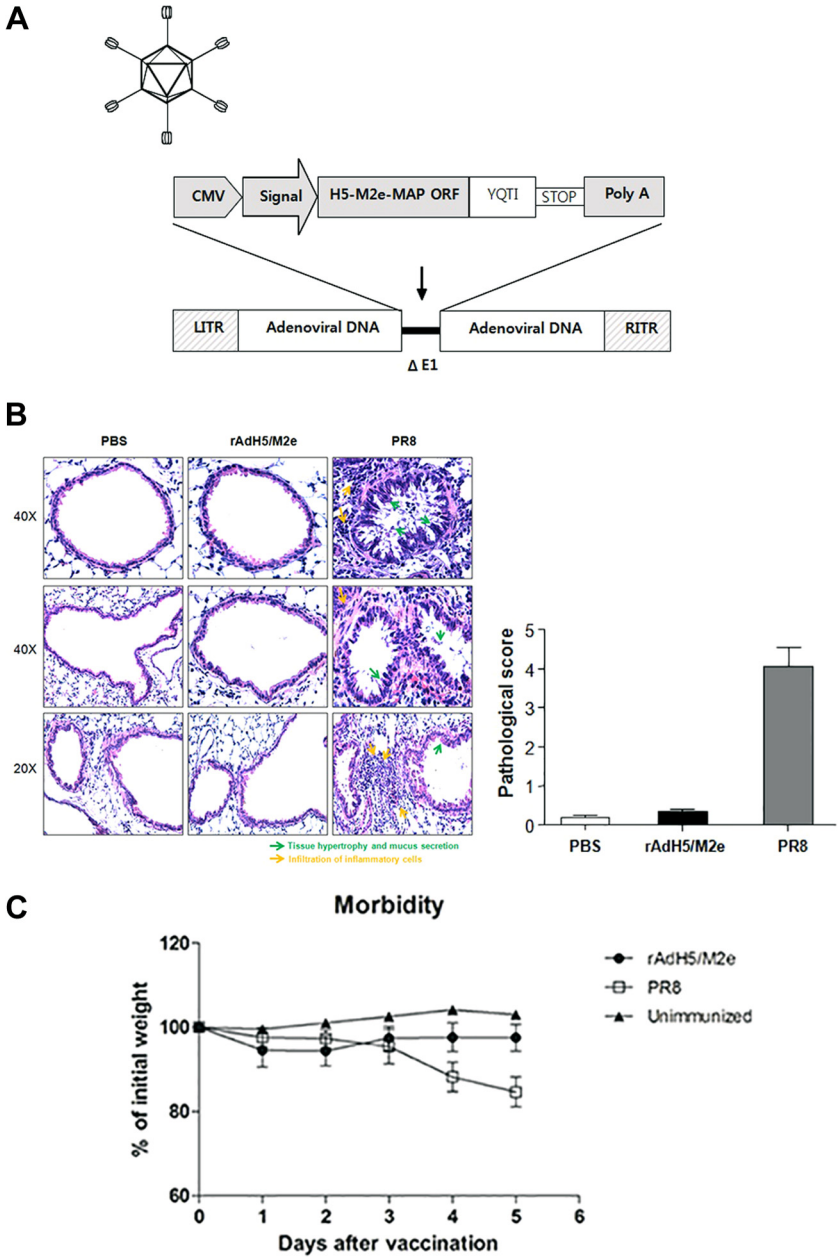

FIG 1 Vaccine design and safety of i.n. immunization. (A) Diagram of adenovirus vector construct containing codon-optimized H5 HA and four tandem M2e genes (rAdH5/M2e). The rAd vectors were designed to induce preferential humoral immunity to the targeted antigens by adding an appropriate endoplasmic reticulum (ER) leader peptide sequence and an endo/lysosome targeting signal. All coding sequences were placed under the control of a cytomegalovirus (CMV) promoter. Translation initiation was facilitated by the ATG codon in a proper Kozak surrounding. BALB/c received rAdH5/M2e or live mouse-adapted influenza virus PR8 via i.n. route. A mock-infected (PBS) group of mice was included as a control. (B) Five days later, the mice were euthanized, and the lungs were collected for histological analysis as described in Materials and Methods. (C) The morbidity was monitored daily. The mean body weight loss upon immunization with rAdH5/M2e ± the standard deviations (SD) of each experimental group of five mice were determined at each indicated time point. The values are the means of five mice per group.

gated antibody (Southern Biotechnologies Associates, Inc., Birmingham, AL) diluted 1:5,000 for 1 h at room temperature, followed by another extensive washing step. Tetramethylbenzidine substrate was added, and the reaction was stopped with an equal volume of 1 M sulfuric acid. The color developed was measured by using a SpectraMax photometer (Molecular Devices) at 450 nm. The assay results were expressed as endpoint titration values, which were determined by the last dilutions that are above cutoff for the assay (i.e., when the optical density at 450 nm reaches the plateau value).

HI assay. The serum samples were treated with receptor-destroying enzyme II (Denka Seiken, Co., Ltd., Tokyo, Japan) at a final dilution of 1:3 before being tested by in a hemagglutination inhibition (HI) assay. Two-fold serially diluted samples were incubated with equal volume containing 8 HA units of viruses in V-shaped-bottom 96-well microtiter plates at 37°C for 1 h. At the end of incubation, freshly prepared 1% chicken red blood cells (CRBC) were added, and the plates were mixed by agitation, covered, and allowed to set for 1 h at 4°C temperature. The HI titers were

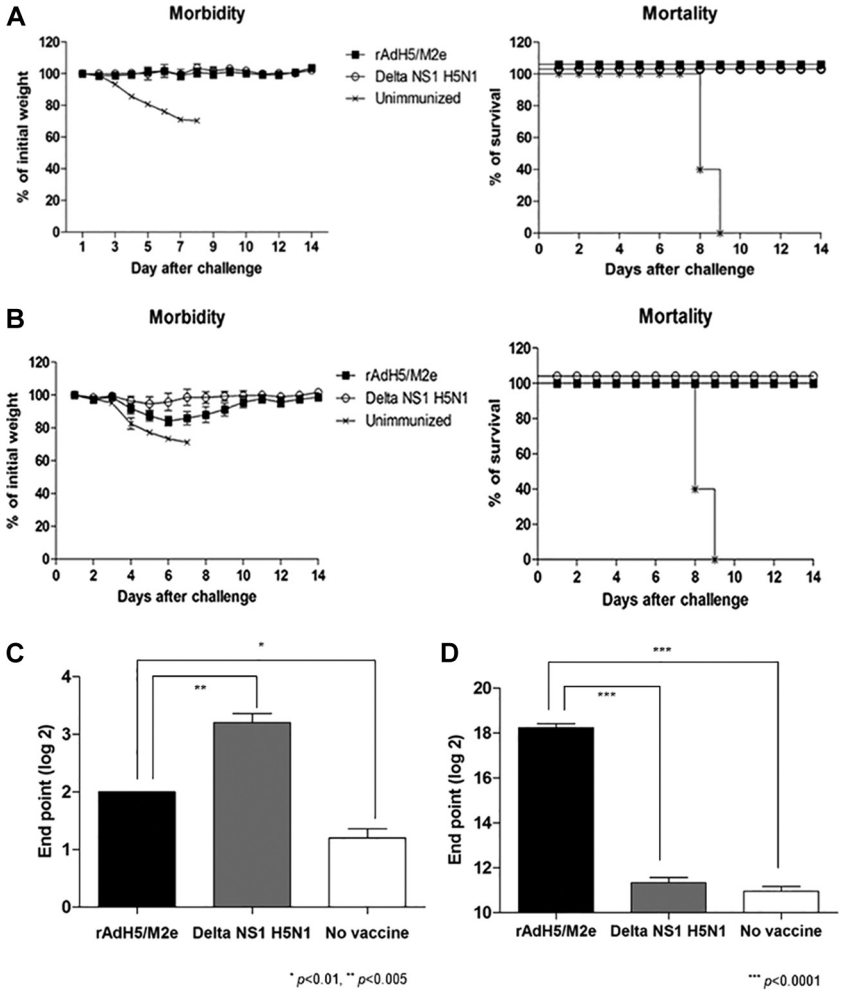

FIG 2 Single i.n. immunization with rAdH5/M2e induces homotypic and heterosubtypic immunity. BALB/c were immunized with rAdH5/M2e via i.n. route. Live-attenuated influenza A virus lacking nonstructural protein NS1 gene, Delta NS1 (H5N1) was included as a positive control. Five weeks later, the mice were i.n. challenged with homotypic A/Aquatic bird/Korea/W81/2005 (H5N2) (A) or heterosubtypic PR8 (H1N1) (B) as described in Materials and Methods. The morbidity and mortality were monitored daily for 2 weeks. Mean body weight ± the SD of each experimental group of five mice was determined at each indicated time point. Mortality is expressed as the mean percentages ± the SD of mice that survived the challenge. The values are the means of five mice per group. The level of HI Abs (C) and M2e-specific Abs (D) in the sera collected on the day before challenge were determined by HI assay and ELISA using M2e-coated plates. The values represent means + the SEM (vertical bars) endpoint ELISA antibody titer determined on five mice per group.

determined by calculating the reciprocal of the last dilution that contained nonagglutinated CRBC. Positive- and negative-control samples were included on each plate.

Preparation of Ig and non-Ig fraction from sera. Immune sera were diluted to 1:2.5 with PBS, added to Amicon Ultra-0.5 Centrifugal filter 100K devices (Millipore), and spun at 14,000 × *g* for approximately 10 to 30 min. The nonimmunoglobulin (non-Ig) fraction containing molecules with molecular masses lower than 100 kDa that went through the filter was collected. The Ig fraction containing molecules with molecular masses higher than 100 kDa was recovered by placing the Amicon Ultra filter device upside down in a clean microcentrifuge tube, followed by spinning for 2 min at 1,000 × *g*.

Determination of virus titers in the lungs by plaque assay. Mice under anesthesia were treated i.n. with 50 μl of 1:5-diluted antisera per animal. Six hours later, the recipient mice were infected with 5× the LD_{50} of mouse-adapted PR8 in 50 μl of PBS per animal. On day 5 after infection, the mice were euthanized. The lungs were removed and homogenized in 1 ml of PBS containing antibiotics. The tissue homogenates were centrifuged, and the supernatants were kept at −70°C until virus titration by standard plaque assay on MDCK cells. The results of the virus titers were expressed as the mean PFU/ml.

Histological examination. Mice under anesthesia were i.n. inoculated with rAdH5/M2e, a sublethal dose of mouse-adapted PR8, or PBS. On day 5, mice were euthanized, and the lungs were perfused with PBS containing gentamicin and fixed in 4% formaldehyde for 1 h at 4°C. Lung tissues were further dehydrated by gradually soaking them in alcohol and xylene, embedded in paraffin, sectioned (5-μm thick), and stained with hematoxylin and eosin (H&E) for histopathological analysis using a digital light microscope (Olympus, Tokyo, Japan). Pathological scores were based on whole tissue integrity (0 to 5), infiltration of inflammatory cells (0 to 5), tissue hypertrophy (0 to 5), and mucus secretion (0 to 5).

Statistics. Statistical analyses were performed using Prism4 (GraphPad, La Jolla, CA). All values were plotted as averages with standard errors of the means (SEM). The Student *t* test and analysis of variance (ANOVA) were used to determine the significant differences between two or multiple sets of experimental data, respectively. *P* values of <0.01, <0.005, and <0.0001 (indicated by asterisks [*, **, and ***, respectively] in the figures) were considered statistically significant.

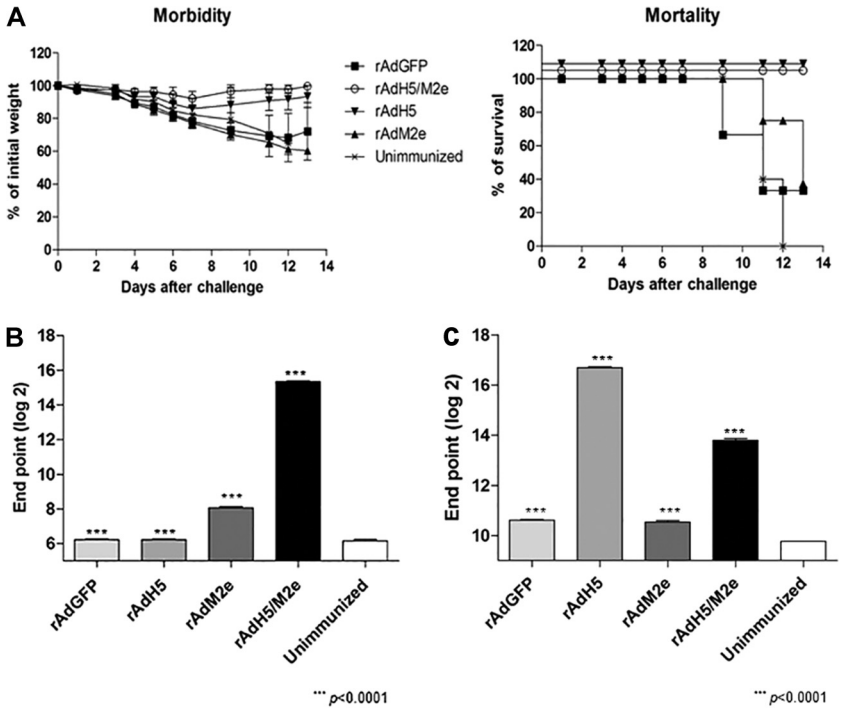

FIG 3 Cross-protection induced by different rAd-vectored vaccine candidates. (A) BALB/c were immunized with 10^7 PFU of rAdGFP, rAdH5, rAdM2e, or rAdH5/M2e/mouse via the i.n. route. Five weeks later, the mice were i.n. challenged with a lethal dose of heterosubtypic PR8 (H1N1). Morbidity and mortality were monitored daily for 2 weeks. The mean body weight ± the SD of each experimental group of five mice was determined at each indicated time point. Mortality is expressed as the mean percentages ± the SD of mice that survived the challenge. The values are means of five mice per group. The level of M2e-specific Abs (B) and H5-specific Abs (C) were determined by ELISA using recombinant M2e or H5N2 whole virus as coating antigens, respectively. The values represent means + the SEM (vertical bars) endpoint ELISA antibody titer determined on five mice per group.

RESULTS

Intranasal administration of rAdH5/M2e did not induce significant histological change in the lungs or morbidity. The rAd containing codon-optimized H5 HA and M2e genes was designed and generated according to the diagram shown in Fig. 1A. To test whether i.n immunization of mice with our Ad vector-based vaccine candidate, rAdH5/M2e (Fig. 1A), induced inflammation in the lungs, we performed histological analysis of the lungs on day 5 postimmunization. As shown in Fig. 1B, no significant histological change could be detected in the lungs of mice that received the vaccine candidate rAdH5/M2e. In contrast, tissue hypertrophy and mucus secretion, along with infiltration of inflammatory cells, were observed in the lungs of mice infected with a sublethal dose of the mouse-adapted influenza virus PR8. Consistent with the H&E staining, rAdH5/M2e vaccination resulted in a low pathological score, while a sublethal dose of PR8 resulted in a pathological score of ~4. Marginal weight loss was seen in mice immunized with our vaccine candidate (Fig. 1C); however, the sublethal dose of PR8 induced considerable weight loss by day 5 postinfection. The results indicate that our vaccine candidate is safe for i.n. immunization in mice.

Single i.n. immunization with rAdH5/M2e induced protection against homotypic and heterosubtypic challenge. BALB/c mice were i.n. immunized with rAdH5/M2e. As a control, a group of mice immunized with live-attenuated influenza A virus with a deletion of the nonstructural protein NS1 gene, the A/Vietnam/1203/04 Delta NS1 (H5N1) that induced HSI (46), was included. As shown in Fig. 2A, a single i.n. immunization with rAdH5/M2e induced complete protection against lethal infection with mouse-adapted A/Aquatic bird/Korea/W81/2005 (H5N2) that shares 94.4% nucleotide sequence homology with HA of A/Vietnam/1203/04 encoded by the rAd vector and the Delta NS1 (H5N1) virus. When challenged with mouse-adapted heterosubtypic PR8 virus, 100% of the immunized mice survived despite the fact that they developed a sign of morbidity, as observed by their body weight loss (Fig. 2B). These results demonstrated that a single i.n. immunization with replication-deficient rAdH5/M2e protected mice from infection with the same virus subtype and induced a high level of HSI to infection with a different IAV subtype. The protection is associated with the vaccine-induced HI (Fig. 2C) and M2e-specific (Fig. 2D) Ab titers.

rAd vector encoding multiple influenza virus antigens induced superior cross-protection. We next compared the levels of HSI induced by different vaccine candidates. Different groups of mice were immunized with rAd encoding only the HA of the H5 virus (rAdH5) or M2e (rAdM2e) or both HA (H5) and M2e (rAdH5/M2e). Upon heterosubtypic challenge with the PR8, mice immunized with the rAd vector encoding both HA and M2e were best protected compared to those immunized with rAd vectors encoding only HA or M2e. Although all mice immunized with rAdH5 survived the challenge, they suffered more body weight loss upon challenge compared to that seen in mice immunized with rAd vector encoding multiple antigens (rAdH5/M2e) (Fig. 3A). rAdH5/M2e induced a superior Ab response to M2e compared to that induced by rAd encoding only M2e (Fig. 3B) but lower Ab responses to HA (Fig. 3C) compared to that induced by

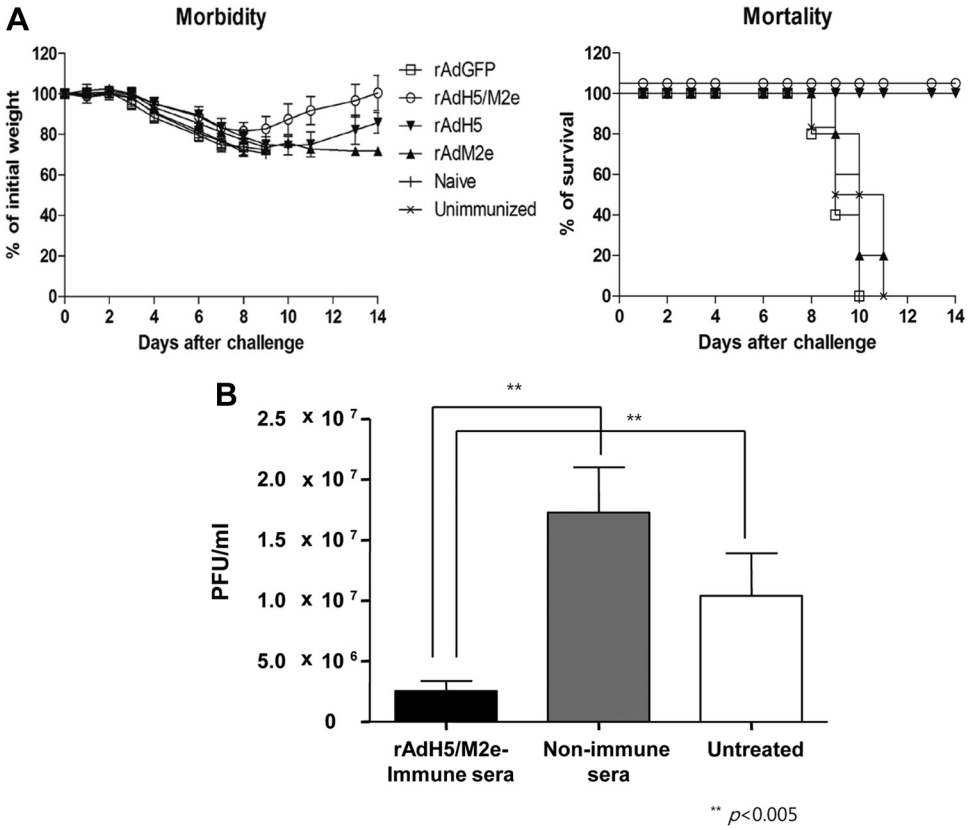

FIG 4 rAd vectors encoding HA elicited cross-protective Abs. (A) Protection against challenge with heterosubtypic virus (H1N1) upon adoptive transfer of immune sera. Anesthetized BALB/c mice received (i.n.) 50 μl of rAdGFP-, rAdH5/M2e-, rAdH5-, or rAdM2e-induced sera. Six hours later, they were infected with 5× the LD_{50} of mouse-adapted PR8 (H1N1) virus. The morbidity and mortality were monitored daily for 2 weeks. The mean body weight ± the SD for each experimental group of five mice was determined at each indicated time point. Mortality is expressed as the mean percentages ± the SD of mice that survived the challenge. (B) Virus titers in the lungs at day 5 postinfection. In selected groups of mice, the virus titers were determined in the lungs on day 5 after infection by standard plaque assay as described in Materials and Methods. The virus titers are expressed as the $\log_{10}$ PFU ± the SEM.

rAd encoding only HA. These results indicated that rAd encoding HA and M2e induced Ab responses to both encoded antigens and that those induced Ab responses contribute additively to HSI.

rAd vectors encoding HA elicited cross-protective Abs. To provide direct evidence for critical contribution of vaccine-induced Abs to HSI, we performed adoptive transfer of antisera from mice immunized with rAdH5/M2e, rAdH5, and rAdM2e. Mice were i.n. immunized with the vaccine candidates. Five weeks after i.n. immunization of mice with the indicated vaccine candidates, sera were collected and i.n. transferred to naive mice. At 6 h after transfer, the recipient mice were infected with a lethal dose of PR8 as a heterosubtypic challenge. As shown in Fig. 4A, only antisera obtained from groups of mice immunized with rAd encoding HA provided protection against heterosubtypic challenge, whereas antisera from mice immunized with rAd encoding M2e or irrelevant green fluorescent protein (GFP) failed to provide protection. Importantly, antisera obtained from mice immunized with rAd vector encoding both HA and M2e provided superior protection compared to those derived from animals immunized with the rAd vector encoding only HA. The cross-protection provided by antisera is probably mediated by control of virus replication in the lungs since virus titers were significantly reduced in the lungs at day 5 after infection compared to those seen in mice treated with sera from unimmunized mice (Fig. 4B). The results suggest that serum-derived Abs generated by rAd vectors encoding HA readily provided cross-protection upon adoptive transfer and that those generated by rAd encoding multiple antigens, including HA and M2e, provided better cross-protection by controlling virus replication in the lungs.

Immunization with rAdH5/M2e induced long-lasting HSI. We further sought to determine whether our vaccine candidate rAdH5/M2e induced long-lasting HSI. Immunized mice were challenged 1 year after immunization with a heterosubtypic (H1N1) virus. As shown in Fig. 5A, complete protection against infection with heterosubtypic virus was observed. The level of HSI is comparable to that seen at 4 weeks after immunization (Fig. 4A). Consistently, sera collected at 1 year after immunization with rAdH5/M2e provided protection of naive mice against infection with either a heterosubtypic or homotypic virus upon adoptive transfer of immune sera (Fig. 5B and C, respectively). The effect was comparable to that induced by sera collected from mice immunized with live virus. In contrast, formalin-inactivated virus failed to generate cross-protective serum Abs.

rAdH5/M2e elicited significant stalk-specific Abs. Since stalk HA-specific Abs contribute to Ab-mediated HSI (22, 47), we determined the levels of stalk-specific Abs in mice immunized with different vaccines using recombinant chimeric H9/1 protein. As shown in Fig. 6, the levels of stalk-specific Abs induced by rAdH5/

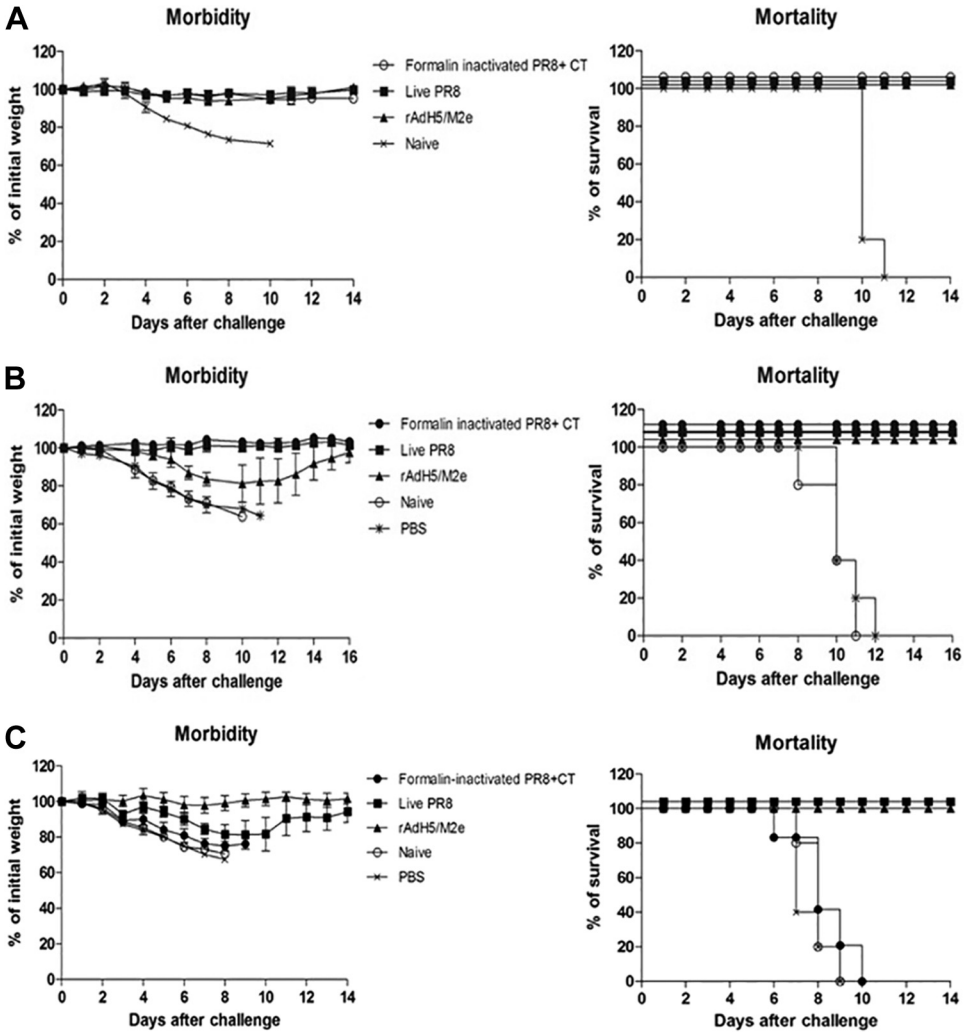

FIG 5 rAdH5/M2e induced long-lasting HSI. BALB/c mice were immunized with live PR8, formaldehyde-inactivated PR8+CT, or rAdH5/M2e. (A) Twelve months later, they were i.n. challenged with 5× the LD_{50} of mouse-adapted PR8 (H1N1) virus. Sera collected before the challenge were adoptively transferred to naive mice. (B and C) Six hours later, the recipient mice were challenged with mouse-adapted PR8 (H1N1) virus (B) or A/Aquatic bird/Korea/W81/2005 (H5N2) virus (C). Morbidity and mortality were monitored daily for 2 weeks. Mean body weight ± the SD of each experimental group of five mice was determined at each indicated time point. Mortality is expressed as the mean percentages ± the SD for mice that survived the challenge. The values are means of five mice per group.

M2e were at levels comparable to those induced by live wild type or attenuated Delta NS1 virus. In contrast, formaldehyde-inactivated PR8 induced a low level of stalk-specific Abs. These results indicate that live influenza virus and rAd-vectored vaccine, but not formalin-inactivated virus, induced pronounced stalk-specific Ab responses that are associated with vaccine-induced HSI.

Adoptive transfer of antiserum fraction containing immunoglobulins provided cross-protection. To determine whether the Abs contained in the antisera were associated with HSI, we separated antisera into Ig and non-Ig fractions using 100-kDa cutoff Amicon Ultra-0.5 Centrifugal filters (Millipore). Adoptive transfer of the Ig fraction, but not the non-Ig fraction, protected naive mice from infection with either heterosubtypic or homotypic viruses (Fig. 7A and B, respectively), suggesting that Abs contained in the antisera mediate the cross-protection.

DISCUSSION

In this study we demonstrated that a rAd vector encoding multiple influenza virus antigens, i.e., H5 HA and M2e induced complete protection against challenge infection with either a heterologous H5N2 or a heterosubtypic H1N1 influenza virus. Our results support earlier findings that gene-based vaccines, including rAd vectors encoding highly conserved influenza virus genes, such as the viral nucleoprotein (NP) and the ion channel matrix protein (M2), conferred protection against lethal challenge (48, 49), and inclusion of HA in the vaccine induced more potent protective immunity against heterologous challenge (35). Although an rAd vector encoding HA had been shown earlier to induce protection against infection with the same subtype (33–36), the cross-protection by the rAd vector encoding HA against other virus subtypes had not been explored until recently. Intranasal vaccination with rAd encoding HA provided complete protection from homologous infection, partial protection against heterologous challenge (50), and cross-protection against infection with other subtypes of the same group of influenza viruses (37). Consistently, our rAd vector encoding H5 HA readily provided cross-protection against infection with an H1 virus that is more potent compared to that

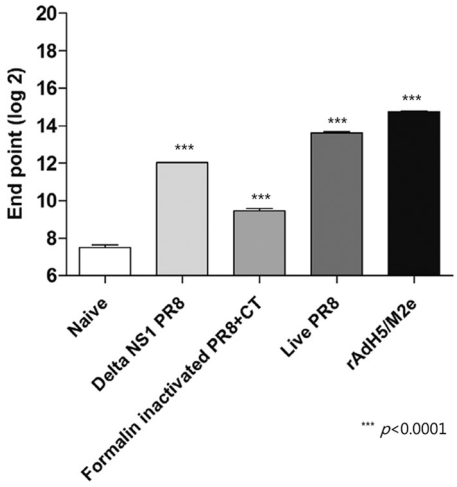

FIG 6 rAdH5/M2e elicited stalk-specific Abs. BALB/c mice were immunized with live PR8, formaldehyde-inactivated PR8+CT, and rAdH5/M2e via the i.n route. Four weeks later, sera were collected, and the levels of stalk-specific Abs were determined using cH9/1 protein-coated ELISA plate. Live-attenuated influenza A virus lacking nonstructural protein NS1 gene Delta NS1 (H1N1) was included as a positive control. The values represent the mean + the SEM (vertical bars) end-point ELISA antibody titer determined on five mice per group.

induced by rAd vector encoding the conserved M2e. Of note, substantial cross-protection elicited by rAdM2e could be only observed after three immunizations (data not shown). Although HA is highly variable and vaccines targeting HA have been designed for the induction of protection against infection with the same subtype, it has been shown recently that vaccination based on priming with DNA encoding HA and boosting with seasonal vaccine or rAd encoding HA stimulated the production of broadly neutralizing Abs that are directed to the conserved stalk domain of HA (24, 51). Here, we demonstrated that a single i.n. immunization with rAd vector encoding H5 HA readily generated significant levels of stalk-specific Abs (Fig. 6), along with complete cross-protection. Importantly, the rAd vector encoding multiple antigens HA and M2e induced superior HSI, suggesting the additive effect of immune responses specific for conserved epitopes. Indeed, whereas Abs against the HA stalk prevent the release of viral genetic material into the cells by blocking virus and host cell membrane fusion (52), the Abs against M2e mediate the lysis of virus-infected cells by antibody-dependent cellular cytotoxicity in the presence of NK cells and/or macrophages bearing the Fc gamma receptor (FcγR) (53, 54). The two processes complementarily contribute to the protection by limiting virus infection and spread, respectively. Of note, the rAd encoding M2e, along with codon-optimized HA, induced a significantly higher level of M2e-specific Abs (Fig. 3B) compared to that induced by rAd encoding only M2e, suggesting the effect of codon-optimized HA in the rAd vector on the stimulation of M2e-specific immune responses. However, the level of HA-specific Abs induced by the rAd vector encoding codon-optimized HA and M2e is not improved compared to that generated by rAd encoding HA only. Thus, the rAd vector encoding HA and M2e induced a higher level of Abs toward M2e and a moderate level of Abs to HA compared to the antigen-specific Ab responses induced by rAd vectors encoding either M2e

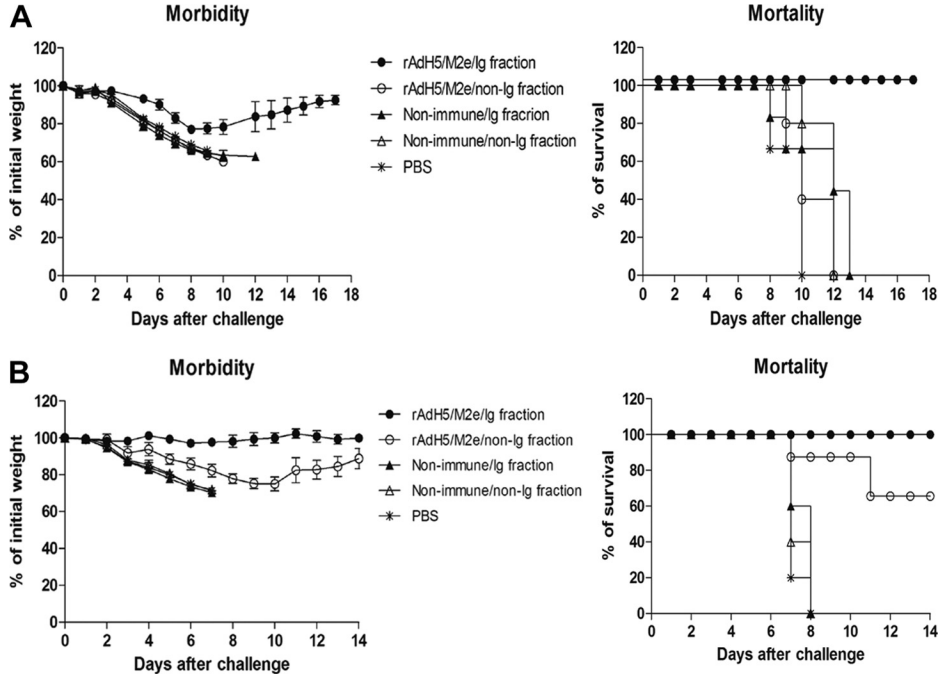

FIG 7 Adoptive transfer of immune serum fractions containing immunoglobulins provided cross-protection. (A) Protection against challenge with heterosubtypic virus (H1N1) upon adoptive transfer of an Ig fraction; (B) protection against challenge with homotypic virus (H5N2) upon adoptive transfer of an Ig fraction. BALB/c mice were i.n. inoculated with 10^7 PFU of rAdH5/M2e/50 μl. Sera were collected 4 weeks after immunization. Immunoglobulin (Ig) and non-immunoglobulin (non-Ig) fractions were separated as described in Materials and Methods. Naive mice were i.n. inoculated with the Ig or non-Ig fraction. Six hours later they were infected with 5× the LD_{50} of mouse-adapted PR8 (H1N1) virus (A) or A/Aquatic bird/Korea/W81/2005 (H5N2) (B) virus. Morbidity and mortality were monitored daily for 2 weeks. The mean body weights ± the SD of each experimental group of five mice were determined at each indicated time point. Mortality is expressed as the mean percentages ± the SD of mice that survived the challenge. The values are means of five mice per group.

or HA, suggesting that Ab response to HA is more potent than that against M2e in cross-protection.

The HA encoded by the H1 challenge virus and the H5 HA encoded by the rAdH5/M2e belong to the phylogenetic group 1 of IAV HA subtypes. Whether vaccination with rAdH5/M2 induces protection against challenge with group 2 subtype influenza viruses (such as H3 or H7 viruses) remains to be determined. It is likely that this vaccine candidate could induce cross-protection against a group 2 virus since the vaccine induced high levels of M2e-specific Abs and despite group 1 and 2 HA subtypes sharing less homology in the sequence and structure of the HA stalk domains.

Although rAd vectors are effective at induction of T cell responses that play a role in HSI (55, 56), our results indicate that Abs induced by the rAd-vectored vaccines readily mediate HSI since adoptive transfer of antisera containing specific Abs conferred protection against infection with another subtype. This is supported by further findings that adoptive transfer of an Ig-containing fraction, but not of a non-Ig-containing fraction of antisera provided protection (Fig. 7). Of note, the non-Ig-containing fraction of antisera was able to confer partial protection against challenge with H5 virus (Fig. 7B), indicating that rAdH5/M2e may trigger other unidentified host factors involved in protection against H5 virus. The question is under investigation in our laboratory. The results obtained from experiments involving adoptive transfer of antisera are consistent with those obtained from the challenge of immunized animals. Antisera derived from mice immunized with rAdH5/M2e provided protection superior to that mediated by antisera obtained from mice immunized with rAd encoding either HA or M2e. Thus, the results indicated that rAd vectors encoding HA generated cross-protective Abs that mediate HSI upon adoptive transfer. The adoptive transfer of Abs via the i.n. route to provide protection against influenza virus infection has been used in an earlier study (42). The method is more sensitive since it requires small amounts of antisera to achieve protection compared to the amount of antisera required for passive transfer via the intraperitoneal route (57).

In practice, it is important to develop a vaccine that induces not only protection shortly after immunization but also long-lasting protective immunity. To this end, we found that mice immunized with rAd-vectored vaccines at least 12 months earlier survived heterosubtypic challenge (Fig. 5). The long-lasting protective immunity is associated with the persistence of cross-protective Abs, since high levels of specific Abs were detected in the sera obtained from mice 1 year after immunization (data not shown). Adoptive transfer of such antisera readily provided HSI (Fig. 5B and C).

Although conserved Ab epitopes in the HA stalk (HA2 domain) were described many years ago (20), vaccine approaches targeting the conserved HA stalk were recently reevaluated, with promising results generated in preclinical animal models (21, 22, 58). Since our rAd encoding HA provided readily some level of cross-protection against H1 virus, we speculated that the cross-protection could relate to the vaccine-induced Abs against the stalk domain of HA. Indeed, our rAdH5/M2e induced significant levels of stalk-specific Abs (Fig. 6) that were comparable to the level induced by the live virus but not inactivated vaccine. Repeated immunizations with rAdH5/M2e significantly increased the levels of stalk-specific Abs (data not shown), indicating that immunity induced by previous immunization with the rAd vector did not prevent a boosting effect of subsequent immunization with rAd-vectored vaccines. This is in accordance with the finding that nasal delivery, in contrast to intramuscular injection, of an Ad-based vaccine bypasses preexisting immunity to the vaccine carrier and improves the immune response in mice (59). The findings indicate that i.n. vaccination with rAd can bypass preexisting immunity to Ad in the majority of the human population, making nasal Ad-based vaccines useful in humans. Our results show that rAd-vectored influenza vaccines containing the HA gene are superior in the induction of stalk-specific Abs that play an important role in HSI. The question of why rAd-vector encoding HA induced better stalk-specific Abs is under investigation in our laboratory. It is possible that the rAd-vectored vaccine expressed a larger amount of HA than that by live influenza virus and the rAd-expressed stalk HA retains the native structure of influenza virus-expressed HA. Thus, our vaccine candidate that is capable of inducing stalk-specific and M2e-specific Abs and cross-protection against other influenza virus subtypes offers a new universal influenza vaccine candidate for further clinical evaluation. Since the vaccine-induced antisera mediated cross-protection upon adoptive transfer, the vaccine could be used to stimulate cross-protective Abs for prevention and treatment of seasonal or pandemic influenza with immediate effect in individuals who fail to respond to or receive the vaccine in due time. The rAd vaccine candidate offers a new tool to control influenza outbreaks, including pandemics.

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