M. Mejima, K. Kashima, M. Kuroda, N. Takeyama, S. Kurokawa, Y. Fukuyama, H. Kiyono, K. Itoh, T. Mitsui, Y. Yuki	Development of selection marker-free rice-based oral cholera toxin B-subunit vaccine and characterization of location and structure of transgene by using whole genome resequencing analysis.	Plant Cell Tiss. Org. Cult.	120	35-48	2015
K. Kashima, M. Mejima, S. Kurokawa, M. Kuroda, H. Kiyono, Y. Yuki	Comparative whole-genome analyses of selection marker-free rice-based cholera toxin B-subunit vaccine lines with wild-type lines.	BMC Genomics	16	48	2015
Ainai A, Tamura S, Suzuki T, van Riet E, Ito R, Odagiri T, Tashiro M, Kurata T, Hasegawa H.	Intranasal vaccination with an inactivated whole influenza virusvaccine induces strong antibody responses in serum and nasal mucus of healthy adults.	Hum Vaccin Immunother.	9(9)	1962-70	2013
Y. Fukuyama, Y. Yuki*, Y. Katakai, N. Harada, H. Takahashi, S. Takeda, M. Mejima, S. Joo, S. Kurokawa, S. Sawada, H. Shibata, EJ. Park, K. Fujihashi, D. Briles, Y. Yasutomi, H. Tsukada, K. Akiyoshi, H. Kiyono:	Nanogel-based pneumococcal surface protein A nasal vaccine induces microRNA-associated Th17 cell responses with neutralizing antibodies against Streptococcus pneumoniae in macaques	Mucosal Immunol.		In press	2015

T. Azegami , H. Itoh H. Kiyono ,Y. Yuki	A Novel Transgenic Rice-based Vaccine	Arch. Immunol. Ther. Ex.	63	87-99	2015
福山賀子 幸　義和	粘膜ワクチンの現状—経鼻ワクチンを中心に—	医学のあゆみ	253	15033-15038	2015
Sasaki I, Hoshino K, Sugiyama T, Yamazaki C, Yano T, Iizuka A, Hemmi H, Tanaka T, Saito M, Sugiyama M, Fukuda Y, Ohta T, Sato K, Ainai A, Suzuki T, Hasegawa H, Toyama-Sorimachi N, Kohara H, Nagasawa T, Kaisho T.	Spi-B is critical for plasmacytoid dendritic cell function and development.	Blood.	120(24)	4733-43	2012
van Riet E, Ainai A, Suzuki T, Hasegawa H.	Mucosal IgA responses in influenza virus infections; thoughts for vaccine design.	Vaccine.	30(40)	5893-900	2012
Senchi K, Matsunaga S, Hasegawa H, Kimura H, Ryo A.	Development of oligomannose-coated liposome-based nasal vaccine against human parainfluenzavirus type 3.	Front Microbiol.	4	346	2013

Senchi K, Matsunaga S, Hasegawa H, Kimura H, Ryo A.	Development of oligomannose-coated liposome-based nasal vaccine against human parainfluenzavirus type 3.	Front Microbiol.	4	346	2013
Ainai A, Tamura S, Suzuki T, van Riet E, Ito R, Odagiri T, Tashiro M, Kurata T, Hasegawa H.	Intranasal vaccination with an inactivated whole influenza virusvaccine induces strong antibody responses in serum and nasal mucus of healthy adults.	Hum Vaccin Immunother.	9(9)	1962-70	2013
Niikura K, Matsunaga T, Suzuki T, Kobayashi S, Yamaguchi H, Orba Y, Kawaguchi A, Hasegawa H, Kajino K, Ninomiya T, Ijiro K, Sawa H.	Gold nanoparticles as a vaccine platform: influence of size and shape on immunological responses in vitro and in vivo.	ACS Nano.	7(5)	3926-38	2013
Sakai K, Ami Y, Tahara M, Kubota T, Anraku M, Abe M, Nakajima N, Sekizuka T, Shirato K, Suzaki Y, Ainai A, Nakatsu Y, Kanou K, Nakamura K, Suzuki T, Komase K, Nobusawa E, Maenaka K, Kuroda M, Hasegawa H, Kawaoka Y, Tashiro M, Takeda M.	The host protease TMPRSS2 plays a major role in in vivo replication of emerging H7N9 and seasonal influenza viruses.	J Virol.	88(10)	5608-16	2014

van Riet E, Ainai A, Suzuki T, Kersten G, Hasegawa H.	Combatting infectious diseases; nanotechnology as a platform for rational vaccine design.	Adv Drug Deliv Rev.	74	28-34	2014
Hasegawa H, van Reit E, Kida H.	Mucosal immunization and adjuvants.	Curr Top Microbiol Immunol.	386	371-80	2015
Inoue, Y., Kubota-Koketsu, R., Yamashita, A., Nishimura, M., Ideno, S., Ono, K., Okuno, Y., Ikuta, K.	Induction of anti-influenza immunity by modified green fluorescent protein (GFP) carrying hemagglutinin-derived epitope structure.	J Biol Chem	288	4981-4990	2013
Yasugi, M., Kubota-Koketsu, R., Yamashita, A., Kawashita, A., Du, A., Sasaki, T., Nishimura, M., Misaki, R., Kuhara, M., Boonsathorn, N., Fujiyama, K., Okuno, Y., Ikuta, K.	Human monoclonal antibodies broadly neutralizing against influenza B virus.	PloS Pathog	9	e1003150	2013

Sasaki, T., Setthapramote, C., Kurosu, T., Nishimura, M., Asai, A., Omokoko, MD., Pipattanaboon, C., Pitaksajjakul, P., Limkittikul, K., Subchareon, A., Chaichana, P., Okabayashi, T., Hirai, I., Leaungwutiwong, P., Misaki, R., Fujiyama, K., Ono, K., Okuno, Y., Ramasoota, P., Ikuta, K.	Dengue virus neutralization and antibody-dependent enhancement activities of human monoclonal antibodies derived from dengue patients at acute phase of secondary infection.	Antiviral Res	98	423-431	2013
Yasugi, M., Kubota-Koketsu, R., Yamashita, A., Kawashita, A., Du, A., Misaki, R., Sasaki, T., Kuhara, M., Boonsathorn, N., Fujiyama, K., Okuno, Y., Nakaya, T., Ikuta, K.	Emerging antigenic variants at the antigenic site Sb in pandemic A(H1N1)2009 influenza virus in Japan detected by a human monoclonal antibody.	PloS One	8	e77892	2013
Ideno, S., Sakai, K., Yunoki, M., Kubota-Koketsu, R., Inoue, Y., Nakamura, S., Yasunaga, T., Okuno, Y., Ikuta, K.	Immunization of rabbits with synthetic peptides derived from a highly conserved β-sheet epitope region underneath the receptor binding siteof influenza A virus.	Biologic: Targets and Therapy	7	233-241	2013

Ohshima, N., Kubota-Koketsu, R., Iba, Y., Okuno, Y., Kurosawa, Y.	Binding near the sialic acid-binding pocket and neutralizing both H1N1 and H5N1 viruses.	PloS One	9	e87305	2014
Lee, PS., Ohshima, N., Stanfield, RL., Yu, W., Iba, Y., Okuno, Y., Kurosawa, Y., Wilson, IA.	Receptor mimicry by antibody F045-092 facilitates universal binding to H3 subtype of influenza virus.	Nature Com.	5	3614	2014
Kumagai, T., Nakayama, T., Okuno, Y., Kase, T., Nishimura, N., Ozaki, T., Miyata, A., Suzuki, E., Okafuji, T., Ochiai, H., Nagata, N., Tsutsumi, H., Okamatsu, M., Sakoda, Y., Kida, H., Ihara, T.	Humoral immune response to influenza A(H1N1)pdm2009 in patients with natural infection and in vaccine recipients in the 2009 pandemic.	Viral Immunology	27	368-374	2014
Haredy, AM., Yamada, H., Sakoda, Y., Okamatsu, M., Yamamoto, N., Omasa, T., Mori, Y., Kida, H., Okamoto, S., Okuno, Y., Yamanishi, K.	Neuraminidase gene homology contributes to the protective activity of influenza vaccines prepared from the influenza virus library.	J Gen Virol	95	2365-2371	2014

Ⅳ. 研究成果の刊行物・別冊

（主要なもの）

Vaccine 30 (2012) 4160–4166

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

RNAi suppression of rice endogenous storage proteins enhances the production of rice-based *Botulinum* neutrotoxin type A vaccine

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ARTICLE INFO

Article history:
Received 1 March 2012
Received in revised form 14 April 2012
Accepted 18 April 2012
Available online 1 May 2012

Keywords:
Rice
Nasal vaccine
MucoRice
RNAi
Botulinum neutrotoxin

ABSTRACT

Mucosal vaccines based on rice (MucoRice) offer a highly practical and cost-effective strategy for vaccinating large populations against mucosal infections. However, the limitation of low expression and yield of vaccine antigens with high molecular weight remains to be overcome. Here, we introduced RNAi technology to advance the MucoRice system by co-introducing antisense sequences specific for genes encoding endogenous rice storage proteins to minimize storage protein production and allow more space for the accumulation of vaccine antigen in rice seed. When we used RNAi suppression of a combination of major rice endogenous storage proteins, 13 kDa prolamin and glutelin A in a T-DNA vector, we could highly express a vaccine comprising the 45 kDa C-terminal half of the heavy chain of botulinum type A neurotoxin (BoHc), at an average of 100 μg per seed (MucoRice-BoHc). The MucoRice-Hc was water soluble, and was expressed in the cytoplasm but not in protein body I or II of rice seeds. Thus, our adaptation of the RNAi system improved the yield of a vaccine antigen with a high molecular weight. When the mucosal immunogenicity of the purified MucoRice-BoHc was examined, the vaccine induced protective immunity against a challenge with botulinum type A neurotoxin in mice. These findings demonstrate the efficiency and utility of the advanced MucoRice system as an innovative vaccine production system for generating highly immunogenic mucosal vaccines of high-molecular-weight antigens.

1. Introduction

Although several plants have been shown to be useful for vaccine production [1], there is accumulating evidence that the seed crop rice is one of the most suitable systems for vaccine production, storage, and delivery [2]. We have previously developed rice expressing the B subunit of cholera toxin (CTB) vaccine, MucoRice-CTB, which possesses mucosal immunogenicity and prevents diarrhea in the event of *V. cholerae* and heat-labile enterotoxin-producing enterotoxigenic *Escherichia coli* challenges [3–6]. This rice-based vaccine has proven to be stable at room temperature for three years and thus could be used as a cold-chain-free vaccine [3,6].

In this study, we co-introduced antisense sequences specific for genes encoding endogenous rice storage proteins to block expression of these proteins and allow space for the increased accumulation of vaccine antigen in rice seed [7]. A previous report showed the feasibility of increasing the accumulation of an endogenous seed storage protein, cruciferin, by using an antisense sequence to reduce the production of another authentic seed protein (napin) in *Brassica napus* seeds [8]. In general, there are two types of protein storage organelles, called protein bodies (PB-I and -II), in rice seeds. Alcohol-soluble prolamins are expressed in PB-I (10, 13 16 kDa) and alkali-soluble glutelins (A and B) are expressed in PB-II [9–11]. To examine whether suppression of the production of prolamins or glutelins can effectively increase the expression of a transgene-encoded vaccine antigen, we investigated the effects of RNAi suppression of 13 kDa prolamin and/or glutelin A in a T-DNA vector on expression of a candidate vaccine.

To increase the versatility of the MucoRice system, it is necessary to develop high-yield vaccines for antigens with high molecular

Abbreviations: Ab, antibody; BoHc, a nontoxic subunit fragment of *Clostridium botulinum* type-A neurotoxin; BoNT/A, *C. botulinum* neurotoxin type-A; CT, cholera toxin; CTB, cholera toxin B-subunit; ELISA, Enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; LPS, lipopolysaccharide; mCTA/LTB, A subunit of mutant cholera toxin E112K with the pentameric B subunit of heat-labile enterotoxin from enterotoxigenic *Escherichia coli*; PB, protein body; PBS, phosphate-buffered saline; RNAi, RNA interference; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SIgA, secretory IgA; T-DNA, transfer DNA.

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http://dx.doi.org/10.1016/j.vaccine.2012.04.064

weight. In the original study, we successfully expressed a low-molecular-weight CTB antigen (monomer, 11 kDa) at 30 μg per seed [3]. Here, we chose a nontoxic 45 kDa fragment of the C-terminal half of the heavy chain of botulinum neurotoxin type A (BoHc) to use as an example of a high-molecular-weight vaccine antigen to evaluate the advanced MucoRice expression system. Although the botulinum neurotoxin is known as oral poisons and is absorbed from the gut to reach peripheral nerve terminals via the blood circulation, the toxin also acts as an inhalant poison, which is absorbed from the airway [12]. The Hc fragment of type A (BoHc) has been successfully used as a nasal vaccine against botulism in mice and nonhuman primates [13].

Here, we could successfully express high yields of high molecular-weight BoHc in a soluble form with the use of an optimized RNAi vector. Because the rice-based BoHc vaccine (or MucoRice-BoHc) was water-soluble and could be purified easily by standard gel filtration, our results demonstrate that advanced MucoRice system can be used for the preparation of purified antigen for nasal immunization and the induction of protective immunity against a neurological toxin.

2. Materials and methods

2.1. DNA construction, transformation of rice plants, and purification of rice-based BoHc

The sequences encoding BoHc were synthesized with optimized codon usage for rice [3] and inserted into a binary T-DNA vector (pZH2B/35SNos) [14] with an overexpressing cassette of BoHc and a combination cassette for RNAi suppression of either 13 kDa prolamin or glutelin A or both storage proteins (Fig. 1A) as described previously [7]. A RNAi casette containing no RNAi trigger sequences for rice endogenous storage proteins was called pZH2BiK. RNAi cassettes containing RNAi trigger sequences for the suppression of the genes encoding 13 kDa prolamin and glutelin A were constructed and called pZH2Bik45 and pZH2BikG1B, respectively. The RNAi trigger sequence for the gene encoding 13 kDa prolamin was a 45 bp fragment of rice 13 kDa prolamin gene comprising coding sequence 1–45. The RNAi trigger sequence for glutelin gene was a 129 bp fragment of the rice glutelin A gene comprising coding sequence 142–270. The *Acs* I–*Mul* I fragment of the BoHc expression cassette was subcloned into pZH2BiK, pZH2Bik45, pZH2BikG1B, and pZH2Bik45-G1B. The expression vectors were used to transform a japonica variety of rice, *Nipponbare*, by using an *Agrobacterium*-mediated method described previously [3] and the recombinant BoHc produced was termed MucoRice-BoHc. The rice expressing BoHc together with a combination cassette for RNAi suppression of both 13 kDa prolamin and glutelin A was polished and extracted by using PBS and then purified by using gel filtration on a Sephadex G-100 column.

2.2. Preparation of recombinant proteins

A recombinant BoHc was constructed and produced by use of the *E. coli* expression system as previously described [13]. A nontoxic form of chimeric mucosal adjuvant that combines the A subunit of mutant cholera toxin E112K with the pentameric B subunit of heat-labile enterotoxin from enterotoxigenic *E. coli* (mCTA/LTB) was constructed and produced by use of the *Brevibacillus choshinensis* expression system as previously described [15]. The level of LPS contamination in the purified BoHc and mCTA/LTB (<10 endotoxin units/mg protein) were measured by using a Limulus Test (Wako).

2.3. Protein analyses

Total seed protein was extracted from transgenic rice plant seeds as described previously [3]. Briefly, seeds of rice plants were ground to a fine powder by using a Multibeads shocker (Yasui Kikai, Osaka, Japan) and extracted in the sample buffer (2% [w/v] SDS, 5% [w/v] β-mercaptoethanol, 50 mM Tris–HCl [pH 6.8], and 20% [w/v] glycerol) and the proteins were separated by SDS-PAGE followed by Western blot analysis with rabbit anti-BoHc antibody (Ab), which was established in our laboratory using *E. coli*-derived recombinant BoHc. The level of BoHc accumulated in the rice seeds was determined by densitometry analysis of a Western blot against a standard curve generated with the use of purified *E. coli*-derived BoHc, as previously described [3].

2.4. Immunohistochemical and immune electronmicroscopic analyses

To microscopically evaluate the localization of BoHc in the MucoRice-BoHc seed, a frozen section of the rice seed was reacted with polyclonal rabbit anti-BoHc Ab and visualized with the use of 3,3′-diaminobenzidine. We confirmed that normal rabbit IgG as a control showed no immune-reactivity in MucoRice-BoHc seed. The distribution of BoHc expressed in rice seeds was analyzed by using immunoelectron microscopy with polyclonal rabbit anti-BoHc Ab as described previously [16].

2.5. Immunization, sample preparation and ELISA for detection of BoHc-Ab

To examine the mucosal immunogenicity of MucoRice-BoHc, the purified material of MucoRice-BoHc (100 μg) alone, *E. coli*-derived rBoHc (100 μg) alone, MucoRice-BoHc (25 μg) with or without CT (1 μg, List Biological Laboratories, Campbell, CA) or MucoRice-BoHc (25 μg) with mCTA/LTB (10 μg) dissolved in 20 μl of PBS, or PBS vehicle alone was intranasally immunized in mice (10 μl/nostril, $N = 10$) on 3 occasions at 1 wk intervals. The serum and nasal wash were collected prior to immunization, and 1 wk after each immunization. BoHc-specific Ab responses were determined by using BoHc-specific enzyme-linked immunosorbent assay (ELISA) as described previously [17]. Endpoint titers were expressed as the reciprocal log2 of the last dilution that gave an OD450 of 0.1 greater than that of the negative control.

2.6. Preparation of botulinum neurotoxin A (BoNT/A)

BoNT/A from *C. botulinum* type-A 62 was purified from the culture supernatant as previously described [18]. The toxicity of purified BoNT/A (1.1×10^8 mouse i.p. LD_{50}/mg protein) was assayed by time to death after intravenous injection into mice [19].

2.7. Neutralizing assay

To analyze the protective activity of antigen-specific mucosal Ab immune responses induced by the use of purified MucoRice-BoHc as a nasal vaccine against toxin-induced neurological death, we performed a toxin challenge study, as described previously with some modification [17]. Briefly, the immunized mice were intraperitoneally challenged with 100 ng (1.1×10^4 i.p. LD_{50}) or 500 ng of BoNT/A (5.5×10^4 i.p. LD_{50}) diluted in 100 μl of 0.2% gelatin/PBS, and their survival was observed for 7 days.

2.8. Data analysis

Differences between groups were assessed by Tukey's *t*-test for ELISA data and the log-rank (Mantel–Cox) test for survival analyses. *P* values < 0.05 were considered to indicate statistical significance.

3. Results

3.1. Establishment of rice-expressed botulinum type A neurotoxin vaccine

To increase the levels of high-molecular weight vaccine antigens, such as BoHc, that can be accumulated in rice grains, we constructed T-DNA vectors containing expression cassettes with four combinations of RNAi triggers specific for different storage protein genes together with a vaccine gene cassette (Fig. 1). Several independent transgenic rice lines were generated for each of the four types of MucoRice and the BoHc accumulation levels in seeds were determined by densitometry analysis of Western blots. For each of the four types of MucoRice, the plant line with the highest levels of BoHc antigen accumulated in the seed was selected and advanced to the T3 generation by self-crossing to obtain homozygous lines.

SDS-PAGE showed that the content of 13 kDa prolamin and/or glutelin in MucoRice BoHc lines were approximately half that of the WT (Fig. 2B). Examination of the expression levels of BoHc by densitometry analysis revealed that the expression level of BoHc in rice seeds suppressing both 13 kDa prolamin and glutelin A reached an average of 100 μg per seed, whereas rice seeds carrying the vector with no RNAi trigger obtained an average of 10 μg BoHc per seed. Seeds carrying vectors that suppressed 13 kDa prolamin alone or glutelin A alone obtained an average of 10 μg or 30 μg BoHc per seed, respectively. All further experiments were performed with MucoRice-BoHc derived from rice suppressing both 13 kDa prolamin and glutelin A.

When MucoRice-BoHc was extracted by PBS, densitometry analysis showed that an average 85% of all the MucoRice-BoHc was recovered in PBS (Fig. 2C). Because the MucoRice-BoHc was soluble in PBS, we were able to purify the protein from polished rice without the aleurone layer by using gel filtration (Fig. 2C). The yield of purified BoHc from the total amount of harvested rice seeds was approximately 68% (an average of 68 μg per seed). The level of LPS contamination in purified MucoRice-BoHc was less than 10 endotoxin units/mg protein, which was equivalent to that in highly purified rBoHc from the *E. coli* expression system.

3.2. MucoRice-BoHc accumulated in the cytoplasm between protein bodies in rice seeds

Because expression of BoHc was under the control of the 13 kDa prolamin-specific promoter as a rice-seed-specific promoter (Fig. 1), we examined the location of BoHc expression in MucoRice-BoHc seeds. The results of immunohistochemistry showed that BoHc accumulated in the endosperm cells under

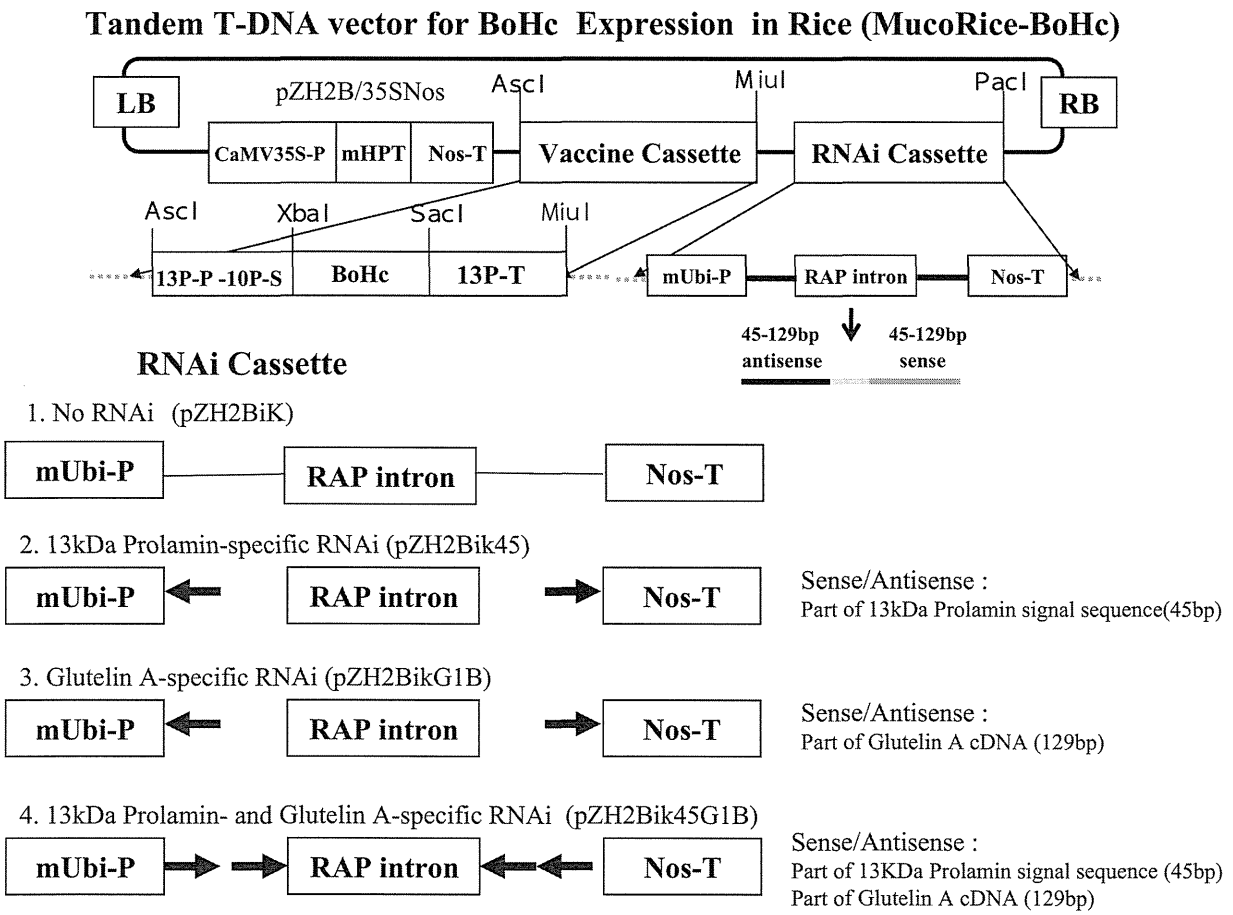

Fig. 1. T-DNA plasmids for RNAi suppression of storage proteins and overexpression of BoHc in rice seed. We constructed a tandem T-DNA plasmid containing a BoHc overexpression cassette with BoHc vaccine antigen sequences controlled by the promoter of rice 13 kDa prolamin, and a combination cassette of RNAi triggers for suppression of major rice endogeneous storage proteins, 13 kDa prolamin and/or glutelin controlled by the ubiquitin promoter. CaMV35S-P, cauliflower mosaic virus 35S promoter; mHPT, mutant hygromycin phosphotransferase; 13P-P, 13 kDa prolamin promoter; 10P-S, signal sequence of 10 kDa prolamin; 13P-T, 13 kDa prolamin terminator; Nos-T, nos terminator; RAP intron, rice aspartic protease intron; Ubi-P, ubiquitin promoter; LB, T-DNA left border; RB, T-DNA right border.

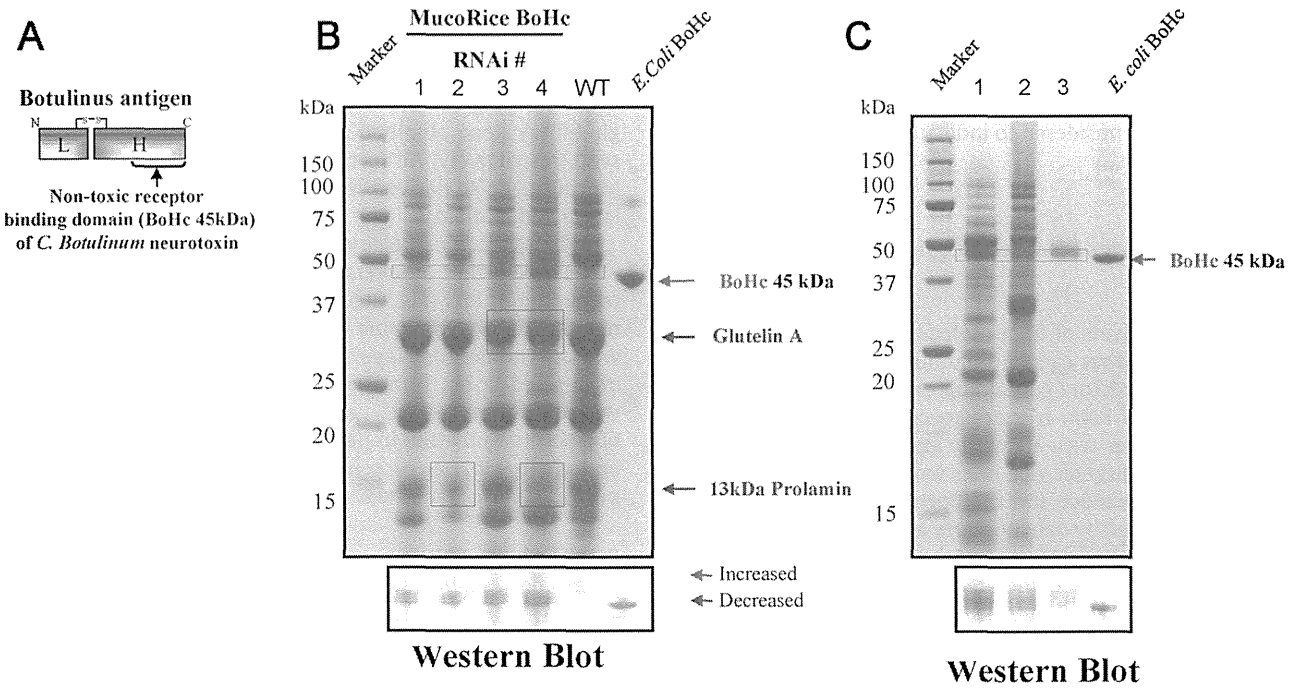

Fig. 2. Expression and purified MucoRice-BoHc. (A) Conceptual scheme of BoHc antigen expressed in MucoRice or *E. coli*. (B) SDS-PAGE analysis comparing protein expression among MucoRice-BoHc lines containing various T DNA plasmids: #1, no RNAi suppression; #2, RNAi suppression of 13 kDa prolamin; #3, RNAi suppression of glutelin A; #4, RNAi suppression of 13 kDa prolamin and glutelin A. WT indicates wild-type rice, and *E. coli* BoHc indicates recombinant BoHc from *E. coli* system. The results showed predominant expression of MucoRice-BoHc with a molecular weight of approximately 48 kDa (red arrowhead, *E. coli* derived BoHc with 45 kDa). Endogenous rice proteins (blue arrows, glutelin A and 13 kDa prolamin) were reduced in MucoRice-BoHc with RNAi suppression of glutelin and/or 13 kDa prolamin compared with levels in non-transformed WT rice. Western blotting revealed that a transgenic protein of 48 kDa specifically reacted with anti-BoHc Ab. (C) The samples were analyzed by SDS-PAGE and Western blot with rabbit anti-BoHc. 1, extract from MucoRice-BoHc transgenic #4 seed (with RNAi suppression of both 13 kDa prolamin and glutelin A) powder produced by using PBS; 2, extract from the MuocRice-BoHc #4 seed powder produced by re-extracting the PBS extract with SDS-sample buffer [2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 50 mM Tris–HCl (pH 6.8)]; 3, purified MucoRice-BoHc, which was isolated from PBS-extract of the MucoRice-BoHc #4 seed powder by using a gel filtration on Sephadex G-100 column. rBoHc indicates BoHc purified from *E. coli*.

the aleurone layer in MucoRice-BoHc seeds, whereas immunereactivity was absent in wild-type rice seeds (Fig. 3A).

Furthermore, immune-electron-microscopy analysis of MucoRice-BoHc seeds showed that the vaccine antigens were unexpectedly expressed in cytoplasm at the interspace between PB-I and PB-II (Fig. 3B). It is interesting to note that destruction of PB-II was found in MucoRice-BoHc seeds but not WT rice. Because expression level of rice storage proteins including 13 kDa prolamin and glutelin A in MucoRice-BoHc seeds were suppressed (Fig. 2B), the RNAi knockdown on MucoRice system most likely accounts for the abnormal storage organelle formation.

3.3. Nasal MucoRice-BoHc induces not only systemic but also mucosal antigen-specific Ab immune responses

To examine whether purified MucoRice-BoHc antigen maintained sufficient immunogenicity to induce a protective Ab response, mice were nasally immunized with PBS (vehicle control) or 100 μg of purified MucoRice-BoHc or rBoHc from an *E. coli*–BoHc expression system. Antigen-specific Ab responses were assessed by using *E. coli*-derived BoHc as a coating antigen for ELISAs. After nasal immunization, the levels of BoHc-specific IgG Ab titers were not significantly different between mice nasally immunized with MucoRice-BoHc and those nasally immunized with *E. coli*-derived rBoHc (Fig. 4A). However, a detectable level of rBoHc-specific secretory IgA (SIgA) Ab titers was found in the nasal washes of mice that were nasally immunized with MucoRice-BoHc (Fig. 4A).

To confirm whether the immunogenicity of MucoRice-BoHc was sufficient for the induction of antigen-specific mucosal immunity, mice were nasally immunized with 25 μg of purified MucoRice-BoHc with or without CT or nontoxic chimera mCTA/LTB as mucosal adjuvants. Both nasal MucoRice-BoHc with CT and nasal MucoRice-BoHc with mCTA/LTB induced brisk rBoHc-specific serum IgG and nasal IgA Ab immune responses (Fig. 4B).

3.4. Nasal MucoRice-BoHc induces protective immunity against botulinum neurotoxin

Next, a challenge test with BoNT/A was performed on all BoHc-vaccinated mice and control mice to examine the quality of BoHc-specific Ab induced. Mice that were vaccinated with 100 μg of purified MucoRice-BoHc or *E. coli*-derived rBoHc without the presence of mucosal adjuvant, but not the control mice, were partially protected against the high lethal dose (100 ng, 1.1×10^4 i.p. LD_{50}) of intraperitoneally injected BoNT/A (Fig. 5A). The mice that were nasally immunized with 25 μg of purified MucoRice-BoHc with CT or mCTA/LTB were completely protected against the extraordinarily high lethal dose (500 ng, 5.5×10^4 i.p. LD_{50}) of intraperitoneally injected BoNT/A, since the mice nasally immunized with 25 μg of MucoRice-BoHc or PBS only failed to protect against the same amount of toxin (Fig. 5B). In the group of mice that were nasally immunized with 25 μg of MucoRice-BoHc with CT or mCTA/LTB, there were no clinical signs of toxin-associated disease over a 1 wk observation period following the BoNT/A challenge test.

4. Discussion

One of our major goals was the adoption of RNAi technology to advance the MucoRice system by co-introduction of antisense sequences specific for endogenous rice storage proteins. We used

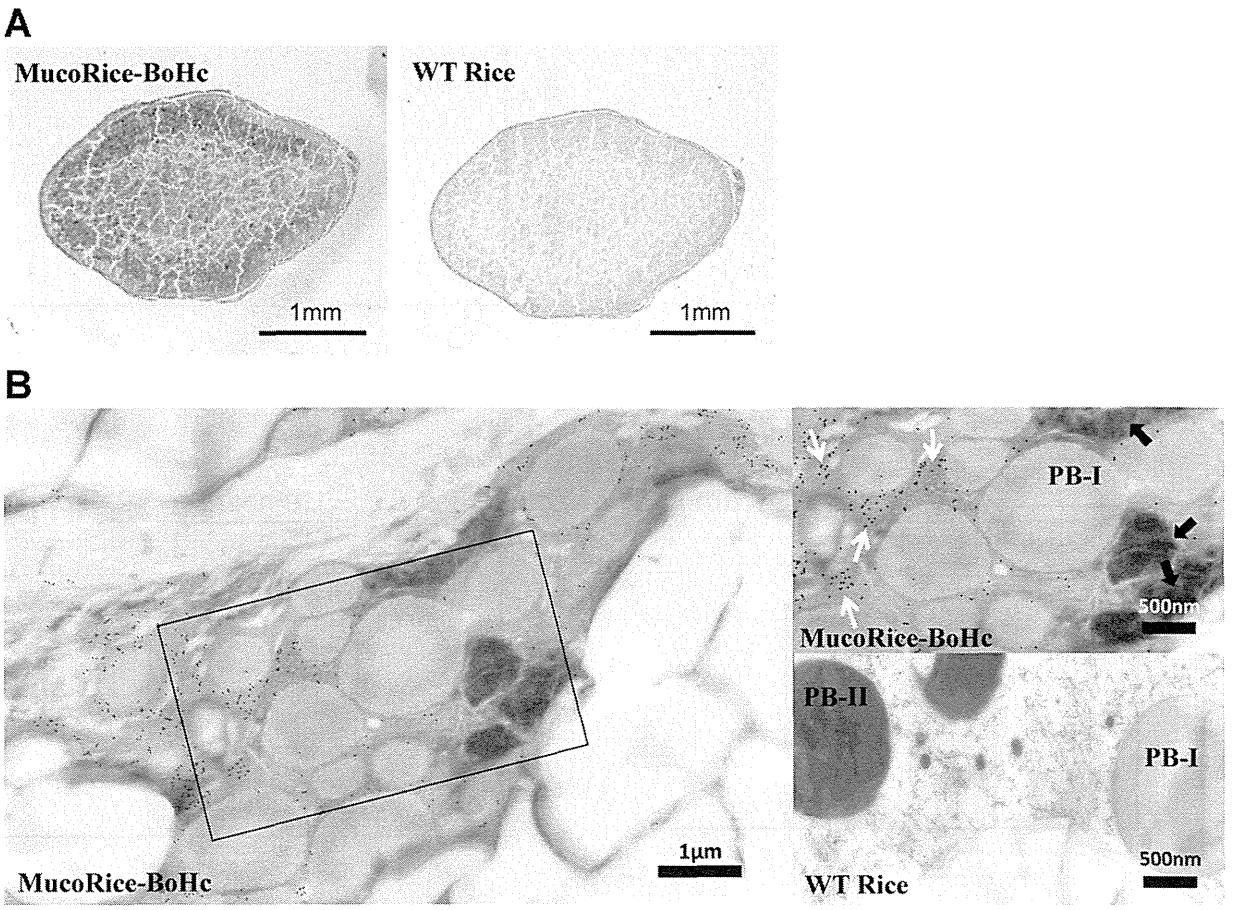

Fig. 3. Immunohistochemical and immune electronmicroscopic analyses of MucoRice-BoHc seed. (A) Immunohistochemical analysis showed that BoHc had accumulated in the whole MucoRice-BoHc seed, whereas it was not detected in a WT-rice seed. (B) Immune electronmicroscopy of MucoRice-BoHc showed that BoHc in the endosperm cells of MucoRice-BoHc was observed as black spots (white arrows). BoHc was predominantly localized in cytoplasm as the interspace between PB-I and PB-II. Destruction of PB-II structure was found in MucoRice-BoHc seed (black arrows) when compared with WT rice seed.

a high-molecular-weight protein vaccine antigen – the 45 kDa C-terminal half (BoHc) of botulinum neurotoxin [13,17] – to showcase the practicality of using this system to improve the capacity for vaccine antigen accumulation in rice seed. To optimize the choice of endogenous storage protein to suppress, we performed RNAi suppression of rice 13 kDa prolamin, glutelin A, or both by altering the sequences included in RNAi cassette in the T-DNA vector and then assessed the level of expression of the antigen MucoRice-BoHc. SDS-PAGE and western blot analysis revealed that suppressing both 13 kDa prolamin and glutelin A greatly improved the production of high-molecular-weight vaccine antigen, BoHc (100 μg/seed) when compared the T-DNA vector without RNAi (10 μg/seed) (Fig. 2B). Thus, introduction of the RNAi technology into the MucoRice vaccine antigen expression system allowed a high-molecular-weight vaccine antigen to be expressed in MucoRice with high yields.

Another unique feature of the BoHc antigen produced by using the advanced MucoRice system was its water-solubility. Because proteins in PBs are not soluble in water [10,11], the unique and advanced property of MucoRice-BoHc comes from its expression in cytoplasm between PB-I and PB-II of endosperm cells in rice seed (Fig. 3B). Because we use prolamin promoter and signal for targeting BoHc to PB-I or PB-II in rice endosperm cells, we cannot explain logically why the vaccine antigen accumulated in cytoplasm and not in PBs. However, BoHc antigen location and level of accumulation might be attributed to abnormal storage organelle formation including destruction of PB-II structure caused by the knock-down of glutelin A in the advanced MucoRice system (Fig. 3B). The MucoRice-BoHc from rice powder of polished rice could be easily dissolved in and extracted from PBS and could be purified by a single-step gel filtration without endotoxin contamination. Thus, the high expression level and PBS-solubility of MucoRice-BoHc confer an economic advantage over vaccine products that are expressed in bacteria or other plant systems.

SDS-PAGE analysis indicated that the molecular weight of MucoRice-BoHc (48 kDa) was slightly higher than that of rBoHc from *E. coli* (45 kDa) (Fig. 2B and C). Because there are nine glycosylation-sequon (N-X-T/S) positions in the sequence of BoHc [20], we considered that several sugar chains attach to MucoRice-BoHc at these positions but not to rBoHc from *E. coli*. SDS-PAGE, and Western blot analysis detected a least 3–4 bands corresponding to MucoRice-BoHc, whereas *E. coli*-derived rBoHc was detected as a single band only (Fig. 2B and C). These results suggest that MucoRice-BoHc is a mixture of fully and partially glycosylated protein.

We next examined whether MucoRice-BoHc was highly immunogenic when compared with *E. coli*-derived rBoHc after nasal administration in mice. When mice were nasally immunized with 100 μg of purified MucoRice-BoHc or *E. coli*-derived rBoHc, there are no statistical differences in BoHc-specific IgG immune responses and protective immunity against high lethal dose of a neurotoxin challenge test between mice immunized with MucoRice-BoHc and *E. coli*–derived rBoHc. Taken together, these results demonstrate that the quality of the toxin neutralization

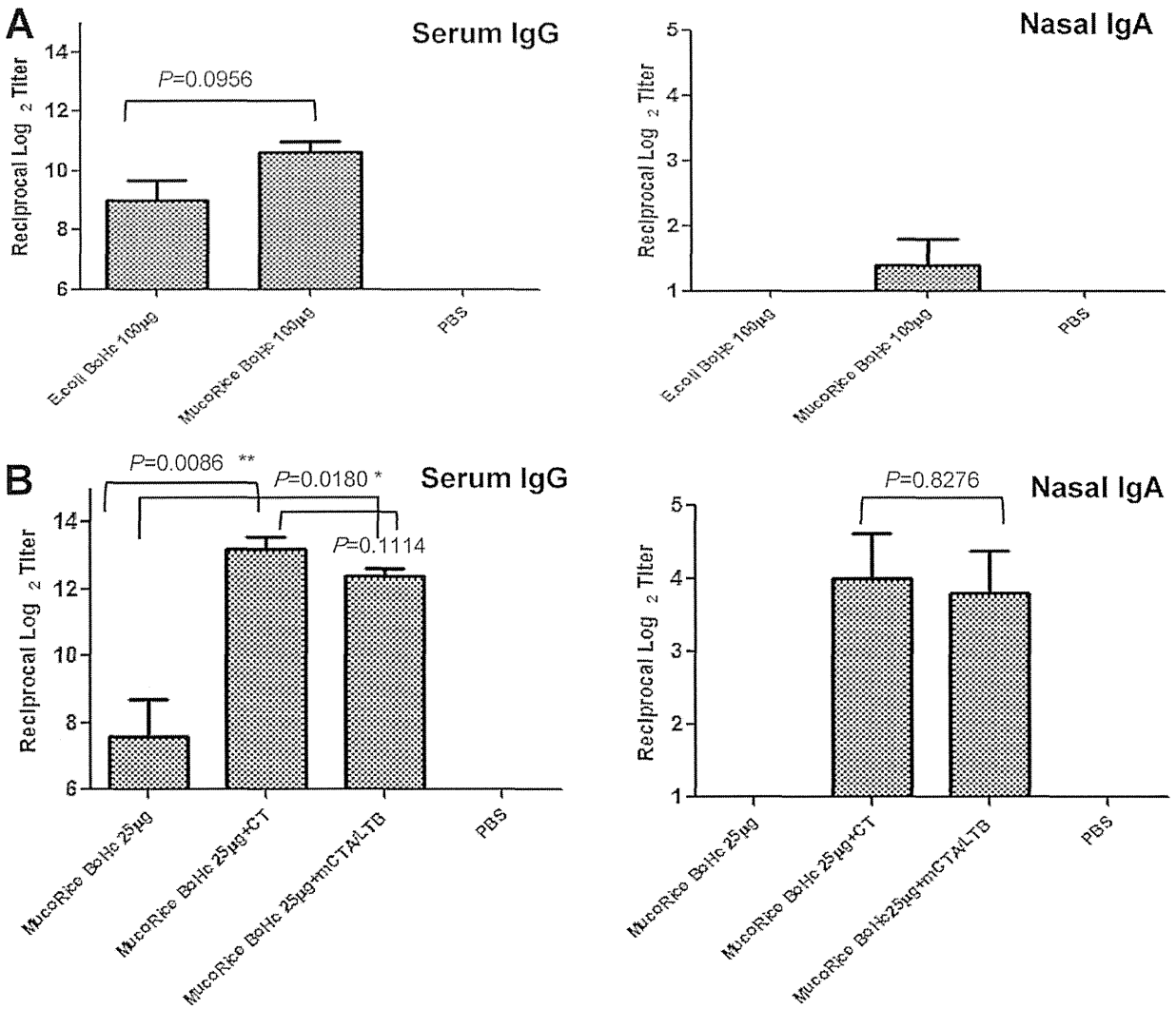

Fig. 4. Immunogenicity of purified MucoRice-BoHc produced by using advanced MucoRice system. (A) Mice were nasally immunized with 100 μg of purified MucoRice-BoHc alone on 3 occasions at 1-week intervals and compared with mice that were nasally immunized with same dose of recombinant BoHc prepared by means of a standard *E. coli* system (*E. coli*-BoHc). (B) In a separate study, mice were immunized with nasal vaccine containing 25 μg purified MucoRice-BoHc with or without mucosal adjuvant cholera toxin (1 μg) or nontoxic chimera adjuvant (mCTA/LTB, 10 μg) on 3 occasions at 1-week intervals. Data are expressed as means ± standard deviation ($N = 10$). The experiments were conducted three times. $^{*}P < 0.05$; $^{**}P < 0.01$.

Ab activity induced by MucoRice-BoHc was comparable to that induced by *E. coli*-derived rBoHc and that the immunogenicity of the MucoRice-BoHc and *E. coli*-derived rBoHc mucosal vaccines was equivalent.

To examine whether MucoRice-BoHc could induce high levels of mucosal immune response when nasally administered together with mucosal adjuvant, mice were immunized with nasal vaccine composed of 25 μg of MucoRice-BoHc and mucosal adjuvant CT or mCTA/LTB. As one might expect, antigen-specific serum IgG and nasal IgA Ab responses were rapidly induced in mice immunized with nasal MucoRice-BoHc plus adjuvant. Further, there was no difference between the antigen-specific immune responses induced by nasal MucoRice-BoHc administered with CT or mCTA/LTB. Native CT is a potent enterotoxin and induces high total and vaccine-specific immunoglobulin E responses; however, use of the mCTA/LTB chimera adjuvant did not have the same effect in our previous study [15]. To confirm the quality of BoHc-specific antibodies induced by MucoRice-BoHc plus CT or mCTA/LTB, we performed a challenge test on vaccinated mice by using an extraordinarily high lethal dose (5.5×10^4 mouse i.p. LD_{50}) of intraperitoneally injected BoNT/A. Regardless of the adjuvant used, the immunized mice survived after the challenge, indicating that they had gained full protection from the neurotoxin. In contrast, the non-immunized mice died within 12 h. These results suggest that MucoRice-BoHc with nontoxic chimera adjuvant CTA/LTB has the potential to be used as a promising nasal vaccine against botulism.

Acknowledgments

This work was supported by grants from the Programs of Special Coordination Funds for Promoting Science and Technology, a Grant-in-Aid for Scientific Research on Priority Areas, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (H.K.); the Ministry of Health, Labor and Welfare of Japan (Y.Y., H.K.); Core Research of Evolutional Science Technology (CREST) of the Japan Science and Technology Agency (JST) (H.K.); New Energy and Industrial Technology Development Organization (NEDO) (Y.Y., H.K.); Young Researcher Overseas Visits Program for Vitalizing Brain Circulation of the Japan Society for the Promotion of Science (JSPS) (H.K., Y.Y.); the Global Center of Excellence Program "Center of Education and

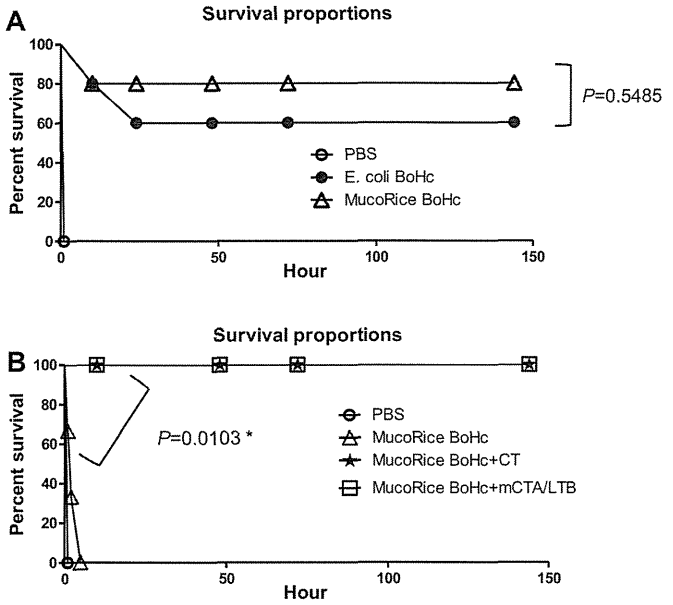

Fig. 5. Induction of protective immunity the against neurotoxin in mice immunized with nasal vaccine containing MucoRice-BoHc and CT or nontoxic mCTA/LTB. (A) Mice immunized with 100 μg of purified MucoRice-BoHc or *E. coli*-derived rBoHc were challenged with a high lethal dose (100 ng, 1.1×10^4 mouse i.p. LD_{50}) of intraperitoneal injection of BoNT/A. (B) Mice immunized with 25 μg of purified MucoRice-BoHc together with CT or mCTA/LTB were challenged with an extraordinarily high lethal dose (500 ng, 5.5×10^4 mouse i.p. LD_{50}) of intraperitoneal injection of BoNT/A. Immunization conditions were described in Fig. 4A and B. The experiments were conducted three times. $^*P < 0.05$; $^{**}P < 0.01$.

Research for Advanced Genome-Based Medicine – For Personalized Medicine and the Control of Worldwide Infectious Diseases" (H.K.); a Research Fellowship from the Japan Society for the Promotion of Science (T.N.); the Japan Foundation for Pediatric Research (D.T.). We are grateful to Drs. T. Masumura, K. Tanaka, and Ms. J.Y. Chen for their technical supports.

References

[1] Paul M, Ma JK. Plant-made immunogens and effective delivery strategies. Expert Review of Vaccines 2010;9:821–33.
[2] Streatfield SJ, Jilka JM, Hood EE, Turner DD, Bailey MR, Mayor JM, et al. Plant-based vaccines: unique advantages. Vaccine 2001;19:2742–8.
[3] Nochi T, Takagi H, Yuki Y, Yang L, Masumura T, Mejima M, et al. Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. Proceedings of the National Academy of Sciences of the United States of America 2007;104:10986–91.
[4] Nochi T, Yuki Y, Katakai Y, Shibata H, Tokuhara D, Mejima M, et al. A rice-based oral cholera vaccine induces macaque-specific systemic neutralizing antibodies but does not influence pre-existing intestinal immunity. Journal of Immunology 2009;183:6538–44.
[5] Yuki Y, Tokuhara D, Nochi T, Yasuda H, Mejima M, Kurokawa S, et al. Oral MucoRice expressing double-mutant cholera toxin A and B subunits induces toxin-specific neutralising immunity. Vaccine 2009;27:5982–8.
[6] Tokuhara D, Yuki Y, Nochi T, Kodama T, Mejima M, Kurokawa S, et al. Secretory IgA-mediated protection against *V. cholerae* and heat-labile enterotoxin-producing enterotoxigenic Escherichia coli by rice-based vaccine. Proceedings of the National Academy of Sciences of the United States of America 2010;107:8794–9.
[7] Kuroda M, Kimizu M, Mikami C. A simple set of plasmids for the production of transgenic plants. Bioscience, Biotechnology, and Biochemistry 2010;74:2348–51.
[8] Kohno-Murase J, Murase M, Ichikawa H, Imamura J. Effects of an antisense napin gene on seed storage compounds in transgenic *Brassica napus* seeds. Plant Molecular Biology 1994;26:1115–24.
[9] Yamagata H, Sugimoto T, Tanaka K, Kasai Z. Biosynthesis of storage proteins in developing rice seeds. Plant Physiology 1982;70:1094–100.
[10] Masumura T, Hibino T, Kidzu K, Mitsukawa N, Tanaka K, Fujii S. Cloning and characterization of a cDNA encoding a rice 13 kDa prolamin. Molecular and General Genetics 1990;221:1–7.
[11] Katsube T, Kurisaka N, Ogawa M, Maruyama N, Ohtsuka R, Utsumi S, et al. Accumulation of soybean glycinin and its assembly with the glutelins in rice. Plant Physiology 1999;120:1063–74.
[12] Fujihashi K, Staats HF, Kozaki S, Pascual DW. Mucosal vaccine development for botulinum intoxication. Expert Review of Vaccines 2007;6:35–45.
[13] Yuki Y, Nochi T, Harada N, Katakai Y, Shibata H, Mejima M, et al. In vivo molecular imaging analysis of a nasal vaccine that induces protective immunity against botulism in nonhuman primates. Journal of Immunology 2010;185:5436–43.
[14] Hajdukiewicz P, Svab Z, Maliga P. The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Molecular Biology 1994;25:989–94.
[15] Kweon MN, Yamamoto M, Watanabe F, Tamura S, Van Ginkel FW, Miyauchi A, et al. A nontoxic chimeric enterotoxin adjuvant induces protective immunity in both mucosal and systemic compartments with reduced IgE antibodies. The Journal of Infectious Diseases 2002;186:1261–9.
[16] Saito Y, Shigemitsu T, Tanaka K, Morita S, Satoh S, Masumura T. Ultrastructure of mature protein body in the starchy endosperm of dry cereal grain. Bioscience, Biotechnology, and Biochemistry 2010;74:1485–7.
[17] Nochi T, Yuki Y, Takahashi H, Sawada S, Mejima M, Kohda T, et al. Nanogel antigenic protein-delivery system for adjuvant-free intranasal vaccines. Nature Materials 2010;9:572–8.
[18] Sakaguchi G. Clostridium botulinum toxins. Pharmacology & Therapeutics 1982;19:165–94.
[19] Kondo H, Shimizu T, Kubonoya M, Izumi N, Takahashi M, Sakaguchi G. Titration of botulinum toxins for lethal toxicity by intravenous injection into mice. Japanese Journal of Medical Science & Biology 1984;37:131–5.
[20] Byrne MP, Smith TJ, Montgomery VA, Smith LA. Purification, potency, and efficacy of the botulinum neurotoxin type A binding domain from *Pichia pastoris* as a recombinant vaccine candidate. Infection and Immunity 1998;66:4817–22.

Journal of Medical Virology 84:336–344 (2012)

Characterization of Neutralizing Antibodies in Adults After Intranasal Vaccination With an Inactivated Influenza Vaccine

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The levels and properties of neutralizing antibodies in nasal wash and serum collected from five healthy adults were examined after intranasal administration of an A/Uruguay/716/2007 (H3N2) split vaccine (45 μg hemagglutinin (HA) per dose; five doses, with an interval of 3 weeks between each dose). Prior to the assays, nasal wash samples were concentrated so that the total amount of antibodies was equivalent to about 1/10 of that found in the natural nasal mucus. Vaccination induced virus-specific neutralizing antibody responses, which increased with the number of vaccine doses given. Neutralizing antibodies were produced more efficiently in the nasal passages than in the serum: A ≥4-fold increase in nasal neutralization titres was observed after the second vaccination in four out of five subjects, whereas a rise in serum neutralization titres was observed only after the fifth vaccination. Nasal and serum neutralizing antibodies were mainly found in the polymeric IgA and monomeric IgG fractions, respectively, after gel filtration. Taken together, these results suggest that intranasal administration of an inactivated split vaccine induces high levels of nasal neutralizing antibodies (primarily polymeric IgA) and low levels of serum neutralizing antibodies (primarily monomeric IgG). ***J. Med. Virol. 84:336–344, 2012.***

KEY WORDS: influenza; vaccine; neutralizing antibody

INTRODUCTION

To prevent influenza, protective immunity must be induced in advance by administration of a vaccine. Currently available inactivated vaccines, detergent disrupted split-viruses, or purified glycoproteins (surface antigen vaccines) are given via parenteral injection [Murphy and Webster, 1996]. Parenteral vaccination, that is, vaccination via the non-mucosal route, induces serum IgG antibodies, which are highly protective against homologous virus infection, but less effective against heterologous virus infection. Thus, intramuscular vaccination of seasonal influenza vaccine would be less effective in protecting against a heterologous virus epidemic.

A large number of studies show that the protective immunity induced by influenza virus infection is mainly mediated by secretory IgA (S-IgA) and IgG antibodies within the respiratory tract. S-IgA is carried to the mucus by transepithelial transport, while serum IgG is transported from the serum to the mucus by diffusion [Murphy and Clements, 1989; Brandtzag et al., 1994; Murphy, 1994; Asahi et al., 2002; Asahi-Ozaki et al., 2004]. S-IgA in the upper respiratory tract prevents viral infection, while IgG supports S-IgA-mediated protection by neutralizing newly-generated viruses [Ito et al., 2003; Renegar et al., 2004]. IgG is the main antibody involved in anti-viral protection in the lungs [Ramphal et al., 1979; Palladino et al., 1995; Renegar et al., 1998; Ito et al., 2003]. Also, polymeric S-IgA neutralizes viruses more effectively than monomeric IgA or IgG [Taylor

Grant sponsor: Japanese Ministry of Health, Labor, and Welfare.

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Received 3 October 2011
DOI 10.1002/jmv.22273
Published online in Wiley Online Library (wileyonlinelibrary.com).

and Dimmock, 1985; Renegar et al., 1998]. The polymeric nature of S-IgA also explains why S-IgA cross-reacts with variant influenza viruses to a greater extent than serum IgG [Tamura et al., 1990, 1991, 1992; Asahi-Ozaki et al., 2004]. Thus, intranasal administration of an inactivated influenza vaccine is advocated to elicit S-IgA and IgG responses and improve the protective efficacy of current vaccination procedures [Tamura and Kurata, 2004; Tamura et al., 2005, 2010].

Several clinical trials have examined the induction of both S-IgA and IgG following intranasal administration of inactivated influenza vaccines, either with or without adjuvant [Kuno-Sakai et al., 1994; Hashigucci et al., 1996; Muszkat et al., 2000; Greenbaum et al., 2002; Durrer et al., 2003; Treanor et al., 2006; Atmar et al., 2007]. The antibody responses after intranasal administration of inactivated influenza vaccines were assessed by measuring hemagglutination inhibition (HI) titres in the serum, and anti-hemagglutinin (HA) IgA and IgG titres in nasal wash samples. They did not measure the titre of neutralizing antibodies, which is considered to be a better criterion for functional protective antibodies. Neutralization titres can directly inhibit the complex process involved in virus replication, which include virus attachment and entry to the host cells, and release of newly-synthesized virus from the infected cells in tissue culture. In addition, a previous study found that HI titres were lower, or higher, than the corresponding neutralization titres, depending on a strain of influenza A or B virus used for the assay [Okuno et al., 1990], whereas other studies show that anti-H5 HI antibodies fail to detect H5N1 viruses [Lu et al., 1982; Rowe et al., 1999]. Thus, neutralizing antibody responses following intranasal administration of an inactivated influenza vaccine remain to be fully characterized.

Therefore, the aim of the present study was to examine the levels and properties of neutralizing-antibodies in nasal wash and serum samples from healthy adults after intranasal administration of an inactivated vaccine (five doses, with an interval of 3 weeks between each dose). The inactivated vaccine used in this study was a concentrated split-virus vaccine (containing 45 μg HA per dose), prepared from the A/Uruguay/716/2007 (H3N2) strain. A concentrated split-virus vaccine was chosen because the vaccine has already been shown to induce mucosal antibody responses after intranasal vaccination [Kuno-Sakai et al., 1994]. To ensure that neutralization titres specific for the A/Uruguay/716/2007 virus were assayed at equivalent levels in both serum and nasal wash samples, the neutralization titres were measured using concentrated nasal wash samples (1 mg/ml total protein) that contained approximately 1/10 of the IgA found in undiluted mucus [Kurono and Mogi, 1987]. The properties of the neutralizing IgA and IgG antibodies induced by intranasal vaccination were then examined, and their relative levels and molecular size were determined.

MATERIALS AND METHODS

Subjects

Five healthy male subjects (P1, P2, P3, P4, and P5) were enrolled in the study (aged 22, 32, 42, 42, and 68 years, respectively, at the time of the first vaccination). All participants had already acquired some degree of immunity to H1N1 and H3N2 influenza A virus subtypes after previous exposure to these viruses and/or as a result of previous vaccinations. Each subject provided informed consent and the study protocol and other relevant documentation were reviewed and approved by the Ethics Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Virus and Vaccine

The A/Uruguay/716/2007 (A/Uruguay; H3N2) influenza virus strain was propagated in the allantoic cavity of 10-day-old embryonated hen's eggs and purified from the allantoic fluid. The $TCID_{50}$ (50% infectious dose in tissue culture) of the virus was estimated as described previously [Tobita et al., 1975; Kadowaki et al., 2000]. In brief, 10-fold serial dilutions of the allantoic fluid containing the virus were inoculated into Madin-Darby canine kidney (MDCK) cells (ATCC No. CCL-34) cells in 96-well culture plates and incubated for 4 days at 37°C in a 5% CO_2 humidified atmosphere. The cytopathic effects in the virus-containing wells were monitored under a microscope and the $TCID_{50}$ was calculated using the Reed–Muench method. The split product virus vaccine was supplied by the Research Foundation for Microbial Disease of Osaka University (BIKEN, Kanonji, Japan). The vaccine was prepared from purified viruses, which were sedimented through a linear sucrose gradient according to the manufacturer's protocol. The viruses were then treated with ether and formalin according to the manufacturer's protocol, which was based on the method of Davenport et al. [1964]. The concentrated split vaccine containing 45 μg HA was the product of a process used to prepare a trivalent vaccine comprising A/H1N1, A/H3N2, and B type vaccines, each containing 15 μg HA.

Vaccinations

All participants were immunized intranasally with a threefold concentrated split H3N2 virus vaccine (A/Uruguay, containing 45 μg HA). Each received five doses, with an interval of 3 weeks between each dose. Intranasal vaccination was performed by spraying 0.25 ml of the split vaccine into each nostril (0.5 ml total) using an atomizer (Keytron, Ichikawa, Japan). The mean droplet diameter was 56.5 μm, ranging in size between 10 μm and 90 μm.

Nasal Wash and Serum Samples

About 100 ml of nasal wash was collected from each participant in polypropylene tubes by washing the

nasal cavity several times using a nose irrigation device (Hananoa; Kobayashi Pharmaceutical, Osaka, Japan) filled with saline solution according to the manufacturer's instructions. Pieces of dental cotton (Dental Cotton Roll; B.S.A. Sakurai, Nagoya, Japan) were then immersed in the collected nasal washes. Dental cotton pieces (containing a combined absorbed volume of about 25 ml of nasal wash) were then placed into a filter insert (Oxi Fil filter insert; TOHO, Tokyo, Japan) with bottoms drilled to create several pores, and placed in 50 ml polypropylene centrifuge tubes. Clean nasal wash was separated from mucopolysaccharides and other debris by centrifugation at 2,200*g* for 5 min at room temperature. This procedure was repeated for the entire 100 ml nasal wash sample from each participant. The pooled, clean nasal wash was then concentrated to a final volume of approximately 0.5 ml using Vivaspin centrifugal concentrators (Vivaspin 20, MWCO = 30,000; Sartorius Stedim Biotech, Aubagne, France). The concentrated nasal wash was stored at −80°C before use.

Quantitation of IgA, IgG and IgM Antibodies and Other Proteins

The levels of human IgA, IgG, and IgM antibodies in the nasal wash and serum samples were estimated using human IgA, IgG, or IgM ELISA kits (Bethyl Laboratories, Montgomery, USA). The level of human serum albumin in the nasal wash samples was estimated using a Human Albumin ELISA kit (Bethyl Laboratories). The protein concentration in the samples was measured using either a BCA Protein Assay Kit, or a Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Yokohama, Japan) according to the manufacturer's instructions.

Neutralization Assays

The level of serum antibodies against the vaccine viruses was examined using micro-neutralization assays as previously described [Belshe et al., 2000; Kadowaki et al., 2000] with minor modifications. In brief, serum samples were treated with a receptor-destroying enzyme (RDE(II); Denka Seiken, Tokyo, Japan) overnight at 37°C and heat-inactivated for 30 min at 56°C before use. The first dilution tested in the assays was 1:10. The concentrated nasal wash samples [1 mg/ml total protein, corresponding to about 1/10 of the total IgA found in nasal mucus (2.20 mg/ml)] [Kurono and Mogi, 1987] were also treated with RDE(II) and heat-inactivated before use. The first dilution tested in the nasal wash assays was 1:20. Twofold serial dilutions of the serum samples were mixed with an equal volume (50 μl) of diluent containing influenza virus equivalent to 100 $TCID_{50}$. Each mixture was added to the wells of a 96-well plate containing a monolayer of MDCK cells. Four control wells were included on each plate and contained either virus or diluent alone. The plates were then incubated for 4 days at 37°C in a 5% CO_2-humidified atmosphere. The monolayer in each well was observed for the presence or absence of cytopathic effects, fixed with 10% formalin for more than 5 min at room temperature, and stained with Naphthol blue black. After the plates were washed and dried, the stained cells were solubilized with 0.1 M NaOH and the absorbance (A) was measured at 630 nm. The average $A_{630\ nm}$ value was determined from quadruplicate virus-infected wells (A_{virus}) and cell culture-only controls (A_{cell}). All values above 50% of the specific signal, calculated using the formula: $X = (1/2) \times (A_{cell} - A_{virus}) + A_{virus}$, were considered positive for neutralization. The titres recorded were the reciprocal of the highest dilution, where A_{630} was >X.

Hemagglutination Inhibition

The antibody responses to the vaccine viruses were examined in serum and nasal washes using HI antibody assays incorporating a microtiter method as described elsewhere [Hierholzer et al., 1969]. All samples were pre-treated with RDE(II) at 37°C for 18 hr, subsequently inactivated at 56°C for 30 min, and mixed with packed red blood cells to remove any nonspecific inhibitors. The starting material for the assays was a 1:10 dilution for the serum samples and a 1:40 dilution for the nasal wash samples.

Fractionation of Nasal and Serum Samples

The concentrated nasal wash samples (100 μl, 6 mg/ml) and diluted serum samples (10-fold dilution, 100 μl, about 6 mg/ml) were fractionated on a Superose 6 10/300 GL gel filtration column using an FPLC-AKTA chromatography system (GE Healthcare, Little Chalfont, UK). The concentrated nasal wash sample was treated with 1 μg/ml of lysozyme (Sigma-Aldrich, St. Louis, MO) for 1 hr at 37°C to decrease the viscosity and then centrifuged using Vivaspin to remove the lysozyme prior to gel filtration. Fractions (each 500 μl) were collected in PBS at a flow rate of 0.1 ml/min; little or no change in the fractionation pattern of the antibodies in the concentrated nasal wash samples was observed following lysozyme treatment. Molecular weight marker proteins (Kit for Molecular Weights 29,000–700,000 Da; Sigma-Aldrich) were eluted under the same conditions to determine the size of each fraction.

RESULTS

Measurement of Neutralization and HI Titres in Concentrated Nasal Wash Samples

The total protein level and the levels of IgA, IgG, and IgM and human serum albumin in 100 ml of unconcentrated nasal wash and in approximately 0.5 ml of concentrated nasal wash are shown in Table I. About 70% of the total nasal wash proteins were lost during the concentration process. Also, a fraction of the higher molecular weight (MW) proteins and lower MW proteins (less than 30 kDa) was lost by

TABLE I. Concentration of IgA, IgG, IgM and HSA in 0.5 ml of Solution Concentrated From 100 ml of Nasal Wash (n = 10)*

Unit	Concentration: Mean ± SD				
	Total protein	IgA	IgG	IgM	HSA
Nasal wash (n = 10)					
Unconcentrated					
mg/100 ml	5.875 ± 1.856	1.132 ± 0.678	0.125 ± 0.057	0.032 ± 0.021	0.531 ± 0.280
Concentrated					
mg/0.43 ± 0.06 ml	1.647 ± 0.549	0.375 ± 0.193	0.093 ± 0.044	0.007 ± 0.006	0.292 ± 0.214
Concentration calculated in terms of total protein (mg/ml)	1.00	0.217	0.057	0.004	0.177

*The concentration was calculated using two nasal wash samples collected from five participants (with a 1 week interval).

adsorption to the cotton and during Vivaspin centrifugation, respectively. However, better recovery was observed for IgA and IgG. When the concentration of the enriched nasal washes was adjusted to 1 mg/ml total protein, the amount of IgA was 0.217 mg/ml. This amount of IgA in the concentrated nasal wash corresponded to about 1/10 of the levels of total IgA recovered from nasal mucus (2.20 mg/ml) by aspiration as reported by Kurono and Mogi [1987] (Table I). In subsequent experiments, neutralization and HI titres in the nasal wash samples were measured using concentrated nasal wash proteins (1 mg/ml of total protein), which contained 1/10 of the IgA found in mucus, to ensure that the nasal and serum neutralization titres were assayed at equivalent levels.

The amount of total IgA and total IgG in the nasal wash samples from each participant varied slightly at each sampling time. Also, the level of total IgA and IgG antibodies did not increase significantly between pre-vaccination and post-vaccination in any of the participants. Thus, the average amount of total IgA or total IgG in the nasal wash samples from the five participants was relatively constant (data not shown).

Neutralizing Antibody Responses in Nasal Wash and Serum Samples

Next, antibody responses in the nasal wash and serum samples were examined in all five study participants. The responses are presented as neutralization titres against the A/Uruguay (H3N2) virus in Table II. The responses recorded in the four young adults (between 18- and 50-years-old) are also shown as geometric neutralization titres (Fig. 1). The nasal wash and serum neutralization titres increased in all participants as the number of vaccinations increased, although the degree of increase differed between participants. In addition, nasal wash neutralization titres increased more rapidly than serum titres. The nasal wash titres showed at least a fourfold increase after the second vaccination in the four young participants (all of whom had a nasal wash neutralization titre of 1:20 or 1:40 before vaccination). By contrast, a fourfold increase in the serum titre was observed only after the fifth vaccination in three of the participants (all of whom had serum titres of <1:10, 1:20, or 1:40 before vaccination). Participant P5, who was 67 years old, showed at least a fourfold increase in nasal wash titre after the fourth vaccination, but no significant

TABLE II. Neutralizing Antibody Responses in Subjects who Received the Threefold Concentrated A/Uruguay/716/2007 (H3N2) Split Vaccine

Weeks (vaccination)	Neutralization titre against A/Uruguay virus (H3N2)[a]									
	P1		P2		P3		P4		P5	
	Nasal wash	Serum	Nasal wash	Serum	Nasal wash	Serum	Nasal wash	Serum	Nasal wash	Serum
0 (1st)	20 (1)	40 (2)	20 (1)	<10 (<0)	40 (2)	20 (1)	20 (1)	<10 (<0)	20 (1)	<10 (<0)
3 (2nd)	80 (3)	160 (4)	20 (1)	<10 (<0)	80 (3)	20 (1)	20 (1)	<10 (<0)	40 (2)	<10 (<0)
6 (3rd)	160 (4)	160 (4)	80 (3)	10 (0)	320 (5)	20 (1)	80 (3)	<10 (<0)	40 (2)	<10 (<0)
9 (4th)	320 (5)	160 (4)	160 (4)	20 (1)	1280 (7)	40 (2)	160 (4)	10 (0)	40 (2)	<10 (<0)
12 (5th)	320 (5)	160 (4)	320 (5)	40 (2)	2560 (8)	80 (3)	80 (3)	10 (0)	80 (3)	<10 (<0)
15	1280 (7)	160 (4)	320 (5)	40 (2)	2560 (8)	80 (3)	160 (4)	20 (1)	160 (4)	10 (0)
28	640 (6)	160 (4)	160 (4)	40 (2)	1280 (7)	80 (3)	N.D.	N.D.	80 (3)	10 (0)

N.D., not done.
[a]Respective values are a reciprocal titre and a geometric titre (10×2^n) in a parenthesis.

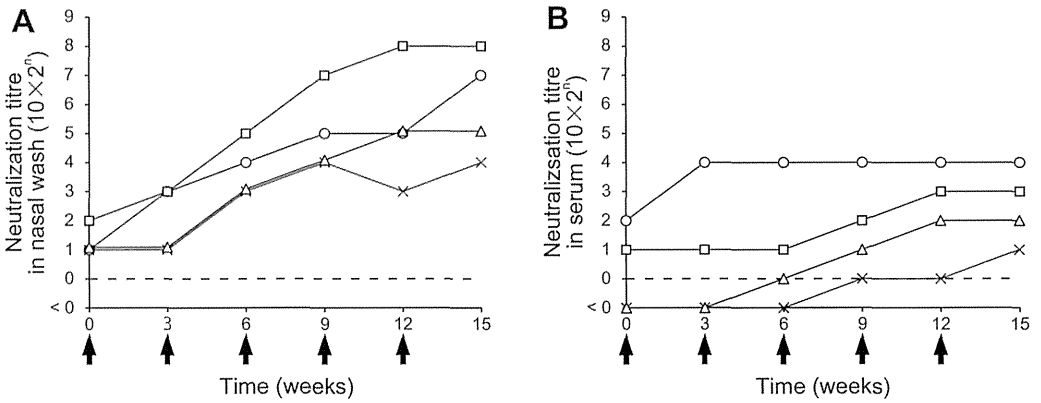

Fig. 1. Nasal wash and serum sample neutralization titres against A/Uruguay (H3N2) (pre- and post-intranasal immunization). Neutralization titres against the A/Uruguay virus in nasal washes (**A**) and serum (**B**) were determined in samples collected from four participants (18–60 years old; P1, open circle; P2, open triangle; P3, open square; and P4, cross). The participants were given five doses of the threefold concentrated A/Uruguay split influenza vaccine intranasally with an interval of 3 weeks between doses (each arrow indicates a point of vaccination). The neutralization titre shown is the geometric titre.

increase was observed in serum titre after five vaccinations. In all participants, the nasal wash and serum titres were largely maintained, even at 16 weeks after the fifth vaccination, at which point the nasal wash titre decreased only slightly, while no decrease was observed in the serum titre (Table II).

HI Antibody Responses in Nasal Wash and Serum Samples

Antibody responses were also examined by measuring the HI titre against the A/Uruguay (H3N2) virus. Table III shows the pre-vaccination HI titres of the nasal wash and serum samples from two participants, and the HI titres 3 weeks after each of the five vaccinations. For each participant, the HI titres were lower than the neutralization titres shown in Table II. The HI titres were approximately 1/4–1/8 the level of the neutralization titres. Statistical correlation analysis of the data presented in Tables II and III showed a strong correlation between the HI titres and the neutralization titres ($r = 0.8699$). Thus, the HI titre correlated with the neutralization titre, although it was less sensitive than the neutralization titre.

TABLE III. Hemagglutinin Inhibition (HI) Antibody Responses in Subjects who Received the Threefold Concentrated A/Uruguay/716/2007 (H3N2) Split Vaccine

	HI titre against A/Uruguay virus (H3N2)[a]			
	P1		P2	
Weeks (vaccination)	Nasal wash	Serum	Nasal wash	Serum
0 (1st)	N.D.	10 (0)	<40 (<2)	<10 (<0)
3 (2nd)	<40 (<2)	20 (1)	<40 (<2)	<10 (<0)
6 (3rd)	<40 (<2)	20 (1)	<40 (<2)	<10 (<0)
9 (4th)	40 (2)	20 (1)	<40 (<2)	10 (0)
12 (5th)	40 (2)	20 (1)	80 (3)	20 (1)
15	160 (4)	40 (2)	80 (3)	20 (1)

N.D., not done.
[a]Respective values are a reciprocal titre and a geometric titre (10×2^n) in a parenthesis.

Fractionation of The Nasal Wash and Serum Samples

The types of antibody present in the nasal wash and serum samples were examined after fractionation on a gel filtration column. The concentrated nasal wash samples (100 μl, about 6 mg/ml) and diluted serum samples (10-fold diluted sera, 100 μl, about 6 mg/ml) were fractionated on a Superose 6 column in PBS. The antibody concentration in each fraction was then measured by ELISA. Figure 2 shows the profiles for IgM, IgA, and IgG antibodies, together with the absorbance values for the total protein in each fraction. The nasal wash samples contained IgM, which comprised less than 1% of the total protein and showed a peak MW of 970 kD; IgA, which comprised about 20% of the total protein and showed a peak MW of about 660 kD; and IgG, which comprised about 6% of the total protein and showed a peak MW of 150 kD. The MW of the nasal IgA (150 kD–900 kD, with a peak MW of 660 kD) appeared to correspond to that of tetrameric IgA (the MW of dimeric IgA is estimated to be about 360 kD). The maximum absorbance value observed in the protein profile (at around 66 kD) was due to the presence of human serum albumin (Fig. 2A).

The serum samples contained IgM, which comprised about 3% of the total protein and showed a peak MW of 970 kD; IgA, which comprised about 6% of the total protein and showed a peak MW of about 150 kD; and IgG, which comprised about 23% of the total protein and showed a peak MW of 150 kD

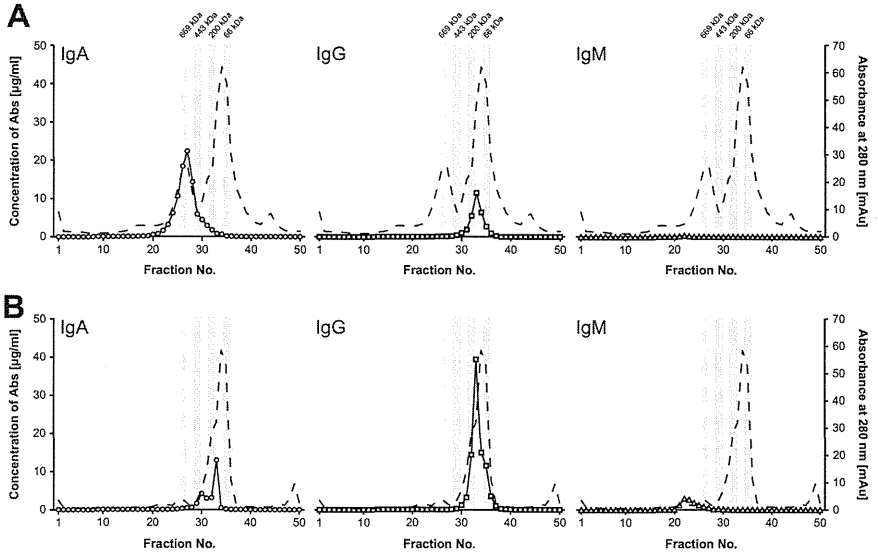

Fig. 2. Fractionation of nasal wash (**A**) and serum (**B**) samples from representative participants on Superose 6 columns. Quantification (μg/ml) of IgA (open circles), IgG (open squares), or IgM (open triangles) antibody levels and the absorbance at 280 nm (mAu, broken line) are shown. The grey zones in the upper part of the curves indicate the positions of the molecular weight markers [thyroglobulin (669 kD), apoferritin (443 kD), β-amylase (200 kD), and bovine serum albumin (66 kD)].

(Fig. 2B). Serum IgA (which showed a lower peak at about 360 kD in addition to a peak at about 150 kD) appeared to comprise both monomeric and dimeric IgA.

Taken together, the results of the fractionation analysis suggests that highly polymeric IgA is the predominant nasal antibody, and can be separated from nasal IgG and IgM. By contrast, the monomeric forms of IgG are the major component of total serum antibodies.

Neutralization Activity of the IgA and IgG Antibodies in The Nasal Wash and Serum Samples

To determine the isotype of the antibodies responsible for the neutralization activity induced by intranasal administration of the inactivated vaccine, nasal wash and serum samples from participant P1, who showed relatively high neutralization titres after the fifth vaccination, were separated on a Superose 6 column and the neutralization titre of the resulting antibody fractions assayed. The nasal polymeric IgA fraction (No. 27) showed a neutralization titre of 1:10, whereas the nasal monomeric IgG fraction (No. 33) showed a reciprocal neutralization titre of <1:10. However, the serum dimeric IgA fraction (No. 30) showed a neutralization titre of <1:10, whereas the serum peak monomeric IgG fraction (No. 33) showed a neutralization titre of 1:10 (Table IV). The respective peak fractions in the nasal wash were then concentrated to 100 μg/ml, and the neutralization activity of the nasal IgA antibodies (a mixture of fractions 26 and 27) was compared with that of the nasal IgG antibodies (a mixture of fractions 33 and 34). The nasal IgA fractions showed a neutralization titre of 1:40, whereas the nasal monomeric IgG fractions showed a neutralization titre of 1:10. Similarly, the neutralization activity of the serum IgA antibodies (100 μg/ml; a mixture of fractions 30 and 31) was compared with that of serum IgG antibodies (a mixture of fractions 33 and 34). The serum IgA fractions showed a neutralization titre of <1:10, whereas the serum

TABLE IV. Neutralization Titre of the IgA and IgG Fractions From the Nasal Wash and Serum Samples Following Separation on Superose Columns

	Neutralization titre[a]			
	Nasal wash		Serum	
	Polymeric IgA	IgG	Dimeric IgA	IgG
A/Uruguay (A/H3N2)				
Peak fraction: Separated on Superose column	10 (0)	<10 (<0)	<10 (<0)	10 (0)
Concentrated fraction (100 μg/ml)	40 (2)	10 (0)	<10 (<0)	10 (0)

The samples were collected from a representative subject vaccinated five times with an interval of 3 weeks between vaccinations.
[a]Respective values are a reciprocal titre and a geometric titre (10×2^n) in a parenthesis.