ies after nasal administration with Rhodamine-conjugated cCHP nanogel holding Alexa 647-conjugated BoHc (Nochi et al., 2010). While BoHc-coupled fluorescence signals were observed in M cells using our previously developed M cell-specific monoclonal antibody (mAb NKM 16-2-4) in NALT, which is an inductive tissue for the airway mucosal immune system, more BoHc was delivered to the apical membrane of the nasal epithelium (Nochi et al., 2010). In addition, consistent with the *in vitro* findings discussed in the previous chapter, the high magnification images showed that cCHP-BoHc was internalized into the nasal epithelium within 1 h following nasal administration and that BoHc was gradually detached from the cCHP nanogel in nasal epithelial cells (Nochi et al., 2010). Furthermore, flow cytometry and immunohistochemical analyses clearly showed that the released BoHc was effectively taken up by $CD11c^+$ DCs located in both the epithelial layer and lamina propria of the nasal cavity within 6 h of administration (Figure 4b; Nochi et al., 2010). These findings showed that, in addition to the delivery of vaccine antigen to classical inductive sites (or NALT) the cCHP nanogel is effective in widely distributing the vaccine antigen within the nasal cavity so that the nasal DCs can initiate activation of NALT-independent immune responses. Additional studies focused on whether respiratory M cells located away from NALT are involved in the NALT-independent induction of the antigen-specific immune response.

Since mucosal vaccination induces antigen-specific immune responses in systemic (e.g., serum IgG) and mucosal (e.g., sIgA) compartments, our study was next directed to address whether the levels of BoNT/A-specific serum antibody responses were induced by intranasal immunization with cCHP-BoHc. Compared with naked BoHc-immunized or control PBS-treated mice, notable levels of BoNT/A-specific serum IgG antibodies were induced in the cCHP-BoHc-vaccinated mice 1 week after the second intranasal immunization (Figure 4c). We then performed toxin challenge experiments to confirm the neutralizing effects of antigen-specific antibodies induced by intranasal immunization with cCHP-BoHc against a native form of BoNT. The mice nasally immunized with cCHP-BoHc were completely protected from a lethal dose (10 μg) of the toxin given nasally (Figure 4d) or systemically (data not shown; Nochi et al., 2010).

The cCHP nanogel was further adapted to the development of nasal vaccine against *S. pneumoniae* (Kong et al.; in 2013), a major causative pathogen of upper respiratory infectious disease (Girard, Cherian, Pervikov, & Kieny, 2005). When PspA, a vaccine candidate antigen prepared from *S. pneumoniae* (Briles et al., 2003), was incorporated into the cCHP nanogel and administered nasally, high titers of PspA-specific serum IgG as well as mucosal IgA antibodies were induced (Figure 5a, b). To evaluate the protective effect of antigen-specific antibody responses induced by cCHP-PspA nasal vaccination against pneumococcus, the vaccinated and control groups of mice were lethally challenged by *S. pneumoniae* (2×10^5 cfu/mouse) by the nasal route under anesthesia at 1 week after the final immunization. All mice immunized with cCHP-PspA survived, as did positive control mice nasally immunized with PspA and the mucosal adjuvant CT (PspA-CT; Figure 5c). PspA-CT is an effective intranasal immunization system for the induction of antigen-specific serum IgG and sIgA responses with protective activity (M. Yamamoto et al., 1998). Survival rates of mice nasally immunized with PspA alone and PBS were as low as 0% and 20%, respectively (Kong et al., 2013). These results indicate that nasal vaccines formulated with cCHP-nanogel effectively induce protective immunity against pathogens, including infectious respiratory diseases and toxins, without co-administration of mucosal adjuvants. Together with data obtained with cCHP nanogel used as a carrier of bacterial toxin and cell-membrane-related

Downloaded by [University of Tokyo] at 18:44 07 August 2013

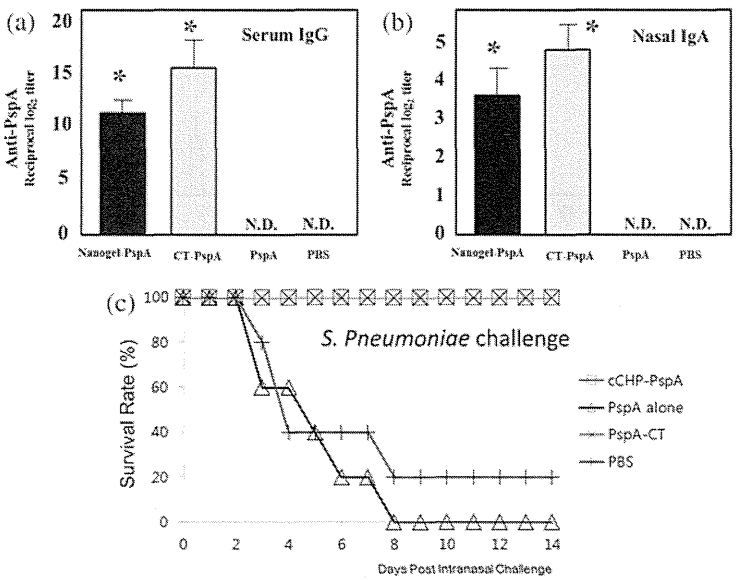


Figure 5. Induction of protective immunity by intranasal immunization with cCHP-pneumococcal surface protein A (PspA). Note: Strong PspA-specific serum IgG (a) and nasal IgA (b) immune responses were induced by intranasal immunization with cCHP–BoHc/A or BoHc with CT as a mucosal adjuvant (CT-PspA), but not by naked BoHc/A or control PBS. (c) Mice intranasally immunized with cCHP–BoHc/A or CT-PspA were protected from nasal challenge with *Streptococcus pneumoniae*, but mice immunized with naked PspA or PBS were not. N.D., not determined.

vaccine candidate antigens, cCHP-nanogel has great potential as a novel universal antigen-delivery vehicle for nasal vaccines (Kong et al., 2013).

Safety issues of nanogel antigen-delivery systems

Intranasal immunization is an effective and logical vaccination regimen for the induction of mucosal immunity in the respiratory tract. The nasally delivered live attenuated influenza vaccine FluMist was approved for healthy individuals aged 2–49 years, in the USA (Yuki & Kiyono, 2009). Although the US Centers for Disease Control and Prevention recommends that children 6 months and older should be vaccinated against influenza, the Food and Drug Administration decided that children under 2 years should not be given FluMist because of an increased risk of wheezing and other side effects possibly associated with nasal administration (Belshe, Lee, Walker, Stoddard, & Mendelmen, 2004). To improve vaccine safety, the subunit or purified recombinant form was considered to be a desirable alternative, but would require a mucosal adjuvant. A human clinical trial in Switzerland showed that an inactivated nasal influenza vaccine used with a heat-labile enterotoxin (LT) adjuvant was associated with facial paralysis (Mutsch et al., 2004). Like LT, CT is a potent mucosal adjuvant for supporting the induction of antigen-specific mucosal immunity. Toxin-based adjuvants, comprised of CT or the B subunit of CT (CTB), are redirected to the olfactory bulb in the CNS when administered via the nasal route in mice (van Ginkel et al., 2000). The results of this study indicate that the deposition of vaccine and/or adjuvant in the CNS via the nasal route might cause side effects. Thus, key objectives in nasal vaccine development are to

Downloaded by [University of Tokyo] at 18:44 07 August 2013

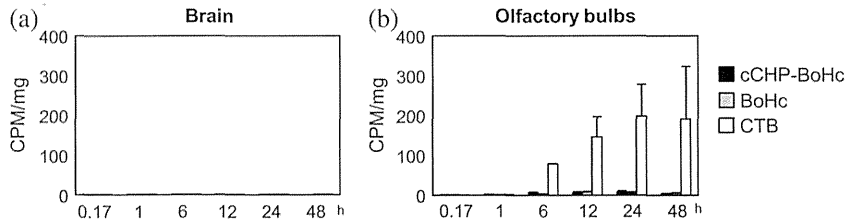


Figure 6. Intranasally administered cCHP-BoHc does not accumulate in the olfactory bulb and brain in mice.
Note: Radioisotope counting assay showed that intranasally administered cCHP nanogel carrying [^{111}In]-labeled BoHc did not accumulate in the brain (a) or olfactory bulbs (b). By contrast, [^{111}In]-labeled cholera toxin B subunit (CTB), which was used as a positive control, had accumulated in the olfactory bulbs at 6 h after intranasal administration.

Downloaded by [University of Tokyo] at 18:44 07 August 2013

investigate the fate of candidate vaccines and/or adjuvant and to examine whether they can reach the CNS with or without adjuvant.

Because the most important issue for nasal vaccine development is to address safety concerns related to the anatomical proximity between the nasal cavity and CNS, we developed an *in vivo* vaccine antigen tracer system with [^{111}In]-labeled BoHc. When cCHP nanogel holding [^{111}In]-labeled BoHc was administered nasally, we observed no transition of the vaccine into the olfactory bulbs or brain for at least 2 days (Figure 6). By contrast, when [^{111}In]-labeled CTB, which is known to reach and accumulate in olfactory tissues, was given nasally at the same dose, significant radioisotope levels were detected in the olfactory bulbs, but not the brain, after at least 6 h (Nochi et al., 2010). These findings indicate that cCHP nanogel can be used as a safe vaccine-delivery system for intranasal immunization without affecting the CNS.

To further confirm the safety and effectiveness of cCHP nanogel nasal delivery systems, we also tested whether the vaccine antigen PspA would deposit in the CNS after nasal administration using cCHP nanogel as the delivery vehicle. [^{111}In]-labeled PspA was administered into the nasal cavity with or without cCHP. There was no obvious accumulation of [^{111}In]-labeled PspA in the olfactory bulbs or brain over the entire observation period (Kong et al., 2013). These results further support cCHP nanogel as a safe vaccine carrier for nasal vaccination. Taken together, we believe that cCHP nanogel is a novel, safe and effective vaccine-delivery vehicle that can be universally used for the development of adjuvant-free nasal vaccines.

Future perspectives

For the development of nanogel-based nasal vaccines for human use, we need to demonstrate proof of concept in higher mammals using an appropriate vaccine candidate antigen. To this end, PspA has been shown to be an ideal candidate vaccine antigen for the control of pneumococcal infection. It is also necessary to demonstrate the induction of systemic and mucosal immune responses with protective immunity by intranasal immunization of nonhuman primates with nanogel used as the delivery vehicle for recombinant PspA. Also, it would be beneficial to investigate whether intranasal immunization of macaques with PspA-nanogel could induce *S. pneumoniae*-specific neutralizing antibodies and provide protection against lethal respiratory challenge with *S. pneumoniae*. We recently established an *in vivo* molecular imaging method for macaques based on an ^{18}F-protein positron emission tomography system (Yuki et al., 2010),

which we would employ to demonstraSte visually the safety of nanogel-based pneumococcal vaccines. In these experiments we would examine the metabolic fate of nasally administered nanogel holding ^{18}F-PspA in macaques in addition to studies determining absorption, distribution, metabolism and excretion patterns, especially in the olfactory bulb and brain.

References

Ayame, H., Morimoto, N., & Akiyoshi, K. (2008). Self-assembled cationic nanogels for intracellular protein delivery. *Bioconjugate Chemistry, 19*, 882–890.

Belshe, R., Lee, M. S., Walker, R. E., Stoddard, J., & Mendelman, P. M. (2004). Safety, immunogenicity and efficacy of intranasal, live attenuated influenza vaccine. *Expert Review of Vaccines, 3*, 643–654.

Briles, D. E., Hollingshead, S. K., Paton, J. C., Ades, E. W., Novak, L., van Ginkel, F. W., & Benjamin, W. H.Jr (2003). Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with *Streptococcus pneumoniae*. *Journal of Infectious Diseases, 188*, 339–348.

Campbell, J. J., Brightling, C. E., Symon, F. A., Qin, S., Murphy, K. E., Hodge, M., Andrew, D. P., Wu, L., Butcher, E. C., & Wardlaw, A. J. (2001). Expression of chemokine receptors by lung T cells from normal and asthmatic subjects. *Journal of Immunology, 166*, 2842–2848.

Czerkinsky, C., Anjuere, F., McGhee, J. R., George-Chandy, A., Holmgren, J., Kieny, M. P., ..., Sun, J. B. (1999). Mucosal immunity and tolerance: Relevance to vaccine development. *Immunological Reviews, 170*, 197–222.

Girard, M. P., Cherian, T., Pervikov, Y., & Kieny, M. P. (2005). A review of vaccine research and development: Human acute respiratory infections. *Vaccine, 23*, 5708–5724.

Hartl, F. U. (1996). Molecular chaperones in cellular protein folding. *Nature, 381*, 571–579.

Holmgren, J., & Czerkinsky, C. (2005). Mucosal immunity and vaccines. *Nature Medicine, 11*, S45–53.

Ikuta, Y., Katayama, N., Wang, L., Okugawa, T., Takahashi, Y., Schmitt, M., ..., Shiku, H. (2002). Presentation of a major histocompatibility complex class 1-binding peptide by monocyte-derived dendritic cells incorporating hydrophobized polysaccharide-truncated HER2 protein complex: Implications for a polyvalent immuno-cell therapy. *Blood, 99*, 3717–3724.

Iwasaki, A. (2007). Mucosal dendritic cells. *Annual Review of Immunology, 25*, 381–418.

Iwata, M., Hirakiyama, A., Eshima, Y., Kagechika, H., Kato, C., & Song, S. Y. (2004). Retinoic acid imprints gut-homing specificity on T cells. *Immunity, 21*, 527–538.

Kabanov, A. V., & Vinogradov, S. V. (2009). Nanogels as pharmaceutical carriers: Finite networks of infinite capabilities. *Angewandte Chemie International Edition (English), 48*, 5418–5429.

Kaetzel, C. S. (2005). The polymeric immunoglobulin receptor: Bridging innate and adaptive immune responses at mucosal surfaces. *Immunological Reviews, 206*, 83–99.

Kim, D. Y., Sato, A., Fukuyama, S., Sagara, H., Nagatake, T., Kong, I. G., ..., Kiyono, H. (2011). The airway antigen sampling system: Respiratory M cells as an alternative gateway for inhaled antigens. *Journal of Immunology, 186*, 4253–4262.

Kiyono, H., & Fukuyama, S. (2004). NALT- versus Peyer's-patch-mediated mucosal immunity. *Nature Reviews. Immunology, 4*, 699–710.

Kong, I., Sato, A., Yuki, Y., Nochi, T., Takahashi, H., Sawada, S., ... Kiyono, H. (2013). Nanogel-based PspA intranasal vaccine prevents invasive disease and nasal colonization by Pneumococcus. *Infection and Immunity, 81*, 1625–1634.

Kunisawa, J., Fukuyama, S., & Kiyono, H. (2005). Mucosa-associated lymphoid tissues in the aerodigestive tract: Their shared and divergent traits and their importance to the orchestration of the mucosal immune system. *Current Molecular Medicine, 5*, 557–572.

Leader, B., Baca, Q. J., & Golan, D. E. (2008). Protein therapeutics: A summary and pharmacological classification. *Nature Reviews. Drug Discovery, 7*, 21–39.

McGhee, J. R., Fujihashi, K., Beagley, K. W., & Kiyono, H. (1991). Role of interleukin-6 in human and mouse mucosal IgA plasma cell responses. *Immunologic Research, 10*, 418–422.

Mora, J. R., Iwata, M., Eksteen, B., Song, S. Y., Junt, T., Senman, B., ..., von Andrian, U. H. (2006). Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science, 314*, 1157–1160.

Downloaded by [University of Tokyo] at 18:44 07 August 2013

Mutsch, M., Zhou, W., Rhodes, P., Bopp, M., Chen, R. T., Linder, T., ... Steffen, R. (2004). Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *New England Journal of Medicine, 350*, 896–903.

Neutra, M. R., Frey, A., & Kraehenbuhl, J. P. (1996). Epithelial M cells: Gateways for mucosal infection and immunization. *Cell, 86*, 345–348.

Neutra, M. R., & Kozlowski, P. A. (2006). Mucosal vaccines: The promise and the challenge. *Nature Reviews. Immunology, 6*, 148–158.

Nochi, T., Yuki, Y., Takahashi, H., Sawada, S., Mejima, M., Kohda, T., ..., Kiyono, H. (2010). Nanogel antigenic protein-delivery system for adjuvant-free intranasal vaccines. *Nature Materials, 9*, 572–578.

Nomura, Y., Ikeda, M., Yamaguchi, N., Aoyama, Y., & Akiyoshi, K. (2003). Protein refolding assisted by self-assembled nanogels as novel artificial molecular chaperone. *FEBS Letters, 553*, 271–276.

Park, J. B., & Simpson, L. L. (2003). Inhalational poisoning by botulinum toxin and inhalation vaccination with its heavy-chain component. *Infection and Immunity, 71*, 1147–1154.

Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., ... Ricciardi-Castagnoli, P. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature Immunology, 2*, 361–367.

Ryan, E. J., McNeela, E., Murphy, G. A., Stewart, H., O'Hagan, D., Pizza, M., ... Mills, K. H. (1999). Mutants of Escherichia coli heat-labile toxin act as effective mucosal adjuvants for nasal delivery of an acellular pertussis vaccine: Differential effects of the nontoxic AB complex and enzyme activity on Th1 and Th2 cells. *Infection and Immunity, 67*, 6270–6280.

Sasaki, Y., & Akiyoshi, K. (2010a). Development of an artificial chaperone system based on cyclodextrin. *Current Pharmaceutical Biotechnology, 11*, 300–305.

Sasaki, Y., & Akiyoshi, K. (2010b). Nanogel engineering for new nanobiomaterials: From chaperoning engineering to biomedical applications. *Chemical Record, 10*, 366–376.

Taguchi, H., Ueno, T., Tadakuma, H., Yoshida, M., & Funatsu, T. (2001). Single-molecule observation of protein-protein interactions in the chaperonin system. *Nature Biotechnology, 19*, 861–865.

Tsuji, K., Hamada, T., Uenaka, A., Wada, H., Sato, E., Isobe, M., ..., Iwatsuki, K. (2008). Induction of immune response against NY-ESO-1 by CHP-NY-ESO-1 vaccination and immune regulation in a melanoma patient. *Cancer Immunology, Immunotherapy: CII, 57*, 1429–1437.

van Ginkel, F. W., Jackson, R. J., Yuki, Y., & McGhee, J. R. (2000). Cutting edge: The mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. *Journal of Immunology, 165*, 4778–4782.

Watanabe, K., Tsuchiya, Y., Kawaguchi, Y., Sawada, S., Ayame, H., Akiyoshi, K., & Tsubata, T. (2011). The use of cationic nanogels to deliver proteins to myeloma cells and primary T lymphocytes that poorly express heparan sulfate. *Biomaterials, 32*, 5900–5905.

Yamamoto, M., Briles, D. E., Yamamoto, S., Ohmura, M., Kiyono, H., & McGhee, J. R. (1998). A nontoxic adjuvant for mucosal immunity to pneumococcal surface protein A. *Journal of Immunology, 161*, 4115–4121.

Yamamoto, S., Kiyono, H., Yamamoto, M., Imaoka, K., Fujihashi, K., Van Ginkel, F. W., ... McGhee, J. R. (1997). A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. *Proceedings of the National Academy of Sciences of the United States of America, 94*, 5267–5272.

Yuki, Y., & Kiyono, H. (2003). New generation of mucosal adjuvants for the induction of protective immunity. *Reviews in Medical Virology, 13*, 293–310.

Yuki, Y., & Kiyono, H. (2009). Mucosal vaccines: Novel advances in technology and delivery. *Expert Review of Vaccines, 8*, 1083–1097.

Yuki, Y., Nochi, T., Harada, N., Katakai, Y., Shibata, H., Mejima, M., ..., Kiyono, H. (2010). In vivo molecular imaging analysis of a nasal vaccine that induces protective immunity against botulism in nonhuman primates. *Journal of Immunology, 185*, 5436–5443.

Downloaded by [University of Tokyo] at 18:44 07 August 2013

Downloaded on August 8, 2013. The Journal of Clinical Investigation. More information at www.jci.org/articles/view/70266

Rice-based oral antibody fragment prophylaxis and therapy against rotavirus infection

Daisuke Tokuhara,[1,2] Beatriz Álvarez,[3] Mio Mejima,[1] Tomoko Hiroiwa,[1] Yuko Takahashi,[1] Shiho Kurokawa,[1] Masaharu Kuroda,[4] Masaaki Oyama,[5] Hiroko Kozuka-Hata,[5] Tomonori Nochi,[1] Hiroshi Sagara,[5] Farah Aladin,[6] Harold Marcotte,[3] Leon G.J. Frenken,[7] Miren Iturriza-Gómara,[8] Hiroshi Kiyono,[1,9] Lennart Hammarström,[3] and Yoshikazu Yuki[1,9]

[1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, University of Tokyo, Tokyo, Japan. [2]Department of Pediatrics, Osaka City University Graduate School of Medicine, Osaka, Japan. [3]Division of Clinical Immunology, Karolinska Institutet at Karolinska University Hospital Huddinge, Stockholm, Sweden. [4]Crop Development Division, NARO Agriculture Research Center, Niigata, Japan. [5]Medical Proteomics Laboratory, The Institute of Medical Science, University of Tokyo, Tokyo, Japan. [6]Enteric Virus Unit, Virus Reference Department, Health Protection Agency, London, United Kingdom. [7]Unilever Research and Development Vlaardingen BV, Vlaardingen, Netherlands. [8]Institute of Infection and Global Health, University of Liverpool, Liverpool, United Kingdom. [9]International Research and Development Center for Mucosal Vaccine, The Institute of Medical Science, University of Tokyo, Tokyo, Japan.

Rotavirus-induced diarrhea is a life-threatening disease in immunocompromised individuals and in children in developing countries. We have developed a system for prophylaxis and therapy against rotavirus disease using transgenic rice expressing the neutralizing variable domain of a rotavirus-specific llama heavy-chain antibody fragment (MucoRice-ARP1). MucoRice-ARP1 was produced at high levels in rice seeds using an overexpression system and RNAi technology to suppress the production of major rice endogenous storage proteins. Orally administered MucoRice-ARP1 markedly decreased the viral load in immunocompetent and immunodeficient mice. The antibody retained in vitro neutralizing activity after long-term storage (>1 yr) and boiling and conferred protection in mice even after heat treatment at 94°C for 30 minutes. High-yield, water-soluble, and purification-free MucoRice-ARP1 thus forms the basis for orally administered prophylaxis and therapy against rotavirus infections.

Introduction

Rotavirus is the leading cause of diarrhea in infants and young children worldwide, causing more than 114 million episodes of diarrhea annually in children under the age of 5 (1–4), 80% of which occur in developing countries (4). More than 600,000 children die annually from rotavirus (RV) infection, and although most RV-related deaths occur in low income countries, in developed countries, thousands of children under 5 years of age are hospitalized every year, including approximately 50,000–80,000 in Japan and 60,000 in the US (2, 3), causing a huge financial burden (2, 5). Currently, there are 2 licensed RV vaccines (Rotarix and RotaTeq) that have been reported to be highly effective (>85%) in reducing severe RV-induced diarrhea in developed countries (6–8). More recent clinical trials in developing countries in Africa and Asia (9–11) have also demonstrated a potential impact of these vaccines, but with a markedly lower efficacy, ranging from 39.3% (RotaTeq in Sub-Saharan Africa) to 61.2% (Rotarix in Malawi and South Africa) (9, 10). Both vaccines have proven to be safe in clinical trials and post-marketing studies, but presently, they are licensed for use only within a very narrow age window (>6 weeks and ≤26 weeks of age) to avoid the risk of intussusception.

Vaccines are often associated with poor efficacy in immunocompromised individuals (12–14). Such individuals may even acquire infections derived from the use of live vaccines. Cases of chronic diarrhea induced by the attenuated RotaTeq vaccine have indeed been recently reported (12–14), and the current RV vaccines are therefore not recommended for use in immunodeficient infants.

The lag time between vaccine administration and induction of an immune response, as well as poor efficacy in immunocompromised individuals, can be critical in highly endemic regions and outbreak situations, such as those occurring in hospital settings (15–18). Furthermore, severely immunocompromised individuals do not mount protective immunity when infected with RV and thus suffer chronic infection (19–22), which can subsequently lead to extraintestinal spread with severe clinical consequences (19–21). There is still a need for alternative strategies that can be used in situations where the available vaccines are not indicated and/or to complement vaccination strategies in situations where efficacy of vaccination alone may not be sufficient.

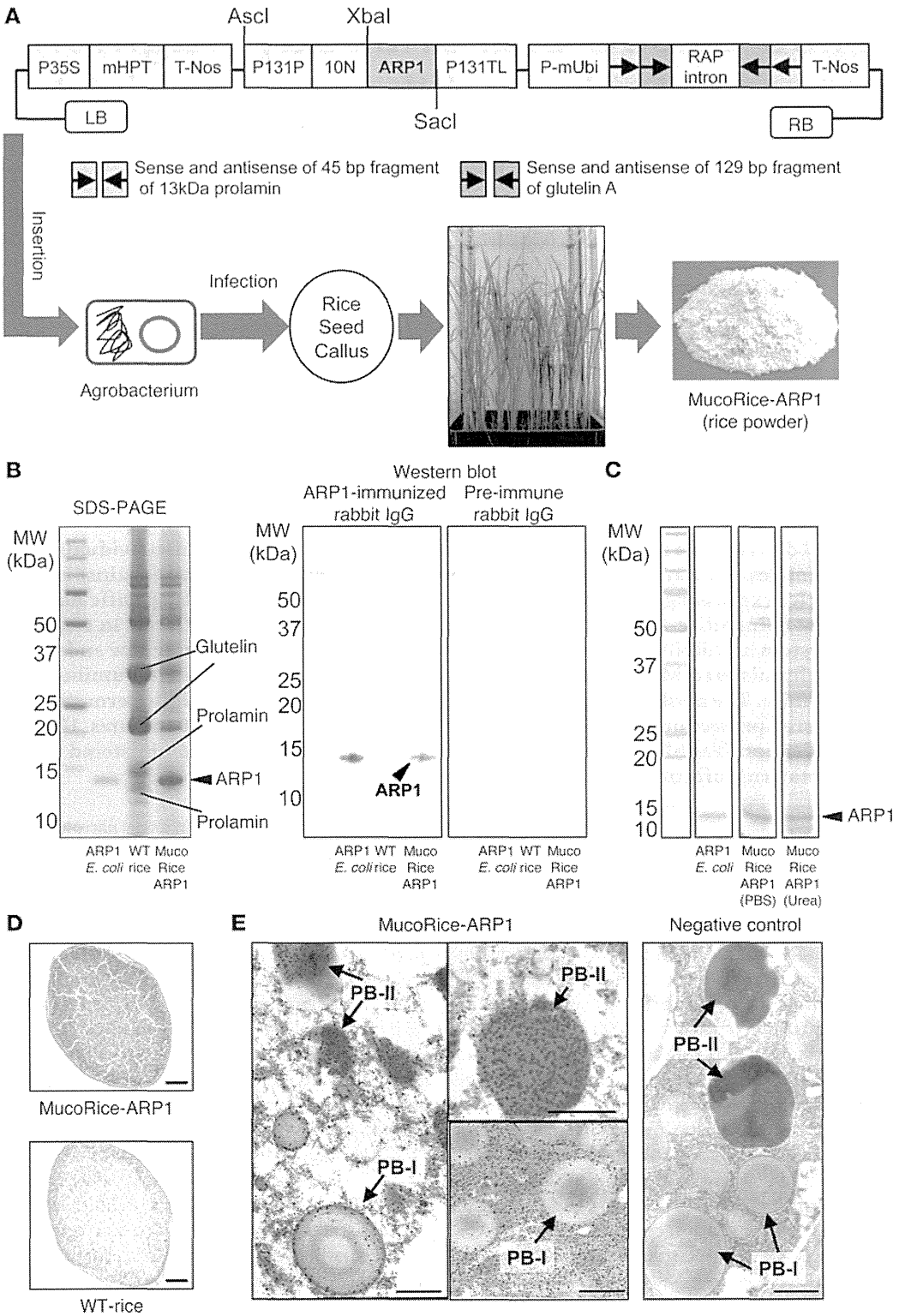
Passive immunotherapy is, at present, the only available intervention that can provide immediate protection. It may thus represent the prophylaxis of choice in highly endemic areas where vaccine responses may be poor or in selected groups of patients such as hospitalized children, immunocompromised individuals, and older infants and children in whom vaccination is contraindicated. It has been previously shown that oral administration of polyclonal antibodies from various sources can be used successfully both as

Conflict of interest: ARP1 has been patented by Unilever and was provided under a material transfer agreement for research relating to the potential to apply the technology to food products. Leon G.J. Frenken owns shares in Unilever. Miren Iturriza-Gómara receives research support provided by GlaxoSmithKline and Sanofi Pasteur MSD.

Note regarding evaluation of this manuscript: Manuscripts authored by scientists associated with Duke University, The University of North Carolina at Chapel Hill, Duke-NUS, and the Sanford-Burnham Medical Research Institute are handled not by members of the editorial board but rather by the science editors, who consult with selected external editors and reviewers.

Citation for this article: *J Clin Invest.* doi:10.1172/JCI70266.

Downloaded on August 8, 2013. The Journal of Clinical Investigation. More information at www.jci.org/articles/view/70266



Downloaded on August 8, 2013. The Journal of Clinical Investigation. More information at www.jci.org/articles/view/70266

Figure 1
Expression and localization of water-soluble ARP1 in transgenic rice. (**A**) Inserted plasmid for overexpression of ARP1 in rice seeds. P35S, CaMV35S promoter; mHPT, modified hygromycin phosphotransferase; P131P, 13-kDa prolamin promoter; 10N, signal sequence of 10-kDa prolamin; P131TL, 13-kDa prolamin terminator; T-Nos, nos terminator; RAP intron, rice aspartic protease intron; P-mUbi, ubiquitin promoter; LB, T-DNA left border; RB, T-DNA right border. (**B**) Production of MucoRice-ARP1. SDS-PAGE showed predominant expression of the transgenic protein with a molecular weight of approximately 12 kDa (arrowhead). Original rice proteins (arrows; 22- to 23-kDa and 34- to 37-kDa subunits of glutelin and 13-kDa prolamin) in nontransformed WT rice were markedly suppressed in MucoRice-ARP1. Western blotting revealed that a transgenic protein of 12 kDa was specifically detected using the anti-ARP1 antibody. ARP1 *E. coli*, ARP1 purified from *E. coli*. (**C**) Solubility of MucoRice-ARP1. MucoRice-ARP1 (PBS), extracts of MucoRice-ARP1 in PBS; MucoRice-ARP1 (urea), extracts of MucoRice-ARP1 in 8 M urea. (**D**) Immunohistochemistry showed that ARP1 had accumulated throughout the whole MucoRice-ARP1 seed, whereas it was not detected in a WT rice seed. Scale bars: 1 mm. (**E**) Immune electron microscopy showed that ARP1 is observed as black spots (left and middle panels). ARP1 is predominantly localized in the PB-II and the interspace between the PB-I and PB-II (left and middle panels). ARP1 is also slightly found at the surface of PB-I (left panel and lower middle panel). ARP1 is not detected in a WT rice seed used as a negative control (right panel). Scale bars: 1 μm.

prophylaxis and as therapy in children with RV-induced diarrhea (23, 24). However, the production and purification of the antibodies is costly, making it necessary to find alternative approaches.

The variable domain of llama heavy chain antibodies (VHH) consists of a single domain and constitutes the smallest naturally occurring antigen-binding domain known to date (25). In addition to its high binding capacity, VHH has several attractive features, such as resistance to pepsin, acid environment, and heat (26, 27). Furthermore, because of its small size and simple form, it is easy to produce VHH as a recombinant protein with an intact spatial structure (28). An anti-RV VHH (ARP1) produced in yeast and capable of protecting mouse pups against RV-induced diarrhea has been described previously (29). Modified lactobacilli-producing surface-anchored ARP1 were generated and shown to be effective in the same mouse pup model (30). Furthermore, orally administered ARP1 produced by yeast was recently found to be safe and effective in reducing severity of RV induced diarrhea in children in a clinical trial conducted in Bangladesh (S.A. Sarker, M. Jäkel, S. Sultana, N.H. Alam, P.K. Bardhan, M.A. Salam, W. Theis, L. Hammarström, L.G.J. Frenken., unpublished observations). Western blots using a variety of RV strains and recombinant VP6 suggest that ARP1 binds to polymeric VP6 (31). VP6 is the most conserved protein in the RV capsid and contains the group and subgroup determining epitopes (31).

We previously reported that a rice-based vaccine containing the cholera toxin B subunit (MucoRice-CTB) was effective against cholera (32, 33). Recently, we also introduced RNAi technology in an attempt to suppress internal storage rice protein production in order to enhance accumulation of foreign proteins in rice seed (34). In this study, we extended this system further to include a rice-based, orally administered product against RV by producing rice expressing an antibody fragment, ARP1 (MucoRice-ARP1). MucoRice-ARP1 rice powder or rice water offer what we believe are novel approaches to the prevention and treatment of RV-induced diarrhea, which may be used to reduce the medical and economic burden in both developed and developing countries, complement current vaccine-based prophylaxis, and in situations where live attenuated vaccines are contraindicated.

Results

Development of rice-based water soluble ARP1 (MucoRice-ARP1) with a high expression level. Using a binary vector (pZH2Bik45G1B) (35), described in Figure 1A, a codon-optimized *ARP1* gene combined with a RNAi suppression cassette was transfected into rice plants (Nippon-Bare) using *Agrobacterium tumefaciens*–mediated transformation. As shown in Figure 1B, the expression of the prolamin (13 kDa) and glutelin (22–23 kDa and 34–37 kDa) was suppressed and the 12 kDa ARP1 protein was predominantly expressed in the transgenic rice (MucoRice-ARP1). When accumulation of the ARP1 protein in the MucoRice-ARP1 seed was examined by SDS-PAGE and Western blot, using a rabbit anti-ARP1 polyclonal antibody, 1 band (12 kDa) was detected under denaturing conditions (Figure 1B). ARP1 was released from the MucoRice-ARP1 rice powder after PBS addition at room temperature (rice water) as shown in Figure 1C. Using densitometric analysis, with purified yeast-derived ARP1 as a standard, the yield of soluble ARP1 (which is 95% of the total yield) was found to reach an average of 170 μg per seed, representing 11.9% of the total seed protein (0.85% of seed weight). In contrast, transgenic rice, without RNAi suppression of prolamin and glutelin, yielded a low amount of ARP1 (on average 14 μg / seed) (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI70266DS1).

Localization of ARP1 protein in a MucoRice-ARP1 seed. Immunohistochemistry staining showed that MucoRice-ARP1 is expressed throughout the entire seed (Figure 1D). Storage proteins of WT rice seeds generally accumulate into 2 different types of protein bodies (PB), PB-I and PB-II, which contain prolamin and glutelin as their major components, respectively (36). PB-I is resistant to digestive enzyme whereas PB-II is easily digested in the gut (37). When the sections were examined by immune electron microscopy, ARP1 was predominantly localized in PB-II and the cytoplasm between PB-I and PB-II (Figure 1E).

Mass spectrometric analyses of MucoRice-ARP1. Using Q-STAR and Orbitrap mass spectrometry, we determined the full sequence of the 123 amino acids of ARP1 and the N-terminal sequence (serine-arginine) derived from the restriction enzyme site (*XbaI*) necessary for introduction of the ARP1 encoding gene into the binary vector (Supplemental Tables 1 and 2).

Heat stability and long-term room temperature stability of MucoRice-ARP1. MucoRice-ARP1 showed a binding to rhesus RV (RRV) particles similar to that of yeast-derived ARP1 (BAC BV, Naarden, the Netherlands) (Supplemental Figure 2). In order to determine the heat stability of MucoRice-ARP1, ARP1 (from rice and yeast) and bovine hyperimmune colostrum (HBC) (38) were boiled for 10, 20, and 30 minutes. After 30 minutes of boiling, a loss in the binding capacity to RV using ELISA of between 33% and 57% was observed in the ARP1 samples. It is worth nothing that rice water containing MucoRice-ARP1 (protein solution from MucoRice-ARP1 powder in PBS) showed a higher heat resistance (33% loss in the binding capacity after 30 minutes of boiling) than the purified ARP1 samples from MucoRice-ARP1 (57% loss) and yeast (46% loss). In contrast, the HBC sample completely lost its binding activity already after 10 minutes of boiling (Supplemental Figure 3).

Furthermore, MucoRice-ARP1, stored at room temperature (>1 year), had a neutralizing activity against RV in vitro equal to

Downloaded on August 8, 2013. The Journal of Clinical Investigation. More information at www.jci.org/articles/view/70266

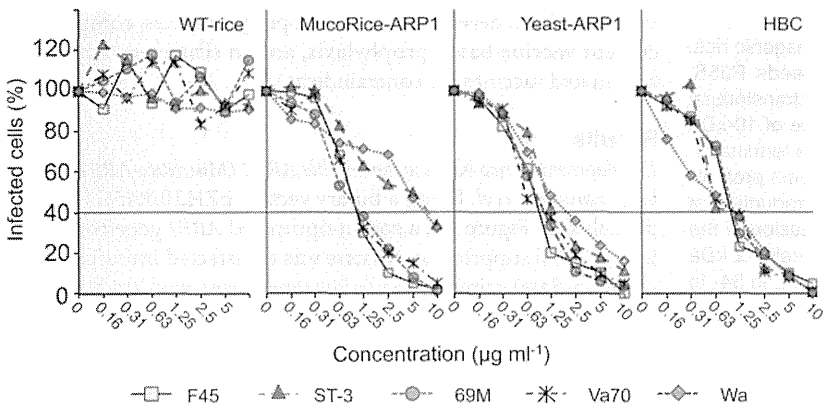


Figure 2
Neutralizing activity of MucoRice-ARP1 against human RV strains. Neutralizing activity of ARP1 samples from different origins and HBC against the human RV strains Wa (G1P[8]), ST-3 (G4P[6]), 69M (G8P[10]), F45 (G9P[8]) and Va70 (G4P[8]) using 2-fold dilutions of MucoRice-ARP1 containing rice water (shown as MucoRice-ARP1), WT rice (used as a negative control), ARP1 purified from yeast (shown as yeast-ARP1), and HBC. Lines indicate the percentage of infected cells related to control wells infected in the absence of added proteins. The horizontal line at 40% infected cells indicates a reduction of 60% in the infectivity of the RV strains.

that of freshly harvested MucoRice-ARP1 when applied as rice water (Supplemental Figure 4).

MucoRice-ARP1 neutralizes human RV strains of different serotypes in vitro. Neutralization assays, preventing infection in MA104 cells, were carried out to determine whether MucoRice-ARP1 was able to detect human RV strains of different serotypes (Figure 2). MucoRice-ARP1 containing rice water neutralized the human RV strains Wa G1P[8], ST-3 G4P[6], 69M G8P[10], F45 G9P[8] and Va70 G4P[8] in a dose-dependent manner. A reduction of more than 60% in the infectivity of the RV strains F45, 69M, and Va70 was observed in vitro using the same dose of MucoRice-ARP1 and purified yeast-derived ARP1 (1.25 μg ml^{-1}). For neutralization of the RV strains ST-3 and Wa, 4 times the amount of rice water of MucoRice-ARP1 (10 μg ml^{-1}) was needed as compared with purified yeast-derived ARP1 (2.5 μg ml^{-1}) (Figure 2).

MucoRice-ARP1 reduced diarrhea in a mouse pup model of RV infection. All 4-day-old mice infected with RRV and given PBS or WT rice developed diarrhea (Figure 3, A–C) whereas none of the noninfected control mice (fed PBS or WT rice) did (Figure 3, A–C). When MucoRice-ARP1 (containing 8.5 μg of ARP1) was prophylactically given to neonatal mice, the percentage of animals with diarrhea on day 2 after RRV inoculation was significantly lower ($P = 0.007$) than in mice receiving PBS or WT rice (Figure 3A). In addition, disease severity in mice treated with MucoRice-ARP1 was significantly lower ($P = 0.022$ and $P = 0.041$) than in mice given PBS or WT rice.

Heat treated MucoRice-ARP1 (for 10 minutes or 30 minutes at 94°C) used prophylactically against RRV-induced diarrhea also significantly ($P = 0.035$) reduced the percentage of animals with diarrhea on day 2 in comparison with mice receiving PBS or WT rice. In addition, disease severity in mice treated with heat-treated MucoRice-ARP1 was significantly lower ($P = 0.041$) than in mice given PBS or WT rice. No significant difference was observed between heat-treated and non-heat treated MucoRice-ARP1 in their ability to reduce the percentage of mice with diarrhea and disease severity (Figure 3A).

As shown in Figure 3B, long-term (>1 year) stored MucoRice-ARP1 (8.5 μg ARP1) applied prophylactically significantly reduced the incidence of diarrhea and disease severity ($P = 0.011$ and $P = 0.008$, respectively on day 2) compared with that of mice receiving PBS and was as effective as freshly harvested MucoRice-ARP1 ($P = 0.297$ and $P = 0.267$, respectively on day 2).

When MucoRice-ARP1 was given therapeutically, 9 hours after RRV inoculation, the percentage of mice with the virus-induced diarrhea and disease severity was also significantly lower ($P = 0.007$ and $P = 0.041$, respectively on day 2) than in the control mice receiving PBS or WT rice (Figure 3C).

Histopathological changes and virus loads in the small intestine of neonatal murine pups. No lesions were seen in any of the control mice receiving PBS and sacrificed on day 3 (Figure 4A). In the RRV-infected neonatal mice that received WT rice water solution or PBS, all of the examined mice showed typical histological signs of RV infection, with accumulation of large vacuoles in the enterocytes lining the surface of the villous tips in the ileum (Figure 4, B and D). Some vacuoles were also detected in the duodenum and the jejunum (data not shown). RV-infected mice treated prophylactically with the MucoRice-ARP1 preparation showed no pathological changes (Figure 4C). When *VP7* mRNA–specific RT-PCR was performed, the level of virus load tended to correlate with the histopathological changes. Thus, the virus load in the intestines of RRV-inoculated mice that received MucoRice-ARP1 was significantly reduced compared with that of mice given PBS ($n = 6$, $P = 0.002$) or WT rice ($P < 0.01$) ($n = 6$, $P = 0.002$) (Figure 4E).

Stability of MucoRice-ARP1 in the neonatal mice gut. Intragastrically administered MucoRice-ARP1 containing rice water (8.5 μg of soluble ARP1) was detected in the small intestine of the mice (4 days after birth) for at least 6 hours (Supplemental Figure 5). In some mice (40%, $n = 5$), MucoRice-ARP1 could be detected even 9 hours after intragastric administration.

MucoRice-ARP1 reduces the viral shedding in SCID mice. Following challenge with RRV, watery diarrhea was observed in all SCID neonatal pups. MucoRice-ARP1 (containing 8.5 μg of ARP1), given therapeutically to SCID pups 9 hours after RRV inoculation, significantly reduced the percentage of mice with diarrhea ($P = 0.022$) and disease severity ($P = 0.038$) compared with SCID pups given PBS or WT rice (Supplemental Figure 6).

Adult SCID mice did not develop overt diarrhea. However, the mice suffered a chronic infection lasting more than 6 weeks, as recognized by viral shedding in feces (Figure 5). Oral administration of 200 mg of MucoRice-ARP1 powder (containing 1.7 mg of soluble ARP1) twice daily for 7 days markedly reduced the number of viral VP7 RNA copies in feces on day 3 and day 9 ($n = 6$, $P = 0.002$ and 0.003) compared with that in mice given PBS or WT rice. No difference was, however, observed after the cessation of MucoRice-ARP1 administration (day 14) (Figure 5). No reduction in viral shedding was observed in RRV-inoculated SCID adult mice treated with WT rice powder compared with mice receiving PBS. These data indicate that MucoRice-ARP1 can transiently, but effectively, decrease the shedding of RV even in immunocompromised adult mice.

Downloaded on August 8, 2013. The Journal of Clinical Investigation. More information at www.jci.org/articles/view/70266

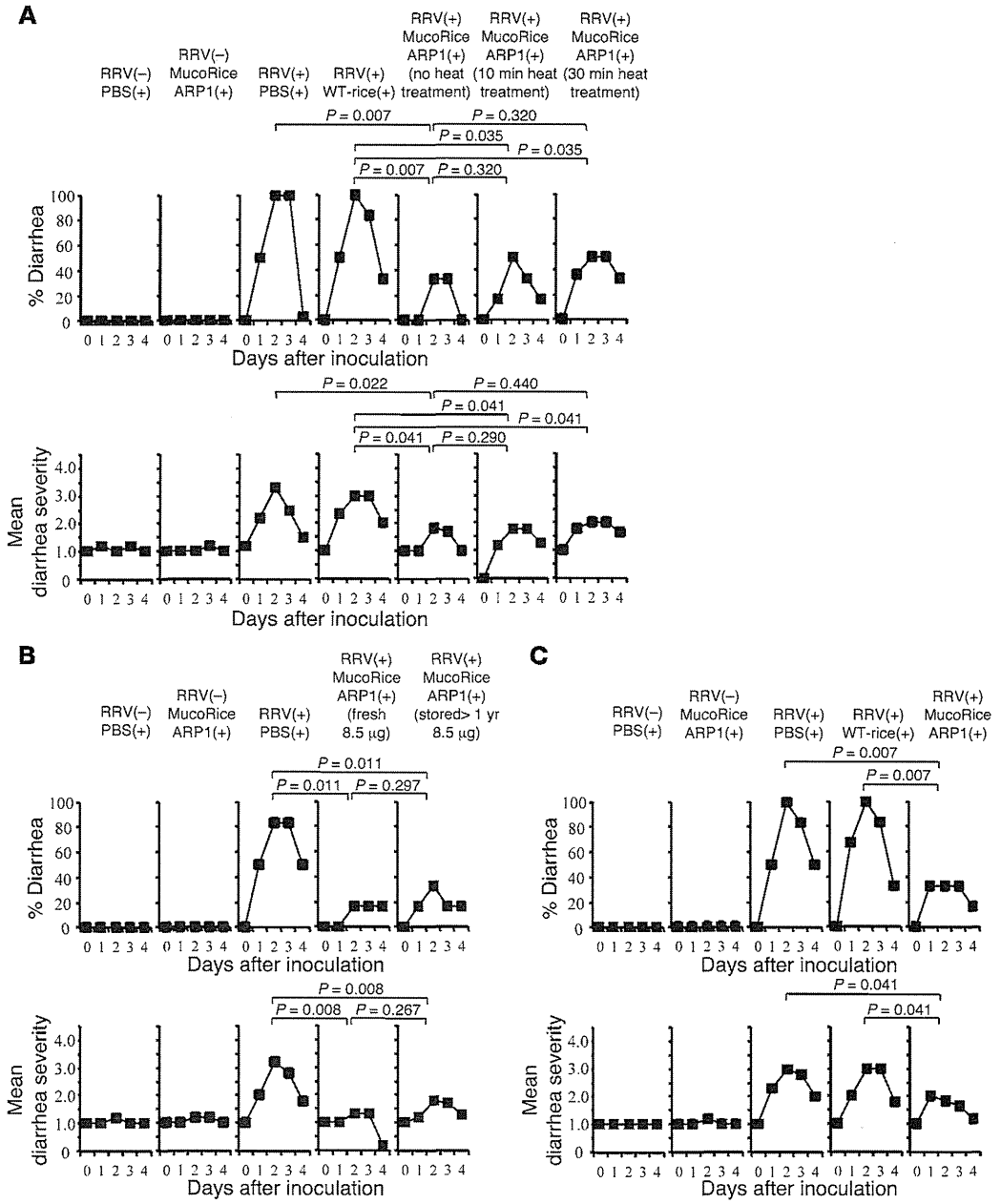


Figure 3

MucoRice-ARP1–mediated protection in a mouse pup model of RV infection. (**A**) Prophylactic administration of MucoRice-ARP1 containing rice water (8.5 μg of ARP1) given to 4-day-old neonatal mouse litters 9 hours prior to RRV inoculation and subsequently given twice daily for 4 consecutive days. Results are shown as percentage of mice with diarrhea and mean diarrhea severity. (**B**) Prophylactic administration of long-term stored MucoRice-ARP1 containing rice water given 9 hours prior to RRV inoculation and subsequently given twice daily for 4 consecutive days. (**C**) Therapeutic administration of MucoRice-ARP1 (8.5 μg of ARP1) given 9 hours after RRV inoculation and subsequently given twice daily for 4 consecutive days. Statistical significance among groups was calculated using the Kruskal-Wallis test followed by the Mann-Whitney *U* test.

Discussion

Although we have previously developed an oral rice-based vaccine expressing botulinum neurotoxin type A against botulism and CTB for cholera/enterotoxigenic *E. coli* (ETEC) (32, 33), expression of a cytokine or antibody such as human IL-10 or single-chain Fv (scFv) from an M cell–specific monoclonal antibody in rice resulted in insoluble proteins. Thus, we could not apply these proteins orally (data not shown). The present study markedly extends the potential of rice plants into an antibody production system. Using the MucoRice technology, we successfully produced the variable domain of a RV-specific llama-heavy chain antibody and showed that oral administration of MucoRice-ARP1 afforded protection in mice. Unique features of this product, including water solubility, high expression level, heat stability, and long-term stability upon storage at room temperature, make MucoRice-ARP1 an attractive ready-to-use, oral, anti-RV product that can easily be distributed without the need for a cold chain.

Downloaded on August 8, 2013. The Journal of Clinical Investigation. More information at www.jci.org/articles/view/70266

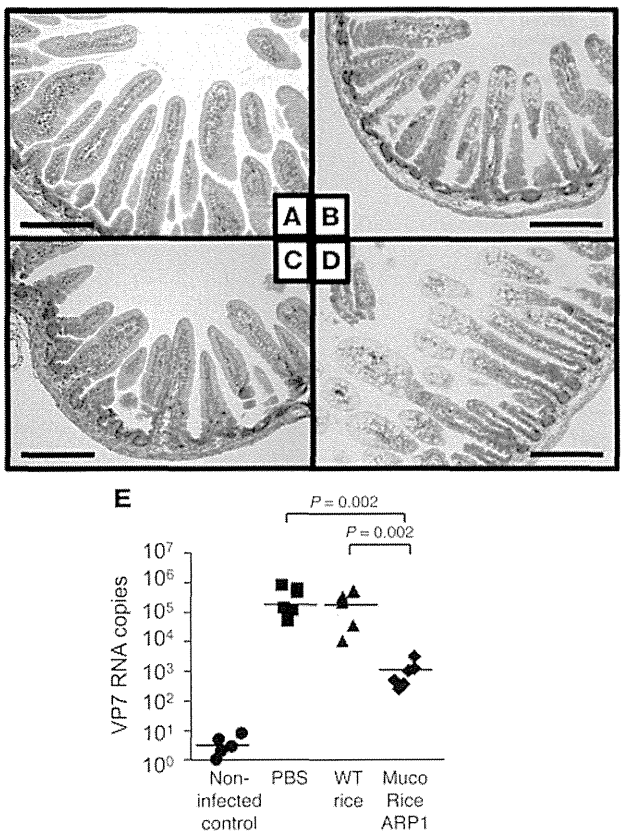


Figure 4
Histopathological examination and quantification of RV in the small intestine after RRV-induced diarrhea. (**A**–**D**) Histological sections of small intestine ileal mucosa from a noninfected mouse treated with PBS (**A**), RRV-infected mouse treated with PBS (**B**), RRV-infected mouse treated with MucoRice-ARP1 (**C**), and RRV-infected mouse treated with WT rice extract (**D**). In the RRV-infected mouse pups treated with PBS (**B**) or WT rice extract (**D**), large vacuoles are seen in the enterocytes lining the surface of the villous tips in the ileum. Histopathological lesions were absent in noninfected control mice (**A**) and pups treated with MucoRice-ARP1 (**C**). Sections were stained with H&E. Scale bars: 100 μm. (**E**) Quantification of RV in the small intestine after MucoRice-ARP1 treatment. Results are given as VP7 RNA copies per weight (mg) of small intestine. Bars indicate the median RV load for each group of animals. Differences in virus load between the MucoRice-ARP1–treated group and the WT rice-fed group or PBS-treated (RRV alone) group were evaluated using the Kruskal-Wallis test followed by the Mann-Whitney *U* test.

The first important aspect of plant-based antibody system is that large amounts of antibodies can be produced at a low cost (39). Various plants have previously been used to produce antibodies and antibody fragments, including scFv, IgG, Fab, and VHH (40–43). The plant-based antibody (fragment) yields are reported as 28–136 mg kg^{-1} (VHH) and 500 mg kg^{-1} (fully assembled IgG) in tobacco leaves (40, 41) and 0.67% (scFv) and 3%–6% (Fab) of the total soluble protein in tobacco seeds (43) and *Arabidopsis thaliana* leaves (42), respectively. As suppression of internal storage protein production may enhance accumulation of foreign proteins (44), we introduced RNAi to the ARP1 production system and developed a T-DNA expression system by cointroduction of antisense genes specific for the 13-kDa prolamin and glutelin storage proteins to minimize their expression in order to allow expansion of space for ARP1 in the rice seeds (33). In comparison with those plant-based antibodies (scFV, IgG, Fab, and VHH) described previously (40–43), the MucoRice system achieved an extremely high yield of soluble antibodies (8.5 g soluble ARP1 kg^{-1} of total weight or 11.9% of total protein) in rice seeds. Most important, MucoRice-ARP1 is highly water soluble (Figure 1C) due to the localization of the ARP1 antibody fragment in the PB-II and cytoplasm fractions, which are the soluble components of rice (Figure 1E).

A second important aspect of the plant-based antibody system is that, in contrast with previous systems, there is no need for purification. As MucoRice-ARP1 originates from edible rice seeds, the obtained rice powder can be directly used as an ingredient for use in a broad range of nutraceuticals applied orally as a rice powder or rice water by simply dissolving the MucoRice-ARP1 rice powder in water (at room temperature), thus eliminating the costs for purification.

A third important aspect is that cold-chain transport and storage are unnecessary, as RV antibodies are primarily needed in developing countries. Antibody-producing tobacco leaves need storage under −20 degrees, and antibody-producing tomatoes need a temperature below 4 degrees. In contrast, antibody-producing cereals such as rice and wheat as well as pea seeds can be stored at room temperature (39). In this study, we demonstrated that MucoRice-ARP1 was stable at room temperature for more than 1 year. Furthermore, in a boiling experiment, extracts from MucoRice-ARP1 demonstrated a higher heat resistance than the purified ARP1 from MucoRice-ARP1 and from yeast-based ARP1, suggesting that the presence of rice proteins in the sample contributes to an increased stability of ARP1. The high stability of MucoRice-ARP1 confers an economic advantage over products that require a cold-chain for distribution and storage. The cost effectiveness of MucoRice-ARP1, added to the fact that rice is one of the most important types of staple food, may contribute to making this product accepted and affordable and thus especially suitable for populations in developing countries where the burden of RV-induced diarrhea is high.

RV-induced diarrhea in humans is caused by RVs belonging to group A. A large number of serotypes have been isolated from infected individuals, although the most common serotypes worldwide are G1P[8], G2P[4], G4P[8], and G9P[8] (45). It has recently been shown that yeast produced ARP1 can neutralize a broad range of virus serotypes in vitro (M37 G1[P6], Wa G1[P8], Rv4 G1[P8], DS-1 G2[P4], P G3[P8], ST-3 G4[P4], Va70 G4[P8], 69M G8[P10], WI-61 G9[P8], F45 G9[P8]) (31). Moreover, it could also bind the recently emerged G8, G10, and G12 strains (31). In this study, we showed that ARP1 produced in rice (MucoRice-ARP1) neutralizes RV strains in a range similar to that of the yeast produced ARP1. Thus, it should be possible to use rice-based ARP1 treatment regardless of geographical differences in strain diversity.

The broad reactivity and neutralizing capacity of ARP1 indicates that it recognizes crossreactive epitopes. The outer layer proteins of RV, VP7 and VP4, both contain neutralizing epitopes, although these tend to be serotype specific (31). The middle layer protein, VP6, is the most abundant and immunodominant viral protein and contains the group and subgroup determining epitopes (31). Iturriza-Gómara et al. recently identified the epitope recognized by ARP1 and showed that it binds to polymeric VP6 (31), thus explaining its broad neutralization capacity. With regard to the role of anti-VP6 antibodies against RV-induced diarrhea, crossreactive anti-RV VP6 antibodies appear to be unable to neutralize infection by immune exclusion both in vitro and in vivo. However, some anti-VP6 secretory IgA bind to RV double

Downloaded on August 8, 2013. The Journal of Clinical Investigation. More information at www.jci.org/articles/view/70266

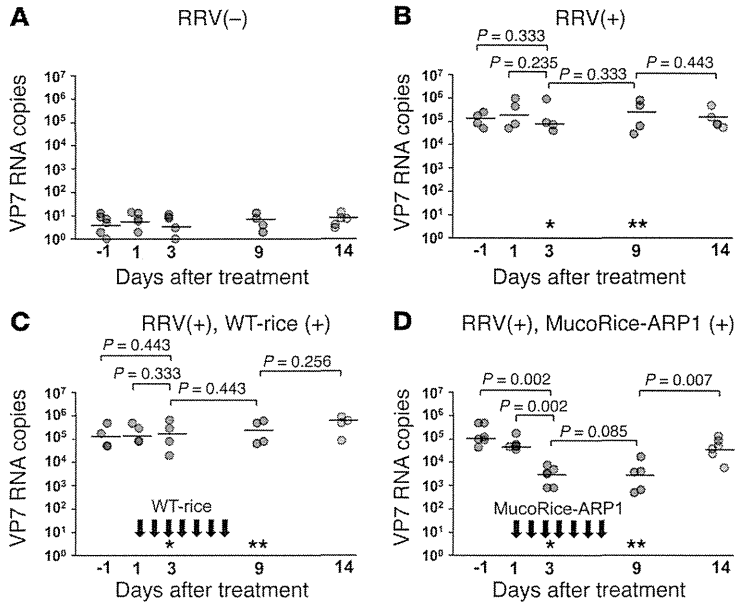


Figure 5

Inhibition of viral shedding after MucoRice-ARP1 treatment in SCID mice with chronic RRV infection. (**A–D**) MucoRice-ARP1 powder (200 mg) containing 1.7 mg of ARP1 was intragastrically administered to mice twice daily for 7 days. Fecal samples were collected on days –1, 1, 3, 9, and 14 (n = 6, each group). Control feces were collected from noninfected SCID mice. Positive control feces were collected from RRV-inoculated SCID adult mice treated with PBS. Bars indicate the median RV shedding level for each group of animals. Results are given as VP7 RNA copies per mg of stool. MucoRice-ARP1–treated mice showed a significantly lower amount of VP7 RNA copies on day 3 (*P = 0.002) and day 9 (**P = 0.003) compared with WT rice–treated or PBS-treated mice. Statistical significance among groups was calculated using the Kruskal-Wallis test followed by the Mann-Whitney U test.

and triple-layered particles, conferring protection by intracellular neutralization following transcytosis in mice and also in vitro using polarized Caco-2 cells (46–48). The mechanism by which these antibodies interfere with RV replication is not yet understood, and it is postulated that they may interfere with early replication events by inducing conformational changes in the VP6 layer, or alternatively, the anti-VP6 antibodies may interfere with the secretory pathways during late replication, preventing virus assembly and release (47). It is also known that not all anti-VP6 antibodies can neutralize RV replication intracellularly (49). Recently, the ARP1 llama-derived antibody that binds specifically to RV VP6 has been shown to bind to RV in ELISA and immune electron microscopy and to neutralize infection with a variety of RV genotypes in vitro using classical neutralization assays and in an in vivo mouse pup model, which suggests that these antibodies are likely to neutralize RV by immune exclusion (31, 50–53). Although the mechanism by which the llama antibodies neutralize infection is not yet understood, it is possible that the VP6-specific VHH could block a conformational change in the outer layer proteins of the RV particle, VP7 and/or VP4, preventing attachment and/or entry of the virus particle, as previously suggested (31, 50). This is supported by existing evidence that the phenotype of the outer layer VP4 can be altered by interactions with different VP7 proteins and of the marked impact of the protein-protein interactions among the constituent proteins of the RV inner, middle and outer layers (54). The neutralizing capacity associated with the recognition of crossreactive epitopes within VP6 of these antibodies may also relate to their small size, as bivalent VHH antibodies showed a much reduced neutralizing activity compared with the monovalent VHH, suggesting that these may have no or limited access to the exposed VP6 of an infectious triple-layered RV particle (50).

A recent clinical trial in Bangladesh proved that yeast-derived ARP1 was effective in reducing the severity of the disease in RV-infected children with no adverse events considered to be related to ARP1 treatment (S.A. Sarker, M. Jäkel, S. Sultana, N.H. Alam, P.K. Bardhan, M.A. Salam, W. Theis, L. Hammarström, L.G.J. Frenken, unpublished observations), and, as there are no differences in the efficacy between the yeast-derived ARP1 and MucoRice-ARP1 in vitro, the latter has a potential to be equally effective in a clinical setting. With regard to the safety of the rice seed component, we have used a rice-based cholera vaccine in 5 cynomolgus macaques and did not observe any adverse events (55, 56). Rice seed itself is an edible plant; thus, taken together with the present results, we consider MucoRice-ARP1 to be safe and to be able to provide effective protection against RV-induced diarrhea. One remaining problem is the dose translation between mouse and human based on the body surface area normalization between animals and human adults (57, 58). The dose for neonatal (17 μg/d) or adult (SCID) mice (3.4 mg/d) translates as 0.7 mg of ARP1/kg/d (82 mg of MucoRice-ARP1 powder per kg/d) and 20.7 mg/kg/d of ARP1 (107 mg/d of MucoRice-ARP1 powder per mg/d), respectively, in 6- to 24-month-old children. In the recent Bangladesh study, in which 6- to 24-month-old children with RV diarrhea were treated, 5–10 mg of ARP1/kg 3 times per day was used, equivalent to a daily ARP1 dose of 15–30 mg/kg/d. Thus, the dose of rice-produced ARP1 we used for neonatal and SCID mice is lower, or at least equivalent, to the dose given to children in the Bangladesh study.

Therapeutic proteins produced using recombinant DNA technologies are generally complex, heterogeneous, and subject to a variety of enzymatic or chemical modifications during expression (59). Since efficacy, clearance, and immunogenicity can be highly dependent on the protein sequence and specific posttranslational modifications, it is important to characterize their structure by determining the sequence of the recombinant protein and amino acid modifications that may affect the safety and activity of therapeutic protein products using analytical tools such as mass spectrometry (60). However, to date there is no report showing the complete amino acid sequence of plant-derived therapeutics, including vaccines, antibodies, or enzymes. Using mass spectrometry, we elucidated the full amino acid sequence of rice-based ARP1, confirming that it does not contain any amino acid modification. The present study is thus the first, to our knowledge, to include this information for the safety and efficacy of the product.

In conclusion, we have developed a high-yield, purification-free, cold-chain–free, and heat stable, rice-based antibody fragment containing product for oral administration that provides protection against RV of different serotypes in vitro and reduction of virus shedding and disease in both immunocompetent and immunodeficient mice. MucoRice-ARP1 offers what we believe is a novel approach to the prevention and treatment of RV-induced diarrhea in both devel-

Downloaded on August 8, 2013. The Journal of Clinical Investigation. More information at www.jci.org/articles/view/70266

oped and developing countries and may provide an alternative to vaccination in individuals in whom current vaccines are contraindicated. It could also complement current vaccination programs by targeting different age groups and situations in which rapid protection or control of spread of infection is required. This technology can also be extended to the production of antibody fragments against other enteric pathogens such as norovirus, and may also be applicable to intestinal diseases beyond infections.

Methods

DNA constructs and transformation of rice plants. ARP1 was selected from a library of VHH fragments generated from llamas immunized with RRV (strain MMU18006, P5B[3], G3) (61), as previously described (29). Briefly, a phage display library was constructed from B lymphocyte cDNA encoding VHHs and selected by biopanning on RRV at a low pH (2.3). ARP1 was previously referred to as 2B10 (29) or VHH1 (30). The gene encoding ARP1 was synthesized with an optimized codon usage for plants and inserted into a binary T-DNA vector (pZH2B/35SNos). This vector contains a cassette for overexpression of ARP1 and a combination cassette for RNAi suppression of production of the major rice endogenous storage proteins, prolamin (13 kDa) and glutelin (Figure 1A and ref. 35). The plasmid was transformed into a japonica variety of rice plants, Nippon-Bare, using a Agrobacterium-mediated method described previously (32).

Protein analyses. Total protein was extracted from transgenic rice plant seeds using a buffer containing 2% (wt/vol) SDS, 8 M urea, 5% (wt/vol) β-mercaptoethanol, 50 mM Tris-HCl (pH 6.8), and 20% (wt/vol) glycerol as previously described (62). The level of ARP1 was determined by Western blot densitometry analysis using purified yeast-derived ARP1 (BAC BV) as a standard. For detection of ARP1, a rabbit anti-ARP1 polyclonal antibody was prepared in our laboratory.

ARP1 was also extracted from MucoRice-ARP1 powder using PBS (rice water) or 8 M urea in PBS at room temperature. After centrifugation, the supernatants were analyzed by SDS-PAGE and Western blot. Purified ARP1 from MucoRice-ARP1 was produced from MucoRice-ARP1 containing rice water, using gel filtration on Sephadex G100 columns (GE Healthcare).

Mass spectrometry. Mass spectrometric analysis was performed as previously described (63). Samples were injected into a nanoflow LC system (Dina; KYA Technologies) and sprayed into a quadrupole time-of-flight tandem mass spectrometer (QSTAR Elite; AB SCIEX). The QSTAR analysis failed to detect 39 amino acids of the C-terminal peptide generated by trypsin because of the large mass (4256 Da) (Supplemental Table 1). To obviate this problem, a linear ion trap Orbitrap mass spectrometer (LTQ Orbitrap Velos; Thermo Fisher Scientific), which enables shotgun proteomics analysis with high resolution and high mass accuracy, was used. The data were analyzed using a Mascot Search Server.

Immune electron microscopy. The distribution of ARP1 in rice seeds was analyzed using immunoelectron microscopy as previously described, with some modification (32). Rice caryopses sections stained with a rabbit anti-ARP1 polyclonal antibody and gold particle–conjugated (18 nm) goat anti-rabbit IgG (Jackson) were examined using transmission electron microscopy (HITACHI). For localization of ARP1 in the seed, a frozen section was stained with rabbit anti-ARP1 polyclonal antibody and visualized using 3,3′-diaminobenzidine.

Binding to RRV by ELISA. ELISA plates were coated with HBC anti-RRV (38) as a capture antibody, followed by RRV (2×10^6 ffu ml^{-1}) and ARP1 (2-fold dilutions between 100 and 12.5 ng ml^{-1}). Biotinylated rabbit anti-VHH K492 antibody (BAC BV), followed by AP conjugated streptavidin (BD Pharmingen), was used for quantification of ARP1 bound to RRV. The assay was developed using para-nitrophenol phosphate (pNPP) (Sigma-Aldrich) as a substrate, and the optical density was read at 405 nm using a Varioskan Flash (Thermo Electron Corporation). HBC was produced by vaccination of pregnant cows in a Swiss dairy farm with human strains of RV, i.e., Wa, RV3, RV5, and ST3, representing serotypes G1 to G4 (38).

In vitro heat stability test. To test the heat stability, samples with 100 ng ml^{-1} of different ARP1 preparations (MucoRice-ARP1 containing rice water, ARP1 purified from MucoRice-ARP1, and ARP1 purified from yeast) and HBC containing 100 ng ml^{-1} of total protein were boiled at 100°C for 10, 20, and 30 minutes. After cooling, 2-fold dilutions of each sample were tested in ELISA as described above for the ARP1-containing samples. For the HBC samples, a rabbit anti-RV K230 antiserum (a gift from Lennart Svensson, University of Linköping, Linköping, Sweden) was used as a capture antibody for RRV. The functional anti-RV IgG antibodies contained in the HBC samples were detected using AP-conjugated goat anti-bovine IgG (H+L) (Jackson ImmunoResearch Laboratories). The percentage of binding activity was calculated in relation to nonboiled samples in the same ELISA plate at a particular concentration before reaching binding saturation (25 ng ml^{-1} ARP1 for ARP1-containing samples and 50 ng ml^{-1} total protein for HBC samples).

In vitro neutralization assay. In vitro neutralization assays were carried out using MA104 cells and the human RV strains Wa G1P[8], ST-3 G4P[6], 69M G8P[10], F45 G9P[8] and Va70 G4P[8] as previously described (64). Briefly, 10^5 MA104 cells ml^{-1} were seeded in 96-well plates. Forty-eight hours later, 2-fold dilutions of antibodies or rice protein preparations were incubated in duplicates with 200 ffu of RVs, and the mixture was subsequently used for infection of the seeded cells. Infected cells were detected by immunofluorescence staining using a monoclonal mouse anti-VP6 antibody (Austral Biologicals) and FITC-conjugated rabbit anti mouse IgG antibodies (Dako). Significant neutralization was defined by a reduction of the infected cells higher than 60% in relation to the control wells.

In vivo assays. Pregnant BALB/c mice were purchased from Japan SLC. Each dam was housed individually with her litter in cages in the same room under negative pressure in the animal facility on a 12-hour light/12-hour dark cycle. Food and deionized water were autoclaved and provided ad libitum.

To determine the level of protection against RRV infection conferred by MucoRice-ARP1 in immunocompetent mice (BALB/c), 4 day-old pups were infected orally using 2×10^7 ffu trypsin-activated RRV ($n = 6$ to 10 per group). Rice water derived from the supernatant of a mixture of MucoRice-ARP1 powder and PBS, containing a total of 8.5 μg of ARP1, was intragastrically administered to the pups 9 hours prior to RRV inoculation and subsequently given twice daily for 4 consecutive days. In order to examine the heat stability and long-term stability at room temperature, MucoRice-ARP1 heat-treated at 94°C for 10 and 30 minutes or stored at room temperature over 1 year was intragastrically applied to the pups prior to RRV inoculation and subsequently given twice daily for 4 consecutive days. In order to examine the therapeutic effects of MucoRice-ARP1, MucoRice-ARP1 was intragastrically given to the pups 9 hours after RRV inoculation and subsequently given twice daily for 4 consecutive days. In the control groups, PBS or MucoRice-ARP1 was intragastrically administered twice daily for 4 consecutive days without RRV inoculation. The pups were examined daily for evidence of RV-induced diarrhea by gentle abdominal palpation. Diarrhea was recorded and scored blindly from 1 to 4 based on stool color, amount, and consistency as described previously, with a minor modification (65). Normal feces or absence of feces were given a score of 1. Exceptionally loose feces were given a score of 2. Loose yellow-green feces were given a score of 3. Watery feces were given a score of 4. A score of 2 or greater was considered diarrhea, as described previously (65). The percentage of mice with diarrhea for each group was calculated by dividing the number of diarrheic samples by the total number of mice scored for diarrhea each day. The diarrhea severity was determined by dividing the sum of all scores by the number of total number of mice scored for diarrhea each day. Finally, we calculated percentage of mice with diarrhea on a daily basis in each group based on accumulated data

Downloaded on August 8, 2013. The Journal of Clinical Investigation. More information at www.jci.org/articles/view/70266

after repeated experiments and compared the percentage of diarrhea and the mean diarrhea severity on day 2 after RRV inoculation among groups using the Kruskal-Wallis test followed by the Mann-Whitney test. Samples of small intestine were collected 3 days after infection for histopathological analysis and viral RNA quantification by real-time PCR against VP7 RNA.

Evaluation of histopathology. To evaluate histopathology, small intestinal samples were collected at 3 days after RRV inoculation ($n = 3$ for each group). Samples from duodenum, jejunum, and ileum were fixed in 4% paraformaldehyde for 12 hours. Subsequently, the samples were transferred to graded ethanol for dehydration, embedded in paraffin wax, and sectioned at 4 μm using a microtome. Sections were stained with H&E (65) and visualized under light microscope. Duodenal, jejunal, or ileal villi were examined for presence of enterocyte injury, inflammation, and vacuolization by a person blinded to the treatment given to the mice.

Quantification of viral RNA. Total RNA was isolated from small intestines of neonatal pups, using TRIzol reagent (Life Technologies) and treated with RNase-free DNase (QIAGEN) following the manufacture's protocol. To evaluate viral shedding in the chronic RRV infection SCID mouse model, fecal specimens were prepared as 10% suspensions with PBS, and total RNA was isolated using TRIzol following the manufacturer's protocol. RV *VP7* mRNA or viral genomic RNA was amplified at 58°C in the presence of 600 nmol l^{-1} primers, 300 nmol l^{-1} probe, and 5 mmol l^{-1} Mn to generate a 121-bp–long amplicon. The sense primer (VP7 forward, 5′-CCAAGGGAAAATGTAGCAGTAATTC-3′; nt 791-815), the antisense primer (VP7 reverse, 5′-TGCCACCATTCTTTC-CAATTAA-3′; nt 891-912), and the probe (5′-6FAMTAACGGCTGATCCAAC-CACAGCACCTAMRA-3′; nt 843-867) were designed on the basis of the *VP7* gene sequence of RRV (GenBank AF295303). Reverse transcription reactions were carried out in a final volume of 20 μl using Superscript III Reverse Transcriptase (Invitrogen) following the manufacturer's protocol. Each RT reaction sample was analyzed by the Light-Cycler 480 System II (Roche Applied Science) following the manufacturer's protocol. A standard curve was generated using a plasmid that contained a RRV *VP7* gene, and the lowest level of detection of the PCR was 10 viral RNA copies. The presence of less than 10 copies of VP7 RNA per weight (mg) was defined as clearance of infection. The ratio of *VP7* gene copy number to the weight of the stool sample (mg) or to the weight of the small intestine segment (mg) was compared among the groups.

In vivo assays in SCID mice. The efficacy of MucoRice-ARP1 against chronic RRV infection was determined in SCID mice. Pregnant C.B-17 SCID/SCID mice were purchased from Nihon Clea Inc. Four-day-old pups were infected orally with 2×10^7 ffu RRV. All of the mice developed diarrhea and chronic infection. The litters were weaned by removing each mouse from the dam at 21 days of age. After inoculation, all mice were confirmed to have low serum immunoglobulin levels by ELISA and examined for pathogen surveillance, but no specific pathogen, including murine norovirus, was found at 5 weeks of age. When mice were 6 weeks old (i.e., 6 weeks after RRV infection), 200 mg of MucoRice-ARP1 powder (containing 1.7 mg of ARP1) or nontransformed WT rice powder was intragastrically administered twice daily for 7 consecutive days. Mice were examined on days −1, 1, 3, 9, and 14 for diarrhea and viral shedding. Diarrhea scores, percentage of mice with diarrhea, and disease severity were measured as described above. Viral shedding was measured by VP7-specific real-time PCR using fecal samples. In order to examine the therapeutic effects of MucoRice-ARP1 in SCID mouse pups, MucoRice-ARP1 was intragastrically given to the pups 9 hours after RRV inoculation and subsequently given twice daily for 4 consecutive days. Diarrhea scores, percentage of animals with diarrhea, and disease severity were measured as described above.

Statistics. Individual data for the percentage of mice with diarrhea, the disease severity scores, and differences in the intestinal virus load as assessed by real-time PCR were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney test. $P < 0.05$ was considered statistically significant.

Study approval. All mouse experiments were approved by the local ethics committee of the Institute of the Medical Science at the University of Tokyo.

Acknowledgments

This work was supported by grants from the Programs of Special Coordination Funds for Promoting Science and Technology, the Grant-in-Aid for Scientific Research on Priority Areas, and the Grant-in-Aid for Scientific Research (A and S) of the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to H. Kiyono); the Ministry of Health, Labor and Welfare of Japan (to Y. Yuki and H. Kiyono); Core Research of Evolutional Science Technology (CREST) of the Japan Science and Technology Agency (JST) (to H. Kiyono); the New Energy and Industrial Technology Development Organization (NEDO) (to Y. Yuki and H. Kiyono); the Young Researcher Overseas Visits Program for Vitalizing Brain Circulation of the Japan Society for the Promotion of Science (JSPS) (to H. Kiyono and Y. Yuki); the Global Center of Excellence Program Center of Education and Research for Advanced Genome-Based Medicine – For Personalized Medicine and the Control of Worldwide Infectious Diseases (to H. Kiyono); a research fellowship of the Japan Society for the Promotion of Science (to T. Nochi); Adaptive and Seamless Technology Transfer Program Through Target-Driven R&D (A-Step) (to Y. Yuki); the Japan Foundation for Pediatric Research (to D. Tokuhara); a Houjinkai fellowship award of the Department of Pediatrics at Osaka City University Graduate School of Medicine (to D. Tokuhara); the Association for Preventive Medicine of Japan (to D. Tokuhara); and the European Union–funded project LACTOBODY (202162). We are grateful to H. Hatai, Y. Chen, and A. Cyubachi for technical support. We thank T. Masumura and K. Tanaka of the Kyoto Prefectural Institute of Agricultural Biotechnology for support. We also thank J. Kunisawa of the Institute of Medical Science, the University of Tokyo, and K. Fujihashi of the University of Alabama at Birmingham for support. We would like to thank N. Pant for technical advice regarding the in vivo neutralization assays and the animal model of rotavirus infection.

Received for publication April 2, 2013, and accepted in revised form June 13, 2013.

Address correspondence to: Yoshikazu Yuki, Department of Mucosal Immunology, The Institute of Medical Science, The University of Tokyo, Tokyo, 108-8639, Japan. Phone: 81.3.5449.5274; Fax: 81.3.5449.5411; E-mail: yukiy@ims.u-tokyo.ac.jp.

1. O'Ryan M, et al. Rotavirus-associated medical visits and hospitalizations in South America: a prospective study at three large sentinel hospitals. *Pediatr Infect Dis J.* 2001;20(7):685–693.
2. Nakagomi T, Nakagomi O, Takahashi Y, Enoki M, Suzuki T, Kilgore PE. Incidence and burden of rotavirus gastroenteritis in Japan, as estimated from a prospective sentinel hospital study. *J Infect Dis.* 2005;192(suppl 1):S106–S110.
3. Fischer TK, et al. Hospitalizations and deaths from diarrhea and rotavirus among children < 5 years of age in the United States, 1993–2003. *J Infect Dis.* 2007;195(8):1117–1125.
4. Parashar UD, Hummelman EG, Bresee JS, Miller MA, Glass RI. Global illness and deaths caused by rotavirus disease in children. *Emerg Infect Dis.* 2003;9(5):565–572.
5. Bernstein DI. Rotavirus overview. *Pediatric Infect Dis J.* 2009;28(suppl 3):S50–S53.
6. Vesikari T, et al. Rotavirus efficacy and safety trial (REST) study team. Safety and efficacy of a pentavalent human-bovine (WC3) reassortant rotavirus vaccine. *N Engl J Med* 2006;354(1):23–33.
7. Ruiz-Palacios GM, et al. Human rotavirus vaccine study group. Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis. *N Engl J Med.* 2006;354(1):11–22.
8. Glass RI, Parashar UD. The promise of new rotavirus vaccines. *N Engl J Med.* 2006;354(1):75–77.
9. Madhi SA, et al. Effect of human rotavirus vaccine

Downloaded on August 8, 2013. The Journal of Clinical Investigation. More information at www.jci.org/articles/view/70266

on severe diarrhea in African infants. *N Engl J Med.* 2010;362(4):289–298.
10. Armah GE, et al. Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in sub-Saharan Africa: a randomised, double-blind, placebo-controlled trial. *Lancet.* 2010;376(9741):606–614.
11. Zaman K, et al. Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in Asia: a randomised, double-blind, placebo-controlled trial. *Lancet.* 2010;376(9741):615–623.
12. Werther RL, Crawford NW, Boniface K, Kirkwood CD, Smart JM. Rotavirus vaccine induced diarrhea in a child with severe combined immune deficiency. *J Allergy Clin Immunol.* 2009;124(3):600.
13. Patel NC, et al. Vaccine-acquired rotavirus in infants with severe combined immunodeficiency. *N Engl J Med.* 2010;362(4):314–319.
14. Uygungil B, Bleesing JJ, Risma KA, McNeal MM, Rothenberg ME. Persistent rotavirus vaccine shedding in a new case of severe combined immunodeficiency: A reason to screen. *J Allergy Clin Immunol.* 2010;125(1):270–271.
15. Rotbart HA, et al. Neonatal rotavirus-associated necrotizing enterocolitis: case control study and prospective surveillance during an outbreak. *J Pediatr.* 1988;112(1):87–93.
16. Cunliffe NA, et al. Healthcare-associated viral gastroenteritis among children in a large pediatric hospital, United Kingdom. *Emerg Infect Dis.* 2010;16(1):55–62.
17. Stelzmueller I, et al. A cluster of rotavirus enteritis in adult transplant recipients. *Transpl Int.* 2005; 18(4):470–474.
18. Krüger W, Stockschläder M, Zander AR. Transmission of rotavirus diarrhea in a bone marrow transplantation unit by a hospital worker. *Bone Marrow Transplant.* 1991;8(6):507–508.
19. Gilger MA, Matson DO, Conner ME, Rosenblatt HM, Finegold MJ, Estes MK. Extraintestinal rotavirus infections in children with immunodeficiency. *J Pediatr.* 1992;120(6):912–917.
20. Oishi I, et al. Serial observations of chronic rotavirus infection in an immunodeficient child. *Microbiol Immunol.* 1991;35(11):953–961.
21. Liakopoulou E, et al. Rotavirus as a significant cause of prolonged diarrhoeal illness and morbidity following allogeneic bone marrow transplantation. *Bone Marrow Transplant.* 2005;36(8):691–694.
22. Saulsbury FT, Winkelstein JA, Yolken RH. Chronic rotavirus infection in immunodeficiency. *J Pediatr.* 1980;97(1):61–65.
23. Sarker SA, et al. Successful treatment of rotavirus diarrhea in children with immunoglobulin from immunized bovine colostrum. *Pediatr Infect Dis J.* 1998;17(12):1149–1154.
24. Sarker SA, et al. Randomized, placebo-controlled, clinical trial of hyperimmunized chicken egg yolk immunoglobulin in children with rotavirus diarrhea. *J Pediatr Gastroenterol Nutr.* 2001;32(1):19–25.
25. Hamers-Casterman C, et al. Naturally occurring antibodies devoid of light chains. *Nature* 1993;363(6428):446–448.
26. van der Linden RH, et al. Comparison of physical chemical properties of llama VHH antibody fragments and mouse monoclonal antibodies. *Biochim Biophys Acta.* 1999;1431(1):37–46.
27. Dolk E, et al. Induced refolding of a temperature denatured llama heavy-chain antibody fragment by its antigen. *Proteins.* 2005;59(3):555–564.
28. Frenken LG, et al. Isolation of antigen specific llama VHH antibody fragments and their high level secretion by *Saccharomyces cerevisiae*. *J Biotechnol.* 2000;78(1):11–21.
29. van der Vaart JM, et al. Reduction in morbidity of rotavirus induced diarrhoea in mice by yeast produced monovalent llama-derived antibody fragments. *Vaccine.* 2006;24(19):4130–4137.
30. Pant N, et al. Lactobacilli expressing variable domain of llama heavy-chain antibody fragments (Lactobodies) confer protection against rotavirus-induced diarrhea. *J Infect Dis.* 2006;194(11):1580–1588.
31. Aladin F, et al. In vitro neutralization of rotavirus infection by two broadly specific recombinant monovalent llama-derived antibody fragments. *PLoS One.* 2012;7(3):e32949.
32. Nochi T, et al. Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. *Proc Natl Acad Sci U S A.* 2007;104(26):10986–10991.
33. Tokuhara D, et al. Secretory IgA-mediated protection against *V. cholerae* and heat-labile enterotoxin-producing enterotoxigenic *Escherichia coli* by rice-based vaccine. *Proc Natl Acad Sci U S A.* 2010; 107(19):8794–8799.
34. Yuki Y, et al. RNAi suppression of rice endogenous storage proteins enhances the production of rice-based Botulinum neutrotoxin type A vaccine. *Vaccine.* 2012;30(28):4160–4166.
35. Kuroda M, Kimizu M, Mikami C. A simple set of plasmids for the production of transgenic plants. *Biosci Biotechnol Biochem.* 2010;74(11):2348–2351.
36. Tanaka K, Sugimoto T, Ogawa M, Kasai Z. Isolation and characterization of two types of protein bodies in the rice endosperm. *Agric Biol Chem.* 1980; 44(7):1633–1639.
37. Tanaka K, Hayashida S, Hongo M. The relationship of the feces protein particles to rice protein bodies. *Agric Biol Chem.* 1975;39(2):515–518.
38. Brüssow H, Hilpert H, Walther I, Sidoti J, Mietens C, Bachmann P. Bovine milk immunoglobulins for passive immunity to infantile rotavirus gastroenteritis. *J Clin Microbiol.* 1987;25(6):982–986.
39. Stoger E, et al. Practical considerations for pharmaceutical antibody production in different crop systems. *Mol Breed.* 2002;9(3):149–158.
40. Ismaili A, Jalali-Javaran M, Rasaee MJ, Rahbarizadeh F, Forouzandeh-Moghadam M, Memari HR. Production and characterization of anti-(mucin MUC1) single-domain antibody in tobacco (*Nicotiana tabacum* cultivar Xanthi). *Biotechnol Appl Biochem.* 2007;47(1):11–19.
41. Giritch A, et al. Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. *Proc Natl Acad Sci U S A.* 2006;103(40):14701–14706.
42. Peeters K, De Wilde C, Depicker A. Highly efficient targeting and accumulation of a F(ab) fragment within the secretory pathway and apoplast of *Arabidopsis thaliana*. *Eur J Biochem.* 2001;268(15):4251–4260.
43. Fiedler U, Conrad U. High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds. *Biotechnol.* 1995; 13(10):1090–1093.
44. Tada Y, Utsumi S, Takaiwa F. Foreign gene products can be enhanced by introduction into low storage protein mutants. *Plant Biotechnol J.* 2003; 1(6):411–422.
45. Kirkwood CD. Genetic and antigenic diversity of human rotaviruses: potential impact on vaccination programs. *J Infect Dis.* 2010;202(suppl 1):S43–S48.
46. Burns JW, Siadat-Pajouh M, Krishnaney AA, Greenberg HB. Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing activity. *Science.* 1996;272(5258):104–107.
47. Corthesy B, et al. Rotavirus anti-VP6 secretory immunoglobulin A contributes to protection via intracellular neutralization but not via immune exclusion. *J Virol.* 2006;80(21):10692–10699.
48. Schwartz-Cornil I, Benureau Y, Greenberg H, Hendrickson BA, Cohen J. Heterologous protection induced by the inner capsid proteins of rotavirus requires transcytosis of mucosal immunoglobulins. *J Virol.* 2002;76(16):8110–8117.
49. Ruggeri FM, Johansen K, Basile G, Kraehenbuhl JP, Svensson L. Antirotavirus immunoglobulin A neutralizes virus in vitro after transcytosis through epithelial cells and protects infant mice from diarrhea. *J Virol.* 1998;72(4):2708–2714.
50. Garaicoechea L, et al. Llama-derived single-chain antibody fragments directed to rotavirus VP6 protein possess broad neutralizing activity in vitro and confer protection against diarrhea in mice. *J Virol.* 2008;82(19):9753–9764.
51. Pant N, et al. Lactobacilli expressing variable domain of llama heavy-chain antibody fragments (lactobodies) confer protection against rotavirus-induced diarrhea. *J Infect Dis.* 2006;194(11):1580–1588.
52. Pant N, et al. Lactobacilli producing bispecific llama-derived anti-rotavirus proteins in vivo for rotavirus-induced diarrhea. *Future Microbiol.* 2011; 6(5):583–593.
53. van der Vaart JM, et al. Reduction in morbidity of rotavirus induced diarrhoea in mice by yeast produced monovalent llama-derived antibody fragments. *Vaccine.* 2006;24(19):4130–4137.
54. Pesavento JB, Billingsley AM, Roberts EJ, Ramig RF, Prasad BV. Structures of rotavirus reassortants demonstrate correlation of altered conformation of the VP4 spike and expression of unexpected VP4-associated phenotypes. *J Virol.* 2003;77(5):3291–3296.
55. Nochi T, et al. A rice-based oral cholera vaccine induces macaque-specific systemic neutralizing antibodies but does not influence pre-existing intestinal immunity. *J Immunol.* 2009; 183(10):6538–6544.
56. Yuki Y, et al. Induction of toxin-specific neutralizing immunity by molecularly uniform rice-based oral cholera toxin B subunit vaccine without plant-associated sugar modification [published online ahead of print April 22, 2013]. *Plant Biotechnol J.* doi:10.1111/pbi.12071.
57. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *FASEB J.* 2008;22(3):659–661.
58. U.S. Department of Health and Human Services. Food and Drug Administration. Guidance for industry-Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. FDA Web site. www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM078932.pdf. Updated July 22, 2005. Accessed June 28, 2013.
59. Jenkins N, Murphy L, Tyther R. Post-translational modifications of recombinant proteins: significance for biopharmaceuticals. *Mol Biotechnol.* 2008;39(2):113–118.
60. Srebalus Barnes CA, Lim A. Applications of mass spectrometry for the structural characterization of recombinant protein pharmaceuticals. *Mass Spectrom Rev.* 2007;26(3):370–388.
61. Stucker G, Oshiro LS, Schmidt NJ. Antigenic comparisons of two new rotaviruses from rhesus monkeys. *J Clin Microbiol.* 1980;11(2):202–203.
62. Takagi H, et al. A rice-based edible vaccine expressing multiple T cell epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses. *Proc Natl Acad Sci U S A.* 2005;102(48):17525–17530.
63. Oyama M, et al. Temporal perturbation of tyrosine phosphoproteome dynamics reveals the system-wide regulatory networks. *Mol Cell Proteomics.* 2009; 8(2):226–231.
64. Svensson L, Finlay BB, Bass D, von Bonsdorff CH, Greenberg HB. Symmetric infection of rotavirus on polarized human intestinal epithelial (Caco-2) cells. *J Virol.* 1991;65(8):4190–4197.
65. Ciarlet M, Conner ME, Finegold MJ, Estes MK. Group A rotavirus infection and age-dependent diarrheal disease in rats: a new animal model to study the pathophysiology of rotavirus infection. *J Virol.* 2002;76(1):41–57.

Human Vaccines & Immunotherapeutics 9:9, 1962–1970; September 2013; © 2013 Landes Bioscience

Intranasal vaccination with an inactivated whole influenza virus vaccine induces strong antibody responses in serum and nasal mucus of healthy adults

Akira Ainai,[1,2] Shin-ichi Tamura,[2] Tadaki Suzuki,[2] Elly van Riet,[1] Ryo Ito,[2,3] Takato Odagiri,[1] Masato Tashiro,[1] Takeshi Kurata[2], and Hideki Hasegawa[2,*]

[1]Influenza Virus Research Centre; National Institute of Infectious Diseases; Tokyo, Japan; [2]Department of Pathology; National Institute of Infectious Diseases; Tokyo, Japan; [3]Biological Science and Technology; Tokyo University of Science; Chiba, Japan

Keywords: influenza virus, intranasal vaccination, neutralizing antibody, haemagglutination-inhibiting antibody, healthy adult volunteer

Abbreviations: S-IgA, secretory IgA; HA, haemagglutinin; NT titer, neutralization titer; HI assay, haemagglutination inhibition assay; HI titer, haemagglutination inhibition titer; HI antibody response, haemagglutination-inhibiting antibody response; GMT, geometric mean titer; ELISA, enzyme-linked immunosorbent assay

Haemagglutination inhibition (HI) and neutralization (NT) titers as well as haemagglutinin (HA) specific antibody responses were examined in 50 healthy adults aged between 22 and 69 y old after two intranasal administrations of an inactivated whole virus vaccine derived from A/Victoria/210/2009 virus (45 μg HA per dose) at 3 week intervals. Serum HI titers after two-doses of the nasal vaccine showed >2.5-fold rise in the ratio of geometric mean titer upon vaccination, >40% of subjects with a ≥4-fold increase in titer and >70% of subjects with a titer of ≥1:40, all parameters associated with an effective outcome of vaccination in the criteria defined by the European Medicines Agency. Serum neutralizing antibody responses correlated with HI antibody responses, although NT titers were about 2-fold higher than HI titers. These high levels of serum responses were accompanied by high levels of HI and neutralizing antibody responses in nasal mucus as measured in concentrated nasal wash samples that were about 10 times diluted compared with natural nasal mucus. Serum and nasal HI and neutralizing antibody responses consisted of HA-specific IgG and IgA antibody responses, with IgG and IgA antibodies being dominant in serum and nasal responses, respectively.

Introduction

Currently available inactivated vaccines, usually whole virus vaccines or sub-virion vaccines, such as detergent-disrupted split-viruses or purified surface glycoprotein vaccines, are injected via the non-mucosal route.[1] These vaccines induce serum IgG antibodies, which are highly protective against homologous virus infections but less effective against heterologous virus infections. However, it has been shown that secretory IgA (S-IgA) and IgG antibodies in the respiratory tract largely contribute to the protective immunity induced by influenza virus infection.[2,3] Moreover, S-IgA antibodies are more cross-reactive against variant influenza viruses than serum IgG antibodies and therefore provide more effective protection against a heterologous virus.[4-9] Thus, intranasal administration of an inactivated influenza vaccine that induces both S-IgA and IgG antibody responses is expected to outperform the protective efficacy of intramuscular or subcutaneous vaccines.[10] Of note, intranasal vaccination would have several additional advantages, since this type of vaccination is needle-free which enables easier administration, is more readily accepted by the recipients, reduces the problems associated with needle waste and prevents the risk of disease transmission through needle reuse.[11]

Several trials have been conducted to augment the induction of both S-IgA and IgG antibodies using intranasal administration of an inactivated influenza vaccine, either with or without an extrinsic adjuvant.[12-21] In several clinical trials, antibody responses were mainly evaluated based on haemagglutination inhibition (HI) titers of the serum and haemagglutinin (HA)-specific IgA and IgG antibody titers estimated by enzyme-linked immunosorbent assay (ELISA) in nasal wash samples. Currently, serum HI titers are used for the evaluation of the efficacy of the seasonal influenza vaccines,[22-24] because levels of protection against viruses that are homologous to the vaccine strain, correlate well

*Correspondence to: Hideki Hasegawa; Email: hasegawa@nih.go.jp
Submitted: 03/20/2013; Revised: 06/05/2013; Accepted: 06/19/2013
http://dx.doi.org/10.4161/hv.25458

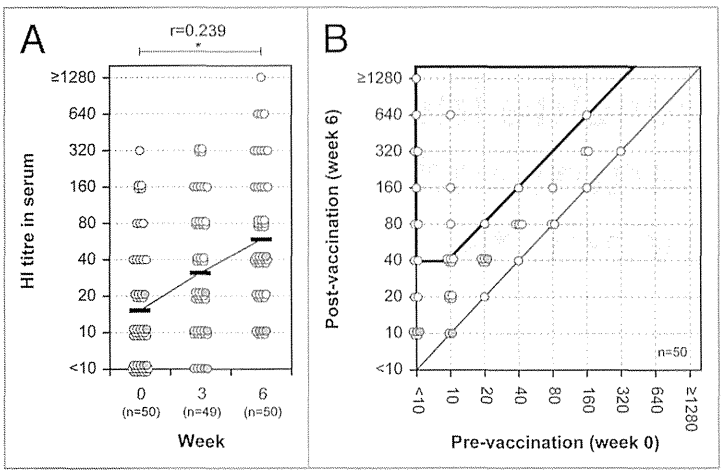


Figure 1. HI antibody responses in serum. (**A**) HI antibody responses before and after primary and secondary vaccination are shown for serum. A paired t test was performed to compare data from week 0 (pre) and 6 (post). Correlation coefficient (r) and p value were calculated. *; $p < 0.05$. (**B**) The relationship of serum HI antibody responses before and after secondary vaccination. The abscissa and ordinate show the pre- and post-vaccination HI titers, respectively. Further, it is shown how these relate to conversion rate and protection rate, which are on the border or within the area marked by the bold line and by the light gray background, respectively. Each circle represents an individual and shows the relation between the pre- and post-vaccination titers. Gray circles indicate subjects between 60 and 69 y-of-age.

with these serum HI titers.[25] However, both serum and nasal antibody responses are involved in protection,[26,27] and together might result in better correlates for protection against heterologous influenza strains. Therefore, it would be useful to measure HI titers in both serum and nasal mucus in humans. In addition, although neutralizing capacity is considered to be a more functional criterion for protection than HI or HA-specific binding, neutralizing antibody responses in nasal wash samples have rarely been assessed. Previous studies show that HI titers may be lower or higher than the corresponding neutralization (NT) titers, depending on the strain of influenza A or B virus used.[28] Other studies show that HI assays using anti-sera failed to detect the H5N1 virus.[29,30] Thus, the efficacy of antibody responses following nasal vaccination should preferentially be assessed by characterizing the HI and NT titers in serum and nasal mucus.

In a previous study, it was shown that neutralizing antibody responses in both serum and nasal mucus were induced in five healthy adults after intranasal administration of a split-virus vaccine derived from A/Uruguay/716/2007 (H3N2) virus (45 μg HA per dose).[31] Neutralizing antibody titers were measured in nasal wash samples, which typically contain about 1/10 the amount of IgA antibody found in natural nasal mucus.[31,32] Virus-specific neutralizing antibody responses were detected in nasal mucus samples from 4 out of 5 subjects, with a rise in NT titer of ≥ 4-fold after the second vaccination.[31] Nasal mucus NT titers appeared to reflect the absolute titers of nasal mucus antibodies and these titers were not affected by the slight variability in the recovery of total antibodies from nasal mucus of different subjects.

Inactivated influenza whole virus vaccines are more immunogenic than split-product vaccines when administered intranasally to mice.[33,34] Similar results were found for humans in clinical trials showing that intranasally administered whole virus vaccines cause enhanced production of both local HA-specific IgA antibodies and serum HI antibodies.[14-16] The higher immunogenicity of the whole virus vaccine may be explained by the adjuvant action of single-stranded viral RNAs that activate toll-like receptor 7. Viral RNA is present in the inactivated virus particles, but is absent in split-product vaccine formulations.[35-38] Together, these reports suggest that inactivated whole virus vaccines can induce more effective immune responses than split-virus vaccines after intranasal vaccination in healthy adults.

In the present study HI and neutralizing antibody responses were examined in serum and nasal mucus samples from 50 healthy adults after two intranasal vaccinations with an inactivated whole virus vaccine derived from A/Victoria/210/2009 (H3N2) virus (A/Victoria vaccine; 45 μg HA per dose) with a 3-week interval. Antibody responses in nasal mucus were assayed using concentrated nasal wash (containing 1 mg/ml total protein).[31,32] HA-specific IgA and IgG antibodies were also examined to characterize the immune response. It was found that two doses of the nasal vaccine induced high levels of HI and neutralizing antibody responses in both serum and nasal mucus. These responses were accompanied by major changes in HA-specific serum IgG and nasal IgA antibody responses, respectively. In addition, the vaccination with the A/Victoria vaccine resulted in a slight increase in HI and NT titers, which were cross-reactive to the A/Sydney/05/1997 (H3N2) virus.

Results

HI and neutralizing antibody responses in serum. HI antibody responses, currently in use as a correlate of protection for the evaluation of vaccine efficacy, were examined in serum samples from the volunteers aged between 22 and 69 y old who received two intranasal vaccinations with an inactivated whole virus vaccine derived from A/Victoria virus strain (containing 45 μg HA) with a 3-week interval. Serum samples were obtained both before vaccination (week 0) and 3 weeks after primary (week 3) and secondary vaccination (week 6). HI titers against A/Victoria virus were increasing after the first vaccination and again after the second vaccination (**Fig. 1A**). The serum HI titers were evaluated using the mean geometric increase between week 0 and 6, the conversion rate as well as the protection rate, which are also used by the EMA and the FDA to review the efficacy of seasonal influenza vaccines (**Table 1**). After two doses of the intranasal vaccination (week 6), serum HI titers fulfilled the criteria of the mean geometric increase (4.25-fold) and the conversion rate (43.5%), as defined by the EMA for the vaccine efficacy in people aged 18–60 y. In addition, serum samples also reached protective levels (76.1%), which were defined as the protection rate by the EMA (**Fig. 1B**

Table 1. Tools currently in use to evaluate vaccine-induced changes in serum HI titer

Name	Description	EMA criteria[a]	FDA criteria[b]
Mean geometric increase	Ratio of the geometric mean titer (GMT) post vaccination to that pre-vaccination	>2.5	No standard
Conversion rate or significant increase in titer [n/N (%, 95% CI)] [c]	Conversion rate: Proportion of subjects showing an increase from a pre-vaccination titer of < 1:10 (non-immune) to a post-vaccination titer of ≥ 1:40 (designated immune state). Significant increase in titer: Proportion of subjects showing a 4-fold or greater increase from a pre-vaccination titer of ‡ 1:10 (designated as a significant increase in titer).	>40%	LL of 95% CI > 40%[d]
Protection rate [n/N (%, 95% CI)] [c]	Proportion of subjects showing a post-vaccination titer of ‡ 1:40	>70%	LL of 95% CI > 70%[e]

[a]European Medicines Agency (EMA) criteria for serum HI antibody responses in people aged 18–60 y. [b]US Food and Drug Administration (FDA) criteria for serum HI antibody responses in adults < 65 y. [c]n = the number of subjests who meet a requirement; n = total number of subjects; 95% CI = the 95% Confidence Interval. [d]Lower limit (LL) of the 95% CI should exceed 40%. [e]Lower limit (LL) of the 95% CI should exceed 70%.

Table 2. Serum HI antibody responses after two doses of the nasal A/Victoria vaccine

	EMA criteria	FDA criteria	Week	Serum[a]
GMT (mean geometric increase)	>2.5	No standard	0	16.2 (1.00)
			6	68.8 (**4.25**[b])
Conversion rate or significant increase in titer [n/N (%, 95% CI)]	>40%	LL of 95% CI > 40%	6	20/46 (**43.5**, 28.5–58.4)
Protection rate [n/N (%, 95% CI)]	>70%	LL of 95% CI > 70%	0	13/46 (28.3, 14.7–41.8)
			6	35/46 (**76.1**, 63.2–88.9)

[a]Serum HI titers of < 1:10 were considered negative and were arbitrarily assigned a titer of 1:5. [b]In bold values that exceed EMA criteria as defined for HI titers.

and Table 2). The subjects over 60 y old produced only low levels of HI titers. These results show that serum HI titers induced by administration of two doses of the nasal A/Victoria vaccine exceeded three of the EMA criteria in people aged 18–60 y.

In addition, neutralizing antibody responses were also examined in the serum samples, since those responses are considered to be more functional in the protection against influenza viruses than HI antibody responses. NT titers against A/Victoria virus were increasing after the first vaccination and again after the second vaccination (**Fig. 2A**). Serum HI titers correlated strongly with serum NT titers ($r = 0.925$, $p < 0.0001$). Among 46 volunteers below 60 y of age, the ratio of GMTs between week 0 and 6 (the mean geometric increase after two doses of the nasal A/Victoria vaccine) in serum NT titers was 8.00 (**Fig. 2A and Table 3**), whereas this ratio in serum HI titers was 4.25 (**Table 2**). These results show that the increase in NT titers is about 2-fold higher than the increase in HI titers (**Fig. 2B**).

These data suggest that a titer of 1:80 for neutralizing antibodies would correspond to a titer of 1:40 for HI antibodies, which is defined as the minimal HI titer providing protection. Using an NT titer of 1:80 temporarily as the lower limit of protection, the enhanced sensitivity of neutralization assay could be corrected so as to tentatively estimate the vaccine efficacy. As shown in **Table 3**, serum NT titers at week 6 showed a 63.0% conversion rate, and an 87.0% protection rate. These results roughly correspond to those obtained in the serum HI titers (**Table 2**), suggesting that NT titers after correcting for the enhanced sensitivity of the NT assay could be used as an indicator to evaluate protective efficacy of the vaccines.

HI and neutralizing antibody responses in nasal mucus. HI and neutralizing antibody responses were also examined in nasal mucus samples from the volunteers who received two intranasal vaccinations with the inactivated vaccine. Nasal mucus samples (containing 1 mg/ml total protein), prepared by concentrating nasal wash samples so that the total amount of IgA was equivalent to about 1/10 of that in natural nasal mucus, were obtained both before vaccination (week 0) and 3 weeks after primary (week 3) and secondary vaccination (week 6). HI and NT titers against A/Victoria virus were increasing after the first vaccination and again after the second vaccination (**Fig. 3A and B**). Among 46 volunteers below 60 y of age, the ratio of GMTs between week 0 and 6 were 3.13 and 5.88 for HI (**Fig. 3A and Table 4**) and NT (**Fig. 3B and Table 4**) titers, respectively. These results show that NT titers in nasal mucus samples are about 2-fold higher than the HI titers (**Fig. 3C**), similar to the relationship between HI and NT titers in serum samples (**Fig. 2B**). Thus, the volunteers who received two doses of intranasal inactivated vaccine induced high levels of HI and neutralizing antibody responses both in nasal mucus and serum.

HA-specific IgA and IgG ELISA antibody responses in serum and nasal mucus. The HA-specific IgA and IgG responses in serum from subjects before and after two intranasal vaccinations with the A/Victoria vaccine were determined by ELISA (**Table 5**). Serum HA-specific IgG and IgA titers were obtained using serum samples starting from a 1:10 dilution. The mean geometric increase of IgG and IgA antibodies after two doses of nasal vaccine (week 6) was estimated to be 2.96-fold and 2.47-fold, respectively (**Fig. S1A and B; Table 5**). The GMTs of IgG responses in serum samples were higher than those of IgA responses at each time point. This indicates that HA-specific IgG, rather than IgA, is likely to be the major isotype responsible for haemagglutination inhibiting and neutralizing activity in serum samples.

The HA-specific IgA and IgG responses from subjects before and after the two intranasal vaccinations were also determined in nasal mucus (Table 5). HA-specific IgG and IgA titers in nasal mucus were assayed using concentrated nasal wash samples (containing 1 mg/ml total protein) which were measured by ELISA starting with a 1:160 dilution. The GMTs of HA-specific IgA responses were higher than those of HA-specific IgG responses at each time point. The mean geometric increase of IgA and IgG antibody after two doses of nasal vaccine (week 6) was estimated to be 3.88-fold and 1.37-fold, respectively (Fig. S1C and D; Table 5). This indicates that HA-specific IgA antibody is most likely the predominant antibody isotype responsible for the haemagglutination inhibiting and neutralizing activity in the nasal mucus. Thus, predominant changes in HA-specific IgA and IgG titers were found in the nasal mucus and serum, respectively.

In addition, relationships between NT titer and HA-specific antibody titer in serum or nasal wash were evaluated. In serum, NT titers correlated well with HA-specific IgG titers ($r = 0.778$, $p < 0.0001$), but not with IgA titers (Fig. S2A and B). Nasal NT titers show a weak correlation with HA-specific IgA titers ($r = 0.473$, $p < 0.001$), but not IgG titers (Fig. S2C and D).

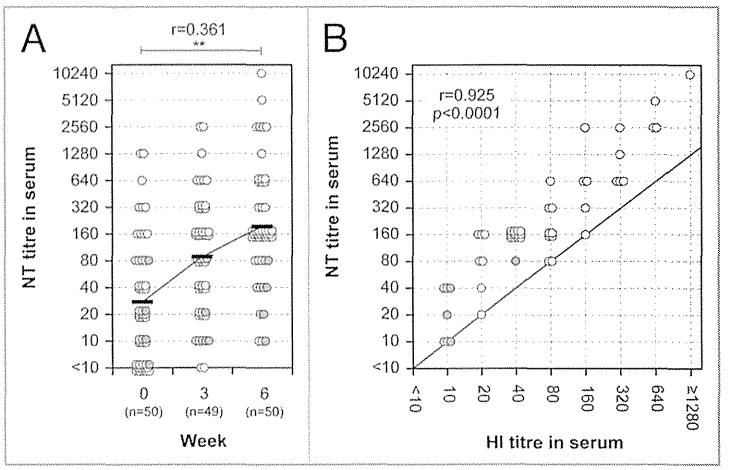


Figure 2. Neutralizing antibody responses and correlation between HI and NT titers in serum. (**A**) Neutralizing antibody responses before and after primary and secondary vaccination are shown. A paired t test was performed to compare data from week 0 (pre) and 6 (post). The correlation coefficient (r) and p value were calculated. **; $p < 0.01$. (**B**) Correlation between HI and NT titers in serum 3 weeks after the secondary nasal vaccination. The abscissa and ordinate show HI and NT titers, respectively. Pearson r value and p value were calculated. Each individual is represented by a circle showing corresponding HI and NT titers. Gray circles indicate subjects between 60 and 69 y-of-age.

HI and neutralizing antibody responses show cross-reactivity with the A/Sydney/05/1997 virus. Cross-reactivity of HI and neutralizing antibody responses that were induced upon vaccination with A/Victoria (H3N2) with the A/Sydney/05/1997 (A/Sydney, H3N2) virus was examined in serum (Table 6). Before vaccination, moderate HI and very high neutralizing antibody responses, were found to be cross-reactive to A/Sydney virus (week 0, GMTs in Table 6), when compared with responses to the homologous A/Victoria virus (Table 2 and 3). The mean geometric increase in the cross-reactive HI and NT titers were very similar and showed a 1.44-fold and a 1.46-fold, respectively. This increase was lower than that directed against the homologous A/Victoria virus (Tables 2, 3, and 6).

The cross-reactive HI and neutralizing antibody responses in nasal mucus samples were also examined using concentrated nasal wash samples. They showed a 1.63-fold and a 2.12 mean geometric increase, respectively. Similar to the responses in serum, this increase was lower than that directed against the homologous A/Victoria virus (Tables 4 and 6).

Clinical observation for adverse reactions. Before and after each vaccination clinical data were compiled from the health check records and personal interviews. None of the subjects experienced systemic adverse effects after the nasal vaccination. Minor complaints included light local reactions (mainly runny nose and nasal congestion) that resolved spontaneously within a few days (data not shown). Thus, the intranasal vaccine was considered to be well tolerated.

Discussion

The present study examined HI antibodies as well as neutralizing antibodies in serum and nasal mucus of 50 healthy adults who received two intranasal doses of an inactivated whole virus vaccine derived from A/Victoria/210/2009 (H3N2) virus (45 μg HA per dose) with an interval of 3 weeks. The two doses of nasal vaccine induced serum HI titers, demonstrated by a >4-fold mean geometric increase, a > 40% conversion rate or significant increase in titer, and a > 70% protection rate in subjects aged 18–60 y (Table 2 and Fig. 1). When the efficacy of vaccination was evaluated using the serum HI antibody responses observed in the present experiments, the serum HI titers exceeded three of the criteria used by the EMA, however, they did not meet the FDA criteria. These results suggest that two intranasal administrations of inactivated whole virus vaccine (45 μg HA per dose) could be a candidate treatment regimen. This vaccination procedure is simple, safe and effective, at least for adults with some immunological memory induced by previous infection or vaccination.

The intranasal A/Victoria vaccination also induced high levels of serum neutralizing antibody responses, measured by the microneutralization assay, of which the sensitivity was about 2-fold higher than that of the HI assay (Table 2 and Fig. 2). When the enhanced sensitivity of the NT assays was corrected in such a way that it could be compared with the antibody response obtained by the HI assays, the neutralizing antibody responses at week 6 showed a mean geometric increase, a conversion rate, and a protection rate which corresponded to those obtained in the serum HI antibody responses in subjects aged 18–60 y (Tables 2 and 3). These results suggest that serum NT titers could be used as an indicator to evaluate protective efficacy of the vaccines.

The present study also examined HI, NT and HA-specific antibody titers in nasal mucus samples, prepared by concentrating nasal wash samples resulting in a total amount of IgA that was equivalent to about 1/10 of that in undiluted nasal mucus. In our previous study, the total protein level and the levels of IgA, IgG and

Table 3. Serum neutralizing antibody responses after two doses of the nasal vaccine

	Time after first vaccination (week)	Serum[a]
GMT (mean geometric increase)	0	28.7 (1.00)
	6	229.7 (8.00)
Conversion rate or significant increase in titer [n/N (%, 95% CI)][b]	6	29/46 (63.0, 48.5–77.6)
Protection rate [n/N (%, 95% CI)][b]	0	14/46 (30.4, 16.6–44.3)
	6	40/46 (87.0, 76.8–97.1)

[a]Serum NT titers of < 1:10 were considered negative and were arbitrarily assigned a titer of 1:5. [b]Conversion rate and protection rate were tentatively estimated by correcting for the relative ratio between GMT of NT titers and that of HI titers; a serum NT titer of 1:80 was considered to be a 1:40 of the lowest protection level in HI titers.

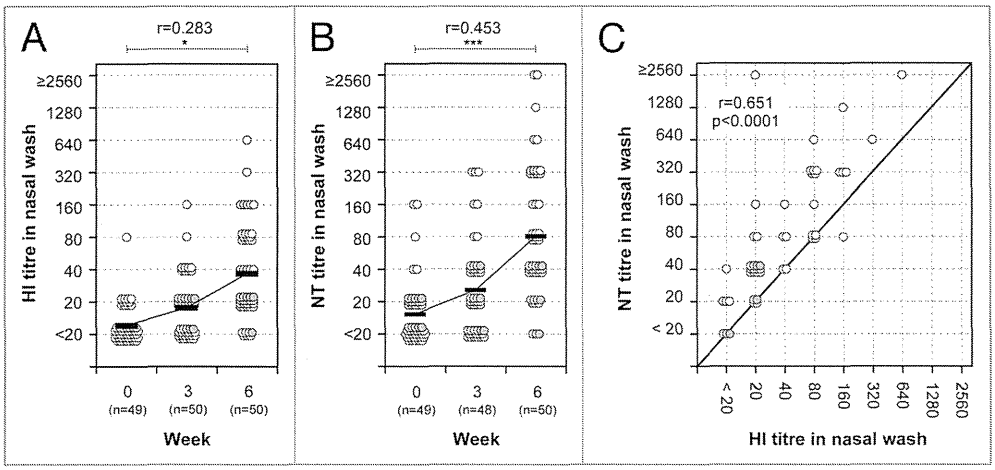


Figure 3. HI and neutralizing antibody responses in nasal mucus. HI (**A**) and NT (**B**) titers before and after primary and secondary vaccination. A paired t test was performed to compare data from week 0 (pre) and 6 (post). The correlation coefficient (r) and p value were calculated. *; $p < 0.05$, ***; $p < 0.001$. (**C**) Correlation between HI and NT titers in nasal wash 3 weeks after the secondary nasal vaccination. The abscissa and ordinate show HI and NT titers, respectively. Pearson r value and p value were calculated. Each circle represents an individual showing corresponding HI and NT titers. Gray circles indicate subjects between 60 and 69 y-of-age.

IgM and human serum albumin before and after concentration of nasal wash samples from several participants were examined.[31] About 70% of the total nasal wash proteins, 67% of IgA and 26% of IgG were lost during the concentration processes. These decreases might be caused by degradation by proteolytic enzymes or the aggregation of immunoglobulin complexes with other materials in the process of concentration. The amount of total IgA and total IgG recovered from each participant varied slightly at each sampling time; however, the average amount was comparable and constant. This means that the concentrated nasal material is sufficiently comparable within different isolations to be used to express relative antibody responses in the nasal wash. Under the described experimental conditions, allowing for small variations in the recovery of total IgA and IgG from the nasal mucus, the specific antibody titers in the nasal wash samples could be considered to be suitable to compare absolute antibody titers in the nasal mucus before and after vaccination.

The two doses of intranasal vaccine induced both nasal HI and NT titers, demonstrated by a 3.13-fold and 5.88-fold mean geometric increase (the ratio of GMTs between week 0 and 6 after the nasal A/Victoria vaccine), respectively (**Fig. 3 and Table 4**). In addition, the nasal vaccine induced HA-specific IgA and IgG antibodies in nasal mucus, demonstrated by a 3.88-fold and 1.37-fold mean geometric increase, respectively (**Table 5**). Thus, the nasal inactivated vaccine induced high levels of nasal HI and neutralizing antibody responses with dominant HA-specific IgA antibody responses, in parallel with high levels of serum HI and neutralizing antibody responses dominated by HA-specific IgG antibody responses (**Table 5**; **Fig. S2**). It is currently unknown how long these antibody responses are maintained in nasal mucus. Nasal HA-specific IgA and IgG antibody responses induced by live attenuated influenza A virus vaccine were shown to persist for at least 12 mo after inoculation in children who had not previously been infected by influenza A virus.[2] However, the duration of antibody responses in nasal mucus induced by intranasal vaccination with an inactivated whole virus vaccine in healthy adults remains to be examined.

Regarding antibody responses, Clements et al. compared the correlation between antibody responses and the degree of protection in adults who received a live attenuated intranasal vaccine followed by a challenge with wild-type influenza virus with those in adults who received an inactivated parenteral vaccine prior to challenge.[39,40] They found that serum HI titers correlated with protection against viral replication after parenteral vaccination, but not after intranasal vaccination. In contrast, intranasal vaccination induced nasal HA-specific IgA antibodies that correlated