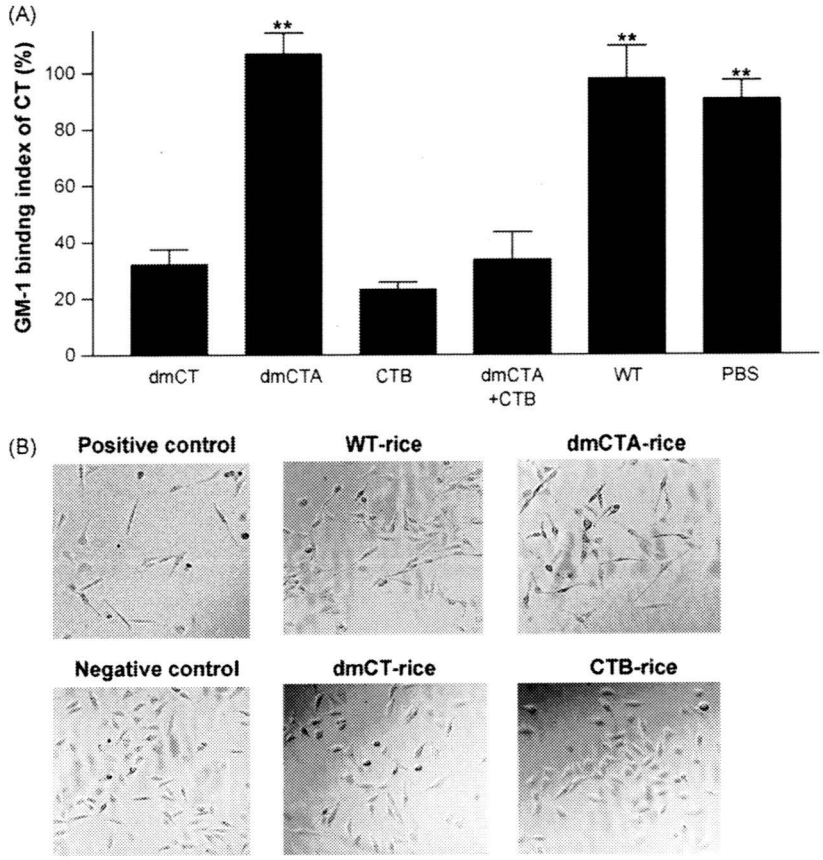

Fig. 5. *In vitro* demonstration of neutralising antibody induction by oral vaccination with MucoRice-dmCT. (A) Neutralising indices, calculated with OD_{450nm} obtained by GM1-ELIZA. Sera of mice immunised with rice-expressed dmCT, CTB, or CTB plus dmCTA, but not with rice-expressed dmCTA, wild-type (WT) rice, or PBS completely blocked the binding of CT to coated GM-1-ganglioside. (B) Elonintesinalgation assay with CHO cells revealed a morphology similar to that of normal cells when the cells were stimulated with CT pretreated with sera from mice immunised with rice-expressed dmCT or CTB. In contrast, there was marked elongation of cells stimulated by CT pretreated with sera from mice immunised with WT rice or rice-expressed dmCTA. Immunisation conditions are as described in Fig. 4.

(Fig. 5B), similar to that induced by the non-pretreated native form of CT. These results revealed that subunit MucoRice-dmCT could induce biologically active antibodies that not only had a GM-1 binding inhibition effect but were also able to neutralise CHO elongation.

Finally, in an *in vivo* CT oral challenge experiment, mice orally vaccinated with powder forms of MucoRice-dmCT, -CTB or -dmCTA plus CTB showed no, to very weak, clinical signs of diarrhoea, whereas those given MucoRice-dmCTA, WT rice or PBS orally developed severe diarrhoea (Fig. 6). The volumes of intestinal fluid in mice immunised with MucoRice-dmCT, -CTB or -dmCTA plus CTB were significantly lower after oral challenge with CT than the volumes in mice given WT rice or demCTA rice or PBS. Interestingly, the reduction in volume of intestinal fluid depended on the level of mucosal CTB-specific IgA antibody but not on that of systemic CTB-specific IgG antibody (Fig. 4). These data demonstrated that

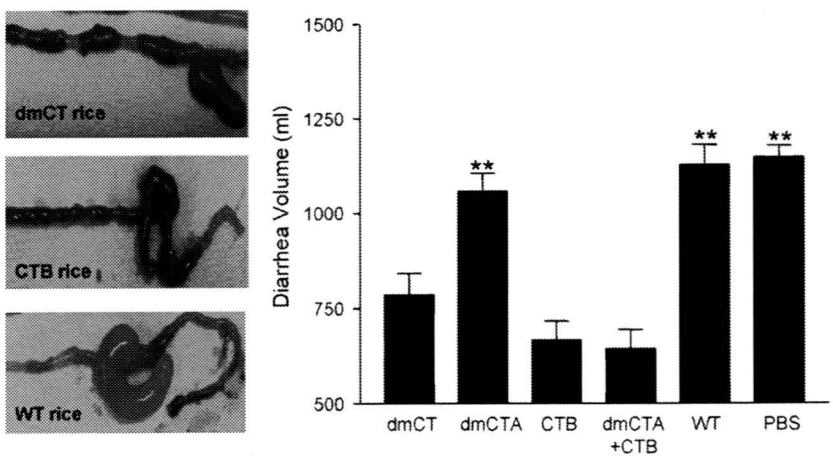

Fig. 6. Induction of protective immunity against CT by oral immunisation with MucoRice-dmCT. Mice immunised with rice-expressed dmCT, CTB, or CTB plus dmCTA, but not with rice-expressed dmCTA, wild-type (WT) rice, or PBS showed no symptoms of diarrhoea and low volumes of intestinal fluid. Immunisation conditions are as described in Fig. 4.

oral vaccination with MucoRice-dmCT could induce a high level of protective antibody response against CT challenge.

4. Discussion

It is well known that oral administration of CT to mice induces strong protective immunity against CT-induced diarrhoea [16]. Although appropriate oral administration of CT itself can protect mice from toxin-induced diarrhoea, it is unsuitable for use in humans because of its toxicity, causing severe diarrhoea if given orally. To overcome this obstacle, a killed *Vibrio cholerae* vaccine combined with recombinant CTB was successfully developed and approved by the European Union in 2004 and in many other regions, including South Asia and South America [17]. Although the killed cholera vaccine has been shown to be useful, further advances are required in the practical aspects of vaccine production and global distribution. For instance, the vaccine is not heat stable; instead, it requires a "cold chain" en route from vaccine manufacture to the field of vaccination [18,19]. In this regard, the rice expression system is recognised as a new form of bioreactor for cost-effective production of large-scale recombinant proteins on an industrial scale and offers a highly practical global strategy for cold-chain and needle-free vaccination against infection [3]. In fact, CTB-expressing transgenic rice seeds (or MucoRice-CTB) preserved for over 1.5 years at room temperature have been shown to induce both systemic and mucosal antigen-specific immune responses for the protection of mice against CT-induced diarrhoea [3]. Our additional and separate data (Tokuhara, D., et al., manuscript in preparation) show that the MucoRice system maintained the immunogenicity of expressed vaccine at room temperature for 2.5 years.

Here, we expressed two major subunits (A and B) of nontoxic mutant CT in a MucoRice system. We used dmCT as an example of nontoxic cholera toxin for rice transgene expression; it has two amino acid substitutions, in the ADP-ribosyltransferase activity centre (E112K) and the carboxyl-terminal KDEL [6]. dmCT lacks ADP-ribosyltransferase activity, has proven unable to move from the Golgi to the endoplasmic reticulum (ER), and does not elicit fluid accumulation in mouse-ligated ileal loops [6]. These results suggest that dmCT is a good and safe candidate for a vaccine against the toxin. To enhance the accumulation of CTB protein in rice, an ER retention signal (KDEL) was fused to the 3′ end of the CTB genes when we originally established rice-expressed CTB or MucoRice-CTB [3]. In this study, not only did we change the KDEL sequence of the carboxyl terminal of dmCT and dmCTA into KDGL, but we also did not add the KDEL sequence to the CTB gene construct (Fig. 1). There is therefore no potential for orally administered dmCTA or CTB to be redirected to the plasma membrane by retrograde transport via the ER and to thus be taken up from the mucosal surface [6]. Removal of the KDEL signal prevents the intracellular trafficking of dmCTA; we consider this prevention to be an important safety issue.

When dmCT was expressed separately as dmCTA and CTB subunits by using different promoters, SDS-PAGE analysis under non-reducing conditions indicated that dmCT formed a CTB pentamer in rice but did not assemble dmCTA and CTB pentamer (Fig. 2C and D). Because dmCTA gave a 26-kDs band (Fig. 2B), the dmCTA was synthesised with a trypsin-sensitive bond that joined the CTA-1 and CTA-2 to make unnicked dmCTA. Authentic cholera toxin gave two CTA bands, CTA (26 kDa) and CTA-1 (20 kDa), under reducing conditions (Fig. 2B), and the band of CTA-1 shifted the complex between CTA and the CTB pentamer under non-reducing conditions, suggesting that there were unnicked and nicked CTs and that the nicked CT could still assemble CTA and CTB pentamer under non-reducing conditions (Fig. 2C, D). Although native purified CT assembles CTA and CTB pentamer, the association of unnicked CT may be easy to break down under non-reducing conditions when compared with that of nicked CT. If this is the case, it is possible that the dmCTA and CTB subunits may assemble as a weak complex in rice seed. The unnatural forms of mdCTA and CTB expression in the rice protein bodies may explain why MucoRice-dmCT possesses oral immunogenicity of not the CTA but the CTB subunit, which is capable of inducing neutralising antibodies against the toxin.

Although dmCT prepared by an *E. coli* expression system is a potent and safe mucosal adjuvant when administered intranasally [6], assembly of CTA and CTB as an intact molecule has been considered important for adjuvant activity. Here, we expressed rice-based dmCT separately as dmCTA and CTB pentamer, which did not assemble together. The separate expression of dmCTA and CTB in the protein body of the rice seed may contribute to the lack of adjuvant activity. Thus, oral immunisation with MucoRice-dmCT did not induce rice-storage-protein-specific immune responses, whereas oral administration of WT rice in the presence of CT (10 μg) induced a rice-storage-protein-specific immune response (Fig. 3A). In addition, oral administration of MucoRice-dmCT did not support the induction of a co-administered TT-specific immune response (Fig. 4B), suggesting that MucoRice-dmCT is a safe vaccine candidate for oral immunisation because it shows no unnecessary adjuvanticity.

When MucoRice-dmCT was administered orally, we found high CTB-specific antibody responses in both the systemic and mucosal compartments (Fig. 4A and B) but no marked CTA-specific antibody responses in either compartment (data not shown). Mucosal CT (10 μg) can induce CTB-specific antibody responses at both systemic and mucosal sites [20]. In our separate study, we found induction of CTA-specific antibody responses at both sites following oral immunisation of CT (Tokuhara, D., et al., manuscript in preparation). Because MucoRice-dmCT did not assemble an intact form of dmCTA and CTB, its oral administration may be unable to induce CTA-specific immune responses. In support of this view, when the mixture of MucoRice-dmCTA and -CTB was tested, CTB- but not CTA-specific antibody responses were induced (Fig. 4). As an alternative possibility, the removal of ADP-ribosyltransferase activity from CTA may contribute to the lack, or reduction, of A-subunit-specific responses. In this case, the 10–20 μg of dmCTA used for oral immunisation may be too small an amount to induce A-subunit-specific responses. It has been shown that mutant forms of CT with loss of ADP-ribosyltransferase activity, such as mCT and dmCT, maintain their adjuvant activity but have lower immunoenhancing potency than that of the native form of the toxin [6,21]. In support of this possibility, the adjuvanticity of CTA-DD, a chimeric adjuvant CTA with a dimer of an immunoglobulin (Ig)-binding fragment of *Staphylococcus aureus* protein A, has been reported to be dependent on ADP-ribosyltransferase activity [15].

To test the quality of the CTB-specific antibody induced by oral vaccination with MucoRice-dmCT, we used both *in vitro* (e.g., GM1-ELISA and CHO-cell elongation assay) and *in vivo* (e.g., CT oral challenge model) neutralising analyses. The results obtained by the neutralising investigation showed that there were no differences in the quality of the CTB-specific antibody responses among mice orally immunised with MucoRice-dmCT, -CTB, or -dmCTA plus CTB (Figs. 5 and 6). Thus, all of the serum samples from the immunised mice showed similar levels of toxin neutralising activity. Importantly, the reduction in the volume of intestinal fluids caused by oral CT challenge depended on the level of CTB-specific mucosal IgA antibodies but not systemic IgG antibodies (Figs. 4 and 6). A reduction in the volume of CT-induced intestinal fluid was always associated with the presence of antigen-specific mucosal IgA antibodies. Our findings further suggest that effective induction of the production of CTB-specific mucosal SIgA antibodies is crucial for preventing the diarrhoea induced by CT. Together

with our previous findings concerning the rice-based CTB oral vaccine (MucoRice-CTB) [8], our current results demonstrate that subunit MucoRice-dmCT has almost the same protective effect as MucoRice-CTB against CT.

In summary, we successfully developed MucoRice expressing two major components, dmCTA and CTB, and we demonstrated that oral vaccination with MucoRice-dmCT induced protective immunity against CT *in vivo*. MucoRice-dmCT vaccine specifically induced CTB- but not CTA-specific serum IgG and mucosal IgA antibodies. Furthermore, it did not induce rice-protein- or co-administered unrelated antigen-specific immune responses when administered orally. These results showed that MucoRice-dmCT had neither adjuvanticity nor oral immunogenicity of CTA. Taken together, our results show that MucoRice can be used as a multicomponent vaccine expression system. Furthermore, because the quality of the protective immunity induced by MucoRice-dmCT against CT was almost the same of that induced by MucoRice-CTB, MucoRice-dmCT could be used as an experimental tool for analyses of other CT-derivative-based rice vaccines.

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References

[1] Streatfield SJ, Jilka JM, Hood EE, Turner DD, Bailey MR, Mayor JM, et al. Plant-based vaccines: unique advantages. Vaccine 2001;19(17–19):2742–8.
[2] Streatfield SJ, Howard JA. Plant-based vaccines. Int J Parasitol 2003;33(5–6):479–93.
[3] Nochi T, Takagi H, Yuki Y, Yang L, Masumura T, Mejima M, et al. Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. Proc Natl Acad Sci USA 2007;104(26):10986–91.
[4] Takagi H, Hiroi T, Yang L, Tada Y, Yuki Y, Takamura K, 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 USA 2005;102(48):17525–30.
[5] Yamamoto S, Takeda Y, Yamamoto M, Kurazono H, Imaoka K, Fujihashi K, et al. Mutants in the ADP-ribosyltransferase cleft of cholera toxin lack diarrheagenicity but retain adjuvanticity. J Exp Med 1997;185(7):1203–10.
[6] Hagiwara Y, Kawamura YI, Kataoka K, Rahima B, Jackson RJ, Komase K, et al. A second generation of double mutant cholera toxin adjuvants: enhanced immunity without intracellular trafficking. J Immunol 2006;177(5):3045–54.
[7] Mekalanos JJ, Swartz DJ, Pearson GD, Harford N, Groyne F, de Wilde M. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature 1983;306(5943):551–7.
[8] Qu le Q, Takaiwa F. Evaluation of tissue specificity and expression strength of rice seed component gene promoters in transgenic rice. Plant Biotechnol J 2004;2(2):113–25.
[9] Goto F, Yoshihara T, Shigemoto N, Toki S, Takaiwa F. Iron fortification of rice seed by the soybean ferritin gene. Nat Biotechnol 1999;17(3):282–6.
[10] Hiei Y, Ohta S, Komari T, Kumashiro T. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 1994;6(2):271–82.
[11] Yuki Y, Byun Y, Fujita M, Izutani W, Suzuki T, Udaka S, et al. Production of a recombinant hybrid molecule of cholera toxin-B-subunit and proteolipid–protein–peptide for the treatment of experimental encephalomyelitis. Biotechnol Bioeng 2001;74(1):62–9.
[12] Kweon MN, Yamamoto M, Watanabe F, Tamura S, Van Ginkel FW, Miyauchi A, et al. A nontoxic chimeric enterotoxin adjuvant induces protective immunity in both mucosal and systemic compartments with reduced IgE antibodies. J Infect Dis 2002;186(9):1261–9.
[13] Arakawa T, Yu J, Chong DK, Hough J, Engen PC, Langridge WH. A plant-based cholera toxin B subunit-insulin fusion protein protects against the development of autoimmune diabetes. Nat Biotechnol 1998;16(10):934–8.
[14] Kothary MH, Claverie EF, Miliotis MD, Madden JM, Richardson SH. Purification and characterization of a Chinese hamster ovary cell elongation factor of *Vibrio hollisae*. Infect Immun 1995;63(7):2418–23.
[15] Agren LC, Ekman L, Lowenadler B, Nedrud JG, Lycke NY. Adjuvanticity of the cholera toxin A1-based gene fusion protein, CTA1-DD, is critically dependent on the ADP-ribosyltransferase and Ig-binding activity. J Immunol 1999;162(4):2432–40.
[16] Lycke N, Erlandsson L, Ekman L, Schon K, Leanderson T. Lack of J chain inhibits the transport of gut IgA and abrogates the development of intestinal antitoxic protection. J Immunol 1999;163(2):913–9.
[17] Hill DR, Ford L, Lalloo DG. Oral cholera vaccines: use in clinical practice. Lancet Infect Dis 2006;6(6):361–73.
[18] Giudice EL, Campbell JD. Needle-free vaccine delivery. Adv Drug Deliv Rev 2006;58(1):68–89.
[19] Varmus H, Klausner R, Zerhouni E, Acharya T, Daar AS, Singer PA. Public health. Grand challenges in global health. Science 2003;302(5644):398–9.
[20] Marinaro M, Staats HF, Hiroi T, Jackson RJ, Coste M, Boyaka PN, et al. Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. J Immunol 1995;155(10):4621–9.
[21] Yamamoto S, Kiyono H, Yamamoto M, Imaoka K, Fujihashi K, Van Ginkel FW, et al. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. Proc Natl Acad Sci USA 1997;94(10):5267–72.

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A Rice-Based Oral Cholera Vaccine Induces Macaque-Specific Systemic Neutralizing Antibodies but Does Not Influence Pre-Existing Intestinal Immunity[1]

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We previously showed that oral immunization of mice with a rice-based vaccine expressing cholera toxin (CT) B subunit (MucoRice-CT-B) induced CT-specific immune responses with toxin-neutralizing activity in both systemic and mucosal compartments. In this study, we examined whether the vaccine can induce CT-specific Ab responses in nonhuman primates. Orally administered MucoRice-CT-B induced high levels of CT-neutralizing serum IgG Abs in the three cynomolgus macaques we immunized. Although the Ab level gradually decreased, detectable levels were maintained for at least 6 mo, and high titers were rapidly recovered after an oral booster dose of the rice-based vaccine. In contrast, no serum IgE Abs against rice storage protein were induced even after multiple immunizations. Additionally, before immunization the macaques harbored intestinal secretory IgA (SIgA) Abs that reacted with both CT and homologous heat-labile enterotoxin produced by enterotoxigenic *Escherichia coli* and had toxin-neutralizing activity. The SIgA Abs were present in macaques 1 mo to 29 years old, and the level was not enhanced after oral vaccination with MucoRice-CT-B or after subsequent oral administration of the native form of CT. These results show that oral MucoRice-CT-B can effectively induce CT-specific, neutralizing, serum IgG Ab responses even in the presence of pre-existing CT- and heat-labile enterotoxin-reactive intestinal SIgA Abs in nonhuman primates. *The Journal of Immunology,* 2009, 183: 6538–6544.

Seven distinct cholera pandemics have occurred since 1817 (1). The first six originated from the Indian subcontinent, whereas the last arose on the island of Sulawesi in Indonesia in 1961 and is still spreading throughout the world (1). These pandemics were all caused by oral infection with *Vibrio cholerae* O1 biotype El Tor; however, a non-O1 serogroup, now categorized as O139, recently appeared and caused a large epidemic of cholera in India and Bangladesh (2). A recent report on cholera in the weekly epidemiological record of the World Health Organization showed that the number of cholera cases dramatically increased in 2006 (236,896 cases, including 6,311 deaths) because of several major outbreaks (3).

Currently, three oral cholera vaccines, Dukoral, Orochol, and the Vietnamese vaccine, have been developed for public use (4). Dukoral, the most widely used cholera vaccine, especially in Europe, consists of four types of inactivated *V. cholerae* O1 plus recombinant cholera toxin (CT)[3] B subunit (CT-B; 5, 6). Orochol contains live attenuated CVD 103-HgR derived from the classical *V. cholerae* Inaba strain with 94% deletion of the toxic activity (7, 8). The Vietnamese vaccine contains inactivated forms of both *V. cholerae* O1 and O139 (9, 10). The primary reason for choosing an oral vaccine against cholera is that oral vaccines induce Ag-specific immune responses in both systemic and mucosal compartments, thereby providing two layers of protective immunity (11–13). Despite the efficacy of these three vaccines, their requirement for "cold-chain" maintenance for preservation is a major concern for their use in the field, especially in developing countries (14). Owing to this difficulty, the development of a "cold-chain-free" oral vaccine is needed (15, 16).

To overcome this concern, we have turned to a foreign protein expression system that uses rice as a vaccine production platform, because rice seeds can be preserved for long periods at ambient temperatures (17). Oral immunization with a rice-based oral vaccine expressing CT-B, named MucoRice-CT-B, successfully induced protective immunity in both systemic and intestinal tissues

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[3] Abbreviations used in this paper: CT, cholera toxin; CT-B, cholera toxin B subunit; PB, protein body; SIgA, secretory IgA Ab; LT, heat-labile enterotoxin; LT-B, heat-labile enterotoxin B subunit; WT, wild type; RT, room temperature; DC, dendritic cell.

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in mice without coadministration of whole-cell *V. cholerae* or mucosal adjuvant, and its immunogenicity was maintained for over 1.5 years in storage at room temperature (17). Another advantage to using the rice expression system for the development of oral vaccines is that the rice seeds possess unique protein storage organelles, the protein bodies (PBs; 18, 19). In particular, the endoplasmic reticulum-derived PB that deposits prolamins, PB-I, is not susceptible to digestive enzymes, and thus can survive in the harsh environment of the gastrointestinal tract (18, 19). The use of an endosperm-specific promoter and a signal peptide in MucoRice-CT-B causes CT-B to be expressed and to accumulate in PBs, making the CT-B highly resistant to digestive enzymes and thus giving it mucosal immunogenicity that induces serum IgG and intestinal secretory IgA Abs (SIgA), which protect against CT (17).

Before testing MucoRice-CT-B in human studies, we designed experiments to assess its immunogenicity in nonhuman primates. As it did in mice (17), it successfully induced CT-protective serum IgG Ab responses in cynomolgus macaques. However, to our surprise, the macaques also had pre-existing CT-reactive intestinal SIgA Abs, which appeared to be maximally expressed without immunization. This provided an opportunity to explore the effects of pre-existing intestinal immunity on the potential use of MucoRice-CT-B as a new-generation oral cholera vaccine in humans.

Materials and Methods

Nonhuman primates

We used serum and fecal extracts from 26 randomly selected, untreated cynomolgus macaques (*Macaca fascicularis*, 1 mo to 29 years old; 6 male, 20 female) bred and housed in two different environments in the Tsukuba Primate Research Center (n = 22, Ibaraki, Japan) and Hamry Company (n = 4, Ibaraki, Japan) to examine whether Abs against CT-B and heat-labile enterotoxin (LT)-B were present before immunization. All other experiments, including the study of MucoRice-CT-B immunization, were performed at the Tsukuba Primate Research Center with four additional cynomolgus macaques (each 5 years old; female). All animal experiments were approved by the Animal Care and Use Committee of the Institute of Medical Science at the University of Tokyo and the Tsukuba Primate Research Center at the National Institute of Biomedical Innovation.

Immunization

MucoRice-CT-B was generated as described previously (17). In brief, the codon-optimized *CTB* gene was inserted into a binary vector (pGPTV-35S-HPT), and the plasmid was transformed into rice (*Oryza sativa* L. cv. Kitaake). After harvest, the seeds were first ground to a fine powder in a Multibeads shocker (Yasui Kikai). Three cynomolgus macaques (no. 001, no. 002, and no. 003) were orally immunized with 667 mg of powdered MucoRice-CT-B, containing 1 mg of CT-B, and one macaque (no.004) was given the same amount of powdered nontransgenic wild-type (WT) rice. The rice powder was suspended in 5 ml of physiologic saline and administered on five occasions at 2-wk intervals under ketamine anesthesia. Six months after the last immunization, the macaques were orally boosted with the same amount of MucoRice-CT-B or WT rice. Finally, to follow up the Ag-specific Ab responses including pre-existing CT-reactive SIgA, 100 μg of CT dissolved in PBS was given orally to all four macaques on three occasions at 2-wk intervals.

Sample collection and gel filtration chromatography

Serum and fecal extracts were collected from the four macaques before immunization; 1 wk after each immunization; and 2, 4, and 6 mo after the last oral immunization with MucoRice-CT-B (Fig. 2). The feces were suspended (20% w/v) in cold PBS containing Complete Protease Inhibitor Cocktail (Roche) and 0.1% sodium azide. After centrifugation, the supernatant was filtered through a 0.45-μm filter (Pall Corporation) and stored at −80°C before use. A 1-ml aliquot of each fecal extract was separated by gel filtration chromatography on a Sephacryl S-500 (GE Healthcare) column (1.5 × 50 cm, Bio-Rad). Each 2-ml fraction collected was used in the CT-specific ELISA and toxin-neutralizing GM1-ELISA. Bovine IgM (Sigma-Aldrich; MW: 90 kDa) and β-lactoalbumin (Sigma-Aldrich; MW: 18.4 kDa) were used as molecular standards for the gel filtration chromatography.

ELISA

The Ag-specific Ab responses were analyzed by ELISA as described previously (17), with some modifications. In brief, 5 μg/ml CT (List Biologic Laboratories), recombinant CT-B, or recombinant LT-B prepared in our laboratory (20) or 20 μg/ml rice storage protein extracted with 0.01% Triton X-100 was used to coat 96-well plates overnight at 4°C. Two-fold serial dilutions of samples were blocked with 1% BSA, added to the plates, and incubated for 2 h at room temperature (RT). For the CT-specific analysis, the samples were then treated with HRP-conjugated goat anti-monkey IgG (Nordic Immunological Laboratory) or HRP-conjugated goat anti-monkey IgA (Cortex Biochem), each diluted 1/1,000, or HRP-conjugated anti-human IgE cross-reacting with monkey IgE (Serotec) diluted 1/10,000, for 1 h at RT. Because our recent and separate murine study showed that free form of GM1 ganglioside in fecal extracts affected the in vitro toxin-neutralizing assay, it was also important to address the presence or absence of GM1 ganglioside in gel-filtrated fecal extracts. The samples were thus also treated with rabbit anti-GM1 ganglioside (Calbiochem) diluted 1/1,000 for 2 h at RT, followed by an HRP-conjugated anti-rabbit IgG (Southern Biotechnology Associates) diluted 1/4,000 for 1 h at RT. The reaction was developed by using TMB Substrate (XPL), and end-point titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an OD_{450} of 0.1 greater than the negative control.

Western blotting

Extracts of rice were prepared with sample buffer containing 2% (w/v) SDS, 8 M urea, 5% (v/v) 2-ME, 50 mM Tris HCl (pH 6.8), and 20% (v/v) glycerol as described previously (17). The rice extracts and CT-B were subjected to SDS-PAGE in a NuPAGE 12% Bis-Tris Gel (Invitrogen) before being transferred to a polyvinylidene difluoride membrane (Millipore). After blocking with 5% skim milk (Wako), the membranes were treated for 1 h at RT with serum diluted 1/500 or undiluted fecal extract obtained before immunization or after the booster dose, followed, respectively, by HRP-conjugated anti-monkey IgG (Nordic Immunological Laboratory) or HRP-conjugated anti-monkey IgA (Cortex Biochem), each diluted 1/500, for 1 h at RT. After washes, the reactions were developed with 3,3-diaminobenzidine substrate (Vector).

Neutralizing assay

A neutralizing assay was performed by using a GM1-ELISA as described previously (17), with some modifications. In brief, serum (10%, v/v) or gel-filtered fecal extract (50%, v/v) was pretreated with CT (50 ng/ml final concentration) for 1 h at RT and then incubated in 96-well plates coated with monosialoganglioside GM1 (5 μg/ml, Sigma-Aldrich) for 1 h at RT. After washes, the plates were incubated with an HRP-conjugated rabbit anti-CT-B Ab (500 ng/ml) prepared in our laboratory (17) for 1 h at RT, and the reaction was detected by using TMB substrate. The inhibitory effect of serum against the binding of CT to GM1 ganglioside was determined by comparison to CT treated with PBS (positive control).

Results

Unimmunized cynomolgus macaques have intestinal SIgA Abs reactive to CT and LT

The cynomolgus macaques used in this study had been bred in a conventional environment and not in a specific pathogen-free environment. Therefore, before immunizing them with MucoRice-CT-B, we first examined whether they already possessed Abs against CT in the sera and fecal extracts. The fecal and serum samples obtained from 22 randomly selected macaques aged from 1 mo to 29 years old had very few to no CT-B–specific Abs in serum (Fig. 1*A*), as expected, because the quarantine record of these animals did not indicate any *V. cholerae* infection (data not shown). However, all of the fecal extracts unexpectedly contained CT-B–reactive intestinal SIgA Abs (Fig. 1*B*). Because CT possesses high homology to LT (21), we next examined whether the intestinal SIgA Abs present in the fecal extracts reacted with LT-B. Although the serum samples did not show any LT-B–reactive IgG Abs (Fig. 1*C*; similar to the reactivity against CT-B), all of the macaques had LT-B–reactive SIgA Abs in their feces (Fig. 1*D*).

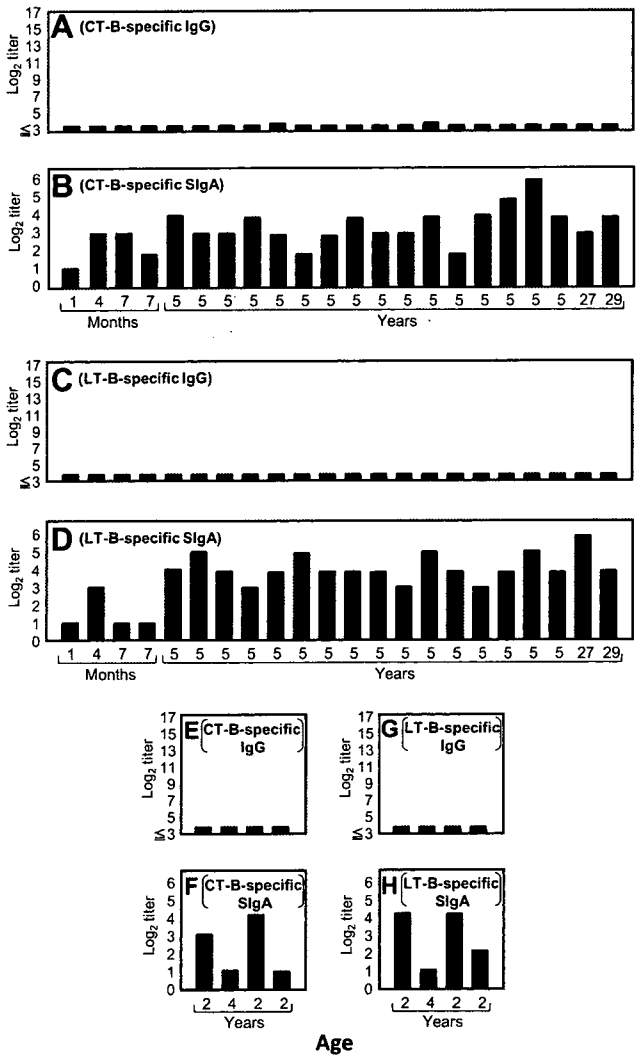

FIGURE 1. Macaques spontaneously acquire intestinal SIgA but not serum IgG Abs specific for CT-B and LT-B. CT-B–specific (*A*, *B*, *E*, *F*) and LT-B–specific (*C*, *D*, *G*, *H*) immune responses in serum (*A*, *C*, *E*, *G*) and fecal extracts (*B*, *D*, *F*, *H*) of 22 randomly selected macaques (*A–D*) and 4 additional macaques housed at a different facility (*E–H*) were examined by ELISA.

To examine whether cynomolgus macaques bred in different housing conditions also had CT-B– and LT-B–reactive SIgA Abs, we randomly selected four additional macaques housed in a different facility. These macaques were 2 to 4 years old, with no record of *V. cholerae* infection. All of these additional macaques also possessed CT-B– and LT-B–reactive intestinal SIgA Abs in their feces (Fig. 1, *F* and *H*) but not serum IgG Abs (Fig. 1, *E* and *G*). Taken together, our results show that macaques acquire CT-B– and LT-B–reactive SIgA Abs in their gastrointestinal immune system under the conventional environment.

Oral immunization of cynomolgus macaques with MucoRice-CT-B induces CT-specific serum IgG Ab responses

To test the immunogenicity of the rice-based vaccine in macaques, we orally immunized three macaques with MucoRice-CT-B, and gave one other macaque nontransgenic WT rice. Five doses of MucoRice-CT-B were given orally at 2-wk intervals as the primary immunization and a booster was given 6 mo after the last immunization (Fig. 2). Serum IgG and intestinal SIgA Abs were measured before immunization and after each dose. Similar to the

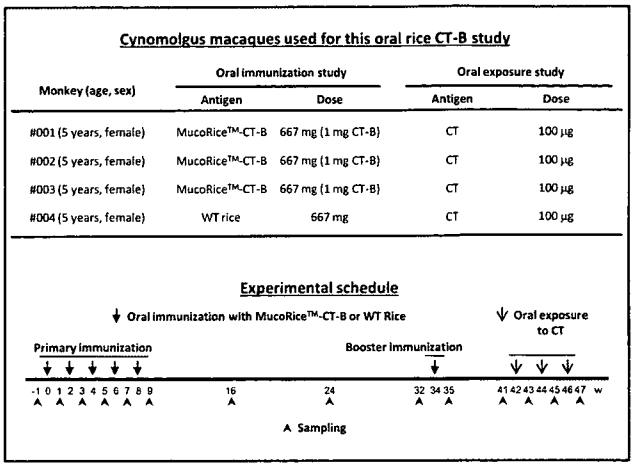

FIGURE 2. Schedule of oral immunization with MucoRice-CT-B. Macaques were orally immunized with 667 mg of MucoRice-CT-B, containing 1 mg of CT-B, or the same amount of wild-type (WT) rice on five occasions at 2-wk intervals. Six months after the fifth immunization, the macaques were given boosters of MucoRice-CT-B or WT rice. All macaques were subsequently given 100 μg of CT orally on three occasions at 2-wk intervals.

26 unimmunized macaques used in the initial study, these four macaques also had pre-existing CT-reactive SIgA Abs with toxin-neutralizing activity but did not have serum IgG Abs (Figs. 3*A* and 5*A*). The aim of this study was to examine whether oral MucoRice-CT-B could induce Ag-specific immune responses in nonhuman primates, which are closer to humans than the rodents used in our previous study (17). After two to three doses of the primary immunization, the levels of Ag-specific serum IgG, but not serum IgA, increased in all macaques immunized with MucoRice-CT-B, but not in the control macaque (Fig. 3*A*). Among the three immunized macaques, no. 001 maintained a high titer of CT-specific Ab responses for more than 6 mo (Fig. 3*A*). Although the Ab levels gradually decreased in the other two macaques after the final immunization, they continuously exceeded the detection limit for 6 mo (Fig. 3*A*). When these macaques were given an oral booster dose of the rice-based vaccine 6 mo after the last immunization, the levels of CT-specific serum IgG Abs immediately recovered to titers higher than those observed after the initial immunization (Fig. 3*A*). These results indicate that MucoRice-CT-B is a potent oral vaccine that is capable of both inducing long-term Ag-specific systemic immunity and eliciting oral booster activity in nonhuman primates.

CT-specific serum IgG Abs induced by MucoRice-CT-B possess toxin-neutralizing activity

To determine the ability of the CT-specific serum IgG Abs induced by oral immunization with MucoRice-CT-B to protect against the toxin, we performed an in vitro neutralizing assay by using a GM1-ELISA, a standard assay for demonstrating the neutralizing activity of CT-specific Abs (17, 22). When CT was preincubated with serum and assayed, the binding of CT to its receptor, GM1 ganglioside, was inhibited by sera from all of the immunized macaques at a level corresponding to the toxin-specific Ab titer, whereas the serum obtained from the control macaque did not show any inhibitory effect (Fig. 3*B*). Although serum from the macaque with the highest Ab responses (no. 001) also showed more neutralizing activity than the sera from the other two immunized macaques, the activity of the sera from these two macaques dramatically increased after the oral booster dose (Fig. 3*B*). Taken

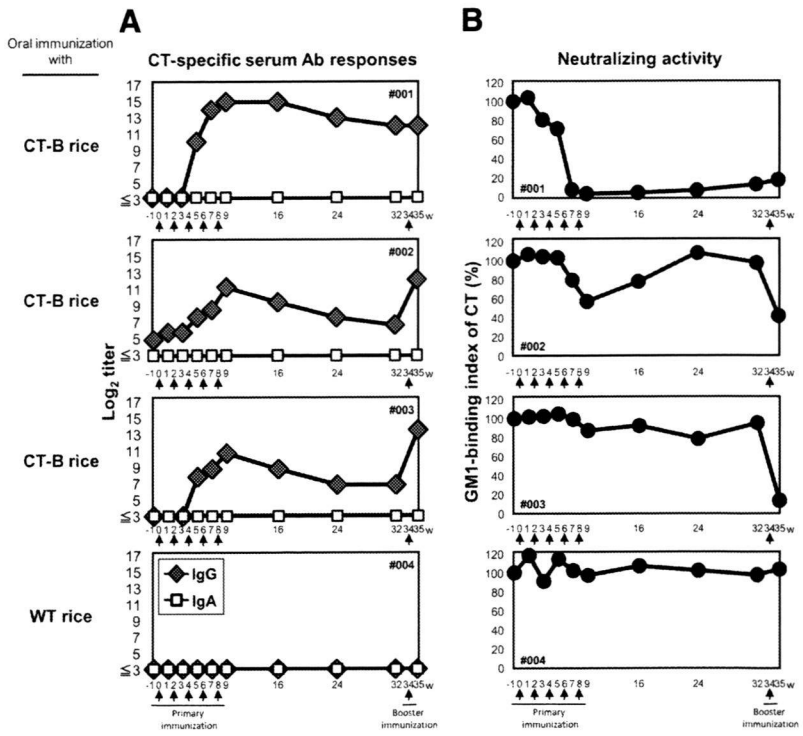

FIGURE 3. Oral vaccination with MucoRice-CT-B induces CT-specific serum IgG Abs with toxin-neutralizing activity. Oral MucoRice-CT-B but not WT rice effectively induced CT-specific serum IgG but not serum IgA Abs for at least 6 mo after the fifth immunization (*A*). Although the titer gradually decreased in two immunized macaques, it rapidly recovered after an oral booster immunization with MucoRice-CT-B (*A*). The serum collected from immunized macaques but not the control macaque inhibited the binding of CT to GM1 ganglioside at a level corresponding to the Ab titer (*B*). The CT-neutralizing activity of the two macaques with decreasing Ab titers after the primary immunization series was dramatically increased after the first oral booster dose (*B*). w = week.

together, these results indicate that oral immunization with MucoRice-CT-B can induce Ag-specific serum IgG Abs that have potential protective activity in nonhuman primates.

Oral immunization with MucoRice-CT-B does not induce IgE Ab responses to rice storage protein

To assess whether oral immunization with MucoRice-CT-B could induce a rice allergy, we examined rice storage protein-specific serum IgE and IgG Ab levels before and during the vaccination study. Rice storage protein-specific serum IgE Abs were barely detected before immunization and were not above the limit of detection after the macaques were orally immunized with the rice-based vaccine or WT rice (Fig. 4*A*). Similarly, all four macaques possessed low levels of rice storage protein-specific serum IgG Abs before immunization, but these levels were not elevated after vaccination (Fig. 4*B*). A subsequent Western blot analysis confirmed that the reactivity of serum IgG Abs against rice storage proteins prolamin and glutelin did not change between the preimmunization and post booster measurements, whereas the reactivity of Abs against CT-B did increase after vaccination (Fig. 4*C*). Taken together, these results suggest that oral MucoRice-CT-B can safely induce protective immunity without causing undesired immune responses.

Oral immunization with MucoRice-CT-B does not increase CT-reactive intestinal SIgA Abs from pre-existing levels

We next assessed whether oral immunization with the rice-based vaccine would increase the spontaneously acquired CT-reactive intestinal SIgA Abs in fecal extracts. Despite the induction of high titers of CT-specific serum IgG Abs, the pre-existing CT-reactive intestinal SIgA Ab titers did not increase even after multiple oral doses of the vaccine (Fig. 5*A*). The booster immunization 6 mo after the last immunization also did not influence the level of CT-reactive intestinal SIgA Abs (Fig. 5*A*). Similarly, a Western blot analysis showed that the reactivity of the SIgA Abs against CT-B did not change from preimmunization levels, even after the booster vaccination (Fig. 5*B*). These findings suggest that oral vaccination with MucoRice-CT-B cannot modulate the pre-existing CT-reactive intestinal SIgA Ab responses.

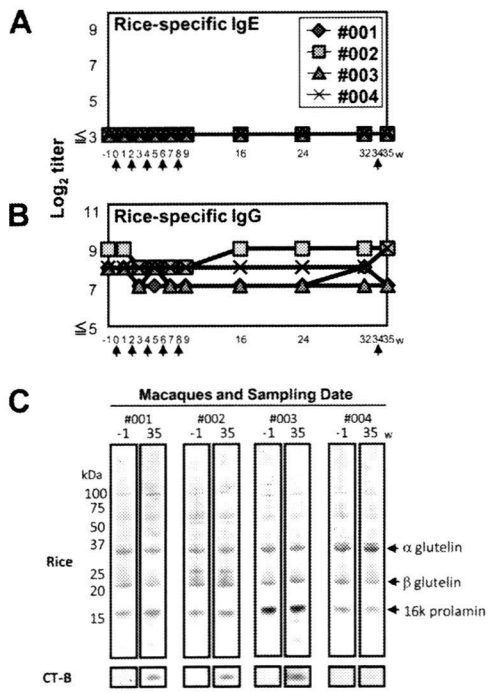

FIGURE 4. Oral immunization with MucoRice-CT-B does not induce rice storage protein-specific immune IgE Ab responses. Very low levels of serum IgE Abs specific for rice storage proteins were detected in each of the macaques orally immunized with MucoRice-CT-B or WT rice (*A*). In addition, rice storage protein-specific serum IgG Ab levels did not increase after multiple vaccinations (*B*). A Western blot analysis also showed that levels of serum IgG Abs to rice storage proteins prolamin and glutelin did not change during the vaccination period, but Abs against CT-B did increase (*C*). w = week.

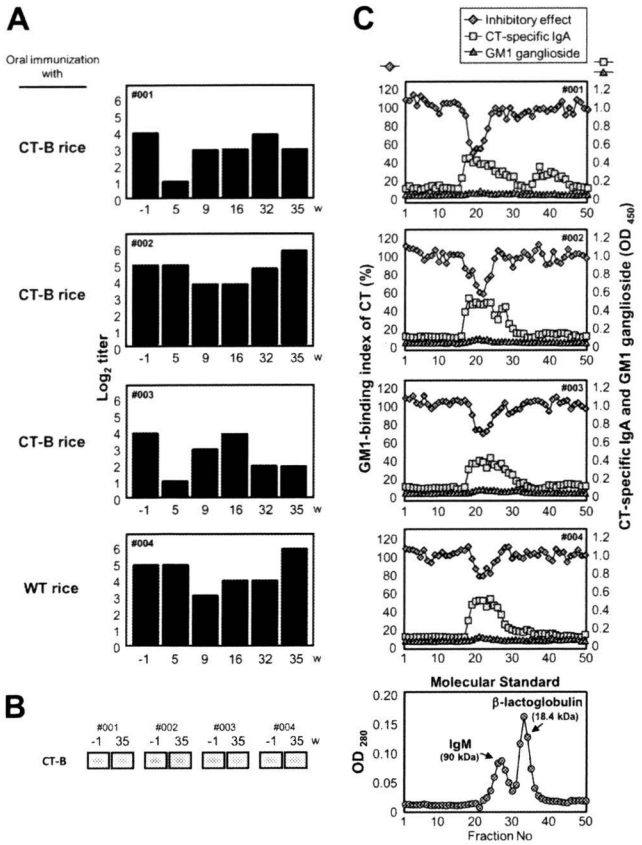

FIGURE 5. Oral immunization with MucoRice-CT-B does not increase spontaneously acquired CT-reactive intestinal SIgA Ab responses, but these SIgA Abs possess toxin-neutralizing activity. Unlike the CT-specific serum IgG Ab responses, CT-specific SIgA Ab responses were not enhanced by oral immunization with MucoRice-CT-B (*A*). Western blot analysis of feces also showed that the reactivity of SIgA Abs to CT-B did not change even after boosting with MucoRice-CT-B (*B*). Fecal extracts collected from the immunized (no. 001, no. 002, and no. 003) and control (no. 004) macaques separated by gel chromatography showed a CT-specific SIgA Ab fraction that corresponded with the toxin-neutralizing activity (inhibitory effect), but did not show a CT-reactive GM1 ganglioside-containing fraction (*C*). The inhibitory effect was calculated in comparison to the control (PBS added instead of sample). Bovine IgM and β-lactoalbumin were used as molecular standards for the gel filtration chromatography (*C*). w = week.

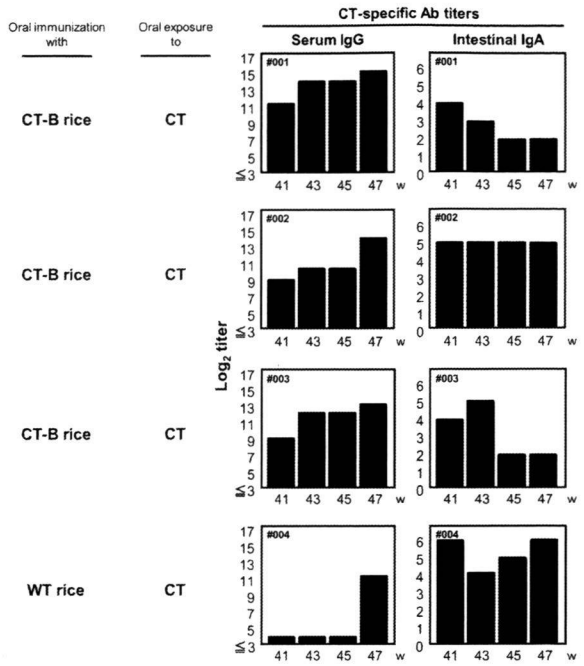

FIGURE 6. Oral administration of CT enhances serum IgG but not CT-reactive intestinal SIgA Ab responses. CT-specific serum IgG Ab responses were sharply increased in the control macaque (no. 004) and showed a further tendency to increase in three macaques previously immunized with MucoRice-CT-B (no. 001, no. 002, and no. 003). In contrast, CT-reactive intestinal SIgA Ab responses did not change consistently in any of the macaques after oral doses of CT. w = week.

Pre-existing CT-reactive intestinal SIgA Abs acquired in a conventional environment possess toxin-neutralizing activity

We recently showed that fecal extracts obtained from naive mice and mice immunized with MucoRice-CT-B contained equivalent levels of abundant, high-molecular mass, CT-reactive GM1 ganglioside derived from dead intestinal epithelial cells; this ganglioside possessed neutralizing activity in vitro but not in vivo (D. Tokuhara, Y. Yuki, T. Nochi, T. Kodama, M. Mejima, S. Kurokawa, Y. Takahashi, M. Nanno, F. Takaiwa, T. Honda, et al., in preparation). To examine whether the pre-existing CT-reactive intestinal SIgA Abs can neutralize the binding of CT to GM1 ganglioside, we first used gel filtration chromatography to separate SIgA Abs from the high-molecular mass form of GM1 ganglioside in the fecal extracts obtained after the final immunization, then assayed the collected fractions by CT- and GM1 ganglioside-specific ELISAs and an in vitro neutralizing assay. However, unlike our observation in the fecal extracts obtained from mice, we did not observe the released GM1 ganglioside in the expected molecular mass fractions obtained from immunized or control macaques (Fig. 5*C*). In contrast, CT-specific intestinal SIgA Abs were observed in the expected fractions of all macaques (Fig. 5*C*), which consisted of the fractions containing polymeric form of the total IgA (data not shown). The weak inhibitory signals were also detected by CT-specific ELISA in some low-molecular mass fractions (fractions 36 to 44) from one immunized macaque (no. 001; Fig. 5*B*). However, these fractions did not contain polymeric or dimeric forms of total IgA (data not shown), suggesting that these signals could be due to nonspecific reactivity of the secondary Ab used in this study. Most importantly, the inhibition of the binding of CT to GM1 ganglioside was observed only in fractions containing the CT-reactive intestinal SIgA Abs (Fig. 5*C*). These results support that the macaques had spontaneously acquired CT-reactive intestinal SIgA Abs possessing toxin-neutralizing activity.

CT-reactive SIgA Ab responses were not affected by oral administration of the native form of CT

Because the primary and booster oral immunization with MucoRice-CT-B did not influence the level of pre-existing CT-reactive intestinal SIgA Abs, we next administered the native form of CT to all macaques to test whether this potent Ag, which is highly immunogenic and possesses strong adjuvant activity (23) in addition to its toxic effects, would alter the SIgA Ab levels (Fig. 2). CT-specific serum IgG Ab responses dramatically increased in the control macaque and showed further increases in the three macaques previously immunized with MucoRice-CT-B (Fig. 6). However, CT-reactive SIgA Ab responses were not consistently altered in either the control or the experimental macaques even after three oral doses of CT (Fig. 6). These results suggest that the pre-existing CT-reactive intestinal humoral immunity that had developed in the conventional housing environment may have already reached immunological plateau levels.

Discussion

A major benefit of oral vaccines is that they induce protective immunity in both the systemic compartment and the aerodigestive mucosa (13). One of most important roles of the mucosal immune system is to elicit Ag-specific IgA Ab production in mucosal tissues and simultaneously to assist in the induction of Ag-specific systemic Ab responses (11). In fact, oral vaccination of cynomolgus macaques with MucoRice-CT-B effectively induced Ag-specific serum IgG Ab responses with toxin-neutralizing activity. In addition, a booster dose of the vaccine enhanced the Ag-specific Ab responses. However, to our surprise, because the macaques already had pre-existing CT-reactive intestinal SIgA Abs and probably permanently maintained them at maximum levels, these SIgA Ab levels were not increased by oral administration of MucoRice-CT-B or even by oral administration of the native form of CT. Considering their housing conditions, it is not likely that the macaques were naturally exposed to *V. cholerae*, and their medical records showed no evidence of *V. cholerae* infection. Although we do not have any definitive explanation of how the macaques may have spontaneously acquired CT-reactive SIgA Abs, CT and LT have high homology (21), and the CT-reactive intestinal SIgA Abs also cross-reacted with LT. It is therefore reasonable to consider that they had been infected by LT-producing enterotoxigenic *Escherichia coli* or homologous unknown bacteria, which may be capable of producing a CT- or LT-like molecule.

In contrast to the pre-existing CT-reactive intestinal SIgA Abs, few or no CT-specific serum IgG Abs were detectable in macaques of any age before oral immunization. The dendritic cells (DCs) in Peyer's patches and isolated lymphoid follicles can retain commensal microbiota sampled by M cells, thereby facilitating the induction of intestinal SIgA Ab responses specific for commensal flora-derived Ags (24). In contrast, commensal-specific immune responses are not induced in the systemic compartments, such as the spleen, because the mesenteric lymph nodes confine the circulation of intestinal commensal-derived Ags to DCs (24). Similar to the commensal microbiota-induced Ag-specific SIgA Ab responses, naturally infecting enterotoxigenic *E. coli* may not be pathogenic for macaques but may still spontaneously stimulate the gastrointestinal (but not systemic) immune system and induce local Ag-specific SIgA Ab production in the intestine. In contrast, the mechanisms that induce the acquired systemic immune system to respond to mucosa-derived Ags may be totally different from those spontaneously acquired mucosal Ab families, including the pre-existing CT-reactive intestinal SIgA Abs, because we recently showed in a separate study that oral immunization of Peyer's patch-deficient mice with the rice-based vaccine induces normal CT-specific serum IgG Ab responses (D. Tokuhara, Y. Yuki, T. Nochi, T. Kodama, M. Mejima, S. Kurokawa, Y. Takahashi, M. Nanno, F. Takaiwa, T. Honda, et al., in preparation). In this regard, it is known that intestinal DCs in the lamina propria directly take up luminal Ags by extending their dendrites (25, 26), and villous M cells also participate in sampling external Ags (27). Thus, another possible explanation for our current results is that MucoRice-CT-B is directly taken up by intestinal DCs and/or villous M cells even in the presence of pre-existing CT-reactive intestinal SIgA Abs, and therefore induces Ag-specific systemic IgG Ab responses through Peyer's patch-independent immunity. Although we do not have any direct evidence to support this hypothesis, it is worth testing in a future study.

IgA is the most abundant Ig produced in our body (11), especially in mucosal tissues, and the production of intestinal IgA is initiated shortly after birth in response to the colonization of commensal microbiota in the gastrointestinal tract (28). However, because the intestinal microbial composition of SIgA-lacking $pIgR^{-/-}$ mice is not completely different from that seen in WT mice (29), the precise immunological role of naturally occurring SIgA Abs is still obscure. $pIgR^{-/-}$ mice are more susceptible to *Salmonella typhimurium* infection than WT mice because they lack naturally occurring bacteria-reactive SIgA Abs (30), suggesting that these SIgA Abs may contribute to the formation of the first protective barrier against mucosal pathogens. It should be noted that macaques are not susceptible to *V. cholerae*, and oral challenge with *V. cholerae* does not cause any cholera symptoms, such as diarrhea (31). In our study, we also found that the macaques did not have diarrhea even after the oral administration of CT (data not shown). Taken together with the previous findings (31), our results suggest that spontaneously acquired CT-reactive intestinal SIgA Abs may play a pivotal role in protecting against *V. cholerae* infection in macaques.

An epidemiological study of 62,285 volunteers in Bangladesh showed that oral vaccination with 1×10^{11} killed *V. cholerae* plus 1 mg of CT-B elicited a 26% reduction in diarrhea for 1 year after the vaccination (32). Similarly, one of three macaques immunized with MucoRice-CT-B retained CT-specific long-term protective immunity in the serum for at least 6 mo after the final vaccination without a booster immunization. Although the Ab level gradually decreased in the other two immunized macaques, it remained above the detection limit, and high titers were rapidly recovered after oral boosting with the rice-based vaccine. These results indicate that oral immunization with MucoRice-CT-B is a suitable strategy not only for inducing long-term immunity, in this case over several months, but also for boosting immunity in nonhuman primates.

Another important revelation of this study is that only 667 mg of MucoRice-CT-B, which contains 1 mg of CT-B and is equivalent to approximately 30 seeds, was sufficient to induce CT-specific serum IgG Ab responses in macaques in our mouse study, we used more than 50 mg of MucoRice-CT-B, containing 75 μg of CT-B, to induce Ag-specific immune responses in mice, even though the body weight of mice is 1/150 that of macaques (17). These facts suggest that MucoRice-CT-B will be effective as a new form of oral vaccine. At same time, we also realize that five oral doses at 2-wk intervals is not a practical schedule for vaccination in the field. Because the present study was the first opportunity to demonstrate whether orally administered MucoRice-CT-B can induce Ag-specific Ab responses in limited numbers of macaques, we chose to use an excessive immunization schedule and therefore could not precisely elucidate the minimum effective dose and frequency of oral MucoRice-CT-B. To address this important issue, we are designing a new series of experiments to test the minimum dose and frequency of oral MucoRice-CT-B that can successfully induce Ag-specific immunity.

In addition, it was interesting to note that macaques harbored rice storage protein-specific IgG Abs in serum obtained before immunization (Fig. 4, *B* and *C*). The response was most likely induced by their dietary chow, which contained small amounts of rice-derived materials. However, it is important to emphasize that the pre-existing rice-specific serum IgG Abs did not increase even after multiple oral immunizations with the rice-based vaccine, and there was no evidence of induction of rice-specific IgE Ab responses (Fig. 4*A*). These results suggest that oral immunization with MucoRice-CT-B did not lead to undesired allergic immune responses even when rice-specific Abs were present in the host. Thus, we conclude that MucoRice-CT-B is a safe, immunogenic oral cholera vaccine for nonhuman primates and should be studied in humans for its possible use as a new-generation cold-chain- and needle/syringe-free vaccine.

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Disclosures

The authors have no financial conflict of interest.

References

1. Faruque, S. M., M. J. Albert, and J. J. Mekalanos. 1998. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. *Microbiol. Mol. Biol. Rev.* 62: 1301–1314.
2. Calain, P., J. P. Chaine, E. Johnson, M. L. Hawley, M. J. O'Leary, H. Oshitani, and C. L. Chaignat. 2004. Can oral cholera vaccination play a role in controlling a cholera outbreak? *Vaccine* 22: 2444–2451.
3. 2007. Weekly epidemiological record. *World Health Organization* 82: 273–284.
4. Lopez, A. L., J. D. Clemens, J. Deen, and L. Jodar. 2008. Cholera vaccines for the developing world. *Hum. Vaccin.* 4: 165–169.
5. Clemens, J. D., D. A. Sack, J. R. Harris, J. Chakraborty, M. R. Khan, B. F. Stanton, B. A. Kay, M. U. Khan, M. Yunus, W. Atkinson, et al. 1986. Field trial of oral cholera vaccines in Bangladesh. *Lancet* 2: 124–127.
6. Sanchez, J. L., B. Vasquez, R. E. Begue, R. Meza, G. Castellares, C. Cabezas, D. M. Watts, A. M. Svennerholm, J. C. Sadoff, and D. N. Taylor. 1994. Protective efficacy of oral whole-cell/recombinant-B-subunit cholera vaccine in Peruvian military recruits. *Lancet* 344: 1273–1276.
7. Kotloff, K. L., S. S. Wasserman, S. O'Donnell, G. A. Losonsky, S. J. Cryz, and M. M. Levine. 1992. Safety and immunogenicity in North Americans of a single dose of live oral cholera vaccine CVD 103-HgR: results of a randomized, placebo-controlled, double-blind crossover trial. *Infect. Immun.* 60: 4430–4432.
8. Cryz, S. J., Jr., M. M. Levine, G. Losonsky, J. B. Kaper, and B. Althaus. 1992. Safety and immunogenicity of a booster dose of *Vibrio cholerae* CVD 103-HgR live oral cholera vaccine in Swiss adults. *Infect. Immun.* 60: 3916–3917.
9. Trach, D. D., J. D. Clemens, N. T. Ke, H. T. Thuy, N. D. Son, D. G. Canh, P. V. Hang, and M. R. Rao. 1997. Field trial of a locally produced, killed, oral cholera vaccine in Vietnam. *Lancet* 349: 231–235.
10. Thiem, V. D., J. L. Deen, L. von Seidlein, G. Canh do, D. D. Anh, J. K. Park, M. Ali, M. C. Danovaro-Holliday, N. D. Son, N. T. Hoa, et al. 2006. Long-term effectiveness against cholera of oral killed whole-cell vaccine produced in Vietnam. *Vaccine* 24: 4297–4303.
11. Kiyono, H., J. Kunisawa, J. R. McGhee, and J. Mestechy. 2008. The mucosal immune system In *Fundamental Immunolology*, 5th Ed. W. E. Paul, ed., Lippincott Williams and Wilkins, Philadelphia, PA, pp. 983–1030.
12. Kunisawa, J., T. Nochi, and H. Kiyono. 2008. Immunological commonalities and distinctions between airway and digestive immunity. *Trends Immunol.* 29: 505–513.
13. Holmgren, J., and C. Czerkinsky. 2005. Mucosal immunity and vaccines. *Nat. Med.* 11: S45–S53.
14. Das, P. 2004. Revolutionary vaccine technology breaks the cold chain. *Lancet Infect. Dis.* 4: 719.
15. Varmus, H., R. Klausner, E. Zerhouni, T. Acharya, A. S. Daar, and P. A. Singer. 2003. Public health: grand challenges in global health. *Science* 302: 398–399.
16. Giudice, E. L., and J. D. Campbell. 2006. Needle-free vaccine delivery. *Adv. Drug Deliv. Rev.* 58: 68–89.
17. Nochi, T., H. Takagi, Y. Yuki, L. Yang, T. Masumura, M. Mejima, U. Nakanishi, A. Matsumura, A. Uozumi, T. Hiroi, et al. 2007. Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. *Proc. Natl. Acad. Sci. USA* 104: 10986–10991.
18. Yamagata, H., and K. Tanaka. 1986. The site of synthesis and accumulation of rice storage proteins. *Plant Cell Physiol.* 27: 135–145.
19. Katsube, T., N. Kurisaka, M. Ogawa, N. Maruyama, R. Ohtsuka, S. Utsumi, and F. Takaiwa. 1999. Accumulation of soybean glycinin and its assembly with the glutelins in rice. *Plant Physiol.* 120: 1063–1074.
20. Yuki, Y., Y. Byun, M. Fujita, W. Izutani, T. Suzuki, S. Udaka, K. Fujihashi, J. R. McGhee, and H. Kiyono. 2001. Production of a recombinant hybrid molecule of cholera toxin-B-subunit and proteolipid-protein-peptide for the treatment of experimental encephalomyelitis. *Biotechnol. Bioeng.* 74: 62–69.
21. Dallas, W. S., and S. Falkow. 1980. Amino acid sequence homology between cholera toxin and *Escherichia coli* heat-labile toxin. *Nature* 288: 499–501.
22. Arakawa, T., D. K. Chang, W. H. Langridge. 1998. Efficacy of a food plant-based oral cholera toxin B subunit vaccine. *Nat. Biotech.* 16: 292–297.
23. Kubota, M., C. J. Miller, K. Imaoka, S. Kawabata, K. Fujihashi, J. R. McGhee, and H. Kiyono. 1997. Oral immunization with simian immunodeficiency virus p55gag and cholera toxin elicits both mucosal IgA and systemic IgG immune responses in nonhuman primates. *J. Immunol.* 158: 5321–5329.
24. Macpherson, A. J., and T. Uhr. 2004. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* 303: 1662–1665.
25. Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J. P. Kraehenbuhl, and P. Ricciardi-Castagnoli. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* 2: 361–367.
26. Niess, J. H., S. Brand, X. Gu, L. Landsman, S. Jung, B. A. McCormick, J. M. Vyas, M. Boes, H. L. Ploegh, J. G. Fox, et al. 2005. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 307: 254–258.
27. Jang, M. H., M. N. Kweon, K. Iwatani, M. Yamamoto, K. Terahara, C. Sasakawa, T. Suzuki, T. Nochi, Y. Yokota, P. D. Rennert, et al. 2004. Intestinal villous M cells: an antigen entry site in the mucosal epithelium. *Proc. Natl. Acad. Sci. USA* 101: 6110–6115.
28. Macpherson, A. J., K. D. McCoy, F. E. Johansen, and P. Brandtzaeg. 2008. The immune geography of IgA induction and function. *Mucosal. Immunol.* 1: 11–22.
29. Sait, L., M. Galic, R. A. Strugnell, and P. H. Janssen. 2003. Secretory antibodies do not affect the composition of the bacterial microbiota in the terminal ileum of 10-week-old mice. *Appl. Environ. Microbiol.* 69: 2100–2109.
30. Wijburg, O. L., T. K. Uren, K. Simpfendorfer, F. E. Johansen, P. Brandtzaeg, and R. A. Strugnell. 2006. Innate secretory antibodies protect against natural *Salmonella typhimurium* infection. *J. Exp. Med.* 203: 21–26.
31. Ivanoff, B., J. L. Tayot, O. Creach, and R. Fontanges. 1978. Experimental cholera in monkeys. *Bull. Soc. Pathol. Exot. Filiales* 71: 240–248.
32. Clemens, J. D., D. A. Sack, J. R. Harris, J. Chakraborty, M. R. Khan, B. F. Stanton, M. Ali, F. Ahmed, M. Yunus, B. A. Kay, et al. 1988. Impact of B subunit killed whole-cell and killed whole-cell-only oral vaccines against cholera upon treated diarrhoeal illness and mortality in an area endemic for cholera. *Lancet* 1: 1375–1379.

EXPERT REVIEWS

Mucosal vaccines: novel advances in technology and delivery

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Mucosal vaccines are considered the most suitable type of vaccines to combat emerging and re-emerging infectious diseases because of their ability to induce both mucosal and systemic immunity. Considerable advances have been made toward the development of mucosal vaccines against influenza virus and rotavirus. Many additional mucosal vaccines are in development, including vaccines against cholera, typhoid, traveler's diarrhea and respiratory infections. In addition to oral and nasal vaccines, transcutaneous (or skin patch) and sublingual immunizations are now part of a new generation of mucosal vaccines. Furthermore, a rice-based oral vaccine (MucoRice™) has been receiving global attention as a new form of cold chain-free vaccine, because it is stable at room temperature for a prolonged period. This review describes recent developments in mucosal vaccines with promising preclinical and clinical results.

KEYWORDS: MucoRice™ • nasal vaccine • oral vaccine • transcutaneous vaccine

Injected vaccines are designed to induce protective immunity in the systemic compartment but are not aimed at providing mucosal immunity as a first line of defense against mucosal pathogens. Vibrio cholerae, Salmonella enterica, enterotoxigenic Escherichia coli (ETEC), Mycobacterium tuberculosis, influenza virus, rotavirus and HIV invade and infect the body at the mucosal surfaces of the digestive, respiratory and reproductive tracts [1,2]. Because mucosal administration of a vaccine can induce both systemic and mucosal antigen-specific immune responses, mucosal vaccination would probably improve the efficacy of current parenterally delivered vaccines and could prevent a range of infectious diseases at the sites where their causative pathogens invade [2].

Several routes of vaccine administration induce both systemic and mucosal immunity. In general, oral administration effectively induces antigen-specific antibody responses in the small intestine, colon and salivary glands in addition to systemic compartments, but generates relatively low levels of antigen-specific IgA antibody responses in the large intestine, tonsils, respiratory tract and female genital tract [3]. Nasal administration of antigen induces potent antigen-specific antibody responses in the oral and nasal cavities and respiratory tract, as well as in systemic compartments, but not in the intestines [4]. Interestingly, nasal immunization also can effectively induce antigen-specific IgA and cytotoxic T lymphocytes (CTLs) in the female genital tract, making it an attractive immunization route for the development of an effective mucosal vaccine against HIV infection and other sexually transmitted diseases [1].

Other than the early development of an oral polio vaccine (OPV), few mucosal vaccines have been available clinically, despite recent progress in understanding the unique basic and clinical aspects of the mucosal immune system. In recent years, only a nasal influenza vaccine and oral rotavirus vaccine are available to the public, but numerous mucosal vaccines are in development worldwide (TABLE 1 & 2). In this review, we concisely introduce the current concepts of mucosal immunity and vaccines and discuss their application to human studies and trials for the development of globally licensed mucosal vaccines.

Vaccine development for the mucosal immune system

We continuously expose our respiratory, digestive and reproductive tracts to both beneficial and pathogenic antigens from the outside world by inhalation, ingestion and sexual contact. The mucosal immune system is equipped with two

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Table 1. Approved mucosal vaccines.

Generic name (brand name)	Sponsor (company)	Infectious disease	Route of administration
Live-attenuated polio vaccine (Oral Polio Vaccine®)	Many	Polio	Oral
Inactivated Vibrio cholerae O1 plus cholera toxin B subunit (Dukoral®)	SBL Vaccine	Cholera	Oral
CVD 103 live-attenuated V. cholerae O1 HgR (Orochol®)	Berna (Swiss Serum and Vaccine Institute)	Cholera	Oral
Live-attenuated Ty21a vaccine (Vivotif®)	Berna (Swiss Serum and Vaccine Institute)	Typhoid	Oral
Live-attenuated cold-adapted influenza virus (FluMist®)	MedImmune	Influenza	Nasal
Live-attenuated monovalent human rotavirus (RotaRix®)	GlaxoSmithKline	Rotavirus	Oral
Live-attenuated pentavalent human–bovine ressortant virus (RotaTeq®)	Merck	Rotavirus	Oral

immunological machineries that mediate the active and quiescent phases of antigen-specific immune responses, both providing a front line of defense against invading pathogens by inducing positive immune responses and also inducing immunologic unresponsiveness to food antigens as a negative immune response (mucosally induced tolerance). The mucosal immune system thus maintains a balance between the host's mucosal surfaces and the external environment [5]. In order to further advance our efforts toward the development of a mucosal vaccine, it is necessary to appreciate and understand the anatomical and functional uniqueness of the mucosal immune system when compared with the better known systemic immune system, which is situated in a totally segregated, germ-free compartment [6].

Antigen-specific mucosal immunity consists of secretory IgA (SIgA) and/or CTL responses and is generally induced by interconnected mucosa-associated inductive and effector sites [7]. When vaccine antigens or pathogens are encountered as a result of ingestion or inhalation, these antigens are taken up by the organized inductive tissues, known as mucosa-associated lymphoid tissues (MALTs) that lie in the digestive and respiratory tracts. The MALTs family includes gut-associated lymphoid tissues (GALTs) such as Peyer's patches (PPs) and isolated lymphoid follicles, nasopharynx-associated lymphoid tissues (NALTs), and bronchus-associated lymphoid tissues. PPs and NALTs are well-characterized members of the MALTs family that are located in the intestinal and respiratory tracts, respectively. Tens of PPs are generally found in the small intestines of rodents and hundreds in the small intestines of humans. NALTs are located at both basal sides of the nasal cavity in rodents. Although there is anatomically no equivalent of NALTs in humans, oropharyngeal lymphoid tissues including the adenoids and palatine tonsils are thought to act as functionally and histologically organized lymphoid structures for the upper respiratory tract [8,9].

In general, MALTs are covered by a follicle-associated epithelium (FAE) that contain professional antigen-sampling cells, known as M cells, that effectively take up antigens from the lumen of the respiratory and digestive tracts. Immediately underneath the FAE, layers of professional antigen-presenting cells or dendritic cells (DCs) are situated for the subsequent capture and processing of antigens taken up by the M cells [10]. MALTs contain a well-organized microarchitecture of B- and T-lymphocyte

Table 2. Mucosal vaccines in development.

Generic name (brand name)	Sponsor (company)	Infectious diseases	Route of administration	Development status
Live-attenuated Vibrio cholerae Peru-15 (Cholera Garde®)	AVANT Immunotherapeutics	Cholera	Oral	Phase II
Live-attenuated Ty800 vaccine	AVANT Immunotherapeutics	Typhoid	Oral	Phase I
Live-attenuated CDV909 vaccine (HolaVax-Typhoid®)	Acambis/Berna	Typhoid	Oral	Phase II
Live-attenuated cold-adapted influenza viris (CAIV-T®)	MedImmune	Influenza	Nasal	Phase III
Live-attenuated b/hPIV3 expressing F protein of RSV	MedImmune	RSV and PIV3	Nasal	Phase I
Heat-labile enterotoxin	Iomai	Traveler's diarrhea	Transcutaneous	Phase II

PIV: Parainfluenze virus; RSV: Respiratory syncytial virus.

zones that can respond to the processed peptide antigens presented by DCs, and that can become antigen-specific effector and memory B and T cells for the generation of an antigen-specific mucosal immune response. Following DC-mediated stimulation, activated $CD4^+$ Th2 cells preferentially induce IgA-committed B cells (IgA^+ B cells) in the germinal center of the lymphoid follicle. After μ(IgM)-to-α(IgA) isotype class switching, these IgA-committed B cells become plasma blasts and then rapidly egress from the MALTs to enter the migration pathway via the connected lymphoid tissues, such as mesenteric and cervical lymph nodes, and continue through the efferent lymphatic system [11]. Finally, these antigen-specific $CD4^+$ T cells and IgA^+ B cells

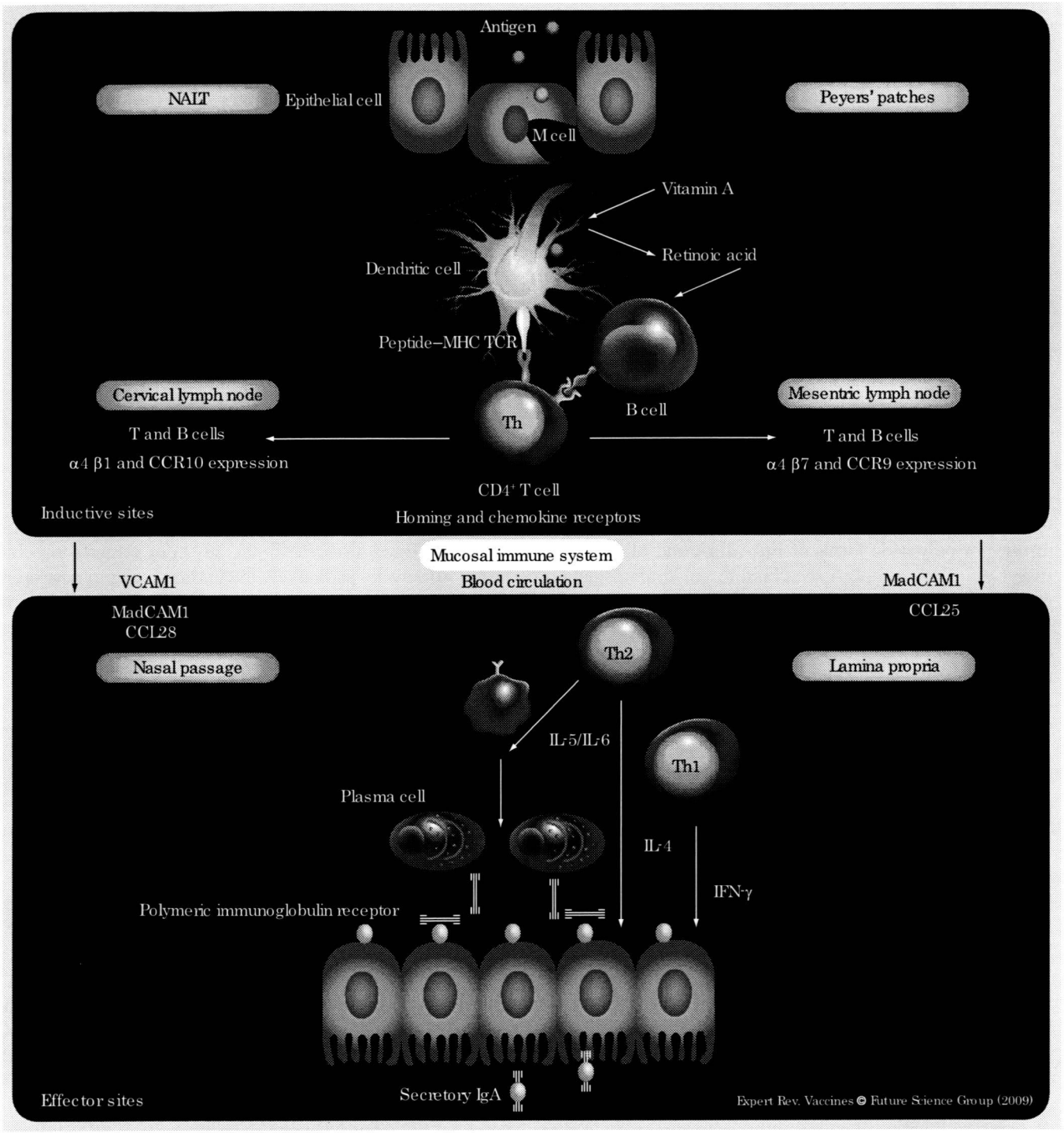

Figure 1. Mucosal immune system for the induction of antigen-specific secretory IgA.
MadCAM: Mucosal addressin cell adhesion molecule; NALT: Nasal-associated lymphoid tissue; TCR: T-cell receptor; VCAM: Vascular cell adhesion molecule.

migrate to effector sites, including the intestinal lamina propria region and nasal passages, through the thoracic ducts and blood circulation (FIGURE 1) [10].

The activated B and T cells migrating from PPs express $\alpha4\beta7$ integrin and CCR9 as guidance molecules, which direct them to the intestinal lamina propria. The integrin interacts with intestinal high endothelial venules expressing mucosal vascular addressin cell adhesion molecule 1 (MadCAM1), and CCR9 enables specific migration to the intestinal lamina propria, where the epithelial cell-produced chemokine CCL25 is expressed [12–14]. In addition to their original antigen processing and presentation capacities, mucosal DCs produce retinoic acids and, therefore, are critical for educating antigen-specific B and T cells to express the gut-imprinting molecules $\alpha4\beta7$ and CCR9 in PPs [15,16]. By contrast, antigen-stimulated B and T cells in NALTs express $\alpha4\beta1$ integrin and CCR10, allowing them to be selectively trafficked to the effector sites of the nose, trachea, and bronchus, where their ligands, VCAM1 and CCL28, are strongly expressed [17]. The same mucosal migration molecules are involved in the trafficking of activated lymphocytes to the genitourinary tract, which can explain the effective induction of antigen-specific immune responses in the genital tract after nasal immunization [18].

When IgA^+ B cells have migrated to the distant effector sites (e.g., intestinal lamina propria), the cells enter into the final differentiation stage to become IgA-producing plasma cells in the presence of IgA-enhancing cytokines such as IL-5 and IL-6, which are produced by Th2 cells for subsequent production of dimeric or polymeric forms of IgA (dIgA and pIgA, respectively) [19]. dIgA and pIgA then bind to polyimmunoglobulin receptors (pIgR) expressed on the basolateral surfaces of epithelial cells for the formation and transport of SIgA [20]. During the time that dIgA (or pIgA) and pIgR complexes are transported to the apical surface, extracellular proteolytic fragments of pIgR are cleaved to become the secretory component, which forms a part of SIgA [21].

Although M cells located in the FAE of PPs and NALTs have been considered an important gateway for the outside environment and subsequent initiation of antigen-specific mucosal immunity against orally or nasally encountered antigens [22], mucosally administered antigens can also be taken up from the mucosal surface, including from columnar epithelial cells such as small intestinal and rectal epithelia, in collaboration with mucosal DCs [23]. For example, mucosal DCs can directly sample antigen from the intestinal lumen by extending dendrites between the tight junctions of intestinal epithelial cells. These intraepithelial DCs then migrate to the mesenteric lymph node for antigen presentation to naive lymphocytes [24]. Recently, we demonstrated that M-like cells in the villous epithelium, which we termed villous M cells, have the potential to act as alternative antigen sampling cells for the initiation of antigen-specific immune responses [25]. The gut immune system contained more antigen sampling sites than we had expected; these included inductive sites (e.g., M cells in the FAE of PPs and isolated lymphoid follicles) and effector sites (e.g., intraepithelial DCs and villous M cells) for the initiation of antigen-specific immune responses. We still do not know whether these mucosal DCs and M cells form a dynamic antigen sampling network that influences the quality or quantity of antigen-specific immune responses in both the mucosal and systemic compartments; however, one has to consider the presence of such a variety of antigen-sampling cells in the intestinal mucosa when developing an oral vaccine.

In addition to DCs in the intestinal epithelium, two kinds of professional antigen-sampling DCs, Langerhans cells (LCs) and submucosal DCs, have been shown to locate within (LCs) and beneath (submucosal DCs) the body surfaces that are covered by stratified epithelial cell layers, such as the skin, oral cavity and sublingual mucosa [23]. These mucosal surfaces are devoid of MALTs structures but are drained by regional lymph nodes. The DC-like LCs can migrate between skin epithelial cells, where they can sample antigen directly from the cell surface [26]. Although LCs stem cells are present in dermis and submucosa, submucosal DCs do not migrate above the basement membrane. These cells migrate to draining lymph nodes, such as cutaneous and proximal lymph nodes, and present antigens to naive lymphocytes. The ability of these DCs to directly sample antigen from skin and oral mucosa surfaces leads to the possibility of transcutaneous [27] or sublingual immunization [28] as alternative strategies for the development of needle/syringe-free vaccines.

Mucosal vaccines

Oral vaccines

Enteric invasion by pathogenic viruses, such as poliovirus and rotavirus, or bacteria, including V. cholerae, ETEC and Salmonella enterica serovar Typhi, are serious threats to public health worldwide, particularly to children living in developing countries [29]. Here, we summarize and discuss mucosal vaccines currently approved or in development for human use against enteric infections (TABLES 1 & 2).

Polio vaccine

The live-attenuated OPV developed by Albert Sabin was the first approved mucosal vaccine [30]. Similar to the injectable inactivated polio vaccine (IPV) developed by Jonas Salk [30], OPV effectively induces systemic antibody immune responses with excellent protection against poliomyelitis, a paralytic disease resulting from the destruction of motor neurons in the CNS [30]. There are three serotypes of poliovirus that recognize a common cellular immunoglobulin-like receptor (CD155) for host cell attachment and entry. All three serotypes cause poliomyelitis [31], and both OPV and IPV are trivalent vaccines that provide good protection against all three serotypes.

Poliovirus is an enterovirus, and infection occurs via the oral route [32]. OPV both produces a higher titer of virus-specific IgG antibodies than IPV and also induces mucosal IgA immune responses in the intestine, the primary poliovirus entry site. OPV therefore prevents infection and transmission of poliovirus at the intestinal epithelium. Despite the advantages of OPV in terms of efficacy and administration, OPV can revert to neurovirulence, causing vaccine-associated paralytic poliomyelitis at a rate of approximately one case in 500,000 administered doses [32].

Although global vaccination with OPV decreased the annual incidence of poliomyelitis in the year 2006 to approximately 2000 cases worldwide [30], outbreaks of paralytic polio caused by the vaccine-derived strains have been reported every year since 2000 [33]. Thus, most industrialized countries have replaced OPV with IPV [30]. Although IPV is currently considered to be a safe vaccine owing to the inability of the vaccine to induce paralytic polio, one of disadvantages of IPV is that it is unable to induce mucosal immunity for the elimination of the virus at the site of invasion. Developing countries continue to use OPV because the oral form provides the same efficacy at a lower cost. Nonetheless, to achieve the global eradication of polio, OPV will have to be improved in terms of its safety and efficacy as well as cost. The development of an inactivated mucosal polio vaccine may be possible and is an attractive goal for overcoming these obstacles, but it may need a novel mucosal adjuvant that supports the induction of excellent polio-specific protective immunity in both the mucosal and systemic compartments.

Cholera vaccine

Diarrheal disease remains a major global health problem. Approximately 4 billion cases of diarrhea occur worldwide each year, resulting in approximately 2.2 million deaths, mostly of children under 5 years of age in developing countries [301]. Approximately half of the cases are caused by pathogenic bacteria that produce enterotoxins. Cholera, which results from infection with V. cholerae, is the most severe of the enterotoxic enteropathies. Injectable vaccination with inactivated V. cholerae was used until the end of the 20th Century. Although the injectable vaccine is still available in the USA, it is no longer recommended because it can induce adverse reactions and offers only partial protection of short duration [34]. The limited efficacy of the injection-type vaccine for the control of V. cholerae infection is predictable, given our current knowledge of the gut mucosal immune system and the nature of the pathogen invasion site.

To overcome the limitations of the injected vaccine, two oral cholera vaccines have been developed and licensed (Table 1). One is a recombinant live-attenuated vaccine (Orochol®). The vaccine strain CVD 103 HgR was constructed by modifying the cholera toxin (CT) gene from V. cholerae Inaba strain 569B [35] by deleting the gene encoding the toxic A subunit of CT, leaving the nontoxic but immunogenic B subunit (CTB). A single oral dose of CVD 103 HgR gave high vibriocidal seroconversion and prevented disease in adult volunteers living in industrialized countries [35,36], but when the vaccine was tested in a large field trial in Thailand and Indonesia, vibriocidal seroconversion was low and no significant protection was observed [35–37]. Therefore, the vaccine is not currently being produced.

The other internationally licensed oral cholera vaccine is an inactivated V. cholerae O1 combined with a recombinant CTB (rCTB-WC, Dukoral®) [35]. rCTB-WC induces both antibacterial and antitoxic SIgA mucosal immunity in the intestinal tract, as well as serum antigen-specific IgG immune responses. Three oral doses of rCTB-WC gave 85% protection for the first 6 months and 50% in the third year in a trial in Bangladesh; protective efficacy of rCTB-WC for children aged 2–5 years after 6 month and reached 26% at 3 years, compared with 63% efficacy for adults and children over the age of 5 years [38–40]. In addition to the direct protection of vaccine recipients, the rCTB-WC vaccine resulted in significant herd protection to neighboring nonvaccinated individuals [41]. The rCTB-WC vaccine was also highly effective in short-term protection against severe cholera in an area of sub-Saharan Africa with a high prevalence of HIV infection [42]. Because of the immune responses to CTB after oral vaccination with rCTB-WC, the vaccine also possessed significant cross-protection against ETEC, which produces a heat-labile enterotoxin (LT) that shares high homology with CT and causes severe diarrhea in children in developing countries and in travelers to those countries [43].

A live oral V. cholerae O1 El or Inaba vaccine, Peru-15® (also known as Cholera Garde®), is currently in development. Peru-15 is also genetically engineered by deleting the native CT gene and introducing the gene encoding CTB [44]. The vaccine has been found to be safe, immunogenic and efficacious in North American volunteers as well as Bangladeshi adults and children [45]. A single dose of oral Peru-15 has provided promising results in a Phase I/II clinical trial in Bangladeshi children aged 9 months to 5 years [46]. Although we have to wait for additional efficacy results from ongoing field trials in developing countries, this form of oral vaccine will probably add another option for the global control of V. cholerae infection.

An ideal cholera vaccine should be a single oral dose that offers a high degree of long-term protection without side effects in children in endemic areas. It should also be an economical and cold chain-free (or refrigeration-free) product, so that the population at potential risk in developing countries can afford the vaccine [34]. It may not be an easy task, but we must develop a vaccine that fulfils these criteria in order to conquer cholera.

Typhoid vaccine

Typhoid fever caused by Salmonella enterica serovar Typhi remains a serious public-health problem, with an estimated 33 million cases and 500,000 deaths annually around the world [47]. The incidence of typhoid fever is the highest in children from 5 to 19 years of age, followed by those 1–5 years of age, in developing countries including areas of Africa, Asia and South America [48]. Travelers from industrialized countries who visit areas where typhoid fever is endemic are also at risk of contracting the disease.

Two types of vaccine are currently available, a purified Vi polysaccharide (PS) injection vaccine and an attenuated Ty21a live oral vaccine [2]. The Vi capsular PS of S. enterica serovar Typhi is an effective vaccine antigen that can induce protective antibody responses against O antigen, leading to the elimination of the bacterium from blood circulation [47]. A trial of single-dose Vi PS parenteral vaccine in South African children (average age: 9 years) gave 70% protection for the first 18 months and 55% protection over 3 years against typhoid fever [49]. Despite the efficacy in young children, the purified Vi PS is considered a poor immunogen for infants because it behaves as a T-cell-independent

antigen, and therefore induces low antigen-specific antibody responses in immunologically immature infants [49]. However, the conjugation of the PS antigen to a protein vaccine antigen improved the generation of protective antibody responses in infants [50]. Thus, current efforts are aimed at the development of a Vi-protein conjugate vaccine [50].

The oral attenuated S. enterica serovar Typhi strain Ty21a vaccine (Vivotif®) was generated by chemical mutagenesis of the wild-type strain Ty2 as a lyophilized live bacteria in an enteric-coated capsule [51]. In clinical trials, the vaccine was initially administered as three doses with 2-day intervals, which provided 67% overall protection against the disease over 3 years, and 62% protection over 7 years [52]. The vaccine was found to elicit Typhi-specific serum IgG and mucosal IgA [53] as well as Th1 and $CD8^+$ CTL responses [54]. Although the attenuated S. enterica Typhi strain Ty21a has been characterized as unable to synthesize the Vi capsular antigen, the mutation has not yet been fully defined, and the possibility remains that this strain could revert to virulence even though no revertants have been isolated so far. In addition, Ty21a requires three to four doses for optimal immunogenicity. [55]. Thus, a single-dose and better defined oral vaccine is highly desirable. Two new attenuated S. enterica serovar Typhi strains designed to overcome the concerns related to oral Ty21a vaccine are currently in advanced clinical trials. S. enterica Typhi Ty800, which has the Salmonella phoP/phoQ virulence region deleted, was shown in a Phase I trial to be a safe and immunogenic typhoid fever vaccine. The single-dose Ty800 vaccine induced brisk serum O-antigen-specific IgA and IgG responses in adult volunteers [56]. A Phase II trial [57] has been successfully completed of another single-dose live attenuated oral vaccine, CVD 909 (HolaVax-Typhoid®), a Salmonella aroC/aroD/htrA deletion mutant that constitutively expresses the Vi antigen. There was no difference in the rates of frequent diarrhea in the 21 days after vaccination among those receiving low and high doses of the vaccine and placebo. Antibody-secreting cells producing lipopolysaccharide (LPS)-specific IgA were detected in 100 and 92% of recipients of the high- and low-dose vaccines, respectively. Serum anti-LPS IgG, lymphocyte proliferation and IFN-γ production against Typhi antigen occurred in a substantial proportion of vaccinees. Additional Phase II or III human studies of these vaccines will be needed to define the immunogenicity of heterologous antigens carried by live S. enterica Typhi vectors.

Rotavirus vaccine

Rotavirus is a major cause of acute gastroenteritis worldwide in children younger than 5 years, causing more than 600,000 deaths annually, mainly in developing countries [58]. Rotaviruses have two surface proteins, VP4 and VP7, which determine the serotype-specific neutralizing antigens P and G, respectively. Although there are 15 G serotypes and 14 P serotypes, only five combinations, G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8], account for 90% of the human rotavirus strains [59]. Thus, these five G–P combinations are theoretically targets for vaccine development.

Rotashield® (Wyeth) was introduced in 1998 as the first licensed live attenuated oral rotavirus vaccine. It was a tetravalent reassortant vaccine composed of rhesus (G3) and human (G1, G2 and G4) rotavirus strains. It was given in a three-dose schedule starting at 8 weeks of age, with two subsequent doses administered at 8-week intervals [60]. Although Rotashield was highly efficacious for the prevention of diarrhea and hospitalization by rotavirus infection, the vaccine might have triggered intussusceptions after the oral vaccination period [60]. The risk of intussusception was estimated at more than one case of intussusception per 32,000 infants during the postimmunization period [61]. Wyeth thus withdrew the vaccine in 1999.

Currently, two live-attenuated oral rotavirus vaccines, Rotarix® (GlaxoSmithKline) and RotaTeq® (Merck), are licensed in 88 and 47 countries, respectively [55]. The two vaccines were designed from Rotashield by using different approaches. Rotarix is an attenuated human rotavirus vaccine that is made of a tissue-culture adapted human isolate, 89–12 (G1P[8]). The 89–12 strain was chosen as a vaccine candidate because natural infection with similar G1P[8] rotaviruses provides broadly cross-reactive neutralizing antibody responses and excellent protection against rotavirus gastroenteritis [62]. In addition, human rotavirus G1P[8] is the predominant strain worldwide. Rotarix is given in a two-dose schedule starting at 6–13 weeks of age, with a second dose administered after a 4- to 8-week interval. The safety and efficacy of Rotarix in a Phase III trial involving 63,225 infants showed that the vaccine was efficacious at protecting infants against severe rotavirus gastroenteritis and was not associated with an increased risk of intussusceptions [63]. The vaccine efficacy was 92% against G1 serotype-specific rotavirus gastroenteritis, whereas the efficacy against G2, G3, G4 and G9 serotype-specific rotavirus gastroenteritis ranged from 41 to 87%.

RotaTeq is composed of five reassortant rotavirus strains, which were derived from a WC3 bovine strain containing two genes, VP4 (P) and VP7 (G), encoded by a rotavirus of human origin. It is given orally in a three-dose schedule starting at 6–12 weeks of age, with subsequent doses administered at 4- to 10-week intervals. A Phase III safety and efficacy trial of RotaTeq involving 70,301 infants showed that the vaccine is efficacious in preventing rotavirus gastroenteritis. The vaccine efficacy against G1, G2, G3, G4 and G9 serotype-specific rotavirus gastroenteritis was 75, 63, 83, 48 and 65%, respectively [64]. The risk of intussusceptions was similar between the recipients of vaccine and placebo.

During the process of developing a safe and effective oral rotavirus vaccine, it was consistently shown that the levels of serum rotavirus-specific IgA measured shortly after either vaccination or natural infection generally reflects the levels of antigen-specific intestinal IgA antibodies and may be the best available marker for protection against rotavirus gastroenteritis [65]. Intestinal rotavirus-specific IgA is probably the most important mechanism for long-term protection against rotavirus gastroenteritis, but the mechanism by which the vaccine elicits protective immunity in humans remains poorly understood [65].

Nasal vaccines

Acute respiratory illness causes the highest burden of disease in the world. In particular, influenza virus, respiratory syncytial virus (RSV), parainfluenza virus (PIV) and M. tuberculosis are major

respiratory pathogens that cause acute respiratory infectious diseases [66]. The mucosal immune system protects against these pathogens, which invade the host via the mucosal surfaces covering the upper and lower airway tracts. To make use of this mucosal immune system, a nasal vaccine has been developed for the flu and is currently available to the public in the USA in addition to the injection-type influenza vaccine. Here, we summarize and discuss mucosal vaccines that are currently approved or in development for human use against respiratory infections (TABLES 1 & 2).

Influenza vaccine

Acute respiratory infections including viruses and bacteria caused nearly 4 million deaths every year in worldwide, mostly in young children and infants in developing countries. The public burden of influenza in the USA is estimated 25–50 million cases and 30,000–40,000 deaths per year. The average global burden of pandemic influenza may be on the order of 1 billion cases [67]. Influenza causes particularly high rates of severe disease in children younger than 5 years of age and adults older than 50 years of age [68]. Influenza viruses are divided into three genera, A, B and C. Influenza A viruses are further divided into subtypes according to antigenicity of the major envelope proteins hemagglutinin (HA) and neuraminidase (NA) [67]. Although there are 15 HA variants and nine NA variants, and thus 135 possible combinations, only viruses of the H1N1, H1N2 and H3N2 subtypes are currently circulating widely in human populations, although the pandemic influenza viruses H5N1, H7N2, H7N7 and H9N2 also cause human disease [67].

Injectable vaccines against influenza have been in common use for a long time. Serum-derived immunoglobulins are the antibodies primarily responsible for the protection of the lower respiratory tract, whereas mucosal antibodies play a critical role in the protection of the upper respiratory tract and are considered to be more important to overall protection against airway infection with influenza [69]. Recently, a nasal live-attenuated, trivalent influenza vaccine, FluMist® (MedImmune), was approved in the USA based on the advanced molecular and cellular knowledge of influenza infection and mucosal immunity. The vaccine contains two type A (H1N1 and H3N2) and one type B attenuated, cold-adapted, temperature-sensitive reassortant influenza virus strains that express the HA and NA surface glycoproteins of the targeted wild-type strains [70]. The vaccine strain can replicate at 25°C, which is the temperature of the nasal surface, but cannot survive at 37°C, the temperature of the lungs [71,72]. FluMist was highly efficacious in a 2-year Phase III trial, with overall protective efficacy of 92%; the vaccine showed 86% protective efficacy against antigenic variants that circulated in the region [70]. The currently licensed formulation of FluMist requires storage at -15°C or less. To overcome this impractical storage requirement, a liquid formulation of cold-adapted trivalent influenza vaccine (CAIV-T) that is stable at refrigerator temperatures of 2–8°C is being evaluated in a Phase III trial [73].

Although FluMist was originally approved only for healthy individuals aged 5–49 years, the US FDA further approved FluMist for children aged 2–5 years in 2007. The US CDC recommends that children 6 months and older should be vaccinated for influenza. However, the FDA decided that children under 2 years of age should not be given FluMist because of an increased risk of wheezing and other side effects possibly associated with the nasal administration [302]. Thus, mucosal vaccines for influenza that can safely be administered to children less than 2 years of age still need to be developed. In addition to the infant population, one must remember that the elderly population of more than 50 years of age is also at major risk for influenza infection and its associated medical complications, and thus the availability of a mucosal influenza vaccine will contribute to improving public health in our aging society.

RSV/PIV vaccines

Respiratory syncytial virus is the most important cause of severe lower respiratory infections in infants worldwide. RSV is estimated to cause approximately 64 million infections each year, and mortality could be as high as 160,000. Almost all children will have been infected with RSV by 2 years of age, and the peak incidence of mortality with RSV infection occurs at less than 3 months of age [74]. The viral surface glycoproteins F and G are major protective antigens. F protein, which is highly conserved in RSV A and B groups, is responsible for fusion of the virus envelope with the target host cell membrane [75]. PIV types 1, 2 and 3 are also important respiratory pathogens in infants and young children. For example, PIV-3 infects approximately 60% of infants before the age of 2 years [74]. The viral surface glycoproteins HA–NA (HN) and F protein are responsible for attachment and fusion of virus to target host cells (e.g., respiratory epithelial cells) [75].

In general, immunity against RSV and PIVs is mediated by humoral antibodies, including secretory antibodies acquired as a result of infection or maternally derived in infants, and cell-mediated immune responses by cells such as CTLs. A variety of immunological approaches against RSV have been tested for the development of a vaccine, but with limited success. For example, young infants frequently fail to respond adequately to vaccination due to immunogenic immaturity. In addition, one formalin-inactivated RSV not only failed to protect infants against RSV infection, but also induced exaggerated clinical responses to wild-type RSV infection in infants who were naive to RSV before vaccination. The early trials resulted in the hospitalization of 80% of vaccinees and two deaths [74].

At present, there is no licensed vaccine against RSV [67,74], although nasal vaccines against RSV are in clinical trials. Nasal immunization with a live attenuated vaccine should induce both systemic and mucosal immunity, and may protect against viral illnesses of the upper and lower respiratory tracts. A live-attenuated vaccine containing a chimeric bovine/human PIV3 expressing the human PIV3 F and HN proteins and the human RSV F protein (b/hPIV3/RSVF) has been produced. Nasal immunization with the b/hPIV3/RSVF vaccine not only induced RSV-neutralizing serum antibodies and protective immunity against RSV challenge in African green monkeys, but also resulted in production of serum antibodies that neutralized hPIV3 and inhibited the hemagglutination of hPIV3 [75]. The b/hPIV3/RSVF vaccine is currently being tested in a Phase I study.

Tuberculosis vaccines

Tuberculosis (TB) is caused by the respiratory pathogen M. tuberculosis and is a major health problem in both developed and developing countries. Globally, TB causes nearly 2 million deaths annually and is the leading cause of death among HIV-infected populations because of the inhibition of the immune system that accompanies HIV infection [303]. The currently available bacillus Calmette–Guérin (BCG) vaccine protects against TB in childhood, but the immunity wanes with age, with the result that this vaccine is ineffective at protecting against adult pulmonary TB. Protective immunity to TB is dependent on strong cellular immune responses involving both $CD4^+$ and $CD8^+$ T cells that have the ability to produce the cellular immunity-enhancing cytokine IFN-γ [76].

New approaches toward the development of vaccine against TB have been reported in preclinical and clinical studies [77,78]. These approaches include using recombinant BCG, live-attenuated strains of M. tuberculosis, subunit vaccine approaches, and nonreplicating viral vector-based systems used alone or in prime–boost regimens. There is increasing evidence that a heterologous prime–boost approach induces higher levels of cellular immunity than homologous boosting with the same vaccine [79]. In addition, there is some experimental evidence that nasal vaccination protects against TB in mice. Although these findings provide strong supportive evidence that nasal vaccination is the best way to attain robust protective immune responses in the lungs, no nasal TB vaccine candidate has reached the clinical trial stage [78].

The TB-specific immunity induced by the current parenteral BCG vaccine is markedly enhanced when it is boosted by nasal administration of an adenovirus vector expressing M. tuberculosis antigen 85A (AdAg85A) in a mouse model. The enhanced protection afforded by nasal AdAg85A correlates with the numbers of IFN-γ-positive $CD4^+$ and $CD8^+$ T cells in the lung [80]. Moreover, nasal immunization with the fusion protein consisting of Ag85B and 6-kDa early secretory antigenic target (ESAT)-6 [81] together with a combined adjuvant composed of immunoimulating complexes [82] and a fusion of the A1 CT subunit with two copies of the D domain from Staphylococcus aureus protein A (CTA1-DD) [83], strongly boosts the pre-existing BCG immunity in mice, promotes an antigen-specific Th1 immune response dominated by IFN-γ-secreting $CD4^+$ T cells, and contributes to protective immunity in the lung [84]. Unlike other toxin-based nasal adjuvants, the CTA1-DD adjuvant itself does not redirect to the CNS after nasal immunization [85]. Thus, the TB mucosal vaccine system with CTA1-DD is a promising candidate for human application.

Conversely, priming with a nasally administered BCG followed by boosting with a parenteral modified vaccinia virus Ankara expressing Ag 85A (MVA85A) also induces high levels of antigen specific $CD4^+$ and $CD8^+$ T cells and protective immunity in mice [86]. Because MVA85A has been shown to be safe and highly immunogenic in a human Phase I study [87], the nasal BCG followed by boosting with MVA85A will probably be further evaluated as a TB vaccine candidate for humans.

New horizons in the development of mucosal vaccines

One of advantages of a mucosal vaccine over the injectable vaccine is, of course, that administration devices such as needles and syringes are not necessary. A departure from the disposable syringes and needles that are currently most commonly used for vaccination would be friendly to both humans and the environment, because it would eliminate the necessity of administration device (e.g., syringe and needle) leading to the secondhand spread of infectious diseases and pain associated with vaccine administration, and the vast amounts of medical waste generated by mass immunizations. Ongoing research into the molecular and cellular mechanisms of surface immunological barrier systems is providing practical strategies for the development of a new generation of mucosal vaccines. Here, we introduce and summarize some of the key discoveries for the development of a new generation of mucosal vaccines for the control of infectious diseases.

Transcutaneous vaccines

In addition to the mucosal immune system, the skin serves as another major surface barrier system with unique and dynamic immune attributes [27]. Transcutaneous immunization (TCI) relies on the application of a vaccine antigen with or without an adjuvant to the outer layer of the skin and subsequent delivery of the vaccine antigen to underlying LCs that serve as antigen-presenting cells [10,27,88]. TCI can induce systemic and mucosal immunity through antibody and mucosal CTL responses [27,89,90]. Recent studies have shown that TCI activates LCs carrying skin-derived antigens and allows them to migrate in a retinoic acid-dependent manner from the skin to the mesenteric lymph nodes, where they present antigen directly to resident lymphocytes for the initiation of antigen-specific mucosal immune response [90,91].

IOMAI Corporation has developed a needle-free skin patch containing a LT from E. coli as a TCI vaccine against traveler's diarrhea. As discussed above, a strain of ETEC is a major cause of traveler's diarrhea. Every year, 27 million travelers and 210 million children suffer from this acute diarrhea, causing 380,000 pediatric deaths. LT is a key pathogenic molecule in approximately two-thirds of cases of ETEC diarrhea [92,93]. Vaccines that induce immunity to LT offer protection against ETEC diarrhea because the toxin produced by the pathogen causes the watery stool [93]. In a double-blind, placebo-controlled trial, 59 volunteer adults received 50 µg of LT or placebo in a patch applied to the arm on days 0, 21 and 42. On day 56, 27 vaccinees and 20 controls were challenged orally with LT^+/ST^+ (heat-stable toxin) ETEC. The LT patch did not prevent ETEC infection, but a single LT patch produced seroconversion in 97% of vaccinated subjects and levels of anti-LT-specific IgG and IgA antibodies were higher than those of the control group following challenge with a virulent LT-expressing ETEC [87]. In order to further investigate the safety and efficacy of a LT-patch vaccine, volunteer travelers to Mexico and Guatemala were vaccinated before travel with two doses of the LT-patch vaccine given at 2–3-week intervals. The LT-patch recipients had shorter episodes of diarrhea with fewer loose stools than those receiving placebo [93]. The results of this Phase II trial suggest that a LT-patch vaccine could protect travelers suffering from diarrhea, but the efficacy of the LT patch

needs further confirmation in a Phase III trial. Vaccine delivery with patches or TCI may allow self-administration, ambient temperature stabilization, and ease of storage, making this approach an attractive needle- and cold chain-free form of vaccination (FIGURE 2).

Plant-based vaccines

Since 1990, plant-based vaccines have been proposed as the next generation of mucosal vaccines [201]. The production of vaccines in plants could have significant advantages over the existing production systems, including cost-effective production, rapid scaling-up production of the proteins, a low risk of contamination by human pathogens, and expression of multiple genes in a plant. Many candidate vaccine antigens, including bacterial diarrhea antigens, hepatitis B antigen, Norwalk virus antigen, cytomegalovirus glycoantigen, Clostridium tetani fragment C and RSV antigen, have been expressed in tobacco, tomato or potato to demonstrate the feasibility of plant-based vaccines [94–97].

Approximately 250 million episodes of acute gastroenteritis due to Norwalk virus occur annually in children and the elderly in the USA, causing vomiting, abdominal cramps, diarrhea, headache and fever. Thus, the development of a plant-based Norwalk virus oral vaccine would benefit public health. Immune responses were investigated in volunteers who ingested 150 g of transgenic potatoes expressing 0.21–0.75 mg of Norwalk virus capsid protein formed into virus-like particles. A total of 19 out of 20 volunteers developed an antigen-specific immune response with a modest titer of serum antibodies [98]. In another study, transgenic corn (2.1 g of plant material as a dose) expressing 1 mg of the B subunit of LT (LTB) was fed to adult volunteers in three doses. Seven of nine volunteers developed LTB-specific serum IgG and four of nine volunteers also developed LTB-specific stool IgA antibodies [99]. These studies demonstrated the feasibility of developing plant-based oral vaccine. Despite their attractiveness, some these plant-based vaccines did not advance to the next stage of development. To further advance the practicality and effectiveness of plant-based vaccines, it is essential to make them stable at room temperature for long periods, able to withstand the harsh environment of the gastrointestinal tract, and targeted to the mucosal inductive tissues, including PP. To overcome these hurdles, we have introduced a rice-based vaccine system (MucoRice™), in which the vaccine is physically and chemically stable and capable of generating protective immunity against enterotoxin such as a CT [100].

In MucoRice, CTB is used as the vaccine antigen [100]. The expressed antigen accumulates in protein bodies, the rice seed organelles for protein storage, which confers resistance against digestive enzyme activity while allowing uptake of the vaccine antigen. A minimal dose of oral antigen is therefore effective at inducing an antigen-specific immune response. In fact, when

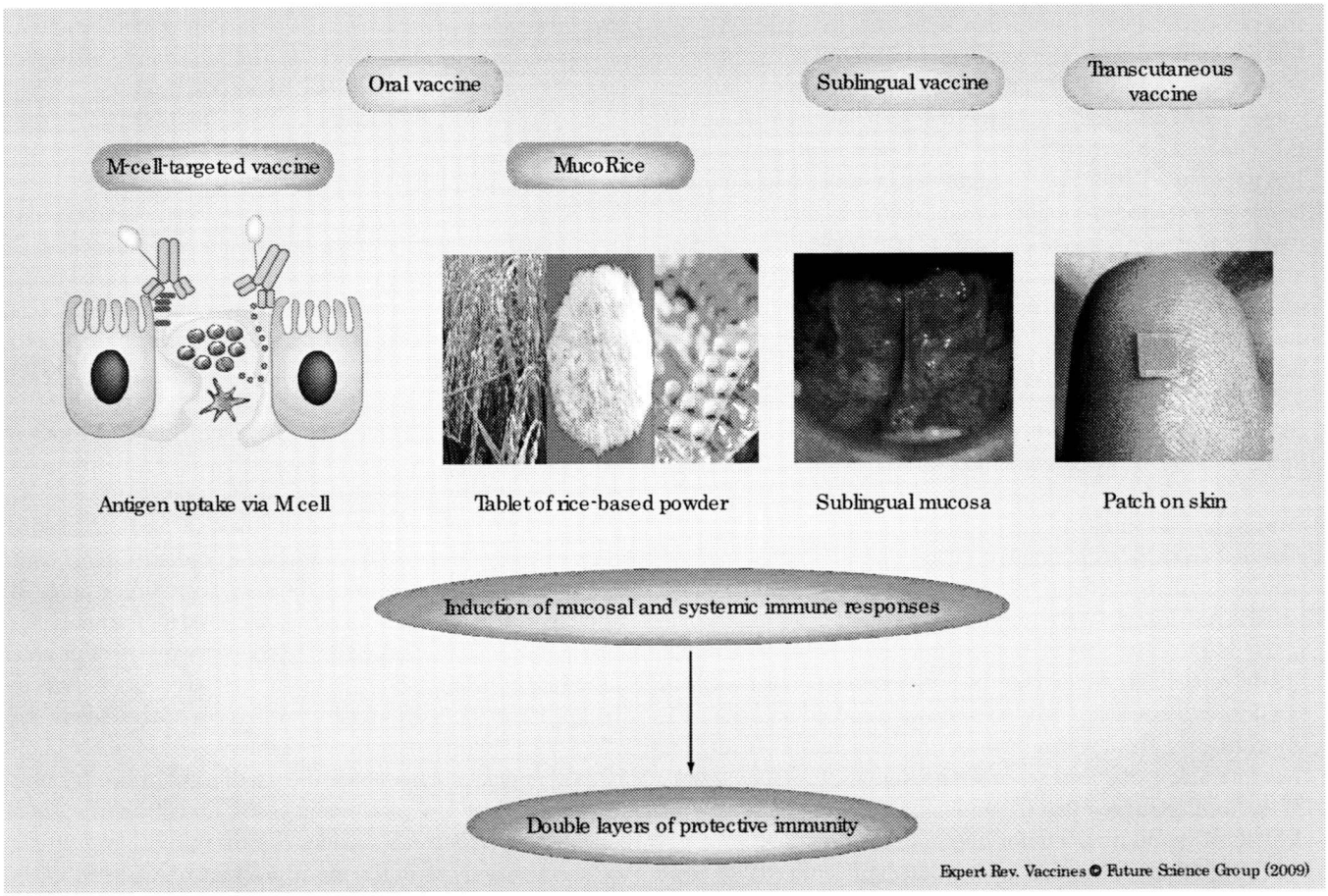

Figure 2. New horizon for the development of needle/syringe-free vaccine.

ingested by mice, MucoRice-expressed CTB was taken up by the M cells covering the PP and induced toxin-specific serum IgG and mucosal IgA antibodies with neutralizing activity. In addition, the rice-based CTB vaccine remained stable and was immunogenic after storage at room temperature for 2 years and was protected from pepsin digestion in vitro. Taken together, these findings suggest that the MucoRice system would be a needle- and cold chain-free oral vaccine against infectious diseases (FIGURE 2) [100].

M-cell-targeted vaccines

M cells possess the ability to take up luminal antigens and are the gateway of the respiratory and digestive immune system, making them attractive targets for vaccine antigen delivery [101]. Several molecules bind preferentially to M cells. For example, Ulex europaeus agglutinin (UEA)-I has specificity for $\alpha(1,2)$ fucose and specifically reacts with murine M cells [102]. The $\sigma 1$ protein derived from reovirus specifically binds to a carbohydrate structure containing $\alpha(2,3)$-linked sialic acid on the membranes of M cells [103]. Vaccination with UEA-1-conjugated [104] or $\sigma 1$-protein-conjugated [105] nasal vaccines induces strong antigen-specific plasma IgG and mucosal IgA responses as well as CTL immunity. However, because UEA-1 also reacts strongly with goblet cells on the intestinal epithelium, the usefulness of UEA-1 as an M-cell-targeting vehicle is limited.

To overcome this obstacle, we established a novel M cell-specific monoclonal antibody (mAb) that selectively recognizes M cells but not goblet cells or epithelial cells [106]. Oral administration of a tetanus toxoid (TT)- or botulinum neurotoxoid (BoNT)-conjugated M cell-specific mAb together with the mucosal adjuvant CT induced high-levels of antigen-specific serum IgG and mucosal IgA responses; TT- or BoNT-conjugated control rat IgG induced no or very low antigen-specific immune responses, and the immune response to even 10-times the amount of noncoupled TT was much lower than that in mice vaccinated with the TT-conjugated M cell-specific mAb. In addition, an oral vaccine formulation of BoNT-conjugated M cell-specific mAb induced protective immunity against a challenge with 10,000-times the LD_{50} dose of botulinum toxin [106]. The results suggest that an M cell-targeted vaccine using the concept of a mAb guiding system could be a useful approach for developing highly effective mucosal vaccines.

Sublingual vaccines

The oral mucosa, including the buccal and sublingual mucosa, may be useful as a delivery site for therapeutic drugs because proteins do not degrade to the same extent in these regions as they do in the intestine [28]. Of the routes of oral administration, the sublingual route has been used for the immunotherapeutic treatment of allergies because antigens are quickly absorbed and enter the bloodstream without passing through the intestine or liver, and are, therefore, able to efficiently elicit antigen-specific tolerance [107,108]. These findings suggest that the sublingual route might be used for delivery of vaccine antigens for infectious diseases.

Sublingual administration of inactivated influenza A/PR8 virus (H1N1) together with a mucosal adjuvant such as CT [28] or non-toxic mCTA/LTB adjuvant (the A subunit of mutant CT with the B subunit of LT) [109,110] induces both systemic and mucosal virus-specific antibody responses as well as CTL responses with protective immunity after respiratory challenge with the A/PR8 virus [28,110]. The studies also showed that the sublingual epithelium harbors a dense lattice of DCs, and that using CT as a mucosal adjuvant mobilizes DCs within the sublingual epithelium. These cells migrate to the proximal draining lymph nodes, such as the submaxillary and superficial cervical lymph nodes, on uptake of the sublingual vaccine antigens. Interestingly, similar to nasal vaccination, sublingual immunization induces antigen-specific immune responses in the female reproductive tract in addition to the respiratory tract and oral/nasal cavity, suggesting that sublingual immunization uses the same cellular trafficking system as nasal immunization [28]. The sublingual mucosa represents a specialized immunological microenvironment favoring the initiation of antigen-specific immune responses [28,110].

A nasal inactivated influenza virus vaccine together with a toxin-based adjuvant (LT) was associated with the incidence of Bell's palsy during human clinical trials [111]. In addition, toxin-based adjuvant such as CT and CTB are redirected to the olfactory bulb in the CNS when administered via the nasal route [112], which has resulted in concerns about side effects in the nervous system. By contrast, inactivated influenza virus with a mucosal adjuvant did not migrate to or replicate in the CNS after sublingual immunization [110]. These findings suggest that sublingual immunization may be another attractive and safe mucosal route for administering influenza vaccines for the generation of influenza-specific neutralizing antibodies and CTL responses (FIGURE 2).

Expert commentary

As discussed above, several live-attenuated forms of mucosal vaccines are already clinically available or are now in or being considered for clinical trials. In general, these live attenuated vaccines can induce potent protective immunity against pathogens because they are strong immunogens and at same time contain natural forms of vaccine adjuvants, such as Toll-like receptor (TLR) ligands (e.g., TLR3, 4 and/or 7) [113]. However, a couple of live-attenuated vaccines, such as the OPV and Rotashield rotavirus vaccines, have been reported to revert to virulent forms or to cause serious side effects. Although these risks are quite low (e.g., one out of 500,000 for OPV [32] and one out of 30,000 for Rotashield [61]), these possibilities must be eliminated in order to safely control infections through vaccination. Further, these unfortunate facts are often revealed after the vaccine is approved for the public [32,61]. Although live-attenuated vaccines induce more potent protective immunity in healthy adults than inactivated or subunit vaccines, the latter may be useful for immunization of the elderly, naive infants and children at high risk of disease.

Another practical reason for the use of some of live-attenuated vaccines is their low production costs. Distribution costs also contribute greatly to the increased total medical cost of global vaccination, and one of the major practical obstacles to vaccination in the field, especially in the developing countries, is the need for refrigerated storage of the vaccine (the cold chain) [114]. The