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A Rice-Based Oral Cholera Vaccine Induces Macaque-Specific Systemic Neutralizing Antibodies but Does Not Influence Pre-Existing Intestinal Immunity¹

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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 μ g 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- μ m 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 -lactalbumin (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 μ g/ml CT (List Biologic Laboratories), recombinant CT-B, or recombinant LT-B prepared in our laboratory (20) or 20 μ g/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

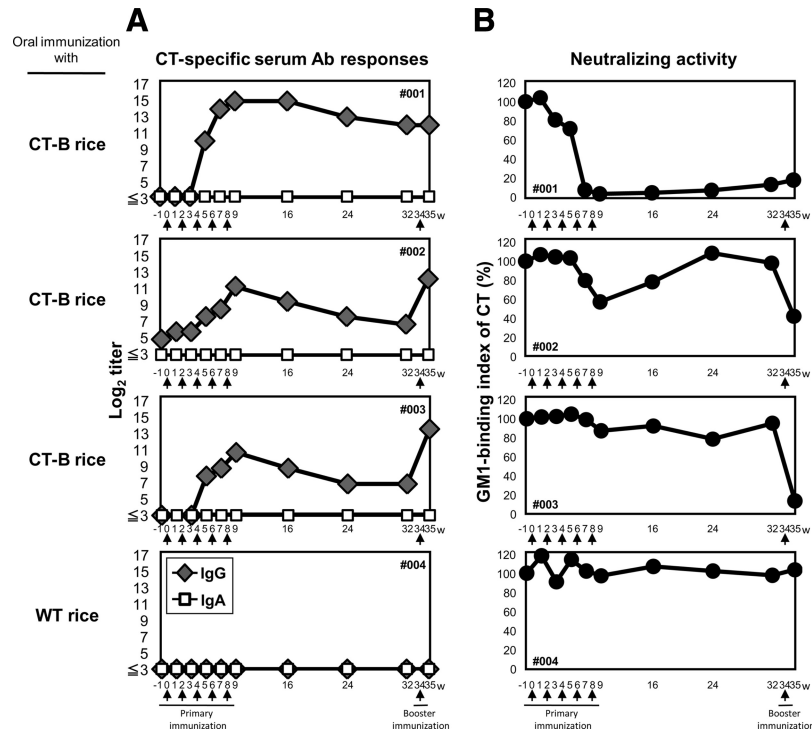
A neutralizing assay was performed by using a GM1-ELISA as described previously (17), with some modifications. In brief, serum (10%, v/v) or gel-filtered fecal extract (50%, v/v) was pretreated with CT (50 ng/ml final concentration) for 1 h at RT and then incubated in 96-well plates coated with monosialoganglioside GM1 (5 μ g/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. 1A), 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. 1B). 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. 1C; similar to the reactivity against CT-B), all of the macaques had LT-B-reactive SIgA Abs in their feces (Fig. 1D).

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. 4A). 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. 4B). 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. 4C). 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. 5A). The booster immunization 6 mo after the last immunization also did not influence the level of CT-reactive intestinal SIgA Abs (Fig. 5A). 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. 5B). These findings suggest that oral vaccination with MucoRice-CT-B cannot modulate the pre-existing CT-reactive intestinal SIgA Ab responses.

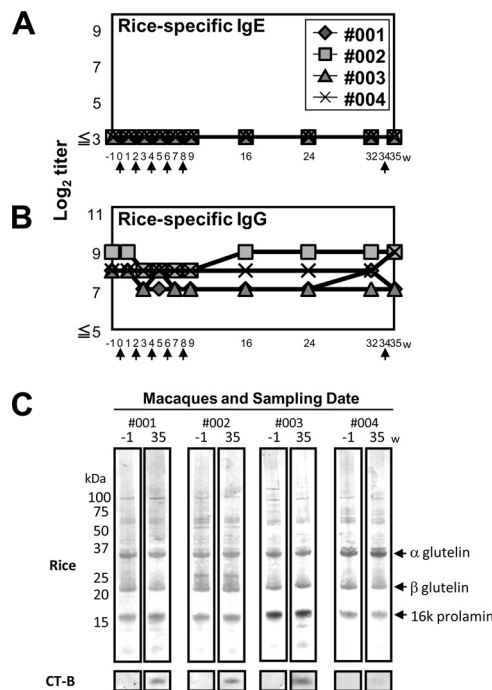


FIGURE 4. Oral immunization with MucoRice-CT-B does not induce rice storage protein-specific immune IgE Ab responses. Very low levels of serum IgE Abs specific for rice storage proteins were detected in each of the macaques orally immunized with MucoRice-CT-B or WT rice (A). In addition, rice storage 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.

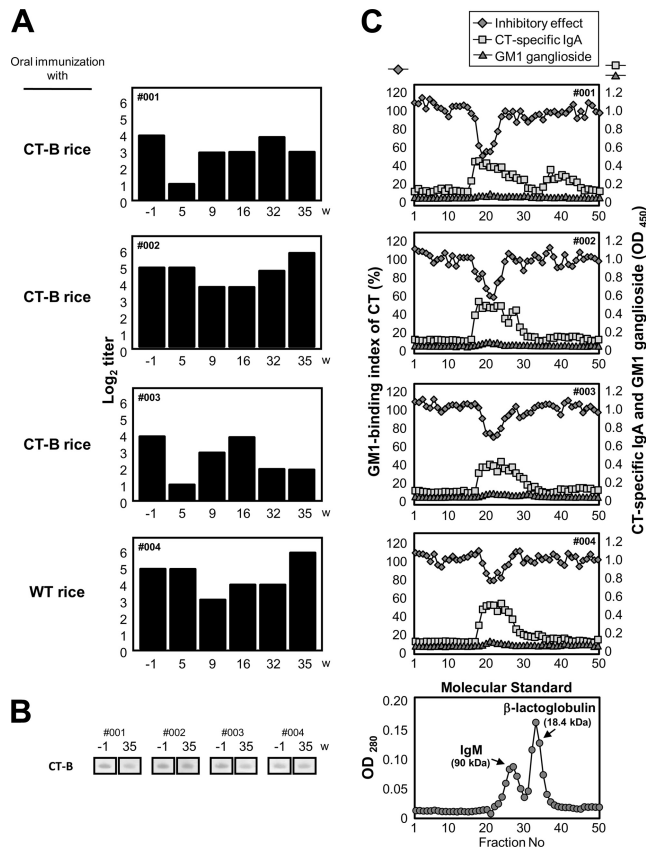


FIGURE 5. Oral immunization with MucoRice-CT-B does not increase spontaneously acquired CT-reactive intestinal SIgA Ab responses, but these SIgA Abs possess toxin-neutralizing activity. Unlike the CT-specific serum IgG Ab responses, CT-specific SIgA Ab responses were not enhanced by oral immunization 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 -lactoglobulin were used as molecular standards for the gel filtration chromatography (C). 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

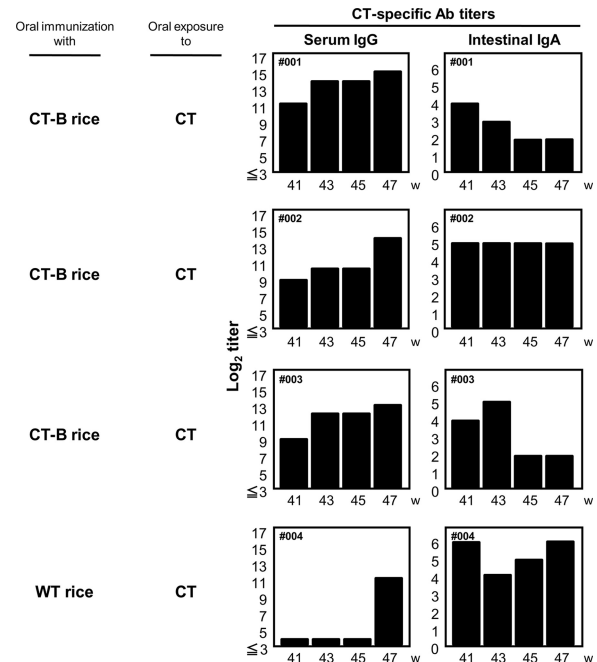


FIGURE 6. Oral administration of CT enhances serum IgG but not CT-reactive intestinal SIgA Ab responses. CT-specific serum IgG Ab responses were sharply increased in the control macaque (no. 004) and showed a further tendency to increase in three macaques previously immunized with MucoRice-CT-B (no. 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.

(Fig. 5C). In contrast, CT-specific intestinal SIgA Abs were observed in the expected fractions of all macaques (Fig. 5C), 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. 5B). 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. 5C). 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, be-

cause 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 μ g 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. 4A). 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.

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