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Author contributions

T.N. and Y.Y. designed and carried out the experiments, analysed the results and wrote the manuscript. Hi.T., S. Kozaki, K.A. and H.K. designed the experiments and wrote the manuscript. Ha.T., S-i.S., M.M., T.K., N.H., N.K., I.G.K., A.S., D.T., S. Kurokawa and Y.T. carried out the experiments.

Additional information

The authors declare no competing financial interests. Supplementary information accompanies this paper on www.nature.com/naturematerials. Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions. Correspondence and requests for materials should be addressed to H.K.

ERRATUM

Nanogel antigenic protein-delivery system for adjuvant-free intranasal vaccines

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This has been corrected in the PDF version of this Letter.

Secretory IgA-mediated protection against *V. cholerae* and heat-labile enterotoxin-producing enterotoxigenic *Escherichia coli* by rice-based vaccine

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Cholera and enterotoxigenic *Escherichia coli* (ETEC) are among the most common causes of acute infantile gastroenteritis globally. We previously developed a rice-based vaccine that expressed cholera toxin B subunit (MucoRice-CTB) and had the advantages of being cold chain–free and providing protection against cholera toxin (CT)–induced diarrhea. To advance the development of MucoRice-CTB for human clinical application, we investigated whether the CTB-specific secretory IgA (SIgA) induced by MucoRice-CTB gives longstanding protection against diarrhea induced by *Vibrio cholerae* and heat-labile enterotoxin (LT)–producing ETEC (LT-ETEC) in mice. Oral immunization with MucoRice-CTB stored at room temperature for more than 3 y provided effective SIgA-mediated protection against CT- or LT-induced diarrhea, but the protection was impaired in polymeric Ig receptor–deficient mice lacking SIgA. The vaccine gave longstanding protection against CT- or LT-induced diarrhea (for ≥6 months after primary immunization), and a single booster immunization extended the duration of protective immunity by at least 4 months. Furthermore, MucoRice-CTB vaccination prevented diarrhea in the event of *V. cholerae* and LT-ETEC challenges. Thus, MucoRice-CTB is an effective long-term cold chain–free oral vaccine that induces CTB-specific SIgA-mediated longstanding protection against *V. cholerae*– or LT-ETEC–induced diarrhea.

cholera toxin B subunit | mucosal vaccine | oral vaccine | plant-made vaccine | MucoRice

Cholera is an acute diarrheal disease leading to death by severe dehydration without appropriate treatment, especially in developing countries (1). Cholera toxin (CT), produced by *Vibrio cholerae*, consists of a B-subunit pentamer (i.e., CTB) and a single A subunit (i.e., CTA) (2). Diarrhea in cholera is induced by the elevation of intracellular cAMP levels in the intestinal epithelial cells by CTA with ADP ribosyltransferase activity after the binding of CT to GM1 ganglioside, expressed on the epithelial cells, via CTB (2). One of the current oral cholera vaccines, Dukoral, consists of recombinant CTB (rCTB) and whole cells of killed *V. cholerae* (CTB-WC) and is the one that has been used the most extensively worldwide (3). The oral CTB-WC vaccine induces both *V. cholerae*– and CTB-specific immune responses, and past epidemiological studies have clearly shown that it reduces the development of diarrhea by 55% to 85% (3, 4). We recently developed a rice-based cholera vaccine expressing CTB (MucoRice-CTB). This vaccine has the advantages of being suited to long-term storage without the need for a cold chain (>1.5 y), and delivery of the vaccine antigen is needle- and syringe-free (5).

To advance the development of MucoRice-CTB for human clinical application, several key issues remain resolved, despite the promising results obtained in our murine studies (5, 6). First, it is necessary to assess the immunogenicity of MucoRice-CTB in nonhuman primates. Our recent study demonstrated that oral MucoRice-CTB can effectively induce antigen-specific neutralizing antibody responses in nonhuman primates (7). Second, despite the generally accepted concept that mucosal vaccine induces antigen-specific secretory IgA (SIgA) production, thus providing a first line of specific defense against mucosal infectious diseases, there is no direct evidence that the CTB-specific SIgA production induced by MucoRice-CTB is essential for protection against CT-induced diarrhea. The fact that nonhuman primates have preexisting protective intestinal immunity and do not develop CT-induced diarrhea (7) makes it uncertain whether MucoRice-CTB–induced CTB-specific SIgA can in fact prevent diarrhea in these animals. Therefore, it is essential to elucidate the significance of the CTB-specific SIgA production induced by MucoRice-CTB in mice. Third, although several oral CTB vaccines have demonstrated the induction of protective immunity against CT-induced diarrhea in mice (5, 8), it remains unclear whether CTB-specific intestinal SIgA responses, including those induced by oral MucoRice-CTB, can protect against diarrhea induced by live *V. cholerae*. Finally, minimal information on the duration of the protective immunity induced by oral MucoRice-CTB vaccine is currently available. To clarify these unresolved key issues, we aimed to (*i*) directly demonstrate whether antigen-specific SIgA production induced by oral MucoRice-CTB is a critical element in protective immunity against CT-induced diarrhea in mice; (*ii*) examine the longevity of MucoRice-CTB–induced primary antigen–specific neutralizing humoral immunity and the effects of oral boosters; and (*iii*) elucidate in vivo whether oral MucoRice-CTB–induced antigen-specific mucosal IgA responses provide protective immunity against diarrhea caused by *V. cholerae*.

In addition to *V. cholerae*, enterotoxigenic *Escherichia coli* (ETEC) is a major cause of bacterial diarrhea in developing countries (9, 10) and a leading cause of travelers' diarrhea in developed countries (11). ETEC produces heat-stable enterotoxin (ST) and/or heat-labile enterotoxin (LT) (2). LT is found in approximately two thirds of cases of ETEC-induced diarrhea (12–14). In addition, previous studies have shown that anti-LT immunity protects against ETEC-induced diarrhea in human (15–17). LT is structurally and biologically similar to CT (2, 18), and several studies have demonstrated cross-protective immunity between CT and LT (19–21). It was therefore an obvious and important question to address whether CT-specific mucosal IgA induced by oral MucoRice-CTB vaccine could provide cross-protective immunity against LT-induced

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diarrhea and, if so, whether it could also provide protection against diarrhea induced by LT-producing ETEC (LT-ETEC).

We demonstrated here that the CTB-specific SIgA response induced by oral MucoRice-CTB is solely responsible for antibody-mediated, cross-protective, long-term immunity against LT- and CT-induced diarrhea; this effectiveness was further extended to *V. cholerae*– and LT-ETEC–induced diarrhea in vivo. These findings enforce the attractiveness and advantages of the cold chain– and needle- and syringe-free MucoRice system and should enable the development of an innovative oral vaccination strategy against *V. cholerae* and LT-ETEC.

Results

MucoRice-CTB–Induced Protection Against CT-Induced Diarrhea Is Impaired in Polymeric Ig Receptor–KO Mice. To examine whether induction of the secretory form of CTB-specific IgA by oral MucoRice-CTB vaccination is a critical element in protection against CT-induced diarrhea, we compared polymeric Ig receptor (pIgR)–KO and WT mice vaccinated orally with MucoRice-CTB. We thus clarified the direct role of CTB-specific SIgA in providing protection against CT-induced diarrhea. MucoRice-CTB–immunized pIgR-KO mice, which lacked the formation and transepithelial transport of SIgA, had significantly lower ($P = 0.0001$) antigen-specific mucosal IgA levels in their intestinal secretions than did immunized WT mice (Fig. 1*A*). In contrast, lack of CTB-specific SIgA formation and transport caused a significant increase ($P < 0.0001$ vs. immunized WT mice) in the serum CTB-specific IgA level in oral MucoRice-CTB–immunized pIgR-KO mice, whereas the antigen-specific serum IgG titer was comparable to that of WT mice orally immunized with MucoRice-CTB (Fig. 1*A*). When the frequency of CTB-specific IgA antibody-forming cells (AFCs) was examined in the small intestinal lamina propria (LP), significantly more antigen-specific IgA AFCs were found in MucoRice-CTB–immunized pIgR-KO mice ($P = 0.0007$) than in MucoRice-CTB–immunized WT mice (Fig. 1*B*). Our finding of large numbers of antigen-specific IgA AFCs in immunized pIgR-KO mice is compatible with the results of a previous study that found a marked accumulation of IgA in the intestinal LP of pIgR-KO mice by immunohistochemical analysis (22). When these two groups (pIgR-KO and WT) of MucoRice-CTB–vaccinated mice were orally challenged with a native form of CT, the immunized WT mice showed protection against CT-induced diarrhea, whereas the pIgR-KO mice developed severe diarrhea ($P = 0.002$ vs. immunized WT mice), despite the presence of high titers of antigen-specific serum IgG and

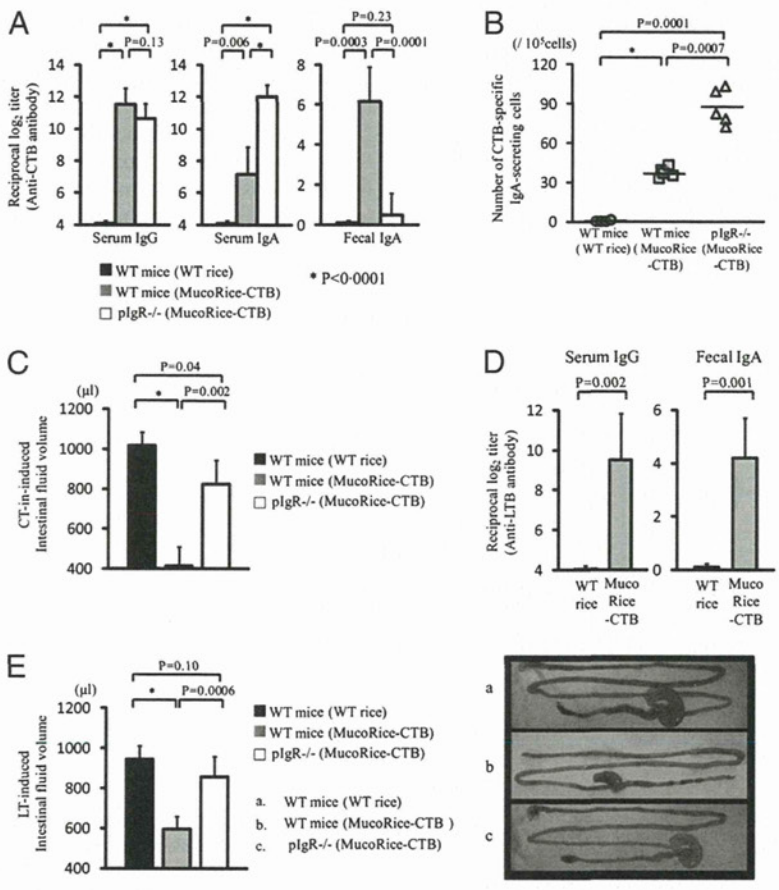


Fig. 1. Critical role of antigen-specific SIgA induced by oral MucoRice-CTB vaccine in protection against CT- or LT-induced diarrhea. Cross-protective antigen-specific antibody immune responses were examined and compared among oral MucoRice-CTB (100 mg)–immunized WT mice (gray columns), oral MucoRice-CTB–immunized pIgR-deficient mice (white columns), and WT rice–fed WT mice (black columns). (*A*) Antibody immune responses against CTB. (*B*) ELISPOT assay. Frequency of CTB-specific IgA AFCs in intestinal LP was elevated in MucoRice-CTB–immunized WT mice (gray squares), and markedly increased in MucoRice-CTB–immunized pIgR-deficient mice (white triangles), but absent in WT rice–fed mice. (*C*) Oral CT challenge (20 μg). WT rice–fed WT mice (black column) or MucoRice-CTB–immunized pIgR-deficient mice (white column) had severe fluid accumulation, whereas MucoRice-CTB–immunized WT mice (gray column) had markedly reduced fluid accumulation. (*D*) Cross-protective specific serum IgG and fecal IgA against LTB were induced in mice by oral MucoRice-CTB immunization. (*E*) Oral LT challenge: 30 μg of LT was intragastrically administered to mice. WT rice–fed WT mice (black column) or MucoRice-CTB–immunized pIgR-deficient mice (white column) had severe fluid accumulation, whereas MucoRice-CTB–immunized WT mice (gray column) had markedly reduced fluid accumulation. Data represent means ± SD. *$P < 0.0001$.

IgA and the increased numbers of CTB-specific IgA AFCs in the intestinal LP (Fig. 1*C*). Taken together, these findings directly demonstrated that CTB-specific SIgA, and not serum antibodies, was responsible for humoral protective immunity against CT-induced diarrhea.

We next clarified the essential role of CTB-specific SIgA by examining whether oral MucoRice-CTB gave protection superior to that of parenteral CTB immunization against CT-induced diarrhea. Comparison of the quantity and quality of antigen-specific protective immune responses, including diarrhea protection, between oral MucoRice-CTB and parenteral rCTB revealed that oral MucoRice-CTB induced the production of not only CTB-specific serum IgG but also CTB-specific SIgA, whereas the injectable vaccine induced only CTB-specific serum IgG production (Fig. S1*A*). The parenterally induced CTB-specific IgG response did not provide sufficient protection against CT-induced diarrhea, but oral MucoRice-CTB offered full protection because of the induction of antigen-specific SIgA responses (Fig. S1*B*). These findings suggest that MucoRice-CTB oral immunization provides protection superior to that from parenteral CTB immunization against experimental cholera because it induces significantly greater production of antigen-specific SIgA ($P = 0.008$).

MucoRice-CTB Induces Cross-Protective Immunity Against LT. Another important aspect of the antigen-specific SIgA induced by oral MucoRice-CTB was the demonstration of cross-reactivity with ETEC-associated toxin (i.e., LT; Fig. 1*D*). Cross-protective serum IgG and fecal SIgA production against B subunit of LT (LTB) was significantly greater ($P = 0.002$ and $P = 0.001$, respectively) in WT mice immunized with MucoRice-CTB than in unimmunized mice. Oral MucoRice-CTB vaccination induced SIgA-mediated protective immunity against LT-induced diarrhea in WT mice, whereas MucoRice-CTB–immunized pIgR-KO mice failed to form cross-reactive SIgA and thus developed severe diarrhea after oral challenge with LT (Fig. 1*E*). These findings demonstrated another advantage of the SIgA responses induced by MucoRice-CTB, whereby oral vaccination induced cross-reactive SIgA-mediated immunity against LT-induced diarrhea.

Long-Lasting Protection and Boosting Effects of MucoRice-CTB Vaccination Against CT-Induced Diarrhea. The duration or memory of protective immunity is another critical issue for further advancement of MucoRice-CTB as a new form of oral vaccine. After three or four primary oral immunizations with MucoRice-CTB, the extent of protective immunity was monitored over a 6-month period. High titers of CTB-specific serum IgG and IgA were maintained during the 6 months (Fig. 2*A*). Levels of CTB-specific SIgA were also high in intestinal secretions, although they gradually decreased during the 6 months: the antibody titer at 6 months was half of the level 1 week after the final immunization (Fig. 2*A*). When a single oral booster MucoRice-CTB vaccination was given, the declining antigen-specific SIgA levels bounced back to high titers within 1 week. The numbers of CTB-specific IgA AFCs in the intestinal LP were thus rapidly and markedly increased after the booster immunization to levels significantly ($P < 0.0001$) greater than in unvaccinated mice or in vaccinated mice 1 or 24 weeks after the last of the first four doses of the primary immunization (Fig. 2*B*). Even though the levels of antigen-specific SIgA had declined by 6 months, partial protection against CT challenge was maintained (Fig. 2*C*). A single oral booster dose of MucoRice-CTB resulted in the recovery of full protection against CT challenge (Fig. 2*C*) and maintained effective protective mucosal immunity for at least another 4 months (Fig. 2*A*). These findings suggested that a CTB-specific-memory type of mucosal SIgA response was induced by oral vaccination with MucoRice-CTB.

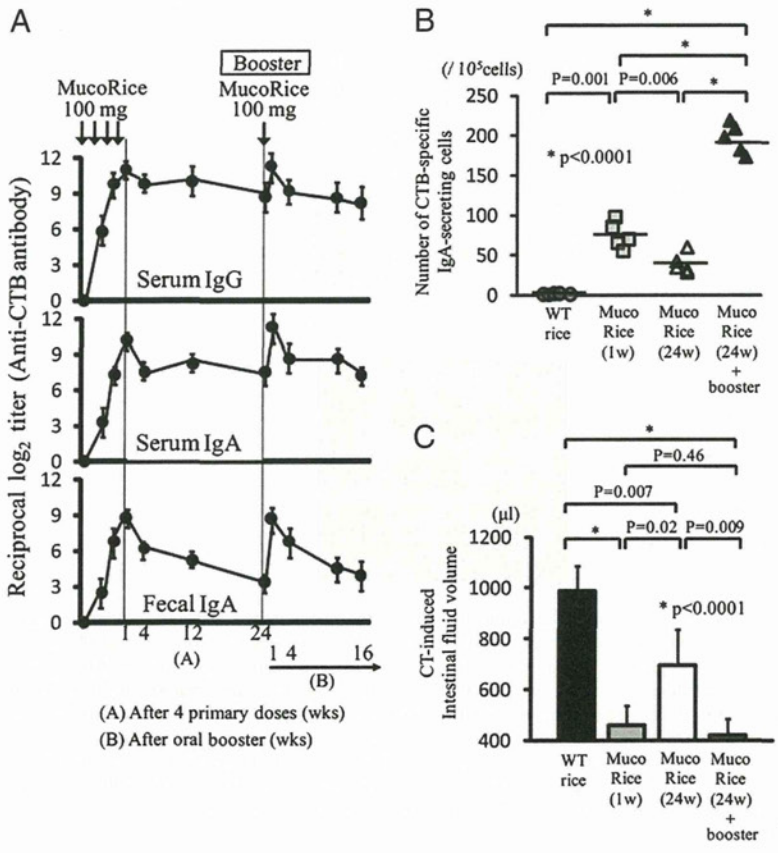


Fig. 2. Induction of long-term SIgA-mediated immunity against toxin by oral MucoRice-CTB vaccine. We examined the longevity of antigen-specific antibody immune responses and protection against CT-induced diarrhea, as well as the boosting effect of oral MucoRice-CTB. Booster (100 mg of MucoRice-CTB containing 150 μg CTB) was administered 24 weeks after the final immunization. One week later, boosted immune responses were measured and monitored for the next 16 weeks. (*A*) Antibody titers were simultaneously evaluated. (*B*) ELISPOT. Numbers of CTB-specific IgA AFCs in the intestinal LP of each mouse were evaluated over the same time period. (*C*) Oral CT challenge. Mice were immunized and challenged over the same time course as described earlier. Data represent means ± SD. *$P < 0.0001$.

MucoRice-CTB Induces Protection Against *V. cholerae*– and LT-ETEC–Induced Diarrhea. We used an intestinal loop bacterial challenge to examine whether oral MucoRice-CTB–induced antigen-specific SIgA provided protection against *V. cholerae*–induced diarrhea. When the small intestines of mice orally vaccinated with MucoRice-CTB were exposed to *V. cholerae*, almost full protection was achieved (Fig. 3). In contrast, most of the mice orally immunized with WT rice developed *V. cholerae*–induced diarrhea. Our preliminary results had shown that although the incidence of diarrhea was low (20–40%) when naive murine intestines were exposed to LT-ETEC, the incidence was sufficient for us to establish the LT-ETEC in vivo challenge model. The incidence of diarrhea was compatible with that in a previous study, which found that 34% of loops tested by using ETEC strains isolated from diarrheic infant mice showed signs of diarrhea (23). Under our experimental conditions, oral MucoRice-CTB vaccination imparted significantly ($P = 0.04$) greater resistance to LT-ETEC challenge than did oral administration of WT rice (Fig. 3). Our findings thus directly demonstrated that oral MucoRice-CTB could induce cross-protective immunity against *V. cholerae*– and LT-ETEC–induced diarrhea.

Discussion

These findings demonstrated the critical role of antigen-specific SIgA responses induced by oral MucoRice-CTB vaccine in long-term cross-protective immunity against *V. cholerae*– and LT-ETEC–induced diarrhea. Thus, these results further reinforced the attractive features of MucoRice-CTB as a new-generation oral vaccine. Our results demonstrated that oral MucoRice-CTB–induced SIgA is a critical protective element in the neutralization of CT- and LT-induced diarrhea. Our comparative study of the quality of oral MucoRice-CTB–induced intestinal SIgA levels and parenteral CTB–induced serum IgG levels showed that the former mucosal immunity plays a more critical role than the latter systemic immunity in protection against CT- and LT-induced diarrhea (Fig. S1). Although previous studies have demonstrated the important role of CT-specific SIgA in protection against CT-induced diarrhea (24, 25), our study shows that induction of CTB-specific SIgA is sufficient for protection against CT-induced diarrhea. When naive mice were orally immunized with CT, production of CTA-specific intestinal SIgA was much lower than that of CTB-specific intestinal SIgA (Fig. S2). In our separate study, we demonstrated that both CTA and CTB are necessary for CHO cells to exhibit the toxic effects of CT (Fig. S3). However, in the inhibition of the CT-induced elongation, CTB- but not CTA-specific antibody alone was sufficient (Fig. S3). These results further indicate that CTB-specific SIgA plays a critical role in protection against CT-induced diarrhea.

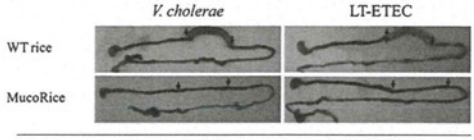
The essential role of CTB-specific SIgA was directly demonstrated by our oral vaccination of pIgR-deficient mice with MucoRice-CTB. In pIgR-deficient mice, the lack of formation in the intestinal LP of CTB-specific SIgA with cross-neutralizing activity, and thus the lack of its secretion into the lumen, resulted in loss of protection against CT- or LT-induced diarrhea (Fig. 1). The critical role of CTB-specific SIgA in the neutralization of CT was further demonstrated by in vitro assay (Fig. S4). When SIgA was purified from intestinal secretions of mice orally immunized with MucoRice-CTB and tested in the two standard in vitro neutralization assays (CHO cell elongation assay and GM1 binding assay), the purified intestinal CTB-specific SIgA effectively neutralized CT. Whereas previous studies have demonstrated the neutralizing ability of CTB-specific serum antibodies in vitro (5, 8), our study directly demonstrates that intestinal CTB-specific SIgA is responsible for humoral immunity in preventing CT- and live gut pathogen–induced diarrhea (Fig. S4).

As a practical aspect of vaccination in the clinical setting, induction of immune memory is another key factor in strategic approaches to the development of a new generation of vaccines against cholera. Our recent and separate study in nonhuman primates showed that the level of CTB-specific humoral immunity was maintained 6 months after oral primary immunization with MucoRice-CTB (7). Our study further provided evidence that oral MucoRice-CTB vaccination could offer long-term protection, because CT-neutralizing antibodies were maintained over a 6-month period in the systemic and mucosal compartments after the final oral primary immunization in mice. In long-term humoral immunity, long-lived plasma cells and memory B cells are key factors (26). Upon antigen rechallenge, memory B cells expand rapidly and differentiate into plasma cells (26). Our results indicated that immunological memory was induced by oral MucoRice-CTB vaccination; thus a single oral booster immunization at 6 months resulted in a rapid increase in levels of CTB-specific neutralizing SIgA and their additional long-term maintenance. Although we need clinical trials to investigate the effectiveness of oral MucoRice-CTB in inducing memory-type immune responses in humans, extrapolation of the mouse lifespan to that of humans suggests that the long-lasting protective immunity (i.e., 6 months) observed in mice will cause MucoRice-CTB to be of practical use in humans.

Another practical advantage of MucoRice-CTB is our original demonstration that refrigerated storage is not necessary for maintenance of immunogenicity through induction of neutralizing antibodies (5). Our uninterrupted investigation has now further demonstrated that oral immunization with MucoRice-CTB stored at room temperature for more than 3 y induces levels of serum and intestinal CTB-specific antibodies comparable to those induced by fresh harvested MucoRice-CTB (Fig. S5). This ability of cold chain–free MucoRice-CTB to induce long-term immune memory offers a global vaccination strategy by which MucoRice-CTB can be supplied to health care facilities at low cost. It can be conveniently stored without refrigeration, even in rural areas of developing countries where populations regularly suffer from *V. cholerae* infection, for primary and/or booster oral immunization against the infection.

The other important aspect of these results is that oral MucoRice-CTB vaccination induced SIgA-mediated cross-protective immunity against LT- and LT-ETEC–induced diarrhea (Figs. 1 and 3). ETEC is an important cause of acute infantile diarrhea and travelers' diarrhea (9–11), and LT-ETEC is found in approximately two thirds of cases of ETEC-associated diarrhea (12–14). Our results suggest that MucoRice-CTB could therefore be used to control a large proportion of ETEC-induced diarrhea.

Oral MucoRice-CTB induced intestinal SIgA–based protective immunity that could neutralize artificially and acutely inoculated large doses of CT or LT in the intestinal canal. In the oral CT challenge model, a bolus of toxin passes through the intestinal canal in



	V.cholerae		LT-ETEC	
	n	Number of mice with positive loop (%)	n	Number of mice with positive loop (%)
WT rice	7	6 (85)	20	7 (35)
MucoRice	7	1 (14)[a]	14	1 (7)[b]

Fig. 3. Oral MucoRice-CTB–induced antigen-specific SIgA provides cross-protective immunity against *V. cholerae*– and LT-ETEC–induced diarrhea. Murine intestinal loop assay using *V. cholerae* (10^9 cells) and LT-ETEC (10^9 cells) was executed in WT mice orally immunized with MucoRice-CTB or WT rice. Unlike WT rice, oral MucoRice-CTB vaccination markedly reduced the incidence of *V. cholerae*– and LT-ETEC–induced diarrhea. When the ratio of fluid to length was greater than 30 μL/cm, the intestinal loop was considered positive for diarrhea. The positive loop ratio is shown in the parentheses as a percentage of the total number of mice examined. (*a*) $P = 0.004$ compared with WT rice–fed mice. (*b*) $P = 0.04$ compared with WT rice–fed mice.

a short time and induces acute diarrhea (27). In the murine intestinal loop assay, inoculated and proliferated bacteria gradually release small amounts of toxin and induce fluid accumulation in the loop 12 to 18 h after inoculation (27). By using these two related but different in vivo models simultaneously, we demonstrated that MucoRice-CTB is a compelling vaccine for inducing effective SIgA-mediated immunity that can control enterotoxin-mediated clinical signs.

A previous study found that intragastric administration of monoclonal LPS-specific IgA, not but CTB-specific IgA, protects against *V. cholerae*–induced death in neonatal mice (28). This study revealed the important role of anti-LPS antibody as a vibriocidal antibody. Moreover, a new modified killed WC oral cholera vaccine was recently reported to be effective in providing 70% protection over a 2-year period (29). However, early studies have shown that the CTB-WC vaccine is initially more effective than the WC vaccine (85% vs. 58% for the initial 4–6-month period) (3, 4), indicating that the induction of anti-CTB antibody has a substantial protective effect against cholera. We showed here that physiologically and continuously secreted CTB-specific SIgA supplied from the gut mucosal immune system was important in protecting against *V. cholerae*– (and LT-ETEC–) induced diarrhea in vivo. We therefore offer an alternative to WC- or LPS-based vaccines. Furthermore, our prevention of LT-ETEC–induced diarrhea by the induction of cross-protective CTB-specific SIgA is not achieved by the WC cholera vaccine.

Transcutaneous immunization with LT supplied in patch form has recently been reported to be protective against ETEC-induced travelers' diarrhea; the increase in serum LT-specific IgA levels induced is correlated with the mucosal immune response (17). A recent study revealed that transcutaneous immunization induces the activity of Ag-specific IgA-secreting cells expressing CCR9 and CCR10 in the small intestine in a retinoic acid–dependent manner and that cross-talk between the skin and gut immune systems might be mediated by langerin(+) dendritic cells in the mesenteric lymph nodes (30). These results provide supportive evidence that our MucoRice-CTB–induced toxin-specific neutralizing SIgA contributes to the induction of protective immunity against CT-producing *V. cholerae* and LT-ETEC in humans. Oral MucoRice-CTB vaccination effectively induces CTB- and LTB-cross-reactive SIgA that most likely does not block colonization by *V. cholerae* and LT-ETEC but strongly inhibits CT- and LT-induced watery diarrhea, which is the clinical sign of greatest concern in *V. cholerae* and LT-ETEC infections.

Previous studies show that CTB can be used as an antigen delivery vehicle for the induction of oral tolerance, whereas CT can be used as an adjuvant agent and can abrogate oral tolerance (31–33). Enhancement of tolerance has been clearly demonstrated when a protein is coupled to CTB and given orally (31, 32). In contrast, CTB does not induce oral tolerance to itself (33). Because MucoRice-CTB at varying doses (18.75–150 μg) induces antigen-specific immune responses against CTB (7), we consider that the MucoRice-CTB does not induce oral tolerance to the CTB itself. MucoRice expressing CTB-based chimeric protein with a foreign antigen (MucoRice-CTB-Ag) may become an effective delivery vehicle for the induction of oral tolerance to the antigen. In fact, rice seed containing CTB-fused allergen-specific T cell epitopes induces oral tolerance to allergen more efficiently than does rice expressing allergen-specific epitopes alone (34). Moreover, conjugation of an antigen to CTB can induce the proliferation of regulatory T cells (35, 36); this may be the mechanism by which the above mentioned rice seed containing the CTB-fused epitopes effectively induces oral tolerance.

In summary, our study has further elucidated the mechanism and practical attractiveness of oral MucoRice-CTB vaccine, as well as its immunological effectiveness. This vaccine is capable of inducing long-term CTB- and LTB-cross-reactive mucosal IgA-mediated protective immunity against *V. cholerae*– and LT-ETEC–induced diarrhea. This feature will be useful in vaccine strategies against outbreaks of not only *V. cholerae* but also LT-ETEC, both in the inhabitants of developing countries and in at-risk travelers in developed countries.

Methods

Animals. Female BALB/c mice (4–7 weeks old) and pIgR KO mice on a BALB/c background were used (22). All of the mice were housed with ad libitum food and water on a standard 12 h/12-h light/dark cycle. All experiments were performed in accordance with the Guidelines for Use and Care of Experimental Animals and approved by the Animal Committee of the Institute of Medical Science of the University of Tokyo.

Vaccine. MucoRice-CTB, a rice-expressed CTB with a KDEL signal at the C-terminal of CTB, was produced as reported previously (5). Rice seeds that had been stored at room temperature for more than 3 y were ground to a fine powder in a Multi-Beads Shocker (Yasui Kikai).

Immunization. Eight-week-old female mice (six per group) were orally given 100 mg of powdered MucoRice-CTB containing 150 μg of CTB by stomach tube a total of three or four times at 2-week intervals (5). To evaluate vaccine booster effects, mice (six per group) were orally given one dose of MucoRice-CTB 6 months after the final primary immunization. In the control group, mice (six per group) were orally given 100 mg of powdered nontransgenic WT rice in distilled water.

ELISA. Serum and fecal extracts were collected 1, 4, 12, 16, and 24 weeks after final oral immunization to assess CTB- and/or LTB-specific antibody immune responses by ELISA. Coating antigens [5 μg/mL rCTB and/or recombinant LTB (rLTB)] were used, as previously described (5). rCTB was expressed in *Bacillus brevis* and purified by using immobilized galactose (Pierce) (5, 37). rLTB was expressed in *Brevibacillus choshinensis* and purified by using immobilized galactose (Pierce) as previously described, with some modification (38).

Enzyme-Linked Immunospot Assay. CTB- and LTB-specific IgA AFCs in the small intestinal LP were evaluated by using an enzyme-linked immunospot (ELISPOT) assay as previously described (39). LP mononuclear cells were isolated as previously described and processed on MultiScreen$_{HTS}$ 96-well filtration plates (Millipore) coated with 5 μg/mL rCTB or rLTB (39).

Neutralizing Assay. An in vivo oral CT or LT challenge test was used as described previously (5). After being fasted for 12 h, mice (12 per group) were orally challenged with 20 μg of CT (List Biological Laboratories) or 30 μg of LT purified from a human ETEC strain in our laboratory. Nine to 12 hours after the challenge, the mice were killed. The small intestine and colon were removed for clinical diarrhea observation and collection of intestinal contents. After centrifugation of the samples, the volume of intestinal water was measured (5).

Bacterial Challenge. An in vivo bacterial challenge, the ligated intestinal loop test, was performed based on a published method, with some modification (28). The bacterial strains used were obtained from the Research Institute for Microbial Diseases (RIMD) Bacterial Culture Collection at Osaka University. RIMD 2203363 is a typical *V. cholerae* strain (El Tor O1 Inaba) of human origin and has been confirmed to secrete CT. RIMD 0509328 is an ETEC strain of human origin confirmed to secrete LT only, without ST. This LT-ETEC strain was selected from among 27 LT-ETEC strains by a reverse-passive latex agglutination test (Denka Seiken) as producing large amounts of LT. *V. cholerae* and LT-ETEC microorganisms were grown overnight in Trypticase Soy Broth (TSB; Becton Dickinson) at 37 °C. *V. cholerae* and LT-ETEC from these cultures were further grown in TSB for 3 h at 30 °C and 4 h at 37 °C, respectively. Bacteria were washed twice in PBS solution to remove secreted toxin and diluted to a concentration of 10^{10} organisms/mL in TSB. Colony-forming units of *V. cholerae* and LT-ETEC were quantified on thiosulfate–citrate–bile salt–sucrose agar plates and on TSB agar plates, respectively.

For the challenge experiments, BALB/c female mice were starved for 36 h but had free access to water. The mice were then anesthetized and subjected to laparotomy. The small intestine was withdrawn and ligated at a distance of approximately 6 cm from the stomach. One loop of 4 to 6 cm was made in each animal. A dose of 10^9 *V. cholerae* cells or LT-ETEC cells in 0.2 mL TSB was delivered into the mouse intestinal loop by syringe. After 12 to 18 h, the challenged mice were killed and the abdomen was reopened. The loops were removed for assessment of the length of each one and the volume of accumulated fluids. The extent of fluid accumulation was expressed as a ratio of the volume (in mL) of accumulated fluid per length (in cm) of the loop. The results were considered positive when the ratio of fluid to length was more than 30 μL/cm (as determined in preliminary studies). Control experiments with 20 normal mice revealed that injection of 0.2 mL of sterile TSB alone into the loop caused no positive reaction in terms of fluid accumulation.

Data Analysis. Data are expressed as mean ± SD. All analyses for statistically significant differences were performed with the Student *t* test.

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Gomisin N enhances TRAIL-induced apoptosis via reactive oxygen species-mediated up-regulation of death receptors 4 and 5

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Abstract. Pharmacological studies have revealed that lignans isolated from *Schisandra chinensis*, including gomisin N, show anticancer, anti-hepatotoxic, anti-oxidative and anti-inflammatory activities. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an important member of the tumor necrosis factor superfamily with great potential in cancer therapy. The present study investigated whether pretreatment with gomisin N significantly enhanced TRAIL-induced cleavage of caspase-3, caspase-8 and PARP-1, which are key markers of apoptosis. Pretreatment with z-VAD-FMK, a pan-caspase inhibitor, was able to inhibit apoptosis enhanced by the combination of gomisin N and TRAIL. These results suggested that gomisin N could promote TRAIL-induced apoptosis through the caspase cascade. In search of the molecular mechanisms, we elucidated that such enhancement was achieved through transcriptional up-regulation of TRAIL receptors, death receptor 4 (DR4) and DR5. Neutralization of DR4 and DR5 could significantly reduce apoptosis induced by gomisin N and TRAIL. We also revealed that gomisin N increased the generation of reactive oxygen species (ROS). N-acetyl cysteine (NAC), an antioxidant, could inhibit ROS production and up-regulation of DR4 and DR5. Overall, our results indicated that gomisin N was able to potentiate TRAIL-induced apoptosis through ROS-mediated up-regulation of DR4 and DR5.

Introduction

The fruit of *Schisandra chinensis* has been used traditionally to alleviate suffering from chronic cough and asthma and also to promote the production of body fluid to quench thirst and arrest sweating in East Asian countries. *S. chinensis* has also been employed in the treatment and prevention of some chronic diseases, such as inflammation, hepatitis and cancer. The major bioactive constituents of *S. chinensis* are lignans, including gomisins A, B, C, E, F, G, K, N and J, schisandrol B, and schisandrin C (1,2). Pharmacological studies revealed that lignans isolated from *S. chinensis* show anticancer, anti-hepatotoxic, anti-oxidative and anti-inflammatory activities (3-5). Gomisins A and N are dibenzo[a,c]cyclooctadiene lignans with R- and S-biphenyl configurations, respectively (6-8). Gomisin A shows anti-apoptotic activity and protects the liver from hepatotoxic chemicals (9). In contrast, gomisin N induces apoptosis of human hepatic carcinoma cells (10), and we have recently reported that gomisin N enhances TNF-α-induced apoptosis via inhibition of the NF-κB and EGFR survival pathways (11).

On the other hand, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily that can initiate apoptosis via the activation of death receptor 4 (DR4) and DR5 (12,13). Since TRAIL induces apoptosis in transformed or tumor cells but not in normal cells, it is considered to be a promising cancer therapeutic agent, better than other TNF superfamily members, such as TNF and Fas ligand (14-17), which have no selectivity for normal and cancer cells. However, many types of cancer cells are resistant to TRAIL-induced apoptosis (18), therefore, it is important to overcome this resistance to expand the ability of TRAIL in cancer therapy. In this study, we focused on whether gomisin N was able to enhance TRAIL-induced apoptosis in HeLa cells and tried to explore the underlying molecular mechanisms.

Materials and methods

Antibodies and reagents. Anti-Bcl-xL, XIAP, Poly (ADP-ribose) polymerase-1 (PARP-1), caspase-8 and caspase-3 antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against Bcl-2, caspase-9, cytochrome-c and β-Actin (C-11) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Recombinant human TRAIL Apo II ligand was obtained from PeproTech, Inc. (Rocky Hill, NJ, USA). Gomisins A and N were purchased

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Table I. Sequences of RT-PCR primers.

Genes	Forward	Reverse
GAPDH	GCACCGTCAAGGTGAGAAC	ATGGTGGTGAAGACGCCAGT
DR4	ACAGCAATGGGAACATAG	GTCACTCCAGGGCGTACAAT
DR5	GCACCACGACCAGAAA	CACCGACCTTGACCAT
DcR1	GTTTGTTTGAAAGACTTCACTGTG	GCAGGCGTTTCTGTCTGTGGGAAC
DcR2	CTTCAGGAAACCAGAGCTTCCCTC	TTCTCCCGTTTGCTTATCACACGC

from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Annexin V was purchased from BioLegend, Inc. (San Diego, CA, USA). Anti-DR4 and anti-DR5 antibodies used for receptor blockage and z-VAD-FMK were obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, USA).

Cell culture and cytotoxicity assay. HeLa cells were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37˚C in 5% CO_2. Cell viability was quantified using the cell proliferation reagent WST-1 {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate} (Dojindo, Kumamoto, Japan). HeLa cells were plated in 96-well microplates at $6x10^3$ cells/wells and then incubated for 24 h. Gomisin N-containing medium was added to the wells, and cells were incubated for 30 min and then stimulated with TRAIL. After 24-h incubation, 10 μl of WST-1 solution was added and absorbance was measured at 450 nm.

Immunoblotting. Cells were treated with gomisin A, gomisin N and TRAIL, and whole-cell lysates were prepared with lysis buffer [25 mM HEPES pH 7.7, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 10% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 10 μg/ml aprotinin and 10 μg/ml leupeptin]. Cell lysates were collected from the supernatant after centrifugation at 14,000 rpm for 10 min. Cell lysates were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to an Immobilon-P-nylon membrane (Millipore). The membrane was treated with Block Ace (Dainippon Pharmaceutical Co., Ltd., Suita, Japan) and probed with primary antibodies. The antibodies were detected using horseradish peroxidase-conjugated anti-rabbit, anti-mouse and anti-goat immunoglobulin G (Dako) and visualized with an enhanced chemiluminescence system (Amersham Biosciences). Some antibody reactions were carried out in Can Get Signal solution (Toyobo).

Analyses of apoptotic cells by Annexin V-FITC. Cells pretreated with gomisin N (100 μM) for 30 min were treated with TRAIL (100 ng/ml) for 6 h. After harvesting, the cells were washed twice with 1,000 μl FACS buffer and resuspended in 500 μl FACS buffer containing 2.5 mM $CaCl_2$ and 1 μg Annexin V-FITC for 15 min in the dark on ice. The samples were analyzed with the FACSCalibur System (BD Biosciences).

Real-time RT-PCR. Total RNAs were prepared using the RNeasy Mini kit (Qiagen). First-strand cDNAs were synthesized by SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNAs were amplified quantitatively using SYBR Premix Ex Taq (Takara). The primer sequences are summarized in Table I (19). Real-time quantitative RT-PCR was performed using an ABI PRISM 7300 sequence detection system (Applied Biosystems). All data were normalized to GAPDH mRNA.

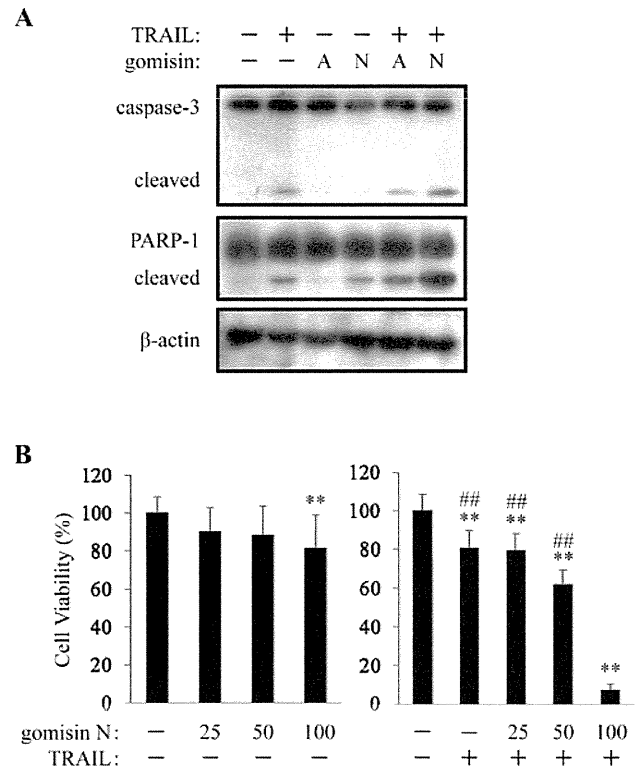

Figure 1. Effects of gomisins A and N on TRAIL-induced cell death. (A) HeLa cells were pretreated with 100 μM gomisin A or gomisin N for 30 min, followed by treatment with TRAIL (100 ng/ml) for 3 h. Cell lysates were collected and subjected to Western blot analysis to detect the cleavage of caspase-3 and PARP-1. (B) HeLa cells were treated with the indicated dosed of gomisin N for 30 min, followed by TRAIL treatment for 24 h. Cell viability was determined by WST-1 assay. Data are presented as the mean ± SD of twelve independent experiments; **P<0.01 vs. control group; $^{\#\#}$P<0.01 vs. combined treatment group with 100 μM gomisin N and TRAIL.

Measurement of intracellular ROS. Reactive oxygen species (ROS) generation was measured by flow cytometry following staining with a chloromethyl derivative of dichloro dihydrofluorescein diacetate (CMH_2DCFDA; Invitrogen). Briefly, HeLa cells pretreated with gomisin N (100 μM) for 30 min