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分担研究報告書

B 細胞亜集団の分化・活性化機構の研究

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研究要旨
B リンパ球は自然抗体産生に関与する B-1 細胞と獲得免疫応答に関与する B-2 細胞に分けられ、両者が粘膜免疫に関わっている。近年、インフルエンザワクチンの経鼻投与が注目されているが、その利点の一つがワクチンと異なる型のインフルエンザウイルスに対する交叉防御性が得られることである。しかし、その機構は不明である。B-1 細胞が産生する自然抗体はもともとすべての型のインフルエンザウイルスに中和活性を示すので、経鼻粘膜免疫に B-1 細胞の関与していることが考えられる。そこでマウス B-1 細胞を精製、培養し、B-1 細胞の活性化機構を試験管内で調べる系を樹立した。また、ヒト粘膜における B-1 細胞を同定する目的で人獣共通の B-1 細胞マーカーの検討を行い、候補としてフォスファチジルコリン含有リポソームによる細胞標識法を確立した。

A. 研究目的

粘膜面にワクチンを投与するメリットは粘膜面が多くの病原体の感染経路になっており、感染局所での免疫反応を誘導できる点である。実際に粘膜は感染防御反応の起こる場として多くの免疫担当細胞が分布している。ワクチンによりこれらの細胞を効率よく活性化するには粘膜面独特の免疫システムを理解しその活性化機構を明らかにする必要がある。

B-1 細胞は B 細胞の亜集団の一つで T 細胞非依存性の抗体産生を行なうほか、感染に関わらず存在する自然抗体の産生に関与している。マウス消化管粘膜では抗体産生細胞の約半分が B-1 細胞由来であることが知られており、B-1 細胞は粘膜免疫に深く関わっている。機能面でも自然抗体は肺炎球菌やインフエンザウイルスにもともと親和性を持つなどの点で注目される。しかし、粘膜面の B-1 細胞がどのように活性化されるか、また、ヒトでの B-1 細胞の存在や役割についてはいまだに明らかにされていない。そこで本研究では 1）B-1 細胞の活性化機構を明らかにするための培養系の確立と 2）ヒト B-1 細胞の同定方法の確立を目的として研究を行った。

B. 研究方法

1）マウス腹腔細胞より B-1 細胞以外の細胞を磁気ビーズ法により除去した。具体的には腹腔マクロファージを FITC 標識 F4/80 抗体、T 細胞を FITC 標識抗 CD3e 抗体、B-2 細胞を FITC 標識抗 CD23 抗体、赤血球を FITC 標識 TER-119 抗体でそれぞれラベルし、抗 FITC 抗体結合マイクロビーズと反応させた。この細胞懸濁液を MACS 磁気カラムにかけ、標識されていない細胞画分を回収した。得られた B-1 細胞を様々な条件で培養し、7 日後にフローサイトメーターにより細胞表面マーカーの解析、ELISA 法により培養上清中

の抗体価の測定をそれぞれ行なった。
2）6-carboxyfluorescein (6CF) を 1M Tris pH 8.0 に溶解し、pH を 7.5 に調整したものに Distearoyl phosphatidyl choline からなるリポソームを懸濁し、6CF 内包リポソームを調製した。マウス腹腔細胞を上記リポソームおよび抗 CD19, 抗 CD23, 抗 CD5 の各蛍光標識抗体と反応させ、洗浄後フローサイトメーターで解析した。

(倫理面への配慮)
マウスを用いる実験は医薬基盤研究所動物実験委員会の承認を得たプロトコールにより行ない、極力苦痛を与えないように配慮した。

C. 研究結果
1）MACS 法で精製した B-1 細胞の純度は約 85%であった。この細胞を 1% BSA 添加 SF-O3 無血清培地で 7 日間培養すると IL-5 と BAFF を添加した条件で CD138 陽性抗体産生細胞が出現し、培養上清中に IgM 抗体が検出された。一方、経鼻ワクチンアジュバントとして効果のある、poly (I:C)または chitosan を IL-5 に加えて添加したものでは CD138 陽性細胞の割合は IL-5 のみを加えたものと違いがなく、培養上清中の IgM 抗体も poly(I:C)を加えたものでわずかに上昇しただけであった。
2）腹腔細胞を 6CF 封入フォスファチジルコリンリポソームで染色すると、CD19 陽性 CD23 陰性 CD5 陽性の B-1a 細胞の一部のみがラベルされ、CD19 陽性 CD23 陽性の B-2 細胞および CD19 陽性 CD23 陰性 CD5 陰性の B-1b 細胞は全くラベルされなかった。

D. 考　　察
1）今回確立した B-1 細胞培養法は支持細胞なし、無血清で行なわれるため、添加物質の B-1 細胞に対する直接の作用を観察することができる。適切な条件下では B-1 細胞が抗体産生細胞に分化することが確かめられ、B-1 細胞分化・活性化機構の解明および B-1 細胞を標的とするアジュバントの探索に極めて有用な系であると言える。また、poly(I:C)と chitosan については B-1 細胞に対する直接の効果がほとんど見られず、経鼻免疫時にはその他の細胞を介して間接的に抗体産生を誘導していることが考えられる。
2）フォスファチジルコリンは細胞膜を構成する成分であり、マウスにおいては B-1 細胞の主要認識抗原である。従ってヒトで B-1 細胞に相当する細胞があれば同じくフォスファチジルコリンに反応することが考えられる。今回、リポソームを用いて B-1a 細胞のみをラベルできたことから同様の方法でヒトの B-1a 細胞をラベルできることが期待される。

E. 結　　論
1）B-1 細胞の活性化を試験管内で再現できる培養系を確立することができた。
2）人獣共通の B-1 細胞の標識方法として蛍光色素内包フォスファチジルコリンリポソームによる染色法を確立した。

F. 研究発表
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G. 知的財産権の出願・登録状況 (予定を含む)

1. 特許取得
   該当なし
2. 実用新案登録
   該当なし
3. その他
   なし

Ⅳ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表平成21年度（2009）

＜雑誌＞

主任研究者：清野宏

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Ⅴ．　研究成果の刊行物・別冊
（主なもの）

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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*).

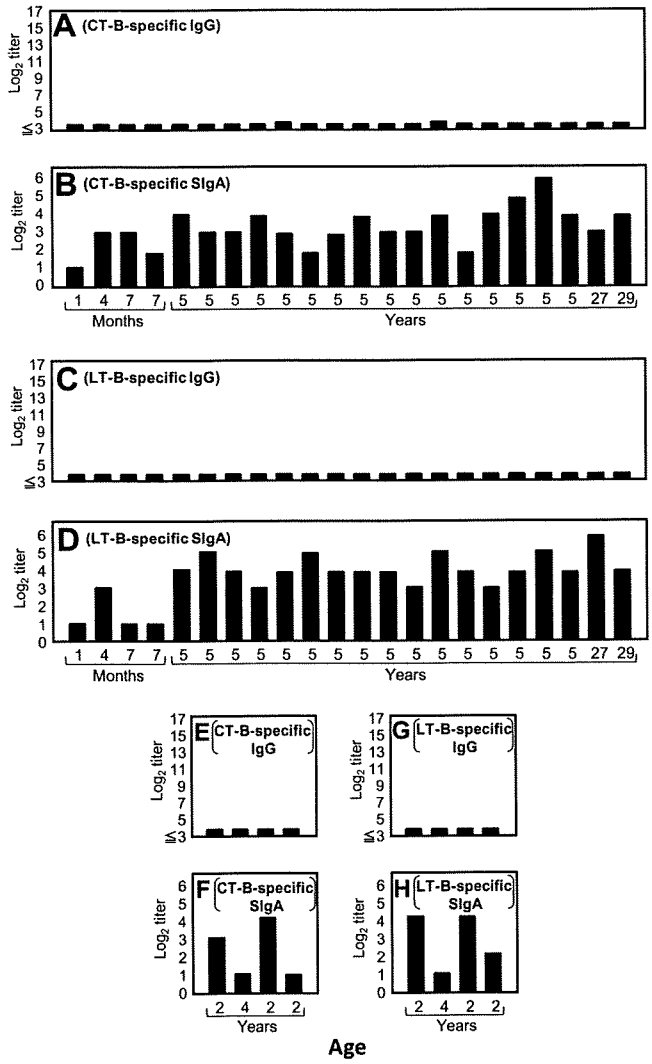

FIGURE 1. Macaques spontaneously acquire intestinal SIgA but not serum IgG Abs specific for CT-B and LT-B. CT-B–specific (*A*, *B*, *E*, *F*) and LT-B–specific (*C*, *D*, *G*, *H*) immune responses in serum (*A*, *C*, *E*, *G*) and fecal extracts (*B*, *D*, *F*, *H*) of 22 randomly selected macaques (*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

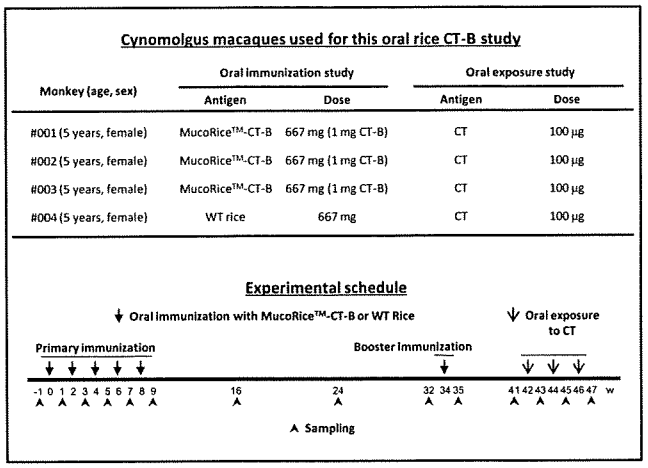

FIGURE 2. Schedule of oral immunization with MucoRice-CT-B. Macaques were orally immunized with 667 mg of MucoRice-CT-B, containing 1 mg of CT-B, or the same amount of wild-type (WT) rice on five occasions at 2-wk intervals. Six months after the fifth immunization, the macaques were given boosters of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

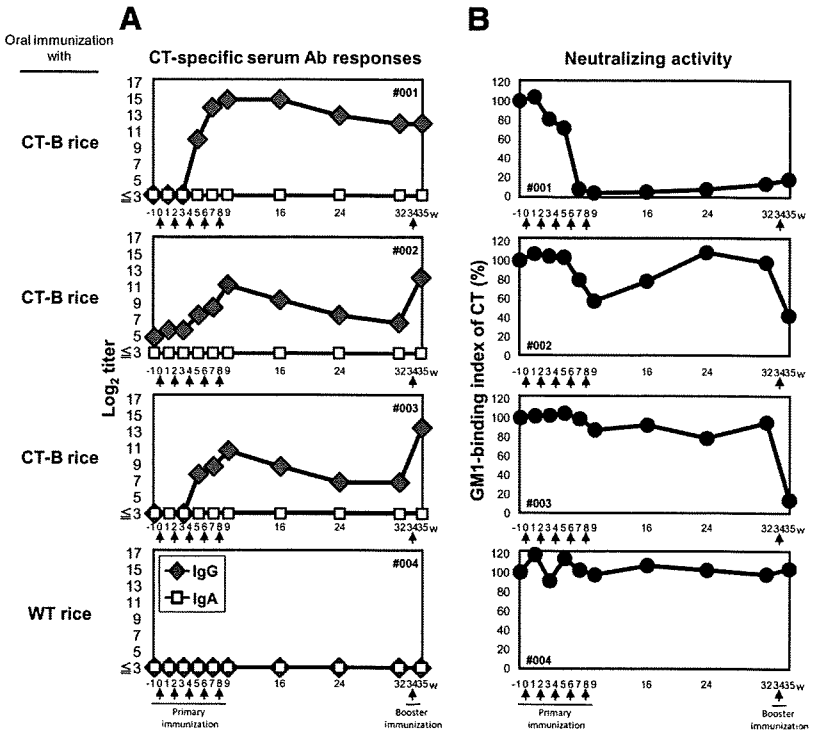

FIGURE 3. Oral vaccination with MucoRice-CT-B induces CT-specific serum IgG Abs with toxin-neutralizing activity. Oral MucoRice-CT-B but not WT rice effectively induced CT-specific serum IgG but not serum IgA Abs for at least 6 mo after the fifth immunization (*A*). Although the titer gradually decreased 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.

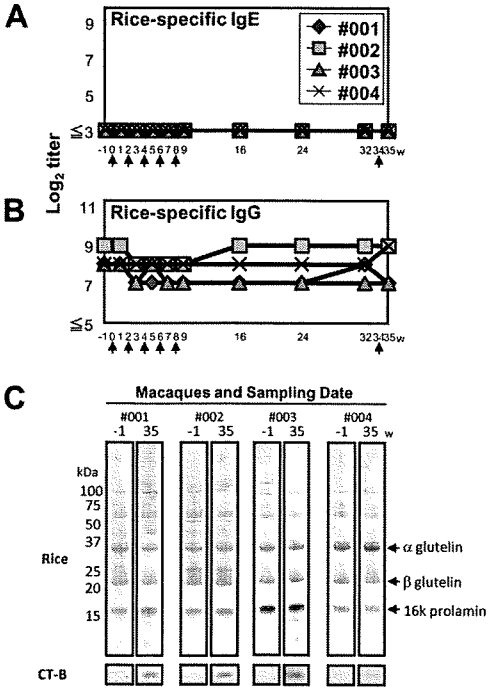

FIGURE 4. Oral immunization with MucoRice-CT-B does not induce rice storage protein-specific immune IgE Ab responses. Very low levels of serum IgE Abs specific for rice storage proteins were detected in each of the macaques orally immunized with MucoRice-CT-B or WT rice (*A*). In addition, rice storage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

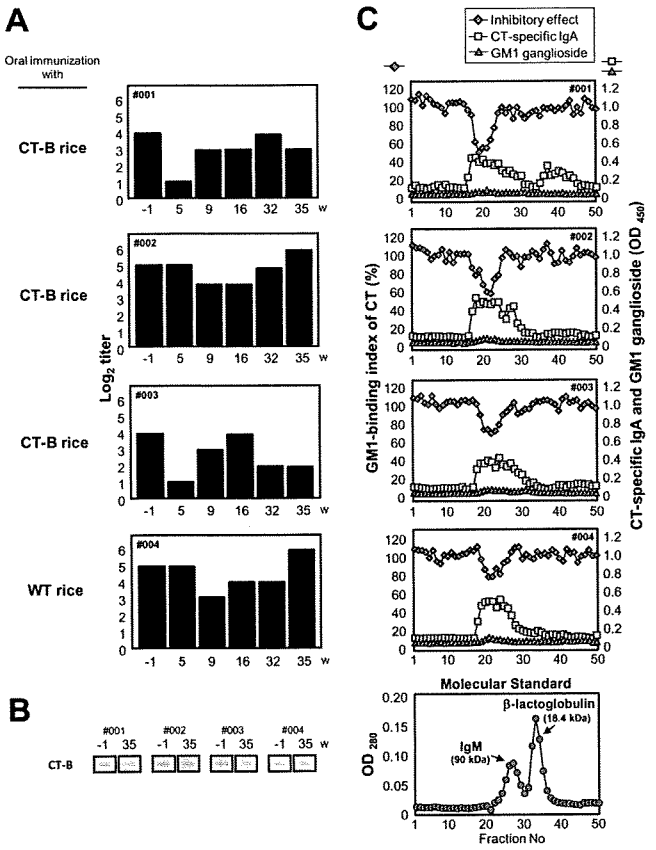

FIGURE 5. Oral immunization with MucoRice-CT-B does not increase spontaneously acquired CT-reactive intestinal SIgA Ab responses, but these SIgA Abs possess toxin-neutralizing activity. Unlike the CT-specific serum IgG Ab responses, CT-specific SIgA Ab responses were not enhanced by oral immunization 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.

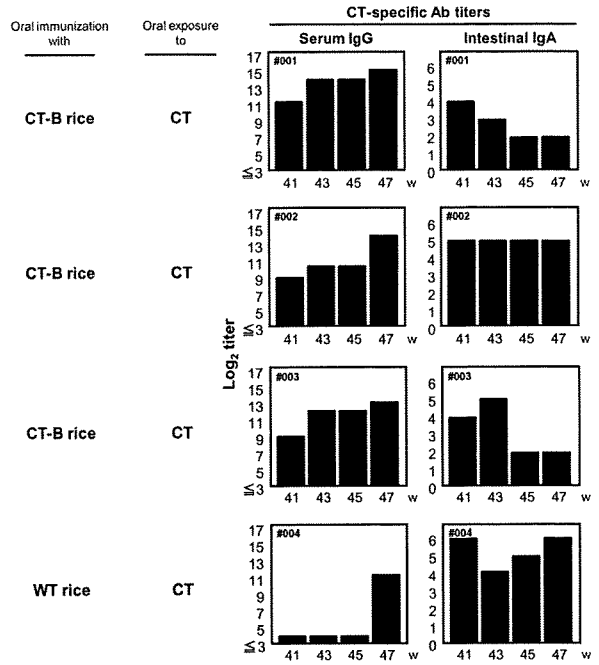

FIGURE 6. Oral administration of CT enhances serum IgG but not CT-reactive intestinal SIgA Ab responses. CT-specific serum IgG Ab responses were sharply increased in the control macaque (no. 004) and showed a further tendency to increase in three macaques previously immunized with MucoRice-CT-B (no.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

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New horizon of mucosal immunity and vaccines

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Progress in the past quarter-century on understanding the molecular, cellular, and *in vivo* components of the mucosal immune system have allowed us to develop a practical strategy for a novel mucosal vaccine. The mucosal immune system can induce secretory IgA (SIgA) and serum IgG responses to provide two layers of defense against mucosal pathogens. For SIgA-mediated immunity in the gastrointestinal tract, the gut-associated lymphoid tissue contains both the tissue-dependent and tissue-independent IgA components. Harnessing the mucosal immune system for vaccine development may help prevent the global health problems caused by enteric infectious diseases. We have therefore combined mucosal immunology and plant biology to create a rice-based mucosal vaccine that requires neither needles and syringes nor refrigeration.

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Introduction

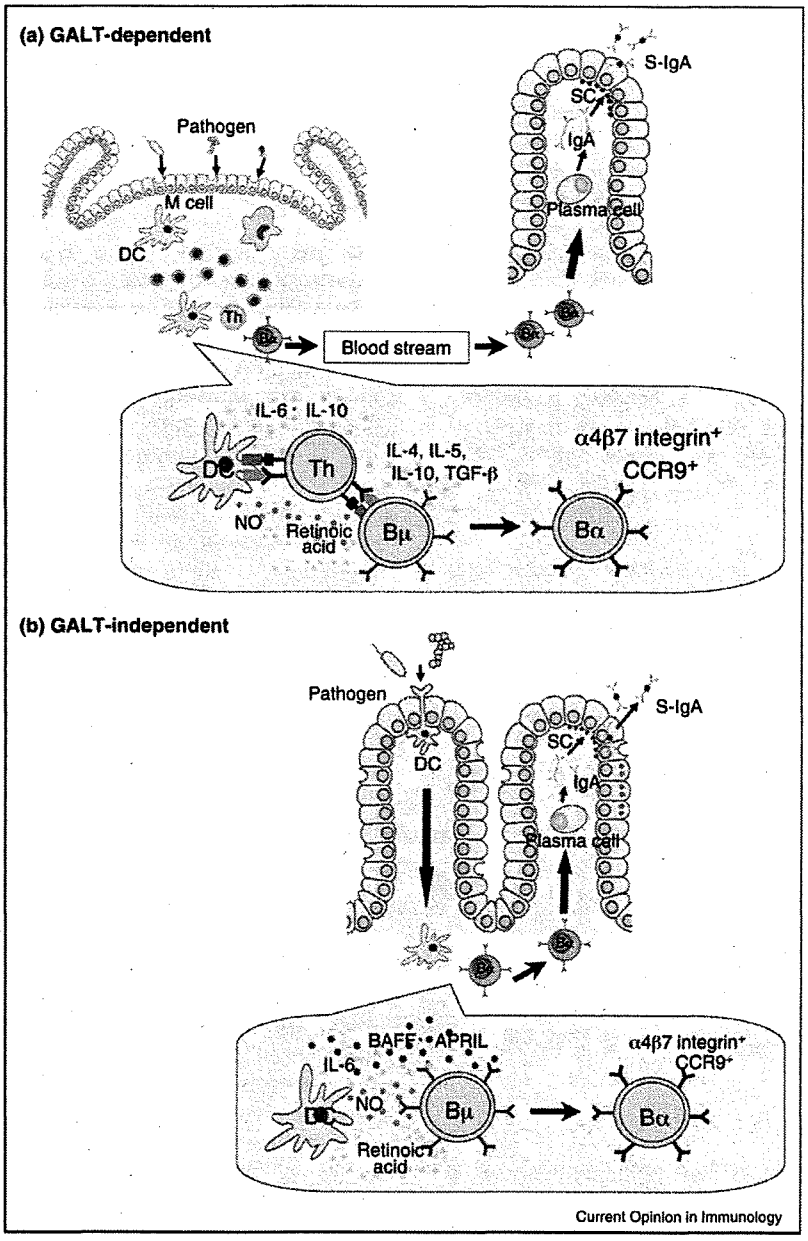
For decades, immunologists have been aiming at understanding the systemic immunity for the prevention of infectious diseases without much attention to one of the host major defensive weapons, the mucosal immune system. Thus, most widely used vaccines to date, such as those for measles, mumps, and rubella, have been aimed at stimulating the systemic immunity to produce serum antibodies against disease-causing organisms. Therefore, most vaccines have been given by injection for induction of effective systemic immunity. However, our present molecular and cellular understanding of the mucosal immune system allows us to consider the use of oral and nasal immunization for induction of antigen-specific immune responses at the mucosal surface as well as systemic compartment [1]. On the basis of the advanced knowledge of the mucosal immune system, it is now plausible to propose that the current injection-type vaccines should be advanced to needle/syringe-free and cold-chain-free forms of mucosal vaccine [2•].

The intestinal immune system for the development of oral vaccine: recent progress

The intestinal immune system consists of specialized local inductive sites, the organized gut-associated lymphoid tissue (GALT), and widely spread effector sites, the diffused intestinal epithelial and lamina propria (LP) regions, both of which are interconnected but separated from outside environment by mucus and epithelial barriers [3••,4]. The first step in the induction of an antigen-specific intestinal immune response is the uptake and transport of antigens across the epithelial barrier. The intestinal epithelium overlying GALT such as Peyer's patches (PP) is specialized to allow the transport of pathogens into the lymphoid tissue. This particular function is carried out by specialized antigen sampling epithelial cells termed M cells [5]. M cells have a folded luminal surface instead of the microvilli present on enterocytes. Unlike enterocytes, M cells do not secrete digestive enzymes or mucus and lack a thick surface glycocalyx. M cells are therefore easily accessible to microorganisms and particle antigens within the gut lumen and are the route by which antigens enter the PP from the lumen. The basolateral membrane of M cells forms pockets containing T and B lymphocytes and dendritic cells (DCs) [5]. Thus, antigens and microorganisms are transcytosed into the M cell pocket and subsequently transferred to underlying professional antigen processing and presenting cells such as DCs for the initiation of antigen-specific mucosal IgA responses. In this regard, our group showed that apical surface of M cells selectively express α(1, 2) fucose-containing carbohydrate moiety, and a monoclonal antibody recognizing the M-cell-specific carbohydrate moiety possessed the ability to efficiently deliver vaccine antigens into M cells and induce brisk antigen-specific mucosal IgA and systemic IgG responses [6•].

Following antigen processing and presentation in inductive sites (GALT), antigen-specific IgA-committed B cells proliferate locally and then migrate via the bloodstream to distant effector sites such as LP of the gut and exocrine tissues such as salivary glands (Figure 1a) [1,3••]. Recent several lines of evidence suggest that PP DCs play a pivotal role in the mucosa-tropism by the induction of gut homing molecules (e.g. α4β7 integrin and CCR9 chemokine receptor) on antigen-primed B and T lymphocytes through the production of retinoic acid from vitamin

Figure 1



The mucosal immune system. There are two distinctive pathways for the production of secretory IgA (SIgA). **(a)** In the conventional secondary lymphoid follicle such as Peyer's patch (PP)-dependent pathway, orally administered antigens are taken up by M cells in follicle-associated epithelium of PP and then processed and presented by dendritic cells (DCs) and macrophages for the generation of helper T (Th) cells and IgA-committed B cells. IL-4, IL-5, IL-10, and TGF-β produced by Th cells and nitric oxide (NO) and IL-6 produced by PP DCs allow B cells to undergo μ to α class switch recombination. Simultaneously, retinoic acid (RA) produced by DCs increases gut homing receptors (α4β7 integrin and CCR9) on antigen-primed Th cells as well as IgA-committed B cells. These antigen-primed Th cells and IgA committed B cells migrate to effector sites such as intestinal lamina propria (LP) for terminal differentiation to IgA producing plasma cells. **(b)** Another lineage of B cells, possibly originated from the peritoneal cavity, is considered as precursor for the generation of IgA, without help of Th cells. In the latter case, LP DCs directly sample antigen from the lumen and present it to the B cells, which under the influence of cytokines such as BAFF, APRIL, and TGF-β1, and NO derived from LP DCs trigger the process of isotype switching and differentiation to IgA producing plasma cells.

A [7], that is, effector memory lymphocytes that arise in response to antigens in the alimentary tract mostly express intestinal migration molecules, particularly the integrin $\alpha4\beta7$, the receptor for mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed by high endothelial venules in the gastrointestinal tracts, and CCR9, the receptor for TECK/CCL25, a chemokine expressed in the small intestine [8,9,10•]. *In vitro* activation of naïve lymphocytes with DCs from PP but not from peripheral lymph nodes (PLNs) or spleen instructs effector lymphocytes with high expression of $\alpha4\beta7$ and CCR9 and the capacity to migrate to the small intestine [8,9]. Importantly, many DCs from PP but few from PLNs or spleen expressed the prerequisite enzyme (retinal dehydrogenase) for oxidative conversion of vitamin A to retinoic acid, and inhibitors of the enzyme rendered intestinal DC incapable of inducing $\alpha4\beta7^+$ lymphocytes [11•]. These tissue-specific consequences of lymphocyte priming in PP explain why vaccination against intestinal infections requires immunization by a mucosal route, particularly oral immunization, because other routes, such as subcutaneous or intramuscular immunization, do not involve mucosal DC with the correct imprinting properties.

The production of IgA at mucosal surfaces is strictly regulated by the coordinated communication consisting of mucosal B cells, mucosal T cells, mucosal DCs, and epithelial cells. Although it has long been recognized that a major source of IgA plasma cells at mucosal surfaces comes from GALT such as PP [1,12], it was recently revealed that some IgA antibodies, particularly commensal microbiota-specific ones, occur even in the absence of GALT [13]. In this setting, DCs located in GALT (e.g. PP) as well as diffused effector tissues (e.g. LP) should be specialized in providing crucial molecular environment for generation of IgA committed B cells as well as their differentiation into IgA producing plasma cells. For example, a $CD11c^+CD11b^+CD8\alpha^-$DC subset isolated from PP, which preferentially produces IL-6 and polarizes antigen-specific T cells to produce Th2 cytokines, promotes IgA production by naïve B cells [14]. Furthermore, a novel PP DC subset for mucosal IgA responses has been identified; these PP DCs produce TNF-α and inducible nitric oxide synthase (iNOS), termed Tip-DCs, and function to induce the production of IgA-committed cells [15•]. Nitric oxide increases TGF-β receptor expression on naïve PP B cells, leading to the enhancement of class-switching recombination to IgA.

In addition to those located in GALT (e.g. PP), a unique population of mucosal DCs in the diffused effector sites (e.g. LP) has been identified and extensively characterized as an important DC subset involved in IgA responses (Figure 1b). One subset of $CD11c^{high}CD11b^{high}$ LP DCs expresses toll-like receptor 5 (TLR5) in the small intestine [16]. When stimulated by the TLR5 ligand bacterial flagellin, $TLR5^+$LP-DCs were able to induce differentiation of IgA committed B cells to IgA^+ plasma cells independent of T cells and GALT *in vivo*. Because $TLR5^+$LP-DCs can synthesize retinoic acid and IL-6 themselves, T cell-independent IgA^+ plasma cells can be generated.

Mucosal DCs located in the LP are likely to further activate B cells through B cell-activating factor of the TNF family (BAFF) and a proliferating-inducing ligand (APRIL), both of which are B-cell-stimulating factors structurally and functionally related to CD40L [17,18]. Growing evidence indicates that this BAFF–APRIL-mediated signaling pathway supports intestinal IgA production in a GALT-independent fashion. Recent data indicate that recognition of pathogen-associated molecular patterns (PAMPs) by TLRs at the intestinal epithelial barrier is essential for the production of BAFF and APRIL by LP DCs [19]. TLR signaling not only stimulates DC production of BAFF and APRIL but also elicits DC expression of iNOS, an enzyme that augments BAFF and APRIL synthesis through the generation of nitric oxide [15•]. BAFF and APRIL induce IgA class switching by activating B cells in cooperation with IgA inducing cytokines released by DCs or other mucosa-associated cells (e.g. macrophages and epithelial cells), including IL-10 and TGF-$\beta1$ [13].

The antigen-specific IgA-committed B cells generated by GALT-dependent and GALT-independent pathways finally differentiate into dimeric IgA producing plasma cells under the influence of another group of IgA inducing cytokines, IL-5 and IL-6 in diffused LP. Dimeric IgA antibodies are, transported across epithelial cells into glandular and mucosal secretions via polymeric Ig-receptor-mediated transcytosis and thus released as a secretory form of IgA (SIgA). Because SIgA antibody appears to be the most important defensive molecule in non-invasive enteric infections such as those caused by *Vibrio cholerae* and enterotoxigenic *Escherichia coli* [2•,20], oral vaccines should effectively stimulate the mucosal immune system to induce antigen-specific IgA responses in the gut.

Cholera is still a global health problem

Despite our recent progress in the molecular and cellular understanding of the mucosal immunity, infectious diseases are responsible for a third of all deaths worldwide, killing at least 15 million people a year [21]. Of these, more than five million are children under five years of age.

The most effective way to reduce morbidity and mortality from infectious diseases is to vaccinate the susceptible population [21]. The impact of vaccination on global health in both developing and developed countries cannot be overemphasized. With the exception of water sanitation, no other modality has had such a major effect on mortality reduction. Nevertheless, at least two million

children die every year from diseases preventable by available, low-cost vaccines [21].

Cholera is an acutely dehydrating, watery diarrheal disease caused by an intestinal infection after ingestion of food or water contaminated with *V. cholerae* [20,22•]. *V. cholerae* is a non-invasive, Gram-negative, motile bacterium that colonizes the epithelial lining of the gut after penetrating the mucous layer. Cholera affects the small intestine through its secreted exotoxin (cholera toxin; CT), which is composed of five receptor-binding B subunits surrounding a catalytic A subunit. CT binds to a specific receptor, monosialosyl ganglioside GM1, on the surface of all nucleated cells, including the brush border of polarized epithelial cells. Once bound, CT is endocytosed, and the A subunit then activates the stimulatory G protein for adenylate cyclase and increases intracellular cyclic AMP, resulting in secretion of chloride and bicarbonate into the small intestine. This secretion causes water to be drawn from the intravascular and extracellular spaces of the body and to be rapidly lost into the gut lumen.

Cholera no longer poses a threat in countries with minimum standards of hygiene but remains a challenge in countries where access to safe drinking water and adequate sanitation cannot be guaranteed [22•,23•]. Many developing countries still face cholera outbreaks or the threat of cholera epidemics.

Protective immune responses against cholera

Protective immunity to cholera is mediated mainly by IgA antibodies produced locally in the intestinal mucosa and secreted onto the mucosal surface [2•,24•]. These antibodies are directed against bacterial adhesins, CT, and lipopolysaccharide, inhibit bacterial colonization and multiplication, and block CT toxicity. IgA, IgG, and IgM antibodies to *V. cholerae* antigens occur in the intestinal lumen of cholera patients, but intestinal IgA antibodies to CT provide the best protective immunity [24•]. Protective antitoxic antibodies in the gut are specific to the B subunit of CT (CT-B), and prevent clinical manifestation by blocking toxin binding to epithelial GM1 ganglioside receptors [24•]. Because of its potent immunogenicity in mucosal tissues, CT has become the model mucosal immunogen and adjuvant. Oral administration of microgram amounts of CT induces SIgA and serum IgG antibodies [1,24•]. Furthermore, CT does not induce oral tolerance and can abrogate oral tolerance to unrelated proteins when administered simultaneously [1].

Currently available cholera vaccines

Currently, two types of oral cholera vaccines have been shown to be safe, immunogenic, and efficacious [22•,23•,24•]. Although these approved vaccines are mainly used by travelers, they are now under consideration for public health use in pandemic areas. Several countries have already attempted to vaccinate populations at high risk of cholera outbreaks [22•,23•,24•].

Dukoral: a killed whole-cell (WC)/rCT-B cholera vaccine

Dukoral consists of killed whole-cell *V. cholerae* O1 with purified recombinant CT-B (WC/rCT-B) [24•]. Dukoral vaccine evokes antitoxic as well as antibacterial mucosal immunity, to provide synergistic cooperative protection against cholera [25]. Clinical trials have been performed in Bangladesh and Peru and in US volunteers. Efficacy trials in Bangladesh have shown that this vaccine is safe and confers 85–90% protection for six months in all age groups (>two years) after administration of two oral doses, 10 to 14 days apart [24•,26]. However, a large volume of liquid (75–150 mL) is needed for individual administration, meaning that the vaccine cannot be given to children under two years of age. The initial protection declined rapidly after six months in young children, but was still about 60% protection of older children (>five years) and adults after one year.

Orochol: a live, attenuated WC cholera vaccine

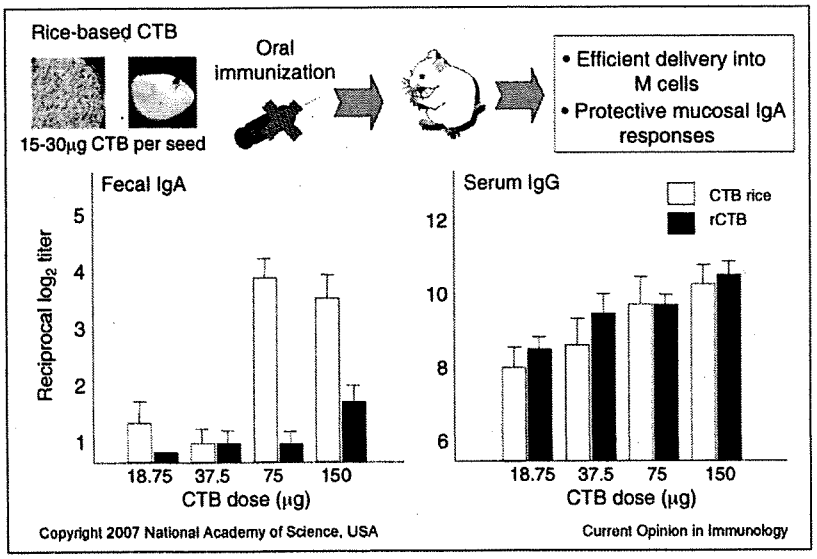
Given that the principal virulence factor of *V. cholerae* is CT itself, the development of a live vaccine deleted for a portion of CT is an obvious first approach. Orochol consists of a live, attenuated, genetically modified *V. cholerae* O1 strain (CVD 103-HgR) that has been engineered to produce CT-B but not CT-A [22•,23•,27]. Placebo-controlled trials in several countries have demonstrated the safety and immunogenicity of Orochol [27,28]. A single dose of this live vaccine conferred good protection (60–100%) in adult volunteers in the USA challenged three months after vaccination. A large field trial performed in Indonesia has not shown convincing protection in a population exposed to cholera long after immunization [22•,29].

Limitation of currently available cholera vaccines and an innovative strategy for a plant-based vaccine

The limitations of available vaccines are apparent in large-scale interventions. The buffer solution requires sterile water for Dukoral, and two doses at separate times require access to the same population twice. In addition, these vaccines require continuous cold storage. These constraints limit the usefulness in pandemic regions or in emergencies because the at-risk populations are mobile and hard to reach.

One innovative approach to improving storage and distribution, as well as to reducing the high production costs associated with antigen purification from traditional expression systems (e.g. microbial and mammalian expression), is to construct plants with transgenic grains that express vaccine antigens. Ideally, these vaccines could be administered to children under two years in a

Figure 2



Mucosal and serum anti-CT-B-specific antibody responses induced by rice-based oral vaccine administration in mice. Orally administered rice-based CT-B (or MucoRice-CTB) was effectively taken-up by PP M cells and induced brisk CT-B-specific serum IgG as well as fecal IgA response in a dose-dependent manner. It was noteworthy that the level of fecal IgA response elicited by oral administration of rice-based CT-B was much higher than that of the equivalent amount of purified recombinant CT-B (rCT-B).

single dose, without water, and would confer long-term protection [2•].

Oral feeding of mice with CT-B and the related, heat-labile enterotoxin B subunit from enterotoxigenic *E. coli* (LT-B) expressed in raw potato induced potent serum IgG as well as mucosal IgA responses against CT-B/LT-B, and protected animals from toxin challenge [30,31]. Furthermore, ingestion of uncooked potato synthesizing LT-B elicited both serum IgG and fecal IgA responses in human volunteers [32]. Antibody-secreting cells derived from GALT were observed in blood of vaccinated volunteers a week after oral vaccination [32]. LT-B antigen expressed in maize could also induce responses in human volunteers [33]. These results demonstrated the attractiveness of plant-based vaccine as a new prototype for needle/syringe free one. However, these potato and maize systems showed rapid antigen degradation in the digestive tract, and consistent dosing was difficult to achieve [34].

MucoRice CT-B: a novel unrefrigerated, needle-free vaccine

Our group recently developed a rice-based oral vaccine expressing CT-B (MucoRice CT-B) as a new possible form of oral cholera vaccine [35•]. Transgenic rice is stabile in the harsh environment of the gastrointestinal tract. Rice seeds possess two kinds of protein storage organelles, protein body I (PB-I) and PB-II. PB-I is alcohol-soluble but water-insoluble, making it resistant to the gastrointestinal environment. To examine the ability of CT-B in rice PB to withstand protease digestion in the stomach, total seed proteins were subjected to pepsin treatment *in vitro*. Prolamins in PB-I were absolutely resistant to the treatment, and ~75% of the CT-B accumulated in rice seed remained intact after pepsin treatment, whereas 90% of the glutelins accumulated in PB-II were digested by pepsin under the same conditions. M cells take up particles smaller than 10 μm [5]. Because PB-I is 1–2 μm in diameter, MucoRice CT-B is taken up by M cells and induces CT-B–specific serum IgG as well as intestinal IgA responses to protect against oral challenges with cholera toxin (Figure 2) [35•]. Furthermore, MucoRice CT-B does not stimulate serum IgG or fecal IgA responses to rice storage proteins [35•], putatively because of its much higher antigenicity. In addition, rice preserved at room temperature for 24 months shows equivalent CT-B–specific mucosal immunogenicity to that of freshly harvested rice, obviating the need for cold storage.

Achieving high levels of recombinant protein in transgenic plants is challenging, with most transgenic proteins of medical interest expressed at 0.01–0.40% of total protein [36]. Our group addressed this challenge by expressing CT-B under the control of the rice-derived promoter, and recombinant CT-B in rice reached 2.1% of the total seed protein [35•].