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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

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ERRATUM

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

Tomonori Nochi, Yoshikazu Yuki, Haruko Takahashi, Shin-ichi Sawada, Mio Mejima, Tomoko Kohda, Norihiro Harada, Il Gyu Kong, Ayuko Sato, Nobuhiro Kataoka, Daisuke Tokuhara, Shiho Kurokawa, Yuko Takahashi, Hideo Tsukada, Shunji Kozaki, Kazunari Akiyoshi and Hiroshi Kiyono

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On the first page of the PDF and printed versions of this Letter originally published, the full list of authors and their affiliations should have been included as shown below:

Tomonori Nochi[1†‡], Yoshikazu Yuki[1†], Haruko Takahashi[2], Shin-ichi Sawada[2], Mio Mejima[1], Tomoko Kohda[3], Norihiro Harada[4], Il Gyu Kong[1], Ayuko Sato[1], Nobuhiro Kataoka[1], Daisuke Tokuhara[1], Shiho Kurokawa[1], Yuko Takahashi[1], Hideo Tsukada[4], Shunji Kozaki[3], Kazunari Akiyoshi[2] and Hiroshi Kiyono[1*]

[1]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan, [2]Department of Organic Materials, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Tokyo 101-0062, Japan, [3]Laboratory of Veterinary Epidemiology, Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka 599-8531, Japan, [4]PET Center, Central Research Laboratory, Hamamatsu Photonics K.K., Shizuoka 434-8601, Japan. [†]These authors contributed equally to this work. [‡]Present address: Division of Infectious Diseases, Center for AIDS Research, University of North Carolina, Chapel Hill, North Carolina 27599, USA. *e-mail: kiyono@ims.u-tokyo.ac.jp.

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Indigenous opportunistic bacteria inhabit mammalian gut-associated lymphoid tissues and share a mucosal antibody-mediated symbiosis

Takashi Obata[a,b], Yoshiyuki Goto[a,b], Jun Kunisawa[a], Shintaro Sato[a], Mitsuo Sakamoto[b], Hiromi Setoyama[c], Takahiro Matsuki[c], Kazuhiko Nonaka[d], Naoko Shibata[a], Masashi Gohda[a], Yuki Kagiyama[a], Tomonori Nochi[a], Yoshikazu Yuki[a], Yoshiko Fukuyama[e], Akira Mukai[f], Shinichiro Shinzaki[f], Kohtaro Fujihashi[e], Chihiro Sasakawa[g], Hideki Iijima[f], Masatoshi Goto[d], Yoshinori Umesaki[c], Yoshimi Benno[b], and Hiroshi Kiyono[a,e,1]

[a]Division of Mucosal Immunology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan; [b]Microbe Division, Japan Collection of Microorganisms, RIKEN BioResource Center, Saitama 351-0198, Japan; [c]Yakult Central Institute for Microbiological Research, Tokyo 186-8650, Japan; [d]Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan; [e]Departments of Pediatric Dentistry and Microbiology, Immunobiology Vaccine Center, University of Alabama at Birmingham, Birmingham, AL 35294-0007; [f]Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan; and [g]Division of Bacterial Infection, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

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The indigenous bacteria create natural cohabitation niches together with mucosal Abs in the gastrointestinal (GI) tract. Here we report that opportunistic bacteria, largely *Alcaligenes* species, specifically inhabit host Peyer's patches (PPs) and isolated lymphoid follicles, with the associated preferential induction of antigen-specific mucosal IgA Abs in the GI tract. *Alcaligenes* were identified as the dominant bacteria on the interior of PPs from naïve, specific-pathogen-free but not from germ-free mice. Oral transfer of intratissue uncultured *Alcaligenes* into germ-free mice resulted in the presence of *Alcaligenes* inside the PPs of recipients. This result was further supported by the induction of antigen-specific Ab-producing cells in the mucosal (e.g., PPs) but not systemic compartment (e.g., spleen). The preferential presence of *Alcaligenes* inside PPs and the associated induction of intestinal secretory IgA Abs were also observed in both monkeys and humans. Localized mucosal Ab-mediated symbiotic immune responses were supported by *Alcaligenes*-stimulated CD11c$^+$ dendritic cells (DCs) producing the Ab-enhancing cytokines TGF-β, B-cell-activating factor belonging to the TNF family, and IL-6 in PPs. These CD11c$^+$ DCs did not migrate beyond the draining mesenteric lymph nodes. In the absence of antigen-specific mucosal Abs, the presence of *Alcaligenes* in PPs was greatly diminished. Thus, indigenous opportunistic bacteria uniquely inhabit PPs, leading to PP-DCs-initiated, local antigen-specific Ab production; this may involve the creation of an optimal symbiotic environment on the interior of the PPs.

Alcaligenes | intratissue habitation | Peyer's patch

The intestine is most frequently exposed to a huge number and a wide variety of environmental antigens, including bacteria and food products. As a result, indigenous bacteria create appropriate homeostatic conditions for physiologic processes such as the production of vitamin K and the metabolism of indigestible dietary carbohydrates and polysaccharides (1). In addition to nutritional mutualism, microbial stimulation is required for full maturation of the host immune system, including intestinal secretory IgA (SIgA) production (2). It was demonstrated that germ-free (GF) mice have an immature mucosal immune system, including hypoplastic Peyer's patches (PPs) and diminished numbers of IgA-producing cells and CD4$^+$ T cells (3). Both naturally occurring and acquired Abs in the intestine are of the IgA isotype. SIgA Abs recognize either T cell-independent or -dependent forms of antigens, which may limit the adherence of commensal bacteria to epithelial cells and prevent their penetration into deeper mucosal and systemic lymphoid tissues (4, 5).

Our current understanding is that commensal bacteria in the lumen and intestinal IgA together create natural cohabitation niches in the gastrointestinal (GI) tract (6). However, the nature and location of these cohabitation niches remain to be elucidated because more than 90% of the intestinal microbes have not been cultured. This limits the ability to perform detailed immunologic and bacteriologic analyses of the cohabitation mechanism between the host immune system and commensal bacteria. However, recent advances in the 16S rRNA gene clone library analysis technique have made it possible to study the composition of symbiotic bacteria in the GI tract (7, 8) and thus allow us to understand the molecular and cell biology of bilateral interactions between the mucosal immune system and the intestinal microbiota.

PPs are an example of well-characterized gut-associated lymphoid tissue and contain a wide variety of immunocompetent cells, including dendritic cells (DCs), macrophages, and B and T cells. The tissues continuously take up gut luminal antigens through M cells, including both beneficial and undesired antigens, and initiate antigen-specific immune responses in the host. The numbers of PPs range from 8 to 10 in the murine, and up to 200 in the human, small intestine (4). In a previous study of the interactions between the GI commensal bacteria and mucosal Ab production, luminal bacteria (e.g., *Enterobacter cloacae*) were shown to be taken up by CD11c$^+$ DCs in the PPs (PP-DCs); this led to the development of the intestinal IgA immune system (9).

Here, we tested the hypothesis that PPs, a major inductive and regulatory site for mucosal immunity (4) and also the entry site for luminal antigens such as indigenous bacteria (9), are one of the intratissue cohabitation niches of the intestinal microbiota necessary for the development of the mucosal immune system. This intratissue colonization may create a state of symbiosis with instructive environmental antigens on the interior of the PPs.

Results

Presence of Indigenous Opportunistic Bacteria on the Interior of PPs. To determine the bacterial composition at the surface and on the interior of PPs in naïve, specific-pathogen-free (SPF) mice, we

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[1]To whom correspondence should be addressed. E-mail: kiyono@ims.u-tokyo.ac.jp.

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IMMUNOLOGY

first used the 16S rRNA gene clone library method. Consistent with a previous report (10), segmented filamentous bacteria were the predominant species detected on the surface of the follicle-associated epithelium covering PPs (Fig. 1*A*). In contrast, several species of indigenous microbiota, including *Alcaligenes* spp., *Ochrobactrum* spp., *Serratia* spp., and *Burkholderia* spp., were detected on the interior of PPs. Of these, *Alcaligenes*, which are opportunistic bacteria (11), were dominant (72%; Fig. 1*A*).

To confirm the presence and localization of *Alcaligenes* on the interior of PPs, we next performed a whole-mount FISH analysis to identify the bacterial distribution in this tissue (12). The microbial cells were visualized by three distinct probes used in several previous studies (12–14) (Table S1). EUB338 is routinely used for detecting bacterial species in an indiscriminate manner (12). ALBO34a is a specific probe for *Alcaligenes* and *Bordetella* (13), and BPA is for *Alcaligenes*, *Burkholderia*, and *Comamonas* (14). Thus, *Alcaligenes* are identified as ALBO34a and BPA double-positive cells.

Consistent with the 16S rRNA analysis (Fig. 1*A*), EUB338-positive bacteria morphologically similar to segmented filamentous bacteria were observed over the entire surface area of PPs covered by wheat germ agglutinin positive (WGA^+) epithelial cells (Fig. 1*B*). ALBO34a and BPA double-positive *Alcaligenes* were detected on the interior of PPs, where WGA^+ epithelial cells were not observed (Fig. 1*B*). Sequential analysis through the *z* axis convincingly showed that *Alcaligenes* were present on the interior of PPs (Movie S1). We also confirmed the presence of *Alcaligenes* by the PCR method in a separate study using the 16S rRNA-gene-targeted group-specific PCR primers for *Alcaligenes*.

In contrast to the preferential localization of *Alcaligenes* in PPs, this species was essentially absent in the diffuse lamina propria (LP) region of the small intestine (Fig. 1*B*), whereas EUB338-positive bacteria were scattered throughout the surface layer of the LP (Fig. S1*A*). Thus, although some antigen-sampling cells [e.g., villous M cells (15) and epithelial DCs (16)] are located in the epithelium covering the more diffuse LP region, it seems that antigen-sampling M cells and DCs in the follicle-associated epithelium of PPs are responsible for the entry of *Alcaligenes*. Furthermore, the presence of *Alcaligenes* inside PPs was demonstrated to be a common feature by the characterization of different species of mice housed in various SPF-maintained experimental animal facilities (Fig. S1*B*). These findings suggest a possibility that commensal bacteria live within the tissues of the organized lymphoid structures associated with the GI tract.

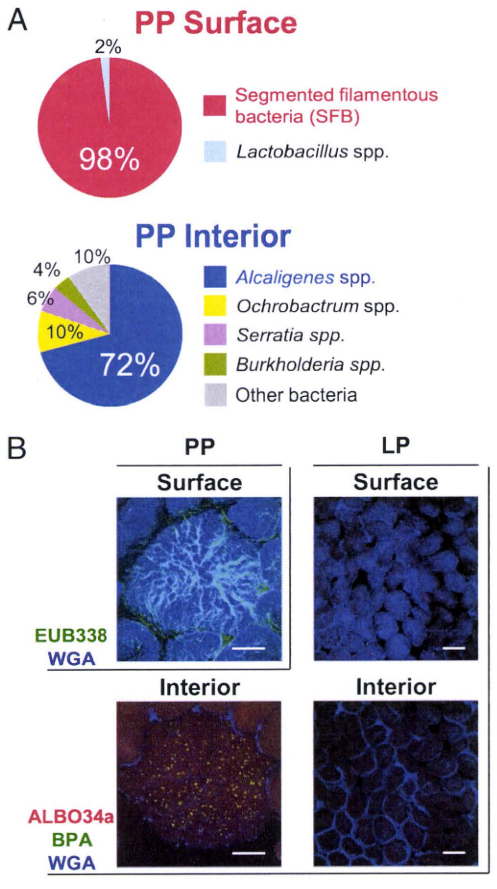

Fig. 1. Microbial distribution in the GI immune compartment. (*A*) Microbial composition at the surface and on the interior of PPs was examined by 16S rRNA gene clone library analysis. (*B*) The presence of *Alcaligenes* was visually analyzed by whole-mount FISH at the surface and on the interior of PPs and LP. Data are representative of five independent experiments. [Scale bars, 100 μm (PP), 150 μm (LP).]

***Alcaligenes*-Ingested PP-DCs Migrate into Mesenteric Lymph Nodes but not Spleen.** We next investigated the fate of *Alcaligenes* inhabiting PPs, and particularly their interactions with mucosal immunocompetent cells. When the microbial populations within DCs purified from different tissues were characterized by the 16S rRNA analysis, *Alcaligenes* were detected within PP-DCs and mesenteric lymph node (MLN) DCs (Fig. 2*A*) but not splenic DCs (Fig. S2). Our findings support the presence of a restricted PP-MLN axis for migration of DCs that have taken up indigenous microbiota and suggest that MLNs act as reinforcement to help prevent intrusions

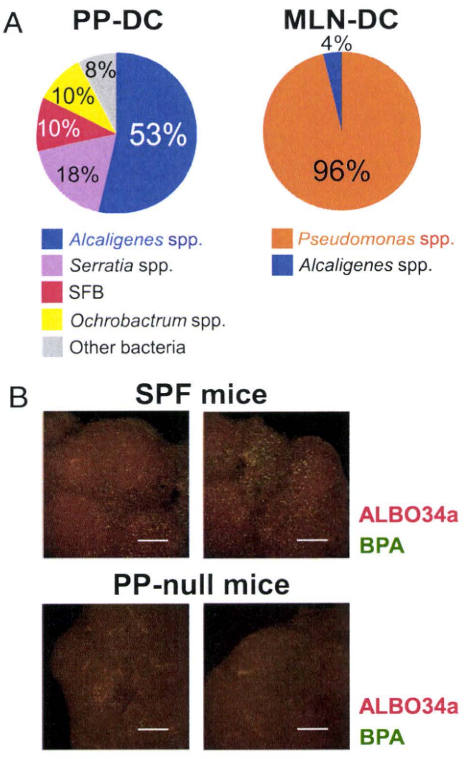

Fig. 2. PP-MLN migration axis for *Alcaligenes*-ingested GI tract DCs. (*A*) $CD11c^+$ DCs were isolated from the PPs and MLNs. Bacterial composition was determined by 16S rRNA gene clone library analysis. (*B*) Whole-mount FISH was performed to detect *Alcaligenes* (yellow) in the MLNs of PP-intact and PP-null mice. The confocal images were sequentially captured at 20-μm intervals along the *z* axis. Data are representative of five independent experiments. (Scale bars, 300 μm.)

by indigenous microbiota into the systemic compartment (17). By using FISH analysis, we also found substantial numbers of *Alcaligenes* in the MLNs of SPF mice (Fig. 2*B*).

To investigate whether PP-DCs are the main source of MLN-DCs harboring *Alcaligenes*, PP-null mice were generated by in utero treatment with an anti-IL-7 receptor α chain mAb (18). In PP-null mice, negligible numbers of *Alcaligenes* were detected in their MLNs (Fig. 2*B*); these bacteria presumably originated from isolated lymphoid follicles (ILFs) (Fig. S1*C* and Movie S2), which resemble PPs and still develop in PP-null mice (19). This result was identical to previous reports showing that PPs are the major sites for uptake of orally inoculated bacteria and the subsequent induction of host immune responses (e.g., *Salmonella typhimurium* and *Helicobacter pylori*) (20, 21).

Preferential Induction of *Alcaligenes*-Specific Mucosal Ab Responses for the Establishment of Symbiosis. To elucidate whether the intratissue presence of *Alcaligenes* and their uptake by PP-DCs affect intestinal mucosal Ab responses, we next examined IgA Ab responses to *Alcaligenes* because IgA is the major isotype of mucosal Abs (4). We used *Alcaligenes faecalis* subsp. *faecalis* NBRC (National Institute of Technology and Evaluation Biological Resource Center) 13111^T, which was the predominant species in the PPs (Fig. S3*A*), for the analysis of antigen-specific immune responses. Substantial amounts of *Alcaligenes*-specific IgA Abs were detected in the feces of SPF mice, whereas GF mice failed to produce this isotype of antigen-specific Abs (Fig. 3*A*, *Left*). No serum IgG Abs specific for *Alcaligenes* were seen in either SPF or GF mice (Fig. 3*A*, *Right*). This result reflected the localization of *Alcaligenes* in PPs, a major mucosal Ab-inductive lymphoid tissue, and not spleen, where systemic IgG Ab responses predominate (Fig. 1 and Fig. S2).

In agreement with this finding, an enzyme-linked immunospot (ELISPOT) assay showed that naïve, SPF mice possessed *Alcaligenes*-specific IgA Ab-forming cells (AFCs) in their intestinal compartments, including PPs and the LP region, but not in the spleen (Table 1). Additionally, no *Alcaligenes*-specific IgG-AFCs were seen in MLNs or spleen (Table 1). *Alcaligenes*-specific IgA-AFCs were more commonly observed in the PPs than in the LP region: more than 2% of IgA-AFCs in the PPs were reactive to *Alcaligenes*, whereas only approximately 0.5% of IgA-AFCs in the LP were specific for *Alcaligenes* (Table 1). This tissue-specific pattern of *Alcaligenes*-specific IgA-AFCs was further confirmed by FACS analysis using GFP-*Alcaligenes* (Fig. S3*B*): 5.3% of IgA-positive B cells (including 2.3% of IgA plasmablasts) were specific for *Alcaligenes* in the PPs, whereas only 1.1% of IgA-positive B cells in the LP were specific for this bacterium (Fig. S3*B*). In addition, when we examined LP-homing properties of local IgA class-switched (or IgA committed) B cells in PPs, *Alcaligenes*-specific IgA^+ B cells expressed fewer gut-homing receptors (α4β7, CCR9, and CCR10) than the rest of the PP-IgA^+ B cells (Fig. S3*C*). Therefore, *Alcaligenes*-specific IgA-committed B cells most likely remained in PPs, which accounted for the presence of elevated *Alcaligenes*-specific IgA-AFCs in PPs compared with LP.

Some intestinal IgA Abs are derived from B1 B cells and recognize T cell-independent antigens commonly expressed by commensal bacteria. Thus, it is possible that *Alcaligenes*-specific IgA Abs show some cross-reactivity with other commensal bacteria. We tested this possibility by FACS analysis and found that *Alcaligenes*-specific Abs did not cross-react with other bacteria (e.g., *Escherichia coli*; Fig. S4*A*). This view was further supported by the analysis of *Alcaligenes*-specific IgA mAb (#3E-12A-6D-3G) developed by fusion of B cells from the PPs of SPF mice. This mAb did not cross-react with *E. coli*. In addition, impaired intestinal IgA Ab responses to *Alcaligenes* were noted in $TCR\beta^{-/-}$ $\delta^{-/-}$ mice (Fig. S4*B*). These data suggest that *Alcaligenes*-specific IgA Abs are mostly derived from B2 B cells producing T cell-dependent, antigen-specific Abs. This agrees with the evidence that PPs are major sites for the induction of intestinal mucosal Ab responses to T cell-dependent microbial antigens regardless of whether the microbes are commensal or pathogenic (4).

Although PPs are thought to play a major role in the induction of IgA-committed B cells and plasmablasts, but not plasma cells (4), these data suggest that a large part of *Alcaligenes*-specific fecal IgA Abs are derived from PP IgA-producing cells in a T cell-dependent manner. In fact, markedly decreased levels of anti-*Alcaligenes* fecal IgA Abs were seen in PP-null mice (Fig. S4*C*). These findings are in agreement with previous reports demonstrating that PP-DCs are involved not only in the class-switching of IgM^+ B cells to IgA^+ ones and the determination of gut-tropism via retinoic acid synthesis (22, 23), but also in regulating IgA secretion in the PPs through the stimulation signal provided by the Ab-enhancing cytokine IL-6 (24). We examined IL-6 production by PP cells from GF mice after treatment with *Alcaligenes* and found that *Alcaligenes* induced mainly PP-DCs to produce substantial levels of IL-6 (Fig. S5*A*). When PP-DCs were isolated from WT mice and cocultured with *Alcaligenes*, the synthesis of the IgA isotype-switching cytokines TGF-β and B-cell-activating factor belonging to the TNF family (BAFF) were also elevated in addition to IgA-enhancing cytokine IL-6 (Fig. S5*B*).

Taken together, these findings suggest that mucosal Abs, including locally produced, antigen-specific IgA Abs, may play a critical role in the intratissue cohabitation of *Alcaligenes* in PPs. Supporting this view, *Alcaligenes* numbers were much lower in the PPs of CBA/N *xid* mice, which exhibit a B cell defect, than in WT mice (Fig. 3*B* and Fig. S6*A*). Further, *Alcaligenes* levels tended to be lower also in PPs of IgA-deficient mice, although no statistically significant differences were observed (Fig. S6*B*). Because the IgA-deficient condition did not lead to the complete removal of PP intratissue *Alcaligenesis*, it is also possible that *Alcaligenes*-

Table 1. Induction of *Alcaligenes*-specific and total AFCs in *Alcaligenes*-associated ex-GF mice

	SPF mice			*Alcaligenes*-associated mice		
Variable	A (Anti-*Alcaligenes*)	B (Total)	A/B × 100 (%)	A (Anti-*Alcaligenes*)	B (Total)	A/B × 100 (%)
IgA-AFCs/10^5 lymphocytes						
PP	28 ± 15	1,304 ± 364	2.10 ± 0.83	10 ± 5	625 ± 307	1.68 ± 0.46
LP	52 ± 12	9,750 ± 3,350	0.57 ± 0.19	12 ± 9	3,133 ± 1,087	0.32 ± 0.20
MLN	2 ± 1	221 ± 64	0.63 ± 0.51	0	20 ± 6	0
Spleen	0	36 ± 8	0	0	15 ± 5	0
IgG-AFCs/10^5 lymphocytes						
MLN	0	13 ± 7	0	0	10 ± 5	0
Spleen	0	15 ± 8	0	1 ± 1	40 ± 18	0.77 ± 1.72

Alcaligenes-specific and total AFCs in SPF and the *Alcaligenes*-associated ex-GF mice were enumerated by ELISPOT assay. Data are expressed as means ± SD (n = 6, respectively).

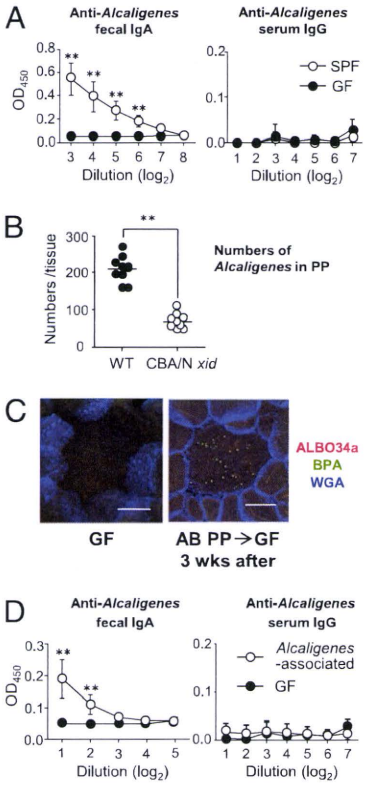

Fig. 3. Preferential induction of *Alcaligenes*-specific mucosal Ab responses in the PPs. (*A*) *Alcaligenes*-specific fecal IgA and serum IgG Ab responses were determined by ELISA. Data are means ± SD (n = 4). (*B*) The numbers of *Alcaligenes* inside PPs were counted in 10 randomly chosen PPs of CBA/N *xid* and WT mice. Data are representative of three independent experiments. Horizontal bar indicates the mean. (*C*) Bacterial distribution on the interior of PPs of GF mice. AB, antibiotic-treated mice. Data are representative of three independent experiments. (Scale bars, 100 μm.) (*D*) *Alcaligenes*-specific fecal IgA and serum IgG Ab responses in the *Alcaligenes*-associated ex-GF mice were measured by ELISA. Data are means ± SD (n = 6). $^{**}P < 0.01$.

specific IgA Abs may not be fully involved in the presence of *Alcaligenes* in PPs. Alternatively, this lack of significant differences may offer another explanation due to the compensation of IgA function by IgM Abs in deficient mice because the numbers of anti-*Alcaligenes* IgM-AFCs was much increased in IgA-deficient mice when compared with WT mice (Fig. S6*C*).

Ability of *Alcaligenes* to Colonize the Interior of PPs. Intratissue cohabitation of *Alcaligenes* in PPs should be addressed formally and directly by the establishment of a gnotobiotic mouse model monoassociated with *Alcaligenes*. The current technology, however, does not permit the isolation and culture of *Alcaligenes* from PPs. Previous studies have shown that *Alcaligenes* have the distinctive feature of being resistant to multiple antibiotics (25, 26), suggesting to us a unique strategy to directly assess the presence of intratissue *Alcaligenes* in PPs. By isolating PPs from antibiotic-treated mice under sterile conditions for the preparation of homogenized tissue and its subsequent oral administration to GF mice, we were able to establish PP-derived, *Alcaligenes*-associated mice. When we examined the antibiotic-treated mice, no bacteria were seen at the intestinal epithelial surface (including the follicle-associated epithelium), whereas *Alcaligenes* were present inside PPs (Fig. S7*A*). Three weeks after oral inoculation, *Alcaligenes* were again noted on the interior of PPs of ex-GF mice (Fig. 3*C*). The colonization of *Alcaligenes* in the PPs of ex-GF mice was further supported by the presence of antigen-specific fecal SIgA but not serum IgG Abs (Fig. 3*D*). A significant increase in antigen-specific IgA- but not IgG-AFCs was also observed in these mice (Table 1). Furthermore, the levels of total IgA were partially increased in the *Alcaligenes*-associated mice (Fig. S7*B*). When we examined PPs of GF mice, the numbers of total IgA-AFCs were 143 ± 45 per 10^5 lymphocytes. On the other hand, the numbers of total IgA-AFCs in PPs isolated from both SPF and the monoassociated mice were 1,304 ± 364 and 625 ± 307, respectively (Table 1). A similar tendency was also seen when total IgA levels were examined in fecal samples taken from monoassociated, GF, and SPF mice (Fig. S7*B*). These findings further suggest that the intratissue habitation of *Alcaligenes* in the PPs may contribute to not only the induction of *Alcaligenes*-specific IgA but also the development of at least a portion of mucosal IgA-associated humoral immunity.

***Alcaligenes* Were Present on the Interior of Monkey and Human PPs.** On the basis of the findings demonstrated by a variety of mouse experiments as described above, we next examined the presence of *Alcaligenes* inside PPs of higher mammals, namely nonhuman primates and humans. This bacterium was observed on the interior of monkey PPs by FISH analysis (Fig. 4*A*, *Left*), and anti-*Alcaligenes* IgA Abs were also detected in the feces of these monkeys (Fig. 4*A*, *Right*). To further demonstrate the intratissue habitation of *Alcaligenes* in monkey PPs, an *Alcaligenes*-specific mAb (#11E-8C-7A, IgM isotype) was developed. Immunohistochemical analysis with *Alcaligenes*-specific mAb #11E-8C-7A showed the presence of this bacterium on the interior of primate PPs (Fig. 4*C*, *Left*). When human PPs were obtained from noninflamed sites of healthy patients who underwent endoscopic biopsy, the intratissue habitation of *Alcaligenes* was demonstrated inside human PPs by FISH

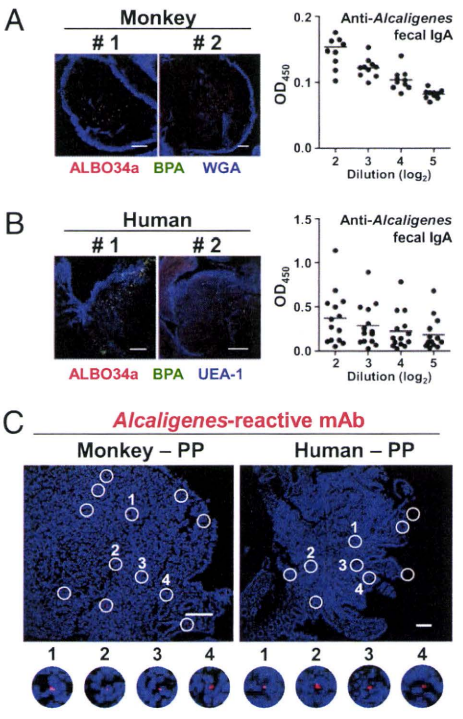

Fig. 4. Intratissue habitation of *Alcaligenes* inside nonhuman primate and human PPs. (*A* and *B*) *Alcaligenes* were detected on the interior of monkey and human PPs by whole-mount FISH (*Left*). *Alcaligenes*-specific fecal IgA Ab responses in monkeys and human were examined by ELISA [*Right*; n = 10 (*A*), n = 14 (B)]. Horizontal bar indicates the mean. (Scale bars, 100 μm.) (*C*) Immunohistochemical analysis was conducted in monkey and human PPs with *Alcaligenes*-reactive #11E-8C-7A mAb and phycoerythrin-labeled anti-mouse IgM Ab. Open circles indicate the presence of *Alcaligenes*. (Scale bars, 100 μm.)

analysis (Fig. 4*B*, *Left*). In addition, anti-*Alcaligenes* fecal IgA Abs were also detected in human fecal samples (Fig. 4*B*, *Right*), consistent with the murine and nonhuman primate studies (Fig. 3*A*, *Left* and Fig. 4*A*, *Right*), The intratissue habitation of *Alcaligenes* in human PPs was further confirmed by the use of *Alcaligenes*-specifc mAb #11E-8C-7A (Fig. 4*C*, *Right*).

Discussion

The present study has revealed a unique aspect of intestinal symbiosis between the host immune system and its indigenous microbiota. In this system some opportunistic bacteria, such as *Alcaligenes*, exploit organized murine mucosal inductive tissues (PPs and ILFs) as their tissue-interior cohabitation niches *in vivo*. The intratissue habitation of *Alcaligenes* was further demonstrated by the analysis of PPs from nonhuman primates and humans. Recently, the microbial composition of mucosa-associated lymphoid tissue (MALT) lymphomas was analyzed by the use of a 16S rRNA method and revealed that *Alcaligenes* were highly detected in those lymphoma tissues (27). This finding also suggests the likelihood that *Alcaligenes* ordinarily inhabit the human mucosal compartment and that the dysregulation of this mutualism in the organized MALT of the host GI tract may contribute to the development of the MALT lymphoma.

The origin of *Alcaligenes* involved in this intratissue colonization remains unknown. *Alcaligenes* are widely present in soil, fresh water, sewage, marine systems, human clinical materials, and the feces of healthy people (11). In this study we attempted to isolate and culture this unique bacterium from PPs of naïve SPF mice, but we unfortunately have not yet developed suitable culture conditions. However, we did confirm that *Alcaligenes faecalis* NBRC 13111^T never entered the PPs after oral inoculation. This may be because *Alcaligenes* can change their morphology, which includes rod-shaped (0.8–1 × 1–2 μm) and coccoid (0.2–1 μm) forms (11). Similarly, *H. pylori* exhibits a coccoid form in the specific environment of the small intestine, which is essential for its selective uptake by PPs and the subsequent induction of antigen-specific and pathogenic $CD4^+$ T cells that cause gastritis (21). Thus, it is possible that a specific form, presumably the coccoid form, of *Alcaligenes* is a prerequisite for its effective transfer into PPs and subsequent establishment of the intratissue cohabitation in the PPs. Supporting this prediction, we detected morphologically small, or presumably coccoid forms of *Alcaligenes* on the surface of the PP (Fig. S8).

An additional observation in the present study was that the numbers of *Alcaligenes* decreased in the absence of B cells and mucosal Abs (Fig. 3*B* and Fig. S6*A*). These results suggest that *Alcaligenes*-specific Abs may play a critical role in the PP tissue colonization by these bacteria. An interesting hypothesis would be that the coccoid form of *Alcaligenes* coated with specific mucosal Abs is selectively taken up by PPs through M cells expressing IgA receptors (28), and formation of the immune complex results in the creation of an appropriate environment for their cohabitation on the interior of PPs.

Another unresolved issue is why *Alcaligenes* exclusively inhabit the PPs. It has already been demonstrated that *Alcaligenes* produce antimicrobial substances inhibiting growth of other bacteria, including multidrug-resistant pathogenic bacteria (29–31). Kalimantacins, antibiotics derived from *Alcaligenes* spp. YL-02632S, were shown to suppress the reproduction of *Staphylococcus* spp., including *Staphylococcus aureus* (29). Further, unique antibacterial compounds produced by *Alcaligenes* spp. FC-88 (30) and M3A (31) were reported to interfere with growth of a wide variety of bacteria, such as *E. coli*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Thus, the presence of *Alcaligenes* spp. in PPs, the active antigen-sampling site, may be beneficial for the host by eliminating other opportunistic and pathogenic bacteria at their portal of entry.

Physiologically, *Alcaligenes* are known to bear a nitric oxide (NO) reductase gene and reduce NO (32), which was recently reported to up-regulate IgA class-switch recombination (33). These findings suggest that *Alcaligenes* possess unique functions to exclusively coexist in the PPs and to create an optimal environment for their cohabitation through the induction and regulation of mucosal Abs. In general, IgM^+ B cells, a major source for μ to α class switching, are a dominant B cell fraction in PPs of naïve mice (≈70%) (34). Under the appropriate molecular environment including TGF-β1, CD40L, and IL-4 (4), these B cells undergo class switching to IgA-committed B cells, and thus ≈5% of the total cells in PPs are IgA^+ B cells (34). Because NO has been shown to be an additional key regulatory molecule for TNFα/iNOS-producing DC (tip-DC) mediated IgA class switching (33), it is interesting to postulate that NO reductase produced by tissue-inhabiting *Alcaligenes* may serve as a regulatory molecule for the creation of an optimal and steady rate of IgA^+ B cell generation in the PPs.

Unexpectedly, we also detected *Pseudomonas* spp. (genetically homologous with *Pseudomonas fluorescens*) and *Stenotrophomonas* spp. (closely related to *Stenotrophomonas maltophilia*) within the systemic- (or splenic-) but not PP-DCs of naïve, SPF mice (Fig. S2). These two bacteria are considered to be nosocomial pathogens with low levels of virulence in the natural cohabitation state (35, 36). It has also been reported that they spontaneously emerge in immunocompromised cancer patients in the absence of contamination from their surrounding environment (37, 38). Therefore, our present findings may be of crucial clinical significance for a possible role of the intratissue cohabitation by commensal opportunistic bacteria in systemic lymphoid tissues. This line of investigation is now being intensively studied in our laboratory to further elucidate the significance of commensal microbiota that inhabits both systemic and mucosal lymphoid tissues.

In summary, the present study has indicated a unique aspect of mutualism of indigenous opportunistic bacteria with the host immune system in the GI tract. By cohabiting within the organized lymphoid tissues (e.g., PPs and ILFs), these bacteria affect the development and maturation of the host mucosal immune system. Further, the PP-inhabiting, commensal microbiota are an additional element that contributes to creating and maintaining immunologic homeostasis in the host. The universality for the concept of intratissue habitation of *Alcaligenes* is shared by mice and primates, and perhaps other mammals, because their presence inside PPs was demonstrated in mice, monkeys, and humans.

Materials and Methods

Animals and Human Samples. BALB/c and C57BL/6 mice were obtained from CLEA Japan. CBA/N *xid* and control DBA/2 mice were purchased from Japan SLC. $TCR\beta^{-/-}$ $\delta^{-/-}$ mice were obtained from the Jackson Laboratory. $IgA^{-/-}$ mice were originally generated by Dr. Gregory Harriman and were kindly provided by the Baylor College of Medicine. Mice were maintained under SPF conditions at the Institute of Medical Science, University of Tokyo and the Immunobiology Vaccine Center, University of Alabama at Birmingham (UAB). GF mouse experiments were performed at the Yakult Central Institute for Microbiological Research. All experiments were conducted in accordance with the guidelines for the Animal Care and Use Committees of the University of Tokyo and UAB.

Nonhuman primate PPs were obtained from cynomolgus macaques housed in the Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation (Tsukuba, Japan). All procedures were conducted in accordance with the guidelines for the Animal Care and Use Committees of the TPRC.

Human PPs were kindly provided by healthy patients without irritable bowel disease who underwent endoscopic biopsy at Osaka University Hospital. All of the subjects provided written informed consent, and the study protocol was approved by the Ethics Committee of Osaka University Graduate School of Medicine (approval no. 08243) and Institute of Medical Science, University of Tokyo (IMSUT) (approval no. 20-67-0331).

16S rRNA Analysis. The 16S rRNA gene was amplified by PCR with two universal primers (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′; 1492R: 5′-GGTTACC-

TTGTTACGACTT-3′) ligated into plasmid vector pCR2.1 and transformed into INVαF′ competent cells by using a TA Cloning Kit (Invitrogen). Plasmid DNA of randomly selected transformants was prepared by using a TempliPhi DNA Amplification Kit (GE Healthcare) and sequenced by using the primers 27F and 520R (5′-ACCGCGGCTGCTGGC-3′). All sequences were examined by BLAST search to identify the closest relatives. Representative nucleotide sequences obtained in this 16S rRNA gene clone library analysis have been deposited in the International Nucleotide Sequence Database (accession nos. AB453241–AB453250).

Whole-Mount FISH Analysis. To detect the domain *Bacteria* or *Alcaligenes*, oligonucleotide probes were purchased from Invitrogen-Molecular Probes (Table S1). Isolated tissue segments were fixed in 4% paraformaldehyde at 4 °C overnight and washed with PBS. Tissues were hybridized in hybridization buffer [0.9 M NaCl, 20 mM Tris-HCl, 45% (ALBO34a, BPA) or 0% (EUB338) formamide, 0.1% SDS, and 10 μg/mL DNA probe] at 60 °C (ALBO34a, BPA) or 42 °C (EUB338) overnight. After washing twice in washing buffer [0.45 M NaCl, 20 mM Tris-HCl, 45% (ALBO34a, BPA) or 0% (EUB338) formamide, and 0.01% SDS] at 60 °C (ALBO34a, BPA) or 42 °C (EUB338) for 10 min, tissue segments were flushed with PBS. Lectin-labeling experiments were performed Alexa Fluor 633-labeled WGA (Invitrogen-Molecular Probes) and biotinylated UEA1 (Vector Laboratories) followed by Alexa 633-conjugated streptavidin (Molecular Probes) at a concentration of 10 μg/mL for 1 h. After being washed with PBS, the tissue samples were mounted and examined by DM IRE2/TCS SP2 confocal microscopy (Leica Microsystems).

Statistical Analysis. Data were expressed as the mean ± SD or SEM and evaluated by an unpaired Student's *t* test. Significance was defined as $P < 0.01$.

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

Daisuke Tokuhara[a,1], Yoshikazu Yuki[a,1], Tomonori Nochi[a], Toshio Kodama[b], Mio Mejima[a], Shiho Kurokawa[a], Yuko Takahashi[a], Masanobu Nanno[c], Ushio Nakanishi[a], Fumio Takaiwa[d], Takeshi Honda[b], and Hiroshi Kiyono[a,2]

[a]Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan; [b]Department of Bacterial Infections, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan; [c]Yakult Central Institute for Microbiological Research, Tokyo 186-8650, Japan; and [d]Transgenic Crop Research and Development Center, National Institute of Agrobiological Sciences, Tsukuba 305-8602, Japan

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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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The authors declare no conflict of interest.

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[1]D.T. and Y.Y. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: kiyono@ims.u-tokyo.ac.jp.

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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

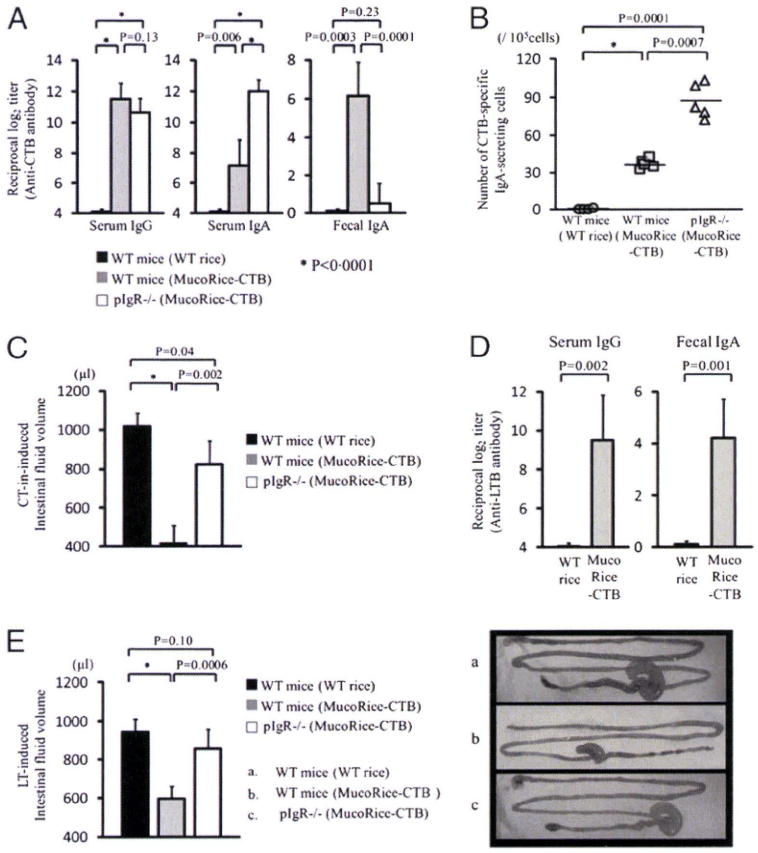


Fig. 1. Critical role of antigen-specific SIgA induced by oral MucoRice-CTB vaccine in protection against CT- or LT-induced diarrhea. Cross-protective antigen-specific antibody immune responses were examined and compared among oral MucoRice-CTB (100 mg)–immunized WT mice (gray columns), oral MucoRice-CTB–immunized 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.

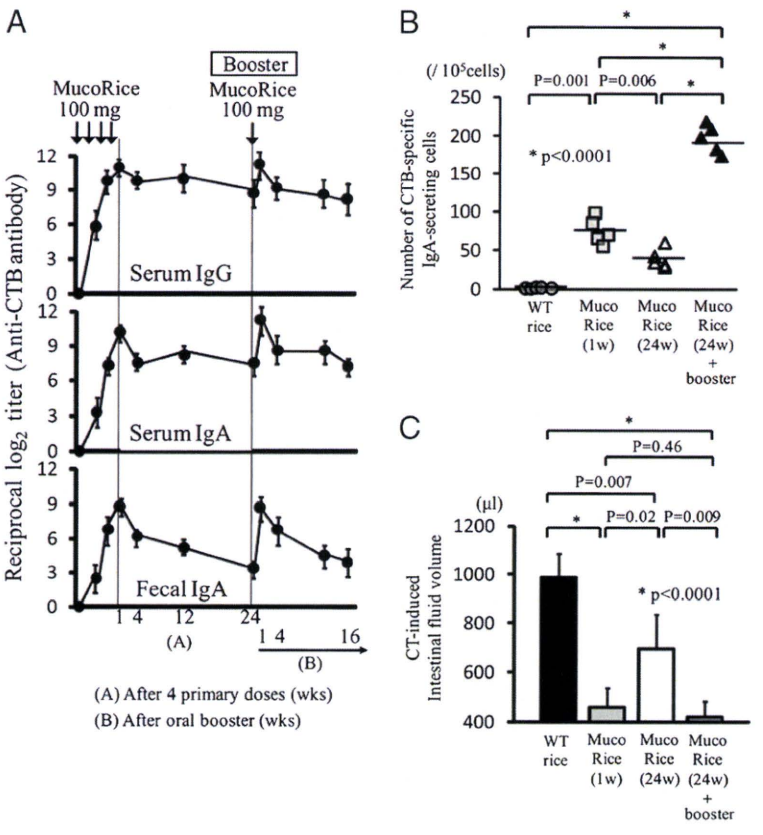


Fig. 2. Induction of long-term SIgA-mediated immunity against toxin by oral MucoRice-CTB vaccine. We examined the longevity of antigen-specific antibody immune responses and protection against CT-induced diarrhea, as well as the boosting effect of oral MucoRice-CTB. Booster (100 mg of MucoRice-CTB containing 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.

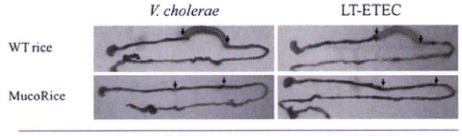
The essential role of CTB-specific SIgA was directly demonstrated by our oral vaccination of pIgR-deficient mice with MucoRice-CTB. In pIgR-deficient mice, the lack of formation in the intestinal LP of CTB-specific SIgA with cross-neutralizing activity, and thus the lack of its secretion into the lumen, 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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Intranasal Administration of Adjuvant-Combined Vaccine Protects Monkeys From Challenge With the Highly Pathogenic Influenza A H5N1 Virus

Takeshi Ichinohe,[1,2] Akira Ainai,[1,3] Yasushi Ami,[4] Noriyo Nagata,[1] Naoko Iwata,[1] Akira Kawaguchi,[1,2] Yuriko Suzaki,[4] Takato Odagiri,[3] Masato Tashiro,[3] Hidehiro Takahashi,[1] David R. Strayer,[5] William A. Carter,[5] Joe Chiba,[2] Shin-ichi Tamura,[1] Tetsutaro Sata,[1] Takeshi Kurata,[1] and Hideki Hasegawa[1,3]*

[1]*Department of Pathology, National Institute of Infectious Diseases, Musashimurayama-shi, Tokyo, Japan*
[2]*Department of Biological Science and Technology, Tokyo University of Science, Chiba, Japan*
[3]*Influenza Virus Research Center, National Institute of Infectious Diseases, Musashimurayama-shi, Tokyo, Japan*
[4]*Department of Experimental Animal Research National Institute of Infectious Diseases, Tokyo, Japan*
[5]*Hemispherx Biopharma, Inc., Philadelphia, Pennsylvania*

The effectiveness in cynomolgus macaques of intranasal administration of an influenza A H5N1 pre-pandemic vaccine combined with synthetic double-stranded RNA (polyI/polyC12U) as an adjuvant was examined. The monkeys were immunized with the adjuvant-combined vaccine on weeks 0, 3, and 5, and challenged with the homologous virus 2 weeks after the third immunization. After the second immunization, the immunization induced vaccine-specific salivary IgA and serum IgG antibodies, as detected by ELISA. The serum IgG antibodies present 2 weeks after the third immunization not only had high neutralizing activity against the homologous virus, they also neutralized significantly heterologous influenza A H5N1 viruses. The vaccinated animals were protected completely from the challenge infection with the homologous virus. These results suggest that intranasal immunization with the Double stranded RNA-combined influenza A H5N1 vaccine induce mucosal IgA and serum IgG antibodies which could protect humans from homologous influenza A H5N1 viruses which have a pandemic potential. ***J. Med. Virol. 82:1754–1761, 2010.***

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KEY WORDS: H5N1 influenza A virus; cynomolgus macaques; adjuvant; intranasal vaccine; IgA

INTRODUCTION

The highly pathogenic avian influenza A subtype H5N1 virus was first discovered to have the capacity to infect humans in Hong Kong in 1997 [Claas et al., 1998; Subbarao et al., 1998]. Influenza A H5N1 virus strains re-emerged subsequently in Southeast Asia, after which they spread across Asia to the Middle East, Europe, and Africa. The World Health Organization has recorded 473 laboratory-confirmed H5N1-infected human cases (including 282 deaths) from January 2003 to February 2010. If influenza A H5N1 viruses can spread from person to person, a pandemic could result. Therefore, the development of effective vaccines against the highly pathogenic avian influenza A H5N1 virus is an urgent public health need.

H5 vaccines, which are prepared from reference strains and delivered by intramuscular injection, will be less effective if the vaccine strains differ from the future pandemic virus strain [Horimoto et al., 2004]. This is because intramuscular injection of a vaccine only induces anti-vaccine IgG antibodies, which are highly protective against homologous virus infections, but less protective against heterologous virus infections [Hasegawa et al., 2007]. This disadvantage of intramuscular vaccination could be circumvented by employing intranasal vaccination, which induces respiratory tract IgA antibodies that are capable of providing protection

Takeshi Ichinohe is the Research Fellow of the Japan Society for the Promotion of Science.

Takeshi Ichinohe's present address is Department of Immunobiology, Yale University School of Medicine, 300 Cedar Street, TAC S640, New Haven, CT 06520.

*Correspondence to: Hideki Hasegawa, Gakuen4-7-1, Musashimurayama-shi, Tokyo 208-0011, Japan.
E-mail: hasegawa@nih.go.jp

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against not only homologous virus infection, but also heterologous virus infections [Asahi et al., 2002; Hasegawa et al., 2007]. Indeed, it has been showed previously in BALB/c mice that intranasal co-administration of an influenza A H5N1 vaccine and synthetic double-stranded RNA (polyI/polyC12U, Ampligen®) as an adjuvant induced both nasal IgA and serum IgG antibodies, and provided protection against infection with not only the homologous influenza A H5N1 virus, but also variant virus strains [Ichinohe et al., 2007a,b]. However, while these experiments using the mouse influenza model provided insights into the effectiveness of intranasal administration of a vaccine against H5 virus infection, primate model experiments would show more directly that the intranasally delivered H5 vaccine could be effective in humans as well. It has been already demonstrated that infection of cynomolgus macaques with influenza A H5N1 viruses can serve as a model for these infections in humans [Rimmelzwaan et al., 2001].

In the present study, the protective effects of intranasal administration of an influenza A H5N1 vaccine together with Amligen as an adjuvant was examined in cynomolgus macaques (*Macaca fascicularis*). The influenza A H5N1 vaccine used was NIBRG14, which is derived from the pathogenic influenza virus A/Vietnam/1194/2004 that had been isolated from a patient with H5N1 influenza. The results suggest that intranasal immunization with the Ampligen-combined influenza A H5N1 vaccine can provide monkeys with cross-protective immunity against influenza A H5N1 virus challenge.

MATERIALS AND METHODS

Influenza Viruses and Animals

The influenza A H5N1 viruses used in this study were A/Vietnam/1194/2004, A/Hong Kong/483/97, and A/Indonesia/6/2005 (A/Indonesia/6/05), all of which were isolated from patients with influenza A H5N1 disease [Gao et al., 1999]. The viruses were propagated in 10-day-old embryonated chicken eggs for 2 days at 37°C and then stored at −80°C before use.

Two male and four female cymomolgus monkeys (*Macaca fascicularis*), aged 3–4 years and weighing 2,130–4,180 g, were used in the experiments (Table I). These monkeys were born and raised in the Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases, Tukuba, Japan, and were maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of National Institute of Infectious Diseases. They were assigned to two groups as shown in Table I. The naïve group consisted of three unvaccinated monkeys (ID numbers 4668, 4669, and 4672), while the immunized group consisted of three vaccinated monkeys (ID numbers 4670, 4671, and 4673). All monkeys were then challenged intranasally with A/Vietnam/1194/04 under Biosafety level 3 containment according to the Guidelines for Animal Experiments Performed at National Institute of Infectious Diseases. All procedures used in this study complied with federal guidelines and were approved by the Institutional Animal Care and Use Committee of National Institute of Infectious Diseases.

Preparation of Vaccine and Adjuvants

Formalin-inactivated whole virus vaccine (NIBRG14) was derived from a recombinant avirulent avian virus that contains a modified hemagglutinin and neuraminidase from the highly pathogenic avian influenza virus strain A/Vietnam/1194/2004 along with other viral proteins from influenza A/PuertoRico/8/34 (A/PR8, H1N1) [Nicolson et al., 2005]. Poly I/Poly $C_{12}U$ (Ampligen®) was provided by Hemispherx Biopharma (Philadelphia, PA).

Immunization With the Vaccine and Virus Challenge

On weeks 0, 3, and 5, the monkeys were anaesthetized with ketamine (0.1 ml/kg) and immunized intranasally with 90 μg of the NIBRG14 vaccine mixed with 500 μg of Ampligen in 0.5 ml of PBS or PBS alone using a spray (0.25 ml in each nostril, DIA, Keytron, Ichikawa, Japan). Two weeks after the final immunization, all

TABLE I. Characteristic Influenza A H5N1 Virus-Associated Symptoms in Mock-Immunized Monkeys and Those Immunized With NIBRG14 and Ampligen

Group	ID	Vaccination	Sex (wt, g)	Challenge virus strain (dose, PFU)	Virus-associated symptoms	Outcome
Mock	4668	Mock	F (2,780)	A/Vietnam/1194/04	Tachypnea, diarrhea	Survival
	4669		F (2,130)	(3×10^5)	Nasal discharge, cough, tachypnea, diarrhea, intention tremor	Survival
	4672		M (3,280)		Nasal discharge	Survival
Vaccine	4670	NIBRG14 (90 μg) + Ampligen (500 μg)	F (3,080)	A/Vietnam/1194/04	None	Survival
	4671		F (2,540)	(3×10^5)	None	Survival
	4673		M (4,180)		None	Survival

ID, monkey identification number; F, female; M, male.

monkeys were infected with 3×10^5 plaque-forming units (PFU) of A/Vietnam/1194/2004, which was suspended in 3 ml of PBS. Of this virus suspension volume, 2.5 ml were applied intratracheally with a catheter (7Fr, Atom Medical, Tokyo, Japan) and 0.5 ml was applied intranasally (0.25 ml into each nostril) with a spray (Keytron, Inc., Chiba, Japan).

Serological Assays

Serum and saliva were collected 0, 1, 2, 3, 4, 5, 6, and 7 weeks after immunization and 0, 2, 5, 9, 12, and 14 days post-infection to measure the levels of Abs specific for the NIBRG14 vaccine. The IgA and IgG antibodies against the NIBRG14 vaccine were measured by an enzyme-linked immunosorbent assay (ELISA) [Ichinohe et al., 2007a]. Briefly, ELISA was conducted on the solid phase (EIA plate; Costar, Cambridge, MA) with the following series of reagents: first, NIBRG14 vaccine; second, serum or saliva; third, goat anti-monkey IgG (γ-chain specific; Alpha Diagnostic Intl., Inc., San Antonio, TX) or goat anti-monkey IgA (α-chain specific; Rockland, Inc.) conjugated with alkaline phosphatase; and fourth, *p*-nitrophenylphosphate. The chromogen produced was measured by determining the absorbance at 405 nm with an ELISA reader.

The virus neutralization activity of the antisera was determined as described previously [Kida et al., 1982]. Briefly, 2-fold serial dilutions of receptor-destroying enzyme (RDE(II); Denka Seiken Co. Ltd, Tokyo, Japan)-treated serum samples (10-fold diluted samples) were mixed with 10^2 $TCID_{50}$ of the virus and incubated at 37°C for 1 hr. The virus and serum mixtures were then inoculated onto confluent Madin-Darby Canine Kidney (MDCK) cell monolayers in 96-well plates and incubated at 37°C. After 1 hr, the inocula were removed and 100 µl of MEM was added to each well. The cells were incubated at 37°C for 4 days and the neutralization titer was determined as the reciprocal of the serum dilution that inhibited the cytopathic effect of the virus by 50%.

Virus Isolation and Virus Titer Measurement

Nasal, throat, and rectal swabs were collected at 0, 2, 5, 9, 12, and 14 dpi in 1 ml MEM containing 2% FBS and antibiotics. Frontal lobe, vertex, cerebellum, brain stem, trigeminal nerve, lung, and ileum tissue samples were collected at 14 dpi and 10% (w/v) tissue homogenates were prepared by using a bead homogenizer in MEM containing 2% FBS and antibiotics. For virus isolation, the swabs and the supernatants of 10% tissue homogenates were inoculated onto MDCK cells in 24-well plates. cytopathic effects were determined under a microscope 3 days later. The samples were considered to be negative for infectious virus when no virus-specific cytopathic effects were observed in the culture.

The virus titer of each swab sample was measured according to the method of Tobita [1975] and Tobita et al. [1975]. Briefly, 200 µl aliquots of serial 10-fold dilutions of the swabs were added to confluent MDCK cell monolayer cultures in six-well plates. After 1 hr of adsorption, each well was overlaid with 2 ml of agar medium. The plate was incubated for 48 hr in a CO_2 incubator and the number of plaques in each well was counted. The virus titers were expressed as the mean PFU/ml ± standard deviations (SD) of triplicate swab samples from each monkey.

Histopathological and Immunohistochemical Analyses

The upper jaw, including the nasal cavity, tonsils, lymph nodes, lung, heart, kidney, liver, spleen, small and large intestine, brain, and spinal cord, was fixed with 10% neutral-buffered formalin, and the upper jaw, including the nasal cavity, was decalcified in EDTA solution. After fixation, the tissues were embedded in paraffin by conventional methods and stained with hematoxylin and eosin (H&E), or subjected to immunohistochemical staining with antiserum against the nucleoprotein from the influenza A/PR8 virus. The specificity of the anti-nucleoprotein Ab and its reactivity to influenza A H5N1 influenza virus have been confirmed previously [Nishimura et al., 2000; Asahi-Ozaki et al., 2006]. Immunohistochemical staining was performed by the biotin-streptavidin-peroxidase method using 3,3′-diaminobenzidine as a substrate.

Statistical Analysis

Comparisons between experimental groups were performed by two-tailed Student's *t*-tests. Values of $P < 0.05$ were considered significant unless otherwise indicated.

RESULTS

Antibody Responses Induced by Intranasal Administration of the Ampligen-Combined Influenza A H5N1 Vaccine

Cynomolgus macaques (*Macaca fascicularis*) were immunized by nasal spraying with the adjuvant-combined vaccine (0.5 ml PBS containing 90 µg of the NIBRG14 whole particle vaccine derived from the Vietnam/1194/2004 strain and 500 µg of Ampligen) on weeks 0, 3, and 5, after which they were challenged by infection with the homologous virus 2 weeks after the third immunization. Their vaccine-specific serum IgG and saliva IgA antibody responses were assayed by ELISA and virus neutralization tests (Fig. 1 and Table II). Serum IgG-ELISA antibody responses became detectable 1 week after the second immunization, peaked 2 weeks after the third immunization, and were maintained at that high level after the challenge infection (Fig. 1A). The mock-immunized control monkeys, which had also been infected with the homologous virus, produced only low IgG antibody responses, which became detectable 1 week after infection (Fig. 1A). In addition, when assayed 2 weeks after the third immunization, the immunized monkeys also evinced serum neutralization antibody activity in neutralization tests

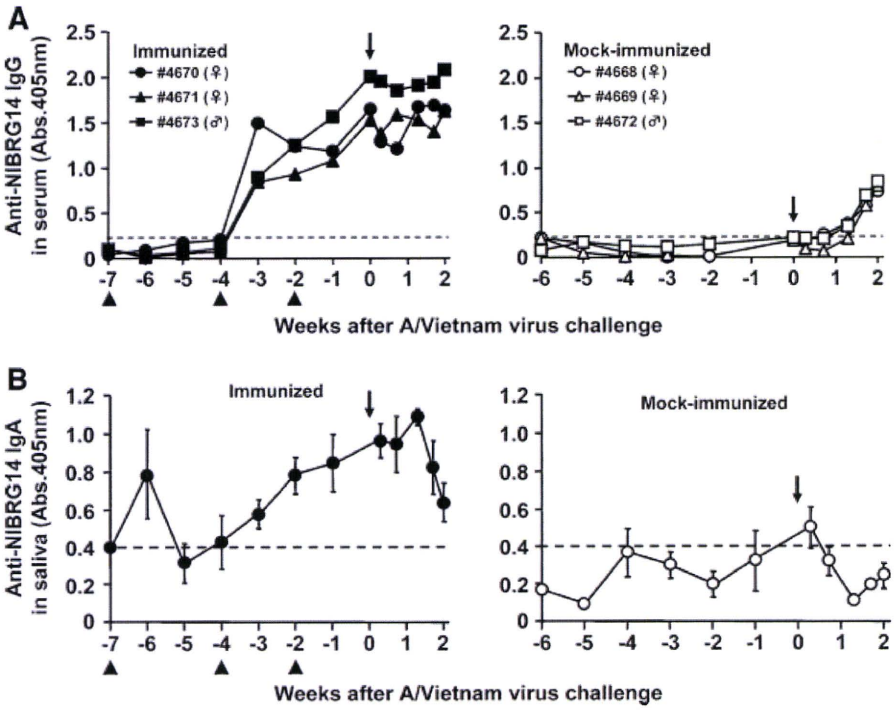

Fig. 1. Changes in the anti-NIBRG14-specific serum IgG (**A**) and saliva IgA (**B**) titers of vaccinated and mock-immunized monkeys after intranasal immunization with the Ampligen-combined NIBRG14 vaccine. The vaccinated (closed symbols) and mock-immunized (open symbols) monkeys were challenged with A/Vietnam/1194/04 virus 2 weeks after the third immunization. Arrowheads below the time scale of each graph indicate the immunization times, while the black arrow in each graph indicates the challenge infection time. The broken line in each graph indicates the background IgG or IgA antibody levels. Each point in the figures in (B) represents the mean ± SE of the combined salivary IgA antibody titers of three monkeys.

using not only the homologous virus A/Vietnam/1194/04, but also heterologous viruses, namely, A/Indonesia/6/05 and A/Hong Kong/483/97 (Table II). The neutralization activity against the homologous and heterologous viruses was high and low, respectively. In contrast, the neutralization activity of the mock-immunized naïve monkeys was below the limit of detection (<10).

Salivary IgA-ELISA antibody responses became detectable 1 week after the second immunization, continued to increase up to the time of challenge, peaked about 1 week after the challenge infection, and decreased thereafter (Fig. 1A). The mock-immunized control monkeys did not produce any detectable saliva IgA-ELISA Ab responses (Fig. 1A). The neutralization activities of the salivary antibodies from the immunized monkeys were below the limit of detection (data not shown).

TABLE II. Serum Neutralizing Antibody Reciprocal Titers Specific for Influenza A H5N1 Viruses

Influenza A H5N1 virus strains	Group	
	Mock-immunized	Vaccine
A/Vietnam/1194/04	<10	40
A/Hong Kong/483/97	<10	10
A/Indonesia/6/05	<10	10

Protection From Challenge With the A/Vietnam/1194/2004 Virus

Next, the ability of intranasal immunization with the Ampligen-combined influenza A H5N1 vaccine to protect the monkeys from challenge with the homologous virus was examined. A monkey was considered protected if the virus could not be isolated from the 0, 2, 5, 9, 12, and 14 dpi nasal, throat or rectal swabs, as determined by testing for cytopathic effects or measuring the virus titer. Table III shows the results of the cytopathic effect assays. The A/Vietnam/1194/2004 virus was not isolated from any of the swabs from the vaccinated monkeys, but was isolated 2 days dpi from the throat swabs of two mock-vaccinated monkeys and from the nose, throat, and rectal swabs of the remaining mock-vaccinated monkey. The virus could also be isolated from the 5 dpi nasal swab of the latter monkey. The results of viral titration were consistent with the cytopathic assay data since they showed that the A/Vietnam/1194/2004 virus could not be isolated from the nasal and throat swabs of any of the vaccinated monkeys, but could be isolated from the 2 and 5 dpi nasal swabs of one mock-vaccinated monkey (Fig. 2A) and from the 2 dpi throat swabs of the other two mock-vaccinated monkeys (Fig. 2B). However, the virus could not be detected in any of the 14 dpi frontal lobe, vertex, cerebellum, brain stem, trigeminal nerve, lung, and

TABLE III. Detection of Influenza Virus in Swabs Obtained From Monkeys Infected With Influenza Virus A/Vietnam/1194/04

Group	Monkey no.	0 dpi			2 dpi			5 dpi			9 dpi			12 dpi			14 dpi		
		N	T	R	N	T	R	N	T	R	N	T	R	N	T	R	N	T	R
Mock	4668	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
	4669	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
	4672	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
Vaccine	4670	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	4671	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	4673	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

N, nasal swab; T, throat swab; R, rectal swab.

ileum tissue samples from vaccinated and mock-vaccinated monkeys (data not shown). Thus, intranasal immunization with the Ampligen-combined influenza A H5N1 vaccine protects monkeys from homologous H5 virus infection.

Pathological Signs of A/Vietnam/1194/2004 Virus Infection

The pathological changes in the lungs of the mock-vaccinated monkeys 14 days after H5 virus infection were compared to those in the lungs of the immunized monkeys. All mock-immunized monkeys developed pneumonia characterized by the destruction of alveoli, lymphocyte infiltration, and proliferation of type II alveolar cells (Fig. 3). In contrast, none of the vaccinated monkeys exhibited active pneumonia, with only scars from the initial infection being observed. Viral antigen was not detected by immunohistochemical staining in the lungs of either the mock-vaccinated or vaccinated monkeys 14 days after infection (data not shown).

To assess clinical signs of infection, the food consumption patterns of the vaccinated and mock-vaccinated monkeys after A/Vietnam/1194/04 virus infection were examined. The mock-immunized monkeys consumed significantly less food on days 1 and 4 after infection ($P < 0.05$) than the vaccinated monkeys (data not shown). Furthermore, the mock-immunized monkeys exhibited significant leukopenia until 5 days post-infection, but this effect was not notable in any of the vaccinated monkeys ($P < 0.001$, data not shown). In addition, the mock-immunized monkeys developed symptoms of tachypnea, diarrhea, nasal discharge, cough, and intention tremor, unlike the vaccinated animals (Table I). However, the body weights and body temperature of all monkeys did not change significantly after A/Vietnam/1194/04 virus infection (data not shown).

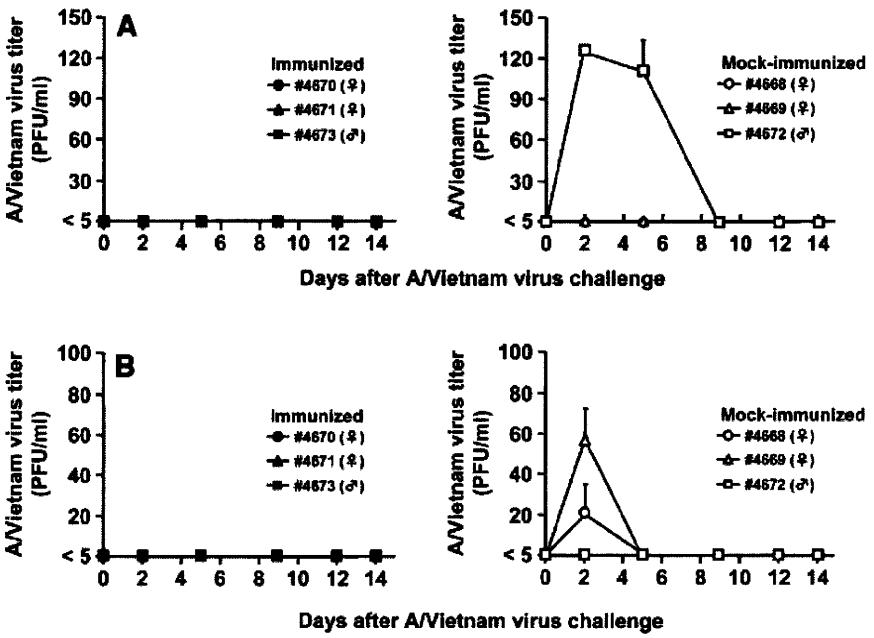

Fig. 2. Changes in the A/Vietnam/1194/04 virus titers in nasal (A) or throat swabs (B) after challenge with the A/Vietnam/1194/04 virus in mock-vaccinated monkeys (open symbols) and in monkeys immunized intranasally with Ampligen-combined NIBRG14 vaccine (filled symbols). Each point represents the mean virus titer (PFU/ml) ± SD of triplicate swab samples from each monkey.

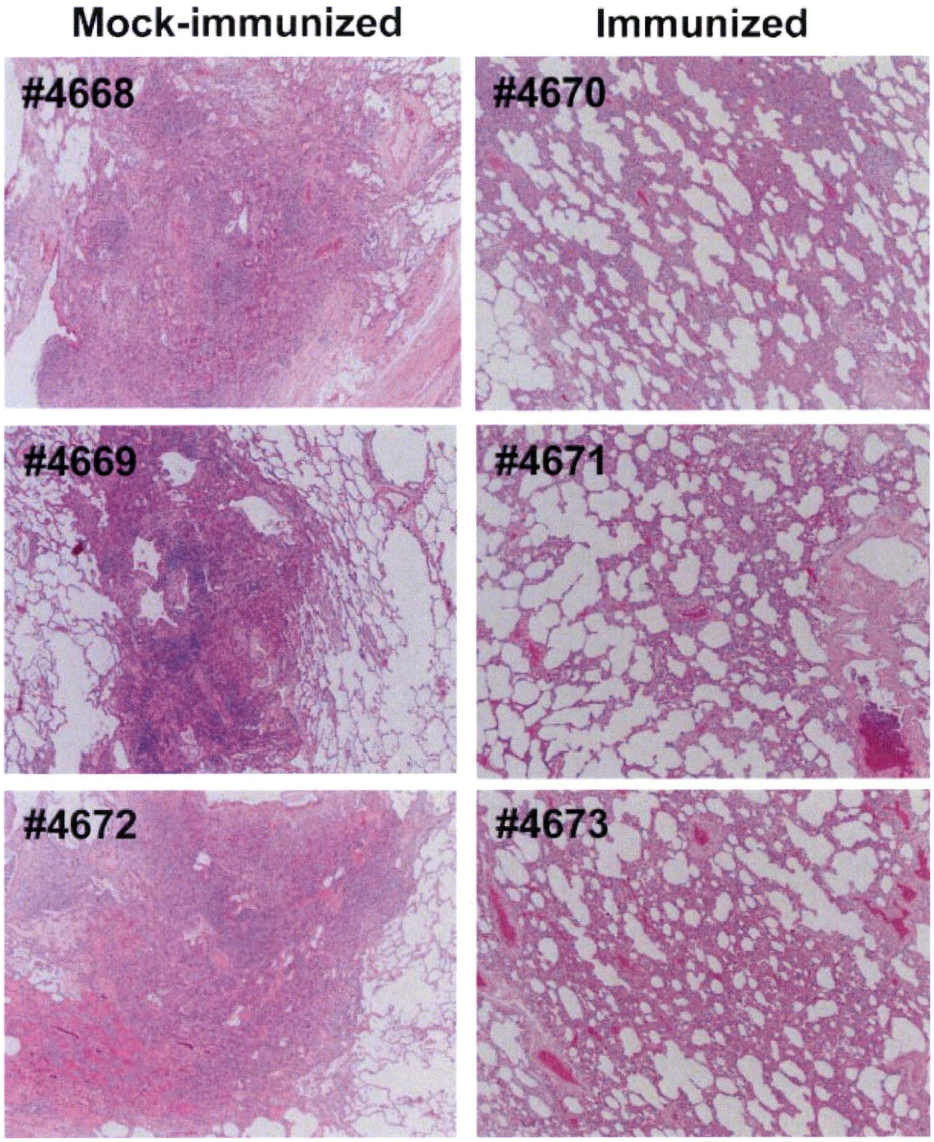

Fig. 3. Histopathological changes in the lungs from monkeys immunized intranasally with the Ampligen-combined NIBRG14 vaccine (#4670, #4671, and #4673) or from mock-immunized control monkeys (#4668, #4669, and #4672) 14 days after challenge with A/Vietnam/1194/04 virus (40×, H&E).

DISCUSSION

In this study, the effectiveness of the influenza A H5N1 vaccine (NIBRG14) in cynomolgus macaques was examined when it was delivered intranasally together with Ampligen. Intranasal vaccinations are known to induce mucosal immune responses by respiratory tract mucosa (which is the initial site of virus infection) and thus could be the most effective immunization strategy to deliver protection from influenza virus infection [Tamura et al., 2005]. However, such a vaccine is more likely to induce effective mucosal antibody responses if it is combined with a potent mucosal adjuvant. While cholera toxin and *Escherichia coli* heat-labile toxin are potent adjuvants that can enhance mucosal immune responses [Tamura et al., 2005], they have several undesirable side-effects in humans, including VIIth cranial nerve dysfunction [Mutsch et al., 2004]. Therefore, for intranasal influenza vaccines in humans, other adjuvants that are both clinically safe and effective should be developed. In this study, Ampligen®, which is a synthetic double-stranded RNA polyI/polyC_{12}U that has both a good safety profile as shown by clinical trials [Unknown, 2004] and good mucosal adjuvant activity in mice when co-administered intranasally with NIBRG14 [Ichinohe et al., 2007a,b] was used.

When administered to the monkeys, the adjuvant-combined vaccine elicited salivary IgA and serum IgG