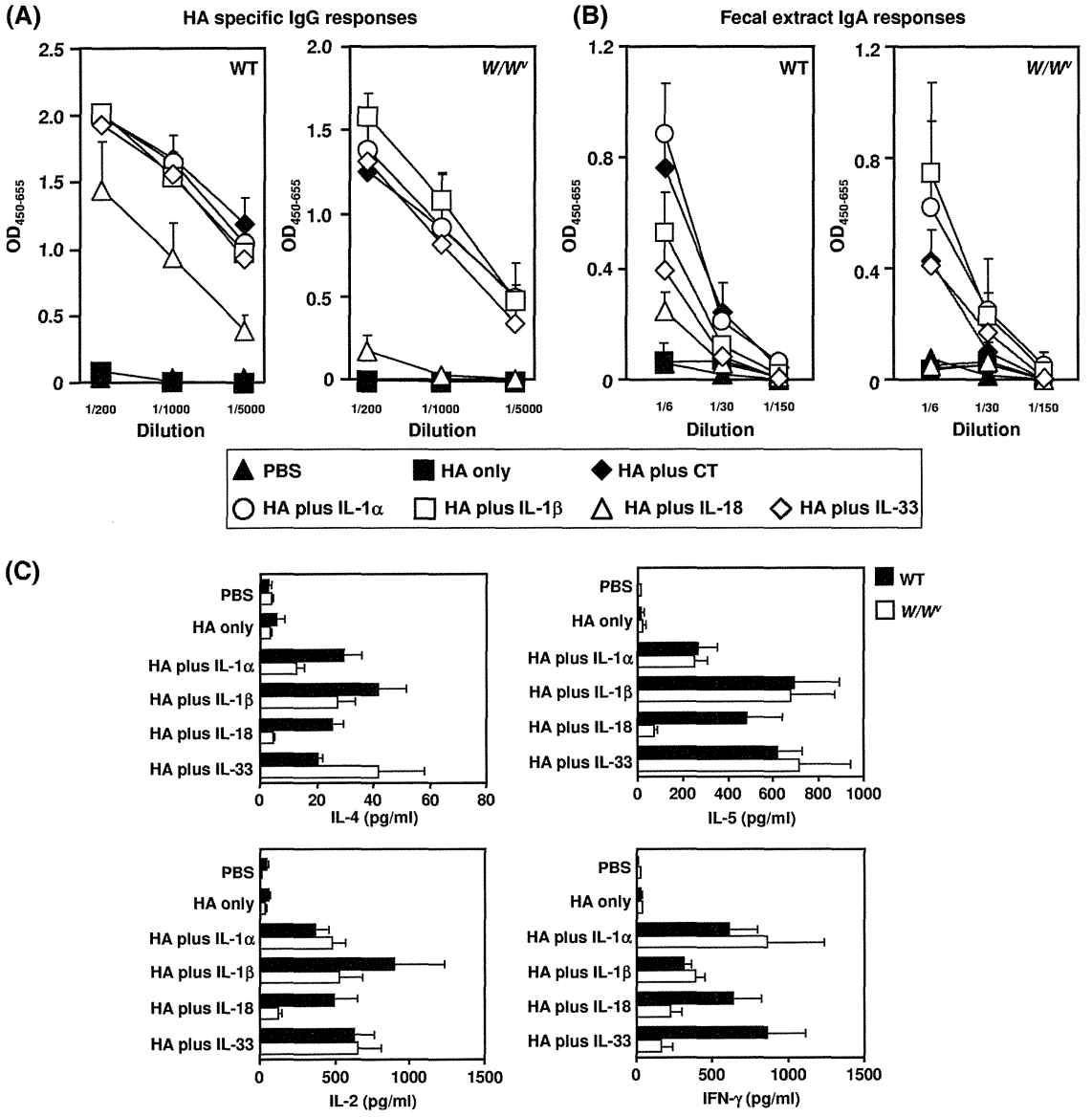

FIG. 7. Role of MCs in induction of rHA-specific immune responses by nasal immunization with rHA plus IL-1 family cytokines. WBB6F1 *W/Wv* and WT mice were immunized intranasally at 0 and 28 days with rHA alone, rHA plus CT (1 μg/mouse), or rHA plus an IL-1 family cytokine (1 μg/mouse). Plasma and fecal extracts were collected 14 days after the final immunization and analyzed by ELISA for rHA-specific IgG in plasma (A) and rHA-specific sIgA in fecal extracts (B). (C) Also, 14 days after immunization, splenocytes from each group of WBB6F1 *W/Wv* and WT mice were cultured with 10 μg rHA/ml. Culture supernatants were harvested after a 3-day incubation, and rHA-specific cytokine production (IL-4, IL-5, IL-2, and IFN-γ) in the culture supernatants was analyzed using a Bio-Plex multiplex cytokine assay. Data are presented as means ± SEM (n = 5).

IFN-γ-mediated Th1-type immunity in mice immunized with rHA plus IL-33 as a mucosal vaccine adjuvant.

DISCUSSION

Of the 26 different IL cytokines studied here, intranasal immunization with rHA plus an IL-1 family cytokine (IL-1α, IL-1β, IL-18, and IL-33) induced the highest levels of rHA-specific systemic IgG. High levels of sIgA were also observed in the mucosa of IL-1 family cytokine-treated mice. However, IL-12 and IL-15 have been reported to promote systemic and mucosal immunity to intramucosally coadministered protein Ags (8, 45), although more frequent immunization was required to produce adjuvant activity. The apparent discrepancy concerning the adjuvant activity of IL-12 and IL-15 in this study and previous reports may be due to differences in immunization regimens and vaccine doses.

For IL-1 family cytokines, we showed that intranasal administration of rHA plus IL-1α, IL-18, or IL-33 induced higher levels of $CD8^+$ CTLs than intranasal administration of rHA alone, whereas the level induced by rHA plus IL-1β was similar to that induced by rHA alone. In agreement with these results, IL-1β has been reported to have a pivotal role in development of Th2-type immune responses (20). A previous report by

Shibuya et al. (36) showed that IL-1α is necessary for optimal Th1 development and IFN-γ secretion in BALB/c mice. In addition, Karupiah et al. (46) showed that IL-18 and IL-12p40 regulate cellular immune responses through $CD8^+$ T-cell activation. Thus, our data are in agreement with previous reports that IL-1α and IL-18 play a pivotal role in inducing Th1-type immune responses. Furthermore, there have been a few reports on the potential of IL-33 to induce a Th1-type immune response (37). In the present study, we showed that of the IL-1 family cytokines, IL-33 induced the highest levels of CTL and IFN-γ^+ cells. We are currently investigating the mechanism of IL-33 in Th1/CTL immunity.

We found that intranasal coadministration of influenza vaccine with IL-1 family cytokines provided protection against influenza viral infection, with IL-1β and IL-18 providing complete protection. It is known that nasal secretions containing locally produced sIgA and serum-derived IgG Abs contribute to forming a first line of defense for combating influenza viral infections (42, 44). Therefore, the prophylactic effects of IL-1 family cytokines may be due mainly to Ab-mediated immunity against influenza virus. Furthermore, previous studies have pointed out the importance of influenza-specific $CD8^+$ CTLs for host recovery from lethal influenza virus infections and protection against further infection (7, 15). Although the mechanism by which IL-18 provided complete protection against influenza remains to be elucidated, high-avidity $CD8^+$ CTLs induced by IL-1α, IL-18, or IL-33 probably confer protection against influenza viral infection. Recently, a requirement for NK cells or NKT cells for control of influenza virus infections was identified (10, 13). Since IL-18 is known to regulate NK and NKT cell activity (4, 38), it is possible that restimulation of these cells may have resulted in the reduction in virus replication and morbidity observed after viral challenge. We are currently investigating the involvement of these cell subsets in the induction of protection against influenza virus by IL-18.

Unfortunately, potent adjuvant action is often correlated with increased toxicity, as exemplified by CT adjuvant, which although it is potent is too toxic for human use. Therefore, one of the major challenges in adjuvant research is to gain potency while minimizing toxicity (17). Intranasal administration of 1 μg of an IL-1 family cytokine for four consecutive days has been shown to induce asthma-like symptoms, including airway hyperresponsiveness and goblet cell hyperplasia in the lungs (26). In contrast, in this study, we found that mice immunized intranasally with IL-1 family cytokines did not exhibit acute toxicity, i.e., there was no cytokine-induced mortality, no obvious weight loss, no abnormal behavior, and no histopathological changes. In addition, use of 0.1 μg of an IL-1 family cytokine as a nasal vaccine adjuvant was still effective at inducing systemic IgG and nasal sIgA Ab responses. Thus, although further safety evaluation is needed, our findings indicate a broad therapeutic utility for IL-1 family cytokines when used as adjuvants for mucosal vaccination.

To develop optimal vaccines for clinical applications, it is important to understand their mechanism of action on the immune system in terms of efficacy as well as safety (23). The present study demonstrates that the enhanced mucosal vaccine adjuvant effect of IL-18 operates via an MC-dependent mechanism. The rHA-specific immune response induced by intranasally administered rHA plus IL-18 in WT mice was significantly reduced in W/W^v mice. In addition, the level of the rHA-specific IFN-γ response in mice intranasally immunized with rHA plus IL-33 was minimal in W/W^v mice. Although studies are needed on the role of MCs in generation of Ag-specific immunity, the studies reported here show that MCs have a role in the effect of IL-18 as an adjuvant and in augmentation of the CTL response induced by IL-33 as a nasal vaccine adjuvant. MC activators (e.g., compound 48/80) have been reported to stimulate protective immune responses against infections (28, 32). In addition, these immune responses are correlated with DC trafficking and lymphocyte recruitment to draining lymph nodes (DLN). Nakae et al. (30) suggested that MC-derived tumor necrosis factor alpha (TNF-α) is required for enhanced recruitment of lymphocytes and DCs to DLN. MC-dependent induction of IL-18 mucosal vaccine adjuvant activity may involve these types of processes. In agreement with this possibility, the IL-18 receptor was highly expressed on the surfaces of MCs but not in nasal passage-associated lymphoid tissue $CD11c^+$ DCs, and IL-18 induced robust TNF-α and IL-6 production from MCs in a concentration-dependent manner *in vitro* (unpublished data). Although further studies are required, IL-18 appeared to exhibit MC-dependent adjuvant activity that was not directly regulated by DC functions, such as DC migration and DC activation.

In summary, IL-1 family cytokines used as mucosal vaccine adjuvants induced two layers of protective immunity when administered intranasally with an influenza virus vaccine Ag, indicating that they may be suitable for use in antiviral nasal vaccines.

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

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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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The authors declare no conflict of interest.

Data deposition: The nucleotide sequences reported in this study have been deposited in the International Nucleotide Sequence Database (accession nos. AB453241–AB453250).

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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.

***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

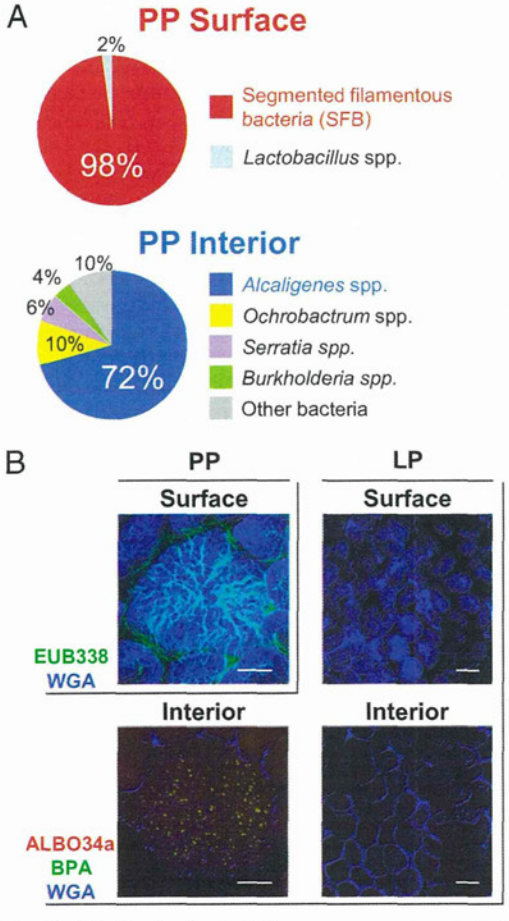

Fig. 1. Microbial distribution in the GI immune compartment. (*A*) Microbial composition at the surface and on the interior of PPs was examined by 16S rRNA gene clone library analysis. (*B*) The presence of *Alcaligenes* was visually analyzed by whole-mount FISH at the surface and on the interior of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

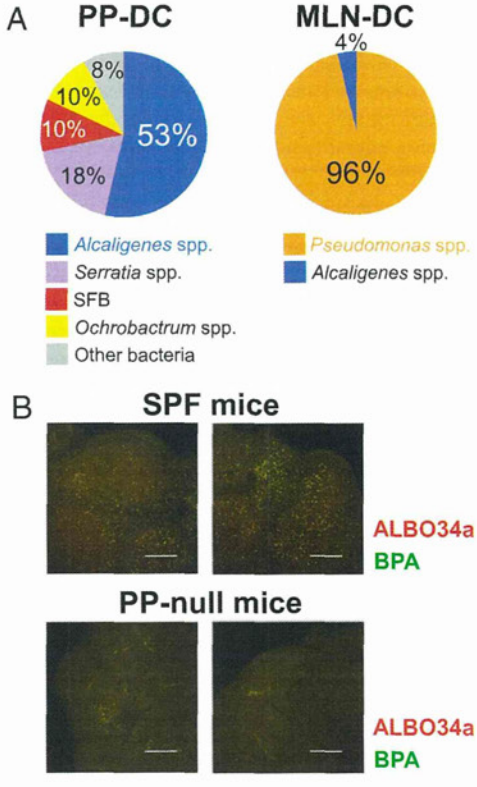

Fig. 2. PP-MLN migration axis for *Alcaligenes*-ingested GI tract DCs. (*A*) $CD11c^+$ DCs were isolated from the PPs and MLNs. Bacterial composition was determined by 16S rRNA gene clone library analysis. (*B*) Whole-mount FISH was performed to detect *Alcaligenes* (yellow) in the MLNs of PP-intact 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).

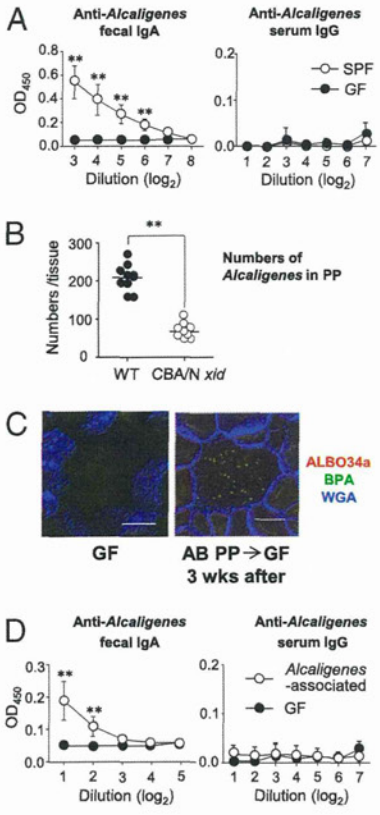

Fig. 3. Preferential induction of *Alcaligenes*-specific mucosal Ab responses in the PPs. (*A*) *Alcaligenes*-specific fecal IgA and serum IgG Ab responses were determined by ELISA. Data are means ± SD ($n = 4$). (*B*) The numbers of *Alcaligenes* inside PPs were counted in 10 randomly chosen PPs of 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 $1{,}304 \pm 364$ 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

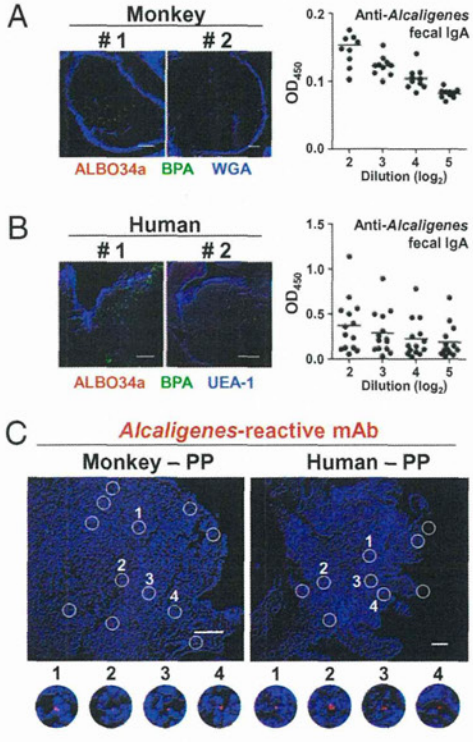

Fig. 4. Intratissue habitation of *Alcaligenes* inside nonhuman primate and human PPs. (*A* and *B*) *Alcaligenes* were detected on the interior of monkey and human PPs by whole-mount FISH (*Left*). *Alcaligenes*-specific fecal IgA Ab responses in monkeys and human were examined by ELISA [*Right*; $n = 10$ (*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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METHODOLOGY Open Access

A simple and inexpensive haemozoin-based colorimetric method to evaluate anti-malarial drug activity

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Abstract

Background: The spread of drug resistance in malaria parasites and the limited number of effective drugs for treatment indicates the need for new anti-malarial compounds. Current assays evaluating drugs against *Plasmodium falciparum* require expensive materials and equipment, thus limiting the search for new drugs, particularly in developing countries. This study describes an inexpensive procedure that is based on the advantage of a positive correlation between the haemozoin level of infected erythrocytes and parasite load.

Methods: The relationship between parasitaemia and the haemozoin level of infected erythrocytes was investigated after converting haemozoin into monomeric haem. The 50% inhibitory concentration (IC_{50}) values of chloroquine, quinine, artemisinin, quinidine and clotrimazole against *P. falciparum* K1 and 9A strains were determined using the novel assay method.

Results: The haemozoin of parasites was extracted and converted into monomeric haem, allowing the use of a colorimeter to efficiently and rapidly measure the growth of the parasites. There was a strong and direct linear relationship between the absorbance of haem converted from haemozoin and the percentage of the parasite ($R^2 = 0.9929$). Furthermore, the IC_{50} values of drugs were within the range of the values previously reported.

Conclusion: The haemozoin-based colorimetric assay can be considered as an alternative, simple, robust, inexpensive and convenient method, making it applicable in developing countries.

Keywords: Anti-malarial, Assay, Haemozoin, Malaria

Background

Malaria is more than just a problem for tropical countries, it also is a major global public health concern. Annually, there are approximately 300 million new malaria infections and millions of deaths worldwide due to malaria [1,2]. Because a vaccine for malaria is not available, chemotherapy is the main treatment. However, the rapid spread of resistance to current quinoline anti-malarials has made malaria a major global and important problem. In addition, artemisinin, from a Chinese herb (*Qinghaosu*) that has been used in the treatment of fevers for more than a thousand years, is now considered an essential component of artemisinin-based combination therapy against drug-resistant malaria [3,4]. However, the malaria parasites have recently been found to be resistant to artemisinin [5-7]. The alarming spread of drug resistance and the limited number of effective drugs for treatment indicates how important it is to find new anti-malarial compounds.

For decades, the anti-malarial activity of a drug has been measured *in vitro* by quantifying the uptake of radioactive substrates by a parasite as a measure of growth and viability in the presence of the test drug [8,9]. Although several *in vitro* methods exist, the ^{3}H-hypoxanthine method [8] is a popular test for novel anti-malarial drugs, but it is labelled with radiation that presents a potential risk to safety, and it relies on

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relatively expensive radio-isotopes and includes multi-step procedures that become increasingly problematic and impractical when the incidence of testing is increased. The other methods, including the PicoGreen [10,11] and the SYBR Green I [12,13] methods, are also considered to be expensive approaches regarding equipment and chemicals. In addition, there are other methods that are based on enzymatic reaction and/or antibodies that specifically detect the presence of histidine-rich protein II or parasite lactate dehydrogenase [14-16]. However, these methods involve multiple complex steps that are too expensive for developing countries, which makes them ill suited for screening potential anti-plasmodial drugs.

During the development and proliferation stage in host erythrocytes, the malaria parasites degrade haemoglobin for use as a major source of amino acids. This is accompanied by the release of free haem. With haem as a prosthetic group of haemoglobin, the iron is in the ferrous state, but free haem loses one electron and assumes the ferric state. This ferric haem could be oxidatively active and toxic to both the host cells and malaria parasites, and can even cause parasite death. Moreover, due to the absence of haem oxygenase, the parasite is unable to cleave haem into an open-chain free haem, which is necessary for cellular excretion [17]. To protect itself, it is necessary for the parasite to convert haem to non-toxic metabolites. Principally, the parasite detoxifies free haem via neutralization with histidine-rich protein 2 [18,19] and degradation with reduced glutathione [20,21], but mostly with crystallization into haemozoin, which is a water-insoluble malarial pigment produced in the food vacuole [19,22]. Therefore, a simple and inexpensive *in vitro* assay was developed based on the colorimetric quantification of haemozoin in infected red blood cells to evaluate the anti-malarial activity of drugs.

Methods

Materials

Chloroquine diphosphate, quinine sulphate, primaquine, clotrimazole, haemin chloride (haem), RPMI 1640 medium, hypoxanthine, and gentamycin were purchased from Sigma Aldrich Chemical Company (Tokyo, Japan). Albumax II (Gibco), and the other chemicals used in the present study were of a high grade. *Plasmodium falciparum* K1 (chloroquine resistant) and 3D7-9A (chloroquine susceptible) strains [23] were provided from Dr Osamu Kaneko and Dr Shusuke Nakazawa, respectively, from the Institute of Tropical Medicine, Nagasaki University, Japan.

Plasmodium cultivation

Plasmodium falciparum K1 and 9A strains were maintained *in vitro* with continuous culture according to a previously described method with a slight modification [24]. The culture medium consisted of RPMI 1640 supplemented with 0.025 mg/ml gentamicin, 0.01 mM hypoxanthine, 23.8 mM $NaHCO_3$, 11 mM glucose and 0.5% albumax II, and adjusted to a pH of 7.3 to 7.4. The parasite was cultured and maintained in a tissue culture flask with complete culture medium containing 5% human erythrocytes. The parasite density was maintained at about 1.5% parasitaemia under an atmosphere of an AnaeroPack sachet (Mitsubishi Gas Chemical Company Inc, Tokyo, Japan) to create 20% CO_2 and remove O_2 (<0.1%) 37°C [25]. Every two days, infected erythrocytes were transferred into fresh medium containing 5% human erythrocyte. The level of parasitaemia was determined by light microscopy on a Giemsa-stained thin blood smear, and parasitized erythrocytes were diluted when parasitaemia was higher than 5% in erythrocytes contained at 5% in culture medium, in order to lower parasitaemia and allow continuous growth. Parasite culture was diluted with fresh uninfected erythrocytes and culture medium to achieve a starting parasitaemia of 2% and a haematocrit of 5%. This final parasite culture was immediately used for anti-malarial assay.

The relationship between parasitaemia and haemozoin level

A culture of the *P. falciparum* K1 strain was serially diluted with uninfected erythrocytes in complete medium to yield a haematocrit of 5% and parasitaemia ranging from 0 to 10%. The serial culture containing 200 μl was prepared independently in triplicate in microtubes, followed by the addition of 800 μl of 2.5% sodium dodecyl sulphate in 0.1 M sodium bicarbonate pH 8.8, then the samples were mixed at room temperature for 15 min. After centrifugation at 13,000 rpm for 10 min, the supernatant was removed. The pellet was washed twice with 800 μl of 2.5% sodium dodecyl sulphate in 0.1 M sodium bicarbonate (pH 8.8), then 200 μl of 5% sodium dodecyl sulphate was added to 50 mM NaOH to convert the haemozoin into haem. After incubation at room temperature for 30 min, the sample (200 μl) was transferred to a 96-well microplate and scanned at 405/750 nm ($A_{405\ nm}$ minus $A_{750\ nm}$) using an IMark microplate reader (Bio-Rad). After the background absorbance of haemozoin was purified of uninfected erythrocytes (5% haematocrit) then subtracted, the amount of haemozoin in the infected erythrocytes was presented as the absorbance at 405/750 nm and then plotted against parasitaemia.

Evaluating the anti-malarial activity of drugs using the haemozoin-based spectrophotometric method

The *P. falciparum* K1 and 9A strains were used to evaluate the anti-malarial activity of quinine, chloroquine,

pyrimethamine, artemisinin and clotrimazole by using the haemozoin-based spectrophotometric method. Stocks of drugs were prepared in dimethyl sulphoxide or phosphate buffer saline (for chloroquine) and were then serially diluted with complete culture medium. To each well of a microplate, 10 μl of serially diluted drug solution was added into 200 μl of final asynchronous parasite culture. Dimethyl sulphoxide or phosphate buffer saline were also tested by adding a similar amount to control wells. The microplates were cultured 72 hr under the conditions described above. The haemozoin of infected erythrocytes was extracted, purified, and quantified, as described above. The 50% inhibitory concentration (IC_{50}) value was calculated by non-linear fitting of the absorbance at 405/750 nm against the logarithm of the drug concentration using the GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Sigmoidal doses with variable slope models were used with the following equation:

$$y = \min + \frac{\max - \min}{1 + 10^{(\{\log IC_{50} - x\} \times Hill\ slope)}}$$

where y is the absorbance at 405/750 nm; x is the logarithm of the drug concentration, min is the absorbance at 405/750 nm measured at time zero (starting point of assay), and max is the maximal absorbance of a particular drug. The Hill slope is the steepness of the curve. The logarithm of the concentration at zero was defined at 2 log lower than the lowest concentration of a particular drug.

Results and discussion

Relationship between the absorbance of the haem content of haemozoin and parasitaemia

The relationship between the haemozoin amount in a parasitized erythrocyte and parasitaemia was revealed by measuring the absorbance of the haem content of the haemozoin obtained from the parasitized erythrocytes after degradation to monomer haem. As shown in Figure 1, the absorption of converted haem showed a direct and linear correlation with the level of parasitaemia. Low and unsynchronized parasitaemia (about 1%) could be detected using this haemozoin-based colorimetric method. The results indicate that this novel assay of parasites is applicable for monitoring parasite growth and for screening new antimalarial compounds.

Haemozoin-based colorimetric assay to determine the IC_{50} values

The IC_{50} values of some anti-malarial drugs were determined with a dose-response experiment using a haemozoin-based colorimetric assay. The result showed that increasing the concentration of anti-malarial drugs resulted in a decreased absorbance at 405/750 nm (Figure 2). The data was best fitted by a typical sigmoidal dose-response model with a variable slope (four parameters) that agreed well with previous reports [26,27].

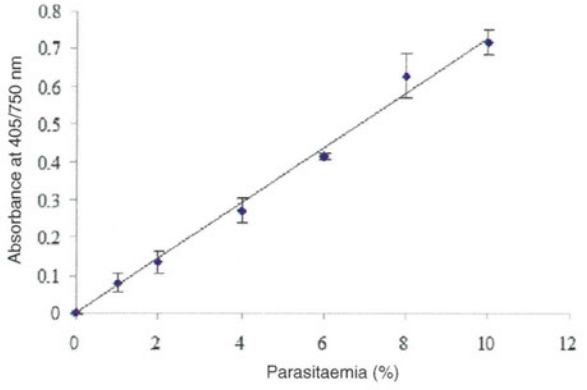

Figure 1 The linear relationship between the haemozoin level of a parasite and parasitaemia. Haemozoin concentration of infected erythrocytes is presented by the absorbance at 405/750 nm of monomeric haem after conversion from haemozoin using an NaOH solution. Absorption values (means ± standard errors of triplicate wells) are plotted against parasitaemia. A well correlated, linear relationship (R^2 = 0.9929) is strong evidence of the sensitivity of the method.

Table 1 summarizes the results of the assay to determine IC_{50} using the haemozoin-based colorimetric method. The *P. falciparum* K1 strain was primarily observed for quinine, chloroquine, clotrimazole, pyrimethamine and artemisinin, with IC_{50} values of 0.258,

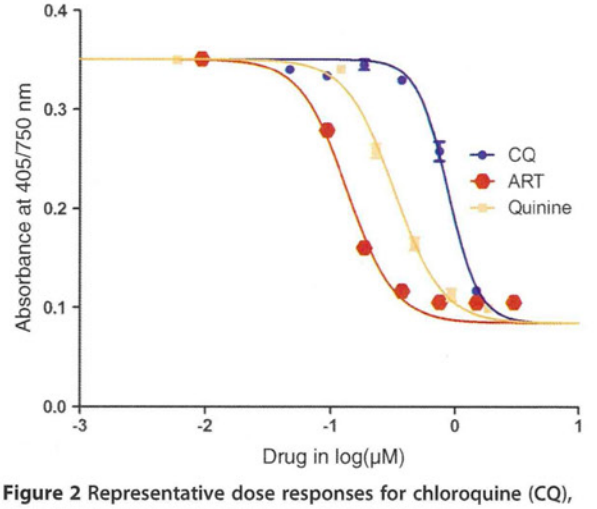

Figure 2 Representative dose responses for chloroquine (CQ), artemisinin (ART) and quinine against the *Plasmodium falciparum* K1 strain. Parasite growth after incubation of parasitized erythrocytes for 72 hr with a drug was measured using a haemozoin-based colorimetric method. The symbols and error bars are the average absorption values at 405/750 nm and standard deviations in triplicate, respectively. The sigmoidal dose response model with a variable slope is the best fit to the data.

Table 1 *In vitro* anti-malarial activities of drugs against chloroquine-susceptible (9A) and -resistant (K1) strains of *Plasmodium falciparum*

Drugs	Mean IC_{50} and 95% CI (μM)	
	P. falciparum K1 strain	*P. falciparum* 9A strain
Quinine	0.258 (0.242 - 0.275)	0.398 (0.307 - 0.516)
Chloroquine	0.873 (0.824 - 0.926)	0.132 (0.082 - 0.211)
Clotrimazole	0.805 (0.689 - 0.939)	1.67 (1.18 – 2.37)
Pyrimethamine	23.03 (18.36 - 28.90)	Not done
Artemisinin	0.139 (0.124 - 0.155)	0.483 (0.376 - 0.622)

Parasitized red blood cells were incubated with different concentrations of drugs for 72 hr and parasite growth was evaluated using the haemozoin-based colorimetric method. The IC_{50} and its 95% confidence interval (95% CI) were calculated from the concentration-response curve of the haemozoin level *vs* the log concentration of a drug.

0.873, 0.805, 23.03, and 0.139 μM, respectively. For the *P. falciparum* 9A strain, pyrimethamine was not evaluated, and the IC_{50} values were 0.398, 0.132, 1.67 and 0.483 μM in succession for quinine, chloroquine, clotrimazole and artemisinin, respectively. The IC_{50} values for quinine, chloroquine, clotrimazole, and pyrimethamine were in a range that was similar to those observed in previous reports [28,29]. On the other hand, the IC_{50} values for artemisinine were higher than previous reports, probably due to asynchronous cultures, ring stage-specific target of artemisinine, accumulation of released hemozoin in the continuous cultures, or several rounds of continuous cultures and cloning of parasite strains in our laboratories. Therefore, further studies are required to compare the novel assay with recent developed methods to validate the accuracy in the screening new antimalarial compounds [30]. Another limitation of the novel method is that it is not easily adaptable for a high throughput screening of anti-malarial drug candidates, which is under-developed using 96-well filter plates [31].

In recent years, the number of laboratories, diagnosis centres and research institutes has risen in developing countries. However, most of them lack the modern equipment and expensive chemicals to apply new methods for screening anti-malarial candidates. In addition, some methods have potential risks of toxicity, so it is prudent to wear disposable gloves at all times when proceeding. Another obstacle is that many laboratories lack the facilities to treat toxic contamination before the toxin is discarded in the environment. The novel anti-malarial assay is safe, non-expensive and easy to apply in laboratories.

Conclusions

The standard curve obtained in this study was strongly linear between the absorbance of monomeric haem converted from haemozoin and the percentage of parasitaemia. The IC_{50} values of chloroquine and quinine obtained from the haemozoin-based colorimetric method are similar to other methods. Even though this report describes specific conditions, the current experiment has introduced an assay that is adaptable to a wide range of conditions. The results also show that using this method has several advantages over using current methods. First, the method is fast and is based on a simple technique that uses a microplate reader, which is available in most laboratories. Second, the assay is based on inexpensive chemicals with no requirement of cold storage. Last but not least, the assay of the inhibition of *P. falciparum* growth using a haemozoin-based colorimetric method is feasible, reproducible, non-toxic, and more convenient than other assays, which makes it particularly useful for developing countries in the screening of novel anti-plasmodials, as a useful high throughput screening method for anti-malarial drug candidates.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NTH and KK developed the idea for the project. TTM and NTH conceived and designed the experiments. TTM, NTH and MNS carried out the laboratory work. TTM, NTH, DTXT, KH, and KK analysed and interpreted the data. TTM, NTH, MNS, KH and KK contributed reagents/materials/analysis tools. . TTM, NTH, DTXT and KK wrote the paper. All authors had full access to all data in the study, read and approved the manuscript.

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