of IgA^+ B cells from the inductive site into the intestinal lamina propria.

Like IgA-committed B cells in GALT, the preferential migration of T cells activated at intestinal inductive tissues (e.g., PP and MLN) to the lamina propria, an intestinal effector site, is mediated by $\alpha4\beta7$ integrin and CCR9 expression [83, 84]. Several studies have demonstrated that only PP- and MLN-derived, but not spleen-derived, DCs are capable of determining the gut tropism of intestinal T cells by the induction of high levels of $\alpha4\beta7$ integrin and CCR9 expression, resulting in the selective migration to the intestinal lamina propria [85-87]. In this context, a recent study has identified that retinoic acid is dominantly expressed by mucosal DCs determining the mucosal T cell tropism [88]. Thus, mucosal T cells educated by orally inoculated antigen presented by mucosal DCs tend to migrate into distant intestinal effector sites by obtaining the mucosal trafficking molecules (e.g., $\alpha4\beta7$ integrin and CCR9). In this regard, it would be interesting to examine whether the selective homing of intestinal B cells is also operated by intestinal DCs.

It is already known that a similar pathway composed of adhesion molecules and chemokines regulates a restricted distribution of B cells to the upper respiratory tract, especially the nasal cavity. It was previously reported that nasal immunization induced up-regulation of $\alpha4\beta1$ integrin, but not $\alpha4\beta7$ integrin, and CCR10, but not CCR9 expression, allowing the selective trafficking of B cells to nasal passage epithelium expressing their ligands, VCAM1 and CCL28, respectively (Fig. **4**) [89, 90]. It is interesting to note that the same molecules are involved in the trafficking of NALT- stimulated B cells to the genito-urinary tract (Fig. **4**) [91], which may explain why high levels of antigen-specific immune responses are induced in the genital tract after nasal immunization [92, 93]. Additionally, L-selectin expression on B lymphocytes was observed to be elevated after nasal but not oral immunization [94]. Conversely, B cells primed at systemic sites express only very low levels of $\alpha4\beta7$ integrin but high levels of $\alpha4\beta1$ integrin and L-selectin [30], resulting in the up-regulation of responses to the CXCR3 ligands which originate from inflammatory sites. Taken together, the complex interactions between chemokines and tissue-specific adhesion molecules determine the mucosal T and B cell tropism from the inductive tissues to the effector tissues.

IgA Production into Lumen as a Missile Molecule Against Microorganisms

When IgA-committed B cells migrate into effector sites, they are influenced by IgA-enhancing cytokines such as IL-5 and IL-6 to differentiate into IgA plasma cells (Fig. **2**) [95, 96]. To achieve the transport of IgA across the epithelial layer, the mucosal immune system selectively formulates secretory IgA (S-IgA). In order to form S-IgA antibodies, the J-chain and poly Ig receptor (pIgR) are essential. Produced by B cells, the J chain is a small polypeptide that regulates dimer/polymer formation of IgA and IgM, but not of other types of Ig [97]. pIgR is expressed on the basal membrane of ECs [98]. Mucosal dimeric or polymeric IgA containing J-chain shows a high affinity for the basolaterally expressed epithelial pIgR, thereby accelerating the internalization and transport of the complex to the apical site via transcytosis [99]. In both J-chain knockout and pIgR knockout mice, serum IgA levels rose while fecal IgA levels fell [100, 101]. In order to induce S-IgA at the diffused effector site, a mucosal internet must be formed through the cooperation of three types of cells: 1) dimeric/polymeric IgA-committed B cells originating in the inductive tissues, 2) Th2-type cells producing IgA-enhancing cytokines (IL-5 and IL-6) and 3) pIgR-expressing ECs. These cells must collaborate if S-IgA is to reach the lumen side of the mucosa where it can act as a first line of defense against invading pathogens.

CTL Plays a Pivotal Role in the Protection of Mucosal Sites

Although there is no doubt that S-IgA plays a pivotal role in protecting the mucosal surface from aerodigestive tract infection, the mucosal immune system is equipped with multiple layers of protective immunity. A sizeable body of evidence suggests that mucosal T cells harbor cytolytic activity and thus are capable of killing cells infected with virus or bacteria [102-104]. Like IgA-producing B cells, most of these mucosal T cells are thought to be derived from the CMIS-dependent induction pathway. Peripheral CTLs mainly recognize cytoplasmic antigen presented by MHC class I molecules following cytoplasmic antigen processing [105, 106]. Like the peripheral CTLs, mucosal CTLs in the inductive and effector sites are largely composed of $TCR\alpha\beta^+$ and $CD8^+$ lymphocytes and recognize the antigen derived from the cytosolic antigen as a complex with MHC class I molecules. Interaction with mucosal DCs at the inductive tissues (e.g., NALT and PPs) induces the clonal expansion of antigen-specific T cells and also determines a T cell tropism by the induction of adhesion molecules and chemokine receptors ($\alpha4\beta7$ integrin and CCR9) that allow for a selective migration to mucosal effector sites (e.g., intestinal lamina propria and the nasal passage) (Figs. **2** and **4**) [85-88]. Once mucosal $CD8^+$ T cells have encountered the specific antigen, they express pore-forming protein, perforin, and cytolytic granules containing granzyme proteases and so become capable of cytotoxic activity against pathogenic target cells [107].

CMIS-INDEPENDENT INDUCTION PATHWAY

It has been generally accepted that CMIS is a major pathway for the induction of antigen-specific mucosal immune responses. However, because antigen-specific immune responses have been

induced in PP- and/or ILF-null mice following oral immunization [9, 49, 108], the existence of alternate pathways has been proposed. Indeed, a number of those pathways have been identified, especially in the gastrointestinal tract, where multi-layers of IgA-mediated immunity provide a first line of defense against invading pathogens.

Alternative Gateways for Antigen Sampling from the Intestinal Lumen

As mentioned above, FAE-associated M cells at inductive tissues are thought to be a principal gateway for the uptake of antigen from the lumen and for the initiation of Ag-specific immune responses. Thus, M cells have been thought to be always developed at and associated with the organized mucosal lymphoid tissues. However, alternative antigen-sampling pathways have been reported. At least three different scenarios have been offered regarding this alternative antigen-encountering pathway (Fig. **2**). First, we have recently identified M cells on intestinal villous epithelium (villous M cells) not in proximity to PP [109]. Intestinal villous M cells are developed in various PP-/ILF-null mice and are capable of taking up bacterial antigens. The discovery of villous M cells has not only shed light on a novel gateway for antigen uptake into the intestine but has also suggested the possibility of a previously unsuspected route of pathogenic invasion.

Secondly, a recent study identified a unique DC population among intestinal ECs [110]. These intraepithelial DCs express tight junction-associated proteins (e.g., occuludin, claudin 1 and zona occuludens 1) and thus are capable of extending their dendritical arm between ECs. A previous study had already demonstrated that CD18-expressing phagocytes were involved in an M cell-independent pathway for bacterial invasion [111]. By protruding dendrites into the lumen, mucosal DCs located between ECs take up gut antigens and then present them to T and/or B cells.

The third pathway for antigen uptake is EC itself. There is evidence to suggest that ECs could process and then present antigens to T cells via MHC class I as well as class II molecules [112]. In addition to sampling a wide variety of foreign antigens, the mucosal immune system must contend with the high number of apoptotic ECs that result from the frequency with which the epithelium is replaced. Although most of these apoptotic ECs are ceded by the epithelium to the lumen, some of these apoptotic ECs have been shown to be potentially immunogenic and transportable to T cell areas of MLNs by mucosal DCs [113, 114]. These findings suggest that the intestinal immune system is equipped with at least two distinct gateways, one of which is located in the organized compartment and the other in the diffused compartments which serve as a portal to outside environments. Thus, M cells developed in the FAE of PP and ILF belong to the former gateway, while villous M cells and intraepithelial DCs are affiliated with the latter. It would be intriguing, of course, to examine how these two gateways, located in such distinctively different immunological environments (e.g., organized lymphoid structure and diffused connective tissue structure), cooperate in the induction of antigen-specific immune responses. For pathogens, the second entry site might be the more attractive for infection, since there they will not be directly exposed to organized lymphoid structures.

B1 Cells are Another Source for Mucosal IgA

Two lineages of murine B cells, B1 and B2 cells, have been identified, which can be distinguished by their cell surface markers (e.g., B220, IgM, IgD, CD5, and Mac-1), origins, and growth properties [115]. As we mentioned above, some IgA plasma cells are derived from conventional B cells (B2 cells) originating from the MALTs (e.g., PP, IEL and NALT), while B1 cells are preferentially observed in mucosal effector sites like the lamina propria and the nasal passages as well as the peritoneal and pleural cavity [116]. Our previous results demonstrated that IL-5, a well-known IgA-enhancing cytokine, supported the differentiation of both B1 and B2 cells into IgA plasma cells [116]. In contrast, mucosal EC-derived IL-15 promoted the proliferation and differentiation into IgA-producing cells of B1 but not of B2 cells [117]. Thus, treatment with anti-IL-15 antibody resulted in the severe paucity of B1 cells at effecter sites such as the intestinal lamina propria and the nasal passage, but did not affect B2 cell levels [117]. Further, it was shown that B2 but not B1 cells developed at organized inductive tissues such as PP and ILF, while both B1 and B2 cells were found in the effector tissues such as the intestinal lamina propria. Based on these findings, it seems that intestinal B1 cells migrate from non-inductive tissues, presumably in the peritoneal cavity, to the effector sites, where they further differentiate into IgA plasma cells under the influence of IL-5 and IL-15. Regarding B1 cell migration, a previous study using *aly/aly* mice that carried a point mutation in the NIK demonstrated a complete absence of B cell population in the intestinal lamina propria but elevated B cell levels in the peritoneal cavity [118]. Additionally, *aly/aly* peritoneal B cells exhibit defective *in vitro* chemotatic responses to CCL21 and CXCL13, implying that the NIK-mediated pathway is involved in B1 cell mucosal migration, which might be operated by specific but not yet identified chemokine receptor(s).

Not only do cell surface markers and points of origin distinguish B1 and B2 cells, but also their different antigen recognition patterns. For example, B1 cells are thought to be specialized in responding to T cell-independent antigens conserved on common pathogens like DNA and phosphatidylcholine, whereas B2 cells require activation by DCs and Th cells [119-121]. Thus, IgA production from B1 cells was noted in MHC class II-

deficient mice as well as TCR β- and δ chain-deficient mice [122, 123]. Of note, about 65% of fecal bacteria were reactive with B1-derived IgA and 30% of bacteria were bound with B2-derived IgA, indicating that S-IgA derived from B1 cells recognized a large population of commensal bacteria [124]. Since, as mentioned above, the T cell-independent IgA antibodies originating from B1 cells possess antigen reactivity to conserved bacterial products, they completely prevented the attachment of commensal bacteria. Thus, it is assumed that the specialized role of B1-derived IgA is not to protect from pathogenic bacterial invasion but rather to maintain the mucosal homeostasis by preventing the attachment of commensal bacteria. In contrast, B2 cell-originated IgA is a key protective antibody against pathogenic microorganisms.

IEL, a Unique Mucosal T Cell Population, Bridges Innate and Acquired Immunity

A unique subset of IELs also characterizes CMIS-independent mucosal immunity. IELs are located at every four to nine ECs and are mainly composed of heterogeneous groups of T cells which may be distinguished by their usage of TCR as well as CD4 and CD8 [125]. Most $CD8^+$ IELs are either TCRγδ- or TCRαβ-positive cells with homodimeric CD8αα in addition to the classical TCRαβ positive CD8αβ T cells [126]. Similar to $CD8^+$ T cells at the periphery and lamina propria, CD8αβ IELs are developed at the thymus and migrate specifically into the mucosal compartments by the selective expression of CCR9 and α4β7 integrin [127]. In contrast to the thymus-dependent CD8αβ IEL T cells, at least some populations of CD8αα IEL T cells, such as TCRγδ T cells, are thought to be thymus-independent and thus developed in gut-associated CPs [19]. The main population of CP cells displayed c-kit, IL-7R, and CD44-positive, but lineage marker (CD3, B220, Mac-1, Gr-1 and TER-119)-negative lympho-hematopoietic stem cell phenotypes [20]. Consistent with the IL-7R expression on CP lymphocytes, gut epithelium-derived IL-7 has been shown to be important in the induction of CD8αα IEL T cells and CP maturation, since $IL\text{-}7^{-/-}$ mice do not have TCRγδ IEL and CP [128, 129]. Additionally, an *in vivo* progeny study demonstrated that they can generate both TCRαβ and TCRγδ IELs without thymic influence [20, 130]. However, other studies questioned the thymus-independent nature of IELs and implied that both CD8αα TCRαβ and TCRγδ IEL originated from the thymus [131-133]. Thus, it remains a subject of debate whether IEL develops independently of the thymus. However, still considered as a key member of the GALT network, CP serves as one of the important immunological sites of development for IELs that form the mucosal intranet with ECs.

The experiments using MHC class $I^{-/-}$ mice also demonstrated that CD8αβ IELs were restricted by MHC class I, while CD8αα IELs were not [134]. Based upon these findings, it can be postulated that CD8αβ IELs exhibit a high degree and CD8αα IELs a low degree of cytotoxic activity against MHC class I-associated, non-self cytoplasmic antigen. The number of CD8αα IELs was markedly reduced in β2-microglobulin (β2m)-deficient mice, implying that non-classical MHC molecules might contribute to the antigen presentation to CD8αα IELs [135]. This hypothesis is supported by the fact that intestinal ECs express several non-classical MHC molecules including thymus leukemia antigen (TL), Qa-1, Qa-2, CD1, and MHC class I-related molecules (MICA and MICB), and IELs express their ligands like a $V\gamma1V\delta1^+$, NKG2D, and CD8αα [136]. It has been generally accepted that these responses mediated by non-classical MHC molecules are induced promptly after infection without any peptide specificity for virus or bacteria [137, 138]. IELs then seem to provide a bridge between rapid innate responses, which may be CMIS-independent, and more time-consuming acquired immune responses, which may be CMIS-dependent [139].

DEVELOPMENT OF MUCOSAL VACCINE

Because of several advantages they offer over systemic vaccination, mucosal vaccines (e.g., nasal or oral vaccine) have become in recent decades an increasingly attractive option for the prevention and control of infection by emerging and re-emerging microorganisms [140]. Perhaps because the restricted absorption system and the fluid secretions which characterize mucosal surfaces preclude the efficient uptake of antigen into MALT after oral or nasal immunization with vaccine antigen alone, oral and nasal immunization have thus far failed to induce adequate antigen-specific immune responses. One obvious means to overcome this problem would be the development of an effective antigen delivery vehicle targeted to the MALT, especially to M cells. We outline the various approaches to the development of such an antigen delivery vehicle system below.

Genetically Modified Live Microorganisms for Antigen Delivery

Historically, vaccine has been prepared from microorganisms inactivated by formaldehyde or β-propiolactone. The inactivated vaccine is capable of inducing humoral immune responses but not cell-mediated immune responses such as CTL responses, since this type of vaccine generally induces MHC class II-mediated immune responses following their processing in lysosomal compartments [141]. For CTL responses, in contrast, cytoplasmic antigen processing and subsequent MHC class I-mediated antigen presentation are requisite [105, 106].

Attenuated live microorganisms are also capable of inducing immune responses and have already been utilized for mucosal vaccines (e.g., poliovirus,

Salmonella typhi Ty21a, and *Vibrio cholerae*) [142]. As mentioned above, some microorganisms naturally express ligands for M cells which facilitate their targeted invasion of host cells including M cells [10]. However, since vaccines using live pathogens sometimes result in undesirable disease symptoms, the attenuation of those pathogens must be complete and irreversible. Recent progress in genetic technology has helped to ensure vaccine safety and enabled us to develop recombinant attenuated vaccines expressing heterologous antigen [143, 144]. In the attenuated recombinant vaccines, several genes determining pathogenicity have been mutated and a gene encoding a heterologous antigen has been inserted. Microorganisms considered as candidates for the mucosa-targeted delivery of DNA encoding heterologous vaccine antigens include non-pathogenic *Lactobacilli*, attenuated strains of *S. typhi*, *Vibrio Cholerae*, *Shigella flexneri*, *Y. enterocolitica*, and *Listeria monocytogenes* [143]. Some of these mucosal delivery systems (e.g., *Salmonella typhi* Ty21a expressing *Shigella sonnei* O antigen and *Salmonella typhi* isolate Ty2 expressing hepatitis B antigen) have already been tested in humans [145, 146]. Based upon these findings, the genetically modified microorganisms seem to have proven capable of inducing immune responses without reverting back to a more virulent form capable of triggering disease symptoms, even in the hostile circumstances of the mucosal lumen. They are able to bind to the apical membrane of M cells using the innate ligand for M cells, thereby inducing humoral and cell-mediated immunity without any undesirable side effects. Such a mucosa-targeted vaccine delivery system is not limited to DNA, but can, in a bacteria-based form, also deliver peptide or protein antigen [147]. In such a bacteria-based system, intrinsic secretion systems (e.g., type III secretion system of *Yersinia* and *Salmonella*, and α-hemolysin secretion system of *E. coli*) are used to deliver the antigen into the cytoplasm to induce MHC class I-restricted antigen presentation, eventually leading to antigen-specific CTL induction.

Application of an Inert Synthetic Delivery System and a Hybrid Delivery System to Mucosal Vaccines

A variety of delivery systems have been developed using inert mucosal antigens including lipid-based particles such as liposomes and ISCOMs, as well as biodegradable polymer-based particles [148]. Encapsulation of antigens within particles is widely used to protect them from degradation in the harsh environment of the gastrointestinal lumen. Evidence suggests that several physical factors of the synthetic particles (e.g., size, hydrophobicity, and surface charge) determine the efficiency of the selective delivery of the encapsulated antigen to M cells [149]. Mucosal antigen delivery could be optimized if those particle features were modified by the addition of chemical or biological bioadhesins. For example, chemical mucoadhesive molecules (e.g., carboxy vinyl polymer) were used to elongate particles containing protein antigen, thereby improving their durability in the intestinal lumen [150]. Appropriate lectins, microbial and viral adhesions, and immuloglobulins have also been widely exploited to gain or enhance access to M cells [151]. Intestinal ECs possess a cell surface glycocalyx composed of membrane-anchored glycoconjugates. Several studies have demonstrated that Ulex europaeus 1 (UEA1), a lectin specific for α-L-fucose residues, binds almost exclusively to the apical surface of M cells of murine PPs [152, 153]. In these studies, the unique reactivity of UEA1 to M cells allowed for the selective delivery of microparticles to M cells after oral administration, leading to the successful induction of mucosal and systemic immune responses [154, 155].

Because a diverse range of microorganisms express a ligand for M cells, as noted above, another approach using bioadhesins is to apply microbial adhesins to mediate M cell binding for the targeted delivery of synthetic particles. As expected given the selectivity of ligands, enhanced antigen uptake was attained by coating polystyrene nanoparticles with *Yersinia*-derived invasin, a ligand for β1 integrins on the apical site of M cells [58, 156]. Viral as well as bacterial products can be utilized. Reovirus has been known to invade through M cells using 45-kDa viral haemagglutinin σ1 protein [157]. It was recently demonstrated that mucosal immune responses were significantly increased by coupling a plasmid DNA encoding HIV gp160 with a reovirus-derived σ1 protein capable of targeting M cells [158, 159]. We have also developed a hybrid antigen delivery vehicle using fusogenic liposome, which is composed of synthetic liposome and Sendai virus (also known as a haemagglutinating virus of Japan) [160]. The nasal administration of protein antigen via fusogenic liposomes resulted in its preferential delivery to NALT ECs, including M cells [161]. After nasal administration of fusogenic liposomes containing HIV glycoprotein 160 (gp160), equally striking results were seen, with high levels of gp160-specific serum IgG and mucosal IgA induced in saliva, fecal extracts and nasal and vaginal washes [93]. Also, because nasally administered fusogenic liposome delivers the antigen directly into the cytoplasm, antigen-specific CTL responses are also induced [93, 161, 162]. In addition to fusogenic liposomes, several other virus-like particles (e.g., hepatitis E virus and Sindbis virus) have been developed for nasal and oral vaccination [163, 164]. In addition to such viral systems, bacterial ghosts composed of intact bacterial envelopes may represent another non-living carrier system for mucosal vaccines [165]. By using hybrid delivery systems and bacterial ghosts, antigen can be selectively delivered to M cells without running the risk of the complications posed by live microbial and viral delivery systems. Because it is both effective and safe, the hybrid delivery system could mark an important step towards a feasible mucosal vaccine.

CONCLUDING REMARKS

This review has been aimed at summarizing the unique anatomical, developmental and immunological aspects of MALT, especially those of NALT, BALT, and GALT in the aerodigestive tracts. MALT serves as multi-functional immunological sites for the initiation of the molecular and cellular regulation of mucosal B cell- and T cell-mediated S-IgA and cell-mediated immunity as a first line of defense against invading pathogens. Nasal and/or oral immunization targeted to M cells in the aerodigestive tract induce antigen-specific IgA antibody and/or CTL responses at mucosal surfaces. Although mucosal immunization presents many advantages over systemic immunization, it does not effectively induce protective immunity when the protein antigen alone is delivered via the nasal and/or intestinal mucosa. To fully profit from the potential of the mucosal immune system, nasal and/or oral vaccinations require the co-administration of a mucosal adjuvant or the use of mucosal antigen delivery vehicles. Accordingly, much research has been focused on developing safe and effective mucosal adjuvants and vaccine delivery systems.

Recently, it was learned that PPs and NALT have distinct organogenesis programs. PP genesis requires the IL-7R- and LTβR-mediated signaling cascades and occurs in the prenatal period, while NALT genesis is independent of the IL-7R/LTβR signaling cascades and occurs postnatally. This discovery of two distinct organogenesis programs for PP and NALT could have important ramifications for our understanding of the mucosal immune system. In experiments using MALT-deficient mice (e.g., PP-null mouse), key molecules regulating organogenesis were disrupted, revealing PP-dependent and -independent mucosal induction pathways for the induction of antigen-specific immunity. These surprising observations challenge our conception of the mucosal immune system, revealing that it is more complex and nuanced than our current schemas suggest. A more profound and comprehensive molecular and cellular understanding of the mucosal immune system and mucosal organogenesis could facilitate the design of mucosal immune therapies and mucosal vaccines, thereby proving a real boon to public health.

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LIST OF ABBREVIATIONS

AID	=	Activation-induced cystidine deaminase
APC	=	Antigen- presenting cell
BALT	=	Bronchus-associated lymphoid tissue
CLN	=	Cervical lymph node
CMIS	=	Common mucosal immune system
CP	=	Cryptopatch
CSR	=	Class switch recombination
CTL	=	Cytotoxic T lymphocyte
DC	=	Dendritic cell
EC	=	Epithelial cell
FAE	=	Follicle-associated epithelium
GALT	=	Gut-associated lymphoid tissue
GC	=	Germinal center
HEV	=	High endothelial venule
ICOS	=	Inducible co-stimulator
ID2	=	Inhibitor of DNA binding/ differentiation 2
IEL	=	Intraepithelial lymphocyte
ILF	=	Isolate lymphoid follicle
LN	=	Lymph node
LT	=	Lymphotoxin
MAdCAM-1	=	Mucosal vascular addressin cell adhesion molecule 1
MALT	=	Mucosa-associated lymphoid tissues
MHC	=	Major histocompatibility complex
MLN	=	Mesenteric lymph node
NALT	=	Nasopharynx-associated lymphoid Tissue
NIK	=	Nf-κB-inducing kinase
PNAd	=	Peripheral node addressin
pIgR	=	Poly Ig receptor
PP	=	Peyer's patch
RORγ	=	Retinoic acid-related orphan receptor γ
SCID	=	Severe combined immunodeficient
SED	=	Subepithelial dome
S-IgA	=	Secretory IgA
S1P1	=	Sphingosine-1-phosphate receptor type 1
TCR	=	T cell receptor
Th	=	Helper T cells
TNF	=	Tumor necrosis factor
UEA-1	=	Ulex europaeus 1

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A rice-based edible vaccine expressing multiple T cell epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses

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Peptide immunotherapy using multiple predominant allergen-specific T cell epitopes is a safe and promising strategy for the control of type I allergy. In this study, we developed transgenic rice plants expressing mouse dominant T cell epitope peptides of Cry j I and Cry j II allergens of Japanese cedar pollen as a fusion protein with the soybean seed storage protein glycinin. Under the control of the rice seed storage protein glutelin *GluB-1* promoter, the fusion protein was specifically expressed and accumulated in seeds at a level of 0.5% of the total seed protein. Oral feeding to mice of transgenic rice seeds expressing the T cell epitope peptides of Cry j I and Cry j II before systemic challenge with total protein of cedar pollen inhibited the development of allergen-specific serum IgE and IgG antibody and $CD4^+$ T cell proliferative responses. The levels of allergen-specific $CD4^+$ T cell-derived allergy-associated T helper 2 cytokine production of IL-4, IL-5, and IL-13 and histamine release in serum were significantly decreased. Moreover, the development of pollen-induced clinical symptoms was inhibited in our experimental sneezing mouse model. These results indicate the potential of transgenic rice seeds in production and mucosal delivery of allergen-specific T cell epitope peptides for the induction of oral tolerance to pollen allergens.

Japanese cedar pollinosis | peptide immunotherapy | seed-specific expression

Immunotherapy using allergen-specific T cell epitope peptides has been shown to be a safe and effective treatment for the control of IgE-mediated allergic diseases (1–3). Immunodominant epitopes derived from several allergens have been shown to possess therapeutic effects in both animal models and human clinical trials (4–8). Japanese cedar (*Cryptomeria japonica*) pollen is a major cause of pollinosis that elicits allergic disorders such as rhinitis and conjunctivitis in Japan (9). Two major allergens, designated Cry j I and Cry j II, were isolated from the pollen (9–13), and multiple domains of T cell epitope for humans and mice were identified from them (14–16). It has been reported that oral feeding to mice of a chemically synthesized major T cell epitope peptide of Cry j II reduces levels of Cry j II-specific IgE and IgG antibody responses via a decrease in the production of allergy-associated IL-4 in mice (15). These results open new possibilities for the development of allergen peptide-based immunotherapy for the control of Japanese cedar-induced pollinosis. Thus, oral vaccination with the major T cell epitope peptides derived from Cry j I and/or Cry j II pollen allergens is considered to be a practical and effective method of immunotherapy for the inhibition of pollinosis-associated type I hypersensitivity.

Plants have recently been recognized as a form of bioreactor for the cost-effective production of large-scale recombinant proteins (17–19). Compared to other expression systems such as bacteria and mammalian cell cultures, plants have a much lower risk of contamination by human pathogens, such as animal virus and prions (17–19). Furthermore, the edible tissues of plants further provide the significant benefit of achieving a simple method for mucosal delivery of vaccines and immunogens without the need for complicated purification steps (20–22).

Cereal crop seeds are essentially edible tissues and have the capacity to produce relatively large amounts of recombinant products (23, 24). Recombinant products accumulated in seeds have been shown to be stable for 6 months, even when stored at room temperature (19). Rice, a staple food in Asia, can be considered as an attractive system, compared to other cereals, because of its easy storage and processing, high yield, and low production cost (25). A detailed search for a number of promoters, using β-glucronidase (GUS) reporter gene, provided a choice of suitable promoters for the effective expression of transgenes in rice seeds (26). Another advantage of rice plants is that targeting to protein storage vacuoles (protein bodies) provides a greater space for the accumulation of recombinant proteins (27). A soybean glycinin A1aB1b provided one successful instance of high-level accumulation in the protein storage vacuole II (protein body II), reaching ≈5% of the total seed protein (27). Furthermore, the expression level of glycinin A1aB1b was enhanced in low storage protein mutants of rice (28). Based on the progress of molecular analysis of the expression and accumulation of transgene products, rice can be considered a potential candidate for the development of plant-derived edible vaccines.

In this study, we developed transgenic rice plants accumulating mouse T cell epitope peptides specific for pollen allergens of *Cryptomeria japonica* in seeds. To achieve greater accumulation, the T cell epitope peptides of Cry j I and Cry j II were expressed as a fusion protein with the soybean storage protein glycinin A1aB1b. The fusion protein (A1aB1b-Crp-1 and -2) accumulated at a level of 0.5% of the total seed protein. Oral administration of the transgenic rice seeds to mice before systemic challenge with total cedar pollen protein induced oral tolerance with the inhibition of allergen-induced allergy-associated T helper 2 (Th2) cytokine synthesis of IL-4, IL-5, and IL-13 and their supported allergen-specific IgE responses. Furthermore, it resulted in the inhibition of the pollen-induced clinical symptoms of nasal sneezing. These results demonstrate the efficacy of T cell epitope peptides expressed in transgenic rice seeds for oral delivery and induction of oral tolerance against pollen allergen-specific responses.

Methods

Plasmid Construction and Rice Transformation. Two major T cell epitopes, KQVTIR1GCKTSSS (residues 277–290 of Cry j I) and RAEVSYVHVNGAKF (residues 246–259 of Cry j II) (15, 16),

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Abbreviation: Thn, T helper n.

See Commentary on page 17255.

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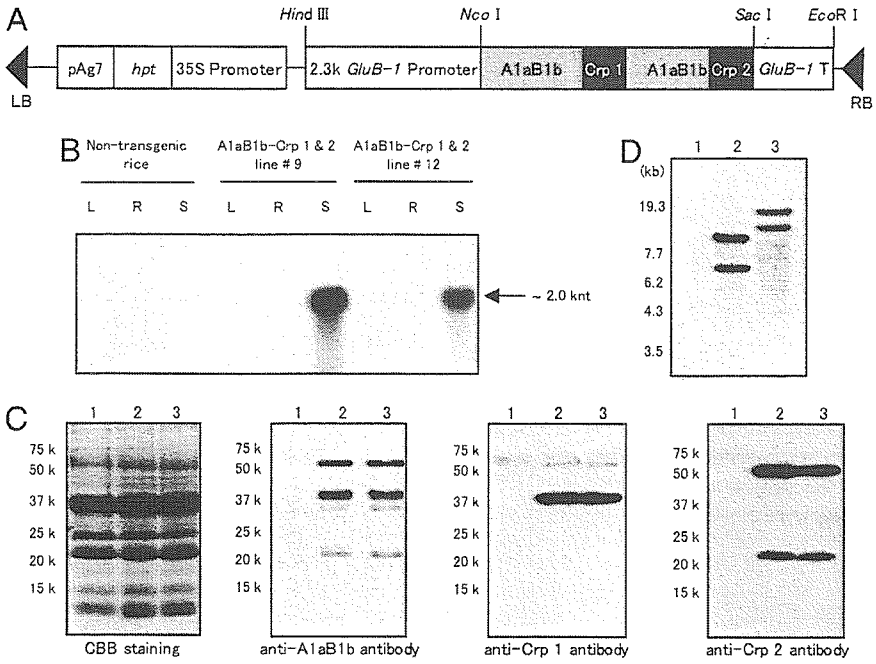

Fig. 1. Expression of A1aB1b-Crp-1 and -2 in transgenic rice. (*A*) Schematic representation of the transformation plasmid. The DNA fragment coding for the A1aB1b-Crp-1 and -2 protein was placed under the control of rice seed major storage protein glutelin 2.3-kb *GluB-1* promoter. The *hpt* gene was used for the selection of transgenic rice plants. *GluB-1*, rice glutelin *GluB-1*; 35S, cauliflower mosaic virus 35S promoter; *hpt*, hygromycin phosphotransferase gene; pAg7, agropine synthase polyadenylation signal sequence; RB, right border; LB, left border. (*B*) Northern blot analysis. Total RNA was isolated from leaves (L), roots (R), or developing seeds (S) of nontransgenic and A1aB1b-Crp-1 and -2 transgenic lines 9 and 12. (*C*) Western blot analysis of total protein extracted from seeds. Lane 1, nontransgenic rice; lane 2, A1aB1b-Crp-1 and -2 transgenic line 9; lane 3, A1aB1b-Crp-1 and -2 transgenic line 12. Anti-glycinin antibody, anti-Crp-1 antibody, or anti-Crp-2 antibody was used for the detection of A1aB1b-Crp-1 and -2 protein. (*D*) Southern blot analysis. Genomic DNA isolated from young leaves of rice plants was digested with SacI and fractionated by electrophoresis on 0.8% agarose gel. Lane 1, nontransgenic rice; lane 2, A1aB1b-Crp-1 and -2 transgenic line #9; lane 3, A1aB1b-Crp-1 and -2 transgenic line 12.

named Crp-1 and -2, respectively, were inserted into variable regions in acidic and basic subunits of glycinin A1aB1b (29, 30). Fifteen amino acid residues (residues 293–307 of A1aB1b) in the acidic subunit and eight amino acid residues (residues 488–495 of A1aB1b) in the basic subunit were substituted by the Crp-1 and -2 T cell epitopes, respectively, resulting in the recombinant protein A1aB1b-Crp-1 and -2. The construction of the A1aB1b-Crp-1 and -2 gene sequence was carried out by two stages of PCR amplification. A DNA sequence coding for the acidic subunit (residues 1–292 of A1aB1b) was amplified by PCR from the pUGluBGly plasmid (27) with a set of oligonucleotides −103 and Crp1R, which added a DNA sequence coding for the Crp-1 peptide at the 3′ end of the acidic subunit of A1aB1b sequence. The other sequence coding for the basic subunit (residues 308–487 of A1aB1b) was PCR-amplified by using the primer set Crp1F and M13-RV, which provided DNA sequences coding for the Crp-1 and -2 peptides at the 5′ and 3′ end of the basic subunit of A1aB1b sequence, respectively. These two DNA fragments were then annealed and amplified by overlap PCR with −103 and M13-RV primers to generate the complete DNA fragment coding for the A1aB1b-Crp-1 and -2 protein. This product was placed under the control of the 2.3-kb *GluB-1* promoter, and the plant expression cassette was then inserted into a binary vector pGPTV-35S-HPT (26). The resultant expression plasmid (Fig. 1*A*) was introduced into the rice genome (*Oryza sativa* L. cv Kitaake) by *Agrobacterium tumefaciens*-mediated transformation as described (26).

Southern and Northern Blot Analysis. Genomic DNA was prepared from young leaves by using the cetyltrimethylammonium bromide (CTAB) extraction method (28). Total RNA was extracted by the phenol/chloroform extraction method (28) from frozen rice seeds, leaves, or roots. Southern and Northern blot analyses were carried out by using standard methods (28). Hybridizations were performed at 65°C by using ^{32}P-labeled full-length A1aB1b-Crp-1 and -2 probes.

Detection of A1aB1b-Crp-1 and -2 Protein. Rice seeds were ground to a fine powder by using a Multibeads shocker (Yasui Kikai, Osaka, Japan), and total seed protein was extracted with an extraction buffer containing 4% (wt/vol) SDS, 8 M urea, 5% (wt/vol) 2-mercaptoethanol, 50 mM Tris·HCl (pH 6.8), and 20% (wt/vol) glycerol as described (28). Total seed protein was separated by using SDS/12% or 15% PAGE, and then transferred to Hybond-P poly(vinylidene difluoride) membranes (Amersham Pharmacia) for Western blot analysis. To confirm the accumulation of Crp-1 and -2 T cell epitope peptides in transgenic rice seeds, anti-Crp-1 peptide and anti-Crp-2 peptide antibodies were raised in rabbit (Qiagen, Tokyo). A rabbit anti-glycinin A1aB1b antibody had been prepared previously (27). The membranes were probed with one of the primary antibodies, and then incubated with a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Promega) for visualizing signals. Accumulation levels of A1aB1b-Crp-1 and -2 protein were determined by the quantitative dot immunoblotting with anti-glycinin A1aB1b antibodies as described (27).

Mouse Feeding Experiments. A first group of eight BALB/c male mice at 6 weeks of age (CLEA Japan, Tokyo) was orally fed with 200 mg of fine powder of rice seeds containing 70 μg of A1aB1b-Crp-1 and -2 protein suspended in 1.0 ml of PBS once a day over a period of 4 weeks. A second group of mice was fed with equal amounts of seed powder from nontransgenic rice plants. For a third group of mice, PBS was administered as a control experiment. All mice were then i.p. challenged twice at weeks 4 and 5 with 0.1 mg of total protein extracts of Japanese cedar pollen (Cosmo Bio, Tokyo) adsorbed on 5 mg of aluminum hydroxide (alum) (Cosmo Bio) in 500 μl of PBS. At the first challenge at week 4, recombinant mouse IL-4 (R & D Systems) was mixed with the allergen solution at 0.1 μg per mouse to maximize the induction of allergen-specific IgE responses. Our preliminary study demonstrated that the coadministration of IL-4 resulted in the acceleration of allergen-specific IgE responses compared with the case when IL-4 was not coadministered.

ELISA. At week 7 of the experiment, mice were bled to allow measurements of total and allergen-specific antibodies by ELISA as described (31, 32) with a slight modification. Immunoplates (Nalge Nunc) were coated with 2 μg/ml anti-mouse IgE or anti-mouse IgG antibody (Southern Biotechnology, Birmingham, AL). After washing and blocking of the plates, serial dilutions of serum were added

to the wells, which were then washed. For detection of allergen-specific antibodies, total protein extracts of pollen were biotinilated according to the manufacturer's procedure (Pierce) and added to the wells as a secondary antibody. Total IgE levels in serum were measured by a sandwich ELISA as described (31). After washing the plates, streptavidin–horseradish peroxidase conjugate (Pierce) was added to the wells, and the reaction was developed with peroxidase substrate solution (Moss, Pasadena, MD). The last serum dilution yielding an OD_{450} value of 0.1 over the background was recorded as the endpoint titer for each sample.

T Cell Proliferation and Cytokine Assay. $CD4^+$ T cells were purified from splenocytes at week 7 of the experiment by MACS beads separation using anti-mouse CD4 Ab-conjugated magnetic beads (Miltenyi Biotec). The cells were cultured at 1×10^5 cells per well together with gamma-ray-irradiated splenic antigen-presenting cells at 5×10^5 cells per well for 6 days at 37°C with or without 20 μg/ml total protein extracts of pollen in 96-well plates. Our preliminary study showed that a cedar pollen protein concentration of 20 μg/ml resulted in the optimal dose for the induction of maximum allergen-specific $CD4^+$ T cell responses among the different doses tested (e.g., 4–40 μg/ml). Each well was then pulsed with 0.25 μCi of [^{3}H]thymidine (Amersham Pharmacia; 1 Ci = 37 GBq) for the last 22 h of incubation, and the cells were harvested to allow measurement of radioactivity levels. At the same time, the other aliquots of cells were incubated under identical conditions for 5 days to assess the different Th1- and Th2-type cytokine production by ELISA as described (33).

Histamine Assay. To examine the levels of serum histamine, mice were challenged at week 7 with an i.p. injection of 0.1 mg of total protein extracts of pollen adsorbed on 5 mg of alum. Within 10 min after the injection, blood was taken and histamine levels were determined by using an enzyme immunoassay kit (Neogen, Lexington, KY).

Clinical Symptoms of Pollen Allergy. To examine the effect of A1aB1b-Crp-1 and -2 rice seeds induced oral tolerance for the inhibition of pollen allergen triggered clinical symptoms associating with pollinosis, other sets of mice were fed with the experimental and control rice seeds as described above. These mice were presensitized with pollen allergen via systemic route at weeks 4 and 5. At week 7 through week 8 of the experiment, these mice were then challenged once a day with 20 μl of 1 μg/ml total protein extracts of pollen dissolved in PBS via the intranasal route as described (34). Sham-challenged mice were nasally administered with 20 μl of PBS in the same manner. Nasal symptoms were evaluated by counting the number of sneezes observed in the 5 min after the last nasal challenge at week 8.

Statistics. The significance of the differences (e.g., P values) between groups was evaluated by the Mann–Whitney U test.

Results

Development of Transgenic Rice Plants Accumulating A1aB1b-Crp-1 and -2 Protein in Seeds. Thirty independent transgenic rice plants were generated, and accumulation levels of the A1aB1b-Crp-1 and -2 protein in seeds were examined by immunoblot analysis. Transgenic lines 9 and 12, which showed high levels of accumulation of A1aB1b-Crp-1 and -2 protein at the level of 7 μg per grain ($\approx$0.5% of total seed protein), were selected and proceeded to the T_3 generation by self-crossing to obtain homozygous lines.

To examine the tissue-specific expression of A1aB1b-Crp-1 and -2 gene, total RNA extracted from leaves, roots, and maturing seeds were subjected to Northern blot analysis. The transcript of the A1aB1b-Crp-1 and -2 gene was only detected in maturing seeds, whereas no band was found in the leaves or roots of transgenic lines 9 and 12 (Fig. 1*B*). These results indicate that the A1aB1b-Crp-1 and -2 gene is specifically expressed in seeds under the control of the 2.3-kb *GluB-1* promoter.

Next, total seed protein was extracted for analysis of A1aB1b-Crp-1 and -2 protein expression by Western blot (Fig. 1*C*). We previously demonstrated that the glycinin A1aB1b expressed in the endosperm of transgenic rice was synthesized as a precursor form and then posttranslationally processed into two mature subunits, the acidic and basic subunits (27). As shown in Fig. 1*C*, three signals for the precursor, the acidic and basic subunits with molecular masses of 56, 35, and 21 kDa, respectively, were detected in A1aB1b-Crp-1 and -2 transgenic lines by using anti-glycinin A1aB1b antibody. This result indicates that the A1aB1b-Crp-1 and -2 protein was expressed and posttranslationally processed in a similar manner to the native glycinin A1aB1b (27). The accumulation of Crp-1 and -2 T cell epitope peptides in the A1aB1b-Crp-1 and -2 protein was further confirmed by immunoblot analysis using the peptide specific anti-Crp-1 and anti-Crp-2 antibodies (Fig. 1*C*). It was shown that the glycinin acidic subunit (35 kDa) and the precursor (56 kDa) were recognized by the anti-Crp-1 antibody, whereas the glycinin basic subunit (21 kDa) and the precursor (56 kDa) were detected by the anti-Crp-2 antibody. These results clearly indicated that the Crp-1 and -2 peptides were expressed as fusion protein with A1aB1b and processed into the acidic and basic subunits of A1aB1b, respectively.

Integration of the A1aB1b-Crp-1 and -2 gene into the rice genome was confirmed by Southern blot analysis. Because the SacI restriction enzyme cuts only once in the transformation plasmid, the number of bands indicates the number of copies of the A1aB1b-Crp-1 and -2 gene integrated into the rice genome. At least two copies of A1aB1b-Crp-1 and -2 gene were estimated to be present in transgenic rice lines 9 and 12 (Fig. 1*D*).

Oral Feeding of Transgenic Rice Seeds Prevents the Development of Allergen-Specific IgE and IgG Responses. In the control group of mice fed with PBS, i.p. challenge with the pollen allergen elicited significant allergen-specific IgE and IgG responses (Fig. 2*A* and *C*). Oral feeding of nontransgenic rice seeds did not affect the high levels of allergen-specific IgE antibody response (Fig. 2*A*). In contrast, the level of serum allergen-specific IgE was significantly reduced in the group of mice fed with transgenic rice seeds accumulating A1aB1b-Crp-1 and -2 protein when compared to those in the control groups of mice fed with PBS or nontransgenic rice seeds ($P < 0.01$) (Fig. 2*A*). The levels of total IgE antibodies in serum were similar among the three groups of mice (Fig. 2*B*). In the case of allergen-specific IgG responses, the antibody titers were decreased in mice orally immunized with A1aB1b-Crp-1 and -2 rice seeds when compared to those in the control groups of mice ($P < 0.01$) (Fig. 2*C*). In addition, a dominant allergen-specific IgG1 subclass with some IgG2a and IgG2b antibodies were all decreased in the experimental group of mice (Table 1, which is published as supporting information on the PNAS web site). These results suggest that oral administration of A1aB1b-Crp-1 and -2 seeds inhibits a dominant Th2 cell-mediated antibody with some Th1-involved antibody responses to pollen allergens.

Oral Feeding of Transgenic Rice Seeds Inhibits Allergen-Specific T Cell Proliferation and IgE-Associated Th2 Cytokine Responses. To examine the effect of oral feeding of rice seeds on allergen-specific T cell responses, $CD4^+$ T cells were isolated from the spleens of experimental and control mice and were stimulated *in vitro* with or without pollen allergen. Oral immunization with A1aB1b-Crp-1 and -2 rice seeds greatly suppressed the allergen-specific T cell proliferative responses when compared to those in the control mice (75% suppression, $P < 0.01$) (Fig. 2*D*). To further demonstrate the effect of oral feeding of transgenic seeds on the inhibition of allergen-specific $CD4^+$ T cell responses, we next examined levels of Th1 and Th2 cytokine synthesis (Fig. 3). The amounts of Th1 and Th2 cytokines produced in the culture supernatants of allergen-

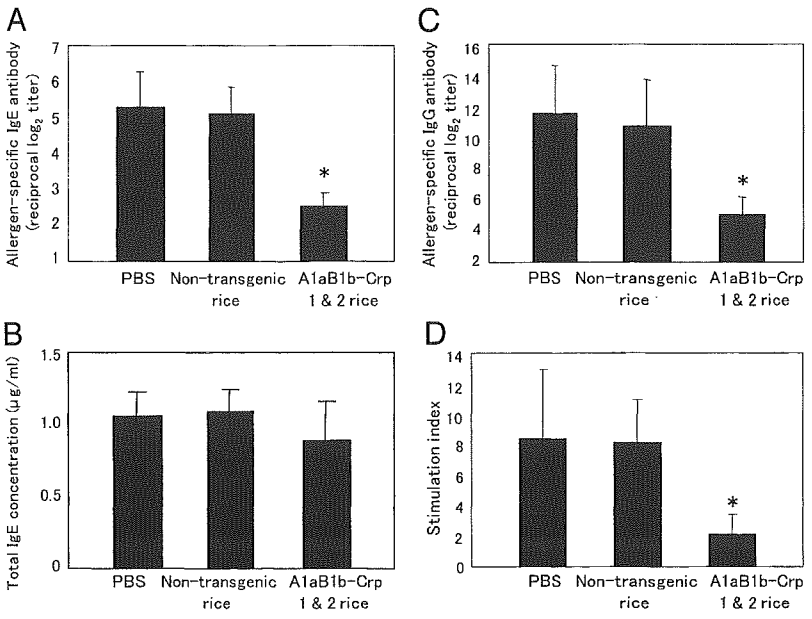

Fig. 2. Inhibition of allergen-specific serum IgE, IgG, and $CD4^+$ T cell responses by oral administration of A1aB1b-Crp-1 and -2 rice seeds. Levels of allergen-specific IgE (*A*), total IgE (*B*), and allergen-specific IgG (*C*) were examined in serum of mice fed with PBS, nontransgenic rice seeds, or A1aB1b-Crp-1 and -2 rice seeds before systemic challenge with total protein extracts of pollen. Allergen-specific splenic $CD4^+$ T responses (*D*) were expressed as stimulation index calculated as the ratio of [cpm of cells cultured in the presence of allergen]/[cpm of cells cultured in the absence of allergen]. Data are expressed as mean ± SD. *, $P < 0.01$ for the group of mice fed with A1aB1b-Crp-1 and -2 rice seeds in comparison with the group of mice fed with PBS or nontransgenic rice seeds.

stimulated $CD4^+$ T cells were measured by ELISA. In the control groups of mice fed with PBS or nontransgenic rice seeds, high quantities of Th2 cytokines associating with IgE-mediated immune responses such as IL-4, IL-5, and IL-13 were produced in the culture supernatants. In the group of mice fed with A1aB1b-Crp-1 and -2 rice seeds, levels of allergic reaction-associated cytokines IL-4, IL-5, and IL-13 were significantly lower than those of control groups of mice (85%, 86%, and 78% suppression, respectively; $P < 0.01$) (Fig. 3). Both Th2-associated IL-10 and Th1-associated IFN-γ cytokines were not induced significantly by this allergic response-inducing system. However, their levels were also decreased in the group of mice fed with A1aB1b-Crp-1 and -2 rice seeds (75% and 59% suppression, respectively; $P < 0.01$) (Fig. 3). The levels of IL-6 were not drastically changed between the three groups. These findings specifically demonstrate that oral immunization of A1aB1b-Crp-1 and -2 rice seeds effectively induced the state of oral tolerance where the inhibition of IgE-associated Th2 cytokines, including IL-4, IL-5, and IL-13, was achieved at the level of allergen-specific $CD4^+$ T cells.

Inhibition of Levels of Histamine Released in Serum of Mice Orally Fed with A1aB1b-Crp-1 and -2 Rice Seeds. Next, we examined the levels of serum histamine release, one of the inflammatory mediators released at degranulation of mast cells associating with IgE-mediated responses (35). Mice were bled within 10 min of the challenge with pollen allergen at week 7 of the experiment. The levels of histamine released in serum were as high as ≈60 ng/ml in mice fed with PBS or nontransgenic rice seeds (Fig. 4*A*). On the other hand, this high level of histamine release was dramatically reduced to ≈20 ng/ml in mice orally immunized with the A1aB1b-Crp-1 and -2 rice seeds (Fig. 4*A*). The results show that oral administration of rice seeds containing A1aB1b-Crp-1 and -2 protein was effective in the induction of oral tolerance for the inhibition of allergy-associated immune responses including those of Th2 cell-mediated IgE response and histamine release.

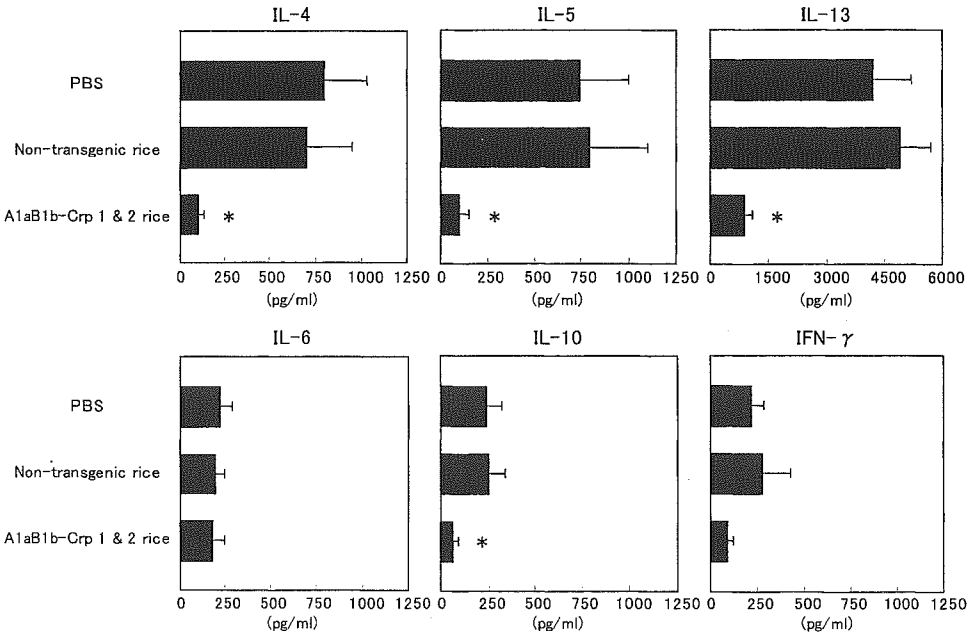

Fig. 3. Inhibition of allergen-induced Th2 cytokine production by splenic $CD4^+$ T cells isolated from mice fed with A1aB1b-Crp-1 and -2 rice seeds. Splenic $CD4^+$ T cells were cultured with or without total protein extracts of pollen as described earlier. Levels of Th1 and Th2 cytokines in cell-free culture supernatants of $CD4^+$ T cells were assayed by ELISA. Data are presented as mean ± SD. *, $P < 0.01$ for the group of mice fed with transgenic rice seeds in comparison with the group of mice fed with PBS or non-transgenic rice seeds.

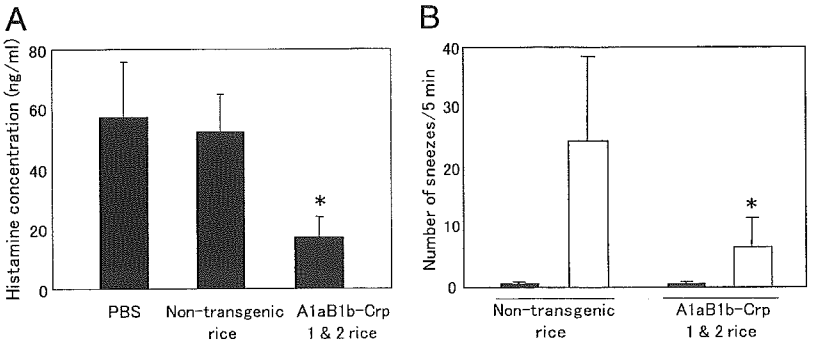

Fig. 4. Serum histamine levels (*A*) and the number of sneezes (*B*) were inhibited in the group of mice fed with A1aB1b-Crp-1 and -2 rice seeds. The number of sneezes was counted in the 5 min after the last nasal challenge at week 8 (white bars). Sham-challenged mice were nasally administered with 20 μl of PBS in the same manner (black bars). Data are expressed as mean ± SD. *, $P < 0.01$ for the group of mice fed with A1aB1b-Crp-1 and -2 seeds in comparison with the group of mice fed with nontransgenic rice seeds.

Inhibition of Pollen-Induced Allergic Symptoms in Mice Orally Fed with A1aB1b-Crp-1 and -2 Rice Seeds. To examine the effect of orally fed A1aB1b-Crp-1 and -2 rice seeds on the development of clinical symptoms of pollinosis, we adopted an experimental murine sneezing model (34). Mice were orally fed with nontransgenic or A1aB1b-Crp-1 and -2 rice seeds and i.p. presensitized with total protein extracts of pollen. Mice were then intranasally challenged with the pollen protein extracts. Significant nasal symptoms of sneezing developed in the group of mice fed with nontransgenic rice seeds (Fig. 4*B*). In contrast, the number of sneezes was reduced in the group of mice fed with A1aB1b-Crp-1 and -2 rice ($P < 0.01$) (Fig. 4*B*). Nasal challenge with PBS did not induce any nasal symptoms of sneezing (Fig. 4*B*). These findings demonstrated that oral administration of A1aB1b-Crp-1 and -2 rice seeds was effective in the induction of tolerance against pollen allergen leading to the inhibition for the development of allergic symptoms of sneezing in nasal tract.

Discussion

Adaptation of the concept of oral tolerance has been considered as a fundamental strategy for the development of immunotherapy for the prevention and/or treatment of allergic diseases (36). The mechanism of oral tolerance has not yet been precisely clarified; however, oral immunization of allergens is known to induce a state of systemic unresponsiveness to the administered allergens (36). To avoid unwanted anaphylactic reactions being elicited during the desensitization process using allergens, the use of T cell epitope peptides has been shown to be an attractive approach (4–8). T cell epitope peptides are incapable of binding to allergen-specific IgE antibody molecules on the surface of mast cells, so the administration of high doses of T cell epitope peptides is theoretically possible without inducing anaphylactic side effects (2). The efficacy of tolerance induction was shown to depend on the dose of allergens administered (7); thus, immunotherapy with T cell epitope peptides is expected to be both safe and effective in the treatment of allergic diseases (2). In this study, we developed transgenic rice plants expressing T cell epitope peptides in seeds and examined whether oral feeding of the transgenic rice seeds to mice could prevent the development of allergic responses against pollen allergens of Japanese cedar. Our results demonstrate that oral immunization of the transgenic rice seeds expressing A1aB1b-Crp-1 and -2 protein resulted in the generation of systemic unresponsiveness with a reduction of allergen-specific Th2-mediated IgE responses and histamine release.

It has been demonstrated that the direct production of short peptides such as T cell epitope peptides with lengths of 10–20 aa is difficult for most expression systems of eukaryotic and prokaryotic cells (37). Therefore, our efforts in this study were initially focused on the expression of Crp-1 and -2 T cell epitope peptides in transgenic rice seeds. We adopted a strategy in which Crp-1 and -2 peptides were expressed as parts of the soybean seed storage protein glycinin by inserting them into highly variable regions of acidic and basic subunits of glycinin A1aB1b (29, 30). The recombinant protein was successfully expressed in rice seeds; however, the maximum level of A1aB1b-Crp-1 and -2 accumulation (0.5% of total seed protein) was lower than that of A1aB1b (5% of total seed protein) (27). One possible explanation for this result is that the insertion of T cell epitopes into variable regions of A1aB1b potentially influences secondary structure formation or interaction between acidic and basic subunits in A1aB1b-Crp-1 and -2, which may cause the lower accumulation levels of A1aB1b-Crp-1 and -2. When expressed in transgenic rice seeds under the control of the glutelin *GluB-1* promoter, glycinin A1aB1b was synthesized as a preproglycinin and posttranslationally processed into acidic and basic subunits (27). The synthesized glycinin A1aB1b was localized in protein body II, in which ≈30% of glycinin was assembled with glutelin (27). In this study, A1aB1b-Crp-1 and -2 protein was synthesized as a precursor form and then posttranslationally processed into acidic and basic subunits in a similar manner to the glycinin A1aB1b (Fig. 1*C*) (27). These results suggest that A1aB1b-Crp-1 and -2 protein accumulated in protein body II, although there is possibility that the insertion of T cell epitope peptides into A1aB1b may affect the intracellular localization of A1aB1b-Crp-1 and -2 protein in the endosperm cells. When anti-Crp-1 peptide antibody was used as a probe, the precursor signal of A1aB1b-Crp-1 and -2 (56 kDa) was weaker than those obtained by the anti-glycinin and anti-Crp 2 peptide antibodies (Fig. 1*C*). These results might be explained by the difference in binding affinity of anti-Crp-1 peptide antibody to the Crp-1 peptide accumulated in two distinct forms, the precursor and mature acidic subunit.

Two regions of pollen allergens (p277–290 of Cry j I and p246–259 of Cry j II) have been identified as major T cell epitopes in BALB/c mice (15, 16). Previously, one of the major T cell epitope peptides, p246–259 of Cry j II, was chemically synthesized and was orally administered to mice before systemic challenge with Cry j II (15). It was shown that the Cry j II-specific IgE response was significantly decreased (74% suppression) in mice orally immunized with the synthetic T cell epitope peptide. Furthermore, both Th1 and Th2 cytokine production was inhibited in the group of mice fed orally with Cry j II peptide compared to the control group of mice fed with PBS (15). In the present study, to evaluate the efficacy of newly generated transgenic rice seeds expressing Cry j I and Cry j II T cell epitope peptides for the induction of systemic unresponsiveness to pollen allergens of Japanese cedar, a group of mice was fed with the transgenic rice seeds and two other groups of mice were orally administered with nontransgenic rice seeds or PBS. We chose total protein extracts of pollen as allergen for the systemic challenge of mice to assess the effectiveness of transgenic rice seeds for taking account of future applications in the clinical treatment of pollen allergy. We further thought that this challenge method has a benefit to examine bystander tolerance effects to additional T cell epitopes. This line of detailed investigation requires further study.

It has been reported that patients with Japanese cedar pollinosis exhibit a high titer allergen-specific IgE response (38). Allergen-specific IgE antibodies have been shown to play a major biological role for the induction of pollen-associated allergic responses (38,

39). In this study, pollen allergen-specific IgE levels were significantly decreased by oral feeding of transgenic rice seeds accumulating A1aB1b-Crp-1 and -2, whereas the levels of total IgE antibodies were similar among the three groups of mice (Fig. 2 *A* and *B*). In addition, oral administration of A1aB1b-Crp-1 and -2 seeds did not affect OVA-specific $CD4^+$ T cell proliferative responses (Fig. 5, which is published as supporting information on the PNAS web site). These results indicate that oral feeding of transgenic rice seeds induces pollen allergen-specific T cell unresponsiveness. Furthermore, it is important to note that glycinin-specific IgG and IgE antibodies were not detected in the sera of control and experimental groups of mice (data not shown).

The production of $CD4^+$ Th2-type cell derived allergen-specific cytokines, IL-4, IL-5, and IL-13, was dramatically inhibited by oral feeding of transgenic rice seeds (Fig. 3). These Th2-type cytokines were shown to be involved in the process of IgE production. IL-4 and IL-13 stimulate and regulate Ig class switching to IgE (39–41) and IL-5 drives the proliferation and differentiation of B cells into antibody-secreting plasma cells (42, 43). The inhibition of these IgE-associated cytokine responses is one of the important factors for the control of allergen-specific IgE synthesis. Our results show that successful inhibition of these cytokine responses offers effective oral immunization by A1aB1b-Crp-1 and -2 rice seeds for the suppression of IgE-mediated hypersensitive allergic reactions. The production of IL-10 was also inhibited in the group of mice fed with transgenic rice seeds, which is consistent with a previous report describing that IL-10 is not required for induction of oral tolerance to OVA (44). On the other hand, it was recently reported that the suppression of allergic diseases in allergen immunotherapy is associated with the increased levels of IL-10 (45, 46). This conflict might be caused by different experimental designs. Further studies are required to examine this controversial result on the role of IL-10 in the induction of oral tolerance.

Allergy-associated inflammatory mediators such as histamine released by mast cells are known to cause immediate symptoms of type-I allergy (35). Allergen-IgE complex formation on the surface of mast cells triggers degranulation of mast cells leading to the histamine release (35). Thus, the reduction of allergen-specific IgE antibody levels can be an effective strategy for the suppression of histamine release by mast cells. In our study, levels of both allergen-specific IgE antibody and serum histamine release were significantly reduced in the group of mice fed orally with A1aB1b-Crp-1 and -2 rice seeds compared with the control groups (Figs. 2*A* and 4*A*). These results suggest that oral immunization of A1aB1b-Crp-1 and -2 rice seeds is effective in the suppression of allergen-specific IgE responses, which further inhibit histamine release by blocking the formation of the allergen-IgE complex. In addition, using the experimental mouse model of pollen allergy, we have shown here that oral feeding of A1aB1b-Crp-1 and -2 rice seeds inhibits the development of nasal allergic symptoms (Fig. 4*B*). Our findings provide further evidence of a significant potential benefit of A1aB1b-Crp-1 and -2 rice seeds for the prevention of the development of IgE-mediated allergic symptoms without any signs of the anaphylactic side effects.

The seed-expression system possesses several advantages for the production of recombinant proteins, such as simplicity of administration, low risk of contamination with animal pathogens, and low cost for production and long storage at room temperature (17–22, 25). Here, we showed that the status of systemic unresponsiveness associated with the inhibition of allergen-specific Th2-type and IgE responses was achieved by oral feeding of recombinant protein containing Crp-1 and -2 T cell epitope peptides without any purification step. Therefore, rice seeds could serve as an effective and new vehicle for the mucosal delivery of pharmatheutical molecules. Further clinical trials will be required to extend our findings for the development of rice-based edible vaccines as a peptide immunotherapy for the control of allergy.

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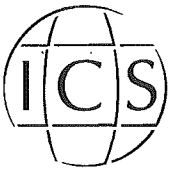

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Linkage between innate and acquired immunities at the mucosa

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Abstract. The epithelium covering mucosal tissues consists of epithelial cells (ECs) and intraepithelial lymphocytes (IELs), which constitutes the first line of defense against infection by microorganisms, through mucosal innate and acquired immunity. One of the principal roles of the mucosal epithelium is to form a strong physical barrier such as tight junction to prevent microbial penetration. While adhesion mechanisms between ECs have been extensively studied, there are few investigations on the mechanisms between ECs and IELs. Our findings indicate that a homophylic adhesion molecule termed epithelial cell adhesion molecule (Ep-CAM) is also expressed in IELs in addition to ECs, thus forming a physical interaction between ECs and IELs. In addition, we also characterized the expression and function of Toll-like receptors (TLRs) in mucosal epithelium using human corneal epithelial cells (HCEs). TLRs are mainly expressed by antigen presenting cells (APCs) and recognize pathogen-associated molecular patterns (PAMPs). However, responsiveness via TLR2 and TLR4 in HCEs was impaired, presumably due to restricted expression of the TLRs in the cytoplasm. These findings suggest that mucosal epithelium may create a state of tolerance in order to avoid unnecessary response to environmental and commensal antigens via TLRs. However, the mucosal surfaces that cover gut and respiratory lymphoid tissues such as Peyer's patch and nasopharynx-associated lymphoid tissue (NALT) are equipped with the gateway system which effectively uptake the outside antigens via M cells to initiate both the positive and negative immune responses. We recently identified M cell-like cells that are located within the villous epithelium and involved in antigen-transport into the lamina propria. Therefore, we designated them as villous M cells. These findings suggest that mucosal

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immune system is equipped with multiple layers of induction/suppression mechanisms for the regulation of mucosal innate and acquired immunity.

Keywords: IEL; TLR; M cell; Epithelial cell adhesion molecule; (Ep-CAM)

1. Adhesion molecules between intestinal epithelial cells (IECs) and intraepithelial lymphocytes (IELs)

Intestinal epithelial cells (IECs) are originated from and are maintained by a small number of pluripotent stem cells residing in crypts and show a life span of 2 to 4 days [1]. IECs have long been known to absorb ingested nutrients and transport them into the circulation. More recently, IECs have also been found to act as an important immunological barrier against pathogens or non-self-antigens. To form the physically firm defensive barrier, mucosal epithelial cells are welded with each other by a number of adhesion mechanisms. For example, tight junctions consisting of occludin [2] and the family of claudin [3,4] have been shown to play a central role in sealing the intercellular space between epithelial cells [5–7]. E-cadherin, another adhesion molecule expressed by epithelial cells, contributes to a homophilic adhesion in a Ca^{2+}-dependent manner [8]. Epithelial cell adhesion molecules (Ep-CAM), which offer a Ca^{2+}-independent adhesion, also show a homophilic adhesive function among epithelial cells [9,10]. The adhesive structure mediated by Ep-CAM among epithelium is localized at the neighboring of E-cadherin, and these molecules form a junction called intermediate junction.

In addition to mucosal epithelial cells, high numbers of lymphocytes termed intraepithelial lymphocytes (IELs) are also integrated within the mucosal epithelium and believed to form the immunological barrier together with epithelial cells. The majority of the IELs are $CD3^+$ T cells that bear either αβ or γδ T cell receptor (TCR) and approximately 80% of the $CD3^+$ T cells belong to the CD8 subset [11]. In mice, IELs are found to be present every six epithelial cells [11]. The ratio of IEL expressing αβ TCR (αβIEL) and γδIEL is quite different from that observed in other systemic T cells. Because IELs are located adjacent to IECs, cell-to-cell communications between IECs and IELs would be important to maintain the epithelial barrier. For example, IECs are capable of producing IL-7 [12] and IL-15 [13], which are important for the stimulation and development of γδIELs, while γδIELs express keratinocyte growth factor (KGF), which stimulates the growth of IECs [14]. In addition, γδIELs have unique features to regulate mucosal immune system. For example, γδIELs are involved in the induction and regulation of antigen-specific IgA because $TCR\delta^{-/-}$ mice induce significantly lower levels of immune responses in both mucosal and systemic sites when immunized with antigens in combination with a mucosal adjuvant [15]. Furthermore, γδIELs are likely to regulate oral tolerance, a state where induction of systemic immune response is suppressed [16].

However, minimal information is currently available regarding the cellular and molecular mechanisms underlying the physical cell-to-cell interaction between epithelial cells and IELs. Recently, $\alpha_E\beta_7$ integrin was found to mediate T cell adhesion to epithelial

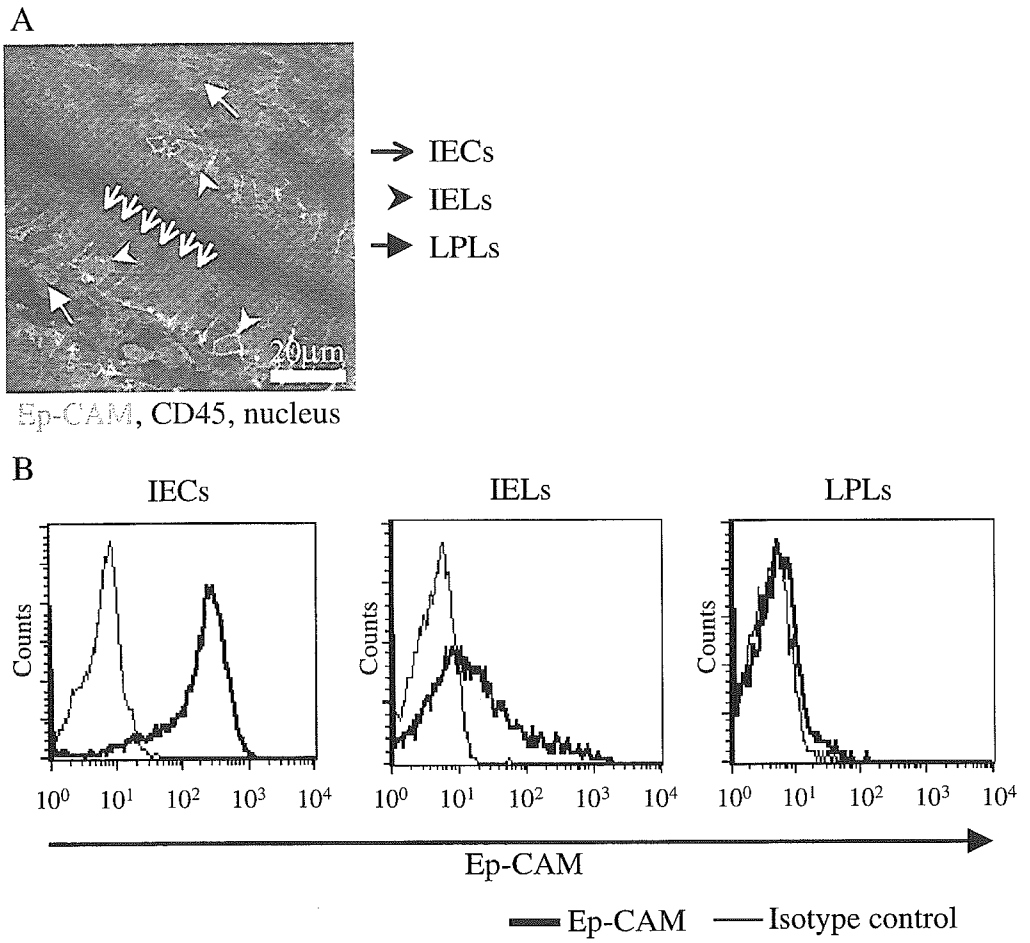

Fig. 1. Expression of epithelial cell adhesion molecule (Ep-CAM) in IECs, IELs and lamina propria lymphocytes (LPLs). Ep-CAM is expressed in $CD45^+$ IELs as well as IECs but not $CD45^+$ LPLs (A). Flow cytometric analysis also indicates that isolated IECs and IELs but not LPLs are reacted with anti-Ep-CAM monoclonal antibody (mAb).

cells through its binding to E-cadherin which was expressed selectively on epithelial cells [17–19]. In fact, the number of IELs was significantly decreased in α_E integrin-deficient mice [20]. These findings suggest that more complicated adhesive mechanisms are involved in the adhesion between IECs and IELs. Our novel findings indicate that Ep-CAM is also expressed by IELs as well as IECs and forms a homophilic adhesive structure between the two [21] (Fig. 1). Most recently, it was reported that IELs also expressed junctional adhesive molecules such as occludin and E-cadherin [22]. These suggest that adhesive mechanisms offered by IECs and IELs play an essential role in the constitution of a firm physical barrier at the mucosa.

2. Role of Toll-like receptors (TLRs) at the mucosa

The mucosal immune system has developed under the dual pressure for protecting the host from pathogenic infections and establishing symbiosis with otherwise harmless commensal microorganisms. Cells that constitute the first line of the protection through innate immunity are the epithelial cells that separate inner and outer body. In this regard,

several molecules such as Toll-like receptors (TLRs) [23], defensins [24,25], and nucleotide oligomerization domains (NODs) [26,27] have been identified and shown to play a role in the host protection against microbial infections. For example, TLRs, the mammalian homologues of *Drosophila* Toll, recognize pathogen-associated molecular patterns (PAMPs) such as cell wall components and nucleic acids, and signal to trigger anti-microbial innate immunity [23]. Currently, the TLR family consists of at least 11 members (TLR1–11). Among them, most investigated are TLR2 and TLR4. Both of the TLRs are expressed in IECs and contribute to the recognition of peptideglycans (PGNs) and lipoproteins of Gram-positive bacteria [28], and recognition of LPS [29], a cell wall component of Gram-negative bacteria, respectively.

We have focused on the role of TLRs in ocular mucosal immunity and have shown that human corneal epithelial cells (HCEs) express TLR2 and TLR4 proteins intracellularly, but not at the surface level, and therefore unresponsive to the respective TLR ligands, PGN and LPS [30] (Table 1). Similar results have also been obtained with IECs; IECs are poorly responsive to PGN and LPS due to their expression of TLR2 and TLR4 at quite low levels [31,32]. Taken together, these findings suggest that there will be a negative regulatory mechanism(s) for mucosal TLRs by which host is protected from undesired induction of inflammation in response to commensal microorganisms.

The impaired surface expression of TLR2 and TLR4 in the HCEs and IECs also raises the possibility that epithelial cells have a defect in the transport of TLRs to the plasma membrane. In this point, a recent study clearly demonstrated that MD2, an accessory protein of TLR4, is essentially required for the transport of TLRs to the cell surface as well as responsiveness of the cells to LPS [33]. As expected, IECs were found defective in the expression of MD2 [34]. Moreover, expression of TLRs was also shown to be regulated by an endoplasmic reticulum chaperone gp96 [35]. Thus, our current interest is aimed at determining whether or not HCEs express these expression regulatory molecules.

In addition to the regulation at surface expression level, cellular responsiveness to TLR ligands seems to be regulated at the level of signal transduction. Hornef et al. have provided evidence that intestinal crypt epithelial m-IC_{cl2} cells can internalize LPS in a clathrin-dependent pathway and respond to the LPS through cytoplasmic TLR4 [36]. This suggests a potential mechanism for intracellular recognition of TLR ligands by normal epithelial cells in an emergency such as pathogenic bacterial penetration. However, forced introduction of LPS into the HCEs resulted in no activation of NF-κB, the common TLR-signaling-associated transcription factor [30], suggesting that there may be a mechanism(s) by which signal transduction through TLRs is hampered. Accordingly, NF-κB-mediated inflammatory cytokine synthesis was not induced either. One explanation for the

Table 1
Expression of TLR2 and TLR4 proteins by human corneal epithelial cell line (HCE-T) and primary human corneal epithelial cells (HCEs)

Cells	TLR2		TLR4	
	Intracellular	Surface	Intracellular	Surface
HCE-T line	+	–	+	–
Primary HCEs	+	–	+	–

impaired responsiveness of the HCEs to LPS may be attributed to lack of gp96 in the HCEs since it is likely involved in the transport of TLR4 to the Golgi apparatus, the site where cytoplasmic TLR4 interact with internalized LPS [36]. Another explanation might be the presence of an inhibitor(s) for TLR-mediated signaling in the HCEs. Recently, Tollip, a Toll/IL-1R (TIR) domain-containing inhibitory protein [37], and TIR8/SIGIRR, single Ig IL-1R-related molecule [38], were identified as inhibitors for TLR signaling. Both of the inhibitors are highly expressed in IECs that poorly respond to LPS [34,38]. Moreover, mice deficient in TIR8/SIGIRR are more susceptible to dextran sodium sulfate (DSS)-induced colitis [39]. Thus, high expression of TLR-signaling inhibitors may limit TLR signaling and thereby control the TLR-mediated induction of inflammation at the mucosa.

In addition to TLR2 and TLR4, IECs were also shown to express TLR5, which recognizes bacterial flagellin at both apical and basolateral surfaces [40]. We are currently examining the expression as well as responsiveness in terms of the other TLRs in the HCEs. However, at least in the responsiveness, given that prolonged exposure of IEC lines to LPS or lipotechoic acid (LTA) leads not only to tolerance, but also to cross-tolerance to the other TLR ligands [41], the HCEs might be hyporesponsive to other PAMPs even if they express other members of TLRs.

Taken all together, these findings suggest that responsiveness of mucosal epithelial cells are negatively regulated by decreased surface expression of TLRs or increased intracellular expression of inhibitors or both in order to maintain the symbiosis with commensal microorganisms.

3. Antigen sampling sites at mucosal epithelium: Peyer's patch M cells and villous M cells

The uniqueness that distinguishes mucosal immunity from systemic immunity is characterized by the production of secretory IgA. Mucosa-associated lymphoid tissues (MALT) including Peyer's patches (PP) located in the small intestine have been considered to play a crucial role for the induction of antigen-specific mucosal immune responses and therefore called inductive site. With regard to the elicitation of specific mucosal immune responses, antigens must be transported across the epithelial layer of PP. Indeed, the follicle-associated epithelium (FAE) that lines mucosal lymphoid follicles contains M cells, specialized epithelial cells that are capable of uptaking foreign antigens. During more than 30 years from the discovery of M cells, some morphological, biochemical and biological features of M cells have been clarified. They have (I) a specific reactivity to the lectin *Ulex europaeus* agglutinin-1 (UEA-1), (II) short and irregular microvilli, (III) an endocytic activity to take up bacteria as well as macromolecules, (IV) a pocket structure, which allows a cluster of immunocompetent cells to be located in closer proximity to the lumen, and (V) low enzymatic activities of alkaline phosphatase and lysozyme [42]. Although it has been believed for a long time that M cells exist only in FAE, evidence have been provided that M cell-like cells are occasionally seen within villous epithelium adjacent to the lymphoid follicle [43] and UEA-1^+ cells are observed within the intestinal villous epithelium [44]. However, the functional properties of these cells remain unclear. Furthermore, our group recently demonstrated that antigen-specific IgA production was

observed in PP null mice [45]. Taken together, these findings imply that M cells capable of sampling antigens also exist in villous epithelium.

Recently, we found epithelial cells that were located within the small intestinal villous epithelium and showed characteristics similar to those observed in conventional M cells (Fig. 2), and thus designated them as villous M cells [46]. Interestingly, villous M cells were located in the tip regions of intestinal villi of not only wild type mice but also various PP null mice such as in utero LTβR-Ig-treated, $LT\alpha^{-/-}$, $TNF/LT\alpha^{-/-}$ and $Id2^{-/-}$ mice. These findings provide evidence that villous M cells develop independently of organized mucosal lymphoid tissues, although development of M cells has been thought to be associated with that of these tissues. Furthermore, our results suggest that villous M cells can serve as an additional gateway for the entry of pathogenic bacteria such as *Salmonella typhimurium* and *Yersinia pseudotuberculosis*, both of which are known to invade through M cells. In addition, production of humoral antigen-specific antibodies was normally induced in $TNF/LT\alpha^{-/-}$ mice, which lack PP and isolated lymphoid follicles (ILF), following oral administration of antigens such as recombinant *Salmonella* expressing the immunogenic C fragment of tetanus toxin. Similarly, M cells that are not associated with lymphoid follicles have been identified within respiratory epithelium in horse [47]. These findings also support the notion that M cells constantly exist in a variety of mucosal epithelium independently of association of the epithelium with lymphoid follicles, and play a role in the initiation of antigen-specific immune responses.

Another gateway for antigen sampling at the mucosa could be mucosal dendritic cells (DCs). Recent studies have clearly demonstrated that DCs residing within or adjacent to the intestinal villous epithelium take up luminal bacteria directly or by extending their arms across the epithelial layer, respectively [48,49].

Thus, various ways for antigen sampling may simultaneously occur at the entire mucosal surface and contribute to the induction of antigen-specific immune responses.

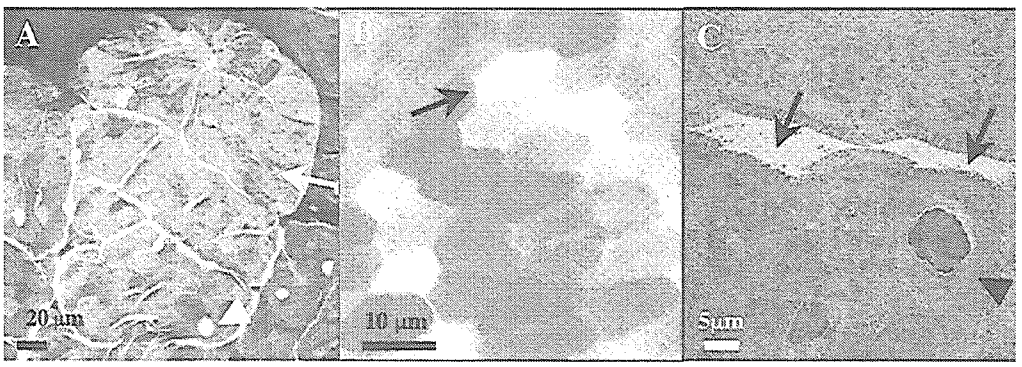

Fig. 2. Morphological characterization of small intestinal villous M cells in naïve BALB/c mice. (A) A confocal image of whole mount preparation of the small intestine stained with UEA-1-TRITC and WGA-FITC. Villous M cells are positive singly for UEA-1 (red, arrow), enterocytes singly for WGA (green), and goblet cells doubly for UEA-1 and WGA (yellow, arrowhead). (B) A light microscopic view of the whole mount preparation tested for alkaline phosphatase activity (red/pink) and alcian blue staining (blue). Villous M cells are doubly negative for alkaline phosphatase activity and alcian blue staining (white, arrow). (C) A transmission electron microscopic image of the villous epithelium. Villous M cells bear short stub-like microvilli at the luminal surface (arrows) and hold a mononuclear cell (arrowhead) in the pocket. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)