2.8. Data analysis

Differences between groups were assessed by Tukey's *t*-test for ELISA data and the log-rank (Mantel–Cox) test for survival analyses. *P* values < 0.05 were considered to indicate statistical significance.

3. Results

3.1. Establishment of rice-expressed botulinum type A neurotoxin vaccine

To increase the levels of high-molecular weight vaccine antigens, such as BoHc, that can be accumulated in rice grains, we constructed T-DNA vectors containing expression cassettes with four combinations of RNAi triggers specific for different storage protein genes together with a vaccine gene cassette (Fig. 1). Several independent transgenic rice lines were generated for each of the four types of MucoRice and the BoHc accumulation levels in seeds were determined by densitometry analysis of Western blots. For each of the four types of MucoRice, the plant line with the highest levels of BoHc antigen accumulated in the seed was selected and advanced to the T3 generation by self-crossing to obtain homozygous lines.

SDS-PAGE showed that the content of 13 kDa prolamin and/or glutelin in MucoRice BoHc lines were approximately half that of the WT (Fig. 2B). Examination of the expression levels of BoHc by densitometry analysis revealed that the expression level of BoHc in rice seeds suppressing both 13 kDa prolamin and glutelin A reached an average of 100 μg per seed, whereas rice seeds carrying the vector with no RNAi trigger obtained an average of 10 μg BoHc per seed. Seeds carrying vectors that suppressed 13 kDa prolamin alone or glutelin A alone obtained an average of 10 μg or 30 μg BoHc per seed, respectively. All further experiments were performed with MucoRice-BoHc derived from rice suppressing both 13 kDa prolamin and glutelin A.

When MucoRice-BoHc was extracted by PBS, densitometry analysis showed that an average 85% of all the MucoRice-BoHc was recovered in PBS (Fig. 2C). Because the MucoRice-BoHc was soluble in PBS, we were able to purify the protein from polished rice without the aleurone layer by using gel filtration (Fig. 2C). The yield of purified BoHc from the total amount of harvested rice seeds was approximately 68% (an average of 68 μg per seed). The level of LPS contamination in purified MucoRice-BoHc was less than 10 endotoxin units/mg protein, which was equivalent to that in highly purified rBoHc from the *E. coli* expression system.

3.2. MucoRice-BoHc accumulated in the cytoplasm between protein bodies in rice seeds

Because expression of BoHc was under the control of the 13 kDa prolamin-specific promoter as a rice-seed-specific promoter (Fig. 1), we examined the location of BoHc expression in MucoRice-BoHc seeds. The results of immunohistochemistry showed that BoHc accumulated in the endosperm cells under

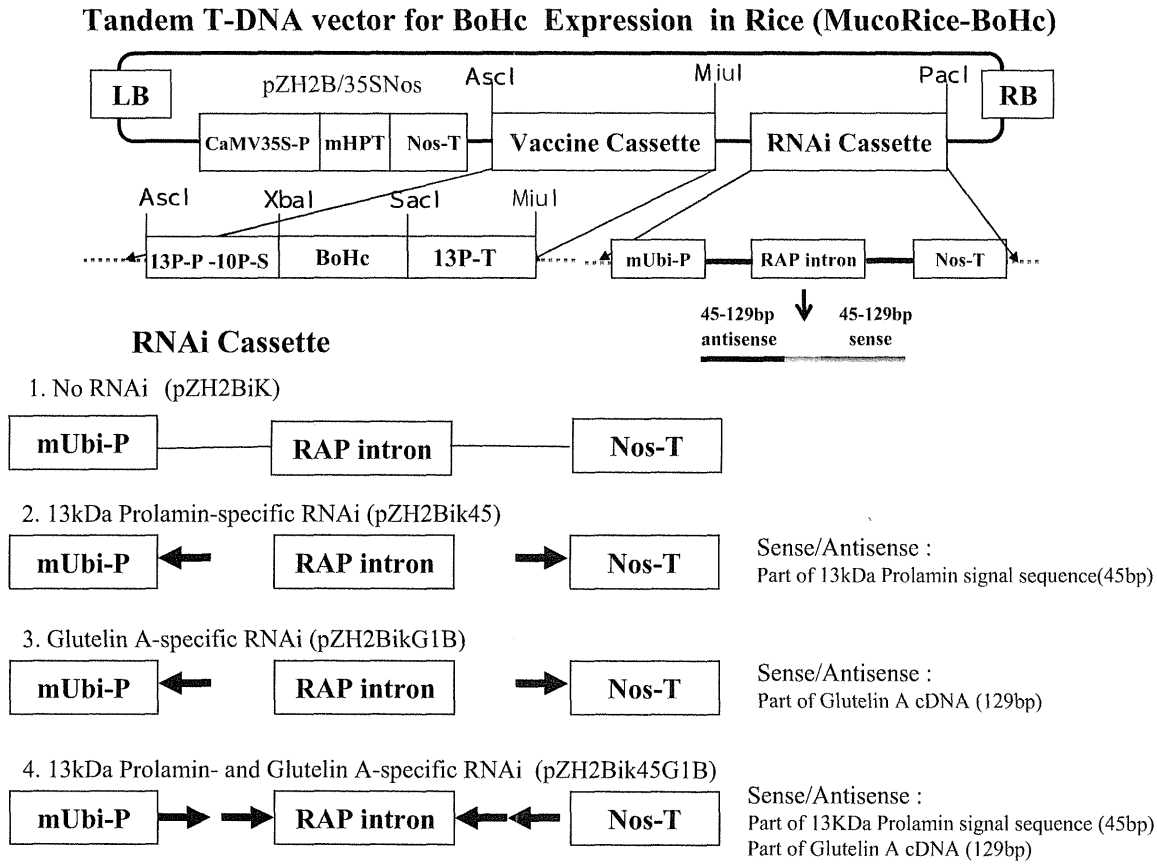

Fig. 1. T-DNA plasmids for RNAi suppression of storage proteins and overexpression of BoHc in rice seed. We constructed a tandem T-DNA plasmid containing a BoHc overexpression cassette with BoHc vaccine antigen sequences controlled by the promoter of rice 13 kDa prolamin, and a combination cassette of RNAi triggers for suppression of major rice endogeneous storage proteins, 13 kDa prolamin and/or glutelin controlled by the ubiquitin promoter. CaMV35S-P, cauliflower mosaic virus 35S promoter; mHPT, mutant hygromycin phosphotransferase; 13P-P, 13 kDa prolamin promoter; 10P-S, signal sequence of 10 kDa prolamin; 13P-T, 13 kDa prolamin terminator; Nos-T, nos terminator; RAP intron, rice aspartic protease intron; Ubi-P, ubiquitin promoter; LB, T-DNA left border; RB, T-DNA right border.

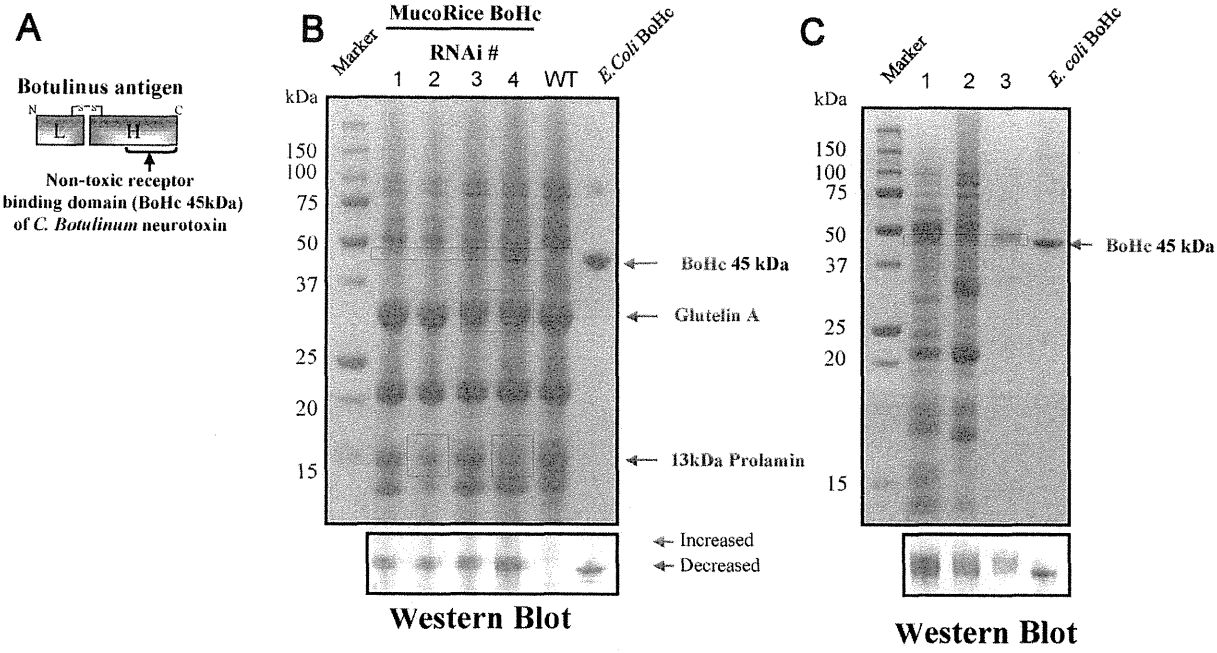

Fig. 2. Expression and purified MucoRice-BoHc. (A) Conceptual scheme of BoHc antigen expressed in MucoRice or *E. coli*. (B) SDS-PAGE analysis comparing protein expression among MucoRice-BoHc lines containing various T DNA plasmids: #1, no RNAi suppression; #2, RNAi suppression of 13 kDa prolamin; #3, RNAi suppression of glutelin A; #4, RNAi suppression of 13 kDa prolamin and glutelin A. WT indicates wild-type rice, and *E. coli* BoHc indicates recombinant BoHc from *E. coli* system. The results showed predominant expression of MucoRice-BoHc with a molecular weight of approximately 48 kDa (red arrowhead, *E. coli* derived BoHc with 45 kDa). Endogenous rice proteins (blue arrows, glutelin A and 13 kDa prolamin) were reduced in MucoRice-BoHc with RNAi suppression of glutelin and/or 13 kDa prolamin compared with levels in non-transformed WT rice. Western blotting revealed that a transgenic protein of 48 kDa specifically reacted with anti-BoHc Ab. (C) The samples were analyzed by SDS-PAGE and Western blot with rabbit anti-BoHc. 1, extract from MucoRice-BoHc transgenic #4 seed (with RNAi suppression of both 13 kDa prolamin and glutelin A) powder produced by using PBS; 2, extract from the MuocRice-BoHc #4 seed powder produced by re-extracting the PBS extract with SDS-sample buffer [2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 50 mM Tris–HCl (pH 6.8)]; 3, purified MucoRice-BoHc, which was isolated from PBS-extract of the MucoRice-BoHc #4 seed powder by using a gel filtration on Sephadex G-100 column. rBoHc indicates BoHc purified from *E. coli*.

the aleurone layer in MucoRice-BoHc seeds, whereas immune-reactivity was absent in wild-type rice seeds (Fig. 3A).

Furthermore, immune-electron-microscopy analysis of MucoRice-BoHc seeds showed that the vaccine antigens were unexpectedly expressed in cytoplasm at the interspace between PB-I and PB-II (Fig. 3B). It is interesting to note that destruction of PB-II was found in MucoRice-BoHc seeds but not WT rice. Because expression level of rice storage proteins including 13 kDa prolamin and glutelin A in MucoRice-BoHc seeds were suppressed (Fig. 2B), the RNAi knockdown on MucoRice system most likely accounts for the abnormal storage organelle formation.

3.3. *Nasal MucoRice-BoHc induces not only systemic but also mucosal antigen-specific Ab immune responses*

To examine whether purified MucoRice-BoHc antigen maintained sufficient immunogenicity to induce a protective Ab response, mice were nasally immunized with PBS (vehicle control) or 100 μg of purified MucoRice-BoHc or rBoHc from an *E. coli*–BoHc expression system. Antigen-specific Ab responses were assessed by using *E. coli*-derived BoHc as a coating antigen for ELISAs. After nasal immunization, the levels of BoHc-specific IgG Ab titers were not significantly different between mice nasally immunized with MucoRice-BoHc and those nasally immunized with *E. coli*-derived rBoHc (Fig. 4A). However, a detectable level of rBoHc-specific secretory IgA (SIgA) Ab titers was found in the nasal washes of mice that were nasally immunized with MucoRice-BoHc (Fig. 4A).

To confirm whether the immunogenicity of MucoRice-BoHc was sufficient for the induction of antigen-specific mucosal immunity, mice were nasally immunized with 25 μg of purified MucoRice-BoHc with or without CT or nontoxic chimera mCTA/LTB as mucosal adjuvants. Both nasal MucoRice-BoHc with CT and nasal MucoRice-BoHc with mCTA/LTB induced brisk rBoHc-specific serum IgG and nasal IgA Ab immune responses (Fig. 4B).

3.4. *Nasal MucoRice-BoHc induces protective immunity against botulinum neurotoxin*

Next, a challenge test with BoNT/A was performed on all BoHc-vaccinated mice and control mice to examine the quality of BoHc-specific Ab induced. Mice that were vaccinated with 100 μg of purified MucoRice-BoHc or *E. coli*-derived rBoHc without the presence of mucosal adjuvant, but not the control mice, were partially protected against the high lethal dose (100 ng, 1.1×10^4 i.p. LD_{50}) of intraperitoneally injected BoNT/A (Fig. 5A). The mice that were nasally immunized with 25 μg of purified MucoRice-BoHc with CT or mCTA/LTB were completely protected against the extraordinarily high lethal dose (500 ng, 5.5×10^4 i.p. LD_{50}) of intraperitoneally injected BoNT/A, since the mice nasally immunized with 25 μg of MucoRice-BoHc or PBS only failed to protect against the same amount of toxin (Fig. 5B). In the group of mice that were nasally immunized with 25 μg of MucoRice-BoHc with CT or mCTA/LTB, there were no clinical signs of toxin-associated disease over a 1 wk observation period following the BoNT/A challenge test.

4. Discussion

One of our major goals was the adoption of RNAi technology to advance the MucoRice system by co-introduction of antisense sequences specific for endogenous rice storage proteins. We used

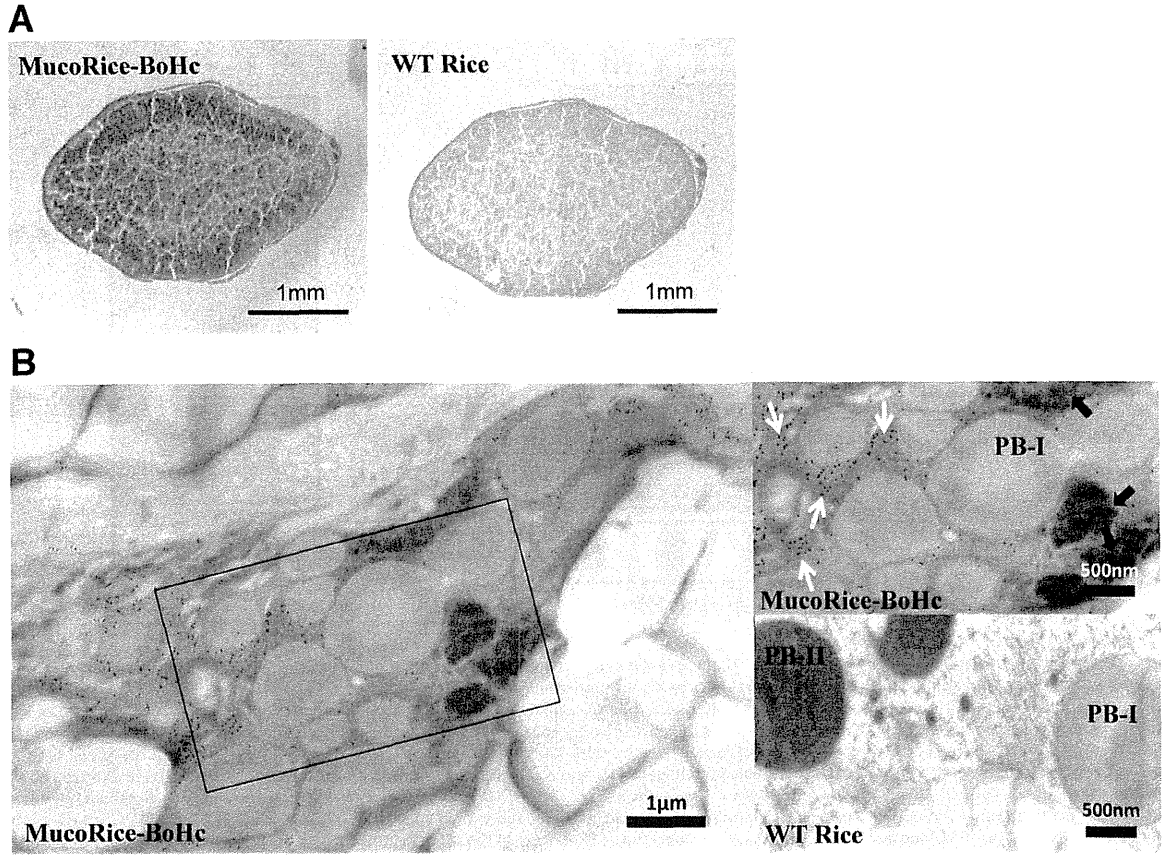

Fig. 3. Immunohistochemical and immune electronmicroscopic analyses of MucoRice-BoHc seed. (A) Immunohistochemical analysis showed that BoHc had accumulated in the whole MucoRice-BoHc seed, whereas it was not detected in a WT-rice seed. (B) Immune electronmicroscopy of MucoRice-BoHc showed that BoHc in the endosperm cells of MucoRice-BoHc was observed as black spots (white arrows). BoHc was predominantly localized in cytoplasm as the interspace between PB-I and PB-II. Destruction of PB-II structure was found in MucoRice-BoHc seed (black arrows) when compared with WT rice seed.

a high-molecular-weight protein vaccine antigen – the 45 kDa C-terminal half (BoHc) of botulinum neurotoxin [13,17] – to showcase the practicality of using this system to improve the capacity for vaccine antigen accumulation in rice seed. To optimize the choice of endogenous storage protein to suppress, we performed RNAi suppression of rice 13 kDa prolamin, glutelin A, or both by altering the sequences included in RNAi cassette in the T-DNA vector and then assessed the level of expression of the antigen MucoRice-BoHc. SDS-PAGE and western blot analysis revealed that suppressing both 13 kDa prolamin and glutelin A greatly improved the production of high-molecular-weight vaccine antigen, BoHc (100 μg/seed) when compared the T-DNA vector without RNAi (10 μg/seed) (Fig. 2B). Thus, introduction of the RNAi technology into the MucoRice vaccine antigen expression system allowed a high-molecular-weight vaccine antigen to be expressed in MucoRice with high yields.

Another unique feature of the BoHc antigen produced by using the advanced MucoRice system was its water-solubility. Because proteins in PBs are not soluble in water [10,11], the unique and advanced property of MucoRice-BoHc comes from its expression in cytoplasm between PB-I and PB-II of endosperm cells in rice seed (Fig. 3B). Because we use prolamin promoter and signal for targeting BoHc to PB-I or PB-II in rice endosperm cells, we cannot explain logically why the vaccine antigen accumulated in cytoplasm and not in PBs. However, BoHc antigen location and level of accumulation might be attributed to abnormal storage organelle formation including destruction of PB-II structure caused by the knock-down of glutelin A in the advanced MucoRice system (Fig. 3B). The MucoRice-BoHc from rice powder of polished rice could be easily dissolved in and extracted from PBS and could be purified by a single-step gel filtration without endotoxin contamination. Thus, the high expression level and PBS-solubility of MucoRice-BoHc confer an economic advantage over vaccine products that are expressed in bacteria or other plant systems.

SDS-PAGE analysis indicated that the molecular weight of MucoRice-BoHc (48 kDa) was slightly higher than that of rBoHc from *E. coli* (45 kDa) (Fig. 2B and C). Because there are nine glycosylation-sequon (N-X-T/S) positions in the sequence of BoHc [20], we considered that several sugar chains attach to MucoRice-BoHc at these positions but not to rBoHc from *E. coli*. SDS-PAGE, and Western blot analysis detected a least 3–4 bands corresponding to MucoRice-BoHc, whereas *E. coli*-derived rBoHc was detected as a single band only (Fig. 2B and C). These results suggest that MucoRice-BoHc is a mixture of fully and partially glycosylated protein.

We next examined whether MucoRice-BoHc was highly immunogenic when compared with *E. coli*-derived rBoHc after nasal administration in mice. When mice were nasally immunized with 100 μg of purified MucoRice-BoHc or *E. coli*-derived rBoHc, there are no statistical differences in BoHc-specific IgG immune responses and protective immunity against high lethal dose of a neurotoxin challenge test between mice immunized with MucoRice-BoHc and *E. coli*–derived rBoHc. Taken together, these results demonstrate that the quality of the toxin neutralization

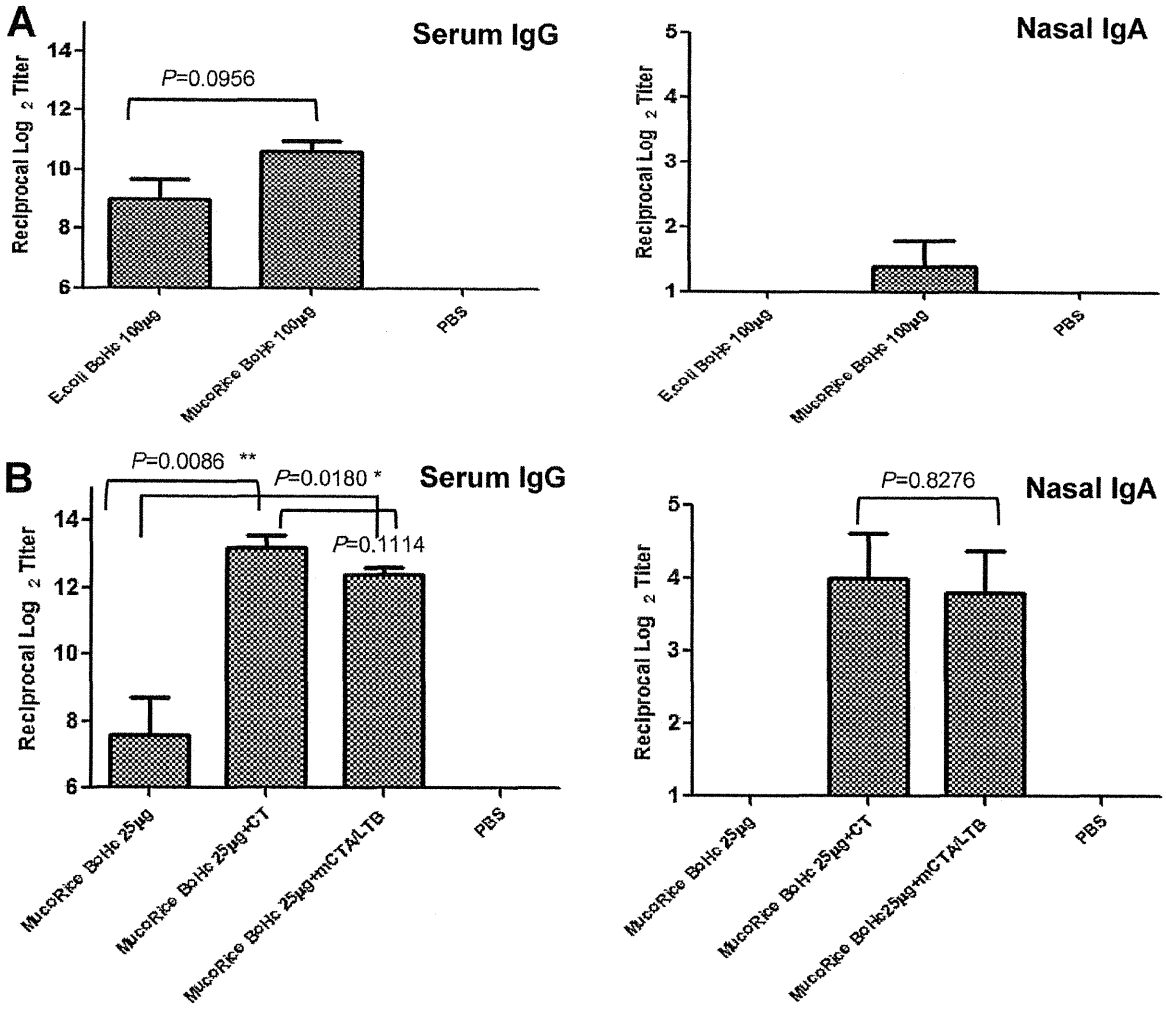

Fig. 4. Immunogenicity of purified MucoRice-BoHc produced by using advanced MucoRice system. (A) Mice were nasally immunized with 100 μg of purified MucoRice-BoHc alone on 3 occasions at 1-week intervals and compared with mice that were nasally immunized with same dose of recombinant BoHc prepared by means of a standard *E. coli* system (*E. coli*-BoHc). (B) In a separate study, mice were immunized with nasal vaccine containing 25 μg purified MucoRice-BoHc with or without mucosal adjuvant cholera toxin (1 μg) or nontoxic chimera adjuvant (mCTA/LTB, 10 μg) on 3 occasions at 1-week intervals. Data are expressed as means ± standard deviation ($N = 10$). The experiments were conducted three times. $^{*}P < 0.05$; $^{**}P < 0.01$.

Ab activity induced by MucoRice-BoHc was comparable to that induced by *E. coli*-derived rBoHc and that the immunogenicity of the MucoRice-BoHc and *E. coli*-derived rBoHc mucosal vaccines was equivalent.

To examine whether MucoRice-BoHc could induce high levels of mucosal immune response when nasally administered together with mucosal adjuvant, mice were immunized with nasal vaccine composed of 25 μg of MucoRice-BoHc and mucosal adjuvant CT or mCTA/LTB. As one might expect, antigen-specific serum IgG and nasal IgA Ab responses were rapidly induced in mice immunized with nasal MucoRice-BoHc plus adjuvant. Further, there was no difference between the antigen-specific immune responses induced by nasal MucoRice-BoHc administered with CT or mCTA/LTB. Native CT is a potent enterotoxin and induces high total and vaccine-specific immunoglobulin E responses; however, use of the mCTA/LTB chimera adjuvant did not have the same effect in our previous study [15]. To confirm the quality of BoHc-specific antibodies induced by MucoRice-BoHc plus CT or mCTA/LTB, we performed a challenge test on vaccinated mice by using an extraordinarily high lethal dose (5.5×10^4 mouse i.p. LD_{50}) of intraperitoneally injected BoNT/A. Regardless of the adjuvant used, the immunized mice survived after the challenge, indicating that they had gained full protection from the neurotoxin. In contrast, the non-immunized mice died within 12 h. These results suggest that MucoRice-BoHc with nontoxic chimera adjuvant CTA/LTB has the potential to be used as a promising nasal vaccine against botulism.

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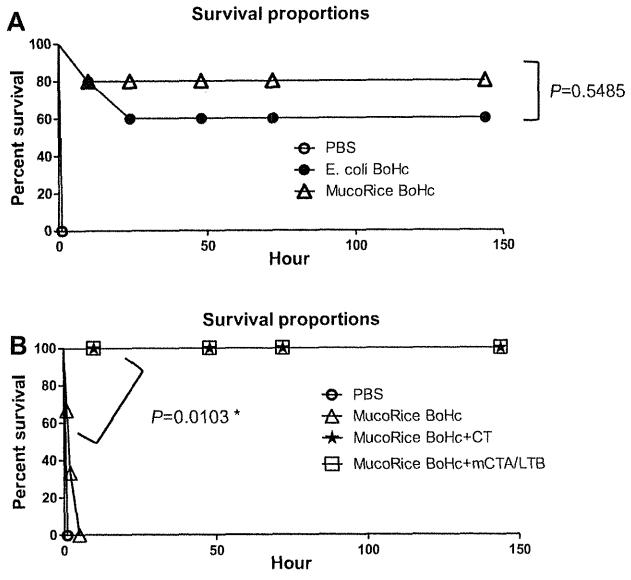

Fig. 5. Induction of protective immunity the against neurotoxin in mice immunized with nasal vaccine containing MucoRice-BoHc and CT or nontoxic mCTA/LTB. (A) Mice immunized with 100 μg of purified MucoRice-BoHc or *E. coli*-derived rBoHc were challenged with a high lethal dose (100 ng, 1.1×10^4 mouse i.p. LD_{50}) of intraperitoneal injection of BoNT/A. (B) Mice immunized with 25 μg of purified MucoRice-BoHc together with CT or mCTA/LTB were challenged with an extraordinarily high lethal dose (500 ng, 5.5×10^4 mouse i.p. LD_{50}) of intraperitoneal injection of BoNT/A. Immunization conditions were described in Fig. 4A and B. The experiments were conducted three times. $^*P < 0.05$; $^{**}P < 0.01$.

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Reviews • POST SCREEN

Immune regulation and monitoring at the epithelial surface of the intestine

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The intestinal enterocytes and other epithelial cells create physical barriers, including tight junctions and mucus layers. These cells also actively transport antibodies across the epithelium and simultaneously produce antimicrobial peptides and enzymes. These functions maintain intestinal homeostasis by allowing the selective absorption of nutrients and simultaneously preventing pathogenic infections. Recent evidence has revealed that both host-derived factors (e.g., cytokines) and gut environmental factors (e.g., commensal bacteria, dietary materials, and their metabolites) regulate the physical and immunological functions of the epithelium. Understanding the interactions between host cells and these environmental factors should help us to develop new strategies to prevent and treat immune diseases of the intestine.

The surface of the gastrointestinal tract is covered by a single layer of epithelium that separates the outside world from interstitial tissues. The intestinal epithelium is mainly composed of absorptive enterocytes (ECs) but also includes enteroendocrine, goblet, and Paneth cells [1]. Cross-communication among these cells enables the selective absorption of nutrients while simultaneously preventing the penetration of antigens and pathogens. The defense against pathogenic materials is at least partly achieved by the physical barriers of the epithelium, which include tight junctions and mucus layers. A large number of pathogens disrupt these barriers to access deeper tissues for dissemination [2,3]. The barriers also contribute to the establishment and maintenance of mucosal homeostasis. Indeed, a leaky intestinal barrier is one of the characteristics of chronic intestinal inflammatory diseases, such as inflammatory bowel disease and celiac disease [4,5].

Intestinal tissues also show intense immunological activity, and ECs contribute to the intestinal immune system by transporting and processing antibodies and associated antigens, by producing immunologically functional molecules, and by interacting with immunocompetent cells in the intestine [6]. Accumulating evidence has revealed that both host-derived factors (e.g. cytokines) and gut environmental factors (e.g. commensal bacteria, dietary materials, and their metabolites) engage in molecular crosstalk with the intestinal epithelium and affect intestinal barrier function and immune responses [7,8]. In this review, we focus on the immunological functions of ECs in the intestine and their regulation by commensal bacteria and dietary materials.

Physical barriers at the intestinal epithelium

Tight junctions

ECs provide a physical barrier to prevent the paracellular transport of luminal antigens and pathogens. Tight junctions are multifunctional complexes that are crucial for the maintenance of barrier integrity because they form a seal between adjacent ECs [9]. The tight junction regulates the absorption of nutrients, ions, and water while preventing the entry of pathogens into the host.

Tight junctions are composed of numerous interacting cellular proteins, including claudin, occludin, and zonula occludens (ZO) proteins (Fig. 1). Claudin and occludin are transmembrane proteins that seal the paracellular space between adjacent ECs. Among

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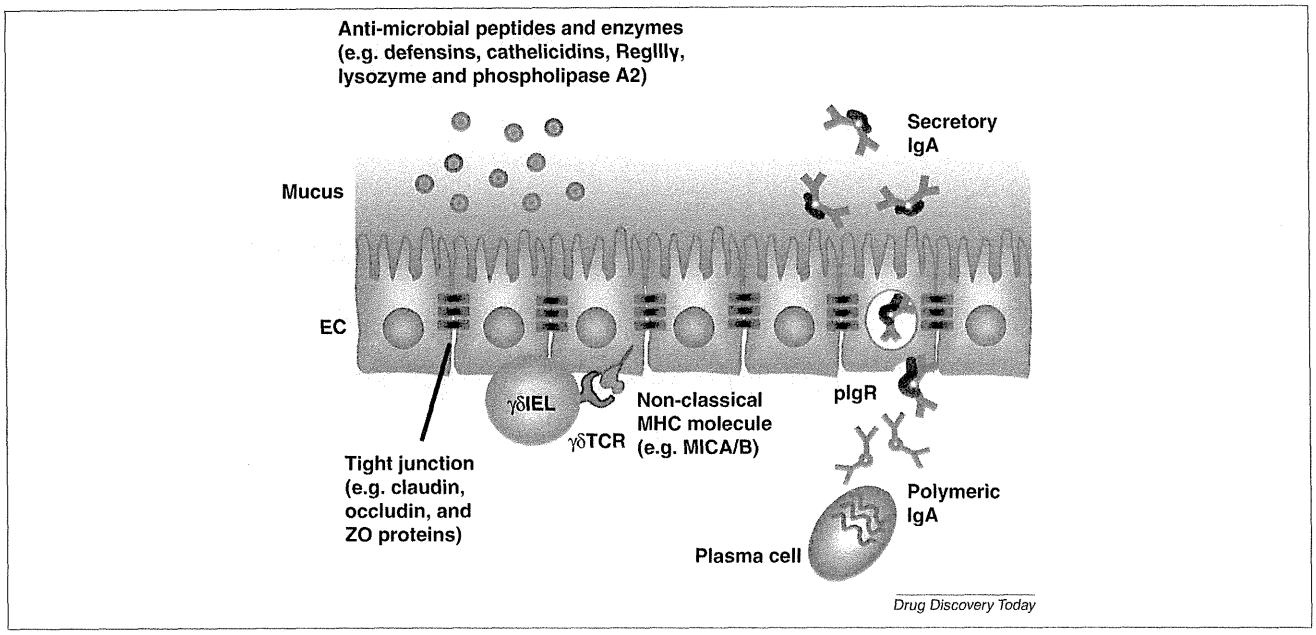

FIGURE 1

Physical and immunological barriers mediated by ECs. ECs (including Paneth cells) produce several molecules that create physical barriers in the intestine. They also produce antimicrobial peptides and enzymes, such as defensins, cathelicidins, RegIIIγ, lysozyme, and phospholipase A2 to kill the bacteria and establish a mucus layer to prevent bacterial attachment to the ECs. Tight junctions among ECs prevent bacterial penetration between the cells. ECs also have immunological functions. They express polymeric immunoglobulin receptor (pIgR), which binds and transports polymeric IgA produced from plasma cells into the intestinal lumen. ECs exposed to stresses (e.g. infection or cancer) express non-classical MHC molecules (e.g. MICA/B). MICA/B acts as a ligand for γδ T cell receptors, which are uniquely expressed on intraepithelial lymphocytes (γδIELs). *Abbreviations*: EC, enterocytes; MHC, major histocompatibility complex; ZO, zonula occludens.

the various types of claudins, claudin-1, -2, -3, -4, -5, -7, -8, -12, -15, -18, -20, and -23 are expressed in the intestinal epithelium [10,11]. ZO proteins are adaptors that connect transmembrane proteins; in particular, ZO-1 interacts with the claudin proteins and with F-actin in the intestinal ECs [12,13].

The physical barriers created by ECs are at least partly regulated by the immunological stimulation provided by commensal bacteria and dietary materials. Indeed, commensal and probiotic bacteria, their metabolites, food extracts, and dietary materials (e.g. fatty acids, polysaccharides, and flavonoids) have been shown to promote intestinal barrier integrity by increasing the expression of tight junction proteins [10].

Mucus

The mucus layer has been recognized as an important component in the intestine (Fig. 1). Mucin 2 (MUC2), a large glycoprotein characterized by variable O-linked glycans, is abundantly expressed by goblet cells located in the intestinal epithelium [14]. Generally, mucus can be divided into two layers. Although both layers have similar protein composition, the outer mucus layer is loose, whereas the inner mucus layer adheres firmly to the surface of the ECs. The firm mucus in the inner layer is an efficient barrier against pathogens [15]. In addition to the physical and biological barrier function of mucus, mucus also ensures the concentration of antimicrobial peptides and IgA antibodies at the surface of ECs. As similar to tight junctions, mucus expression is regulated by commensal bacteria, and the mucus layer of germ-free mice is thicker than that of specific pathogen-free mice [15].

Production of antimicrobial molecules at the epithelium

Antimicrobial peptides

The epithelium also secretes a variety of antimicrobial peptides [e.g. defensins, cathelicidins, and RegIIIγ (Fig. 1)]. The production of these peptides is mainly mediated by ECs and Paneth cells [16]. Paneth cells reside at the base of the crypt regions of the intestine, where they constitutively produce α-defensins. This does not require bacterial stimulation, because Paneth cells produce normal amounts of α-defensin in germ-free mice [17]. By contrast, ECs require microbial stimulation for the production of β-defensins [16]. ECs also produce cathelicidin, the expression of which is regulated by short-chain fatty acids produced when polysaccharides are metabolized by fermenting bacteria [18]. Both defensins and cathelicidin are cationic small peptides that exhibit antimicrobial activity by damaging and permeabilizing the bacterial cell membrane by pore formation [19].

RegIIIγ is a C-type lectin produced by ECs and Paneth cells in the ileum, where it kills Gram-positive bacteria by binding to surface-exposed carbohydrate moieties of peptidoglycans [20]. Commensal bacteria, especially Gram-negative bacteria, induce RegIIIγ expression on ECs, and a recent study demonstrated that MyD88 intrinsically expressed on ECs controls the production of RegIIIγ, which establishes the physical separation between the microbiota and the intestinal epithelial surface [21].

Unlike RegIIIγ, which specifically targets Gram-positive bacteria, bactericidal and/or permeability-increasing protein (BPI) shows antimicrobial activity against Gram-negative bacteria. The high affinity of BPI for lipopolysaccharide (LPS) leads to the

destabilization of the outer membrane of Gram-negative bacteria and also neutralizes LPS-induced inflammation [22].

Antimicrobial enzymes

Antimicrobial activity is also mediated by bacteriolysis enzymes (e.g. secretory phospholipase A2 and lysozyme). Phospholipase A2 is a small enzyme produced by Paneth cells that degrades bacterial phospholipids and subsequently disrupts the integrity of Gram-positive and -negative bacteria [23]. Phospholipase A2 enzyme activity is normal in the intestine of germ-free rats [24], but caloric restriction increases the gene expression of lysozyme and phospholipase A2 [25]. Therefore, it is likely that nutritional conditions rather than commensal bacteria regulate the activity of these antimicrobial enzymes in the intestine. Lysozyme is produced by Paneth cells and ECs. Its bactericidal activity derives from its cleavage of the glycosidic linkage between *N*-acetylglucosamine and *N*-acetyl muramic acid of peptidoglycan. Because Gram-positive bacteria express more peptidoglycan than Gram-negative bacteria, lysozyme acts preferentially on Gram-positive bacteria.

Transport of antibodies through ECs

IgA transport mediated by polymeric immunoglobulin receptors

One function of the epithelial immune barrier is to transport antibodies across the barrier. ECs express polymeric immunoglobulin receptors (pIgR) for the transport of polymeric forms of IgA (pIgA) and IgM (pIgM) in the basal-to-apical direction in association with an extracellular proteolytic fragment of the pIgR (known as the secretory component) [26]; together, the IgA and the secretory component form secretory immunoglobulin A (S-IgA). After S-IgA is secreted into the intestinal lumen, it inhibits adherence of pathogens to host ECs in the intestine and neutralizes pathogenic toxins by binding to their biologically active sites (Fig. 1) [27]. Additionally, IgA is able to exclude antigens and pathogens from the intestinal secretions while it is transported through ECs, and it also prevents viral replication inside ECs [28,29].

In addition to the function of S-IgA in the immunosurveillance, several lines of evidence demonstrate that S-IgA has a key role in preventing the penetration and/or growth of commensal bacteria [30]. These functions of S-IgA achieve the immune responses against commensal bacteria restricted in the intestinal but not systemic immune compartments in normal mice, while IgA-deficient mice exhibited systemic IgG responses against commensal bacteria [31–33]. A recent study also demonstrated that, in the absence of IgA, commensal bacteria-derived stimulation induced the increased expression of interferon-regulated genes in the ECs for the compensatory immunosurveillance with simultaneous reduction of lipid metabolism-related Gata4-regulated genes, which resulted in the lipid malabsorption and decreased lipid deposition [34]. Thus, S-IgA mediates the regulation between ECs and commensal bacteria, which is important not only for the maintenance of immunological homeostasis but also for metabolism [34].

Neonatal Fc receptor for IgG transport

Another receptor for immunoglobulin is the neonatal Fc receptor for IgG (FcRn). Although early studies in rodents indicated that FcRn was responsible for the passive acquisition of IgG neonatally, subsequent studies indicated that FcRn is also expressed by adult human epithelium and antigen-presenting cells in the intestine and thus is not strictly limited to neonatal life [35]. Unlike pIgR mentioned above, human FcRn binds IgG and the transport pathway is bidirectional, both apical to basal and basal to apical [36]. The bidirectional transport of IgG enables retrieval of intestinal antigens in a complex with IgG into the intestinal lamina propria, where the antigen and/or IgG complexes are subsequently taken up by antigen-presenting cells to prime T cell responses [37].

Intraepithelial T lymphocytes

The epithelium also includes lymphocytes that are commonly termed intraepithelial lymphocytes (IELs) [38]. IELs reside between the basolateral surfaces of ECs, and one IEL occurs for every 4–10 ECs in the small intestine and for every 30–50 ECs in the large intestine.

Most IELs are T cells. As similar to T cells observed at other sites (e.g. spleen and intestinal lamina propria), some portions of IELs express αβ T cell receptors and act as cytotoxic T lymphocytes by recognizing antigenic peptides presented by classical major histocompatibility complex (MHC) molecules on pathogenic ECs (e.g. microbe-infected cells) and killing them by producing cytotoxic molecules (e.g. perforin and granzymes) [38]. Other IELs express the γδ T cell receptor (and are therefore known as γδIELs) and show minimal pathogen-specific activity [38,39]. The innate immune function of γδIELs enables the rapid removal of infected ECs. To recognize the infected ECs, non-classical MHC molecules, such as MHC class I chain-related protein A/B (MICA/B) in human, act as ligands for γδIELs. MICA/B is generally not expressed on ECs, but is induced by stresses such as heat shock and microbial infections. The activated γδIELs then synthesize an array of cytokines, including interleukin (IL)-2, IL-3, IL-6, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β, and cytotoxic molecules, such as perforin, granzyme, and Fas ligand to kill the microbe-infected ECs [38].

Epithelium senses signals from commensal bacterial in the regulation of T cell differentiation in the intestine

The immune system requires interactions with commensal bacteria for its development. Toll-like receptors (TLRs) act as sensors of commensal bacteria although they were initially discovered as pathogen recognition receptors. ECs express several kinds of TLRs and the ligands from commensal bacteria promote immunological functions of Ecs, such as IgA transport, tight junctions, and expression of antimicrobial peptides [40]. Of note, ECs have unique expression profiles and spatially restricted distribution (apical vs. basolateral) of TLRs together with unique underlying signaling pathways, which enables the prevention of deleterious inflammatory responses in the intestine [40].

Because commensal bacteria express shared molecules which act as a ligand of TLRs, it was previously thought that unspecified commensal bacteria indiscriminately induced the development of the immune system; however, accumulating evidence has demonstrated that individual species of commensal bacteria have specific roles in the determination of immunological balance by regulating T cell differentiation in the intestine [8]. ECs have an important role in this pathway.

Segmented filamentous bacteria induce the differentiation of Th17 cells

Several groups have shown that segmented filamentous bacteria (SFB) induce components of the active immune system, including IgA-producing cells, γδIELs, and IL-17-producing T (Th17) cells [41–43]. SFB colonization on ECs results in the production of serum amyloid A, which acts on intestinal dendritic cells (DCs) to enhance the production of IL-6 and IL-23 [43]. Because these two cytokines are Th17 cell-inducing cytokines, the immunological environment mediated by SFB, ECs, and DCs results in the preferential induction of Th17 cells in the intestine.

Preferential induction of Treg cells in the colon by Clostridium *clusters IV and XIVa*

Another form of crosstalk between ECs and commensal bacteria in the regulation of T cell differentiation is mediated by *Clostridium* clusters IV and XIVa (also known as the *Clostridium leptum* and *coccoides* groups) [44]. By contrast to the effects of SFB, colonization by *Clostridium* clusters IV and XIVa induces regulatory T (Treg) cells in the colon to achieve quiescent immunity. *Clostridium* clusters IV and XIVa form a thin colonizing layer on the epithelium, where they enhance the release of the active form of TGF-β by increasing the expression of matrix metalloproteinases that convert latent TGF-β into the active form. Because TGF-β is an essential cytokine for the differentiation of Treg cells from naive T cells, colonization with these *Clostridium* species converts non-Treg cells into Treg cells locally in the colon with little effect on thymus-derived Treg cells.

Dietary metabolites regulate intestinal immunity through the epithelium

Nutritional materials also influence intestinal immunity, and commensal bacteria are involved in metabolizing indigestible dietary materials into biologically active metabolites. Dietary materials (e.g. polysaccharides, vitamins, and lipids) and their metabolites contribute to the regulation of intestinal immunity (Fig. 2).

Polysaccharides

Dietary polysaccharides and endogenous mucus in the intestine are digested and metabolized into short-chain fatty acids, such as acetate, butyrate, and propanoate, by bacterial fermentation. These short-chain fatty acids are an energy source for ECs and affect immune cell functions. For example, acetate and butyrate maintain epithelial barrier function by stimulating the release of mucin and by facilitating the maintenance of epithelial integrity [45,46]. Acetate and butyrate also regulate the proliferation of ECs and their production of cytokines [47,48]. In addition, acetate modulates the immunological function of neutrophils that express G-protein-coupled receptor 43 [GPR43, also known as free fatty acid receptor 2 (FFAR2)], a receptor for the short-chain fatty acids. Neutrophils lacking GPR43 show decreased levels of phagocytic activity and lower production of reactive oxygen species, but also are more responsive to chemoattractants such as C5a and inflammatory chemokines [49]. Consistent with these findings, intestinal inflammation is exacerbated in GPR43-deficient mice.

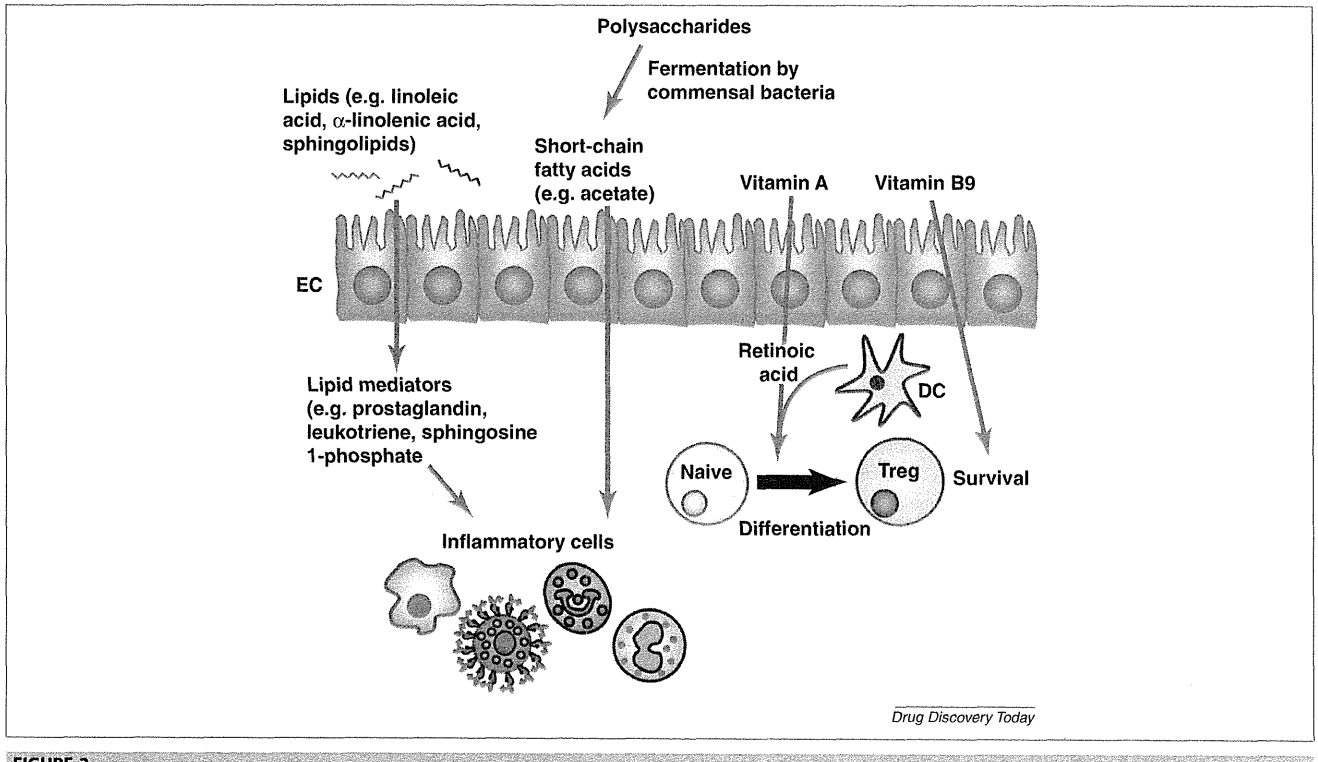

FIGURE 2

Dietary materials in the regulation of EC functions. Dietary lipids are metabolized into lipid mediators, and short-chain fatty acids are generated by fermentation of polysaccharides by commensal bacteria. These products positively or negatively regulate the functions of inflammatory cells. ECs also absorb vitamin A, and both ECs and dendritic cells (DCs) metabolize vitamin A into retinoic acid, which preferentially induces regulatory T (Treg) cells from naive T cells. The differentiated Treg cells require vitamin B9 for their survival. *Abbreviation:* EC, enterocytes.

Vitamins

Vitamins are supplied by both the diet and commensal bacteria. Several lines of evidence have shown that vitamins are involved in regulating immune responses through the epithelium. For example, retinoic acid, a metabolite of vitamin A, is involved in the preferential induction of regulatory T cells and the inhibition of Th17 cells [50]. Both ECs and DCs in the intestine are the major cell types that express retinaldehyde dehydrogenase, a key enzyme for the conversion of vitamin A into retinoic acid, suggesting that the unique gut environment mediated by ECs, DCs, and vitamin A preferentially induces Treg cells for maintaining quiescent immunity in the intestine. Because it was reported that Treg cells enhanced the differentiation of IgA^1 B cells in the intestine [51,52] and retinoic acid induced the expression of gut-homing molecules (e.g. CCR9 and $\alpha4\beta7$ integrin) on IgA-committed B cells as well as T cells [53,54], it is likely that retinoic acid directly and indirectly enhances intestinal IgA responses.

Vitamin B9 is another important vitamin in the maintenance of Treg cells. Vitamin B9 receptor (folate receptor 4) is exclusively expressed on Treg cells and can therefore be used as a cell surface marker of Treg cells [55]. We recently showed that vitamin B9 is an essential survival factor for Treg cells [56]. Indeed, Treg cells differentiate from naive T cells but fail to survive in vitamin B9-reduced conditions. Because vitamin B9 is supplied from both the diet and commensal bacteria, and dietary vitamin B9 is predominantly absorbed by ECs in the jejunum and duodenum, depletion of dietary vitamin B9 results in the reduction of Treg cells in the small intestine.

Lipids

Dietary lipids also involved in the regulation of intestinal immune responses. The ratio of omega-3 polyunsaturated fatty acids (ω-3 PUFA) to ω-6 PUFA in the diet may determine the presence and/or levels of inflammatory conditions. Dietary linoleic acid is the parent fatty acid of ω-6 PUFA which is metabolized into proinflammatory lipid mediators, whereas ω-3 PUFA, which is derived from dietary linolenic acid, is metabolized into anti-inflammatory mediators [57]. A possible molecular mechanism is that ω-3 PUFA exert anti-inflammatory effects through binding to GPR120, which is mostly expressed by macrophages, thereby inhibiting the production of inflammatory cytokines [58].

Another lipid metabolite with important immunological function is sphingosine 1-phosphate (S1P), which regulates cell trafficking, activation, and survival. Intestinal tissues contain higher levels of sphingolipids, including S1P, than other tissues and diet could be a major source of sphingolipids in the intestine, especially sphingomyelin from meat, milk, eggs, and fish [59]. Because ECs express alkaline sphingomyelinase and ceramidase to degrade dietary sphingomyelin into ceramide and sphingosine, respectively, and also express several key enzymes in the production of S1P from ceramide and sphingosine (e.g. sphingosine kinase), it is possible that ECs produce ceramide, sphingosine, and S1P for the regulation of intestinal immune responses.

Concluding remarks

ECs in the intestine have both physical and immunological barrier functions, which are achieved by immunological communication with both immunocompetent cells and gut environmental factors (e.g. commensal bacteria, dietary materials, and their metabolites). Elucidation of the complex networks established by commensal bacteria, dietary molecules, and the host immune system will provide new insights in gut environment-based mucosal immunology and should lead to new strategies to prevent and treat infectious and immune diseases in the intestine.

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ORIGINAL RESEARCH

Single systemic administration of Ag85B of mycobacteria DNA inhibits allergic airway inflammation in a mouse model of asthma

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Abstract: The immune responses of T-helper (Th) and T-regulatory cells are thought to play a crucial role in the pathogenesis of allergic airway inflammation observed in asthma. The correction of immune response by these cells should be considered in the prevention and treatment of asthma. Native antigen 85B (Ag85B) of mycobacteria, which cross-reacts among mycobacteria species, may play an important biological role in host–pathogen interaction since it elicits various immune responses by activation of Th cells. The current study investigated the antiallergic inflammatory effects of DNA administration of Ag85B from *Mycobacterium kansasii* in a mouse model of asthma. Immunization of BALB/c mice with alum-adsorbed ovalbumin followed by aspiration with aerosolized ovalbumin resulted in the development of allergic airway inflammation. Administration of Ag85B DNA before the aerosolized ovalbumin challenge protected the mice from subsequent induction of allergic airway inflammation. Serum and bronchoalveolar lavage immunoglobulin E levels, extent of eosinophil infiltration, and levels of Th2-type cytokines in Ag85B DNA-administered mice were significantly lower than those in control plasmid-immunized mice, and levels of Th1- and T-regulatory-type cytokines were enhanced by Ag85B administration. The results of this study provide evidence for the potential utility of Ag85B DNA inoculation as a novel approach for the treatment of asthma.
Keywords: immunotherapy, asthma, Ag85B, mycobacteria, allergy

Introduction

Asthma is characterized by airway hyperresponsiveness to a variety of specific and nonspecific stimuli, chronic pulmonary inflammation with eosinophilia, excessive mucus production, and high serum immunoglobulin E (IgE) levels. T-helper-2 (Th2) cells are thought to play a crucial role in the initiation, progression, and persistence of asthma in association with the production of interleukin-4 (IL-4), IL-5, and IL-13.[1–3] Bronchoalveolar lavage (BAL) T-cells from human asthmatics have been reported to express elevated levels of IL-4 and IL-5 messenger ribonucleic acid (mRNA).[4,5] Although the correction of this deviation to Th2-type immune responses is considered to be necessary to achieve therapeutic and preventive effects on asthma, it is not sufficient to obtain therapeutic effects in many cases. Another subset of T-cells, T-regulatory (Treg) cells, has been reported to be important in the development of allergic diseases such as asthma.[6] Many studies have suggested that effective immunotherapy for allergic diseases is associated with immune deviation from a disease-promoting Th2 response towards a Th1 response, with Treg cells having appropriate functions.[7] However, the induction of both subsets of cells – Th1 and Treg cells – for the treatment of asthma using immunological strategic tools is very difficult.

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Administration of mycobacteria, including the bacillus Calmette–Guerin, has been thought to be effective for preventing the development of asthma by induction of Th1-type immune responses and inhibition of IgE by the production of IL-21 from natural killer T-cells.[8–10] However, the relationship between bacillus Calmette–Guerin infection or mycobacteria immunization and asthma in humans is controversial because of the many causative factors affecting the induction of immune responses by mycobacteria, eg, human genetic background, mycobacteria strains, and environmental factors (reviewed in Arnoldussen et al).[11] From these findings, bacterial products from mycobacteria for immunotherapy against allergic disease should eliminate the harmful effects of host genetic factors, environmental factors, and strain specificity of mycobacteria.

Antigen 85B (Ag85B) is one of the most dominant protein antigens secreted from all mycobacterial species and has been shown to induce substantial Th cell proliferation and vigorous Th1 cytokine production.[12] Moreover, the induction of Th1-type immune responses by immunization of Ag85B was enhanced by presensitization with bacillus Calmette–Guerin.[13,14] From these findings, the effectiveness of Ag85B DNA as immunotherapy for tumor disease and as a vaccine adjuvant for infectious disease, by its ability to induce Th1-type immune responses, was also reported.[13,14] The current study investigated whether Ag85B DNA from *Mycobacterium kansasii* can inhibit the development of allergic airway inflammation as a novel immunotherapy.

Material and methods

Induction of allergic inflammation in mice

BALB/c female mice used in this study were handled according to ethical guidelines approved by the Institutional Animal Care and Use Committee of National Institute of Biomedical Innovation, Japan. The mice were sensitized to ovalbumin (OVA; Sigma-Aldrich, St Louis, MO) and challenged with aerosolized OVA according to a modification of the method of Nishikubo et al.[15] Briefly, mice were subcutaneously immunized with 10 μg OVA complexed with alum on days zero and 14. On days 21–25 after the first immunization, mice were challenged with an aerosol of 5% OVA in phosphate-buffered saline in a chamber for 20 minutes.

Administration of DNA

Mice were intraperitoneally administered 50 μg plasmid DNA encoding Ag85B DNA once on day –7, zero, 14, or 21. An empty plasmid vector (pcDNA™ 3.1; Life Technologies, Carlsbad, CA) was used as a control (Figure 1A).

BAL fluid collection

BAL fluid was obtained by injecting and recovering two 0.5 mL aliquots of phosphate-buffered saline via a tracheal cannula. BAL fluid and sera were collected 25 days after the first OVA immunization. Cells in the BAL fluid were counted using a hematocytometer, and the differentials were determined by utilizing light microscopy to count 300 cells on Cytospin® preparations (Thermo Fisher Scientific, Waltham, MA). The concentration of inflammatory protein was measured by Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA).

Quantitation of IgE

IgE levels in sera were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the procedure recommended by the manufacturer (Shibayagi Co, Ltd, Shibukawa, Japan).

Determination of cytokine production

Lymphocytes obtained from thoracic lymph nodes of immunized mice (5×10^6) were cultured with 10 μg/mL OVA in 24-well culture plates at a volume of 2 mL. After incubation at 37°C in a humidified incubator (5% carbon dioxide) for 48 hours, culture supernatants were collected and analyzed for production of interferon-γ (IFN-γ; Life Technologies) or IL-4 (Quantikine®; R&D Systems, Minneapolis, MN) by an ELISA assay according to the manufacturer's protocol (Life Technologies). The amounts of IL-5 and IL-13 in BAL fluid were also measured by an ELISA kit (R&D Systems) 25 days after the first OVA immunization.

Detection of cytokine mRNA from lymphocytes using real-time polymerase chain reaction

Total RNA was purified from OVA-stimulated or fetal calf serum (control)-stimulated spleen cells using Isogen (Nippon Gene Co, Ltd, Tokyo, Japan) following the manufacturer's instructions. For the real-time reaction, a reverse transcription system (Promega Corporation, Fitchburg, WI) was used. Polymerase chain reaction was performed in a total volume of 50 μL of 1 × polymerase chain reaction buffer (Takara Shuzo, Kyoto, Japan) containing 0.5–1.0 μg of complementary DNA, 0.25 mM of each deoxyribonucleotide triphosphate, 2 μM of each primer, and 2.5 U of *Taq* DNA polymerase (Takara Shuzo). The specific primer pairs used were described previously.[15] The samples were amplified for 30–35 cycles under the following conditions: annealing for 30 seconds at 56°C, extension for 1 minute at 73°C, and denaturation for 30 seconds at 93°C. The reaction products were

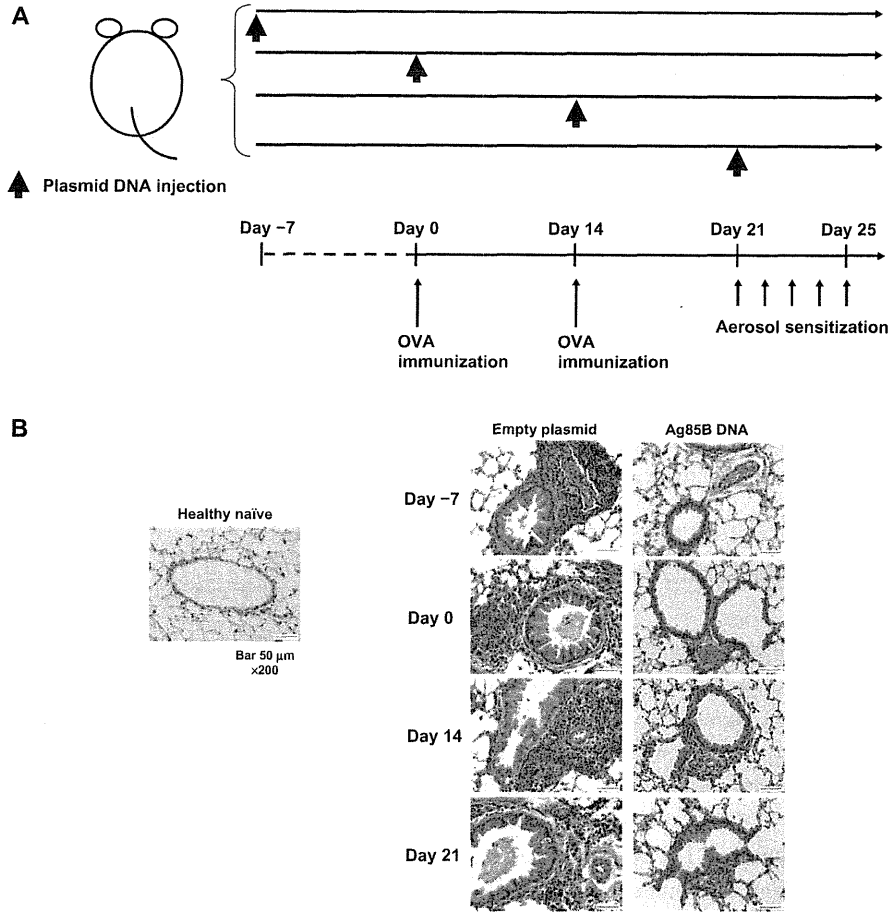

Figure 1 Inhibition of the development of allergic inflammation in lungs by administration of Ag85B DNA vaccine. (**A**) Experimental design used to investigate the effects of Ag85B DNA vaccine on OVA-induced asthma. Mice were subjected to an OVA sensitization scheme,[15] and 50 μg of Ag85B DNA vaccine was intraperitoneally injected once on days −7, 0, 14, or 21. A control plasmid was also administered on the same day. (**B**) Results of histopathological examination of lungs of mice that had been administered Ag85B DNA or control DNA. All tissues were obtained 25 days after the first OVA immunization. The tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.
Abbreviations: Ag85B, antigen 85B; OVA, ovalbumin.

analyzed on 2% agarose, Tris-buffered ethylenediaminetetraacetic acid gel. Photographs of the gels were scanned, and band intensities were measured using a densitometer (CS Analyzer 3.0; ATTO Corporation, Tokyo, Japan). The quantity of cytokine mRNA was determined by the ratio of cytokine and beta actin band intensities. The profiles shown are representative of three independent experiments.

Histopathological examinations

Histopathological examinations of the lungs of the mice that had been administered Ag85B DNA or control DNA were performed. All tissues were obtained 25 days after the first OVA immunization. The tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Results for healthy naïve mice and control plasmid DNA-immunized mice are also shown.

Statistical analysis

Statistical analyses were performed using the Mann–Whitney U test and the Kruskal–Wallis test. Values are expressed as mean ± standard deviation. A 95% confidence limit was considered to be significant ($P < 0.05$).

Results

Inhibition of the development of allergic inflammation in the lung by administration of Ag85B DNA

Mice were sensitized to OVA and challenged with aerosolized OVA as described previously.[11] These mice were intraperitoneally administered 50 μg plasmid DNA encoding Ag85B once on day −7, zero, 14, or 21. An empty plasmid vector (pcDNA 3.1) was used as a control (Figure 1A).

Histopathological examinations of the lungs of mice injected with Ag85B DNA or control DNA and the lungs of healthy naïve mice were performed 25 days after the first inoculation of the plasmid. The lungs of mice that were administered Ag85B DNA on days –7, zero, and 14 did not show any pathological abnormalities compared with those of healthy naïve mice, but the lungs of mice that were administered Ag85B DNA on day 21 showed mild inflammation due to infiltration of eosinophils (Figure 1B). Mice administered the control plasmid did not show any inhibitory effects on the development of allergic inflammations. These results indicated that Ag85B DNA administration was effective for inhibiting the development of allergic inflammation, especially in the early phase of antigen sensitization.

Marked inhibition of allergic immune responses by administration of Ag85B DNA

The levels of protein, total cells, eosinophils, lymphocytes, and neutrophils in BAL fluid from mice immunized with Ag85B DNA vaccine were significantly lower than those in BAL fluid from mice vaccinated with control DNA (Figure 2A–F). Administration of Ag85B DNA also resulted in a significant reduction in the level of OVA-specific IgE (Figure 2G). The concentrations of Th2-type cytokines (IL-5 and IL-13) in BAL fluid from mice immunized with Ag85B DNA vaccine were significantly lower than those in BAL fluid from control mice (Figure 3A and B). These inhibitory effects on the development of allergic inflammation were correlated with day of Ag85B DNA injection. Injection on an early day was more effective for inhibiting the development of allergic inflammation. These results were also confirmed by histopathological observation.

Effects of Ag85B DNA administration on the production of IL-4 and IFN-γ in response to OVA

The production of OVA-specific cytokines in lymph node cells after in vitro stimulation with OVA were assessed. The lymphocytes obtained from thoracic lymph nodes were stimulated in vitro with OVA for 48 hours. IL-4 and IFN-γ levels were measured in culture supernatants by ELISA. The level of IL-4 in culture supernatants from cells of Ag85B DNA-immunized mice was much lower than in culture supernatants from cells of control mice (Figure 4A). On the other hand, the production level of IFN-γ in Ag85B DNA-immunized mice was significantly higher than in control DNA-immunized mice (Figure 4B).

Expression of cytokine mRNA in pulmonary lymph node cells after stimulation with OVA

The production of OVA-specific cytokines was also confirmed by mRNA levels of Th1-type cytokines (IFN-γ, IL-2, and IL-12) and Th2-type cytokines (IL-4, IL-5, and IL-13) (Figure 5A–C). Lymph node cells from Ag85B DNA vaccine-immunized mice showed strong IFN-γ, IL-2, and IL-12 expression and weak IL-4, IL-5, and IL-13 expression of mRNA, whereas control DNA-immunized mice showed the completely opposite results. The cells from control mice showed strong mRNA expression of Th2-type cytokines and weak mRNA expression of Th1-type cytokines (Figure 5A–C). It has been reported that therapeutic effects against asthma by administration of the culture supernatant of *M. vaccae* were derived from Treg cells by the induction of IL-10 and transforming growth factor-β.[16] In the current study, mRNA expression levels of IL-10 and transforming growth factor-β in lymph node cells obtained from mice immunized with Ag85B DNA were much higher than those in lymph node cells obtained from control mice after in vitro stimulation with OVA (Figure 5A and D). Another Th17 cell lineage, which is associated with allergen-induced airway allergic inflammation, was also assessed by the mRNA expression of cytokines. In the current experiment, mRNA expression of IL-17 was seen in both control DNA-immunized and Ag85B-DNA immunized mice after stimulation with OVA, with no difference in the mRNA expression levels of IL-17 between these groups (Figure 5A and E). The mRNA expression of IL-23 was also assessed since IL-23 is associated with the maturation of Th17 cells.[17] Expression of IL-23 mRNA was observed at the same level in all samples (Figure 5A and E). Inhibitory effects on the development of allergic inflammation are readily obtained in a mouse model of asthma through the administration of Ag85B DNA. These effects of immunotherapy by Ag85B DNA are due to activation of the immune responses of Th1 and Treg cells and inhibition of the responses of Th2 cells as a result of the enhancement of responses of Th1 and Treg cells.

Discussion

Current treatments of nonspecific immunosuppressive therapy for asthma, such as administration of glucocorticoids, are not satisfactory. Although these treatments are highly effective for controlling disease, most patients must continue to take these drugs throughout their lives. Moreover, these drugs have side effects, and asthma cannot be controlled by these drugs in up to 30% of patients. Given the high prevalence

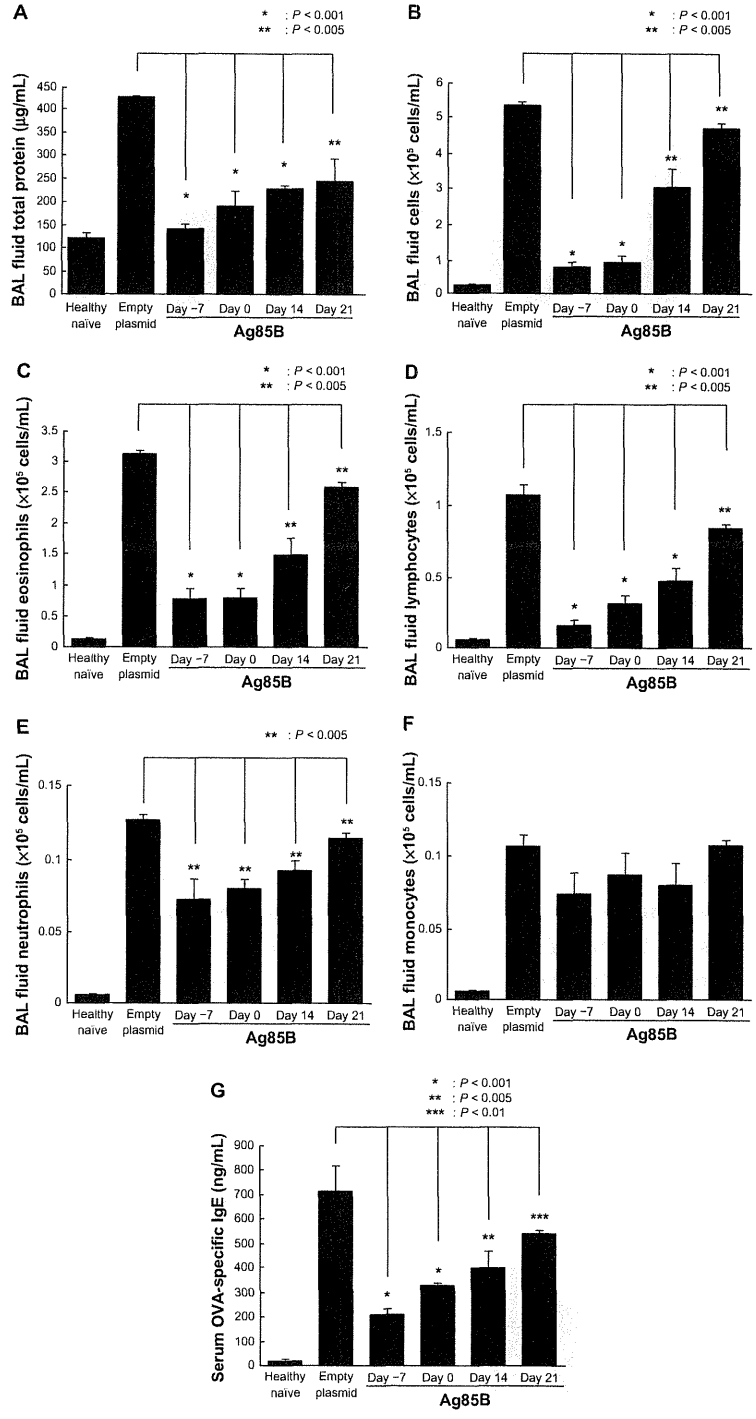

Figure 2 Marked inhibition of the development of allergic inflammation by administration of Ag85B DNA. BAL fluid was obtained by injecting and recovering two 0.5 mL aliquots of phosphate-buffered saline via a tracheal cannula. Cells in the lavage fluid were counted using a hematocytometer, and the differentials were determined by utilizing light microscopy to count 300 cells on Cytospin® preparations (Thermo Fisher Scientific, Waltham, MA). The concentration of inflammatory protein was measured by Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA). Results for healthy naïve mice and control plasmid DNA-immunized mice are also shown. (**A**) Total protein, (**B**) number of cells, (**C**) eosinophils, (**D**) lymphocytes, (**E**) neutrophils, and (**F**) monocytes in BAL fluid from experimental animals were investigated. (**G**) The degrees of ovalbumin-specific immunoglobulin E responses in sera collected from experimental mice were also analyzed. Immunoglobulin E levels in sera were measured using enzyme-linked immunosorbent assay kits according to the procedure recommended by the manufacturer (Shibayagi Co, Ltd, Shibukawa, Japan). BAL fluid and sera were collected 25 days after the first ovalbumin immunization.

Notes: Data are representative of at least three independent experiments; values shown are the means and standard deviations of five mice per group; statistical analysis was performed using the Mann–Whitney U test and the Kruskal–Wallis test.

Abbreviations: Ag85B, antigen 85B; BAL, bronchoalveolar lavage.

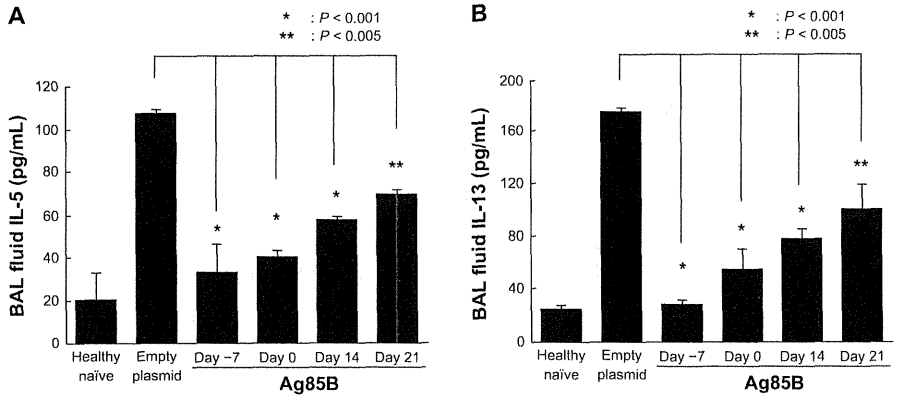

Figure 3 IL-5 and IL-13 production in BAL fluid. Amounts of (**A**) IL-5 and (**B**) IL-13 in BAL fluid were measured using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) 25 days after the first ovalbumin immunization.
Notes: Data are representative of at least three independent experiments; values shown are means and standard deviations of five mice per group; statistical analysis was performed using the Mann–Whitney U test and the Kruskal–Wallis test.
Abbreviations: Ag85B, antigen 85B; BAL, bronchoalveolar lavage; IL, interleukin.

of this disease, improved and more effective therapeutic strategies are needed. The results of many studies have suggested that effective immunotherapy for allergic disease is associated with immune deviation from a disease-promoting Th2 response towards a Th1 response, with Treg cells having appropriate functions (reviewed in Takeda et al).[18] In the current study, the applicability of plasmid encoding complementary DNA of Ag85B from mycobacteria DNA to gene therapy of asthma was assessed. Although the introduced DNA is expressed predominantly by somatic cells, it is known that a relatively small but biologically significant number of dendritic cells are transfected with the inoculated DNA.[19–21] Moreover, it was recently reported that systemic inoculation of a plasmid DNA may cause dendritic cell activation through direct transfection into dendritic cells.[22] It was demonstrated that inhibitory effects on the development of allergic inflammation are readily obtained in a mouse model of asthma through the administration of Ag85B DNA, even with only a single administration before or after antigen sensitization.

The mechanism of immune responses induced by Ag85B remains unclear. Various products having adjuvant activities, eg, lipopolysaccharide, cytosine-phosphodiester-guanine motif, and polyinosinic:polycytidylic acid, involve toll-like receptors (TLRs) and show augmentation of Th1-type immune responses.[18] It was previously reported that plasmid DNA encoding Ag85B stimulated the expression of TLR2, TLR3, and TLR4 mRNA. One possibility is that the induction

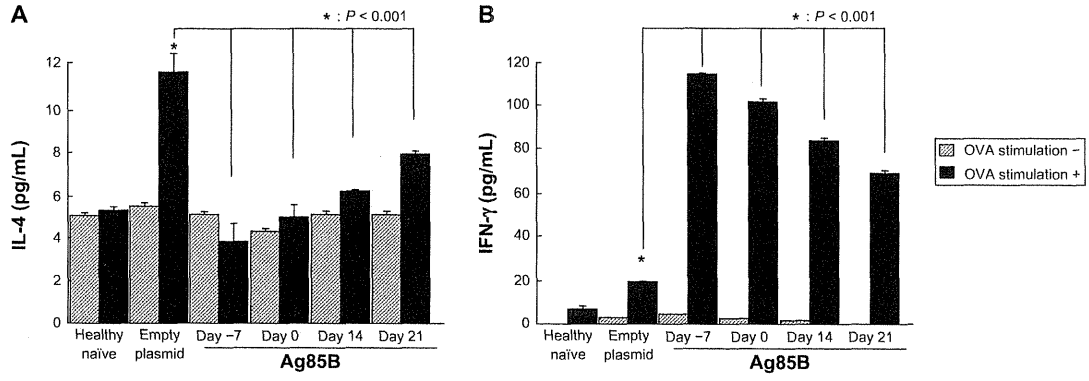

Figure 4 IFN-γ and IL-4 production in culture supernatant. Amounts of (**A**) IFN-γ and (**B**) IL-4 in culture supernatant were measured by enzyme-linked immunosorbent assay 25 days after the first OVA immunization. Spleen cells from immunized mice (5×10^6) were cultured with 10 μg/mL OVA in 24-well culture plates at a volume of 2 mL. After incubation at 37°C in a humidified incubator (5% carbon dioxide) for 96 hours, culture supernatants were quantified by using a standard enzyme-linked immunosorbent assay kit (Life Technologies, Carlsbad, CA).
Notes: Data are representative of at least three independent experiments; values represent mean and standard deviation of ten mice per group; statistical analysis was performed using the Mann–Whitney U test and the Kruskal–Wallis test.
Abbreviations: Ag85B, antigen 85B; IFN-γ, interferon-γ; IL-4, interleukin-4; OVA, ovalbumin.

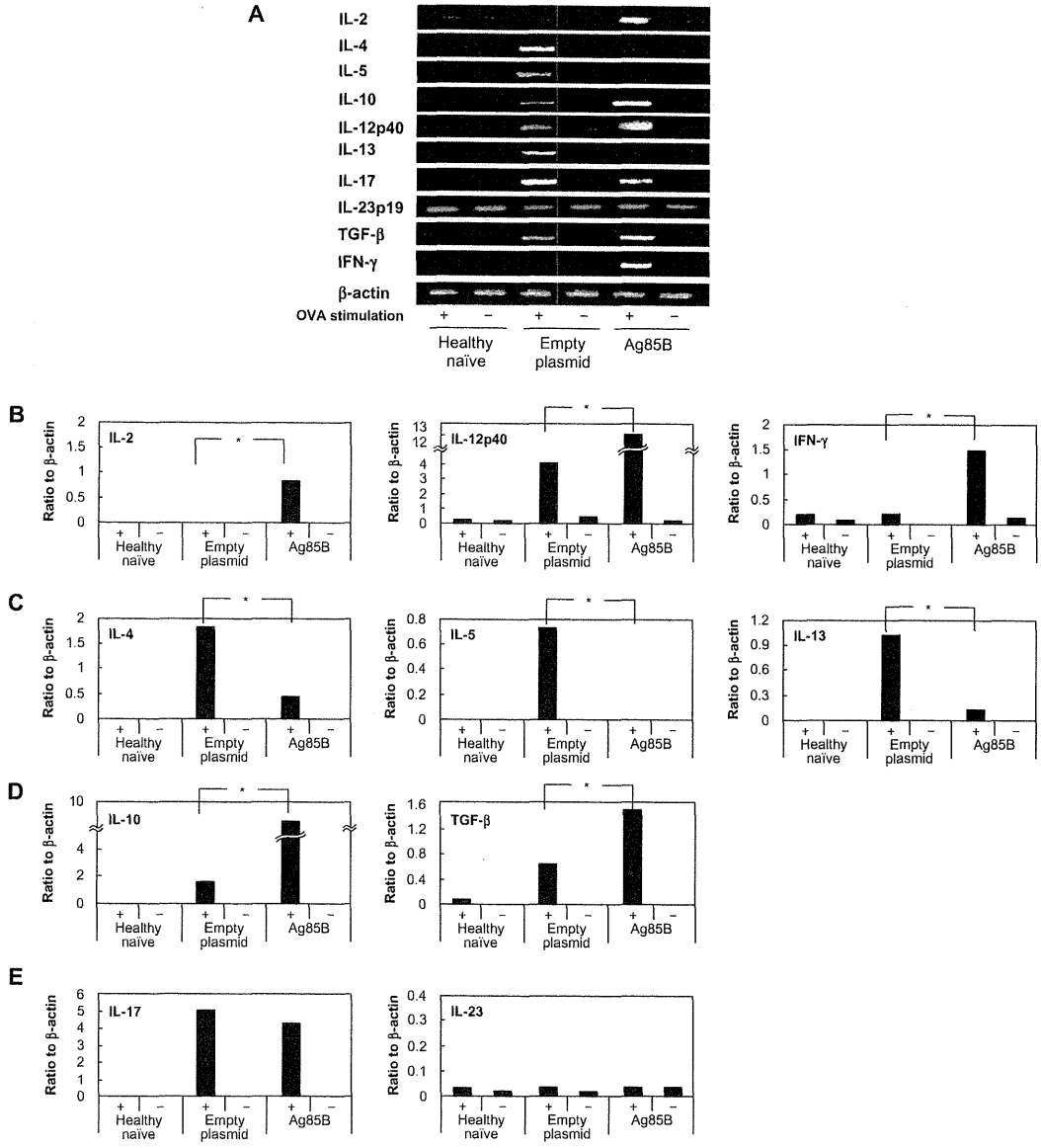

Figure 5 Detection of cytokine messenger ribonucleic acid from lymphocytes using real-time polymerase chain reaction. Spleen cells were stimulated in vitro with OVA for 1 day in culture. Spleen cells stimulated with fetal calf serum were used as controls. Total ribonucleic acid was purified from the OVA-stimulated or fetal calf serum (control)-stimulated spleen cells using Isogen (Nippon Gene Co, Ltd, Tokyo, Japan) following the manufacturer's instructions. For the real-time reaction, a reverse transcription system (Promega Corporation, Fitchburg, WI) was used. Polymerase chain reaction was performed in a total volume of 50 μL of 1 × polymerase chain reaction buffer (Takara Shuzo, Kyoto, Japan) containing 0.5–1.0 μg of complementary DNA, 0.25 mM of each deoxyribonucleotide triphosphate, 2 μM of each primer, and 2.5 U of *Taq* DNA polymerase (Takara Shuzo). The specific primer pairs used were previously described.[15] The samples were amplified for 30–35 cycles under the following conditions: annealing for 30 seconds at 56°C, extension for 1 minute at 73°C, and denaturation for 30 seconds at 93°C. (**A**) The reaction products were analyzed on 2% agarose, Tris-buffered ethylenediaminetetraacetic acid gels. (**B–E**) Photographs of the gels were scanned, and band intensities were measured using a densitometer (CS Analyzer 3.0; ATTO Corporation, Tokyo, Japan). The quantity of cytokine messenger ribonucleic acid was determined by the ratio of cytokine and beta actin band intensities.

Notes: *$P < 0.005$; the profiles are representative of three independent experiments; statistical analysis was performed using the Mann–Whitney U test and the Kruskal–Wallis test.

Abbreviations: Ag85B, antigen 85B; IFN-γ, interferon-γ; IL, interleukin; OVA, ovalbumin; TGF-β, transforming growth factor-β.

of Th1-type immune responses by Ag85B is involved in innate immune responses. From this result, the activation of Th1 and Treg cells by Ag85B administration was thought to be involved in responses through stimulation of TLR2, TLR3, and TLR4, but not TLR9.[14] Various proteins derived from pathogens promote Th1 responses through stimulation of TLRs and subsequently through secretion of cytokines.[18] It has also been reported that TLR signaling induces not only Th1-type immune responses but also secretion of various cytokines from Treg cells.[23–27] Moreover, recent studies have

indicated that Th1 cells produce IL-10 as well as Th1-type cytokines by Notch regulation-dependent signal transducer and activator of transcription-4 signaling.[28] From these findings, effective immunotherapy by induction of both Th1 cell and Treg cell responses is thought to be possible by using appropriate materials. In fact, an asthma model of mice immunized with culture supernatant of mycobacteria, *M. vaccae*, showed Th1 and Treg responses.[16] The results of the current study suggest that the administration of Ag85B DNA has several potential advantages due to the activation of Th1 and Treg cells for the prevention and treatment of asthma.

Immunization with mycobacteria or mycobacteria products has been reported to inhibit the development of allergic disease.[29–32] However, various causative factors affect immune responses by mycobacteria. It was reported as a notable point that the efficacy of mycobacteria in preventing allergic inflammation of asthma was strongly affected by *Nramp1* alleles.[33] Several host genetic factors, including natural resistance-associated macrophage protein 1 (NRAMP1),[34] vitamin D receptor (VDR),[35,36] and Mendelian susceptibility to mycobacterial disease,[37] have been reported to be involved in responses to mycobacteria (reviewed in Casanova and Abel).[38] Differences in immune responses induced by different mycobacteria strains have also been reported. The differential immune responses were mediated by lipid-extracted molecules of mycobacteria.[39] Moreover, environmental factors are important for immune responses induced by mycobacteria in therapy for atopic diseases.[40,41] Presensitization of mycobacteria in the natural environment affects the induction of Th1-type immune responses by mycobacteria vaccination.[9,42,43] However, the specific components of mycobacteria that inhibit the development of allergic responses have not been reported. Ag85B is a single component of mycobacteria, and this product might not be affected by various other mycobacteria factors involved in immune responses. In fact, Th1-type immune responses induced by Ag85B are not affected by *Nramp* in mice.[44,45]

Wu et al demonstrated the effects of intranasal administration of Ag85B in a mouse model of asthma.[46] It was previously reported that Ag85B has strong adjuvant activities involving Th1 immune responses.[14] Intranasal administration of a plasmid DNA (DNA vaccine) with adjuvant activities has been considered to be inappropriate for human use. Intranasal inactivated influenza vaccine, with adjuvant, induced Bell's palsy in humans. Therefore, intranasal inactivated influenza vaccine with adjuvant is no longer in clinical use.[47] Systemic administration of a plasmid DNA (DNA vaccine) is better than intranasal administration if the same effects of the plasmid DNA can be induced. The current study demonstrated the usefulness of Ag85B DNA vaccine and provided evidence of the potential utility of Ag85B DNA vaccine for the prevention and treatment of asthma, even with only a single systemic administration before or after antigen sensitization.

Conclusion

The correction of immune response should be considered in the prevention and treatment of asthma. Ag85B has potential utility for the prevention and treatment of asthma even with only a single administration.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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