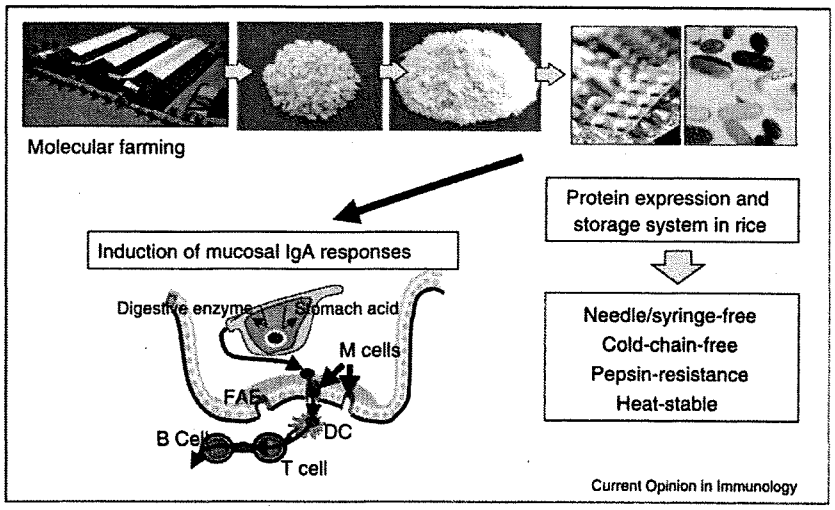
Figure 3



Overview of rice-based mucosal vaccine development; departure from edible vaccine to plant-based oral vaccine. Rice-based oral vaccine system possesses several advantages of circumventing limitations inherent to the currently available injection-type vaccines, including requirement of professional skill and syringe/needle for administration, cold-chain (or refrigeration storage), and physicochemical-stability. These properties are suitable for the prevalence of global vaccine development for many emerging and re-emerging infectious diseases. However, highly sophisticated and closed soilless farming facility with artificial sunlight should be suitable technical advancement for the rice-based transgenic vaccine system.

Although, ingestion of the tablet (or powder) form of plant-based vaccines, even transgenic rice, has to overcome several social and practical concerns, including public confusion and hesitation about genetically modified plants in general, unease about segregating transgenic vaccine plants from food plants, and questions about the consistency of antigen concentration in different lots of transgenic plants. These issues need to be addressed by scientific evidence and improved technology. For example, the development of a soilless molecular farming facility with artificial sunlight will address segregation issues. The term 'edible vaccine' has expanded the development of novel oral vaccines [37,38], but has also created unnecessary confusion that the vaccine could be a part of other food products and not a separate medical preparation. Thus, we propose to use the precise term 'plant-based oral vaccine' (Figure 3). The scientific community in vaccinology, immunology, and plant biology must provide new experimental results and technological advancements to address these public concerns.

Conclusion

The MucoRice system circumvents limitations of other forms of oral vaccine, including physicochemical stability and the requirement for continuous refrigeration. MucoRice CT-B is stable at room temperature for 24 months and does not require purification. Rice plants do not scatter pollen as widely as other crops modified to produce vaccines, for example, maize and wheat, and so have less risk of contaminating non-transgenic crops. Nevertheless, a closed facility would be required for production of the MucoRice vaccine. A rice-based oral vaccine offers a practical global strategy for unrefrigerated, needle-free vaccination against important infectious diseases not only for cholera but also for other re-emerging and emerging infectious diseases, including influenza, botulism, and anthrax.

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Review

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EXPERT REVIEWS

Mucosal vaccines: novel advances in technology and delivery

Expert Rev. Vaccines 8(8), 1083–1097 (2009)

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Mucosal vaccines are considered the most suitable type of vaccines to combat emerging and re-emerging infectious diseases because of their ability to induce both mucosal and systemic immunity. Considerable advances have been made toward the development of mucosal vaccines against influenza virus and rotavirus. Many additional mucosal vaccines are in development, including vaccines against cholera, typhoid, traveler's diarrhea and respiratory infections. In addition to oral and nasal vaccines, transcutaneous (or skin patch) and sublingual immunizations are now part of a new generation of mucosal vaccines. Furthermore, a rice-based oral vaccine (MucoRice™) has been receiving global attention as a new form of cold chain-free vaccine, because it is stable at room temperature for a prolonged period. This review describes recent developments in mucosal vaccines with promising preclinical and clinical results.

KEYWORDS: MucoRice™ • nasal vaccine • oral vaccine • transcutaneous vaccine

Injected vaccines are designed to induce protective immunity in the systemic compartment but are not aimed at providing mucosal immunity as a first line of defense against mucosal pathogens. *Vibrio cholerae*, *Salmonella enterica*, enterotoxigenic *Escherichia coli* (ETEC), *Mycobacterium tuberculosis*, influenza virus, rotavirus and HIV invade and infect the body at the mucosal surfaces of the digestive, respiratory and reproductive tracts [1,2]. Because mucosal administration of a vaccine can induce both systemic and mucosal antigen-specific immune responses, mucosal vaccination would probably improve the efficacy of current parenterally delivered vaccines and could prevent a range of infectious diseases at the sites where their causative pathogens invade [2].

Several routes of vaccine administration induce both systemic and mucosal immunity. In general, oral administration effectively induces antigen-specific antibody responses in the small intestine, colon and salivary glands in addition to systemic compartments, but generates relatively low levels of antigen-specific IgA antibody responses in the large intestine, tonsils, respiratory tract and female genital tract [3]. Nasal administration of antigen induces potent antigen-specific antibody responses in the oral and nasal cavities and respiratory tract, as well as in systemic compartments, but not in the intestines [4]. Interestingly, nasal immunization also can effectively induce antigen-specific IgA and cytotoxic T lymphocytes (CTLs) in the female genital tract, making it an attractive immunization route for the development of an effective mucosal vaccine against HIV infection and other sexually transmitted diseases [1].

Other than the early development of an oral polio vaccine (OPV), few mucosal vaccines have been available clinically, despite recent progress in understanding the unique basic and clinical aspects of the mucosal immune system. In recent years, only a nasal influenza vaccine and oral rotavirus vaccine are available to the public, but numerous mucosal vaccines are in development worldwide (TABLE 1 & 2). In this review, we concisely introduce the current concepts of mucosal immunity and vaccines and discuss their application to human studies and trials for the development of globally licensed mucosal vaccines.

Vaccine development for the mucosal immune system

We continuously expose our respiratory, digestive and reproductive tracts to both beneficial and pathogenic antigens from the outside world by inhalation, ingestion and sexual contact. The mucosal immune system is equipped with two

Table 1. Approved mucosal vaccines.

Generic name (brand name)	Sponsor (company)	Infectious disease	Route of administration
Live-attenuated polio vaccine (Oral Polio Vaccine®)	Many	Polio	Oral
Inactivated *Vibrio cholerae* O1 plus cholera toxin B subunit (Dukoral®)	SBL Vaccine	Cholera	Oral
CVD 103 live-attenuated *V. cholerae* O1 HgR (Orochol®)	Berna (Swiss Serum and Vaccine Institute)	Cholera	Oral
Live-attenuated Ty21a vaccine (Vivotif®)	Berna (Swiss Serum and Vaccine Institute)	Typhoid	Oral
Live-attenuated cold-adapted influenza virus (FluMist®)	MedImmune	Influenza	Nasal
Live-attenuated monovalent human rotavirus (RotaRix®)	GlaxoSmithKline	Rotavirus	Oral
Live-attenuated pentavalent human–bovine ressortant virus (RotaTeq®)	Merck	Rotavirus	Oral

immunological machineries that mediate the active and quiescent phases of antigen-specific immune responses, both providing a front line of defense against invading pathogens by inducing positive immune responses and also inducing immunologic unresponsiveness to food antigens as a negative immune response (mucosally induced tolerance). The mucosal immune system thus maintains a balance between the host's mucosal surfaces and the external environment [5]. In order to further advance our efforts toward the development of a mucosal vaccine, it is necessary to appreciate and understand the anatomical and functional uniqueness of the mucosal immune system when compared with the better known systemic immune system, which is situated in a totally segregated, germ-free compartment [6].

Antigen-specific mucosal immunity consists of secretory IgA (SIgA) and/or CTL responses and is generally induced by interconnected mucosa-associated inductive and effector sites [7]. When vaccine antigens or pathogens are encountered as a result of ingestion or inhalation, these antigens are taken up by the organized inductive tissues, known as mucosa-associated lymphoid tissues (MALTs) that lie in the digestive and respiratory tracts. The MALTs family includes gut-associated lymphoid tissues (GALTs) such as Peyer's patches (PPs) and isolated lymphoid follicles, nasopharynx-associated lymphoid tissues (NALTs), and bronchus-associated lymphoid tissues. PPs and NALTs are well-characterized members of the MALTs family that are located in the intestinal and respiratory tracts, respectively. Tens of PPs are generally found in the small intestines of rodents and hundreds in the small intestines of humans. NALTs are located at both basal sides of the nasal cavity in rodents. Although there is anatomically no equivalent of NALTs in humans, oropharyngeal lymphoid tissues including the adenoids and palatine tonsils are thought to act as functionally and histologically organized lymphoid structures for the upper respiratory tract [8,9].

In general, MALTs are covered by a follicle-associated epithelium (FAE) that contain professional antigen-sampling cells, known as M cells, that effectively take up antigens from the lumen of the respiratory and digestive tracts. Immediately underneath the FAE, layers of professional antigen-presenting cells or dendritic cells (DCs) are situated for the subsequent capture and processing of antigens taken up by the M cells [10]. MALTs contain a well-organized microarchitecture of B- and T-lymphocyte

Table 2. Mucosal vaccines in development.

Generic name (brand name)	Sponsor (company)	Infectious diseases	Route of administration	Development status
Live-attenuated *Vibrio cholerae* Peru-15 (Cholera Garde®)	AVANT Immunotherapeutics	Cholera	Oral	Phase II
Live-attenuated Ty800 vaccine	AVANT Immunotherapeutics	Typhoid	Oral	Phase I
Live-attenuated CDV909 vaccine (HolaVax-Typhoid®)	Acambis/Berna	Typhoid	Oral	Phase II
Live-attenuated cold-adapted influenza viris (CAIV-T®)	MedImmune	Influenza	Nasal	Phase III
Live-attenuated b/hPIV3 expressing F protein of RSV	MedImmune	RSV and PIV3	Nasal	Phase I
Heat-labile enterotoxin	Iomai	Traveler's diarrhea	Transcutaneous	Phase II

PIV: Parainfluenze virus; RSV: Respiratory syncytial virus.

zones that can respond to the processed peptide antigens presented by DCs, and that can become antigen-specific effector and memory B and T cells for the generation of an antigen-specific mucosal immune response. Following DC-mediated stimulation, activated CD4+ Th2 cells preferentially induce IgA-committed B cells (IgA+ B cells) in the germinal center of the lymphoid follicle. After μ(IgM)-to-α(IgA) isotype class switching, these IgA-committed B cells become plasma blasts and then rapidly egress from the MALTs to enter the migration pathway via the connected lymphoid tissues, such as mesenteric and cervical lymph nodes, and continue through the efferent lymphatic system [11]. Finally, these antigen-specific CD4+ T cells and IgA+ B cells

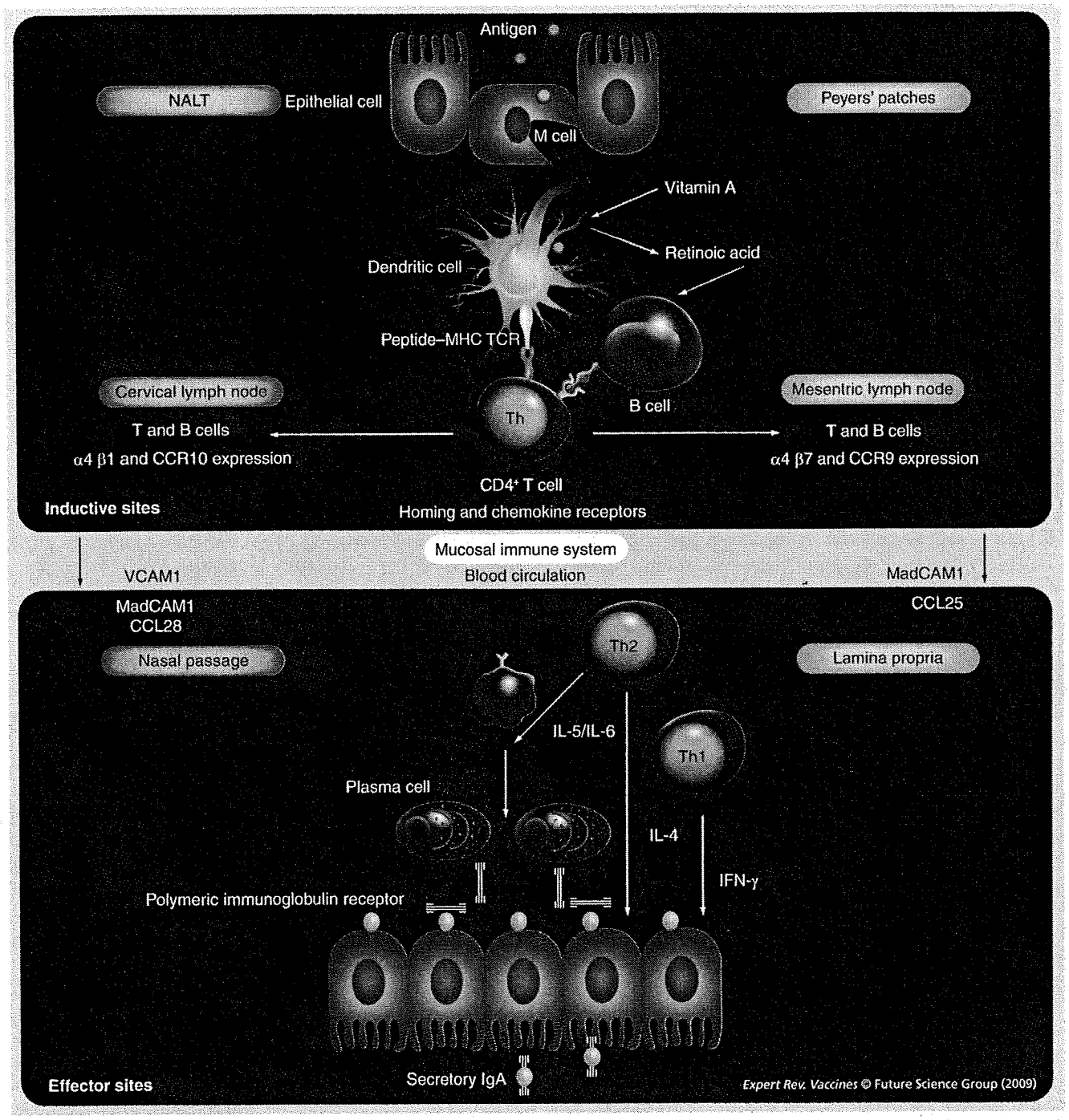

Figure 1. Mucosal immune system for the induction of antigen-specific secretory IgA.
MadCAM: Mucosal addressin cell adhesion molecule; NALT: Nasal-associated lymphoid tissue; TCR: T-cell receptor; VCAM: Vascular cell adhesion molecule.

migrate to effector sites, including the intestinal lamina propria region and nasal passages, through the thoracic ducts and blood circulation (Figure 1) [10].

The activated B and T cells migrating from PPs express $\alpha4\beta7$ integrin and CCR9 as guidance molecules, which direct them to the intestinal lamina propria. The integrin interacts with intestinal high endothelial venules expressing mucosal vascular addressin cell adhesion molecule 1 (MadCAM1), and CCR9 enables specific migration to the intestinal lamina propria, where the epithelial cell-produced chemokine CCL25 is expressed [12–14]. In addition to their original antigen processing and presentation capacities, mucosal DCs produce retinoic acids and, therefore, are critical for educating antigen-specific B and T cells to express the gut-imprinting molecules $\alpha4\beta7$ and CCR9 in PPs [15,16]. By contrast, antigen-stimulated B and T cells in NALTs express $\alpha4\beta1$ integrin and CCR10, allowing them to be selectively trafficked to the effector sites of the nose, trachea, and bronchus, where their ligands, VCAM1 and CCL28, are strongly expressed [17]. The same mucosal migration molecules are involved in the trafficking of activated lymphocytes to the genitourinary tract, which can explain the effective induction of antigen-specific immune responses in the genital tract after nasal immunization [18].

When IgA^+ B cells have migrated to the distant effector sites (e.g., intestinal lamina propria), the cells enter into the final differentiation stage to become IgA-producing plasma cells in the presence of IgA-enhancing cytokines such as IL-5 and IL-6, which are produced by Th2 cells for subsequent production of dimeric or polymeric forms of IgA (dIgA and pIgA, respectively) [19]. dIgA and pIgA then bind to polyimmunoglobulin receptors (pIgR) expressed on the basolateral surfaces of epithelial cells for the formation and transport of SIgA [20]. During the time that dIgA (or pIgA) and pIgR complexes are transported to the apical surface, extracellular proteolytic fragments of pIgR are cleaved to become the secretory component, which forms a part of SIgA [21].

Although M cells located in the FAE of PPs and NALTs have been considered an important gateway for the outside environment and subsequent initiation of antigen-specific mucosal immunity against orally or nasally encountered antigens [22], mucosally administered antigens can also be taken up from the mucosal surface, including from columnar epithelial cells such as small intestinal and rectal epithelia, in collaboration with mucosal DCs [23]. For example, mucosal DCs can directly sample antigen from the intestinal lumen by extending dendrites between the tight junctions of intestinal epithelial cells. These intraepithelial DCs then migrate to the mesenteric lymph node for antigen presentation to naive lymphocytes [24]. Recently, we demonstrated that M-like cells in the villous epithelium, which we termed villous M cells, have the potential to act as alternative antigen sampling cells for the initiation of antigen-specific immune responses [25]. The gut immune system contained more antigen sampling sites than we had expected; these included inductive sites (e.g., M cells in the FAE of PPs and isolated lymphoid follicles) and effector sites (e.g., intraepithelial DCs and villous M cells) for the initiation of antigen-specific immune responses. We still do not know whether these mucosal DCs and M cells form a dynamic antigen sampling network that influences the quality or quantity of antigen-specific immune responses in both the mucosal and systemic compartments; however, one has to consider the presence of such a variety of antigen-sampling cells in the intestinal mucosa when developing an oral vaccine.

In addition to DCs in the intestinal epithelium, two kinds of professional antigen-sampling DCs, Langerhans cells (LCs) and submucosal DCs, have been shown to locate within (LCs) and beneath (submucosal DCs) the body surfaces that are covered by stratified epithelial cell layers, such as the skin, oral cavity and sublingual mucosa[23]. These mucosal surfaces are devoid of MALTs structures but are drained by regional lymph nodes. The DC-like LCs can migrate between skin epithelial cells, where they can sample antigen directly from the cell surface [26]. Although LCs stem cells are present in dermis and submucosa, submucosal DCs do not migrate above the basement membrane. These cells migrate to draining lymph nodes, such as cutaneous and proximal lymph nodes, and present antigens to naive lymphocytes. The ability of these DCs to directly sample antigen from skin and oral mucosa surfaces leads to the possibility of transcutaneous [27] or sublingual immunization [28] as alternative strategies for the development of needle/syringe-free vaccines.

Mucosal vaccines

Oral vaccines

Enteric invasion by pathogenic viruses, such as poliovirus and rotavirus, or bacteria, including *V. cholerae*, ETEC and *Salmonella enterica* serovar Typhi, are serious threats to public health worldwide, particularly to children living in developing countries [29]. Here, we summarize and discuss mucosal vaccines currently approved or in development for human use against enteric infections (Tables 1 & 2).

Polio vaccine

The live-attenuated OPV developed by Albert Sabin was the first approved mucosal vaccine [30]. Similar to the injectable inactivated polio vaccine (IPV) developed by Jonas Salk [30], OPV effectively induces systemic antibody immune responses with excellent protection against poliomyelitis, a paralytic disease resulting from the destruction of motor neurons in the CNS [30]. There are three serotypes of poliovirus that recognize a common cellular immunoglobulin-like receptor (CD155) for host cell attachment and entry. All three serotypes cause poliomyelitis [31], and both OPV and IPV are trivalent vaccines that provide good protection against all three serotypes.

Poliovirus is an enterovirus, and infection occurs via the oral route [32]. OPV both produces a higher titer of virus-specific IgG antibodies than IPV and also induces mucosal IgA immune responses in the intestine, the primary poliovirus entry site. OPV therefore prevents infection and transmission of poliovirus at the intestinal epithelium. Despite the advantages of OPV in terms of efficacy and administration, OPV can revert to neurovirulence, causing vaccine-associated paralytic poliomyelitis at a rate of approximately one case in 500,000 administered doses [32].

Although global vaccination with OPV decreased the annual incidence of poliomyelitis in the year 2006 to approximately 2000 cases worldwide [30], outbreaks of paralytic polio caused by the vaccine-derived strains have been reported every year since 2000 [33]. Thus, most industrialized countries have replaced OPV with IPV [30]. Although IPV is currently considered to be a safe vaccine owing to the inability of the vaccine to induce paralytic polio, one of disadvantages of IPV is that it is unable to induce mucosal immunity for the elimination of the virus at the site of invasion. Developing countries continue to use OPV because the oral form provides the same efficacy at a lower cost. Nonetheless, to achieve the global eradication of polio, OPV will have to be improved in terms of its safety and efficacy as well as cost. The development of an inactivated mucosal polio vaccine may be possible and is an attractive goal for overcoming these obstacles, but it may need a novel mucosal adjuvant that supports the induction of excellent polio-specific protective immunity in both the mucosal and systemic compartments.

Cholera vaccine

Diarrheal disease remains a major global health problem. Approximately 4 billion cases of diarrhea occur worldwide each year, resulting in approximately 2.2 million deaths, mostly of children under 5 years of age in developing countries [301]. Approximately half of the cases are caused by pathogenic bacteria that produce enterotoxins. Cholera, which results from infection with *V. cholerae*, is the most severe of the enterotoxic enteropathies. Injectable vaccination with inactivated *V. cholerae* was used until the end of the 20th Century. Although the injectable vaccine is still available in the USA, it is no longer recommended because it can induce adverse reactions and offers only partial protection of short duration [34]. The limited efficacy of the injection-type vaccine for the control of *V. cholerae* infection is predictable, given our current knowledge of the gut mucosal immune system and the nature of the pathogen invasion site.

To overcome the limitations of the injected vaccine, two oral cholera vaccines have been developed and licensed (Table 1). One is a recombinant live-attenuated vaccine (Orochol®). The vaccine strain CVD 103 HgR was constructed by modifying the cholera toxin (CT) gene from *V. cholerae* Inaba strain 569B [35] by deleting the gene encoding the toxic A subunit of CT, leaving the nontoxic but immunogenic B subunit (CTB). A single oral dose of CVD 103 HgR gave high vibriocidal seroconversion and prevented disease in adult volunteers living in industrialized countries [35,36], but when the vaccine was tested in a large field trial in Thailand and Indonesia, vibriocidal seroconversion was low and no significant protection was observed [35–37]. Therefore, the vaccine is not currently being produced.

The other internationally licensed oral cholera vaccine is an inactivated *V. cholerae* O1 combined with a recombinant CTB (rCTB-WC, Dukoral®) [35]. rCTB-WC induces both antibacterial and antitoxic SIgA mucosal immunity in the intestinal tract, as well as serum antigen-specific IgG immune responses. Three oral doses of rCTB-WC gave 85% protection for the first 6 months and 50% in the third year in a trial in Bangladesh; protective efficacy of rCTB-WC for children aged 2–5 years after 6 month and reached 26% at 3 years, compared with 63% efficacy for adults and children over the age of 5 years [38–40]. In addition to the direct protection of vaccine recipients, the rCTB-WC vaccine resulted in significant herd protection to neighboring nonvaccinated individuals [41]. The rCTB-WC vaccine was also highly effective in short-term protection against severe cholera in an area of sub-Saharan Africa with a high prevalence of HIV infection [42]. Because of the immune responses to CTB after oral vaccination with rCTB-WC, the vaccine also possessed significant cross-protection against ETEC, which produces a heat-labile enterotoxin (LT) that shares high homology with CT and causes severe diarrhea in children in developing countries and in travelers to those countries [43].

A live oral *V. cholerae* O1 El or Inaba vaccine, Peru-15® (also known as Cholera Garde®), is currently in development. Peru-15 is also genetically engineered by deleting the native CT gene and introducing the gene encoding CTB [44]. The vaccine has been found to be safe, immunogenic and efficacious in North American volunteers as well as Bangladeshi adults and children [45]. A single dose of oral Peru-15 has provided promising results in a Phase I/II clinical trial in Bangladeshi children aged 9 months to 5 years [46]. Although we have to wait for additional efficacy results from ongoing field trials in developing countries, this form of oral vaccine will probably add another option for the global control of *V. cholerae* infection.

An ideal cholera vaccine should be a single oral dose that offers a high degree of long-term protection without side effects in children in endemic areas. It should also be an economical and cold chain-free (or refrigeration-free) product, so that the population at potential risk in developing countries can afford the vaccine [34]. It may not be an easy task, but we must develop a vaccine that fulfils these criteria in order to conquer cholera.

Typhoid vaccine

Typhoid fever caused by *Salmonella enterica* serovar Typhi remains a serious public-health problem, with an estimated 33 million cases and 500,000 deaths annually around the world [47]. The incidence of typhoid fever is the highest in children from 5 to 19 years of age, followed by those 1–5 years of age, in developing countries including areas of Africa, Asia and South America [48]. Travelers from industrialized countries who visit areas where typhoid fever is endemic are also at risk of contracting the disease.

Two types of vaccine are currently available, a purified Vi polysaccharide (PS) injection vaccine and an attenuated Ty21a live oral vaccine [2]. The Vi capsular PS of *S. enterica* serovar Typhi is an effective vaccine antigen that can induce protective antibody responses against O antigen, leading to the elimination of the bacterium from blood circulation [47]. A trial of single-dose Vi PS parenteral vaccine in South African children (average age: 9 years) gave 70% protection for the first 18 months and 55% protection over 3 years against typhoid fever [49]. Despite the efficacy in young children, the purified Vi PS is considered a poor immunogen for infants because it behaves as a T-cell-independent

antigen, and therefore induces low antigen-specific antibody responses in immunologically immature infants [49]. However, the conjugation of the PS antigen to a protein vaccine antigen improved the generation of protective antibody responses in infants [50]. Thus, current efforts are aimed at the development of a Vi-protein conjugate vaccine [50].

The oral attenuated *S. enterica* serovar Typhi strain Ty21a vaccine (Vivotif®) was generated by chemical mutagenesis of the wild-type strain Ty2 as a lyophilized live bacteria in an enteric-coated capsule [51]. In clinical trials, the vaccine was initially administered as three doses with 2-day intervals, which provided 67% overall protection against the disease over 3 years, and 62% protection over 7 years [52]. The vaccine was found to elicit Typhi-specific serum IgG and mucosal IgA [53] as well as Th1 and $CD8^+$ CTL responses [54]. Although the attenuated *S. enterica* Typhi strain Ty21a has been characterized as unable to synthesize the Vi capsular antigen, the mutation has not yet been fully defined, and the possibility remains that this strain could revert to virulence even though no revertants have been isolated so far. In addition, Ty21a requires three to four doses for optimal immunogenicity. [55]. Thus, a single-dose and better defined oral vaccine is highly desirable. Two new attenuated *S. enterica* serrovar Typhi strains designed to overcome the concerns related to oral Ty21a vaccine are currently in advanced clinical trials. *S. enterica* Typhi Ty800, which has the *Salmonella phoP/phoQ* virulence region deleted, was shown in a Phase I trial to be a safe and immunogenic typhoid fever vaccine. The single-dose Ty800 vaccine induced brisk serum O-antigen-specific IgA and IgG responses in adult volunteers [56]. A Phase II trial [57] has been successfully completed of another single-dose live attenuated oral vaccine, CVD 909 (HolaVax-Typhoid®), a *Salmonella aroC/aroD/htrA* deletion mutant that constitutively expresses the Vi antigen. There was no difference in the rates of frequent diarrhea in the 21 days after vaccination among those receiving low and high doses of the vaccine and placebo. Antibody-secreting cells producing lipopolysaccharide (LPS)-specific IgA were detected in 100 and 92% of recipients of the high- and low-dose vaccines, respectively. Serum anti-LPS IgG, lymphocyte proliferation and IFN-γ production against Typhi antigen occurred in a substantial proportion of vaccinees. Additional Phase II or III human studies of these vaccines will be needed to define the immunogenicity of heterologous antigens carried by live *S. enterica* Typhi vectors.

Rotavirus vaccine

Rotavirus is a major cause of acute gastroenteritis worldwide in children younger than 5 years, causing more than 600,000 deaths annually, mainly in developing countries [58]. Rotaviruses have two surface proteins, VP4 and VP7, which determine the serotype-specific neutralizing antigens P and G, respectively. Although there are 15 G serotypes and 14 P serotypes, only five combinations, G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8], account for 90% of the human rotavirus strains [59]. Thus, these five G–P combinations are theoretically targets for vaccine development.

Rotashield® (Wyeth) was introduced in 1998 as the first licensed live attenuated oral rotavirus vaccine. It was a tetravalent reassortant vaccine composed of rhesus (G3) and human (G1, G2 and G4) rotavirus strains. It was given in a three-dose schedule starting at 8 weeks of age, with two subsequent doses administered at 8-week intervals [60]. Although Rotashield was highly efficacious for the prevention of diarrhea and hospitalization by rotavirus infection, the vaccine might have triggered intussusceptions after the oral vaccination period [60]. The risk of intussusception was estimated at more than one case of intussusception per 32,000 infants during the postimmunization period [61]. Wyeth thus withdrew the vaccine in 1999.

Currently, two live-attenuated oral rotavirus vaccines, Rotarix® (GlaxoSmithKline) and RotaTeq® (Merck), are licensed in 88 and 47 countries, respectively [55]. The two vaccines were designed from Rotashield by using different approaches. Rotarix is an attenuated human rotavirus vaccine that is made of a tissue-culture adapted human isolate, 89–12 (G1P[8]). The 89–12 strain was chosen as a vaccine candidate because natural infection with similar G1P[8] rotaviruses provides broadly cross-reactive neutralizing antibody responses and excellent protection against rotavirus gastroenteritis [62]. In addition, human rotavirus G1P[8] is the predominant strain worldwide. Rotarix is given in a two-dose schedule starting at 6–13 weeks of age, with a second dose administered after a 4- to 8-week interval. The safety and efficacy of Rotarix in a Phase III trial involving 63,225 infants showed that the vaccine was efficacious at protecting infants against severe rotavirus gastroenteritis and was not associated with an increased risk of intussusceptions [63]. The vaccine efficacy was 92% against G1 serotype-specific rotavirus gastroenteritis, whereas the efficacy against G2, G3, G4 and G9 serotype-specific rotavirus gastroenteritis ranged from 41 to 87%.

RotaTeq is composed of five reassortant rotavirus strains, which were derived from a WC3 bovine strain containing two genes, *VP4* (P) and *VP7* (G), encoded by a rotavirus of human origin. It is given orally in a three-dose schedule starting at 6–12 weeks of age, with subsequent doses administered at 4- to 10-week intervals. A Phase III safety and efficacy trial of RotaTeq involving 70,301 infants showed that the vaccine is efficacious in preventing rotavirus gastroenteritis. The vaccine efficacy against G1, G2, G3, G4 and G9 serotype-specific rotavirus gastroenteritis was 75, 63, 83, 48 and 65%, respectively [64]. The risk of intussusceptions was similar between the recipients of vaccine and placebo.

During the process of developing a safe and effective oral rotavirus vaccine, it was consistently shown that the levels of serum rotavirus-specific IgA measured shortly after either vaccination or natural infection generally reflects the levels of antigen-specific intestinal IgA antibodies and may be the best available marker for protection against rotavirus gastroenteritis [65]. Intestinal rotavirus-specific IgA is probably the most important mechanism for long-term protection against rotavirus gastroenteritis, but the mechanism by which the vaccine elicits protective immunity in humans remains poorly understood [65].

Nasal vaccines

Acute respiratory illness causes the highest burden of disease in the world. In particular, influenza virus, respiratory syncytial virus (RSV), parainfluenza virus (PIV) and *M. tuberculosis* are major

respiratory pathogens that cause acute respiratory infectious diseases [66]. The mucosal immune system protects against these pathogens, which invade the host via the mucosal surfaces covering the upper and lower airway tracts. To make use of this mucosal immune system, a nasal vaccine has been developed for the flu and is currently available to the public in the USA in addition to the injection-type influenza vaccine. Here, we summarize and discuss mucosal vaccines that are currently approved or in development for human use against respiratory infections (Tables 1 & 2).

Influenza vaccine

Acute respiratory infections including viruses and bacteria caused nearly 4 million deaths every year in worldwide, mostly in young children and infants in developing countries. The public burden of influenza in the USA is estimated 25–50 million cases and 30,000–40,000 deaths per year. The average global burden of pandemic influenza may be on the order of 1 billion cases [67]. Influenza causes particularly high rates of severe disease in children younger than 5 years of age and adults older than 50 years of age [68]. Influenza viruses are divided into three genera, A, B and C. Influenza A viruses are further divided into subtypes according to antigenicity of the major envelope proteins hemagglutinin (HA) and neuraminidase (NA) [67]. Although there are 15 HA variants and nine NA variants, and thus 135 possible combinations, only viruses of the H1N1, H1N2 and H3N2 subtypes are currently circulating widely in human populations, although the pandemic influenza viruses H5N1, H7N2, H7N7 and H9N2 also cause human disease [67].

Injectable vaccines against influenza have been in common use for a long time. Serum-derived immunoglobulins are the antibodies primarily responsible for the protection of the lower respiratory tract, whereas mucosal antibodies play a critical role in the protection of the upper respiratory tract and are considered to be more important to overall protection against airway infection with influenza [69]. Recently, a nasal live-attenuated, trivalent influenza vaccine, FluMist® (MedImmune), was approved in the USA based on the advanced molecular and cellular knowledge of influenza infection and mucosal immunity. The vaccine contains two type A (H1N1 and H3N2) and one type B attenuated, cold-adapted, temperature-sensitive reassortant influenza virus strains that express the HA and NA surface glycoproteins of the targeted wild-type strains [70]. The vaccine strain can replicate at 25°C, which is the temperature of the nasal surface, but cannot survive at 37°C, the temperature of the lungs [71,72]. FluMist was highly efficacious in a 2-year Phase III trial, with overall protective efficacy of 92%; the vaccine showed 86% protective efficacy against antigenic variants that circulated in the region [70]. The currently licensed formulation of FluMist requires storage at -15°C or less. To overcome this impractical storage requirement, a liquid formulation of cold-adapted trivalent influenza vaccine (CAIV-T) that is stable at refrigerator temperatures of 2–8°C is being evaluated in a Phase III trial [73].

Although FluMist was originally approved only for healthy individuals aged 5–49 years, the US FDA further approved FluMist for children aged 2–5 years in 2007. The US CDC recommends that children 6 months and older should be vaccinated for influenza. However, the FDA decided that children under 2 years of age should not be given FluMist because of an increased risk of wheezing and other side effects possibly associated with the nasal administration [302]. Thus, mucosal vaccines for influenza that can safely be administered to children less than 2 years of age still need to be developed. In addition to the infant population, one must remember that the elderly population of more than 50 years of age is also at major risk for influenza infection and its associated medical complications, and thus the availability of a mucosal influenza vaccine will contribute to improving public health in our aging society.

RSV/PIV vaccines

Respiratory syncytial virus is the most important cause of severe lower respiratory infections in infants worldwide. RSV is estimated to cause approximately 64 million infections each year, and mortality could be as high as 160,000. Almost all children will have been infected with RSV by 2 years of age, and the peak incidence of mortality with RSV infection occurs at less than 3 months of age [74]. The viral surface glycoproteins F and G are major protective antigens. F protein, which is highly conserved in RSV A and B groups, is responsible for fusion of the virus envelope with the target host cell membrane [75]. PIV types 1, 2 and 3 are also important respiratory pathogens in infants and young children. For example, PIV-3 infects approximately 60% of infants before the age of 2 years [74]. The viral surface glycoproteins HA–NA (HN) and F protein are responsible for attachment and fusion of virus to target host cells (e.g., respiratory epithelial cells) [75].

In general, immunity against RSV and PIVs is mediated by humoral antibodies, including secretory antibodies acquired as a result of infection or maternally derived in infants, and cell-mediated immune responses by cells such as CTLs. A variety of immunological approaches against RSV have been tested for the development of a vaccine, but with limited success. For example, young infants frequently fail to respond adequately to vaccination due to immunogenic immaturity. In addition, one formalin-inactivated RSV not only failed to protect infants against RSV infection, but also induced exaggerated clinical responses to wild-type RSV infection in infants who were naive to RSV before vaccination. The early trials resulted in the hospitalization of 80% of vaccinees and two deaths [74].

At present, there is no licensed vaccine against RSV [67,74], although nasal vaccines against RSV are in clinical trials. Nasal immunization with a live attenuated vaccine should induce both systemic and mucosal immunity, and may protect against viral illnesses of the upper and lower respiratory tracts. A live-attenuated vaccine containing a chimeric bovine/human PIV3 expressing the human PIV3 F and HN proteins and the human RSV F protein (b/hPIV3/RSVF) has been produced. Nasal immunization with the b/hPIV3/RSVF vaccine not only induced RSV-neutralizing serum antibodies and protective immunity against RSV challenge in African green monkeys, but also resulted in production of serum antibodies that neutralized hPIV3 and inhibited the hemagglutination of hPIV3 [75]. The b/hPIV3/RSVF vaccine is currently being tested in a Phase I study.

Tuberculosis vaccines

Tuberculosis (TB) is caused by the respiratory pathogen *M. tuberculosis* and is a major health problem in both developed and developing countries. Globally, TB causes nearly 2 million deaths annually and is the leading cause of death among HIV-infected populations because of the inhibition of the immune system that accompanies HIV infection [303]. The currently available bacillus Calmette–Guérin (BCG) vaccine protects against TB in childhood, but the immunity wanes with age, with the result that this vaccine is ineffective at protecting against adult pulmonary TB. Protective immunity to TB is dependent on strong cellular immune responses involving both $CD4^+$ and $CD8^+$ T cells that have the ability to produce the cellular immunity-enhancing cytokine IFN-γ [76].

New approaches toward the development of vaccine against TB have been reported in preclinical and clinical studies [77,78]. These approaches include using recombinant BCG, live-attenuated strains of *M. tuberculosis*, subunit vaccine approaches, and nonreplicating viral vector-based systems used alone or in prime–boost regimens. There is increasing evidence that a heterologous prime–boost approach induces higher levels of cellular immunity than homologous boosting with the same vaccine [79]. In addition, there is some experimental evidence that nasal vaccination protects against TB in mice. Although these findings provide strong supportive evidence that nasal vaccination is the best way to attain robust protective immune responses in the lungs, no nasal TB vaccine candidate has reached the clinical trial stage [78].

The TB-specific immunity induced by the current parenteral BCG vaccine is markedly enhanced when it is boosted by nasal administration of an adenovirus vector expressing *M. tuberculosis* antigen 85A (AdAg85A) in a mouse model. The enhanced protection afforded by nasal AdAg85A correlates with the numbers of IFN-γ-positive $CD4^+$ and $CD8^+$ T cells in the lung [80]. Moreover, nasal immunization with the fusion protein consisting of Ag85B and 6-kDa early secretory antigenic target (ESAT)-6 [81] together with a combined adjuvant composed of immunoimulating complexes [82] and a fusion of the A1 CT subunit with two copies of the D domain from *Staphylococcus aureus* protein A (CTA1-DD) [83], strongly boosts the pre-existing BCG immunity in mice, promotes an antigen-specific Th1 immune response dominated by IFN-γ-secreting $CD4^+$ T cells, and contributes to protective immunity in the lung [84]. Unlike other toxin-based nasal adjuvants, the CTA1-DD adjuvant itself does not redirect to the CNS after nasal immunization [85]. Thus, the TB mucosal vaccine system with CTA1-DD is a promising candidate for human application.

Conversely, priming with a nasally administered BCG followed by boosting with a parenteral modified vaccinia virus Ankara expressing Ag 85A (MVA85A) also induces high levels of antigen specific $CD4^+$ and $CD8^+$ T cells and protective immunity in mice [86]. Because MVA85A has been shown to be safe and highly immunogenic in a human Phase I study [87], the nasal BCG followed by boosting with MVA85A will probably be further evaluated as a TB vaccine candidate for humans.

New horizons in the development of mucosal vaccines

One of advantages of a mucosal vaccine over the injectable vaccine is, of course, that administration devices such as needles and syringes are not necessary. A departure from the disposable syringes and needles that are currently most commonly used for vaccination would be friendly to both humans and the environment, because it would eliminate the necessity of administration device (e.g., syringe and needle) leading to the secondhand spread of infectious diseases and pain associated with vaccine administration, and the vast amounts of medical waste generated by mass immunizations. Ongoing research into the molecular and cellular mechanisms of surface immunological barrier systems is providing practical strategies for the development of a new generation of mucosal vaccines. Here, we introduce and summarize some of the key discoveries for the development of a new generation of mucosal vaccines for the control of infectious diseases.

Transcutaneous vaccines

In addition to the mucosal immune system, the skin serves as another major surface barrier system with unique and dynamic immune attributes [27]. Transcutaneous immunization (TCI) relies on the application of a vaccine antigen with or without an adjuvant to the outer layer of the skin and subsequent delivery of the vaccine antigen to underlying LCs that serve as antigen-presenting cells [10,27,88]. TCI can induce systemic and mucosal immunity through antibody and mucosal CTL responses [27,89,90]. Recent studies have shown that TCI activates LCs carrying skin-derived antigens and allows them to migrate in a retinoic acid-dependent manner from the skin to the mesenteric lymph nodes, where they present antigen directly to resident lymphocytes for the initiation of antigen-specific mucosal immune response [90,91].

IOMAI Corporation has developed a needle-free skin patch containing a LT from *E. coli* as a TCI vaccine against traveler's diarrhea. As discussed above, a strain of ETEC is a major cause of traveler's diarrhea. Every year, 27 million travelers and 210 million children suffer from this acute diarrhea, causing 380,000 pediatric deaths. LT is a key pathogenic molecule in approximately two-thirds of cases of ETEC diarrhea [92,93]. Vaccines that induce immunity to LT offer protection against ETEC diarrhea because the toxin produced by the pathogen causes the watery stool [93]. In a double-blind, placebo-controlled trial, 59 volunteer adults received 50 µg of LT or placebo in a patch applied to the arm on days 0, 21 and 42. On day 56, 27 vaccinees and 20 controls were challenged orally with LT^+/ST^+ (heat-stable toxin) ETEC. The LT patch did not prevent ETEC infection, but a single LT patch produced seroconversion in 97% of vaccinated subjects and levels of anti-LT-specific IgG and IgA antibodies were higher than those of the control group following challenge with a virulent LT-expressing ETEC [87]. In order to further investigate the safety and efficacy of a LT-patch vaccine, volunteer travelers to Mexico and Guatemala were vaccinated before travel with two doses of the LT-patch vaccine given at 2–3-week intervals. The LT-patch recipients had shorter episodes of diarrhea with fewer loose stools than those receiving placebo [93]. The results of this Phase II trial suggest that a LT-patch vaccine could protect travelers suffering from diarrhea, but the efficacy of the LT patch

needs further confirmation in a Phase III trial. Vaccine delivery with patches or TCI may allow self-administration, ambient temperature stabilization, and ease of storage, making this approach an attractive needle- and cold chain-free form of vaccination (Figure 2).

Plant-based vaccines

Since 1990, plant-based vaccines have been proposed as the next generation of mucosal vaccines [201]. The production of vaccines in plants could have significant advantages over the existing production systems, including cost-effective production, rapid scaling-up production of the proteins, a low risk of contamination by human pathogens, and expression of multiple genes in a plant. Many candidate vaccine antigens, including bacterial diarrhea antigens, hepatitis B antigen, Norwalk virus antigen, cytomegalovirus glycoantigen, *Clostridium tetani* fragment C and RSV antigen, have been expressed in tobacco, tomato or potato to demonstrate the feasibility of plant-based vaccines [94–97].

Approximately 250 million episodes of acute gastroenteritis due to Norwalk virus occur annually in children and the elderly in the USA, causing vomiting, abdominal cramps, diarrhea, headache and fever. Thus, the development of a plant-based Norwalk virus oral vaccine would benefit public health. Immune responses were investigated in volunteers who ingested 150 g of transgenic potatoes expressing 0.21–0.75 mg of Norwalk virus capsid protein formed into virus-like particles. A total of 19 out of 20 volunteers developed an antigen-specific immune response with a modest titer of serum antibodies [98]. In another study, transgenic corn (2.1 g of plant material as a dose) expressing 1 mg of the B subunit of LT (LTB) was fed to adult volunteers in three doses. Seven of nine volunteers developed LTB-specific serum IgG and four of nine volunteers also developed LTB-specific stool IgA antibodies [99]. These studies demonstrated the feasibility of developing plant-based oral vaccine. Despite their attractiveness, some these plant-based vaccines did not advance to the next stage of development. To further advance the practicality and effectiveness of plant-based vaccines, it is essential to make them stable at room temperature for long periods, able to withstand the harsh environment of the gastrointestinal tract, and targeted to the mucosal inductive tissues, including PP. To overcome these hurdles, we have introduced a rice-based vaccine system (MucoRice™), in which the vaccine is physically and chemically stable and capable of generating protective immunity against enterotoxin such as a CT [100].

In MucoRice, CTB is used as the vaccine antigen [100]. The expressed antigen accumulates in protein bodies, the rice seed organelles for protein storage, which confers resistance against digestive enzyme activity while allowing uptake of the vaccine antigen. A minimal dose of oral antigen is therefore effective at inducing an antigen-specific immune response. In fact, when

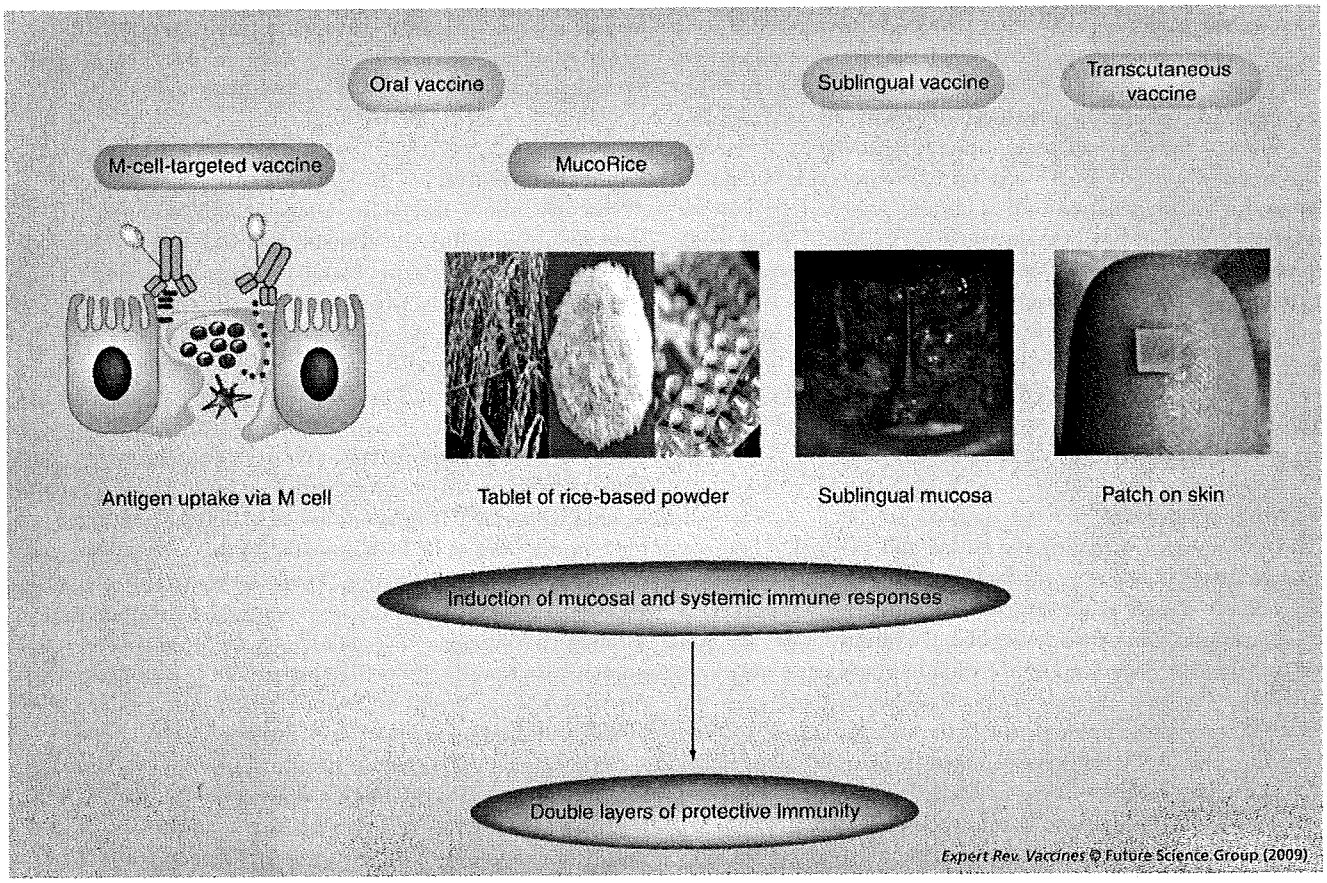

Figure 2. New horizon for the development of needle/syringe-free vaccine.

ingested by mice, MucoRice-expressed CTB was taken up by the M cells covering the PP and induced toxin-specific serum IgG and mucosal IgA antibodies with neutralizing activity. In addition, the rice-based CTB vaccine remained stable and was immunogenic after storage at room temperature for 2 years and was protected from pepsin digestion *in vitro*. Taken together, these findings suggest that the MucoRice system would be a needle- and cold chain-free oral vaccine against infectious diseases (Figure 2) [100].

M-cell-targeted vaccines

M cells possess the ability to take up luminal antigens and are the gateway of the respiratory and digestive immune system, making them attractive targets for vaccine antigen delivery [101]. Several molecules bind preferentially to M cells. For example, *Ulex europaeus* agglutinin (UEA)-I has specificity for α(1,2) fucose and specifically reacts with murine M cells [102]. The σ1 protein derived from reovirus specifically binds to a carbohydrate structure containing α(2,3)-linked sialic acid on the membranes of M cells [103]. Vaccination with UEA-1-conjugated [104] or σ1-protein-conjugated [105] nasal vaccines induces strong antigen-specific plasma IgG and mucosal IgA responses as well as CTL immunity. However, because UEA-1 also reacts strongly with goblet cells on the intestinal epithelium, the usefulness of UEA-1 as an M-cell-targeting vehicle is limited.

To overcome this obstacle, we established a novel M cell-specific monoclonal antibody (mAb) that selectively recognizes M cells but not goblet cells or epithelial cells [106]. Oral administration of a tetanus toxoid (TT)- or botulinum neurotoxoid (BoNT)-conjugated M cell-specific mAb together with the mucosal adjuvant CT induced high-levels of antigen-specific serum IgG and mucosal IgA responses; TT- or BoNT-conjugated control rat IgG induced no or very low antigen-specific immune responses, and the immune response to even 10-times the amount of noncoupled TT was much lower than that in mice vaccinated with the TT-conjugated M cell-specific mAb. In addition, an oral vaccine formulation of BoNT-conjugated M cell-specific mAb induced protective immunity against a challenge with 10,000-times the LD_{50} dose of botulinum toxin [106]. The results suggest that an M cell-targeted vaccine using the concept of a mAb guiding system could be a useful approach for developing highly effective mucosal vaccines.

Sublingual vaccines

The oral mucosa, including the buccal and sublingual mucosa, may be useful as a delivery site for therapeutic drugs because proteins do not degrade to the same extent in these regions as they do in the intestine [28]. Of the routes of oral administration, the sublingual route has been used for the immunotherapeutic treatment of allergies because antigens are quickly absorbed and enter the bloodstream without passing through the intestine or liver, and are, therefore, able to efficiently elicit antigen-specific tolerance [107,108]. These findings suggest that the sublingual route might be used for delivery of vaccine antigens for infectious diseases.

Sublingual administration of inactivated influenza A/PR8 virus (H1N1) together with a mucosal adjuvant such as CT [28] or non-toxic mCTA/LTB adjuvant (the A subunit of mutant CT with the B subunit of LT) [109,110] induces both systemic and mucosal virus-specific antibody responses as well as CTL responses with protective immunity after respiratory challenge with the A/PR8 virus [28,110]. The studies also showed that the sublingual epithelium harbors a dense lattice of DCs, and that using CT as a mucosal adjuvant mobilizes DCs within the sublingual epithelium. These cells migrate to the proximal draining lymph nodes, such as the submaxillary and superficial cervical lymph nodes, on uptake of the sublingual vaccine antigens. Interestingly, similar to nasal vaccination, sublingual immunization induces antigen-specific immune responses in the female reproductive tract in addition to the respiratory tract and oral/nasal cavity, suggesting that sublingual immunization uses the same cellular trafficking system as nasal immunization [28]. The sublingual mucosa represents a specialized immunological microenvironment favoring the initiation of antigen-specific immune responses [28,110].

A nasal inactivated influenza virus vaccine together with a toxin-based adjuvant (LT) was associated with the incidence of Bell's palsy during human clinical trials [111]. In addition, toxin-based adjuvant such as CT and CTB are redirected to the olfactory bulb in the CNS when administered via the nasal route [112], which has resulted in concerns about side effects in the nervous system. By contrast, inactivated influenza virus with a mucosal adjuvant did not migrate to or replicate in the CNS after sublingual immunization [110]. These findings suggest that sublingual immunization may be another attractive and safe mucosal route for administering influenza vaccines for the generation of influenza-specific neutralizing antibodies and CTL responses (Figure 2).

Expert commentary

As discussed above, several live-attenuated forms of mucosal vaccines are already clinically available or are now in or being considered for clinical trials. In general, these live attenuated vaccines can induce potent protective immunity against pathogens because they are strong immunogens and at same time contain natural forms of vaccine adjuvants, such as Toll-like receptor (TLR) ligands (e.g., TLR3, 4 and/or 7) [113]. However, a couple of live-attenuated vaccines, such as the OPV and Rotashield rotavirus vaccines, have been reported to revert to virulent forms or to cause serious side effects. Although these risks are quite low (e.g., one out of 500,000 for OPV [32] and one out of 30,000 for Rotashield [61]), these possibilities must be eliminated in order to safely control infections through vaccination. Further, these unfortunate facts are often revealed after the vaccine is approved for the public [32,61]. Although live-attenuated vaccines induce more potent protective immunity in healthy adults than inactivated or subunit vaccines, the latter may be useful for immunization of the elderly, naive infants and children at high risk of disease.

Another practical reason for the use of some of live-attenuated vaccines is their low production costs. Distribution costs also contribute greatly to the increased total medical cost of global vaccination, and one of the major practical obstacles to vaccination in the field, especially in the developing countries, is the need for refrigerated storage of the vaccine (the cold chain) [114]. The

cost of preserving the cold chain for currently used vaccines has been estimated at approximately US$300 million a year [88]. In addition, inappropriate disposal of needles and syringes poses a threat of environmental contamination and increases the risk of secondhand use, which can lead to the spread of secondary infectious diseases [88]. Thus, we must develop mucosal vaccines, which do not require dangerous administration devices or a cold chain, to combat emerging and re-emerging infectious diseases while addressing issues of cost, safety and the environment.

Five-year view

Because mucosal administration of vaccines can induce both systemic and mucosal immune responses, mucosal vaccination might improve the efficacy of current parentally delivered vaccines and provide a basis for preventing a range of infectious diseases that are initiated at the mucosal surfaces of the digestive, respiratory and reproductive tracts. However, some inactivated or subunit-type vaccines, which offer attractive safety profiles, are generally poor immunogens when given mucosally [6]. Thus, we propose a system of delivery of vaccine antigen to M cells. The development of M-cell-targeting systems for antigen delivery could increase the efficacy of mucosal vaccines for the induction of antigen-specific systemic and mucosal immune responses. To this end, we believe that the MucoRice system is an attractive and promising strategy for vaccination without the need for needles, syringes, or a cold chain [100], and the development of an M cell-targeted MucoRice vaccine may be the most promising approach to create an effective mucosal vaccine.

Financial & competing interests disclosure

The work was supported by the grants from Bill & Melinda Gates Foundation in US, Scientific Research Priority Areas and Global Center Excellence from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Ministry of Health and Labor of Japan, the Ministry of Economy, Trade and Industry of Japan (Hong Kong) and Bio-oriented Technology Research Advancement Institution in Japan (Saitama). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Key issues

- Other than FluMist™, no nasal vaccines are currently available against most bacterial respiratory pathogens, including *Streptococcus pneumonia, Haemophilus influenza, Mycobacterium tuberculosis* and *Bordetella pertussis*.
- Transcutaneous and sublingual vaccines are nonclassical mucosal vaccines that can effectively induce both mucosal and systemic immunity for the prevention of infectious diseases.
- Creation of a novel mucosal-targeting system, such as M cell- and/or mucosal dendritic cell-targeted vaccine delivery, is a key issue for the development of effective mucosal vaccines.
- Mucosal adjuvants must also be developed for the induction of effective protective immunity against emerging and reemerging infectious diseases.
- Cold chain-free and needle/syringe-free vaccines are needed, especially in developing countries. A rice-based vaccine, MucoRice™, is currently one of the best candidates for achieving this goal.
- Registered mucosal vaccines are still few in number. Public-health requires effective and safe mucosal vaccines to control both emerging and re-emerging infectious diseases.

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Vaccine 27 (2009) 5982–5988

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Oral MucoRice expressing double-mutant cholera toxin A and B subunits induces toxin-specific neutralising immunity

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ARTICLE INFO

Article history:
Received 30 April 2009
Received in revised form 10 July 2009
Accepted 22 July 2009
Available online 7 August 2009

Keywords:
Rice-based vaccine
MucoRice
Oral vaccine
Cholera
Cold-chain-free
Mutant cholera toxin

ABSTRACT

Rice-expressed cholera toxin B (CTB) subunit is a cold-chain-free oral vaccine that effectively induces enterotoxin-neutralising immunity. We created another rice-based vaccine, MucoRice, expressing non-toxic double-mutant cholera toxin (dmCT) with CTA and CTB subunits. Western-blot analysis suggested that MucoRice-dmCT had the shape of a multicomponent vaccine. Oral administration of MucoRice-dmCT induced CTB- but not CTA-specific serum IgG and mucosal IgA antibodies, generating protective immunity against cholera toxin without inducing rice-protein-specific antibody responses. The potency of MucoRice-dmCT was equal to that of MucoRice-CTB vaccine. MucoRice has the potential to be used as a safe multicomponent vaccine expression system.

1. Introduction

To prepare for the successful execution of future global vaccination programs, it is essential that we consider creating a new generation of vaccines that do not require refrigeration storage and traditional syringes and needles for vaccination. The use of transgenic plant-derived recombinant protein is a promising strategy that combines innovation and knowledge of mucosal immunology and plant biotechnology to produce such suitable plant-based vaccines for global immunisation [1,2]. The potential benefits include cost-effective and rapid up-scaling of production, expression of multiple genes at one time, and lower risk of contamination with human pathogens in the preparation of vaccine antigens. Furthermore, plants are suitable for foreign protein production and storage and as oral delivery options for subunit-type vaccines to induce protective immunity against infectious diseases via the mucosal immune system [1,2]. Among several plant-based vaccines developed, grains such as corn, wheat and rice have recently attracted interest for vaccine production, storage and delivery systems for oral immunisation. As a vaccine antigen production system, rice seed has advantages over other grains, including easier storage and processing and greater yield; moreover, the rice plant has self-crossing ability [3]. In addition, a rice transformation system has been established and the full genome sequence elucidated, enabling rice genetic information to be easily applied to the creation of a gene-manipulated product [3,4].

We recently developed a rice-based oral cholera toxin (CT) B (CTB)-subunit vaccine (MucoRice-CTB) that has many practical advantages over most traditional injection-type vaccines and other plant-based oral vaccines [3]. The rice-based oral vaccine is stable at room temperature for several years and is protected from digestive enzymes in the harsh conditions of the gastrointestinal tract. When MucoRice-CTB was given orally, the vaccine induced antigen-specific antibodies with toxin-neutralising activity [3]. Here, to demonstrate the development of a multicomponent vaccine as part of a rice-based vaccine antigen expression system, we produced transgenic rice seed expressing the A and B subunits

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doi:10.1016/j.vaccine.2009.07.071

of a nontoxic double-mutant cholera toxin (dmCT), which contained two amino acid substitutions, of the ADP-ribosyltransferase active centre (E112K) and carboxyl-terminal KDEL (E112K/KDGL) in the A subunit (dmCTA) [5,6]. We then examined whether oral vaccination with this seed would effectively induce enterotoxin-neutralising immunity. Although dmCT is considered safe and nontoxic, exhibiting no ADP-ribosyltransferase activity and participating in normal intracellular trafficking [6], it retains the biological capacity to enhance antibody immune responses against co-administered antigens [6]. Our strategy was aimed at utilising these unique characteristics of dmCT by inserting a dmCTA-specific gene into the rice genome to develop rice expressing dmCTA in addition to the original CTB, thus yielding a multicomponent vaccine, MucoRice-dmCT.

2. Materials and methods

2.1. DNA construction and transformation of rice plants

A double mutant of the CT gene (dmCT E112K/KDGL) was modified to a suitable codon optimisation form for rice seed by introducing two potent mutations into the ADP-ribosylation activity centre and C-terminal KDEL [6,7]. The modified dmCTA subunit and B-subunit genes for the dmCT gene were cloned as individual ORFs flanked with plant elements to facilitate the transcription of each subunit. The dmCTA subunit and dmCT cassettes were assembled. The dmCTA subunit cassette consisted of a *GluB-4* promoter/signal sequence followed by the rice-optimised dmCTA (E112K/KDGL) with a Nos terminator [8]. The dmCT cassette consisted of both a dmCTA subunit cassette and a B subunit cassette, which comprised a GluB-1 promoter/signal sequence followed by a rice-optimised B subunit with a GluB-1 terminator [8]. Finally, each cassette was cloned into the binary vector pGPTV-35S-HPT [4]. The resulting plasmids for dmCT and dmCTA were individually transformed in rice plants, *Oryza sativa* L. 'Kita-ake' [9], by using an *Agrobacterium*-mediated method described previously [10]. Rice-expressed CTB with a KDEL signal at the C-terminal of CTB was produced as reported previously [3].

2.2. DNA and protein analyses

Using the cetyltrimethylammonium bromide (CTAB) extraction method, genomic DNA was extracted from the leaf tissues of transgenic rice, and the integration of the dmCT gene into the genomic DNA was analysed by PCR [4]. Total seed protein was extracted from the seeds as described previously [3]. Briefly, seeds of rice plants were ground to a fine powder by using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). Seeds were extracted under reducing conditions in 2% (w/v) SDS, 8 M urea, 5% (w/v) β-mercaptoethanol, 50 mM Tris–HCl (pH 6.8) and 20% (w/v) glycerol before being separated by SDS-PAGE with a 15–25% gradient polyacrylamide gel (Daiichi Pure Chemical, Tokyo, Japan). Under non-reducing conditions, seeds were extracted in 0.1% (w/v) SDS, 50 mM Tris–HCl (pH 6.8) and 20% (w/v) glycerol before being separated by SDS-PAGE. The gel was subsequently transferred to Hybond-P PVDF membranes (GE Healthcare) for Western-blot analysis with 5 μg/ml rabbit anti-mCTA antibody or rabbit anti-CTB prepared in our laboratory. Accumulation levels of CTA or CTB were determined by densitometric analysis of the Western blots against a standard curve generated with the use of rmCTA (E112K) or rCTB expressed in *Bacillus brevis* and purified in our laboratory [5,11,12]. Antibody to rmCTA or rCTB was raised from rabbits immunised with the respective recombinant protein in our laboratory.

2.3. Oral immunisation and assessment of antibody responses by ELISA

An oral immunisation study was performed in 6-week-old BALB/c mice (CLEA, Tokyo, Japan). On five occasions at 2-week intervals, mice (five mice per group) were orally immunised with 50 mg of CTB-transgenic rice, with a corresponding dose of CTB at 75 μg; or with 200 mg of dmCT-transgenic rice, with a corresponding dose of dmCTA at 20 μg and CTB at 50 μg; or with 50 mg of CTB-transgenic rice plus 200 mg of dmCTA-transgenic rice, with a corresponding dose of CTB at 75 μg plus dmCTA at 10 μg; or with 250 mg of dmCTA-transgenic rice, with a corresponding dose of dmCTA at 12.5 μg; or with either 200 mg of non-transgenic rice dissolved in 1 ml of PBS or with PBS alone, as controls. To examine the adjuvant effect of transgenic rice, mice were orally immunised three occasions at a week interval with 250 μg tetanus toxoid (TT, kindly provided by The Research Foundation for Microbial Diseases of Osaka University, Suita, Osaka, Japan) alone in 1 ml of PBS or with TT together with 200 mg of rice-expressed dmCT, 100 mg of rice-expressed CTB, 200 mg of wild-type (WT) rice or 10 μg of CT (List Biological Laboratories, Campbell, CA). One week after the final immunisation, serum and faecal extracts were collected and CTB-, CTA-, TT- or rice-storage protein-specific immunoglobulin responses were measured by ELISA with 5 μg/ml of rCTB, rmCTA or TT or 20 μg/ml rice-storage protein extracted with 0.01% Triton X-100, as described previously [3].

2.4. Analyses of neutralising antibody and protection activity

CT (50 ng/ml) was added to serially diluted sera collected from immunised mice. The sera were then subjected to GM1-ELISA as previously described, with some modifications [13]. Briefly, 96-well plates (Thermo, Milford, MA) coated with 5 μg/ml of monosialoganglioside GM1 (Sigma) were incubated with CT that had first been treated with serum from immunised mice and then with an HRP-conjugated Rabbit anti-CTB antibody prepared in our laboratory [3]. The colour of the solutions was developed by the addition of TMB substrate (Moss, Pasadena, MD), and absorbance was measured at a wavelength of 450 nm. In addition, a CHO cell (line ATCC, CCL-61) assay [14] was performed with serum to which 50 ng/ml of CT had been added. After 14 h of stimulation of the cells in 5% CO_2 in a humidified incubator at 37 °C, morphological changes were observed under a microscope. In addition, we performed an *in vivo* challenge experiment with CT, as described previously [3]. The vaccinated mice were orally challenged with 20 μg of CT. After 14 h the mice were examined for clinical signs of diarrhoea and the volume of intestinal water was measured.

2.5. Data analysis

Data were expressed as means ± standard deviation. All analyses for statistically significant differences were performed with Tukey's *t* test, with P values < 0.01 considered to indicate significance (**).

3. Results

3.1. Development of rice-expressed nontoxic double-mutant cholera toxin (dmCT)

The two genes encoding dmCTA and CTB were generated as shown in Fig. 1. We chose to introduce the dmCTA and CTB genes separately into the same rice as rice-expressed dmCT in order to prove the antigenicity, and lack of adjuvanticity, of dmCT. Using codons preferentially used for translation of several rice-seed-protein genes, both genes were optimised for expression in the

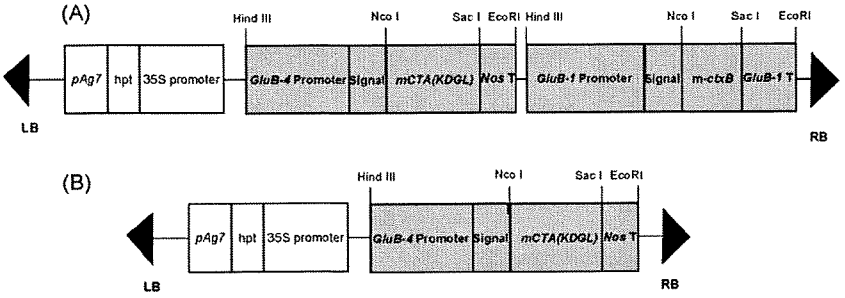

Fig. 1. Schematic representation of transformation plasmids for the development of MucoRice-dmCT. The DNA fragments coding dmCT (A) and dmCTA (B) were placed under the control of the rice-seed major storage protein glutelin GluB-4 promoter and/or glutelin GluB-1 promoter. 35S promoter: Cauliflower mosaic virus 35S promoter; hpt: hygromycin phosphotransferase gene; LB: left border; pAg7: agropine synthase polyadenylation signal sequence; RB: right border.

transgenic rice seed. Rice seed storage protein glutelin 1.4-kb GluB-4 promoter/signal peptides and 2.3-kb GluB-1 promoter/signal peptides were translationally fused to the dmCTA and CTB genes, respectively, to achieve endosperm-specific expression. The dmCT (dmCTA and CTB) or dmCTA alone of these chimeric genes was cloned into the plant expression vector pGPTV-35S-HPT (Fig. 1A, dmCT; Fig. 1B, dmCTA). Following *Agrobacterium*-mediated transformation of Kita-ake rice plants, several independent transgenic rice lines were generated for each of the two constructs (dmCT and dmCTA), and accumulation levels in the seed were examined by immunoblot analysis. For each antigen, one plant line that had the highest levels of antigen accumulated in the seed was selected and proceeded to the T3 generation by self-crossing to obtain homozygous lines. Integration of the dmCTA and CTB genes into the rice genome was examined by PCR amplification; each gene was PCR-amplified in the plant line in the case of the dmCTA or CTB construct, whereas no signal was amplified in non-transgenic rice (data not shown).

To examine the accumulation of dmCTA and CTB in transgenic rice seed, total seed protein was extracted under either reducing (Fig. 2A and B) or non-reducing (Fig. 2C and D) conditions from mature seed and analysed by SDS-PAGE followed by Western blotting analysis with anti-rmCTA (Fig. 2 B and D) or anti-rCTB (Fig. 2A and C) antibodies. Under reducing conditions, a 26-kDa band was recognised by the mCTA-specific antibody in the total seed protein of the dmCT and dmCTA lines (Fig. 2B), whereas authentic cholera toxin gave two bands, at 26 and 20 kDa, which corresponded to CTA and CTA1, respectively (Fig. 2B). CTA is synthesised with a trypsin-sensitive bond that joins the CTA1 and CTA2 pieces; each piece is itself bonded to the other with a disulphide bond [15]. In the dmCT and CTB lines, 11-kDa and 14-kDa bands were detected by CTB-specific antibody; however, the accumulation levels of CTB in the dmCT line were less than those in the CTB line (Fig. 2A). The 14-kDa bands estimated from electrophoresis were larger than the authentic and monomeric CTB band (10 kDa), probably because of the addition of all, or part of, the *GluB-1* signal peptide at the N-terminus of CTB and/or plant-based glycosylation.

Under non-reducing conditions, several bands of CTB protein of high molecular weight (about 37–50 kDa) were detected in the total seed protein from dmCT and CTB lines by Western-blot analysis with CTB-specific antibody (Fig. 2C). These findings indicated that CTB proteins were produced and accumulated in both transgenic rice lines of MucoRice-dmCT and -CTB as part of the assembly of the pentameric structure, which consisted of two types of monomer. Unlike the case with authentic CT, a 60-kDa band was not detected with either anti-CTA (Fig. 2C) or anti-CTB (Fig. 2D) antibodies when MucoRice-dmCT was examined, suggesting that the mutant toxin assembled in MucoRice-dmCT was not of authentic size. However, notably, the two subunits were definitively and independently expressed in the transgenic rice seed.

The levels of accumulation of dmCT and dmCTA expressed in the seeds were quantified by densitometric analysis with known amounts of purified rmCTA and rCTB as standards (data not shown). CTB was accumulated in rice-expressed dmCT at 5 μg per seed, whereas the quantity of rice-expressed CTB with the KDEL signal was as high as 30 μg per seed. CTA accumulated in rice-expressed dmCT at 2 μg per seed and in rice-expressed dmCTA at 1 μg per seed. The differences in expression level between dmCT and

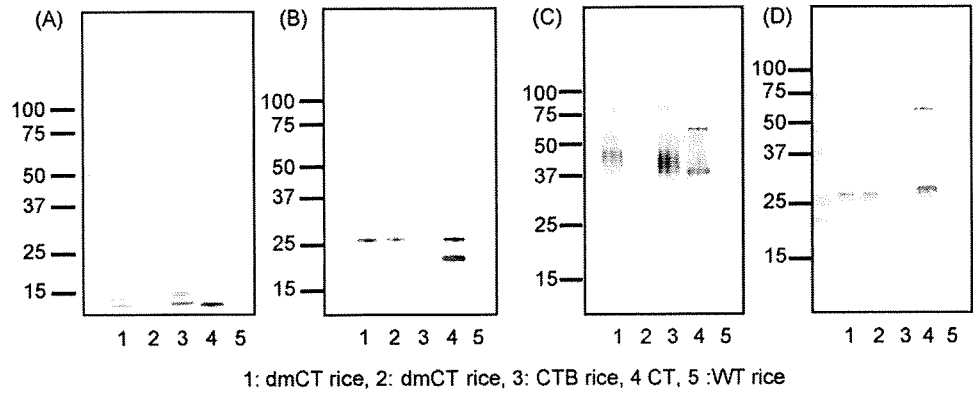

Fig. 2. Analysis of CT subunit expression in MucoRice-dmCT. Western-blot analysis under reducing conditions (A and B) revealed that rice-expressed dmCT and CTB gave 11-kDa and 14-kDa bands on detection with specific anti-CT-B antibody (A), and rice-expressed dmCT and CTA gave 26-kDa bands on detection with specific anti-CTA antibody (B), whereas authentic CT gave an 11-kDa band for CTB, and 26-kDa and 20-kDa bands for CTA and CTA1, respectively (A and B). Western-blot analysis under non-reducing conditions (C and D) revealed that rice-expressed dmCT and -CTB gave 37–50-kDa bands upon detection with specific anti-CT-B antibody (C), and rice-expressed dmCT and CTA gave a 26-kDa band on the detection with specific anti-CTA antibody (D), whereas authentic CT gave bands of 26-kDa (CTA), 37–50-kDa (CTB pentamer), and 60-kDa (CT) (C and D).