Mucosal vaccines: wisdom from now and then

Hiroshi Kiyono[1,2,3], Yoshikazu Yuki[1], Rika Nakahashi-Ouchida[1] and **Kohtaro Fujihashi[4,5]**

[1]Division of Mucosal Immunology, IMSUT Distinguished Professor Unit, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan
[2]Department of Immunology, Graduate School of Medicine, Chiba University, Chiba 260-0856, Japan
[3]Department of Medicine, School of Medicine and CU-UCSD Center for Mucosal Immunology, Allergy and Vaccines, University of California, San Diego, San Diego, CA 92093, USA
[4]Division of Clinical Vaccinology, International Research and Development Center for Mucosal Vaccines, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan
[5]Department of Pediatric Dentistry, The University of Alabama at Birmingham, Birmingham, AL 35294, USA

Correspondence to: H. Kiyono; E-mail: kiyono@ims.u-tokyo.ac.jp

Abstract

The oral and nasal cavities are covered by the mucosal epithelium that starts at the beginning of the aero-digestive tract. These mucosal surfaces are continuously exposed to environmental antigens including pathogens and allergens and are thus equipped with a mucosal immune system that mediates initial recognition of pathogenicity and initiates pathogen-specific immune responses. At the dawn of our scientific effort to explore the mucosal immune system, dental science was one of the major driving forces as it provided insights into the importance of mucosal immunity and its application for the control of oral infectious diseases. The development of mucosal vaccines for the prevention of dental caries was thus part of a novel approach that contributed to building the scientific foundations of the mucosal immune system. Since then, mucosal immunology and vaccines have gone on a scientific journey to become one of the major entities within the discipline of immunology. Here, we introduce our past and current efforts and future directions for the development of mucosal vaccines, specifically a rice-based oral vaccine (MucoRice) and a nanogel-based nasal vaccine, with the aim of preventing and controlling gastrointestinal and respiratory infectious diseases using the interdisciplinary fusion of mucosal immunology with agricultural science and biomaterial engineering, respectively.

Keywords: delivery system, infectious disease, MucoRice, nanogel, SIgA

Introduction

In higher mammals, the mucosal immune system consists of an integrated immunological network of tissues, lymphoid and mucous membrane-associated cells and effector molecules. Along with cytokines, chemokines and their receptors, secretory IgA (SIgA, dimeric antibodies of the IgA isotype that contain the secretory component) are key players in mucosal immunity and appear to function in synergy with innate cells [e.g. NK cells, innate lymphoid cells (ILCs), mucosal-associated invariant T (MAIT) cells and γδT cells] and anti-microbial molecules (e.g. defensins, lysozyme, mucus and surfactant) (1–3). In the mammalian host, organized secondary lymphoid tissues have evolved in the upper respiratory (UR) and gastrointestinal (GI) tracts to facilitate antigen uptake, processing and presentation for the initiation of antigen-specific immune responses. To this end, nasopharyngeal-associated lymphoid tissue (NALT) and gut-associated lymphoid tissue (GALT) have been the most well-characterized mucosa-associated secondary lymphoid tissues (1–3).

Collectively, NALT and GALT constitute a mucosa-associated lymphoreticular tissue (MALT) network (1–3), which, although integrated, is at best only partially understood, especially in humans. MALT shares the common molecular and cellular characteristics of inductive tissue that initiates antigen-specific immune responses, owing to its lymphoepithelial covering that contains active antigen-sampling M cells (1–3). It encompasses immunologically organized regions that include several features: the subepithelium (dome), enriched with antigen-presenting cells (APCs) including dendritic cells (DCs), B-cell zones with germinal centers and adjacent T-cell areas with APCs and high endothelial venules (HEVs) for entry and egress of lymphocytes recirculation and migration (1–3). MALT is thus considered to be a commander site for integrated mucosal immune cell circulation and communication.

Resident at the mucosal effector sites [e.g. airway-digestive tract lamina propria (LP) regions and glandular tissues

(e.g. salivary and lachrymal glands)] originated from MALT are the antigen-specific CD4-positive (CD4+) Th1 cells, Th17 cells and CD8+ cytotoxic T lymphocytes (CTLs) responsible for mucosal cell-mediated immunity (CMI)/CTL functions (1–3), as well as CD4+ Th2 cells and B lymphocytes responsible for dimeric and polymeric IgA antibody synthesis (1–3). Of importance, these polymeric IgA antibodies produced in the LPs are transported from the basal side of epithelial cells via the polymeric Ig receptor to luminal sites on mucosal surfaces where the molecular form of SIgA antibodies plays a key role in the creation of healthy mucosal environments, preventing pathogen invasion and forming a salubrious location for commensal microflora (1–3).

Historical insights into the contribution of dental science to mucosal immunology

As a result of the advance of modern technology and the sophisticated achievements of science, mucosal immunology has become a core entity uniting the biomedical fields of immunology, microbiology, allergology, pathology and the science of nutrients and metabolism. An era that began in the mid-1960s to 1970s saw major scientific efforts directed toward understanding the regional immune system, known as 'local immunity', although the presence of the immune system at the mucosal surface of the digestive tract had previously been postulated (4–6).

IgA antibodies, which play central roles in mucosal immunity, were originally found in the external secretions including saliva by Tomasi *et al.* in the mid-1960s (4, 6). They showed that human parotid saliva (and other nonvascular fluids) contains large amounts of IgA relative to IgG and that these IgA antibodies differ in chemical and immunological properties from serum IgA (4–6). Several investigators, including those in our group, with backgrounds in dentistry and oral biology, recognized the important relationship between the oral cavity as the beginning of the digestive tract and the large quantities of IgA antibodies (~9200 mg) generated in the salivary glands and ingested through saliva (750–1000 ml) each day (7).

During the same era, it was reported that *Streptococcus mutans* is a causative pathogen for developing dental caries (8, 9). One could thus hypothesize that the induction of *S. mutans*-specific SIgA in salivary fluids would be a scientifically logical and significant strategy—this led to efforts to develop caries vaccines (10). Our laboratory showed that oral administration of whole, killed *S. mutans* induced both antigen-specific salivary IgA and serum IgG antibodies (11–13). These strategies and achievements made by several research groups in the fields of dental science and oral biology opened up a new world of immunology where the mucosal immune system was elucidated and understood, allowing the knowledge thus gained to be used as the basis for development of mucosal vaccine strategies.

Mucosal vaccination as a sensible strategy for the prevention of infectious diseases

Currently, most licensed vaccines available for human use are administered through systemic routes by injection using syringes and needles. The traditional route of vaccination effectively induces antigen-specific, protective immune responses in the systemic compartment; however, it essentially elicits only weak or absent antigen-specific immune responses at mucosal surfaces, where the majority of pathogens, including the recent pandemic virus SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), replicates at the mucosal surface and invades the host (3). Thus, although the systemic route of vaccination that is currently and routinely used can unequivocally induce protective immunity within the body and is thus useful to prevent infectious illness from worsening (14–16), it is not suitable for defending against the invasion of harmful mucosal pathogens upon their entry by inhalation, ingestion or sexual contact (14–16).

In contrast, mucosal vaccination with appropriate delivery vehicles or co-administered with an adjuvant could successfully induce protective mucosal immune responses and thus prevent actual pathogenic infection at mucosal entry sites (14–16). Since mucosal vaccination can also elicit an antigen-systemic immune response equivalent to that induced by injection, it leads to the induction of dual layers of protective immunity at the mucosal surface and in the systemic compartment (14–16). If pathogens leak into the body through the mucosal barrier, mucosal vaccine-induced systemic immunity provides a second layer of protection against pathogens (14–16).

In addition to their effectiveness in inducing a double layer of protective immunity, mucosal vaccines offer several advantages over injectable vaccines. For example, mucosal vaccination may not require a trained health professional for administration. Furthermore, it is environmentally friendly, creating less medical waste when compared with injectable vaccines. Moreover, mucosal vaccines will most likely decrease costs, avoiding needlestick injuries and transmission of blood-borne diseases and cause less physical and psychological discomfort (14). Despite these considerable merits, only limited numbers of oral and nasal vaccines—against poliovirus, rotavirus, *Salmonella typhi*, *Vibrio cholerae* and influenza virus—are currently available for clinical use in humans. Most of these licensed mucosal vaccines involve either attenuated or gene-modified live or killed forms of whole micro-organisms (14). No mucosal vaccine that delivers a component (subunit) or purified form of antigen is yet available for clinical use.

One of the main reasons for this could be that these types of vaccine formulation require appropriate antigen-delivery vehicles and/or mucosal adjuvants that are suitable and tolerable in aero-digestive environments, inducing antigen-specific humoral (e.g. SIgA) and cell-mediated (e.g. CTLs) immune responses. Mucosal environments are indeed generally harsh and facilitate the degradation of antigens because of intrinsic physiologic mechanisms such as the presence of digestive enzymes (e.g. pepsin), clearance mechanisms (e.g. peristaltic action, ciliary movement, sneezing and mucus secretion) and physiologic and biologic barriers (e.g. gastric acid, mucins, serous secretions and tight junctions) (14, 16). The presence of these natural (or innate) defense mechanisms makes it difficult for mucosal vaccines to elicit antigen-specific immune responses.

Normal, healthy conditions also force the mucosal immune system to create and maintain a quiescent state of immune homeostasis (tolerance), since mucosal surfaces are continuously exposed to innumerable ingested and inhaled environmental antigens and allergens that could elicit hyper-immune responses [17–19]. Since there is an accumulated scientific foundation that shows the benefits of mucosal vaccines despite these difficulties and hurdles, numerous investigators including our group have spent time and effort developing novel mucosal antigen-delivery systems and adjuvants. Recent scientific advances, achieved through the sharing of knowledge and technologies among different fields of science, are helping to overcome several hurdles in the development of mucosal vaccines. The following sections introduce and discuss our strategies and efforts to develop oral and nasal subunit vaccines, using the integration and fusion of mucosal immunology, agricultural science and biomedical engineering to facilitate effective induction of mucosal immunity that can prevent bacterial infections in the GI and respiratory tracts.

MucoRice, a new-generation rice-based oral vaccine

The mucosal immune system has been extensively investigated over the past three decades and the concept of the common mucosal immune system (CMIS) is an extremely important element in the design of mucosal vaccines [1–3]. In addition to the presence of the CMIS, the presence of compartmentalization of mucosal immune responses is generally accepted [20]. For example, oral immunization mainly induces antigen-specific immune responses in the digestive tract, whereas nasal immunization resulted in the induction of specific immunity in the respiratory and genital tracts [20]. This evidence needs to be considered as part of our strategy for developing mucosal vaccines.

Diarrheal disease remains a global health issue. It is estimated that there were 1.3–4.0 million cases of cholera and 20 000–140 000 cholera-related deaths worldwide each year during the period from 2008 to 2012 [21]. In addition, pandemic diarrhea and travelers' diarrhea caused by the heat-labile enterotoxin (LT) of enterotoxigenic *Escherichia coli* are significant concerns [22]. Since children in low-income countries are the major victims of diarrheal diseases, a needle/syringe-free oral vaccine strategy that could elicit mucosal immunity in the GI tract is most likely to be ideal in terms of convenience of administration. Furthermore, such an oral vaccine should allow cold-chain-free storage and be low cost, taking into consideration the infrastructure of developing countries.

We have therefore made a combined effort to develop a new generation of subunit oral vaccines for the prevention of diarrheal diseases [23–25]. To accomplish our goal, we employed a plant-based delivery system since transgenic plant-based vaccines have been developed and drawn attention due to their practicality, safety and low cost [15, 16, 26]. Carrot, potato, rice, soybean, tobacco and tomato have been used to incorporate bacterial [e.g. the B subunit of LT (LTB) or cholera toxin (CTB) or *Yersinia pestis*] and viral (hepatitis B virus, rotavirus or norovirus) components in the form of edible vaccines [24, 27–33].

Among the various plant candidates, rice may be the most suitable antigen-expressing plant since the rice seed [especially the protein body (PB)] is resistant to digestion by gastric acid [23–25] and thus may be stably and effectively delivered to the gut immune system (e.g. GALT) for the initiation of antigen-specific immune responses. Furthermore, protein expressed in rice seeds is stable for a prolonged time in the absence of refrigerated storage [23–25] and thus can allow a cold-chain-free vaccine to be created. Rice can thus be considered a viable candidate for the creation of cold-chain-free and needle-free vaccines.

In this regard, we have developed transgenic rice that expresses and accumulates CTB in the rice seeds, mainly in the PBs; this is called MucoRice-CTB and it demonstrates significant potency as a cereal plant-based vaccine [23–25]. The gene encoding CTB was transduced into rice seeds using an *Agrobacterium*-mediated method. Each transgenic seed contains ~30 μg of recombinant protein, which accumulates in the rice PB-I and PB-II [23]. The PB-I is water-insoluble and thus only dissolves in organic solvents, whereas PB-II is known to be a water-soluble protein, suggesting that these PBs act as a natural capsule for the oral delivery of vaccine antigens to the gut immune system including GALT.

Thus, when MucoRice-CTB is administered orally it is reasonable to anticipate that CTB protein is released from PB-II immediately and that subsequent slow and delayed release might occur from PB-I. Indeed, oral immunization with MucoRice-CTB resulted in the induction of CTB-specific mucosal SIgA and systemic IgG antibody responses in experimental animal models even after MucoRice-CTB was stored at room temperature for 3 years [23]. Thus, MucoRice-CTB-vaccinated mice were protected from watery diarrhea when orally challenged with the toxin. Similarly, mice given oral MucoRice-CTB showed significant protection from *V. cholerae*-induced diarrhea [25]. Of importance, MucoRice-CTB vaccination induced cross-protective immunity against LT intoxication because of the close similarity between CTB and LTB in the subunit responsible for delivery of the toxic A subunit into intestinal epithelial cells [25]. These results suggest that MucoRice-CTB could be a potential vaccine against intoxication caused by enterotoxigenic *E. coli*, a major cause of travelers' diarrhea and severe diarrhea in children in developing countries.

MucoRice-CTB phase I human trial showing immunogenicity and safety

As MucoRice-CTB elicited protective CTB-specific antibody production in non-human primates (cynomolgus macaques) [24], we have begun to develop good manufacturing practice (GMP)-grade MucoRice-CTB, using a closed clean hydroponic harvesting facility to ensure that the vaccine remains uncontaminated and that the gene-modified plants are isolated from the natural environment [34] (Fig. 1).

Using this successful, GMP-qualified product (MucoRice-CTB), a double-blind, randomized, placebo-controlled, dose-escalation phase I study to evaluate the safety, tolerability and immunogenicity of MucoRice-CTB (clinical trial registration: UMIN000018001) was conducted at IMSUT Hospital, The Institute of Medical Science, The University of Tokyo,
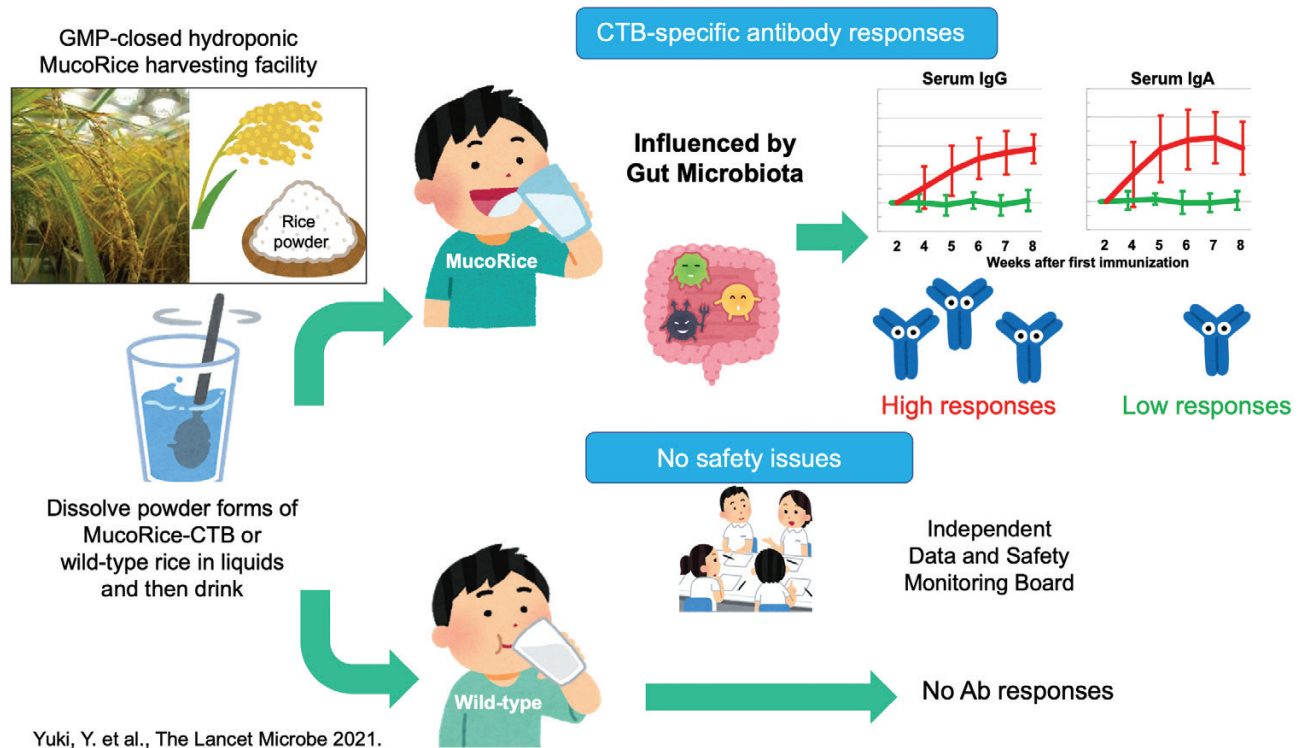
Fig. 1. Needle/syringe-free and cold-chain-free MucoRice-CTB oral vaccine made from rice: proof of immunogenicity and safety in humans. Adapted, with permission, from ref. (35).

Tokyo, Japan (30) (Fig. 1). MucoRice-CTB was administered in four oral doses at 2-week intervals to healthy, adult male Japanese volunteers. A total of 226 participants were initially recruited and 60 healthy Japanese male volunteers between 20 and 40 years of age without any international travel-related episode of diarrhea at the time of informed consent were enrolled. The study comprised three cohorts (1, 3 or 6 g of MucoRice-CTB), with each cohort comprising 20 subjects. MucoRice-CTB induced neutralizing antibodies against diarrheal toxins in a gut microbiota-dependent manner, without major adverse events (35). When metagenomic analysis of study participants was performed using bacterial DNA from fecal samples, high responders had a gut microbiota of higher diversity with indications of the presence of *E. coli* and *Shigella* spp., compared with non/low-responders (35) (Fig. 1). On the basis of this phase I investigation, MucoRice-CTB qualified as a safe and promising plant-based oral vaccine that can advance to large-scale clinical trials in the future.

The essentials of nasal vaccines

In addition to oral vaccines, nasal vaccination is another attractive strategy, which is based on molecular and cellular understanding of the nasopharyngeal and respiratory immune systems, providing a scientific foundation for the induction and regulation of antigen-specific immune responses by the airway immune system (36, 37). According to the concept of compartmentalization of the mucosal immune system, a nasal spray or nasal immunization elicits antigen-specific immune responses in the upper and lower respiratory tracts (20). Furthermore, it has been shown that nasal vaccination can induce vaccine antigen-specific humoral (SIgA and serum IgG) immunity and cell-mediated (CTL) immunity in the genital tract, oral cavity and the intestinal mucosa of mice and non-human primates (38–42).

These reports suggest that nasal vaccination could be an all-embracing, ideal strategy to prevent pathogen invasion at most mucosal surfaces; however, as with oral vaccines, nasal vaccines face obstacles due to the presence of chemical and physical barriers such as digestive enzymes, serous secretions, ciliary movement and sneezing. In this regard, nasal vaccines also require a unique delivery system and/or adjuvants to induce antigen-specific protective immunity. In addition to these considerations, one must pay attention to the safety of nasal vaccines since the anatomical and histological features of the nasal cavity show the presence of termini of the central nervous system (CNS) including olfactory nerves and olfactory epithelial cells (43). Indeed, a serious effect (Bell's palsy) was reported in a phase I human trial using a nasal influenza virus subunit vaccine consistent with LT functioning as a mucosal adjuvant (44).

In this regard, our efforts have been aimed at the development of a novel nasal vaccine-delivery system that resists the harsh environment of the nasal mucosa and does not require any mucosal adjuvants achieved through the combination of mucosal immunology and biomaterial engineering.

The nanogel-based nasal delivery system

Adding a bioadhesive gel that increases residence time, extends antigen release and allows its retention by nasal epithelial cells has facilitated induction of influenza-specific

SIgA antibody responses in nasal washes (45). Bioadhesive nanometer-sized (~40 nm) polymer hydrogels (nanogels) have been developed and have attracted growing interest as nanocarriers, especially in the field of drug delivery (46). Cholesteryl-group-bearing pullulan (CHP) forms physically cross-linked nanogels through self-assembly of associating polymers in water (47, 48). The CHP nanogels trap various proteins or nucleic acids, mainly by hydrophobic interactions, and acquire chaperone-like activity since the proteins and nucleic acids are trapped inside a hydrated nanogel polymer network (nanomatrix) without aggregating and are gradually released in their native form (48).

We have advanced the applicability of the unique bio-material CHP to nasal delivery by the addition of amino acids that convert it to a cationic nanogel, cCHP, since the electronic environment of the nasal epithelium is negatively charged (49). The cCHP has been shown to effectively attach to the nasal cavity with prolonged release of vaccine antigen to the nasal mucosal immune system for the initiation of an antigen-specific immune response (50–52). Nasal administration of a cCHP nanogel containing the C-terminal fragment of the H chain (Hc) of botulinum neurotoxin type A (BoNT/A) was shown to continuously adhere to the nasal epithelium (50). In addition, Hc-BoNT/A released from the cCHP nanogel was effectively taken up by nasal DCs and subsequently induced antigen-specific SIgA and serum IgG antibody responses that protected against BoNT/A intoxication (50). Importantly, cCHP-based nanogels are a safe nasal delivery system since this strategy does not induce any toxicity in the CNS including the deposition of released antigens from cCHP in the nasal cavity (50).

A cCHP nanogel vaccine containing pneumococcal surface protein A to prevent pneumococcal infection

Building on the findings discussed above (50), we next developed a cCHP nanogel nasal vaccine for the prevention of *Streptococcus pneumoniae* infection, as pneumococcal infection is a major UR tract infectious disease that causes severe illness and mortality in children and the elderly (53). Although two licensed injectable vaccines [23-valent pneumococcal polysaccharide vaccine (PPSV) and 13-valent polysaccharide conjugate vaccine (PCV)] have been released on the market, these vaccines cannot induce *S. pneumoniae*-specific mucosal SIgA antibody responses, which most likely play essential roles in blocking bacterial attachment to respiratory epithelial cells, thus preventing further invasion into the host including the lungs.

It has been shown that the currently available polysaccharide vaccines were effective in eliminating the carriage and transmission of vaccine-targeted capsular types of infection caused by *S. pneumoniae* (54, 55). However, the use of these polysaccharide-based vaccines has resulted in strain replacement (or seroconversion) in both colonization and disease (54, 55). We thus took advantage of the current situation that the polysaccharide vaccination can control infection caused by the prevalent strains of *S. pneumoniae* for the development of a next-generation nasal vaccine that can provide broad immunity against numerous strains of *S. pneumoniae* in both the respiratory mucosa and systemic

compartments, providing dual protection against pneumococcal infection.

A superior approach, which would be expected to eliminate virtually all nasal colonization, involves the use of cross-reactive surface proteins that could protect against colonization by strains regardless of their capsular types. It has been shown that vaccines containing pneumococcal surface protein A (PspA) can provide protective immunity against pneumococcal colonization (56–58). Similarly, we have shown that PspA-based nasal vaccines induce PspA-specific mucosal SIgA and systemic IgG antibody responses that provide significant protective immunity in both young and aged mice (59–61). To this end, we employed PspA as a vaccine antigen using the cCHP nanogel delivery system (cCHP-PspA) to develop a safe and effective nasal vaccine—a next-generation *S. pneumoniae* vaccine that protects against pneumococcal infection.

When mice were nasally immunized with cCHP-PspA, PspA was continuously delivered to the nasal epithelium and was thus effectively captured by nasal DCs for antigen presentation, leading to the initiation of antigen-specific immune responses (51). In this regard, increased levels of PspA-specific SIgA antibody responses were seen in nasal washes and the level of PspA-specific IgG antibodies was also significantly elevated in serum and bronchial fluids (51). In addition, PspA-specific antibodies possessed beneficial and functional properties that protect against *S. pneumoniae*. When mice given nasal cCHP-PspA nanogel vaccine were challenged with a lethal dose of *S. pneumoniae*, significantly reduced bacterial growth was seen in the lungs and nasal cavity, resulting in 100% protection (51).

To further advance the nanogel-based PspA vaccine in the direction of human use, rhesus macaques were nasally immunized with cCHP-PspA (52). Significantly increased levels of PspA-specific mucosal SIgA and serum IgG antibody responses were noted (52). These cCHP-PspA nanogel-induced PspA-specific antibodies possess neutralizing activity against *S. pneumoniae* (52). These immunological data suggested that nasal cCHP-PspA nanogel vaccine can induce protective immune response in both mucosal and systemic compartments of non-human primates.

As PspA proteins consist of three domains and two of these, a coiled-coil alpha-helical domain (αHD) and a proline-rich domain (PRD), are exposed at the surface of *S. pneumoniae*, both domains are vital for the induction of protective immunity (62–66). According to sequence data, the αHD shows six clades, whereas the PRD consists of three distinguishing groups (62–66). In this regard, we have recently developed a trivalent PspA-based nasal nanogel vaccine formulation covering the majority of the different serotypes of *S. pneumoniae* infection (Fig. 2) (67). Indeed, non-human primates given nasal trivalent PspA-cCHP using a newly developed nasal device showed increased levels of PspA-specific IgG antibody responses in serum and bronchoalveolar lavage fluid (BALF) that provided protection from intratracheal pneumococcal challenge with different serotypes of *S. pneumoniae* (Fig. 2) (68).

To explore the safety aspects of nasal cCHP-PspA nanogel vaccine, the fate of PspA nasally delivered by cCHP was examined and tracked using a combination of positron
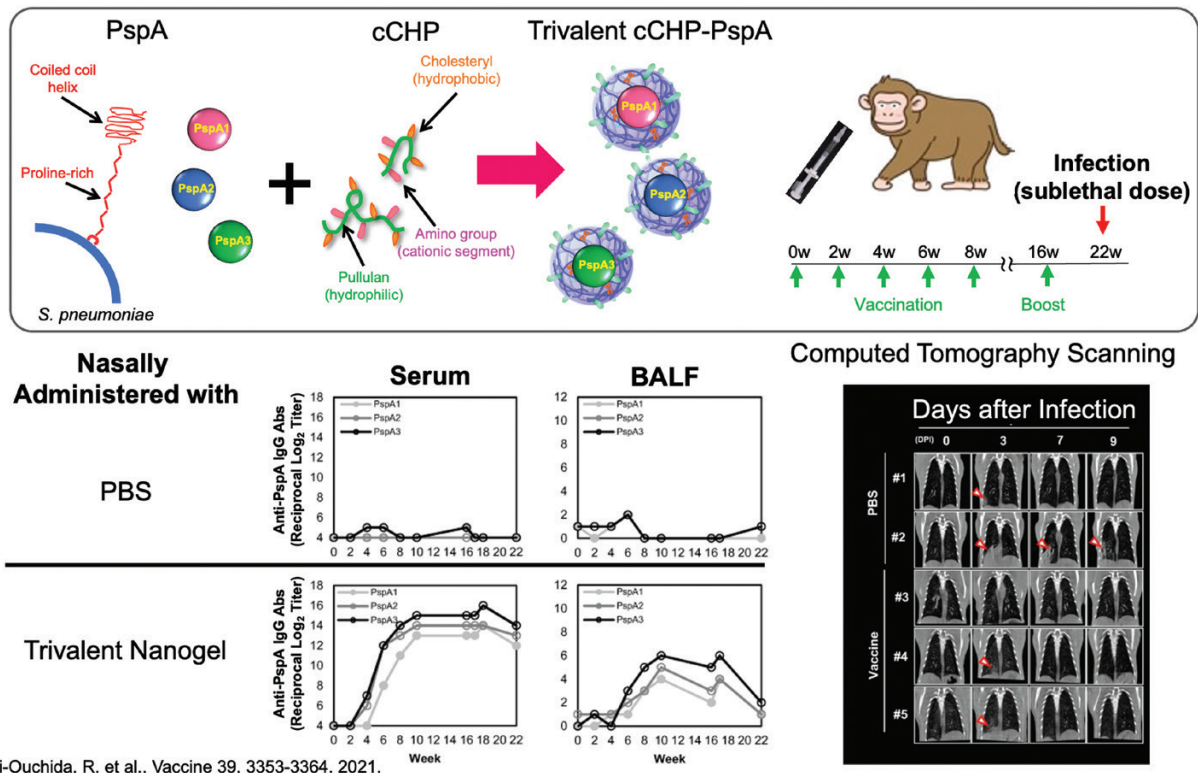
Fig. 2. Trivalent cCHP-PspA nanogel nasal vaccine successfully elicits PspA-specific protective immunity against *S. pneumoniae* infection. PBS, phosphate-buffered saline. Adapted, with permission, from ref. (68).

emission tomography (PET) and MR imaging. cCHP-PspA resulted in prolonged retention in the nasal epithelium (e.g. for as long as 6 h), compared with nasal PspA alone. More importantly, the nanogel-delivered PspA did not migrate and accumulate into the olfactory bulbs and epithelium or the brain of macaques nasally vaccinated with cCHP-PspA (52).

Taken together, these results suggest that PspA-based cCHP nanogel is a safe and effective nasal vaccine candidate for near-future human clinical trials to demonstrate the safety and effectiveness of the cCHP-PspA nasal vaccine as a new-generation *S. pneumoniae* vaccine.

Conclusion

The majority of currently licensed vaccines, including the revolutionary SARS-CoV-2 mRNA vaccines that have been developed, is injectable and effectively induces protective IgG antibody responses; however, it will most likely fail to prevent pathogen entry at mucosal surfaces. Cases of SARS-CoV-2 infection are thus seen in individuals vaccinated with injectable mRNA vaccinates (69). In contrast, mucosal vaccines that induce two layers of antigen-specific immune protection would be ideal and provide significant benefits to society. These layers are at the sites where pathogens invade (e.g. mucosal epithelium) and in the systemic circulation by means of antigen-specific SIgA and serum IgG antibodies, respectively.

Although the development of a new generation of safe and effective mucosal vaccines is time-consuming and faces

several difficult but potentially solvable challenges, our recent creation of two distinct oral and nasal vaccines using novel delivery systems (MucoRice and cCHP nanogel) could facilitate and potentially accelerate the licensing of mucosal vaccines in the near future. Finally, if the readers of this review article wish to learn more details of the past, present and future of 'mucosal vaccines', it might be worth mentioning that a textbook that summarizes recent progress in the development of mucosal vaccines has recently been published (70).

References

1 Kurashima, Y. and Kiyono, H. 2017. Mucosal ecological network of epithelium and immune cells for gut homeostasis and tissue healing. *Annu. Rev. Immunol.* 35:119.

2 Kurashima, Y., Yamamoto, D., Nelson, S. *et al.* 2017. Mucosal mesenchymal cells: secondary barrier and peripheral educator for the gut immune system. *Front. Immunol.* 8:1787.

3 Boyaka, P. N. and Fujihashi, K. 2018. Host defenses at mucosal surfaces. In Rich, R. T., Fleisher, T. A., Shearer, W. T. *et al.*, eds, *Clinical Immunology.* 5th edn, p. 20.1. Elsevier, San Diego, CA.

4 Chodirker, W. B. and Tomasi, T. B., Jr. 1963. Low-molecular-weight rheumatoid factor. *J. Clin. Invest.* 42:876.

5 Tomasi, T. B., Jr, Tan, E. M., Solomon, A. *et al.* 1965. Characteristics of an immune system common to certain external secretions. *J. Exp. Med.* 121:101.

6 Tomasi, T. B., Jr and Zigelbaum, S. 1963. The selective occurrence of gamma-1a globulins in certain body fluids. *J. Clin. Invest.* 42:1552.

7 Kiyono, H. and Fujihashi, K. 2021. Nasopharyngeal and oral immune system. In Smith, P. D., Blumberg, R. S. and MacDonald, T. T., eds, *Principal of Mucosal Immunology*. 2nd edn, p. 293. Garland Science, Taylor & Francis Group, LLC, Abingdon, UK.

8 Hirasawa, M., Kiyono, H., Babb, J. L. *et al.* 1980. Virulence of *Streptococcus mutans*: *in vivo* reversion of a low-virulence mutant results in partial displacement and pathogenesis. *Infect. Immun.* 27:1003.

9 Hamada, S. 1986. Overview of the biology of *Streptococcus mutans*. In Hamada, S., Michalek, S. M., Kiyono, H. *et al.*, eds, *Molecular Microbiology and Immunology*, p. 7. Elsevier, Amsterdam, The Netherlands.

10 Kurita-Ochiai, T., Hashizume-Takizawa, T., Kobayashi, R. *et al.* 2020. Mucosal vaccines for oral disease. In Kiyono, H. and Pascual, D. W., eds, *Mucosal Vaccines*, p. 649. Elsevier, London, UK.

11 Michalek, S. M., McGhee, J. R., Mestecky, J. *et al.* 1976. Ingestion of *Streptococcus mutans* induces secretory immunoglobulin A and caries immunity. *Science* 192:1238.

12 Kiyono, H., Michalek, S. M., Mosteller, L. M. *et al.* 1982. Enhancement of murine immune responses to orally administered haptenated *Streptococcus mutans*. *Scand. J. Immunol.* 16:455.

13 Saito, M., Otake, S., Ohmura, M. *et al.* 2001. Protective immunity to *Streptococcus mutans* induced by nasal vaccination with surface protein antigen and mutant cholera toxin adjuvant. *J. Infect. Dis.* 183:823.

14 Azegami, T., Yuki, Y. and Kiyono, H. 2014. Challenges in mucosal vaccines for the control of infectious diseases. *Int. Immunol.* 26:517.

15 Fujkuyama, Y., Tokuhara, D., Kataoka, K. *et al.* 2012. Novel vaccine development strategies for inducing mucosal immunity. *Expert Rev. Vaccines* 11:367.

16 Yuki, Y. and Kiyono, H. 2009. Mucosal vaccines: novel advances in technology and delivery. *Expert Rev. Vaccines* 8:1083.

17 Czerkinsky, C., Anjuere, F., McGhee, J. R. *et al.* 1999. Mucosal immunity and tolerance: relevance to vaccine development. *Immunol. Rev.* 170:197.

18 Fujihashi, K. and Kiyono, H. 2009. Mucosal immunosenescence: new developments and vaccines to control infectious diseases. *Trends Immunol.* 30:334.

19 Fujihashi, K. and McGhee, J. R. 2004. Mucosal immunity and tolerance in the elderly. *Mech. Ageing Dev.* 125:889.

20 Holmgren, J. and Czerkinsky, C. 2005. Mucosal immunity and vaccines. *Nat. Med.* 11(4 Suppl.):S45.

21 Ali, M., Nelson, A. R., Lopez, A. L. *et al.* 2015. Updated global burden of cholera in endemic countries. *PLoS Negl. Trop. Dis.* 9:e0003832.

22 Girard, M. P., Steele, D., Chaignat, C. L. *et al.* 2006. A review of vaccine research and development: human enteric infections. *Vaccine* 24:2732.

23 Nochi, T., Takagi, H., Yuki, Y. *et al.* 2007. Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. *Proc. Natl Acad. Sci. USA* 104:10986.

24 Nochi, T., Yuki, Y., Katakai, Y. *et al.* 2009. A rice-based oral cholera vaccine induces macaque-specific systemic neutralizing antibodies but does not influence pre-existing intestinal immunity. *J. Immunol.* 183:6538.

25 Tokuhara, D., Yuki, Y., Nochi, T. *et al.* 2010. Secretory IgA-mediated protection against *V. cholerae* and heat-labile enterotoxin-producing enterotoxigenic *Escherichia coli* by rice-based vaccine. *Proc. Natl Acad. Sci. USA* 107:8794.

26 Streatfield, S. J., Jilka, J. M., Hood, E. E. *et al.* 2001. Plant-based vaccines: unique advantages. *Vaccine* 19:2742.

27 Thanavala, Y., Mahoney, M., Pal, S. *et al.* 2005. Immunogenicity in humans of an edible vaccine for hepatitis B. *Proc. Natl Acad. Sci. USA* 102:3378.

28 Li, J. T., Fei, L., Mou, Z. R. *et al.* 2006. Immunogenicity of a plant-derived edible rotavirus subunit vaccine transformed over fifty generations. *Virology* 356:171.

29 Santi, L., Giritch, A., Roy, C. J. *et al.* 2006. Protection conferred by recombinant *Yersinia pestis* antigens produced by a rapid and highly scalable plant expression system. *Proc. Natl Acad. Sci. USA* 103:861.

30 Zhang, X., Buehner, N. A., Hutson, A. M. *et al.* 2006. Tomato is a highly effective vehicle for expression and oral immunization with Norwalk virus capsid protein. *Plant Biotechnol. J.* 4:419.

31 Jiang, X. L., He, Z. M., Peng, Z. Q. *et al.* 2007. Cholera toxin B protein in transgenic tomato fruit induces systemic immune response in mice. *Transgenic Res.* 16:169.

32 Moravec, T., Schmidt, M. A., Herman, E. M. *et al.* 2007. Production of *Escherichia coli* heat labile toxin (LT) B subunit in soybean seed and analysis of its immunogenicity as an oral vaccine. *Vaccine* 25:1647.

33 Rosales-Mendoza, S., Soria-Guerra, R. E., López-Revilla, R. *et al.* 2008. Ingestion of transgenic carrots expressing the *Escherichia coli* heat-labile enterotoxin B subunit protects mice against cholera toxin challenge. *Plant Cell Rep.* 27:79.

34 Kashima, K., Yuki, Y., Mejima, M. *et al.* 2016. Good manufacturing practices production of a purification-free oral cholera vaccine expressed in transgenic rice plants. *Plant Cell Rep.* 35:667.

35 Yuki, Y., Nojima, M., Hosono, O. *et al.* 2021. Oral MucoRice-CTB vaccine for safety and microbiota-dependent immunogenicity in humans: a phase 1 randomised trial. *Lancet Microbe* doi:10.1016/S2666-5247(20)30196-8

36 Sato, S. and Kiyono, H. 2012. The mucosal immune system of the respiratory tract. *Curr. Opin. Virol.* 2:225.

37 Nakahashi-Ouchida, R., Yuki, Y. and Kiyono, H. 2017. Development of a nanogel-based nasal vaccine as a novel antigen delivery system. *Expert Rev. Vaccines* 16:1231.

38 Kobayashi, R., Kohda, T., Kataoka, K. *et al.* 2005. A novel neurotoxoid vaccine prevents mucosal botulism. *J. Immunol.* 174:2190.

39 Imaoka, K., Miller, C. J., Kubota, M. *et al.* 1998. Nasal immunization of nonhuman primates with simian immunodeficiency virus p55gag and cholera toxin adjuvant induces Th1/Th2 help for virus-specific immune responses in reproductive tissues. *J. Immunol.* 161:5952.

40 Joo, S., Suwanto, A., Sato, A. *et al.* 2019. A role for the CCR5-CCL5 interaction in the preferential migration of HSV-2-specific effector cells to the vaginal mucosa upon nasal immunization. *Mucosal Immunol.* 12:1391.

41 Sato, A., Suwanto, A., Okabe, M. *et al.* 2014. Vaginal memory T cells induced by intranasal vaccination are critical for protective T cell recruitment and prevention of genital HSV-2 disease. *J. Virol.* 88:13699.

42 Yoshino, N., Lü, F. X., Fujihashi, K. *et al.* 2004. A novel adjuvant for mucosal immunity to HIV-1 gp120 in nonhuman primates. *J. Immunol.* 173:6850.

43 Fujihashi, K., Koga, T., van Ginkel, F. W. *et al.* 2002. A dilemma for mucosal vaccination: efficacy versus toxicity using enterotoxin-based adjuvants. *Vaccine* 20:2431.

44 Couch, R. B. 2004. Nasal vaccination, *Escherichia coli* enterotoxin, and Bell's palsy. *N. Engl. J. Med.* 350:860.

45 Amidi, M., Romeijn, S. G., Verhoef, J. C. *et al.* 2007. N-trimethyl chitosan (TMC) nanoparticles loaded with influenza subunit antigen for intranasal vaccination: biological properties and immunogenicity in a mouse model. *Vaccine* 25:144.

46 Gilmore, J. L., Yi, X., Quan, L. *et al.* 2008. Novel nanomaterials for clinical neuroscience. *J. Neuroimmune Pharmacol.* 3:83.

47 Kageyama, S., Kitano, S., Hirayama, M. *et al.* 2008. Humoral immune responses in patients vaccinated with 1-146 HER2 protein complexed with cholesteryl pullulan nanogel. *Cancer Sci.* 99:601.

48 Nomura, Y., Ikeda, M., Yamaguchi, N. *et al.* 2003. Protein refolding assisted by self-assembled nanogels as novel artificial molecular chaperone. *FEBS Lett.* 553:271.

49 Mall, M., Gonska, T., Thomas, J. *et al.* 2003. Modulation of Ca^{2+}-activated Cl^- secretion by basolateral K^+ channels in human normal and cystic fibrosis airway epithelia. *Pediatr. Res.* 53:608.

50 Nochi, T., Yuki, Y., Takahashi, H. *et al.* 2010. Nanogel antigenic protein-delivery system for adjuvant-free intranasal vaccines. *Nat. Mater.* 9:572.

51 Kong, I. G., Sato, A., Yuki, Y. *et al.* 2013. Nanogel-based PspA intranasal vaccine prevents invasive disease and nasal colonization by *Streptococcus pneumoniae. Infect. Immun.* 81:1625.

52 Fukuyama, Y., Yuki, Y., Katakai, Y. *et al.* 2015. Nanogel-based pneumococcal surface protein A nasal vaccine induces microRNA-associated Th17 cell responses with neutralizing antibodies against *Streptococcus pneumoniae* in macaques. *Mucosal Immunol.* 8:1144.

53 Gray, B. M., Converse, G. M., 3rd and Dillon, H. C., Jr. 1980. Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. *J. Infect. Dis.* 142:923.

54 Hsu, H. E., Shutt, K. A., Moore, M. R. *et al.* 2009. Effect of pneumococcal conjugate vaccine on pneumococcal meningitis. *N. Engl. J. Med.* 360:244.

55 Singleton, R. J., Hennessy, T. W., Bulkow, L. R. *et al.* 2007. Invasive pneumococcal disease caused by nonvaccine serotypes among Alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. *JAMA* 297:1784.

56 Arulanandam, B. P., Lynch, J. M., Briles, D. E. *et al.* 2001. Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody-mediated opsonization and protective immunity against *Streptococcus pneumoniae* infection. *Infect. Immun.* 69:6718.

57 Briles, D. E., Hollingshead, S. K., Paton, J. C. *et al.* 2003. Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with *Streptococcus pneumoniae. J. Infect. Dis.* 188:339.

58 Ogunniyi, A. D., Grabowicz, M., Briles, D. E. *et al.* 2007. Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae. Infect. Immun.* 75:350.

59 Fukuyama, Y., King, J. D., Kataoka, K. *et al.* 2011. A combination of Flt3 ligand cDNA and CpG oligodeoxynucleotide as nasal adjuvant elicits protective secretory-IgA immunity to *Streptococcus pneumoniae* in aged mice. *J. Immunol.* 186:2454.

60 Fukuyama, Y., King, J. D., Kataoka, K. *et al.* 2010. Secretory-IgA antibodies play an important role in the immunity to *Streptococcus pneumoniae. J. Immunol.* 185:1755.

61 Yamamoto, M., Briles, D. E., Yamamoto, S. *et al.* 1998. A nontoxic adjuvant for mucosal immunity to pneumococcal surface protein A. *J. Immunol.* 161:4115.

62 McDaniel, L. S., Ralph, B. A., McDaniel, D. O. *et al.* 1994. Localization of protection-eliciting epitopes on PspA of *Streptococcus pneumoniae* between amino acid residues 192 and 260. *Microb. Pathog.* 17:323.

63 Yother, J. and White, J. M. 1994. Novel surface attachment mechanism of the *Streptococcus pneumoniae* protein PspA. *J. Bacteriol.* 176:2976.

64 Hollingshead, S. K., Becker, R. and Briles, D. E. 2000. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae. Infect. Immun.* 68:5889.

65 Daniels, C. C., Coan, P., King, J. *et al.* 2010. The proline-rich region of pneumococcal surface proteins A and C contains surface-accessible epitopes common to all pneumococci and elicits antibody-mediated protection against sepsis. *Infect. Immun.* 78:2163.

66 Vadesilho, C. F., Ferreira, D. M., Gordon, S. B. *et al.* 2014. Mapping of epitopes recognized by antibodies induced by immunization of mice with PspA and PspC. *Clin. Vaccine Immunol.* 21:940.

67 Yuki, Y., Uchida, Y., Sawada, S. I. *et al.* 2021. Characterization and specification of a trivalent protein-based pneumococcal vaccine formulation using an adjuvant-free nanogel nasal delivery system. *Mol. Pharm.* 18:1582.

68 Nakahashi-Ouchida, R., Uchida, Y., Yuki, Y. *et al.* 2021. A nanogel-based trivalent PspA nasal vaccine protects macaques from intratracheal challenge with pneumococci. *Vaccine* 39:3353.

69 Keehner, J., Horton, L. E., Pfeffer, M. A. *et al.* 2021. SARS-CoV-2 infection after vaccination in health care workers in California. *N. Engl. J. Med.* 384:1774.

70 Kiyono, H. and Pascual, D. W. 2020. Mucosal vaccines: innovation for preventing infectious diseases. In Kiyono, H. and Pascual, D. W., eds, *Mucosal Vaccines*. 2nd edn, p. 1. Elsevier-Academic Press, London, UK.