

REVIEW



## Noninvasive vaccination against infectious diseases

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### ABSTRACT

The development of a successful vaccine, which should elicit a combination of humoral and cellular responses to control or prevent infections, is the first step in protecting against infectious diseases. A vaccine may protect against bacterial, fungal, parasitic, or viral infections in animal models, but to be effective in humans there are some issues that should be considered, such as the adjuvant, the route of vaccination, and the antigen-carrier system. While almost all licensed vaccines are injected such that inoculation is by far the most commonly used method, injection has several potential disadvantages, including pain, cross contamination, needlestick injury, under- or overdosing, and increased cost. It is also problematic for patients from rural areas of developing countries, who must travel to a hospital for vaccine administration. Noninvasive immunizations, including oral, intranasal, and transcutaneous administration of vaccines, can reduce or eliminate pain, reduce the cost of vaccinations, and increase their safety. Several preclinical and clinical studies as well as experience with licensed vaccines have demonstrated that noninvasive vaccine immunization activates cellular and humoral immunity, which protect against pathogen infections. Here we review the development of noninvasive immunization with vaccines based on live attenuated virus, recombinant adenovirus, inactivated virus, viral subunits, virus-like particles, DNA, RNA, and antigen expression in rice in preclinical and clinical studies. We predict that noninvasive vaccine administration will be more widely applied in the clinic in the near future.

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## Introduction

Although some infectious diseases, including malaria, meningitis, diphtheria, hepatitis B, tetanus, polio, measles, mumps, and rubella, are now controlled in developed countries due to the development of vaccines and good management of healthcare, there is still a need to improve all healthcare systems and reduce the economic disparities between developed and developing countries.<sup>1</sup> Several outbreaks in developing countries of infectious diseases, such as HIV/AIDS, malaria, tuberculosis, dengue, influenza, and other respiratory diseases, occur each year and are responsible for more than 54% of total mortality. In developing countries, strategies are urgently needed to prevent these diseases as are new approaches to developing specific drugs and protective vaccines, which are currently unavailable for some diseases. The available vaccines, such as for hepatitis B and measles, elicit specific humoral and cellular immunity and protect both immunized individuals and their neighbors.<sup>2–4</sup> However, these diseases are still seriously threatening to human life, especially in developing countries.<sup>5</sup> WHO vaccination campaigns have decreased mortality, but improvements in vaccine administration and storage are still needed.

The overwhelming number of vaccinations administered worldwide over the last half century have been by injection.

Such vaccinations have been responsible for preventing millions of deaths per year from infectious diseases. Nevertheless, there are reasons to consider routes of vaccination not involving injection. Of the possible vaccination methods, injection has the advantages of low cost and quick delivery, and has an excellent long-term record for safety in use across billions of doses administered worldwide over more than the past half-century.<sup>6</sup> However, most needle-based vaccines require a cold chain, which is expensive, due to the need for cold storage rooms and cold transportation to maintain the vaccine at the proper temperature.<sup>7</sup> Temperature changes could destroy freezing- or heat-sensitive vaccine vials, which would then need to be destroyed. A study in Bangladesh demonstrated that, while a cold chain could be maintained during storage of polio vaccine at the district level and at health facilities, there were temperature fluctuations during transport. Temperatures  $>8^{\circ}\text{C}$  (with a maximum of  $21^{\circ}\text{C}$ ) were detected in 5 of the 23 refrigerators and 3 of 6 transported cold boxes when the vaccines were carried from the district to local depots. Moreover, 17% of the vaccine carriers could not reach some remote destinations. In addition, in African, Latin American, and some Asian countries, the electrical supply is not reliable and will require extra

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investment in generators or conversion to alternative sources, such as solar energy, to provide consistent cold chain storage.<sup>8</sup>

Furthermore, hypodermic injection is not easy to perform at home by the patients themselves and requires the help of trained nurses.<sup>9</sup> Moreover, hypodermic injection may result in pain for patients, thus potentially leading to needle phobia, especially in children.<sup>10,11</sup> The improper disposal and reuse of syringes contaminated with blood-borne pathogens are also essential safety concerns, especially in developing countries.<sup>12,13</sup>

In recent decades, needle-free vaccine delivery systems have been developed to improve safety, especially to healthcare providers and the community, after concerns regarding pandemics and bioterrorism. Oral, intranasal (i.n.), and transcutaneous vaccinations are promising noninvasive immunization methods that stimulate mucosal immunity, which is one of the most important components of the immune response. Mucosal immunity not only protects against respiratory infections, such as influenza, but also against systemic infections, such as tuberculosis (TB) and HIV.<sup>14</sup> Vaccine delivered directly to the mucosal surface is rapidly and widely distributed and enhances mucosal, cellular, and humoral immune responses.<sup>15</sup> I.n. vaccination against respiratory and gastrointestinal pathogens offers several theoretical and practical benefits, such as avoiding the need for extensive purification from bacteria and significantly reducing the cost of training healthcare workers. Furthermore, noninvasive vaccination eliminates needlestick injuries and exposure of health workers to blood-borne pathogens, such as HIV and hepatitis B, and there is no need to dispose of large quantities of needles, which is a difficult problem in developing countries.<sup>16</sup> In contrast to mucosal immunization, transcutaneous vaccines are not degraded by enzymes or digestive acid,<sup>17</sup> and microneedle patches are easy to carry during travel. In fact, beyond the application in vaccine delivery, microneedle-mediated vaccination also induces more efficient immunity, and it has been widely used in the treatment of diabetes, Alzheimer's diseases, seborrheic keratosis, and tumors.<sup>18-21</sup>

Here we describe advances in oral, i.n., and transcutaneous vaccination at different stages of development, including pre-clinical and clinical trials and licensed vaccines. We focus on vaccine technologies that are based on noninvasive immunization with live attenuated virus, recombinant virus, inactivated virus, viral subunits, virus-like particles, DNA, RNA, and antigen expression in rice.

### Noninvasive vaccines induce mucosal and systemic immunity

The epithelial coating of mucous membranes constitutes the largest immunologic organ, which has a greater probability of infection if the microorganism crosses at least one of the body's protective mucosal barriers. These barriers are the respiratory tract, gut, genital tract, conjunctiva, and urinary system. The protection of mucosal membranes is conferred by the humoral and T cell responses as well as the innate immune response, and this system is collectively known as mucosa-associated lymphoid tissue (MALT).<sup>22</sup> MALT immunization is similar to systemic immunization, which depends on efficient antigen sampling and uptake, antigen presentation by antigen-presenting cells (APCs), and the production of effector and memory B

and T cells.<sup>23</sup> MALT can be subdivided into gut-associated lymphoid tissue (GALT), which includes lymph nodes, Peyer's patches, and isolated lymphoid follicles, and nasopharyngeal-associated lymphoid tissue (NALT), which includes tonsils/adenoids, inducible bronchus-associated lymphoid tissue, and lymph nodes.

Unlike systemic immunization, mucosal immunization depends on the secretion of IgA by plasma cells.<sup>24</sup> Locally, secreted IgA (sIgA) binds to the Ig receptor (pIgR) at the basolateral surface of mucosal epithelial cells and is then actively transported into the lumen by transcytosis,<sup>25,26</sup> where it regulates viral and bacterial infections at the oral and i.n. mucosal surface. The sIgA antibodies bind to pathogens to inhibit their interaction with, and uptake by, epithelial cells but deliver the pathogens to antigen-presenting cells (APCs).<sup>27</sup> In GALT locations, the precursors of IgA plasma cells are generated in localized zones of organized lymphoid induction sites and gut lymph nodes.<sup>28,29</sup> These structures are found in the small intestine and appendix, where the B cells are activated at the germinal centers (GCs). The generation and maturation of IgA-producing B cells are promoted by follicle DCs, T follicular helper cells, and macrophages, which secrete cytokines, such as TGF- $\beta$ , IL-10, and IL-4,<sup>30</sup> and help the B cells to generate long-lasting memory cells with high levels of somatic mutation.<sup>31,32</sup> The NALT in humans is localized in the salivary glands, the paired palatine and tubal tonsils, and the unpaired pharyngeal and lingual tonsils and has similar functions as GALT. The tonsils consist of follicular germinal centers, the extrafollicular areas, the mantle zones, and the reticular crypt epithelium on the surface. The epithelium contains M cells, DCs, naïve B and T cells, and memory B cells. Unlike GALT, the tonsils generate plasma cells with a production of 70% IgG and 30% IgA. However, the paired tubal tonsils and unpaired pharyngeal tonsils produce sIgA, which blocks entry into the epithelia cells. Like GALT, the germinal centers are associated with somatic mutation and differentiation into B memory cells.<sup>33</sup>

In addition, it has been observed that mucosal and transcutaneous immunization induce systemic immune responses that are further enhanced by activated dendritic cell (DC) migration from the mucosa to the lymph nodes and spleen.<sup>27</sup> It has also been observed that, with the help of L-selectin on the cell surface,<sup>34</sup> systemic IgG B cells migrate into the mucosa from bone marrow, and, vice versa, that induced antigen-specific T and B cells leave the Peyer's patches via the efferent lymphatics through thoracic duct lymph and are disseminated to the systemic circulation. They then enter the mucosa of the gut, respiratory system, salivary and mammary glands, and reproductive tract.<sup>35,36</sup>

### Oral vaccination preclinical applications

Oral vaccination is the best method for vaccine administration because of the ease of regulatory compliance and low cost. Oral immunization is a standard needle-free technique that has been used especially in vaccine campaigns against influenza (children), poliomyelitis, rotavirus, typhoid fever, cholera, and other diseases.<sup>22</sup> However, only a few vaccines have an oral formulation, since there are issues that may affect protective immune responses. For example, antigen should travel intact to the

lumen of the intestine without degradation by the acidic environment of the stomach.<sup>37,38</sup> Otherwise, the antigen concentration could diminish from levels capable of inducing an immune response to an anergy response. However, a good vaccine like the poliomyelitis vaccine passes through the digestive tract to the intestine, where the M cells in the Peyer's patches (PPs) of the GALT<sup>37</sup> lack a mucus layer on their apical side and efficiently transport oral vaccines to APCs by transcytosis.<sup>27,39</sup> In fact, APCs are essential cells for the initiation of local and systemic immunity; therefore, developing vaccine carriers targeting APCs is a good strategy to improve the immune response.<sup>3</sup>

Oral delivery has been tested in development of viral and bacterial vaccines that were attenuated by repeated passages in tissue culture or by chemical mutagenesis. This approach was first demonstrated in a mouse model by sublingual immunization with a live attenuated Japanese encephalitis virus vaccine and a recombinant modified vaccinia virus, which induced virus-specific IFN- $\gamma$ <sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells. However, IL-17<sup>+</sup> CD4<sup>+</sup> T cells were also increased after sublingual immunization.<sup>40</sup>

Intriguingly, DNA vaccines delivered by live attenuated *Salmonella typhimurium* (JOL911) effectively protected mice from lethal infection by H1N1 influenza virus, while i.n. administration did not. However, virus copy numbers in the lungs were lower following oral and i.n. immunization compared with the lungs of the PBS-vaccinated control group.<sup>41</sup> In another study, attenuated *S. typhimurium* was used to orally deliver a *Trichinella spiralis* paramyosin DNA vaccine to mice. The orally vaccinated mice were effectively protected from *T. spiralis* infection, and their antibody responses showed significant mucosal sIgA and systemic IgG2a and a significant increase in Th1 (IFN- $\gamma$ , IL-2) and Th2 (IL-4, -5, -6, and -10) cytokines.<sup>42</sup> Oral immunization with antigens carried by engineered viruses has demonstrated that GALT elicits both humoral and cellular immune responses that protect against several infectious diseases. The most frequently used engineered virus is adenovirus, which has been used in phase I clinical trials. Several reports have demonstrated that adenovirus vaccine can be administered orally to prevent different kinds of infection. Lubeck developed human adenovirus type 7 (Ad7) and type 4 (Ad4) vaccines containing hepatitis B surface antigen. After oral immunization of chimpanzees with these two vaccines, the animals generated significant antibody responses and effective protection against hepatitis B virus.<sup>43</sup> In another study, skunks and foxes were vaccinated with an Ad5 vaccine expressing the rabies glycoprotein gene. The vaccine was instilled into the mouth cavity, resulting in a 100% survival rate for street rabies virus-challenged animals.<sup>44</sup> Furthermore, studies with mice orally vaccinated with a replication-deficient adenovirus (Rad68) vaccine containing the measles virus nucleocapsid protein generated a significant splenic cytotoxic T cell response (in 70% of the mice) and antibody response (in 89% of the mice).<sup>45</sup> Oral administration of adenovirus vaccine also protected the mice from HIV, Ebola, influenza virus, and botulism.<sup>46-49</sup> In another study by Lin et al., vaccination with live attenuated gastroenteritis virus incorporating CpG DNA in pigs enhanced the IgA level in the intestinal tract and the IgG level in the serum after oral immunization.<sup>50</sup>

Currently, influenza vaccines are administered intramuscularly or intradermally, which induces mainly humoral responses against hemagglutinin (HA) and neuraminidase (NA) proteins. These proteins are polymorphic, especially in the exposed domains subject to the immune system.<sup>51,52</sup> Nevertheless, these routes of immunization are less effective in stimulating mucosal immunity,<sup>53,54</sup> and a split-flu vaccine in combination with the adjuvant methylglycol chitosan and/or CRX601 administered sublingually was recently found to improve the systemic and mucosal immune responses equivalently or to a greater extent than intramuscular vaccination.<sup>55</sup>

Subunit vaccines based on virus-like particles (VLPs) that self-assemble from viral structural proteins and related antigens is another technology used to stimulate GALT and induce protection.<sup>56</sup> This type of vaccine demonstrated that oral immunization with recombinant *Bacillus subtilis* expressing cholera toxin B subunits and *Helicobacter pylori* urease B spores contributes to a reduction in the *H. pylori* load.<sup>57</sup>

As mentioned above, one of the problems of vaccination in developing countries is maintaining a cold chain to preserve vaccines. To solve this problem, Borde et al. developed a liquid-killed, multivalent whole-cell-plus-enterotoxin-B-subunit oral vaccine against enterotoxigenic *E. coli* that induced an effective immune response in mice.<sup>58</sup> This vaccine is given together with dmLT (an enterotoxin-derived adjuvant) as a dry-powder vaccine formulation that is especially suitable for low-income countries.

Another problem for vaccination is the acidic conditions of the gastrointestinal tract, which could degrade protein, DNA, and polysaccharide vaccines, leading to weak immune responses and poor protection against pathogens. New carriers are needed to design available vaccine payloads, with effective release within their residence time in the small intestine and optimization of the adjuvant capabilities of the delivery vehicles,<sup>41</sup> and some researchers have studied this kind of delivery vehicle. For example, Yagnik, et al. constructed a recombinant *Lactococcus lactis* bacterium that expressed protein A of *Shigella dysenteriae* type 1 in its outer membrane, which elicited a higher immune response after oral administration than by the i.n. route.<sup>59</sup> In another study, Oliveira et al. delivered a plasmid DNA vaccine expressing the Rho1 GTP protein of *Schistosoma mansoni* with chitosan nanoparticles. *In vivo* experiments suggested that oral administration increases the expression of modulatory IL-10 and thereby reduces liver pathology.<sup>60</sup> Moreover, green-synthesis silver nanoparticles formulated with an H5N1 DNA vaccine generated effective antibody and cellular immunity in chicken against influenza.<sup>61</sup> In fact, oral vaccine administration has several protective effects besides the control of infectious diseases. For example, the cholera toxin B subunit, a component of a licensed oral cholera vaccine, increased mucosal healing in the colon after oral administration.<sup>62</sup> Also, hepcortepenlisimut-L, a drug used as a liver cancer vaccine, has proven to be safe, effective, and fast-acting against hepatocellular carcinoma following oral administration.<sup>63</sup>

Interestingly, a transgenic rice-based vaccine has been developed, which has proven to be safe and resistant to the acidic environment in the stomach.<sup>64</sup> It has been demonstrated that rice-based allergic vaccines as well as cholera and diarrhea vaccines can be delivered efficiently by oral

administration.<sup>65–67</sup> Taken together, these results suggest that oral vaccine administration has great potential for protection against different diseases.

### Clinical trials for oral vaccines

Several clinical trials for oral vaccines have been conducted and are listed in Table 1. However, these vaccines have not been approved by the US FDA. Rotarix and RotaTeq are two vaccines that have been internationally approved by WHO; however, they have been shown to be less effective (in 40–70% of subjects) in third world countries.<sup>68</sup> The live attenuated rotavirus vaccine Rotavin-M1 was evaluated for safety and immunogenicity, and the IgA seroconversion rate of Rotavin-M1 was found to be comparable to the licensed vaccine Rotarix.<sup>69</sup> However, infants who received Rotavin-M1 shed virus in their stool at a lower frequency (44–48%) than those who received Rotarix (65%). More infants are expected to be enrolled in subsequent trials.

Another vaccine in clinical trials is designed against enterotoxigenic *E. coli* (ETEC), which is endemic in developing countries and may produce an infectious diarrhea. Chen *et al.* conducted a phase I clinical trial to demonstrate the safety and immunogenicity of the peru-15pCTB vaccine against ETEC infection. This study showed that antitoxin antibody seroconversion increased fourfold compared with baseline. Furthermore, the vaccine was safe up to  $1 \times 10^{10}$  CFU.<sup>70</sup> Recently, an oral recombinant adenovirus influenza vaccine induced significant increases in haemagglutination inhibition and high titers of microneutralization antibodies with only mild adverse

effects.<sup>71</sup> Moreover, Euvichol is an inactivated bivalent oral cholera vaccine tested during a phase I clinical trial in healthy South Korean adult males that induced a seroconversion rate of 95% and 45% for *Vibrio cholera* O1 and O139, respectively.<sup>72</sup>

*H. pylori* is associated with gastritis, peptic ulcer, and gastric adenocarcinoma and is present in the gastric mucosa in at least half of the world population. Until now, there has been no effective vaccine. However, an oral recombinant *H. pylori* vaccine substantially reduced infection by this bacterium in clinical trials that had enrolled 4464 children from China.<sup>73</sup>

One more vaccine assessed in a phase I clinical trial is an adenovirus vaccine that expresses both an avian influenza A hemagglutinin and a TLR3 ligand. This vaccine exhibited cytotoxic T cell responses and IFN- $\gamma$  production that were similar to the placebo group.<sup>74</sup> Efficient immunogenicity and fewer adverse effects were also found in these clinical trials. However, more participants are needed to further evaluate these vaccines before they receive final approval.

### Licensed oral vaccines

The poliomyelitis is a disease caused by three poliovirus serotypes (P1, P2, and P3). The virus enters through the mouth, goes to the gastrointestinal tract, then to the lymph nodes and may travel to the central nervous system, where the virus replicates in the motor neural cells, resulting in motor neuron destruction causing muscle weakness and paralysis.<sup>75</sup> The first immunotherapy was developed in the 1950s by Hammon *et al.*, who used immunoglobulins of individuals recovered from poliovirus infection and injected into patients with active

**Table 1.** Clinical trials for oral immunization.

Type of vaccine	Sponsor institution	Stages	Clinical registration	Time
Live attenuated <i>Shigella</i> vaccine	University of Maryland	Phase 1	NCT01531530	2012
Live attenuated enterotoxigenic <i>Escherichia coli</i> (ETEC) vaccine	TD Vaccines A/S	Phase 1	NCT00901654	2009
Live attenuated typhoid vaccine	International Centre for Diarrhoeal Disease Research, Bangladesh	Phase 2	NCT01019083	2009
Live attenuated cholera vaccine	International Vaccine Institute	Phase 2	NCT00741637	2008
Live attenuated <i>Shigella sonnei</i> vaccine	NIAID	Phase 1	NCT01336699	2011
Live attenuated <i>Salmonella</i> vaccine	University of Maryland	Phase 1	NCT01129453	2010
Live attenuated <i>Shigella a</i> vaccine	PATH	Phase 1, Phase 1	NCT02934178, NCT01813071	2016, 2013
Live attenuated HRV vaccine	National Institute of Hygiene and Epidemiology, Vietnam	Phase 2	NCT01377571 <sup>69</sup>	2011
Live attenuated typhoid fever vaccine	Avant Immunotherapeutics	Phase 2	NCT00498654	2007
Live attenuated ETEC–cholera vaccine	NIAID	Phase 1	NCT00654108 <sup>70</sup>	2008
Adenoviral <i>Norovirus</i> vaccine	Vaxart, Inc.	Phase 1, Phase 1	NCT02868073, NCT03125473	2016, 2017
Adenoviral influenza vaccine	Vaxart, Inc.	Phase 1, Phase 1	NCT02547792, NCT01688297 <sup>71</sup>	2015, 2012
Adenovirus vaccine	PaxVax, Inc.	Phase 1	NCT03160339	2017
Replication-competent adenovirus HIV vaccine	PaxVax, Inc.	Phase 1	NCT02771730	2016
Replicating Ad26-vectored HIV-1 vaccine	International AIDS Vaccine Initiative	Phase 1	NCT02366013	2015
Adeonoviral RSV vaccine	Vaxart	Phase 1	NCT02830932	2016
Inactivated cholera vaccine	International Centre for Diarrhoeal Disease Research, Bangladesh	Phase 1	NCT01019083	2009
Inactivated polio vaccine	Bilthoven Biologicals	Phase 3	NCT02766816	2016
Inactivated <i>Shigella</i> vaccine	PATH	Phase 1	NCT01509846	2012
Inactivated cholera vaccine	International Centre for Diarrhoeal Disease Research, Bangladesh	Phase 2, Phase 1/2	NCT02742558, NCT02823899	2016, 2016
Inactivated enterotoxigenic <i>Escherichia coli</i> (ETEC) vaccine	U.S. Army Medical Research and Materiel Command	Phase 3	NCT02556996	2015
Inactivated <i>Vibrio cholerae</i> vaccine	EuBiologics Co.,Ltd	Phase 1	NCT01707537 <sup>72</sup>	2012
Live attenuated/inactivated cholera vaccine	University of Maryland	Phase 2	NCT02145377	2014
Recombinant <i>Helicobacter pylori</i> vaccine	Jiangsu Province Centers for Disease Control and Prevention	Phase 3	NCT02302170 <sup>73</sup>	2014
HIV-1 MN peptide vaccine	NIAID	Phase 1	NCT00000798	2001
Avian influenza DNA vaccine	Vaxart	Phase 1	NCT01335347 <sup>74</sup>	2011

HRV, human rotavirus; RSV, respiratory syncytial virus; ETEC, enterotoxigenic *Escherichia coli*; NIAID, National Institute of Allergy and Infectious Diseases.

disease. This passive immunization was 80% effective reducing the effects of poliomyelitis, but there were not enough plasma to stop the pandemic.<sup>76</sup>

The first candidate polio vaccine was developed by Hilary Koprowski and colleagues. This poliovirus vaccine was attenuated in cotton rats with low pathogenicity in rhesus monkeys. It was first tested in 20 healthy individuals, and they were all seroconverted and did not get sick.<sup>77</sup> The first licensed vaccine (inactivated poliovirus vaccine, IPV) contains P1, P2 and P3 serotypes, which were grown in Vero cells (monkey kidney cells) and inactivated with formaldehyde. The volunteers who were immunized showed seroconversion with 3 doses.<sup>78</sup> This vaccine was developed by Sack et al., and it was licensed for serotypes P1 and P2 in 1961 and type 3 in 1963.<sup>79</sup> The oral poliovirus vaccine (OPV) was the second vaccine, composed of the subtypes P1, P2 and P3 after the repeated passage of the virus in tissue cultures of cynomolgus monkey kidney at sub-physiological temperatures. The subtype viruses are able to infect cells in the gut but is unable to replicate in the neuron system. The vaccine was developed by Sabin et al., and it was licensed in 1962. OPV vaccine was tested in several countries (Netherlands, Mexico, Japan, USSR, Brazil, Cuba, etc.) before its license.<sup>80</sup> Due to its low cost, it became the most used polio vaccine worldwide. However, OPV can cause vaccine-associated paralytic poliomyelitis (VAPP) especially in developing countries with a risk of 4.7 cases per million births. VAPP were associated with subtype 2 poliovirus.<sup>81</sup> This vaccine was discontinued in the USA in 2000. The VAPP has not been observed using the IPV. OPV is used in many countries. However, WHO recommends that all countries using only OPV add at least 1 dose of IPV to the schedule.<sup>82</sup> The IPV and OPV are licensed in the USA but only IPV is currently used. The most recent version of IPV contains three serotypes of polio vaccine viruses, but grown single component in Vero cells, inactivated with formaldehyde, and has trace amounts of antibiotics as a preservative. Both polio vaccines have saved millions of lives and have prevented deformities, which result in significant high cost in health care especially in developing countries.

Adenovirus types 4 and 7 cause acute respiratory disease, which is a threat to the health of US military personnel.<sup>83</sup> A vaccine for adenovirus types 4 and 7 (BL 125296/0) is orally administered and after 26 days in a clinical trial showed a seroconversion rate of 94.5% and 93.8% against types 4 and 7, respectively.<sup>84</sup> The protective effect of this vaccine was associated with serotype-specific neutralizing antibodies. While there were adverse effects, such as nasal congestion, cough, sore throat, headache, abdominal pain, arthralgia, nausea, and diarrhea, there was not a significant difference from the placebo group. Vaxchora is an FDA-approved oral vaccine for individuals of ages 18–64 years traveling to cholera-endemic areas. This vaccine was 90.3% effective after 10 days and 79.5% effective after 90 days against *V. cholerae* O1, and the immune mechanism is associated with vibriocidal antibody titers.<sup>85</sup>

Rotarix<sup>TM</sup>, an orally administered vaccine, is used widely to protect from gastroenteritis and reduce its occurrence after hospitalization.<sup>86,87</sup> A clinical study showed that after one month of a two-dose series, 86.5% of 787 recipients of Rotarix were seroconverted compared with 6.7% of placebo recipients. A pentavalent vaccine, RotaTeq, was also shown to protect

against rotavirus gastroenteritis at different levels of severity by increased IgA seroconversion.<sup>88,89</sup> Both Rotarix and RotaTeq are more suitable for protecting against severe gastroenteritis (effective for 90% of patients) while less suitable against mild infection (effective for 60–75% of patients).<sup>90</sup> However, it has been documented that the use of RotaTeq and Rotarix has been associated with the development at a very low rate of intussusception (~1:40,000 doses), and shedding from the vaccinee's stools, which may result in the infection of immunocompromised individuals.<sup>27</sup>

*Salmonella typhi* causes typhoid fever, an acute, febrile, enteric disease, which continues to be important in many parts of the world.<sup>91</sup> Vivotif is another vaccine against gut pathogens and is administered by the oral route and is indicated for immunization against *S. typhi* for adults and children older than 6 years. This capsule vaccine should be administered in four doses to induce a broad immune response, mainly including serum IgG and IgA responses, mucosal antibodies, CD4<sup>+</sup> T cells, and CD8<sup>+</sup> cytotoxic T cells.<sup>22,92–94</sup> However, most of these licensed vaccines are not recommended for infants due to the lack of safety data for attenuated virus vaccines, and only rotavirus vaccines are currently used in infants.<sup>27</sup>

The oral killed-cholera vaccines, Shanchol and Dukoral, are safe, immunogenic, effective in the clinic, and are recommended by WHO. However, they are not available in the US as of this writing. It was found that vibriocidal antibodies against *V. cholera* O1 Inaba, *V. cholerae* O1 Ogawa, and *V. cholerae* O139 were effectively induced in adults, toddlers, and younger children.<sup>95</sup> In addition, Dukoral is safe and immunogenic in Peruvian and North American volunteers.<sup>96,97</sup> Sriskeyi et al. showed that TLR–MyD88 signaling may mediate immune responses to the Dukoral vaccine.<sup>98</sup> Furthermore, antigen-specific memory B-cell responses were not detected in Dukoral-treated individuals, which explains the brief period of protection conferred by this vaccine.<sup>99</sup>

### Preclinical evaluation of intranasal vaccination

Several diseases, such as influenza and pneumococcal disease, threaten human life and are transmitted by the nasal route. Nasal tissue is also an excellent route for vaccine administration, which has the advantage of requiring lower doses and without exposure to extreme pH or digestive enzymes. Separate from the main salivary glands and tonsil tissue, there are locally draining lymph nodes that lie under the respiratory epithelium of the nasal cavity, where antigens are also transported to DCs, macrophages, and B cells by M cells. B lymphoblasts migrate to and proliferate in the germinal centers where mucosal sIgA and systemic IgG are induced.<sup>100</sup> In parallel, mature DCs bearing antigens migrate to the follicular B cell zone and interfollicular T cell zone and activate T cells and B lymphoblasts by presenting antigens to them. The activated T and B cells then enter the bloodstream to initiate systemic immunity at distant sites.<sup>101</sup> Therefore, i.n. immunization can induce potent immunity, producing effective protection against infectious disease while helping to achieve widespread vaccination in developing countries.

Studies of i.n. administration of vaccines have been conducted using live attenuated influenza vaccines, which have

generated protective immune responses. Fan *et al.*, demonstrated in rhesus macaques that i.n. vaccination with cold-attenuated H5N1 virus induces neutralizing antibodies and an HA-specific CD4<sup>+</sup> T cell immune response that is fully protective. Yang *et al.* also found that ferrets and mice immunized by i.n. administration with live attenuated BJ/AA ca virus produced high levels of sIgA that was protective against challenge with A/Beijing/501/2009 or A/California/07/2009 viral strains.<sup>102-104</sup> Furthermore, an engineered PR8 influenza virus was generated that expressed the receptor-binding subdomain of botulinum neurotoxin, resulting in a vaccine that protected mice from botulinum and influenza at the same time using i.n. administration.<sup>105</sup> Moreover, an attenuated live viral vaccine expressing a functional species-specific artificial microRNA (PR8-amiR-93NP) was safe and led to cross protection for mice from heterologous influenza virus strains after i.n. immunization.<sup>106</sup>

Although vaccines induce protective immune responses against infectious diseases, the adjuvant helps to accelerate and enhance antigen-specific immune responses. Huang *et al.*, combined several adjuvants, including PELC, which is a squalene-based water-in-oil-in-water emulsion stabilized by Span<sup>®</sup> 85 (sorbitan trioleate), and poly(ethylene glycol)-block-poly(lactide-co- $\epsilon$ -caprolactone) (PEG-b-PLACL) together with LD-indolicidin or alum and inactivated influenza virus to formulate a vaccine that increased influenza-specific serological protection after i.n. vaccination.<sup>107</sup>

In addition, i.n. vaccines against other viruses and bacteria have been developed. For example, when used for i.n. immunization, an rAd5-based vaccine combined with  $2.0 \times 10^8$  IFU Ad5-IFN $\alpha$  as adjuvant facilitated the development of robust specific antibodies and increased survival in guinea pigs exposed to experimental Ebola virus.<sup>108</sup> Malley *et al.* showed that delivery of an inactivated non-encapsulated pneumococcal vaccine and cholera toxin as adjuvant elicited protection against nasopharyngeal colonization and invasive disease from encapsulated pneumococci.<sup>109</sup> Moreover, the combined immunization of formalin-inactivated influenza vaccine and cholera toxin significantly reduced the death rate in mice after lethal infection by influenza A virus followed by group A streptococci. These results showed the importance of preventing secondary bacterial infections after prior non-lethal influenza exposure.<sup>110</sup>

It was also demonstrated that both i.n. and sublingual administration of inactivated polio vaccines contributes to the generation of polio-specific serum IgA in saliva, fecal extracts, and intestinal tissues and to IgA-producing B cells in mouse spleens. The level of polio-specific IgG induced by mucosal immunity was much higher than that induced by the intramuscular route.<sup>111</sup> Interestingly, Harakuni *et al.* showed that the immune effects of i.n. administration on the mucosa against an unrelated Japanese encephalitis virus are better than those resulting from administration with oral and transcutaneous immunization.<sup>112</sup> These results suggest that it is necessary to develop a more suitable method of noninvasive administration.

Likewise, subunit vaccines have been developed for delivery by i.n. administration, which was found to protect mice from the threat of *Yersinia pestis*. For example, Arnaboldi *et al.* developed a tobacco mosaic virus (TMV)-based delivery platform in which i.n. immunization of a subunit vaccine

consisting of *Y. pestis* virulence factors, including F1 and LcrV, with TMV significantly reduced the morbidity and mortality of *Y. pestis*, while subunit vaccines lacking TMV did not.<sup>113</sup> Other i.n. vaccine candidates have been tested with encouraging results. One study showed that a lipopeptide complex including a conserved extracellular domain of matrix protein 2 (M2e) of influenza A virus induced the generation of IgA and IgG2b antibodies and reduced the mortality of influenza-challenged mice without adjuvant.<sup>114</sup> Even when tested without adjuvant, an i.n. vaccine delivery consisting of a nanometer-sized hydrogel (composed of a cationic cholesteryl group-bearing pullulan [cCHP]) with a nontoxic subunit fragment of *Clostridium botulinum* type A neurotoxin BoHc/A strongly increased serum IgG and sIgA antibody responses.<sup>115</sup> Furthermore, the expressed fusion protein, which included M2e and cholera toxin subunit B in a vaccine delivered by the i.n. route, induced specific IgA and IgG antibodies as well as T cell and memory B cell responses and protected mice against heterologous influenza virus.<sup>116</sup> Recombinant vaccines administered with i.n. immunization have also been widely studied. Leishmanial recombinant protein combined with cholera toxin protects mice from *Leishmania* infection by inducing IFN- $\gamma$  production following i.n. administration.<sup>117</sup> Another study found that immunization with recombinant DnaJ (Hsp40) protein induced effective immune antibodies and the release of IL-10, IFN- $\gamma$ , and IL-17A against *Streptococcus pneumoniae*.<sup>118</sup> Interestingly, non-pathogenic *Lactococcus lactis* NZ9000, expressing pneumococcal immunogenic proteins, protected against *S. pneumoniae* and elicited innate and adaptive immune responses.<sup>119</sup>

Replication-deficient recombinant human adenovirus serotype rAd5 vectors were also constructed to express the N gene of the pneumonia virus of mice (PVM) pathogenic strain J3666. Its i.n. administration causes a PVM-specific CD8 response rather than increased serum IgG, which may result in the effective protection of mice after PVM challenge.<sup>120</sup>

The effects of VLP vaccines administered by i.n. immunization have also been widely investigated. A modular murine polyomavirus-like particle delivered by i.n. immunization was used to display the group A streptococcus (GAS) antigen J8i, which significantly induced J8i-specific IgG and IgA antibodies and reduced GAS colonization in the throat.<sup>121</sup> Cai *et al.* showed that a combination of respiratory syncytial virus (RSV) fusion protein VLPs and glycoprotein VLPs increases protection against live RSV, with a reduction in lung viral replication and histopathology damage.<sup>122</sup> However, some adjuvants, such as Toll-like receptor 3, ricin toxin B, TLR7 and nine of its agonists, and murabutide, need to be applied to increase the immune response of VLP vaccines.<sup>123-126</sup> Following i. n. delivery of TLR7 and its nine agonists with Norwalk virus, VLPs produce a more robust and broad spectrum of immune responses than by oral administration.<sup>125</sup>

Other vaccines, such as those involving DNA immunized by the i.n. route, need adjuvants, such as enterohemorrhagic *E. coli*-secreted proteins and IL-6, to increase the immune response.<sup>127,128</sup> Currently, DNA vaccines can be delivered using several biomaterials, such as the copolymer polyethyleneimine and hollow Ag@SiO<sub>2</sub> nanoparticles.<sup>129-131</sup> In the same way, mRNA can be transfected into both dividing and non-dividing cells without entering the nucleus, resulting in higher gene expression. Furthermore, mRNA vaccines may overcome

specific mutations that frequently occur in patients. Li et al. developed a system using a cationic cyclodextrin-modified polyethyleneimine 2 k conjugate with encoded HIV gp120 mRNA. This system delivered by i.n. immunization provides strong systemic and mucosal immune responses, cytokine production, and a balanced Th1/Th2/Th17 T helper cell distribution. These vaccines also overcame the nasal epithelial barrier to increase mRNA paracellular delivery.<sup>132</sup>

### Clinical trials for intranasal vaccines

MEDI-534 is a vaccine designed for delivery by the i.n. route that is currently undergoing a clinical trial. It combines a live attenuated respiratory syncytial virus (RSV) with parainfluenza-3 virus (PIV3), which causes lower respiratory tract illness in young children. This clinical trial demonstrated that MEDI-534 has a potential immunogenic effect in both seropositive and seronegative children.<sup>133-135</sup> Furthermore, after three doses of MEDI-534, the children were found to be 100% seroresponsive to PIV3 but only 50% to RSV. It was demonstrated that a mutation generated in the transcription termination poly A gene sequence upstream of the bPIV3N gene and the RSV fusion protein F open reading frame reduced RSV F expression. Although there was an association between this mutation and a lower rate of RSV seroconversion, a larger sample size is needed for definitive confirmation.<sup>136</sup>

A live attenuated i.n.-administered pertussis vaccine, BPZE1, induced significant memory B cell responses against pertussis

toxin, filamentous haemagglutinin, and pertactin in 7 colonized subjects out of 36 subjects who had received BPZE1.<sup>137,138</sup> Moreover, BPZE1 had a protective effect against RSV, in part, by markedly increasing IL-17 cytokines produced by CD4<sup>+</sup> T cells.<sup>139</sup> The peptide vaccine Vacc-4x was used for HIV therapy and generated T cell responses and mucosal and systemic humoral responses. However, different doses led to different immune responses; hence, the clinical dosage and mechanisms of immune regulation need to be further elucidated.<sup>140</sup>

Atmar et al. conducted a clinical trial to assess the effect of a norovirus-like particle vaccine against norovirus, which causes epidemics and sporadic acute gastroenteritis. This study showed reduced frequencies of gastroenteritis and infection by Norwalk virus and increased IgA seroconversion.<sup>141</sup> A Norwalk VLP vaccine also enhanced the levels of specific IgG antibodies in subjects that had received a 100- $\mu$ g vaccine dose and enhanced the levels of IgA antibody-secreting cells in subjects that received a 50- $\mu$ g or 100- $\mu$ g vaccine dose.<sup>142</sup> Despite limitations with regard to subject age, pregnancy, and health issues of the currently licensed vaccines, an i.n. proteasome-adjuvanted trivalent vaccine increased seroconversion and protection against infection.<sup>143</sup> The clinical trials that have been conducted for i.n. vaccines are listed in Table 2.

### Licensed i.n. vaccines

The US FDA-approved, i.n.-delivered, licensed vaccines are the influenza A (H1N1) 2009 monovalent pandemic flu vaccine

**Table 2.** Clinical trials for intranasal immunization.

Type of vaccine	Sponsor institution	Stages	Clinical registration	Time
Live attenuated RSV/PIV3 vaccine	MedImmune LLC	Phase 1	NCT00345670 <sup>133</sup>	2006
		Phase 1/2	NCT00767416 <sup>134</sup>	2008
		Phase 1	NCT00493285 <sup>135</sup>	2007
		Phase 1/2	NCT00686075 <sup>136</sup>	2008
Live attenuated influenza vaccine	Beijing Chaoyang District Centre for Disease Control and Prevention	Phase 1	NCT02665871	2016
		Phase 1 Phase 2	NCT00112112 NCT00344305	2005 2006
Live attenuated influenza vaccine	PATH Vaccine Solutions	Phase 2	NCT01625689	2012
Live attenuated influenza vaccine	University of Colorado, Denver	Phase 2	NCT02474901	2015
Live attenuated <i>Bordetella pertussis</i> vaccine	GRIEMHMRF	Phase 1	NCT03137927	2017
Unmodified live attenuated Sendai virus vaccine	St. Jude Children's Research Hospital	Phase 1	NCT00186927	2005
Live attenuated <i>B. pertussis</i> vaccine	Institut National de la Santé Et de la Recherche Médicale, France	Phase 1, Phase 1	NCT01188512, <sup>137-139</sup> NCT02453048	2010 2015
Adenovirus-vectored influenza vaccine	Altimmune, Inc.	Phase 1	NCT00755703	2008
Adenovirus vaccine	NINAD	Phase 1	NCT01806909	2013
Live attenuated/inactivated influenza vaccine	St. Jude Children's Research Hospital	Phase 1	NCT00906750	2009
Inactivated influenza virus vaccine	NanoBio Corporation	Phase 1, Phase 1	NCT01333462, NCT01354379	2011
				2011
Inactivated influenza vaccine	MedImmune LLC	Completed	NCT00808808	2008
RSV subunit vaccine	Mucosis BV	Phase 1	NCT02958540	2016
Pneumococcal subunit vaccine	Genocea Biosciences, Inc.	Phase 2	NCT02116998	2014
HIV gag peptides vaccine	Oslo University Hospital	Phase 1/2	NCT01473810 <sup>140</sup>	2011
Recombinant RSV vaccine	Bavarian Nordic	Phase 1	NCT02864628	2016
Norwalk virus-like particle vaccine	Takeda	Phase 1/2	NCT00973284 <sup>141</sup>	2009
Norwalk virus-like particle vaccine	LigoCyte Pharmaceuticals, Inc.	Phase 1	NCT00806962 <sup>142</sup>	2008
Liposomal-based influenza vaccine	Hadassah Medical Organization	Phase 1/2	NCT00197301	2005
Proteasome-adjuvanted influenza vaccine	Hvivo	Phase 1/2	NCT02522754 <sup>143</sup>	2015

RSV, respiratory syncytial virus; PIV3, parainfluenza virus type 3; GRIEMHMRF, Gamaleya Research Institute of Epidemiology and Microbiology, Health Ministry of the Russian Federation.

and the FluMist® trivalent and quadrivalent seasonal flu vaccines. These vaccines activate the immune responses of individuals of ages 2–49 years, who are protected against influenza disease caused by the pandemic (H1N1) 2009 virus, while the FluMist trivalent vaccine was designed against two influenza A and one influenza B strains.<sup>144</sup> The first approved FluMist quadrivalent vaccine was intranasally delivered against H1N1 and H3N2 2009 virus and B strains by Yamagata and Victoria,<sup>145,146</sup> who demonstrated that the quadrivalent and trivalent vaccines are safe and immunogenic.<sup>146</sup> Vaccination with FluMist quadrivalent vaccine may increase protection against both B strains covered by the trivalent vaccine.<sup>146</sup> Another intranasal live attenuated vaccine, Nasovac-S, has been approved in India, as its safety and efficacy were confirmed after administration to a large population.<sup>147</sup>

Even though intranasal vaccines are alternatives to replace the injectable ones and have shown vaccine efficacy, there was an inactivated intranasal influenza vaccine, known as Nasalflu licensed in Switzerland, which was removed from the market because of its association with relative risk of Bell's palsy. This vaccine contained hemagglutinins from three influenza viruses (A/Bayern H1N1, B/Beijing, and A/Wuhan H3N2) and *E. coli* heat-labile enterotoxin (HLT). The prelicensure clinical trials of this vaccine showed no sign of Bell's palsy in 1218 vaccine recipients.<sup>148,149,150</sup> However, Mutsch et al. conducted a matched case-control research and a case-series analysis in German-speaking regions of Switzerland after the vaccine was licensed in Switzerland, and they found a strong association between the inactivated intranasal influenza vaccine and Bell's palsy. The disease was only observed in patients who received the intranasal vaccine but not the ones inoculated with the parenteral influenza vaccines.<sup>150</sup> They estimated that the relative risk of Bell's palsy was 19 times the risk in the controls, corresponding to 13 excess cases per 10,000 vaccinees within 1 to 91 days after vaccination. These kinds of cases have indicated the importance of preclinical safety studies, wherein it requires longer period of observation.<sup>150</sup>

### Transcutaneous vaccination

Transcutaneous vaccination is delivery through the skin and has several advantages, such as that the epidermis contains several types of immune cells, including DCs, langerin<sup>+</sup> cells (LCs), T lymphocytes, NK cells, macrophages, and mast cells.<sup>151,152</sup> Epidermal Langerhans cells are special DCs, responsible for controlling immune responses in the skin, and they are defined by their localization in the epidermis and the expression of CD1a and langerin (CD207). The latter molecule is a receptor of C-type lectin, which recognizes the pathogen-associated molecular pattern (PAMP) on the surface of different pathogens. After the interaction, followed by langerin-mediated endocytosis, the LC migrates to the lymph nodes to activate the immune response.<sup>153,154</sup> Transcutaneous immunization may enable efficient presentation of the antigen to the immune cells of the skin, inducing a rapid and efficient immune response.

One of obstacles for transcutaneous immunization is the stratum corneum (the dead cell layer) of the skin, which is difficult to penetrate. Several studies have designed different

adjuvants and delivery systems using different chemical and physical approaches to improve immune responses without the use of needles. Microneedle patches effectively deliver antigens into APCs of the epidermal and mucosal compartments. Although mucosal and systemic immunity are induced by transcutaneous immunization, the specific mechanisms are still not well understood.<sup>155</sup> Belyakov et al. demonstrated that the migration of DCs and LCs carrying an HIV peptide construct with CT, LT, or CpG oligodeoxynucleotides to the lungs induced a significant cytotoxic T lymphocyte (CTL) response in the systemic circulation and the strongest response in the lung after transcutaneous priming and transcutaneous boosting,<sup>155</sup> and these results may partially explain mucosal immunity. During this process, the LCs induced Th1 and Th2 responses after treatment with antigen-delivering microneedle vaccination. This effect was confirmed in a study in which LC-depleted mice were vaccinated with microneedles coated with subunit influenza vaccine. The results showed that 95% of the vaccine was eliminated from the skin of wild type mice, whereas 65% of the vaccine was eliminated in LC-KO mice. This mouse strain also had impaired humoral and cellular immune responses, with reduced Th1 and Th2 profiles in comparison with wild type mice.<sup>156</sup>

Due to the rapid development of microfabrication manufacturing, immunization with microneedles has recently received increased attention in the vaccine field. Currently, microneedle types include solid (for direct tissue pretreatment), drug-coated, deep, dissolving, and hollow.<sup>157</sup> Several materials, including polymers, colloidal silica, ceramics, steel, glass, sugar, hydrogel, and alumina, are used for microneedle fabrication.<sup>158–164</sup> Each type of microneedle employs different mechanisms for vaccine delivery. Solid microneedles are generally made of stainless steel with a diameter of 500  $\mu\text{m}$ , which directly generates skin pores and allows the penetration of topical vaccine through the surface of the pretreated skin into the body,<sup>165</sup> while drug-coated microneedles are used to release the drug slowly into the skin.<sup>166</sup> One of the materials used in drug-coated microneedles is poly(L-lactide) (PLLA), which is melted on silicone micro-molds with pyramidal cavities at 200°C, compressed by a hot press, and cooled to RT. Later, the microneedles are coated with lidocaine hydrochloride monohydrate. The investigators found that delivery efficiency into porcine skin was  $69.1 \pm 15.1\%$ ,  $77.2 \pm 13.5\%$ , and  $84.0 \pm 6.8\%$  after application at 1, 2, and 5 min, respectively, and that the lidocaine was stable for 3 weeks at different temperatures.<sup>166</sup>

In general, non-dissolvable microneedles are made of inorganic materials, such as metal, silicon, glass, and ceramics, while dissolvable microneedles are made from water-soluble biopolymers, such as sulfate dextran and chondroitin, in which the drugs are applied as a suspension that dissolves into the skin.<sup>167</sup> In fact, the eye may also be a target organ for microneedle application.<sup>168</sup> This technology can be applied to the delivery of DNA, viral capsid subunits, and inactivated and live attenuated vaccines. The delivery of live attenuated vaccines has several disadvantages, since the vaccine needs to be stored and distributed at low temperatures, and reconstitution is required before administration.<sup>169</sup> However, measles virus, which still greatly threatens the morbidity and mortality of children worldwide,<sup>170</sup> combined with excipient trehalose delivered by steel microneedles generates immunity against measles



comparable to subcutaneous injection.<sup>171</sup> Furthermore, these microneedle measles patches are significantly protected from loss of titer for 30 days at room temperature, which meets the WHO requirements for lyophilized vaccines.<sup>171,172</sup>

Edens et al. further developed a dissolving microneedle patch fabricated with sucrose to evaluate the measles vaccine in rhesus macaques.<sup>173</sup> They showed that subcutaneous injection and microneedle delivery generated equivalent titers of neutralizing antibodies. However, microneedle patches sustained a higher level of activity and thermostability with increasing temperature.<sup>174</sup> In addition, Vrdoljak et al. vaccinated live recombinant adenovirus and modified vaccinia Ankara vectors by transcutaneous delivery using solid microneedle patches,<sup>175</sup> which resulted in virus delivery and infection and elicited humoral or CD8<sup>+</sup> T cell responses comparable to that produced by intradermal injection. Similarly, an adenoviral vector vaccine delivered by microneedle conferred robust protective immunity against Zika virus and malaria.<sup>176,177</sup> Motivated by the fact that the efficacy of live attenuated oral rotavirus vaccines is lower in developing countries, Wang et al. showed that the administration of inactivated rotavirus vaccine with Micro-nJet600<sup>®</sup> microneedles in neonatal gnotobiotic piglets generates comparable protective effects as intramuscular injection.<sup>178</sup>

Muller et al. developed a high-density microprojection array, the Nanopatch, which delivers inactivated poliovirus vaccine into the skin. These microneedle arrays led to the generation of neutralizing antibodies against poliovirus in 100% of the rats treated.<sup>179</sup> Furthermore, influenza subunit vaccines were administered by microneedle patches to increase humoral immunity.<sup>180,181</sup> The influenza subunit vaccine coated onto microneedle patches elicited an effective immune response comparable to intramuscular injection in guinea pigs.<sup>182</sup>

Microneedles have also been used to deliver recombinant antigen vaccines, in which anthrax antigen delivered by microneedle induces the same effective immune response and protection as intramuscular injection.<sup>182</sup> Furthermore, recombinant trimeric soluble hemagglutinin derived from the A/Aichi/2/68 virus generates a higher immune response, including more antigen-specific Th1 cells, a greater mucosal antibody response, and a higher survival ratio than unmodified hemagglutinin.<sup>180</sup> Likewise, VLP vaccines confer effective protection against influenza using the microneedle route. It was shown that microneedle delivery of H5N1 influenza VLPs results in a sustained B and T cell response. Intriguingly, application to human skin induced the CD207<sup>+</sup> Langerhans cells to migrate toward the basement membrane.<sup>183</sup> Kim et al. showed that heterologous VLPs incorporating the influenza virus M2 extracellular domain generated a broad cross-protective effect against H1N1, H3N2, and H5N1.<sup>184</sup>

In another study, Hooper et al. developed an experimental smallpox DNA vaccine that included four vaccinia virus genes (4pox), which was delivered using plasmid DNA-coated microneedle arrays and induced a greater antibody response than a live virus vaccine delivered by scarification.<sup>185</sup> Moreover, vaccination with a DNA vaccine encoding influenza hemagglutinin (HA) using microneedles generated better protection against viral challenge, enhanced humoral and cellular immunity, and enabled dose sparing compared with intramuscular injection.<sup>186,187</sup> Although

DNA vaccines are thermostable, inexpensive, and rapidly manufactured, their use under clinical conditions is limited due to their insufficient immunogenicity.<sup>188,189</sup> To solve this problem, different kinds of non-viral delivery systems, including cationic lipids, polymers, and liposomes, combined with microneedle delivery have been used for DNA vaccines.<sup>190-192</sup> Kim et al. used a pH-responsive polyelectrolyte to deliver polyplex-based DNA vaccines, which generated robust humoral and memory immune responses.<sup>193</sup> Recently, a DNA vaccine coated onto PLGA/PEI or PLGA-PLL/ $\gamma$ PGA nanoparticles and administered with microneedle patches effectively reduced the risks of H1N1 and Ebola virus, respectively.<sup>194,195</sup> Arya et al. showed that a DNA vaccine for rabies dissolved in microneedle patches generated a robust immune response, providing the potential to protect individuals from biting infection—at least to some extent.<sup>196</sup> Co-stimulation with A/PR8 influenza hemagglutinin DNA and an A/PR8 inactivated virus vaccine delivered by microneedle patches produced significant protection against A/PR8 and 2009 H1N1 virus. This study demonstrated that a single immunization with a microneedle vaccine or by intramuscular injection induced rapid memory responses with high HAI titers upon heterologous virus challenge, inducing protective immunity.<sup>197</sup>

Currently, microneedle-mediated vaccine delivery has also been investigated in the clinic, and several studies have been completed (Table 3). However, the number of clinical trials of microneedle vaccines has been much fewer than the number of trials for oral or i.n. vaccines. Rouphael *et al.* conducted a phase I clinical trial for inactivated influenza vaccine (fluvirin) administered by microneedle. It was shown that the adverse reactogenicity is mild and transient after dissolvable microneedle administration, and patients exhibited an increased neutralizing antibody titer, seroconversion rate, and level of seroprotection similar to intramuscular injection.<sup>198</sup> Microneedle patches administered by participants themselves also generated increased immune responses.<sup>198</sup> However, no microneedle-related vaccines have been licensed to date.

**Table 3.** Clinical trials for transcutaneous immunization.

Type of vaccine	Sponsor institution	Stages	Clinical registration	Time
Inactivated influenza vaccine	Georgia Institute of Technology	Phase 1	NCT02438423 <sup>198</sup>	2015
Inactivated influenza vaccine	The University of Hongkong	Phase 1	NCT01049490	2010
Inactivated influenza vaccine	NanoPass Technologies Ltd	Phase 1	NCT00558649	2007
Inactivated influenza vaccine	The University of Hong Kong	Phase 1	NCT01304563	2011
Inactivated influenza vaccine	Assistance Publique – Hôpitaux de Paris	Phase 1/2	NCT01707602	2012
Hepatitis B vaccine	The University of Hongkong	Phase 2/3	NCT02621112	2015
Varicella zoster virus vaccine	The University of Hongkong	Phase 1	NCT02329457	2014
Inactivated polio vaccine	Eastern Virginia Medical School	Phase 2	NCT01686503	2012
Fluzone intradermal vaccine	National Institute of Allergy and Infectious Diseases	Phase 1	NCT01518478	2012

## Further improvements

Vaccination is an important approach to controlling infectious diseases, especially in developing countries where technology and sanitary conditions are less advanced. The most common infectious diseases are malaria, TBC, HIV, and gastroenteritis produced by drinking or eating contaminated water or food. The ability to induce a balanced immune response after immunization is determined by several factors, including the route of immunization, the microorganism target organ, the nature of the antigens, the immunological vehicles, and the cytokine and T cell responses. The formulation of vaccine antigens is a problem in developing countries, where the conditions for both storage and transportation of vaccines to the countryside are suboptimal, and the cold chain may fail at either of these steps.<sup>8,199</sup> Most of the vaccines are intramuscular with good results protecting millions of people, and where their administration is not needle-free, especially in developing countries where syringes are reused, it may be dangerous for both medical personnel and patients.

In this review, we have made the case that needle-free vaccines, especially those for mucosal delivery and transcutaneous immunization, are good alternatives, which induce systemic and mucosal immune responses and activate sIgA/IgG plasma B cells and T cells that pass through the lymphatic system to infection sites. However, further studies are needed to resolve some remaining issues. Currently, several clinical trials of needle-free vaccination systems are being conducted using oral, i. n., and microneedle administration, which are showing effective protection against pathogens. However, there are only a few licensed oral and i.n. vaccines that are noninvasive compared with the very large majority of vaccines that are injected. According to the clinical trial results for licensed vaccines, adverse effects may occur. Moreover, because the safety of the attenuated mucosal virus is not easy to evaluate in infants, new vaccines should be carefully evaluated before use in infants. Although there are some significant advantages of vaccination by the oral and i.n. routes, they face unique challenges. Oral vaccination can be affected by low pH, proteolytic enzymes, and biological barriers in the gastrointestinal environment.<sup>3</sup> Furthermore, the short absorption time may constrain the absorption of related vaccines.<sup>200</sup> Considering the degradation of vaccines, the vaccine dose should be increased to generate comparable effects as parenteral injection,<sup>38</sup> and this may reduce vaccine efficacy. Therefore, there are more stringent requirements for the vaccine carrier to achieve an efficient immune response, as a larger dose may lead to risks of immune tolerance, and constant antigen stimulation of the GI tract may limit the response of the GALT to biohazard antigens rather than protecting against these antigens.<sup>201,202</sup>

Intranasal vaccines also face short residence times and higher dose requirements.<sup>101</sup> Furthermore, physiological barriers also hinder the absorption of vaccines. Thus, solving these problems and increasing the vaccine efficacy of mucosal vaccines is still a challenge.

The other needle-free delivery system, the microneedle, has had few vaccination clinical trials. Moreover, the microneedles by themselves may not easily break the skin and may cause swelling during insertion, which has in part reduced their

application in the clinic. While a recent study showed that microneedle arrays filled with liposomes loaded with hepatitis B virus vaccine induced robust systemic and mucosal immunity by oral immunization,<sup>203</sup> the alternative route of administration of microneedle arrays should also be considered. Furthermore, vaccines coated onto solid microneedles or dissolving microneedles may gradually dry out, reducing vaccine activity and possibly producing discomfort when used over a longer period. Therefore, microneedle-mediated vaccination also needs more study to improve its application in the clinic.

The mechanisms of invasive vaccines should also be elucidated for rational vaccine improvement. Overall, although non-invasive administration of vaccines still has several unsolved problems, it has a promising future, since it has the potential to reduce the cost of the vaccines in developing countries, reduce the risk of contamination with other needle-borne diseases, and speed vaccine administration during pandemics. In addition, one of the important challenges of the needle-free system is to design delivery vehicles that protect the vaccines, which should improve results in clinical applications.

## Disclosure of potential conflicts of interest

The authors declare that they have no potential conflicts of interest.

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