Expert Opinion

Oral vaccination: where we are?

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As early as 900 years ago, the Bedouins of the Negev desert were reported to kill a rabid dog, roast its liver and feed it to a dog-bitten person for three to five days according to the size and number of bites [1]. In sixteenth century China, physicians routinely prescribed pills made from the fleas collected from sick cows, which purportedly prevented smallpox. One may dismiss the wisdom of the Bedouins or Chinese but the Nobel laureate, Charles Richet, demonstrated in 1900 that feeding raw meat can cure tuberculous dogs – an approach he termed zomotherapy. Despite historical clues indicating the feasibility of oral vaccination, this particular field is notoriously infamous for the abundance of dead-end leads. Today, most commercial vaccines are delivered by injection, which has the principal limitation that recipients do not like needles. In the last few years, there has been a sharp increase in interest in needle-free vaccine delivery; new data emerges almost daily in the literature. So far, there are very few licensed oral vaccines, but many more vaccine candidates are in development. Vaccines delivered orally have the potential to take immunization to a fundamentally new level. In this review, the authors summarize the recent progress in the area of oral vaccines.

Keywords: immunotherapy, mucosal vaccination, native immunity, oral tolerance, vaccine delivery

Expert Opin. Drug Deliv. (2007) 4(4):323-340

1. Introduction

Despite all their limitations, vaccines have brought great benefit to both humans and animals throughout the world, for disease control and, in the case of smallpox, even eradication.

Humans are constantly exposed to microorganisms present in the environment and in our own bodies. Almost all infectious agents (viruses, bacteria, fungi and parasites) enter the host through mucous membranes. Therefore, local immunity, rather than systemic, is essential to protect against naturally transmitted pathogens. Mucosal vaccine delivery could confer local immunity, not only at the site of delivery, but on the surface of other mucosal membranes too, and eventually induce systemic immunity as well [2].

Ordinary needle and syringe vaccinations are associated with unwanted infection in both patients and healthcare workers, and the fear of needles and discomfort affect uptake rates, particularly in children. In the US alone, > 1 million people suffer injury or infection from needles annually, which can require often costly treatment.

Oral delivery is the obvious choice not only for drugs, but also for vaccines, by virtue of its ease of administration and cost. The oral delivery of vaccines has been the Holy Grail for generations of vaccinologists [3]. However, oral vaccination to provide effective mucosal and/or systemic immunity has been historically thought to be largely ineffective. This is mainly due to the fact that antigens undergo proteolytic degradation in the stomach and intestine. Another barrier that needs to be understood is the immune tolerance resulting from antigen feeding.

In the last few years, there has been exponential increase in interest in needle-free vaccine delivery. Oral vaccine technologies have the potential to radically change the face of today's vaccine industry. Safety, efficacy and compliance could be increased

10.1517/17425247.4.4.323 © 2007 Informa UK Ltd ISSN 1742-5247

Table 1. Present oral vaccine preparations.

Vaccine	Target	Agent
Polio	Poliovirus	Live attenuated virus
Sad, sag2	Rabies	Live attenuated virus
Raboral® V-RG	Rabies	Recombinant virus
Rotateq®, RRV-TV, Rotashield®, Tetramune®, RIX 4414	Rotavirus	Live attenuated virus
Ad4 and Ad7	Adenovirus	Live attenuated virus
TEOvac	Smallpox	Live attenuated virus
Vaxihaler-flu	Influenza	Live attenuated virus
V-1 Immunitor	HIV	Killed virus
Vivotif®, ty21a	Typhus	Live attenuated bacteria
Kolera, CVD 103-HgR, Orochol or Mutachol	Cholera	Live attenuated bacteria
Dukoral®	*Vibrio cholerae*, enterotoxigenic *E. coli*	Live attenuated bacteria
WC/rbs	Cholera	Killed whole cell with recombinant B subunit
Biv-WC	Cholera	Killed whole cell
Microb, Saratov	Cholera	Killed whole cell
Om-85	Respiratory infections	Killed whole cell
Kanvakol, Alvakol, and Urvakol	Several types of bacteria	Killed whole cell
O111	*E. coli*	Killed whole cell
Luivac	Respiratory infections	Killed whole cell
Imocur	Respiratory infections	Killed whole cell
Respivax	Respiratory infections	Killed, polybacterial
Biostim or RU 41740	*Klebsiella pneumoniae*	Antigenic preparation
Paspat	Antibacterial	Antigenic preparation
Ribomunyl	Klebsiella, Streptococcus, Haemophilus	Ribosomal fractions
Atit-ap	*Actinobacillus pleuropneumoniae*	Killed bacteria
Moreau Rio de Janeiro	*Mycobacterium tuberculosis*	Live attenuated Bacillus Calmette Guerin

dramatically by new methods that can reliably produce adequate mucosal immune responses. New delivery systems could also result in efficacious, low-cost products for new indications not yet covered by existing vaccines, and increas immunization in countries where uptake has been minimal. Most oral vaccines available today are either attenuated or killed microorganisms that can survive the intestinal degradation either by replicating in the gut or by virtue of having digestion-resistant bacterial walls (**Table 1**). Presently, there are several oral products in development, but extensive human studies are needed to prove that they are safe and effective. In this review, the authors focus on the state-of-the art and recent progress in the area of oral vaccines and the key vaccine components that are under investigation (**Figure 1**).

2. Basics of gut immunity

The immune system associated with mucosal surfaces covers the largest area of the body and comprises a very sophisticated mechanism discriminating between harmless or commensal microorganisms viewed as 'self' and pathogenic or 'non-self' invaders. An immunological reaction at one mucosal site usually results in an immune response at distant mucosal sites – an observation that led to an unified concept of a common mucosal immune system [3]. The types of reaction and their amplitude are not always the same in different mucosal compartments, but precise reasons for this is not well known. The intestine is the largest and most studied compartment of the common mucosal immune system [4]. The human gastrointestinal tract is ~ 15 ft long. The total surface of the folded mucosa, with villi and microvilli is an area which is 600-times larger than a flat-surfaced tube of the same length and diameter. This area is estimated to be ~ 400 m^2 – by comparison the surface of the skin is only 2.3 m^2. The entire epithelial surface of the small intestine is replaced approximately every 5 days.

The base of the mucosa is underlined by so-called gut- or mucosa-associated lymphoid tissue. The lamina propria is rich in intraepithelial lymphocytes, sometimes referred to as diffuse

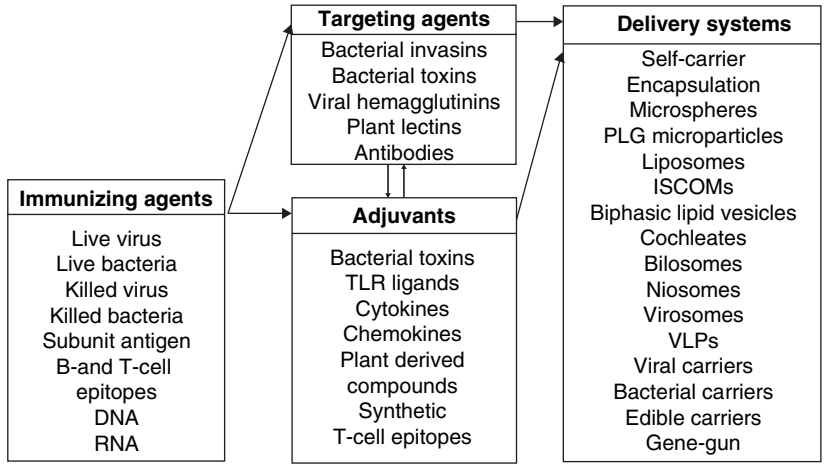

Figure 1. A schematic representation of the key elements of oral vaccines.
ISCOM: Immune stimulating complexes; PLG: Poly(lactide-co-glycolide); TLR: Toll-like receptor; VLP: Virus-like particle.

lymphoid tissue. T cells in the mucosa are either αβ or γδ types. Both CD8 and CD4 cells are found in the mucosa, which also carry B cells, monocytes/macrophages, dendrocytes and other immune cells. For example, most cells responsible for so-called native immunity or regulatory T cells are localized within the mucosa. Gut-associated immune cells represent up to 90% of the total number of immunocompetent cells in a human body. Indeed, lymphocytes circulating at any given moment in the blood, represent only ~ 2% of total lymphocytes [5]. Furthermore, the gut accommodates ~ 80% of all immunoglobulin or Ig-producing cells and releases 2- to 3-times more secretory IgA than the total output of circulating IgG [6].

An obligatory step in the development of the immune responses is the presentation of antigens by specialized accessory cells, termed antigen-presenting cells (APCs). The M-cells of Peyer's patches and dendritic cells are often considered to be the most important APCs in the intestine. This perception is not entirely correct, as enterocytes or intestinal epithelial cells can also serve as fully functional APCs [7]. Humans have only 20 – 30 Peyer's patches, and dendritic cells represent < 1% of the total cell population. Thus, M-cells and dendritic cells constitute a very small fraction of the cells in the intestine. However, under some conditions, intraepithelial Peyer's patch lymphocytes have been shown to induce the conversion of enterocytes into M-cells [8]. Thus, the essential elements of gut immunity are still being learnt, and it is likely that there will yet be some surprises. Nevertheless, the consensus today is that achieving success in the development of oral vaccines will largely depend on a better understanding of mucosal immune regulation.

3. Live oral vaccines

So far, all licensed oral vaccines for human or animal use are composed of attenuated forms of disease-causing pathogens, which can replicate in the mucosa and are capable of eliciting a sustained immune response. The most well-recognized examples of oral viral vaccines are polio (several types based on the original Sabin vaccine); rabies (Street Alabama Dufferin strain or SAD, modified SAD known as SAG2, and vaccinia-rabies recombinant Raboral V-RG [Merial]); rotavirus (RotaTeq® [Merck & Co., Inc.], RotaShield® [Biovirx, Inc.], Tetramune® [Wyeth], Rotarix® [GlaxoSmithKline]); adenovirus (Ad4 and Ad7); smallpox (tableted vaccinia, TEOVac [Virology Center of the Institute of Microbiology of the Ministry of Defense, Russia]); and orally-inhaled influenza vaccine (Vaxihaler-Flu [Vaxihaler-flu]). There are also a few live oral bacterial vaccines, such as typhoid (Vivotif; Ty21a [Crucell]); cholera (Kolera, CVD 103-HgR known as Orochol® [Berna Biotech] or Mutachol® [Crucell]); and a combination of *Vibrio cholerae* and enterotoxigenic *Escherichia coli* vaccine (Dukoral® [Crucell]).

Although most of these vaccines are considered safe, there is an inherent danger that the pathogen may revert back to a virulent form or become unduly pathogenic when used in the wrong target population. For example, the back mutation of the polio vaccine virus caused an epidemic outbreak in Haiti and the Dominican Republic in 2000, which in some cases resulted in paralysis. There have been other vaccine-related complications that were not necessarily caused by virus mutation and, thus, were not anticipated. For example, the rotavirus RRV-TV vaccine was withdrawn from the market shortly after its approval due to an unusual incidence of intussusception in vaccinated children, requiring surgical intervention. RotaTeq, which was approved in 2006, also appears to be associated with intussusception, although it is unknown at this time whether the vaccine is responsible for this observation. A pediatric trial of attenuated Ad4 vaccine caused infections in unvaccinated children and was, therefore, recommended for military use only. The oral rabies vaccine must be used exclusively in animals, and, due to concerns of potential neurological damage, the human exposure is not recommended.

4. Inactivated oral vaccines

As discussed in the previous section, vaccines consisting of weakened pathogens pose a potential risk. Inactivated (killed) oral vaccines are certainly less risky, but their efficacy has always been doubted. Nevertheless, there are killed bacterial oral vaccines on the market and they are not as uncommon as might be thought. In fact, there are an overwhelming number of products marketed in many countries. These include, but are not limited to, a killed whole-cell cholera toxin recombinant B subunit vaccine developed in Sweden (WC/rBS); a simpler version of the cholera vaccine without the recombinant B subunit, manufactured in Vietnam (biv-WC); a Russian tableted cholera bivalent vaccine with Ogawa and Inaba antigens (Microb, Saratov); the sublingual Grazax® grass pollen vaccine (ALK-Abelló); the OM-85 oral vaccine against respiratory infections sold by OM Pharma; Czech-made oral vaccines, Kanvakol (Institute of Microbiology, Olomouc), Alvakol (Institute of Microbiology, Olomouc), and Urvakol (Institute of Microbiology, Olomouc) against several types of bacteria; killed *E. coli* O111 vaccine for diarrhea (Slovakia); the multibacterial vaccine Luivac (Sankyo) against respiratory tract infection from Sankyo; the oral antibacterial vaccine, Imocur (Zambon) to prevent respiratory infections (Zambon); a similar polybacterial, bronchopulmonary vaccine Ismigen (Zambon); Biomunil (Lusofarmaco), which is a bacterial lysate made by Lusofarmaco; a broad-spectrum antigenic preparation from *Klebsiella pneumoniae* (Biostim or RU 41740; Aventis); the Mexican/German antibacterial Paspat (Altana Pharma) made as an oral tablet (Altana Pharma); a French oral preparation, Ribomunyl (Pierre Fabre) or Ribovac (Pierre Fabre) containing ribosomal fractions of Klebsiella, Streptococcus, and Haemophilus (Pierre Fabre); a similar product, D53 (Immucytal), from Italy (Pierre-Fabre Pharma Srl); a tableted lyophilized bacterial lysates Immubron (Bruschettini) and Lantigen B (Bruschettini); Bulgarian polybacterial vaccines Respivax (BulBio-NCIPD Ltd) and Dentavax (BulBio-NCIPD Ltd) for bronchopulmonary and gum infections; the Polish propionibacterium acne vaccine; and several Russian vaccines for a variety of microbial infections [9-13]. Many of these vaccine preparations are used as both therapeutic and prophylactic modalities. Most clinical trials for the registration of these products have never been published, or have been published in non-English language journals, which makes them virtually unknown to most vaccinologists.

There are no commercially available killed oral vaccines against viruses. The only exception is the oral therapeutic AIDS vaccine, V-1 Immunitor (V1) (Immunitor USA Inc.) (Immunitor USA Inc.), manufactured by Immunitor [3]. With the exception of a few products, almost all of the above listed vaccines are marketed as immunomodulators or food supplements for disease immunoprophylaxis [3]. Depending on the country, they are occasionally sold as vaccines. Regardless of their regulatory stature, close attention needs to be paid as to why these products are effective upon oral administration. The elucidation of their mechanism of action may provide valuable clues for the design of effective oral vaccines against other pathogens.

5. Encapsulation of antigens

To create a vaccine that could efficiently rally the immune system, antigens need to be protected from digestive degradation. Several means of protection or delivery are being investigated, some of which are reviewed below (**Table 2**).

The choice of potential encapsulation materials is enormous. The most popular materials used today are poly (lactic-co-glycolic) acid (PLG), polysterene, carboxymethylcellulose, poly(dimethylamino)ethyl methacrylate, polyethylene glycol (PEG), liposomes and so-called cochleates [14-17]. In experiments performed by the authors of the present review, gelatin capsules were originally tested; however, the results were unsatisfying, possibly because of degradation in duodenum (data not published). Starch microparticles for oral delivery of cholera toxin B and human serum albumin have been shown to provide good protection against antigen degradation and induced immune response in mice; however, the same system in humans failed when it was used with diphtheria toxin antigen [18]. A more sophisticated system is composed of microspheres with pH-dependent antigen release. In this system, antigen can be rapidly released in neutral intestinal pH, but not in the acidic milieu of the stomach. Good protection against challenge with such microspheres has been achieved in mice, but it performed unsatisfactory in pigs [2].

Protection of antigen in the stomach has been achieved in ruminants by using alginate microspheres during oral vaccination against rotavirus and experimental vaccination with various proteins and DNAs [19]. Prolonged contact with mucosal membrane was achieved by controlling the size of microparticles. Optimally sized particles (~ 10 µm) have been fixed between villi for longer periods of time [20]. Single oral immunization of mice with hepatitis B-derived B-cell epitope-loaded PLG particles has led to the significant induction of specific anti-HB serum IgG and IgM, and challenge with hepatitis B has been shown to result in the rapid production of anti-HB antibodies from the secondary immune response [20]. On the other hand, for optimal phagocytosis, the particles should be smaller, although the delivery efficiency is size- and species-dependent, as 0.2- to 0.5-µm particles have not been found to be superior to particles in the low micrometer range [21].

The oral delivery of PLG-entrapped antigen has been shown to provide a level of reactivity similar to Freund's adjuvant [22]. Oral vaccination of mice with PLG-ovalbumin microspheres has induced cellular and humoral responses in local and systemic immune systems. Similar results have been reported after oral delivery of PLG particles containing either lysed *Helicobacter pylori* whole cells, *V. cholerae* whole cells, tetanus or ricin toxoids. Protective immunity against challenge has been shown to develop after oral delivery of PLG microspheres with

Table 2. Delivery systems explored for oral delivery.

Delivery systems	Protection of antigen	Targeted delivery	Sustained release	Adjuvanticity
Naked	Weak	Weak	None	None
Live self-carrier	Various	Good, specific	Possible	Possible
Encapsulation	Moderate	Weak	Possible	Not *per se*
pH-driven microspheres	Good, partial	Size dependent	Controllable	Not *per se*
PLG microparticles	Good	Size dependent	Long lasting	Not *per se*
Liposomes	Moderate	Good	None	Weak
ISCOMs	Moderate	Good	None	High
Biphasic lipid vesicles	Moderate	Good	None	High
Cochleates	Moderate	Good	None	Weak
Bilosomes	Good	Good	None	Not *per se*
Niosomes	Good	Good	None	Not *per se*
Virosomes	Good if coated	Good, specific	None	Possible
VLPs	Various	Good, specific	None	High
Viral carriers	Various	Good, specific	Possible	High
Bacterial carriers	Various	Good, specific	Possible	High
Edible carriers	Moderate	Weak	Possible	Possible

ISCOM: Immune stimulating complexes; PLG: poly(lactide-co-glycolide); VLP: Virus-like particle.

Yersinia pestis F1 antigen and *Salmonella typhimurium* phosphorylcholine antigen in animal experiments; however, these results were not reproducible in humans. A few human studies conducted so far have shown only weak protective potency [23].

The oral delivery of PLG particles with an antigen-encoding DNA plasmid can achieve the desired effect after a single round of vaccination. The promise of this method has been demonstrated by the appearance of antigen-specific secretory IgA, induced after oral delivery of HIV-1 antigen DNA [24] and DNA antigens of rotavirus [25]. Despite the demonstrated potential of PLG particles, there is a substantial disadvantage of this system for acid-sensitive antigens: the degradation of PLG microparticles generates low pH carboxylic end groups at the degrading polymer surfaces. This can be avoided by the incorporation of buffering compounds and a higher content of lactides for faster antigen release.

The liposome delivery system does not present this problem, although the incorporation of antigens is technically more difficult and results in a lower antigen load compared with PLG particles. Nevertheless, positive results have been obtained after liposomal delivery of *H. pylori* hsp60 [26], *E. coli* antigens [27], diphtheria and tetanus toxoids [28]. The effectiveness of the liposomal delivery of DNA is comparable to that of PLG. PEG modification of liposomes and double liposome structures further enhance its potential.

The development of the liposomal approach led to Quil A-containing immune stimulating complexes (ISCOMs) of ~ 40 nm in size, which can entrap antigens and efficiently deliver them orally. The ISCOMs possess high adjuvanticity, resulting in potent stimulation of local and systemic immunity, including Th1 and Th2 activity, and a cytotoxic lymphocyte and secretory IgA response. The application of ISCOMs with ovalbumin (OVA) before feeding mice with tolerogenic doses of OVA has been shown to redirect the immune response away from tolerance. The full protection of mice against challenge has been achieved after oral delivery of ISCOMs with herpes simplex and influenza viral antigens [29]. The oral delivery of rotavirus antigen in ISCOMs was shown to efficiently protect gnotobiotic animals against challenge virus [30]. Generally, ISCOMs have intrinsic advantages over PLG particles and liposomes, as they contain potent Quil A adjuvant and are impervious to pH variations. Furthermore, the size of ISCOM particles is favorable for glycocalix penetration and uptake by M-cells and dendritic cells.

A novel delivery system consists of biphasic lipid vesicles (Biphasix™ [Helix BioPharm Corp.] Vaccine-Targeting Adjuvant), which has demonstrated an increased mucosal immune response [31].

6. Targeting the mucosa

To increase antigen concentration on the mucosal surface and duration of contact, the vaccine carriers can be coated with mucosa-binding substances. Non-specific binding can be achieved by altering the surface charge or hydrophobicity. However, it is difficult to maintain these characteristics in the intestinal lumen, which contains a multitude of affecting substances. More stable binding can be achieved by using

bioadhesive substances, which, ideally, should be non-toxic, be poorly immunogenic, and not interfere with the normal microflora of the gut. Among such substances are bacterial toxins, invasins, viral haemagglutinins, plant toxins and lectins.

6.1 Adhesive plant lectins

In theory, it is possible to target different compartments of the mucosa, as carbohydrates on the surface of enterocytes differ in various parts of the intestine and glycocalyx of M-cells from follicle associated epithelium (FAE) enterocytes. Thus, some lectins are able to bind preferentially to different intestinal cells. Ulex europaeus 1 lectin specifically and almost exclusively binds to murine (but not human or rabbit) Peyer's patch M-cells [32]. Therefore, the appropriate lectins for precise M-cell targeting can be selected by comprehensive screening. So far, no lectins that selectively bind human M-cells have been reported, although *Viscum album* and *Sambucus nigra* lectins bind both M-cells and enterocytes of human Peyer's patch FAE [33]; and sialyl Lewis A antigen is expressed mostly by M-cells of human Peyer's patch FAE and by a small subset of FAE enterocytes and goblet cells [34].

6.2 Bacterial adhesins

Certain microorganisms bind to intestinal cells in a very selective manner. For example, LTB-coated particles are predominantly taken up by enterocytes rather than the M-cells, and uptake can be blocked by a ganglioside with specific affinity to LTB. Nevertheless, targeting M-cells provides substantial advantages, particularly when a systemic immune response is desirable. Specific targeting to M-cells can be achieved by using conjugates with bacterial binding sites or attenuated pathogenic bacteria. For example, modified strains of *S. typhimurium* administered as live oral vaccines have been shown to provide protection against challenge with a virulent strain [35], and some mutants of *S. typhi* can be used as a live oral typhoid vaccine for humans [36,37]. *Salmonella choleraesuis* can be used as an oral vaccine against swine septic salmonellosis [38], and *Salmonella enteritidis* against fowl typhoid in chickens [39,40]. Killed pathogenic *V. cholerae* [41,42] or its live attenuated strains [43,44] are used as human oral vaccines against cholera. Killed enterotoxigenic of *E. coli* or its bacterial ghosts are proven to be immunogenic and safe against enterotoxemia and urogenital infections [45,46]. Orally administrated vaccine strains of *E. coli* have protected piglets against postweaning colibacillosis [47]. Similar whole cell approaches with killed or live attenuated bacteria has been shown to work in tuberculosis, shigellosis, pseudomoniasis, Lyme disease, anthrax, *Helicobacter pyloris*, Lawsonia intracellularis in pigs, and even against bacterial coldwater disease in fish.

Some Yersinia species target murine M-cells by invasins, which specifically interact with the β1 integrins of M-cells. Invasin-coated particles are efficiently endocytosed: 13% of invasin-coated nanoparticles are found in the circulation after single vaccine dose, compared with 2% for non-invasin-coated control particles [48]. The Salmonella species can selectively adhere to M-cells, possibly by long polar fimbria, as the transfection of non-piliated bacteria with an *lpf* operon (encoding fimbria), has been shown to enhance their uptake by Peyer's patch [49]. The well-known adjuvanticity of cholera toxin can be attributable in part to the high affinity of its non-toxic B-subunit for the ganglioside GM1, which is present on both M-cells and enterocytes. This toxin B-subunit can enhance liposomal delivery to the intestine [50].

Obviously, bacteria with gut-binding affinity are suitable carriers for a vaccine antigen or DNA. For example, Salmonella served as carrier for the delivery of DNAs or antigens of *Bacillus anthracis*, *H. pylori*, *E. coli*, Campylobacter, Streptococci, *Toxoplasma gondii* and *Shistosoma mansoni*. Viral antigens can also be delivered. Antigens or encoding DNAs of Dengue virus, Newcastle disease virus, porcine respiratory and reproductive syndrome virus, and HIV, have all been tested in this system. Moreover, a similar approach could be successful for the treatment of some forms of cancer, through the oral delivery of DNA-encoding tumor-associated antigen [51-53]. However, in some cases, this approach has produced tolerance, resulting in the abolishment of IgE – this has led to the creation of antiallergic vaccine against pollens [54]. Attenuated strains of other bacteria are suitable for a carrier role (e.g., *Clostridium perfringens* for simian immunodeficiency virus antigen [55], *Listeria monocytogenes* for HIV or SIV antigens [56,57], *V. cholerae*, *Shigella flexneri* and *Yersinia enterocolitica* for enteropathogenic *E. coli* antigens [58,60], and *Mycobacterium bovis* for HIV [61]. Other carriers exists: *Streptococcus gordonii* [62] and non-pathogenic *Lactococcus lacti* [63], which have been tested for the delivery of various antigens.

6.3 Viral adhesins

Oral delivery of some viruses could induce protective immunity. Viral proteins with M-cell-targeting properties have been found in HIV type 1, poliovirus type 1 and reovirus type 1. In a mouse model, the latter virus has been shown to be selectively endocytosed by intestinal M-cells, whereas reovirus type 3 was endocytosed by both M-cells and enterocytes [64]. Hemagglutinin sigma 1 of this virus was shown to be responsible for that interaction [64], and incorporation of this protein into liposomes enhanced their binding to Peyer's patches [65]. Moreover, IgA-directed to this protein has protected mouse intestine from reovirus entry [66]. Selective lectin MAL-II-dependent binding of type 1 (but not types 2 and 3) of reoviruses to rabbit and human M-cells involves the interaction of the type 1 sigma 1 protein with glycoconjugates containing α 2-3-linked sialic acid [67].

Viruses can be exploited as carriers of their own or foreign antigens. Adenovirus carrying the rabies antigen has protected against inhaled rabies virus, and an anticarrier immune response did not affect vaccine boosting [68]. Adeno-associated virus carrying Aβ cDNA (AAV/Aβ) has induced the expression and secretion of Aβ1-43 or Aβ1-21 in the epithelial cell layer of the intestine in amyloid precursor protein transgenic mice. Brain Aβ burden was shown to be significantly

decreased compared with the control, without inflammatory changes, which is promising for the prevention and treatment of Alzheimer's disease [69]. Vaccinia virus expressing the OspA protein of *Borrelia burgdorferi* has been shown to result in high antibody titers to OspA after oral administration; 100% protection of vaccinated mice from challenge was achieved, as well as significant clearance of *B. burgdorferi* from infected ticks fed on vaccinated animals [70]. Canarypox virus has been developed as a safe carrier for mammals, and has demonstrated efficient delivery to the mucosal immune system *F* and *H* genes of the measles virus [71] and canine distemper virus [72].

Intact viral external proteins could be delivered by virus-like particles (VLP). VLPs are self-assembling particles without viral genetic information that are similar in size and conformation to intact virions, but are neither replicating nor pathogenic. VLPs derived from hepatitis E virus could carry foreign DNA, express encoded protein and induce immune response against it [73]. VLP carrying B cell epitopes have been shown to induce a significant secretory humoral response [74]. VLPs can be combined with other delivery systems (e.g., ISCOMs). Lipid envelopes with intercalated viral proteins retaining the antigenic and fusogenic properties of their viral origin but without genetic information, have been developed as virosomes, and have demonstrated good acceptability and protective immune response [75]. Virosomes have been found capable of delivering various antigens, B-cell epitopes, DNA and molecular adjuvants, such as CD40L [76], which is capable of transforming an antigen into superantigen, thus providing a secondary activation signal to immune cells [77]. Sendai virosome-formulated melanoma vaccine has effectively elicited not only a systemic immune response, but also a strong cytotoxic lymphocyte response [78]. Turkeys immunized with avian metapneumovirus virosomes via mucosal intranasal administration have demonstrated a decreased viral load in the respiratory tract of compared with unvaccinated controls, and an increased virus-neutralizing antibody level against avian metapneumovirus was observed in birds vaccinated with virosomes [79]. Virosomes based on vesicular stomatitis [80], Aujeszky disease [81] and Newcastle disease [82] viruses have been prepared and investigated as delivery systems. However, the most researched are immunopotentiating reconstituted influenza virosomes [83], based on which the only two licensed virosomal vaccines (Inflexal® [Crucell] against influenza and Epaxal® [Crucell] against Hepatitis A) were developed, and positive results of their trials were extensively reported [84,85].

Unfortunately, there have been no reports on effective oral virosome-based vaccines so far, but the successful intranasal delivery of antigens [79,86] and DNA [87,88] by virosomes indicates the potential of virosomes for oral delivery by targeting M-cells or enterocytes. The stability of virosomes in the gastrointestinal tract, particularly in the duodenum, should be assured by design optimization or by additional protective delivery system. One option is coating with a modified polysaccharide *o*-palmitoyl mannan to protect the virosomes from bile salts and enzymatic degradation in the gastrointestinal tract, as has been shown with niosomes [89] and bile salt stablilised vesicles (bilosomes) [90].

6.4 Targeting with antibodies

Targeting to M-cells could be achieved by coating vaccine delivery vehicles with immunoglobulins, as some common isotype-non-specific domains of IgA and IgG can adhere to M-cells [91,92]. Liposomes coated with IgA have been shown to induce an enhanced immune response to entrapped ovalbumine [93]. It is possible, that after priming with antigen and minimal specific IgA levels, the boosting can be achieved by enhanced M-cell targeting with IgA–antigen immune complexes. On the other hand, it is possible to design specific antibodies directed against M-cell surface antigens, which can also increase the uptake of a vaccine [94].

7. Adjuvants

Due to potential safety issues associated with live vaccines, most of the modern vaccines are based on killed agents or its subunits. Such vaccines are less immunogenic, and, therefore, need immune potentiating adjuvants.

7.1 Bacterial adjuvants

Despite its potent adjuvanticity, oral administration of Freund's adjuvant did not demonstrate any significant immune response. The bacterial adjuvants appear to be more suitable for oral administration (e.g., *E. coli* thermolabile toxin [LT] and cholera toxin [CT]). LT and its derivatives, LTK63, LTR72 and R192G, have demonstrated an enhanced cellular and humoral immune responses after mucosal vaccination [95,96]. The efficiency of a DNA vaccine encoding LT as an adjuvant has been demonstrated in pigs [97]. Mucosal vaccination against *H. pylori* using LT as a mucosal adjuvant has demonstrated the superiority of an oral route of vaccination over intranasal administration, in terms of gastric IgA, although rectal administration was shown to generate an even better IgA titer [98]. In contrast, B-subunit of CT (CTB) seems to perform better upon systemic and intranasal administration compared with the oral route [99,100]. This is perhaps due to the observation that conjugation or co-administration of the non-toxic B-subunit of CT with antigen leads to oral tolerance [101]. High mucosal adjuvanticity of CT has been shown using bacterial and ISCOM delivery systems [102,103]. In a comparative study between CT and ISCOMs, it was found that CT was a better inducer of mucosal IgA response, and induced a similar systemic immunity following oral immunization. The addition of CT to the oral ISCOM protocol did not stimulate local IgA immunity, nor did it change the quality or magnitude of the systemic responses. However, CT abrogated the induction of oral tolerance stimulated by antigen feeding in IL-12-deficient mice [104]. Recent research has revealed the mucosal adjuvanticity of Brucella lumazine synthase [105], Shigella invasin complex [106], *B. anthracis* edema toxin [107], *Vibrio vulnificus* flagellin FlaB [108], outer

membrane protein of *Neisseria meningitides* [109], and Protollin (*N. meningitidis* outer membrane proteins non-covalently complexed with *S. flexneri* 2a lipopolysaccharide) [110].

7.2 Toll-like receptors ligands as adjuvants

Toll-like receptors (TLRs) are type I transmembrane proteins that recognize microorganisms that come in contact with intestinal tract mucosa, and activate immune cell responses. They play a key role in the innate immune system – the most ancient, conserved component of the immunity. TLRs that are expressed on M-cells and dendritic cells could be efficiently targeted by TLR ligands, which can function as mucosal adjuvants. The TLR-2/6 agonist MALP-2 has demonstrated strong mucosal adjuvanticity for *S. pyogenes* and HIV-1 antigens [111,112]. dsRNA, particularly polyriboinosinic-polyribocytidylic acid (poly(I:C)), has been shown to induce strong mucosal and systemic immunities against Anthrax toxins and bacilli [113,114]. The other TLR ligand – CpG-containing oligodeoxynucleotide (CpG ODN) – has been shown to stimulate a rapid and potent response of CC chemokine macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and RANTES, as well as of CXC chemokines MIP-2 and IP-10, in the vagina and the genital lymph nodes after a single intravaginal administration. In combination with glycoprotein D of herpes simplex virus type 2, CpG ODN intravaginal vaccination in mice has been seen to give rise to a strong antigen-specific Th1-like immune response and both systemic and mucosal IgG antibody responses, as well as protection against an otherwise lethal vaginal challenge with HSV-2 [115].

Systemic and mucosal immunity has also been established via the intranasal and oral routes when CpG ODN is used as an adjuvant [116,117] and high doses of CpG ODN have demonstrated non-toxicity [118]. After mucosal delivery, there is a Th2 immunostimulatory effect associated with the phosphorothioate ODN backbone, and the presence of CpG motifs shifts this towards a Th1 response [119]. The Corixa company's monophosphoryl lipid A, an adjuvant derived from Salmonella minnesota, was recently licensed in Europe as a component of an improved vaccine for hepatitis B (Fendrix®; GSK), and signals via the TLR-4/MD-2 complex. A series of synthetic TLR-4 agonists that are based upon the structure of the major hexa-acylated congener contained within monophosphoryl lipid A, termed the aminoalkyl glucosaminide phosphates, have been produced. Aminoalkyl glucosaminide phosphates stimulate the production of various cytokines by human peripheral blood mononuclear cells *in vitro* and upregulate cell surface markers on monocytes, natural killer cells and B cells [120]. The exploration of TLR signaling mechanism is a promising venue for boosting vaccine performance; however, efficacy, feasibility and safety will remain important subjects for future investigations.

7.3 Cytokine adjuvants

The cascade of an immune response is mediated by cell–cell contact and soluble cytokines. Immunity can be directly or indirectly modulated by introducing cytokines, chemokines or cellular receptor ligands in a vaccine formulation. In one study, a single intranasal administration of IFN-α-adjuvanted influenza vaccine resulted in the full protection of all mice against virus challenge, and vaccine alone was only partially effective (40%). Type I IFN was shown to induce an efficient and long-lasting local and systemic immune response, with a significant increase of antibody titers in all the Ig subclasses, with a particular effect on IgG2a and IgA [121]. Pneumococcal polysaccharide conjugate vaccine with IL-12 mucosal adjuvant has demononstrated enhanced levels of specific antibodies in wild-type (but not IFN-γ [-/-] knockout) mice, the result being the clearance of *S. pneumoniae* and an 89% survival after invasive pneumococcal infection, compared with 22% survival in unvaccinated controls [122]. When IL-12 complexed to liposomes was given orally, shifts to IgG2a and IgG3 and low IgE antibodies occurred concomitantly with enhanced serum IFN-γ. However, oral IL-12 did not result in significant levels of serum IL-12, nor did it alter S-IgA antibody responses, but resulted in higher levels of some Th2-type cytokines when compared with parenteral IL-12 [123]. Treating mice with Flt3 ligand (Flt3L), a dendritic cell growth factor, increased local and systemic responses in the presence of elevated numbers of intestinal dendritic cells with upregulated CD80 and CD86. The combination of Flt3L and IL-1α with oral Ag has been shown to produce a potent immune reaction, rather than a tolerogenic response [124]. granulocyte-macrophage colony-stimulating factor, cytotoxic T lymphocyte antigen-4 [125], MIP-1α [126], CD40L [77] and other cytokines, have been reported as potent molecular adjuvants for parenteral administration; nevertheless, with proper delivery protection, they might serve as mucosal adjuvants and modulators.

7.4 Plant-derived adjuvants

Many plant-derived lectins have demonstrated targeting and adjuvant properties, but their action has been unpredictable. Immunization with *Phaseolus vulgaris* phytohaemagglutinin plus OVA has been shown to elicit a lectin-specific response, but it did not stimulate an enhanced response to OVA compared with the antigen alone. Three mistletoe lectins (MLI – III) from European mistletoe (*Viscum album*) have demonstrated to be potent mucosal adjuvants selectively inducing Th2-type immune responses [127]. Mucosal delivery of tomato lectin has elicited a strong systemic and mucosal antibody response to lectin, but not to antigen; and the administration of wheatgerm agglutinin or *Ulex europaeus* lectin 1 with OVA has stimulated a high serum IgG response to antigen but not to lectins [128]. The detergent-like substance, saponin QS-21, originally from the bark of the South American soap tree, has been used in several vaccine candidates [129]. A *Quillaja saponaria* derivative, Quil A, has been preferred for other vaccine candidates, particularly for ISCOM complex preparations [130]. The Quillaja saponin derivative, GPI-0100, potentiated higher serum and mucosal antibody responses in

combination with LT in one study [131]. Gypsophila *sp*. saponin administered together with the antigen orally sensitized animals with high efficiency in another study [132].

Although ISCOMs have been used primarily for parenteral immunization, saponins from *Quillaja saponaria* and *Chenopodium quinoa* have been tested for mucosal applications [133,134]. The crude *Quillaja saponin* extracts function as potent mucosal adjuvants and are more effective than CTB/CT. Crude saponins are routinely used by the food and beverage industry at concentrations greater than those required for adjuvants, and, as such, they have a better safety profile than bacterial enterotoxins [135]. The screening of 267 different Chinese and Japanese medicinal herbs for mucosal adjuvant activity with influenza HA vaccine in mice has revealed that extract from the root of *Polygala tenuifolia* contains potent mucosal adjuvants, which have been purified and identified as onjisaponins A, E, F and G [136]. The apiogalacturonanic pectin of duckweed (*Lemna minor*), Lemnan LM, has stimulated increased levels of both serum IgG1 and IgG2a subclasses and intestinal IgA, and appeared to act via induction of both Th1- and Th2-type responses [137]. An aqueous extract from the fruit of *Solanum torvum* has been shown to significantly increase intestinal, fecal and pulmonary sIgA, but serum IgG titres does not change significantly [138]. Ricin B, the non-toxic galactose/*N*-acetylgalactosamine-binding subunit of ricin, induces higher levels of IgG1 than IgG2a, suggesting the presence of a Th2 response [139]. Soluble factors from birch (*Betula alba*) pollen have activated human dendritic cells, with an increase in Th2 cells and a reduced Th1 cell-polarizing capacity, and selectively inhibited IL-12 p70 production of lipopolysaccharide- or CD40L-activated dendritic cells, whereas IL-6, IL-10 and TNF-α remained unchanged [140]. The oral administration of the macromolecular components of a herbal immunomodulator isolated from an aqueous ethanolic extract of mixed *Thujae summitates*, *Baptisiae tinctoriae radix*, *Echinaceae purpureae radix* and *Echinaceae pallidae radix* has been shown to significantly enhance mucosal immune response [141]. Clear anti-inflammatory immunomodulation has also been demonstrated by the administration of *Ginkgo biloba* and *Momordica charantia* (bitter gourd) [142,143]. Furthermore, genetically engineered plants have been shown to express potent mucosal adjuvants of non-plant or other plant origin [144].

7.5 Synthetic adjuvants

Several synthetic compounds have been shown to possess mucosal adjuvanticity. Threonyl muramyl dipeptide and adamantylamide dipeptide has demonstrated high mucosal adjuvanticity in simian, murine and rabbit models. Capric acid has also enhanced antigen-specific serum IgG of a limited antigen dose [145]. A novel polycationic sphingolipid, *N*-palmitoyl D-erythro-sphingosyl carbamoyl-spermine (ceramide carbamoyl-spermine [CCS]), has been shown in mice to elicit strong local humoral and cellular responses, resulting in protective immunity; vaccine formulated with CCS was equivalent or superior to the commercial vaccine co-administered with cholera toxin as an adjuvant [146]. 1,25-Dihydroxyvitamin D3 co-administered with monovalent inactivated poliovirus vaccine significantly enhances systemic and mucosal immunity in mice [147]. Avridine, a synthetic lipoidal amine, incorporated in liposomes with viral antigen has enhanced the remote-site IgA antibody response in the respiratory tract without concomitant serum antibody response or side effects [148].

7.6 Epitope adjuvants

The new trend in vaccinology is the notion of short immunogenic peptides or epitopes used as adjuvants. Although B cell and cytotoxic T-cell epitopes need to be tailored for specific infectious agent, the so-called Th-cell epitopes could serve as an adjuvant in vaccine formulations regardless of the nature of the agent. Most of these epitopes are of microbial origin (also known as superantigens) or belong to the family of heat-shock proteins. Exogenous helper epitopes, such as amino acid residues 830 – 843 of the tetanus toxoid, 1 – 20 of tuberculosis bacteria heat-shock protein 65, 54 – 65 of rubella protein E2-4, and 35 – 48 of trachoma heat-shock protein 60, have been investigated in an hepatits B virus *S* gene DNA vaccine formulation and have shown potentiation of humoral immunity [149]. Another example of a helper epitope is MSP1 (DYDVVYLKPLAGMYK) of *Plasmodium vivax* [150]. These epitopes can now be synthesized by computing their affinity to specific MHC-II molecules. For example, the use of sequence-based immunoinformatics tools (BIMAS and SYFPEITHI) have revealed testis cancer NY-ESO-1-derived epitope 134 – 148, which induces specific CD4+ T cell responses restricted to HLA-DRB1 subtypes *0101, *0301, *0401 and *0701, with 40% prevalence in the Caucasian population [151]. Another DR-restricted Th cell epitope known as PADRE has shown its universal helper activity in many studies [152], with activity ~ 1000-times higher than average natural Th-cell epitopes [153]. The immunization of patients with recurrent or residual cervical carcinoma with HPV16 cytotoxic T-cell epitope and PADRE has revealed a strong helper response to PADRE even in the absence of a detectable cytotoxic response [154]. The mucosal adjuvanticity of PADRE has been demonstrated in HLA-A*0201 transgenic mice [155]. Parenteral immunization with the short ELDKWA peptide of HIV gp41, combined with PADRE, has produced a strong mucosal immune response in one study [156]. Similar results were obtained with the PADRE-ASREAK sequence of streptococcal M protein [157]. Despite the fact that PADRE was engineered for human DR, the authors of the present review have previously been able to elicit a good response with SARSCoV B cell epitope and PADRE-encoding DNA in C57BL, but not in BALB/c mice (data not published), although others have shown PADRE activity in BALB/c mice [158]. The oral delivery of cytotoxic epitopes and PADRE-encoded DNA has shown partial protection against gastric cancer [159]. The high potency of PADRE has also been demonstrated in autoimmune induction against self IL-12/23 p40 in a model of experimental IL-23-dependent encephalomyelitis [160]. The control of immunity on a

molecular level by epitopes, which now can be predicted with better certainty, is certainly worthy of pursuit, provided that delivery means will be in place to guarantee their protection.

8. Edible vaccines

Research into this type of vaccine is the fastest growing new approach to oral delivery. Notwithstanding the experience of the Bedouins or Chinese, modern vaccinology had to finally realize that 'we are what we eat'. This may appear as a simple concept, but the modern motion of edible vaccines did not appear until pioneering work of Charles J. Arntzen, who purposefully expressed vaccine antigens in transgenic plants.

Since then, various plants have been shown to be capable of expressing immunogenic proteins (e.g., soybean [161], potato [162] and tobacco [163,164]). Plant-made measles vaccine has demonstrated an antimeasles immune response [135], particularly, when used as an oral boost after DNA vaccine priming [165]. Positive results have been demonstrated with *Arabidopsis thaliana* expressing *Mycobacterium tuberculosis* ESAT-6 antigen [166]. Corn expressing swine transmissible gastroenteritis virus antigen has induced a Th1 immune response [167] bivalent, plant-based oral vaccine against hepatitis B and HIV has been developed, where the immunogenic ENV and GAG epitopes of HIV-1 and the surface antigen (HBsAg) of hepatitis B virus was expressed in tomato plants [168].

Not only plants are suitable for edible vaccines. Yeasts expressing human papilloma virus antigen has induced a vaginal IgA immune response after oral administration [169]. Other yeasts have been used for the oral vaccination of crayfish against white spot syndrome virus [170], and for the oral delivery of the *380R* gene from the red sea bream iridovirus, which is one of the most common viral diseases in cultured marine fish in Japan [171].

In addition, higher forms of carrier organisms are being studied. For example, the silk worm has been used for the oral delivery of an insulin subunit to prevent diabetes in a mouse model [172].

So far, the stability and immunogenicity of orally delivered antigens from plants appears to vary greatly, often producing disappointing results. Further studies on protein engineering to enhance mucosal delivery are needed. The issue of how to deliver an *a priori* defined vaccine dose is another concern, especially from the regulatory point of view. Furthermore, ecological and human risks from field-grown genetically modified plants expressing vaccine components are not fully known. These concerns undermine the interest from vaccine industry, and unless the financial incentive is found, the progress in edible vaccines will be confined to research laboratories.

9. The Yin and Yang of oral vaccination – immunity/tolerance balance

In addition to the problem of the survival of an antigen in the gut, another major hurdle is the phenomenon of oral tolerance that usually occurs when antigen is given orally. The early indications that oral antigen exposure can lead to immune tolerance and suppression of the systemic immune response to subsequent antigenic challenge can be found in seminal works published in late nineteenth and early twentieth centuries by Milton J. Rosenau and John F. Anderson (credited for the foundation of what is now known as the NIH and FDA), and Alexandre Besredka, the successor of Ilya (Elie) Metchnikoff, at the Institute Pasteur. This phenomenon was re-discovered in 1960s when immunology methods became more sophisticated [173,174]. Later studies showed that tolerance does not occur every time the antigen is fed, but that it depends on a multitude of factors, such as age, MHC restriction, delivery site, nature, size and dose of the antigen, antigen uptake and processing and frequency of administration. Still, most immunologists are convinced that antigen feeding will inevitably cause immune anergy. Commonly observed mucosal tolerance to frequently encountered food antigens and nonpathogenic bacterial and viral components supports this notion, and perhaps this is the main reason why this has been accepted without challenge. The persistence of this misconception is illogical, especially when it is considered that highly effective oral vaccines are available. However, even today the mechanism of tolerance has not been fully elucidated, but understanding the principles of mucosal or immune tolerance is critical for the design of effective oral vaccines.

Tolerance is mediated by several immunological mechanisms, including, but not excluding, the induction of regulatory T cells (suppressors) that can downregulate specific immune responses via the production of specific cytokines (e.g., TGF-β, IL-10, IL-4), functional or clonal deletion of effector cells, and antibody-mediated suppression. In most cases, the active immune response is desirable as a result of vaccination. Thus, oral vaccination requires special means to overcome tolerogenic predisposition. Many approaches are being tested to control the tolerance.

It has been reported that large doses or the prolonged consumption of antigens provided or expressed in food are necessary to overcome tolerance [175]. However, frequent exposure to an antigen can push the immune system toward tolerance. Feeding increasing doses of vaccine during each consecutive administration can reduce the blocking effect of antigen-directed IgA [176]. Designing a specially tailored schedule for vaccine administration can help to overcome tolerance. Primary vaccination usually elicits local IgA, which in turn can block the action of an antigen in the boost dose. Immunosupressors given before the boost could diminish the levels of IgA and suppress regulatory cells [176].

The proper stimulation of the local immune response could be achieved by providing mucosal adjuvants, cytokines or interferonogenes, for example. Specific targeting to M-cells versus enterocytes [177] or to a particular subtype of dendritic cells [178] can also abrogate immune tolerance. Dendritic cells could be converted from tolerogenic into immunogenic APCs

Table 3. Antigens employed for the development of oral vaccines.

Acting component	Immunogenicity	Safety	Stability	Cost of manufacturing	Cost of usage
Live virus	High	Low	Low	Moderate	High
Live bacteria	High	Low	Low	Moderate	High
Killed virus	Moderate	High	High	Low	Low
Killed bacteria	Moderate	High	Moderate	Low	Low
Subunit antigen	Moderate	High	Moderate	Moderate	Moderate
B and T epitopes	Moderate	High	Moderate	High	High
DNA	Various	High	Moderate	High	High
RNA	Various	High	Low	High	High

when Flt3 ligand or a combination of Flt3L and IL-1α is given, followed by an antigen stimulus [124].

In some situations, oral vaccination benefits from the phenomenon of tolerance, particularly in diseases characterized by an overdriven immune response (e.g., type I allergies mediated by IgE [179]). Some viral infections, (e.g., severe acute respiratory syndrome and avian flu) provoke an inflammatory immune reaction and, thus, immune suppressors are required as part of the therapy. Moreover, in some situations, viral infections (e.g., feline infectious peritonitis, porcine respiratory reproductive syndrome virus, dengue virus) demonstrate enhanced symptoms of disease among systemically immunized subjects compared with those that are unvaccinated [180,181]. Theoretically, prophylaxis of those infections could benefit from oral tolerance induction. This has been demonstrated in studies of oral vaccination against HIV [182,183], with remarkable therapeutic results and potential prophylactic benefit.

10. Conclusion

There is no doubt, in the opinion of the present authors, that the oral delivery route offers significant advantages over systemic delivery. Oral vaccination is certainly simpler and cheaper than parenteral immunization, and improved safety can be achieved if a vaccine does not contain attenuated pathogens (**Table 3**). In addition, unlike systemic immunization, an oral vaccine can produce a local immune response, which is desirable with most infectious agents. However, it is difficult to make an effective and safe oral vaccine because of the numerous hurdles presented by the gastrointestinal tract. To reliably immunize with peptide or protein vaccines, antigens must be protected, uptake enhanced and the immune tolerance properly controlled. Numerous approaches have been and are evaluated for optimal oral delivery. Except for a few examples, the results have been disappointing. As a rule, vaccines that have worked perfectly in mice have failed to perform in higher animals and/or humans. Most commercially available oral vaccines originate from the period that predates the present boom in oral delivery. Nevertheless, the aggressive research that characterizes the oral vaccination field offers a hope for the future development of safe and effective oral vaccines.

11. Expert opinion

As the building blocks for successful oral immunization are being laid, we can expect significant breakthroughs in the next 5 years, when diverse concepts will start emerging from human and animal trials. It is likely that oral vaccines for veterinary application will be available sooner due to lower regulatory barriers. It is possible that oral and edible vaccine delivery will have greater applicability in developing nations because needle-free injectors, skin patches and nasal delivery costs significantly more and may require special skills that need to be taught in a resource-poor settings.

Edible vaccines are certainly attractive as a concept, but the fact that they are genetically modified plants, and that uniformity in dosing has not been solved, makes them less appealing than traditional oral approaches. Despite the public's positive opinion towards edible vaccines, no commercial product has yet emerged, and it is unlikely that this type of vaccines will be available soon unless radical regulatory policy changes are made. Perhaps the effort directed towards developing these vaccines will be more productive if their animal rather than human use is emphasized as the priority.

Most of the basic work is being conducted at academic institutions; some of this is now in a transition process into small start-up companies, which seldom have product development experience. As major vaccine companies will gradually shift their focus toward needle-free delivery, we may see a change in their attitude towards oral and edible vaccines. If this happens, then oral vaccines will occupy the center stage permanently. Despite the increasingly respectable activity in this field, so far all efforts are merely a lineup of fringe events.

To learn how to make an oral vaccine one could certainly benefit from the hindsight offered by existing oral vaccines, so that the development time for a new generation of vaccines is shortened. Consequently, prior to the start of oral vaccine

development, the investigators should carefully consider prior experience and make a decision as to which delivery technology is likely to have the biggest impact.

Most oral vaccines available today are either attenuated or killed microorganisms that can survive intestinal degradation either by replicating in the gut or by virtue of having digestion-resistant bacterial walls. Attenuated or live vaccines will always be associated with safety concerns. For example, the problems with oral polio or rotavirus vaccines have been described in this review. Killed vaccines do not generally have problems with safety, but their weak point is their efficacy. Nevertheless, multiple bacterial and one viral vaccine are on the market, although they are approved as immunomodulating supplements rather than *bona fide* vaccines. These vaccines should not be dismissed solely because they are not in the proper FDA category. All of them have shown an excellent safety profile and several of them have already been through extended placebo-controlled, randomized trials. Thousands if not millions of people have used them without knowing that, theoretically, these products were not supposed to work.

The ancient Bedouins and Chinese were not burdened with the knowledge of today's science – they did what they knew was working. Nowadays the empirical approach is increasingly out of favor, but 'rational' science has failed to produce effective oral vaccines. Except perhaps cholera vaccine, all other vaccines are in a developmental stage. We believe that the answer to the problem is simple but hidden in plain view. Our biggest drawback is our sophistication. We need to learn to see the forest before the trees.

Presently, there are several new vaccines in development to be delivered orally, but extensive human studies are needed to prove that they are safe and effective. If we finally learn how to effectively orally deliver vaccines, this will transform radically not only the vaccine industry, but also, in an unprecedented manner, global health in general.

Acknowledgments

Many promising oral vaccines are being investigated by our colleagues; however, due to the limitations of this review, we apologize for being unable to cite them all. We thank Professor Tadjbakhsh of Tehran University, Iran, in providing historical reference to the earliest record of oral vaccination. We would appreciate it very much if the readers can provide similar insight and feedback on this exciting topic.

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