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Delivery systems and adjuvants for oral vaccines

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The oral route is the ideal means of delivering prophylactic and therapeutic vaccines, offering significant advantages over systemic delivery. Most notably, oral delivery is associated with simple administration and improved safety. In addition, unlike systemic immunisation, oral delivery can induce mucosal immune responses. However, the oral route of vaccine delivery is the most difficult because of the numerous barriers posed by the gastrointestinal tract. To facilitate effective immunisation with peptide and protein vaccines, antigens must be protected, uptake enhanced and the innate immune response activated. Numerous delivery systems and adjuvants have been evaluated for oral vaccine delivery, including live vectors, inert particles and bacterial toxins. Although developments in oral vaccines have been disappointing so far, in terms of the generation of products, the availability of a range of novel delivery systems offers much greater hope for the future development of improved oral vaccines.

Keywords: adjuvant, delivery system, microparticle, oral vaccine

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1. Introduction: oral vaccines

Vaccine research and development is expanding as vaccines are coming to be regarded as the most effective means of disease control, and global immunisation is a public health priority [1]. As a result of effective immunisation, millions of lives have been saved and countless individuals have been spared the morbidity resulting from infectious diseases. Nevertheless, despite these achievements, many infectious diseases remain endemic over large areas of the world. Furthermore, in addition to the long-term problems posed by established pathogens (e.g., *Vibrio cholerae*, rotavirus, measles), recent outbreaks of diseases caused by agents such as HIV, avian influenza, Ebola and Nipah viruses show that the threat from infectious diseases remains, and this is difficult to predict [2]. As a consequence of the significant disease and mortality that is associated with uncontrolled pathogens, economic progress and development has been seriously restricted in many countries. However, the development of effective vaccines for many pathogens remains a challenging undertaking, particularly when the type of immune response required for protection is unknown (e.g., in the case of HIV). In addition, although challenging, the identification of protective antigens only constitutes the first step in the production of an effective vaccine.

In the recent past, due to significant reactogenicity problems with traditional vaccines (killed or live attenuated organisms and chemically modified toxins), there has been a move away from this approach towards a new generation of vaccines composed of purified subunits. The rationale is that the vaccine comprises only those factors against which a protective immune response should be elicited, and other factors responsible for side effects are omitted. In many cases, the subunits are proteins or peptide antigens. However, the immunogenicity of these vaccines is considerably lower than that of traditional vaccines. This is a result of the presence in traditional vaccines of immunostimulatory factors, such as lipopolysaccharide, peptidoglycan and nucleic acids (e.g., unmethylated CpG oligodeoxynucleotide [CpG] motifs, dsRNA), or to the vaccines being live and/or inducing inflammatory responses by causing local

Box 1. Factors that influence particle uptake across the gastrointestinal tract epithelium.

- Particle size
- Polymer composition
- Particle hydrophobicity
- Particle surface charge
- Dose of particles
- Administration vehicle
- Means of delivery
- Animal species and age
- Fed state of the animals
- Penetration enhancers
- Use of targeting agents

cell or tissue damage. The absence of these immunostimulatory components in subunit vaccines has created a demand for factors that safely enhance immune responses.

Most infectious diseases are caused by pathogens that initially colonise and invade the host at mucosal surfaces. In the case of diarrhoeal diseases and a large number of other bacterial, viral and parasitic diseases, the site of infection is the gastrointestinal tract (GIT). The most effective means of protection against a disease initiated at a mucosal surface is the induction of a specific immune response at that site [3]. This can only be achieved by local delivery of the vaccine, as injection of vaccines does not generally stimulate mucosal immunity. To optimally stimulate an immune response in the gut, the vaccine should be administered to the GIT, and this is most conveniently achieved via the oral route. A live attenuated oral polio vaccine has been successfully and safely used to immunise millions of children, which demonstrates the feasibility of oral vaccination [4].

The oral route would avoid the pain and discomfort that is associated with injections and would eliminate infections caused by inadequately sterilised needles, or needle reuse, which is responsible for the transmission of many infectious diseases. Oral vaccines would also be cheaper to administer, as trained personnel would not be required for immunisation, and vaccine production would be less expensive, as a result of less stringent manufacturing conditions for an oral product. However, in contrast to injections, orally delivered vaccines must cross epithelial cells before an immune response can be elicited. Generally, the efficiency of vaccine uptake across the GIT is poor, mainly due to degradation by proteolytic enzymes and the low efficiency of transport across epithelial cells [5]. Therefore, oral immunisation is relatively ineffective, and delivery systems and adjuvants are required to enhance the immune response. Two approaches that are showing promise are the entrapment of vaccine antigens in delivery systems that can protect them against degradation, and can promote their uptake and the targeting of vaccines to particular regions or cells in the GIT. However, the protection of antigens from degradation and enhancement of their uptake across the epithelium is only the first step. Immunostimulatory factors must be incorporated to activate innate, and subsequently adaptive, immunity in the intestine.

2. Uptake of particulates across the gastrointestinal tract epithelium

Although there is some evidence that mucosal delivery systems such as liposomes [6] and immune-stimulating complexes (ISCOMs) [7] are taken up across the gut, the majority of work in this field has focused on the use of polymeric microparticles [8]. A number of routes of particle uptake across the GIT have been described in the literature, including the villus tips, intestinal macrophages, villus enterocytes and the Peyer's patch (PP) epithelium. Evidence suggests that the PP mucosa-associated lymphoid tissue is the predominant site of uptake for nano- and microparticles. The antigen-sampling microfold/microvillus (M) cells overlying PPs can transport material from the lumen to underlying lymphoid cells [9]. The fate of particles following uptake is influenced by particle size and hydrophobicity, the dose, the delivery vehicle in which particles are suspended, the animal species and age (reviewed in [8,10]; **Box 1**).

3. Immune responses to orally delivered vaccines

The gut-associated lymphoid tissue (GALT) is comprised of individual cells and structures in the intestinal epithelium, PPs, lamina propria and mesenteric lymph nodes [11]. Within this network are inductive sites where antigens are encountered and responses are initiated, and effector sites where local immune responses occur (e.g., the lamina propria regions of the GIT). The inductive and effector sites are linked by a homing system, whereby cells induced by antigen in the GALT migrate via the lymphatics and thoracic duct to the circulation and, subsequently, seed the mucosae (**Figure 1**). As a result, oral vaccination can induce immune responses locally in the gut and at distant mucosal sites, but can also elicit humoral and cellular systemic immune responses. Secretory IgA plays a major role in mucosal defence [12], and the induction of vaccine-specific secretory IgA in the gut may prevent infection.

Dendritic cells (DCs) are found throughout the GIT and are critically important in inducing primary immune responses. DCs have been shown to take up *Escherichia coli* and transport them to mesenteric lymph nodes by a process that is dependent on the chemokine receptor CX3CR1 [13], suggesting a role for these cells in the uptake of killed bacterial vaccines or, indeed, inert delivery systems. In addition to the possibility of targeting M cells for vaccine delivery, it was suggested in 2005 that if specific markers were identified, CX3CR1-positive lamina propria DC could be targeted [13]. It is likely that oral vaccine delivery systems must interact with and activate dendritic cells in the GALT to be effective.

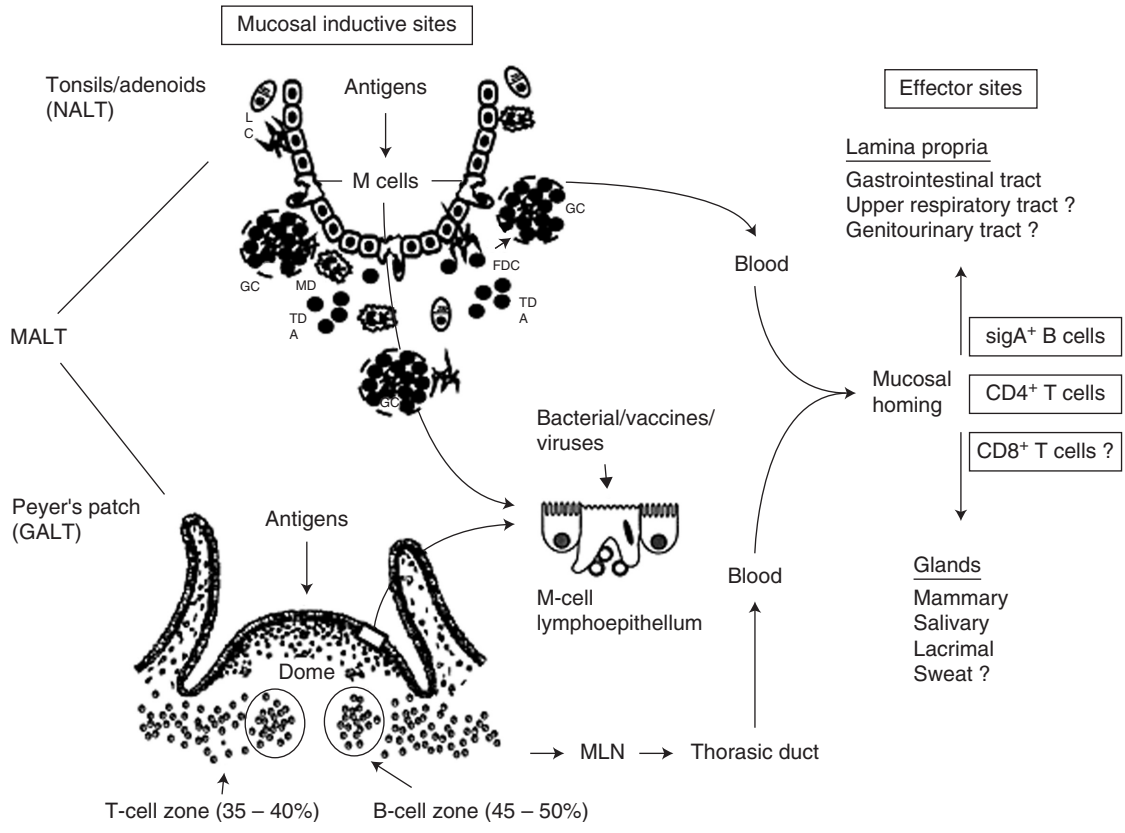

Figure 1. M cells are located in Peyer's patches in the gastrointestinal tract [118]**.** These cells are thought to play an important role in antigen uptake and processing, and possibly in the induction of antigen-specific mucosal immunity in mucosal effector sites. Sites followed by question marks are presumed sites, as data on them are limited.

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FDC: Follicular dendritic cell; GALT: Gut-associated lymphoid tissue; GC: Germinal centre; MALT: Mucosa-associated lymphoid tissue; MO: Macrophage; MLN: Mesentric lymph nodes; NALT: Nasopharynx-associated lymphoid tissue; TD A: T-dependent area.

DCs are also important in the induction of oral tolerance to orally administered antigens, which may have an application in therapies for autoimmune conditions [14].

4. Peyer's patches and microfold/microvillus cells

The PP lumenal epithelium contains enterocytes, goblet cells and M cells. There are fewer mucus-secreting goblet cells present at this site than on the villus epithelium [15], and the M cell glycocalyx is relatively sparse [16]. Combined, these factors may allow greater contact between oral vaccines/delivery systems and the PP follicle-associated epithelium than with the villus epithelium. Furthermore, M cells contain small cytoplasmic vesicles and few lysosomes [17]; the apical membrane expresses reduced levels of hydrolase activity than enterocytes [18] and proteolytic activity may be lower in intestinal PPs than in patch-free zones [19]. Uptake of delivery systems via M cells into the PP is likely to preserve antigenic and immunomodulator integrity due to release into the M-cell pocket [20] and antigens are, therefore, unlikely to enter phagolysosomes [15]. The pocket is an invagination of the M-cell basolateral membrane containing lymphocytes and other lymphoid cells. This capacity of M cells to endocytose and transport protein antigens and inert particles from the lumen into the pocket [21], where macrophages, DC and lymphocytes are located, provides an opportunity for mucosal vaccine delivery.

In addition to their presence in the small intestine, lymphoid follicles have also been described in the caecum [22] and the distal colonic and rectal mucosa of humans [23,24]. There are differences in M-cell surface characteristics at these GIT sites, so there may be potential to specifically target particular gut regions with appropriate ligands. The surface properties of enterocytes can also vary in different gut regions (e.g., surface properties of rectal and colonic epithelial cells differ from small intestinal cells). Rectal vaccine delivery may avoid the low pH and the highly proteolytic conditions in the upper digestive tract, and encouraging results were reported after rectal delivery of antigen associated with lipsosomes [25] and cholera toxin [26].

5. Oral vaccine delivery systems

A wide range of systems has been investigated to enhance oral vaccine delivery, most of which are particulate in nature. The greater efficacy of particulates may relate both to their inherent immune-stimulatory properties, and to their ability to target the PP follicle-associated epithelium and protect entrapped antigens against degradation. Particles are efficiently taken up and processed by antigen-presenting cells, including DCs. In addition to the many particulate delivery systems that have been investigated, a number of bacterial- and plant-derived molecules have also shown significant potential as adjuvants and delivery systems for oral vaccines.

5.1 Live oral vaccine carriers

Many of the parenteral vaccines that are used at present, including measles [27] and Bacille Calmette-Guerin [28], are live attenuated organisms. In addition, the Sabin live attenuated oral polio vaccine is highly effective [4]. Live attenuated oral vaccines have also been licensed against *Vibrio cholerae* and *Salmonella typhi,* and *Salmonella* and *Shigella* spp. are candidate vectors for oral vaccine delivery [29]. However, at present, many important pathogens cannot be successfully attenuated to facilitate the development of live mucosally administered vaccines. Moreover, many organisms are difficult or impossible to grow in culture and some cannot be easily manipulated using existing techniques. In addition, the efficacy of the available live vectors for oral vaccine delivery (e.g., *Salmonella* spp. or polio viruses) in expressing antigens from other pathogens is variable. Consequently, there is interest in the development of novel delivery systems for oral vaccine administration, which can be used to package and deliver antigens. For safety reasons, it would be desirable if these systems were based on non-living carrier systems, rather than modified bacterial or viral vectors. Nonetheless, a number of live attenuated vaccines are under investigation for enteric infections [201].

5.2 Non-living oral vaccine delivery systems

5.2.1 Microparticles

Microparticles have been widely used to deliver vaccines and can induce potent immune responses and protective immunity when administered by injection or mucosally [30]. Microparticles can be prepared from a range of different polymers and can be designed to protect entrapped vaccines against degradation in the gut, to delay gastric transit and/or to target vaccines for uptake into the PP. However, the low efficiency of microparticle uptake across the gut is a major limiting factor. It was proposed that the effectiveness of orally delivered particulate antigens resulted mainly from their greater uptake into intestinal PPs; however, there is increasing evidence, particularly in the case of nanoparticles, that the villus enterocytes also play a significant role in uptake [10].

5.2.2 Poly(lactide-co-glycolide) microparticles

Most of the work on the use of polymers in the formulation of microencapsulated oral vaccines has focused on the aliphatic polyesters: the poly(lactide-co-glycolides [PLGs]). PLGs are the primary candidates for the development of microencapsulated vaccines because they are biodegradable and biocompatible, and have been used in humans for many years as suture material and as controlled-release drug delivery systems [31,32]. PLG polymers have also been extensively evaluated for the development of controlled-release single-dose vaccines [33]. One of the limitations of PLG in relation to vaccine development is that these polymers are only soluble in a limited range of organic solvents, and are insoluble in water. The techniques that are most commonly used to formulate microencapsulated vaccines involve the emulsification of aqueous antigen solutions into organic solvents containing the polymer, and extraction or evaporation of the solvent to form microparticles. A significant problem with PLG microencapsulation of vaccines is the possibility of antigen denaturation on exposure to organic solvents. In addition, during microencapsulation, vaccine antigens may be exposed to high shear stress, aqueous/organic interfaces and elevated temperatures. Nevertheless, a number of proteins have been successfully entrapped in PLG microparticles with little or no deleterious effect on structural and immunological integrity [30].

5.2.3 Immune responses to orally delivered microencapsulated antigens

Oral immunisation of mice with staphylococcal enterotoxin B [34] or ovalbumin (**Figure 2**) [35,36] entrapped in PLG microparticles induced enhanced serum and mucosal antibody responses compared with delivery of the antigens alone. Disseminated secretory IgA responses were induced at mucosal sites, including the gut and the lower genital tract, following immunisation with microencapsulated ovalbumin (OVA) [36]. Microparticles with entrapped OVA have also been shown to induce a systemic cytotoxic T lymphocyte (CTL) response in mice following oral immunisation [37]. Particle size had a significant effect on the immunogenicity of OVA microencapsulated in PLG microparticles. One study indicated that 4-µm microparticles were more effective for the induction of serum antibody responses, but 7-µm particles were superior for mucosal IgA responses [38].

Importantly, a number of studies have demonstrated that oral vaccination with antigens in PLG microparticles can induce protective immune responses. Oral immunisation with microparticle-encapsulated fimbriae from *Bordetella pertussis* protected mice from intranasal challenge [39]. Oral delivery of a PLG-encapsulated vaccine comprising phosphorylcholine conjugated to a protein carrier induced protective immunity against a normally lethal oral challenge with *Salmonella typhimurium* and the level of protection was greater than that induced by intraperitoneal immunisation with Freund's

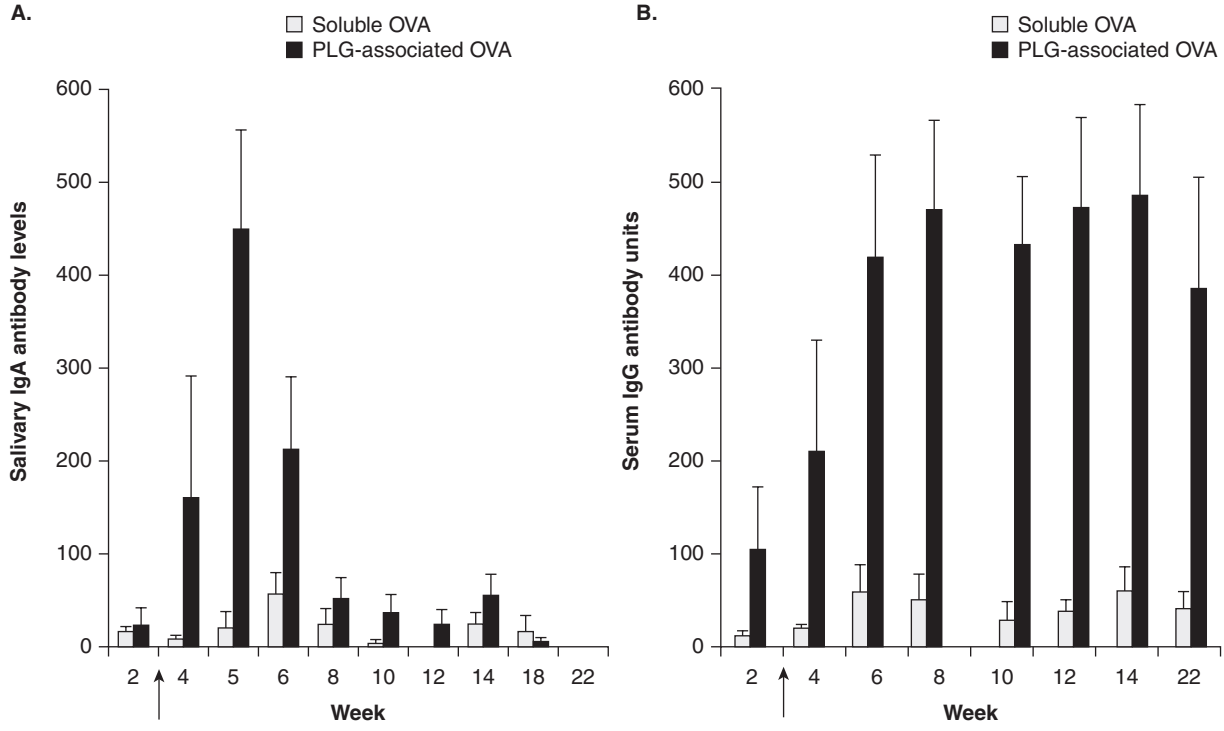

Figure 2. Serum IgG and salivary IgA antibody responses in mice that were orally immunised with PLG/OVA (PLG lactide:glycolide ratio 75:25, poly[vinyl alcohol]-stabilised) or soluble OVA (1 mg). Mice were immunised on days 0, 1 and 2, and boosted on days 21, 22 and 23 (arrow). Serum and saliva samples were taken for analysis, and antibody units were calculated relative to a positive control hyperimmune serum [36]. Arrows refer to the time of booster immunisations. Error bars represent the s.e.m. **A.** Serum IgG. **B.** Salivary IgA.
OVA: Ovalbumin; PLG: Poly(lactide-co-glycolide).

complete adjuvant [40]. An orally administered microencapsulated formalin-treated influenza virus vaccine induced protective immunity in systemically primed mice [41]. Oral immunisation with microparticles containing entrapped simian immunodeficiency virus induced protective immunity in systemically primed macaques against repeated intravaginal challenge with simian immunodeficiency virus [42]. The oral route appeared to be more effective than the intratracheal route, although only a limited numbers of animals were evaluated. When rhesus monkeys were immunised by the intratracheal or oral routes with staphylococcal enterotoxin B, only a limited number of animals were protected against aerosol challenge. A systemic prime was needed to induce optimal protection [43].

As a result of the problems associated with microencapsulation of vaccine antigens, a novel approach of adsorbing antigens onto the surface of PLG microparticles was developed [44]. These microparticles were designed to promote the uptake of adsorbed antigen into APC, and resulted in the induction of potent antibody and T-cell responses against recombinant HIV antigens in mice and in non-human primates [44]. However, as the antigen is exposed on the particle surface this approach is alone unlikely be effective in oral vaccine delivery.

A strategy specifically designed to enhance the efficacy of orally delivered PLG microparticles was the use of enteric coating polymers to stabilise PLG microparticles [45]. OVA encapsulated in these microparticles was protected from proteolytic degradation in simulated gastric and intestinal conditions to a greater degree than in the poly(vinyl alcohol)-stabilised formulation (**Table 1**). Following oral immunisation, an enhanced specific salivary IgA response was induced when OVA was delivered in the enteric polymer-stabilised particles [45]. These and previous data indicated that antigens associated with poly(vinyl alcohol)-stabilised PLG microparticles may be susceptible to protease degradation in the GIT [46]. Nevertheless, all approaches involving encapsulation of antigens into microparticles are likely to suffer from the drawback of low levels of particle uptake across the gut. Whether or not the extent of particle uptake in humans is sufficient to allow the development of an effective oral vaccine is unknown at present. However, if enhancement of particle uptake is required to allow the development of microparticulate oral vaccines, data in rodents suggest that targeting ligands may be effective. In summary, PLG microparticles have shown promise for oral vaccination but are unlikely to be sufficiently potent alone to induce protective immunity. However, their excellent safety profile,

Table 1. Protection of microencapsulated OVA from pepsin- and trypsin-mediated degradation by using enteric polymers as microparticle stabilisers.

Stabiliser (%)	% OVA removed	
	Pepsin (pH 1.2)	Trypsin (pH 7.4)
Polyvinyl alcohol 10%	44.68	47.98
Eudragit®*		
2.5%	35.14	54.39
4%	19.63	38.26
6%	51.35	88.89
Carboxymethyl ethylcellulose		
4%	4.05	28.06
6%	4.39	46.56
8%	13.1	50.81

OVA was microencapsulated in poly(lactide-co-glycolide) microparticles (lactide glycolide ratio 50:50) using an emulsification/solvent evaporation process. Polyvinyl alcohol, Eudragit and carboxymethyl ethylcellulose were used as stabilisers. Microparticles were incubated in simulated gastric or intestinal media for 1 h and OVA was extracted and run on SDS-PAGE gels. Using densitometry, the amount of residual intact OVA was calculated as a percentage of the original amount of OVA in the particles.

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*Röhm & Haas GmbH.

OVA: Ovalbumin; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

ease of formulation and potential for inclusion of additional adjuvants and targeting moieties support the continued evaluation of this system in oral vaccine development.

5.2.4 *Alternative polymeric delivery systems*

Similar approaches to those described for PLG have been applied to microencapsulate antigens into a range of alternative polymers, as PLG is regarded as suboptimal for some antigens due to the use of organic solvents during microparticle formulation.

Live rotavirus was encapsulated in microspheres prepared from anionic polymers and amines, including sodium alginate and spermine hydrochloride [47]. The microspheres were stable in simulated gastric acid, taken up into the GALT and induced enhanced immune responses in comparison to oral delivery of non-encapsulated live or inactivated rotavirus [48]. This process was subsequently used to encapsulate live reovirus for oral administration to suckling mice [49] and induced an immune response, even in the presence of pre-existing maternal antibodies. Oral administration of human serum albumin in starch microparticles induced relatively strong systemic and mucosal immunity [50]. Microparticles formulated using poly-ε-caprolactone have also shown potential as oral delivery systems [51]. Oral administration of antigens encapsulated in microparticles produced using the naturally occurring polysaccharide alginate were shown to induce protective immunity against *Pasturella multocida* and *Streptococcus pneumoniae* [52,53].

Mice were immunised intra-duodenally with mucoadhesive polymer-dispersed microspheres, which facilitated the controlled release of OVA and elicited specific mucosal and serum antibody response [54]. Microspheres prepared from polyanhydride co-polymers of fumaric and sebacic acid displayed strong biological adhesion. These systems increased gastric residence time compared with microspheres that were composed of other polymers. The microspheres were transported across the mucosal absorptive epithelium and the PP follicle-associated epithelium by transcellular and paracellular routes [55]. The advantage of the polyacrylonitrile polymers over alternatives is not clear, although polyacrylonitrile does appear to exhibit strong bioadhesive properties and may be taken up across the intestine to a greater extent than PLG. However, despite claims to the contrary, it is not yet proven that this formulation has any advantages over PLG polymers, and encapsulation generally involves exposure of the entrapped antigens to organic solvents and oil/water interfaces.

5.2.5 *Enteric-coating of vaccines*

An alternative approach to the oral delivery of vaccines, that is not dependent on uptake of particles across the gut, involves the encapsulation of antigens with enteric-coating polymers. The objective is to protect the antigen against gastric degradation and to release it in the intestine. Oral immunisation of rats with *E. coli* heat-labile enterotoxin B subunit (LTB), encapsulated in an enteric-coated formulation, induced an enhanced response compared with delivery of LTB alone [56]. However, for non-living vaccines, simply protecting the antigens against exposure to low pH and proteases in the stomach is unlikely to be sufficient to allow the successful development of oral vaccines. Additional approaches are likely to be necessary to induce potent immune responses to subunit vaccines encapsulated in enteric-coating polymers.

To prevent antigen denaturation on exposure to organic solvents, an alternative process for enteric coating, involving a water-soluble polymer was used [57]. The polymethacrylic co-polymer (Eudragit® L30D; Röhm Pharma) was sprayed onto antigen-coated non-pareil sugar seeds and, following oral immunisation of mice, a specific serum antibody response was induced, in addition to antibody-secreting cells in the gut [57]. In another example of oral immunisation with an enteric coated system, OVA was incorporated in a pH-sensitive microbead with an acrylic-based coating [58]. A comparison of orally administered enterocoated microbead-OVA particles with a formulation of OVA in adjuvant (DETOX-PC), given subcutaneously, showed that the systems elicited comparable antigen-specific lymphoproliferative and CTL responses. Oral immunisation of mice with pneumococcal polysaccharide (type 23F), conjugated to the outer membrane protein complex of *Neisseria meningitidis* (23F-OMPC) that was entrapped within microcapsules,

induced polysaccharide-specific serum antibodies, but delivery of the polysaccharide alone did not [59]. This showed that the conversion from a T-cell independent to a T-cell dependent response, achieved through conjugation was maintained following oral delivery. Although the protection of acid- and protease-labile antigens from gastrointestinal proteolysis is an essential prerequisite of an effective oral vaccine delivery system, this is only one of a number of requirements that include targeting to epithelial cells and activation of innate immunity. Thus, enteric coating alone will not generate an effective oral vaccine delivery system, but may increase the potency of other systems.

5.2.6 *Bioadhesive delivery systems*

Bioadhesion refers to the attachment of a carrier to a specific biological location. Ideally, bioadhesives for oral vaccine delivery should be non-toxic and non-immunogenic, stable under GIT conditions and bind specifically to surface structures of epithelial cells, but not to mucus. To achieve specific bioadhesion between vaccines and the GIT epithelium, targeting molecules may be used. Targeting strategies may be designed to direct vaccines to a specific tissue, cell type or subcellular compartment [60]. Small intestinal transit in humans is usually 3 – 4 h, which is generally too short to allow complete absorption of vaccines from the GIT [61]. Bioadhesive systems may be designed to increase the time available for vaccine interaction with the GIT epithelium. In addition to the small intestine, vaccines may also be taken up from other parts of the digestive tract, particularly the colon. Bioadhesives may, therefore, be designed to interact specifically with particular regions of the GIT.

Enhanced bioadhesion of oral vaccines to the GIT may be achieved using non-specific systems relying on physicochemical interactions or specific systems, in which ligands bind specifically to receptors on the epithelial cell. Non-specific bioadhesive delivery systems based on polymers and microspheres have been widely investigated for drug delivery. However, absorption of water by these systems at mucosal sites resulted in a loss of bioadhesiveness. Certain bioadhesive systems may increase polypeptide or protein uptake across epithelia by passive paracellular diffusion after disrupting intercellular junctions. Hydrophilic polymers and hydrogels can be effective biological adhesives [62]. Bioadhesive particulate systems can additionally confer enhanced immunogenicity on associated antigens. Polymers (polycarbophil) with thiol groups were shown to possess strong adhesive properties [63]. Chitosan is a mucoadhesive polysaccharide of marine origin and is attractive for oral vaccine delivery, as it is regarded as safe and has been shown to promote transmucosal absorption [64]. It was reported that chitosan microparticles were taken up across murine PPs [65]. Oral immunisation with diphtheria toxoid adsorbed on chitosan microparticles induced enhanced systemic and mucosal antibody responses compared with delivery of antigen alone [66]. However, these responses were induced following the administration of six doses of this formulation. The efficacy of chitosan-associated vaccines may be enhanced if the exposed vaccine can be protected from proteolytic degradation. Antigen loaded in chitosan microparticles that were encapsulated in liposomes and niosomes was more immunogenic following oral delivery than antigen in unprotected microparticles [67]. The coating of chitosan microparticles with the enteric polymer Eudragit has also been described [68].

Site-specific vaccine delivery may be achieved using carriers that are resistant to the endogenous gut enzymes, but are degraded by microbial enzymes (azoreductases) in the colon [69]. The good safety record of chitosan and increasing evidence that it can promote adaptive immunity when used parenterally and mucosally suggests that it is a good candidate for inclusion in oral vaccines. However, as with PLG microparticles, application of chitosan alone is unlikely to be sufficiently potent, and will require the inclusion of additional adjuvants.

5.2.7 *Liposomes*

Liposomes are membranous systems comprising of amphipathic molecules such as phospholipids, forming multi- or unilayered vesicles. These systems have been used as delivery vehicles and injectable formulations have been licensed for clinical use [70]. A major advantage of liposomes is that they are composed of natural cell-wall components, such as phospholipids and cholesterol and, thus, are likely to be safe. Molecules with different physicochemical properties (e.g., both hydrophobic and hydrophilic molecules) can be incorporated [70]. Liposomes adsorb to cell membranes and can release their contents following uptake. The incorporation of antigens into liposomes can render them more immunogenic than when delivered alone. Efficient uptake and processing of liposomal vaccines by antigen-presenting cells is likely to be one of the principal factors responsible for their efficacy. Regarding oral delivery, liposomes have been shown to be taken up by PP M cells [71]. It is generally thought that liposomes are relatively unstable in the digestive tract and, therefore, are unsuitable for oral vaccine delivery. Polymerised liposomes are more stable in the GIT [72], and these may offer greater potential as oral delivery systems. To improve efficacy, immunostimulatory molecules such as cytokines can be incorporated, and targeting ligands may be attached to enhance interaction of orally delivered liposomes with the GIT epithelium. Despite the recent use of polymerised liposomes to increase stability, there is little evidence so far to suggest that liposomes have strong potential for oral vaccine development

5.2.8 *Cochleates*

Cochleates are an alternative lipid-based vaccine system composed of phosphatidylserine, cholesterol and calcium. In contrast to liposomes, the structures are lipid bilayer sheets in the shape of a spiral with no internal aqueous spaces. The phospholipid–calcium systems are considered to be non-toxic and non-inflammatory, stable, amenable to lyophilisation and may be effective vaccines when delivered mucosally [73]. Oral

delivery of influenza virus glycoproteins associated with cochleates induced protection following intranasal challenge. In addition, oral delivery of plasmid DNA in these structures elicited specific circulating antibody and CTL responses [73]. The administration of antigens associated with cochleates appears to facilitate cytoplasmic delivery and entry into the MHC class I pathway, allowing the induction of CTL responses. Endocytic uptake of cochleates is also likely to allow MHC class II antigen presentation and the induction of $CD4^+$ T-helper (T_H) cells and antibody responses. Oral delivery of a cochleate-associated HIV peptide stimulated mucosal and systemic humoral and cellular responses [73]. Despite claims made regarding the efficacy of these systems, little work has been carried out so far: particularly comparing cochleates with other non-living oral delivery systems. With the small amount of information available on these systems, there is not a strong case for the exploitation of cochleates as oral vaccine delivery systems.

5.2.9 *Immune-stimulating complexes*

ISCOMs are cage-like structures of 30 – 40 nm in diameter composed of glycosides that are present in Quil A, cholesterol, the vaccine antigen and phospholipids [74]. Quil A saponin, derived from the tree *Quillaja saponaria* Molina, is a strong adjuvant and is important for the efficacy of ISCOMs. To circumvent the problem of Quil A toxicity, a fraction of lower toxicity (QS21) was purified, which is an effective oral vaccine adjuvant in mice [75].

The ISCOM structure formed by hydrophobic interactions between the triterpene glycosides and cholesterol is highly stable [74]. Very small amounts of antigen in ISCOMs are immunogenic, and both humoral and cellular immune responses can be elicited. ISCOM hydrophobicity and the ability of saponins to intercalate into cholesterol-containing membranes may explain the ability of ISCOMs to facilitate antigen uptake and entry into the cell cytosol. Oral immunisation with OVA in ISCOMS primed antigen-specific proliferative and cytokine responses in the spleen and mesenteric lymph nodes [76]. A striking feature of ISCOMs as delivery systems is their ability to elicit MHC class I-restricted CTL responses to associated antigens, including oral immunisation [37]. Relatively strong immune responses and protection were elicited by oral delivery of the influenza A/Sichuan/87 ISCOM vaccine in mice [77]. In 2004, work with a derivative of cholera toxin combined with ISCOMS indicated that the response to orally delivered ISCOMs can be enhanced by including additional adjuvants [78]. The disadvantages of ISCOMs are that the incorporation of many antigens into the structure is difficult, and extensive modification is often required. Nevertheless, the impressive potency of ISCOMS, either when used alone or together with other adjuvants such as toxoids, suggests that these are worthy of further evaluation.

5.3 Oral delivery of DNA vaccines

DNA vaccines have attracted great interest due to advantages including high purity, the capacity to include multiple antigens, cost-effectiveness and suitability for use in the presence of pre-existing maternal immunity. However, DNA vaccine potency is not sufficient in humans [79], necessitating the use of potent delivery systems and adjuvants. PLG microparticles have been proposed as delivery systems to enhance the oral delivery of DNA vaccines [80]. Protective immune responses were elicited following oral immunisation of mice with a rotavirus VP6 DNA vaccine encapsulated in PLG microparticles; the first demonstration that protective immunity could be elicited against an infectious agent by oral administration of a DNA vaccine. Immunisation of mice with DNA vaccines encoding the outer capsid VP4 and VP7 proteins led to a significant reduction in faecal rotavirus antigen after challenge with homologous murine rotavirus, compared with controls [81]. HIV envelope (env)-specific systemic and mucosal antibody and CTL responses were induced by oral vaccination of mice with PLG microparticles containing env-encoded plasmid DNA [82]. Oral administration of PLG-encapsulated DNA encoding HIV glycoprotein 160 also induced specific serum antibodies, and an increased level of specific faecal IgA. In contrast, intramuscular administration of naked or PLG-encapsulated DNA vaccine only induced systemic cellular and humoral responses. Oral immunisation of mice with a PLG-microencapsulated DNA vaccine encoding the hepatitis B virus surface antigen induced antigen-specific CTL and IFN-γ responses in the spleen and GALT [83]. Cationic microparticles have shown significant potential for DNA vaccine delivery [84], and it was suggested that cationic microparticles are optimal for uptake into macrophages and DCs [85]. However, these systems are unlikely to be optimal for oral vaccination, as the DNA is not protected from breakdown in the digestive tract. It has been suggested that chitosan has potential as an oral DNA vaccine delivery system. Oral delivery of DNA encoding the *lac Z* gene and IL-10 associated with *N*-acylated chitosan resulted in gene expression in the intestine [86]. However, as there has been relatively little testing of non-living oral DNA vaccine delivery systems, it is difficult to assess their potential at present.

5.4 Non-particulate systems

In addition to the extensive work carried out on particulate systems as described in Section 5.2, there have also been significant strides forward in the use of non-particulate adjuvants of microbial and plant origin in recent years. Many of these molecules can activate components of the innate immune system via pattern recognition receptors [87]. Some of these molecules are effective adjuvants when simply mixed with the antigen before delivery, whereas others may find application in vaccine targeting.

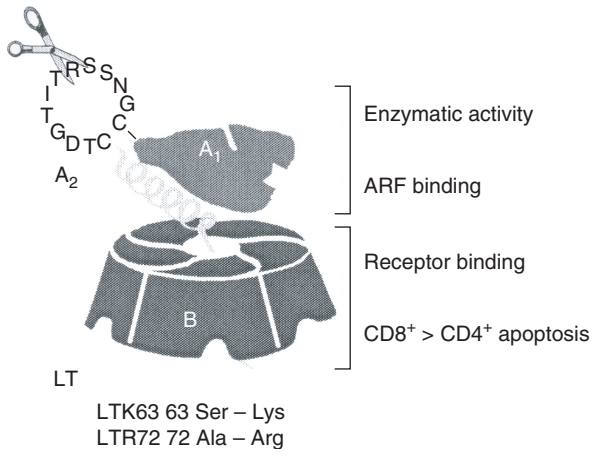

Figure 3. Representation of the structure of *E. coli* heat-labile enterotoxin, showing the A and B subunits, their structural and functional features, and the site of proteolytic cleavage of the loop between the A1 and A2 domains. The B subunit binds to the cellular receptor on epithelial cells and the molecule is internalised. The enzymatically active A subunit is cleaved from the molecule and interacts with soluble ARF, and subsequently induces a permanent activation of adenylyl cyclase and intracellular accumulation of cAMP, through interaction with G proteins. The LT mutants LTK63 and LTR72 have single amino acid substitutions in the enzymatically active A subunit and have significantly reduced toxicity *in vitro* and *in vivo*. Reprinted from RAPPUOLI R, PIZZA M, DOUCE G, DOUGAN G: Structure and mucosal adjuvanticity of cholera and *Escherichia coli* heat-labile enterotoxins. *Immunol. Today* (1999) **20**(11):493-500, with permission from Elsevier.
ARF: ADP-ribosylation factor; LT: Heat labile enterotoxin.

The most widely investigated of these molecules have been members of the A-B class of bacterial toxins. However, CpG DNA, which contains unmethylated CpG dinucleotides in particular base contexts, was also a strong adjuvant for orally delivered vaccines. Tetanus toxoid was administered to mice with CpG oligodeoxynucleotides by the intrarectal, intranasal and oral routes. Strong immune responses were elicited after delivery via all three routes and non-CpG control oligodeoxynucleotides also had adjuvant effects when used at mucosal sites [88]. In 2005, oligonucleotides consisting of a novel 3′-3′-linked structure and synthetic stimulatory motifs, referred to as second-generation immunomodulatory oligonucleotides, were described, which are more stable than CpG DNA in the GIT and can induce more potent mucosal T_H type 1 adjuvant responses [89].

5.4.1 *Cholera toxin and* Escherichia coli *heat-labile enterotoxin*

Cholera toxin (CT) and heat-labile enterotoxin are powerful mucosal immunogens and adjuvants [90]. The B (binding) subunits bind to cellular receptors on epithelial cells and the toxin is internalised (**Figure 3**). In addition to the potent induction of antibody and T_H cell responses, orally administered CT can also promote CTL responses to co-administered antigens [91]. There is great interest in the area of genetically detoxified derivatives of these molecules as mucosal adjuvants. Heat-labile enterotoxin mutants have been produced with potent adjuvant activity, but with negligible or no detectable toxicity [90]. These molecules are effective when administered with antigens by the oral route [92], but generally less so than when nasally administered. The interaction of nasally administered GM1 ganglioside-binding toxins with neuronal tissues [93] may provide a spur to further test these molecules alone, or together with delivery systems via the oral route. Lycke and colleagues have described a fusion of the intact CT A1 subunit with a dimer of an Ig-binding fragment D from *Staphylococcus aureus* protein A (CTA1-DD), which is an effective adjuvant when administered orally [78]. Furthermore, in contrast to wild-type CT or CTB, CTA1-DD did not bind to or accumulate in the nervous tissues of the olfactory bulb when nasally administered [94].

Vibrio cholerae zonula occludens toxin (zot) was an effective adjuvant when co-administered with an antigen by the intranasal route [95], but its application as an adjuvant by the oral route has not been reported. In addition to this adjuvant activity, the B subunits of CT and LT have potential as carrier molecules [96]. For example, oral immunisation with a group B streptococcus capsular polysaccharide–CTB conjugate induced specific antibodies in secretions and in the blood [97].

Oral delivery of antigens can induce antigen-specific oral tolerance, which may potentially be exploited to treat allergies, inflammation and other conditions resulting from immune responses against food antigens or the gut flora. Feeding low doses of myelin basic protein inhibited the induction of experimental autoimmune encephalomyelitis (the murine model for multiple sclerosis) and the efficacy of this approach was enhanced using CTB as a carrier molecule [98]. As bacterial toxins are among the most potent mucosal adjuvants identified, and satisfy the requirements of specific targeting of mucosal cells, activation of innate immunity and efficacy by the oral route, toxoid derivatives remain among the prime candidates for inclusion in oral vaccine delivery systems.

5.4.2 *Lectins*

The most widely studied specific bioadhesives are lectin-like molecules of plant or bacterial origin. Lectins are proteins or glycoproteins of non-immunological origin that bind to sugar structures specifically and with relatively high affinities. Plant lectins are proteins possessing at least one non-catalytic domain, that bind reversibly to a specific mono- or oligosaccharide [99]. Many of these molecules are stable in the GIT and can bind specifically to epithelial cells in the gut. Furthermore, there is evidence that lectins may be translocated across the epithelium and induce immune responses to conjugated or co-administered antigens.

One concern with the use of plant lectins as targeting agents is their immunogenicity. Studies by the authors have shown that plant lectins vary widely in immunogenicity following oral

Table 2. Mean reciprocal antibody titres in sera and secretions of mice after three oral doses of various lectins.

Lectin	IgG1	IgG2a	IgA (saliva)	IgA (vagina)	IgA (nasal wash)	IgA (gut wash)
Ovalbumin	_	_	_	_	_	1.6
Cholera toxin	233245	4134	31	34	36	180
Mistletoe lectin 1	136534	534	20	43	17	229
Tomato lectin	47520	421	0.4	4	0.8	35
Phaseolus vulgaris agglutinin	241	11	_	_	_	1.6
Wheatgerm agglutinin	131	_	_	_	_	_
Ulex europaeus agglutinin 1	671	_	_	_	_	1.2

Mice were immunised orally with 10-µg cholera toxin or 100-µg ovalbumin or plant lectin [100]. End-point titres were determined as the lowest dilution of test sample giving an absorbance of > 0.1 units higher than the control (pre-immune) samples at the same dilution.

delivery to mice [100]. Lectins such as wheatgerm agglutinin, *Ulex europaeus* agglutinin [UEA]-1 and *Phaseolus vulgaris* agglutinin were relatively poorly immunogenic, but mistletoe lectin 1 was a potent immunogen, inducing systemic and mucosal responses that were comparable to those induced by cholera toxin (**Table 2**). Thus, not all lectins are highly immunogenic when administered orally, so molecules may be selected that are effective bioadhesives but poor immunogens. In addition, plant lectins have been identified that are effective adjuvants when co-administered with antigens by the oral route [101]. Oral immunisation of mice with mistletoe lectins and a bystander antigen (OVA) significantly enhanced mucosal and systemic immune responses.

5.4.3 Targeting of oral vaccine delivery systems

When fluorescent nanoparticles were chemically linked to LTB or ConA (concanavalin A) and orally delivered to rats, the particles bound to the surface of, and subsequently crossed, the intestinal villus cells [102]). M cells and enterocytes in different gut regions vary in terms of lectin-binding properties, and this may be exploited for specific vaccine targeting. Investigations into lectin binding to the mouse gut found that a number of fucose-specific lectins (e.g., UEA-1) bound specifically to PP M cells. Polystyrene microspheres (0.5 µm) covalently attached to UEA-1 bound to, and were rapidly absorbed by, PP M cells following oral delivery [103]. Similarly, the association of UEA-1 or wheatgerm agglutinin with polymerised liposomes promoted liposome uptake from the GIT in mice. Although both systems were taken up to a greater extent than the lectin-free liposomes, UEA-1 exhibited the most effective PP targeting [80]. Chemical conjugation of *Lycopersicon esculentum* agglutinin (LEA) to 0.5-µm polystyrene microparticles resulted in enhanced uptake compared with un-conjugated particles after oral delivery [104]. It was reported that uptake was mainly associated with non-lymphoid intestinal tissue. As enterocytes constitute the largest cell population in the intestinal mucosa, it may be easier and more effective to target enterocytes for vaccine delivery. Oral delivery of a hapten complexed to plant lectins induced a significantly enhanced hapten-specific serum antibody response in mice compared with a non-lectin carrier. Thus, lectin-specific binding to M cells may be exploited to enhance the immune response to oral vaccines [105].

Factors involved in microbial adherence and invasion may also have an application in oral vaccine targeting. Fimbriae present on bacterial surfaces can mediate specific interactions with receptors on epithelial cells [106]. When bovine serum albumin was covalently linked to K99 or 987P pili and fed to mice, an enhanced specific serum antibody response was measured in the serum [105]. Pili are used in commercially available animal vaccines that are orally administered, so these molecules may be too immunogenic to use for targeting. As in the case of plant lectins, the immunogenicity of these factors may vary, and this may be a factor in the choice of targeting molecules. Bacterial invasins that mediate internalisation may also have potential to enhance trans-epithelial vaccine transport. Coating of latex microspheres with bacterial factors associated with GIT colonisation/invasion led to enhanced adherence to, and uptake by, epithelial cells [107,108]. The administration of polystyrene microparticles conjugated to the *Yersinia pseudotuberculosis* invasin resulted in enhanced particle uptake from the mouse gut [109]. The *Vibrio cholerae* zot can reversibly open tight junctions in the intestinal mucosa [95].

Soy phosphatidylcholine liposomes were coated with the reovirus σ1 cell attachment protein, which binds to M cells [110]. Using the mouse L929 cell monolayer, specific binding of the liposomes was demonstrated. Furthermore, incubation studies showed a 10-fold increase in the accumulation of coated liposomes compared with uncoated liposomes in PPs. The adsorption of a 64-kDa protein secreted by *Trypanosoma cruzi*

onto bentonite particles facilitated entry of the particles into non-phagocytic HeLa cells [111].

In addition to plant lectins, attempts have been made to exploit endogenous lectins in the GIT for site-specific drug delivery [112]. The lumenal epithelium of the GIT is extensively glycosylated, and this facilitates interactions with many plant and bacterial lectins [60]. Lectins that bind specifically to the epithelial cells may be used to target conjugated antigens, or may be attached to microparticles and other delivery systems, such as liposomes to enhance interaction with gastrointestinal epithelia.

One of the first molecules used as a gut cell-targeting moiety was vitamin B_{12} [102]. The natural uptake mechanism for vitamin B_{12} was used to increase protein absorption from the gut. Vitamin B_{12} forms a complex with an intrinsic factor that binds to a receptor on the small intestinal epithelium. Following binding, the complex is internalised, the intrinsic factor is degraded by cathepsins, and vitamin B_{12} is released. The molecule binds to intracellular transcobalamin with subsequent transcytosis of the complex into the circulation. The carrier capacity of the vitamin B_{12} receptor may be too low for the delivery of many molecules, but this may be addressed by using vitamin B_{12} to target particulate systems containing high vaccine payloads. An additional problem with this system may be interference with nutrient absorption. Although the best candidate molecules for the targeting of oral vaccines may not yet be identified, vaccine targeting has tremendous potential to enhance the efficacy of non-living oral vaccines and this field should receive greater attention.

5.5 Edible vaccines

A recent development with considerable potential for oral vaccine production and delivery is the generation of plants expressing antigenic proteins [113]. Advantages of antigen production in plants include avoidance of the risk of contamination with animal pathogens and the potential for very high production yields. A number of antigens, including LTB, *Streptococcus mutans* surface protein antigen and hepatitis B surface antigen, have been expressed in various plants [113].

So far, the levels of the immune responses elicited to orally administered plant-expressed antigens appear significantly lower than when using adjuvants, such as CT or microparticles. However, the system is attractive due to the ease of production and safety issues. Furthermore, recent studies indicate that antigen expression levels may be substantially increased and antigen may be expressed in more widely consumed food plants, such as tomatoes [113]. In 2005, a double-blind, placebo-controlled clinical trial evaluated the immunogenicity of hepatitis B surface antigen expressed in potatoes that were fed to previously vaccinated individuals. After eating three doses of the uncooked potatoes, serum anti-hepatitis B surface antigen titres increased in 10 out of 16 volunteers [114]. This is a very promising finding, and may support the approach for booster immunisation of already immunised populations. There is no indication so far that edible vaccines can induce immunity of acceptable potency in naive populations, although proponents of the technology suggest that additional factors could be incorporated to improve efficacy.

6. Expert opinion

The oral route is the ideal means to deliver vaccines, due to simple administration, improved safety and the potential to induce mucosal immune responses. However, oral vaccine delivery is complicated by the numerous barriers posed by the GIT. Nevertheless, there are a number of live attenuated and killed whole cell vaccines at various stages of clinical testing against cholera, enterotoxigenic *E. coli*, rotavirus and shigellosis [202]. In contrast, there has been relatively little progress in the clinical development of non-living delivery systems and adjuvants for subunit vaccines. To facilitate effective immunisation with peptide and protein vaccines the antigens must be protected, uptake must be enhanced and the innate immune response activated. Numerous non-living systems have been evaluated as oral vaccine delivery systems, including inert particles, Toll-like receptor agonists and bacterial toxins. Polymeric delivery systems have shown significant potential to enhance responses and can be designed to improve the efficacy of mucosally administered vaccines in a number of ways: antigens may be protected from degradation, concentrated in one area of the GIT for better absorption, retained in the gut for extended time periods, or preferentially targeted to sites of antigen uptake (e.g., PPs). For some polymeric delivery systems it is claimed that they achieve several of these effects simultaneously, although the mechanisms involved are often poorly defined. PLG microparticles have been widely investigated and can induce mucosal and systemic responses in addition to protective immunity against some pathogens. However, further work is needed in a number of areas, including the stabilisation of antigens within the polymeric matrix. Alternative systems such as liposomes and ISCOMS may have some advantages for oral vaccine delivery, but may also have significant limitations. For example, ISCOMS are very effective for the induction of CTL responses, but may not be very stable in the gut and are difficult to manufacture. Approaches that involve modification of antigens (e.g., lipidation) may enhance the immunogenicity of orally delivered antigens and can be used in conjunction with delivery systems such as microparticles.

In addition to these particulate systems, microbial and plant molecules that interact specifically with epithelial cells as targeting agents and/or immunomodulators may be applied as vaccine carriers, adjuvants and immunomodulators in therapies for autoimmune conditions. These molecules have also been used to enhance the interaction of particulate delivery systems with epithelial cells. For example, monophosphoryl lipid A can act as a mucosal adjuvant and enhance the response to liposomal antigens. There is significant interest in the development of therapeutic vaccines to treat existing conditions, including chronic infectious diseases (e.g., diseases caused by herpes simplex virus, HIV, hepatitis B and C, *Helicobacter pylori*), tumours, and allergic and autoimmune conditions (e.g., multiple sclerosis, Type 1 diabetes) [115,116]. An orally delivered *E. coli* heat-labile entero-

toxin mutant was shown to be effective in eradicating an established infection with *H. pylori* [117]. In summary, progress in the field of oral vaccine delivery has been slow so far, but, with increased understanding of innate and adaptive mucosal immunity, as well as the availability of a range of novel delivery systems and adjuvants, there is greater hope for the future development of improved oral vaccines.

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