Recent Advances in Microparticle and Nanoparticle Delivery Vehicles for Mucosal Vaccination

E. A. McNeela and E. C. Lavelle

Abstract The great potential of mucosal vaccination is widely accepted but progress in the clinical development of subunit mucosal vaccines has been disappointing. Of the available approaches, the use of polymer-based microparticles is attractive because these delivery vehicles can be specifically tailored for vaccines and they offer the potential for integration of adjuvant. Here we address recent developments in the use of particulates as mucosal vaccines and the potential of novel targeting strategies, formulation approaches and adjuvant combinations to enhance the efficacy of particle-based mucosal vaccines. This review discusses the current status of mucosal vaccines based on particles and highlights several of the strategies that are currently under investigation for improving their immunogenicity. These include enhancing the stability of formulations in the luminal environment, increasing uptake by specifically targeting particles to mucosal inductive sites, and augmenting immunogenicity through co-formulation with immunostimulatory agents.

Contents

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1 Introduction

The majority of human pathogens infect via the mucosae, including *Vibrio cholerae*, enterotoxigenic *Escherichia coli* (ETEC), influenza virus, human immunodeficiency virus type 1 (HIV-1), rotavirus and respiratory syncytial virus. Mucosal immunization is likely to induce greatest local immunity and prevent infection at sites of pathogen entry (Czerkinsky and Holmgren 2011). Mucosal immunization would obviate many of the problems associated with parenteral vaccination, such as the requirement for sterile needles and trained personnel, and it would improve patient compliance (Giudice and Campbell 2006; Lavelle 2001). Although most of the vaccines currently administered in humans are injected, there are several licensed mucosal vaccines, including oral vaccines against poliovirus, *Salmonella typhi* and *V. cholerae* and an intranasal (i.n.) vaccine for influenza virus (Brandtzaeg and Johansen 2008). These mucosal vaccines are based on killed or live-attenuated microorganisms, and their efficacy has highlighted the potential of mucosal vaccination. Much of the current vaccine research has focused on the development of subunit vaccines that are composed of purified native or recombinant components of microorganisms. Subunit vaccines are safer because they lack contaminants that are often present in whole-cell vaccines and they cannot revert to a virulent form, as reported for the live-attenuated oral polio vaccine (Minor 2009). However, most soluble subunit antigens are poorly immunogenic when administered at mucosal surfaces, and they require appropriate delivery systems and/or adjuvants in order to generate potent immune responses (Lavelle 2005; O'Hagan 1998).

A number of adjuvants and delivery systems have been evaluated for mucosal vaccines, including liposomes, immunostimulatory complexes, CpG oligodeoxynucleotides, bacterial toxins, nanoparticles and microparticles. Microparticles are attractive for mucosal vaccine delivery because they are similar in size to many pathogens that the host immune system has evolved to fight (O'Hagan et al. 2006). As a consequence, these particles and associated antigens may be rapidly taken up by antigen-presenting cells (APC) following immunization. The biodegradable and biocompatible polymers poly(lactide-co-glycolide) (PLG) and poly(lactic acid) (PLA) are the most widely used materials for the construction of microparticles (Cleland 1999). The US Food and Drug Administration (FDA) has approved the use of these materials for various clinical

applications, including resorbable sutures and implants for controlled drug delivery. It has been suggested that the efficacy of microparticles is primarily related to their ability to protect encapsulated antigens, release antigen over extended time periods and to direct antigen to and retain it in local lymphoid tissues (Gupta et al. 1998; Vyas and Gupta 2007). However, recent findings suggest that these particulates can additionally enhance adaptive immunity by triggering innate responses (Sharp et al. 2009).

In the early 1990s, several groups reported the induction of mucosal and systemic immune responses to antigens entrapped in PLA/PLG microparticles after mucosal delivery in mice (Challacombe et al. 1992; Eldridge et al. 1989; Maloy et al. 1994; Moldoveanu et al. 1993). Furthermore, protective immunity against various pathogens, including *Streptococcus pneumoniae* (Fattal et al. 2002; Seong et al. 1999), *Bordetella pertussis* (Cahill et al. 1995; Conway et al. 2001) and *Salmonella typhimurium* (Allaoui-Attarki et al. 1997; Fattal et al. 2002) was generated in rodents by mucosal immunization with PLG microparticulate vaccines. However, despite these early encouraging results, studies in humans have been disappointing, and there are currently no licensed polymer-based microparticle mucosal vaccines (Katz et al. 2003; Lambert et al. 2001; Tacket et al. 1994). Significant improvements in the development of microparticles for human mucosal vaccination are needed, and recent studies have focused on strategies to enhance their efficacy. This review discusses the current status of mucosal vaccines based on particulates and highlights several of the strategies that are currently under investigation for improving their immunogenicity.

2 Mucosal Immunity to PLG/PLA Polymer-Formulated Microparticle Vaccines

The initiation of adaptive immunity following mucosal immunization occurs at specialized sites in the mucosae that contain organized mucosa-associated lymphoid tissue (MALT). Humoral immunity at these sites is mediated by secretory IgA (SIgA), which has been shown to neutralize toxins and to prevent the attachment and entry of pathogens at mucosal surfaces (Macpherson et al. 2008; Mestecky and Russell 2009). Serum IgG antibody responses may also be induced by mucosal immunization. Effectors of cellular immunity in mucosal tissues are $CD8^+$ cytotoxic T lymphocytes (CTL), $CD4^+$ T helper (Th) cells and Natural Killer (NK) cells. Cellular responses can be induced by mucosal immunization with vaccines, such as the oral typhoid vaccine (Salerno-Goncalves et al. 2002). Although vaccines can potentially be delivered via numerous mucosal routes, the majority of microparticle studies have focused on the oral and i.n. routes for vaccine administration.

Many investigators have shown that systemic antibody responses to antigens entrapped within or adsorbed onto PLG/PLA microparticles are greater than

those produced in response to oral or i.n. immunization with soluble antigens (Carcaboso et al. 2003; Carcaboso et al. 2004; Challacombe et al. 1992; Florindo et al. 2009; Kende et al. 2002; Tabata et al. 1996; Vajdy and O'Hagan 2001; Yeh et al. 2002). Furthermore, several studies have reported the augmentation of antigen-specific IgA in mucosal secretions (Challacombe et al. 1992; Florindo et al. 2009; Tabata et al. 1996; Ugozzoli et al. 1998) and the induction of cell-mediated immune responses (Carpenter et al. 2005; Florindo et al. 2009; Moore et al. 1995; Vajdy and O'Hagan 2001) following mucosal delivery of microparticulate vaccines. More importantly, a number of vaccines utilizing PLG/PLA microparticles have elicited protective immunity in mice after oral delivery (Table 1).

From these studies (Table 1), it is apparent that oral immunization of mice with PLG/PLA-associated vaccines generally produces low titers of serum IgG antibodies but results in significant protection against mucosal microbial challenge, which in some studies was greater than that induced by systemic immunization. Therefore, it is important that additional immune parameters, including mucosal, humoral and cellular responses, and especially protective efficacy, be measured when evaluating mucosal vaccines in experimental animals.

One concern with oral vaccination studies is that contamination of the nasal cavity with antigen can occur when vaccine is given in a large volume. Douce et al. (1999) addressed this issue in a study examining the adjuvanticity of detoxified *E. coli* heat-labile toxin (LT) derivatives, and the authors stressed the importance of analyzing individual (rather than pooled) samples from immunized subjects. Furthermore, i.n. administration of vaccine to anaesthetized mice can result in antigen dissemination to the lungs when volumes >20 μl are administered in the naris (Minne et al. 2007; Thompson et al. 1999). These factors should be taken into consideration when designing oral or i.n. vaccination protocols in mice.

Despite encouraging results in mice, there are no studies showing consistent induction of protective immunity in humans following oral delivery of microparticulate vaccines. A study by Tacket et al. (1994) demonstrated antigen-specific jejunal IgA responses in some human subjects orally immunized with ETEC colonization factor antigen encapsulated in PLG microspheres, and one-third of the volunteers were protected against challenge. A subsequent Phase I trial using microencapsulated CS6 ETEC antigen reportedly increased antigen-specific serum IgA and IgG, but the benefits of microparticles over soluble antigen were not clear due to the small number of subjects (Katz et al. 2003). In a more recent study, oral immunization of human subjects with CRM197 diphtheria antigen conjugated to starch microparticles, given as a booster vaccination in previously immunized individuals, failed to significantly increase anti-diptheria toxin neutralizing antibody in serum (Rydell et al. 2006). Nevertheless, the ETEC study (Tacket et al. 1994) indicates that microparticle-based oral vaccines can induce protective immunity against a mucosal pathogen, and hence, support the development of improved microparticle systems.

Table 1 Protective immunity in mice following oral administration of vaccines in PLG/PLA particles

Antigen	Immunization protocol	Outcome	Reference
Pertussis toxoid and filamentous haemagglutinin	100 μg/dose of each orally (500 μl) on weeks 0, 4 and 8	Compared to antigens alone orally; similar or lower serum IgG antibody titers but greater protection post aerosol challenge with *B. pertussis*.	Conway et al. (2001)
DNA encoding HIV envelope glycoprotein	10 μg/dose (volume not specified) orally on days 0, 7 and 14	Compared to i.m. particulate DNA vaccine; similar serum IgG but higher fecal IgA antibody responses. Better protection after intrarectal challenge.	Kaneko et al. (2000)
DNA encoding rotavirus proteins	75 μg/dose (500 μl) orally once	Compared to naked DNA orally; serum IgG antibodies not significantly different. Significantly greater protection after oral challenge.	Herrmann et al. (1999)
Fimbrial protein of *B. pertussis*	10 μg/dose (500 μl) orally once	Compared to alum-formulated i.p. fimbriae; lower serum IgG antibody responses. Similar protection after intranasal challenge with *B. pertussis*.	Jones et al. (1996)
Phosphorylcholine	280 μg/dose (500 μl) orally on days 1, 2, 3, 28, 29 and 30	Compared to i.p. antigen (15 μg/dose); lower serum IgG antibodies. Significantly greater protection after oral challenge with *S. typhimurium*.	Allaoui-Attarki et al. (1997)
Monoclonal antibody to chlamydial antigen	4–6 μg/dose (200 μl) orally twice, 2–3 weeks apart	Compared to s.c. particulate vaccine; similar protection post-ocular infection with *C. trachomatis*.	Whittum-Hudson et al. (1996)

Oral delivery refers to intragastric administration; *i.m.*, intramuscular; *i.p.*, intraperitoneal; *s.c.*, subcutaneous

3 Modifications of Microparticles for Mucosal Vaccine Delivery

There are currently several obstacles for the use of microparticles in mucosal vaccination. These include instability and degradation of the associated antigens in the mucosal lumen and the poor transport of particles across mucosal surfaces. Following mucosal uptake, PLG microparticles degrade slowly by non-enzymatic cleavage into the endogenous metabolites, lactic and glycolic acid. The slow dissolution provides a sustained release of incorporated antigen, which can be adjusted by selecting polymers with particular ratios of lactide and glycolide (Aguado and Lambert 1992; Lin et al. 2000; Watts et al. 1990). The main disadvantage of encapsulating antigen in PLG is that the acids released during

hydrolysis generate a highly acidic microenvironment within the particle, which may denature the vaccine antigen (Park et al. 1995; Takahata et al. 1998). The most common method for manufacture of PLG/PLA microparticle vaccines is the emulsion/solvent evaporation process that utilizes conventional emulsifiers such as poly(vinyl alcohol) (PVA) to stabilize the emulsion. The manufacturing procedure involves the emulsification of antigens in organic solvents, followed by extraction or evaporation to form microparticles. This technique involves high-shear rates, elevated temperatures and the creation of large aqueous/organic solvent interfaces; all of which may degrade entrapped proteins or DNA (Ando et al. 1999; Gupta et al. 1998).

3.1 Attachment of Molecules to Particle Surfaces

One alternative to microencapsulation is to adsorb or covalently link antigen to the particle surface in order to preserve integrity (Coombes et al. 1999; Kazzaz et al. 2000). DNA, in particular, may be significantly damaged and lose its supercoiled structure following the microencapsulation process (Ando et al. 1999). To address this issue, cationic PLG microparticles have been developed for adsorption of negatively-charged DNA (Singh et al. 2000). Intranasal immunization of mice with HIV-1 gag-encoding DNA adsorbed onto cationic PLG microparticles induced potent local Th1 and systemic CTL responses and enhanced systemic antibodies when compared to i.n. immunization with naked plasmid DNA (Vajdy and O'Hagan 2001). Anionic PLG particles have also been developed for adsorption of positively-charged proteins (Kazzaz et al. 2000). Attachment of PLG to a PVA backbone results in three-dimensional branched polymeric structures which may be altered by substitution of PVA with negatively-charged sulfobutylated-PVA or positively-charged diethyl-aminoethyl-PVA derivatives. The negative charge on the surface of sulfobutylated-PVA particles increases antigen adsorption due to electrostatic interactions with positively charged proteins (Dailey et al. 2005). Intranasal or oral delivery of tetanus toxoid (TT) adsorbed to sulfobutylated PVA-graft-PLG particles generated higher serum IgG and IgA antibody titers in mice when compared to mucosal delivery of the same antigen in solution. However, the IgG titers induced after mucosal administration of particles with TT were lower than those elicited by parenteral immunization with the same formulations or with conventional Tetanol® vaccine (Jung et al. 2001).

3.2 Enteric Coating for Intestinal Delivery

Particles expressing vaccine antigen on their surfaces may be effective for i.n. delivery but not for oral administration as the antigen would be exposed to the harsh conditions of the gastrointestinal lumen. The coating of microparticles with

enteric polymers has been shown to be an effective strategy for combating the problem of antigen degradation in the stomach following oral delivery. These enteric polymers, such as the methacrylic acid esters Eudragit® L100 and S100, have a pH-dependent solubility profile and only dissolve in the weakly acidic to alkaline medium of the intestine (Chourasia and Jain 2003). Their application on microparticles can protect surface-expressed or encapsulated antigen from degradation by stomach acid and can facilitate release in specific regions of the small intestine. Enhanced protection of encapsulated ovalbumin (OVA) against proteolysis in simulated gastric fluid was achieved by replacing the conventional PVA stabilizer with carboxymethylethylcellulose (CMEC) or Eudragit L100-55 enteric polymers. Furthermore, significantly greater antigen-specific salivary IgA responses were reported in mice orally immunized with OVA-loaded microparticles stabilized with CMEC compared to PVA (Delgado et al. 1999). More recently, Dea-Ayuela et al. (2006) reported enhanced serum IgG1 antibodies and protection against the nematode parasite *Trichinella spiralis* following three oral immunizations of mice with parasite proteins loaded in enterically-coated particles. Mice immunized with these starch/sugar particles coated with Eudragit® L100 showed a 50% decrease in worm/larvae burden following challenge when compared to non-immunized controls. However, the superiority of enterically-coated microparticles was not apparent in this study as other mice were not immunized with uncoated particles or with parasite antigens alone (Dea-Ayuela et al. 2006).

3.3 Association of Particles with Bioadhesive Agents

The microparticle surface may be modified by coating with bioadhesive polymers or surfactants (Fig. 1). Bioadhesive agents, including polyethylene glycol (PEG) and chitosan, may increase residence time on mucosal surfaces, and consequently, the uptake of particles following mucosal administration.

3.3.1 Polyethylene Glycol

PEG and its derivatives have been used widely for modifying particle surfaces or matrices in order to improve the stability and release profile of encapsulated molecules. The original idea was to provide a protective hydrophilic shell around encapsulated hydrophobic molecules, preventing their rapid clearance and degradation after intravenous delivery (Gref et al. 1994; Woodle and Lasic 1992). However, it is now known that coating particles with PEG is also beneficial for mucosal administration. The effect of PEG coating on the transport of particles across mucosal barriers has been investigated in numerous studies (Cu and Saltzman 2009; Tobio et al. 1998; Tobio et al. 2000; Wang et al. 2008).

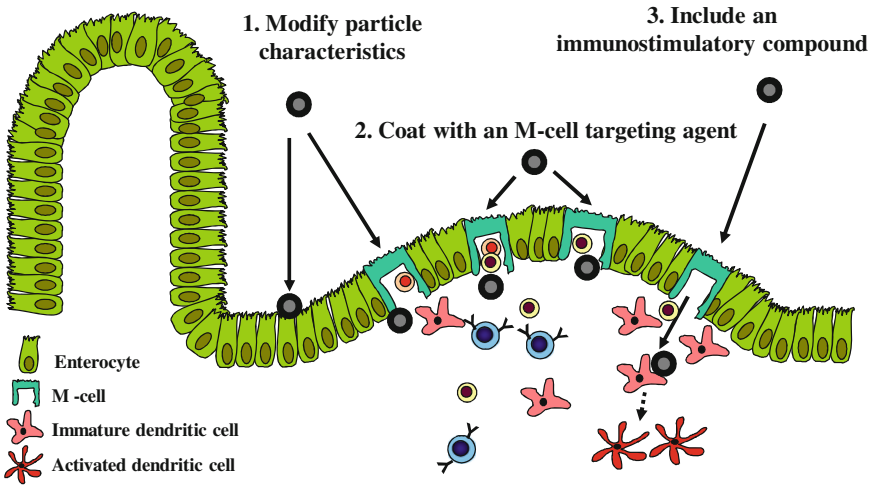

Fig. 1 *Strategies to enhance the efficacy of microparticles and nanoparticles for mucosal vaccine delivery.* 1. Particle characteristics, including size, surface charge and hydrophobicity, may be modified to increase their interaction with the mucosa and the intestinal epithelium. Particles may be enterically coated to protect the associated antigen from degradation under gastric conditions or they may be coated with bioadhesive polymers to enhance antigen bioavailability following mucosal administration. 2. Coating particles with specific targeting agents including lectins, microbial adhesins and antibodies may increase particle uptake by M cells. 3. Adjuvants, such as TLR agonists or other immunostimulatory compounds, may be included to promote DC activation and enhance innate and adaptive immunity to particulate antigen following uptake. It is likely that future mucosal vaccines based on microparticles will use a combination of these strategies to enhance their immunogenicity

Coating the surface of negatively-charged polystyrene nanoparticles with short-length PEG molecules increased their diffusion rate in cervico-vaginal mucus (Lai et al. 2007; Wang et al. 2008). Modification with PEG resulted in neutralization of the negative charge, thereby allowing these particles to diffuse readily in mucus gel when compared to their unmodified, negatively-charged counterparts (Wang et al. 2008). In rats given oral or i.n. immunizations with TT in PLA-PEG nanoparticles, it has been reported that the amount of TT recovered in blood, liver, draining lymph nodes and spleen was significantly higher than that in rats given non-PEGylated particles (Tobio et al. 1998; Tobio et al. 2000). It has also been reported that non-PEGylated particles aggregate strongly on contact with simulated digestive fluid (Tobio et al. 2000) or in aqueous solution containing lysozyme (Vila et al. 2004a). Vila et al. (2004b) also showed that i.n. delivery of TT-loaded PEGylated PLA microparticles in mice enhanced serum IgG and mucosal IgA responses when compared to soluble TT or TT encapsulated in non-PEGylated PLA particles. The authors have suggested that the enhanced immunogenicity of PEGylated particles may be due to decreased aggregation (Vila et al. 2004b).

Particles coated with PEG have also shown potential for mucosal delivery of DNA vaccines. Vila et al. (2002a) reported higher serum IgG antibody titers following i.n.

immunization of mice with a PLA-PEG encapsulated beta-galactosidase plasmid compared to those elicited by the plasmid alone. The PEG-coating of microparticles may also provide a basis for the attachment of ligands onto the particle surface. Garinot et al. (2007) attached integrin-binding, arginine-glycine-aspartate (RGD) peptides to PEGylated PLGA nanoparticles to successfully target human M cell-like epithelial cells in culture. Oral administration of these particles loaded with OVA induced an IgG antibody response in mice. However, RGD-labeling of PEGylated OVA-loaded particles did not produce significantly different OVA-specific IgG titers in serum when compared to the unlabeled particles (Garinot et al. 2007).

3.3.2 Chitosan

Chitosan has also been investigated for the surface modification of PLA/PLG microparticles because of its biocompatibility (Tharanathan and Kittur 2003), mucoadhesive properties (Singla and Chawla 2001) and ability to enhance the nasal absorption of drugs (Illum et al. 1994). In addition, chitosan has been reported to have immunostimulatory properties, including an ability to promote IL-1β and IL-18 cytokine secretion by immune cells (Li et al. 2008). A hydrophilic chitosan coating around nanoparticles was reported to enhance their resistance to aggregation (Vila et al. 2002b). The transport of TT across the nasal mucosa was enhanced by coating nanoparticles with chitosan, although this was less effective than PEG-coating (Vila et al. 2002b). Jaganathan and Vyas (2006) modified the surface of PLGA microspheres with chitosan to render them mucoadhesive and prolong their residence time in the nasal cavity. Intranasal immunization of mice with these surface-modified particles containing recombinant hepatitis B surface antigen (HBsAg) induced systemic and mucosal humoral immunity and cellular immune responses. After two i.n. immunizations, antigen-specific serum IgG antibodies were higher than those induced by chitosan-free HBsAg-particles, and they were comparable to the levels elicited by systemic immunization with an alum-adsorbed vaccine (Jaganathan and Vyas 2006).

3.3.3 Other Bioadhesive Strategies

In addition to chitosan and PEG, several other compounds have been evaluated recently for their ability to enhance the bioadhesive properties of microparticles for mucosal vaccine delivery. Salman et al. (2007) reported higher serum IgG1 and IgG2a titers and mucosal IgA antibodies following oral immunization of mice with thiamine-coated poly(anhydride) particles loaded with OVA, when compared to non-coated particles or to antigen in solution. Florindo et al. (2009) evaluated the immune responses induced after i.n. immunization of mice with PLA particles containing *Streptococcus equi* antigens which had been modified using a range of mucoadhesive polymers (glycol-chitosan and alginate) and absorption enhancers (spermine and oleic acid). Higher serum IgG titers were detected in mice

immunized with the particle formulations compared to free antigen. The greatest IgA responses were observed in the lungs of mice immunized with antigens encapsulated in particles modified with spermine (Florindo et al. 2009). However, in this study a large vaccine volume (50 μl) was administered to anaesthetized mice, which may have resulted in some of the vaccine reaching the lungs.

4 Microparticle Vaccines Based on Alternative Polymers

A number of alternative polymers to PLG and PLA have been evaluated for the encapsulation or adsorption of antigens for mucosal delivery. Biodegradable calcium phosphate (CaP) nanoparticles, generated by combining calcium chloride, sodium phosphate and sodium citrate, have shown potential as parenteral vaccine delivery systems (He et al. 2000). A Phase I study in human volunteers demonstrated the safety of CaP nanoparticles following subcutaneous administration (Morcol et al. 2004). Calcium phosphate particles may additionally act as a mucosal adjuvant (He et al. 2002). Intranasal or intravaginal (i.vag.) immunization of mice with a CaP-based nanoparticle formulation containing a herpes simplex virus-2 (HSV-2) glycoprotein induced greater antigen-specific mucosal IgG and IgA and serum IgG when compared to the antigen alone. Furthermore, mice immunized i.vag. with HSV-2-containing CaP nanoparticles were protected against i.vag. HSV-2 challenge, as assessed by a reduction in clinical pathology when compared to mice immunized with the HSV-2 glycoprotein alone (He et al. 2002).

Intranasal immunization of mice with 1–100 μm polymer-grafted starch microparticles containing entrapped human serum albumin has also induced greater systemic humoral and local cellular immune responses when compared to administration of soluble antigen (Heritage et al. 1998). However, in contrast to CaP or PLG, these microparticles are less well characterized and their safety in humans has not been evaluated. Ideally, in studies evaluating novel microparticles for mucosal vaccine delivery, the immune responses or protective efficacy should be compared to those induced by vaccines based on well-characterized microparticles such as PLG/PLA.

Micro/nanoparticles composed of chitosan have also been used for mucosal vaccine delivery. Oral immunization of mice with DNA nanoparticles, synthesized by complexing chitosan with plasmid DNA encoding a peanut allergen, induced higher secretory IgA and serum IgG2a antibodies and greater protection against allergen-induced hypersensitivity compared to mice immunized with naked DNA alone (Roy et al. 1999). The efficacy of chitosan microparticles for i.n. vaccine delivery was evaluated by Iqbal et al. (2003), who reported the induction of CTL and protection against respiratory syncytial virus infection in mice nasally immunized with chitosan microparticles loaded with DNA encoding respiratory syncytial viral proteins. Sub-lingual administration of OVA-loaded chitosan particles to OVA-sensitized mice was reported to reduce airway hypersensitivity,

eosinophil numbers in bronchoalveolar lavage and OVA-specific Th2-type responses in mediastinal lymph nodes (Saint-Lu et al. 2009). Interestingly, mucoadhesive particles formed from high molecular weight chitosan enhanced tolerance to a greater degree than particles consisting of low molecular weight polymers (Saint-Lu et al. 2009). Recently, the chitosan derivative *N*-trimethyl chitosan chloride (TMC), which has a better solubility profile than chitosan at physiological pH, was also shown to have potential as a mucosal vaccine delivery system. Oral immunization of mice with urease-loaded TMC nanoparticles generated greater serum IgG and intestinal IgA antibody responses when compared to urease antigen alone or urease co-administered with TMC solution (Chen et al. 2008). However, serum IgG titers induced by oral immunization with TMC/urease particles were weaker than those measured after systemic administration of the same vaccine (Chen et al. 2008). Many others have evaluated the efficacy of chitosan particles as mucosal vaccine delivery systems and this subject has been the focus of recent reviews (Bowman and Leong 2006; Kang et al. 2009).

5 Enhancing Particle Uptake Following Mucosal Delivery

The efficacy of microparticulate vaccines following oral or i.n. delivery in mice is partly due to their uptake into local lymphoid aggregates in the intestine or nasal cavity (Almeida and Alpar 1996; Beier and Gebert 1998). Detailed studies have shown that orally administered microparticles are preferentially taken up by specialized antigen-transporting epithelial cells called M cells, which overlie the Peyer's Patches (PP) lymphoid follicles in the small intestine (Jepson et al. 1993a; Jepson et al. 1993b). These M cells are characterized by a basolateral membrane that forms an intraepithelial pocket containing lymphocytes and phagocytic cells (Neutra and Kraehenbuhl 1992). Particulate vaccines taken up by M cells are subsequently transferred to underlying APC for presentation to T cells. Eldridge et al. (1990) demonstrated that microparticles of <10 μm are taken up by lymphoid tissue following oral delivery in mice. Larger particles (5–10 μm) were retained in the PP while smaller particles (<5 μm) left the PP and disseminated within cells (macrophages or DC) through the efferent lymphatics (Eldridge et al. 1990). Subsequently, other authors showed that the efficiency of particle absorption in the intestine increases with decreasing particle size (Damge et al. 1996; Desai et al. 1996; Jani et al. 1992; Sass et al. 1990). Using an in situ rat intestinal loop model, a significantly higher efficiency of uptake was reported for 100 nm PLG particles compared to 500 nm, 1 or 10 μm particles (Desai et al. 1996). Likewise, Jani et al. (1990) reported more efficient absorption of orally-administered polystyrene particles of less than 100 nm in diameter compared to larger particles in rats. However, increased particle uptake may not necessarily correlate with enhanced immune responses to encapsulated or associated antigens. Gutierro et al. (2002) reported that three oral immunizations of mice with bovine serum albumin in 1 μm PLG particles generated greater systemic IgG responses than smaller particles

(200 and 500 nm). In contrast, another study observed higher serum IgG and IgA antibody titers using 100 nm PVA-graft-PLG nanoparticles for the oral delivery of TT compared to particles of 500 nm or 1,500 nm diameter (Jung et al. 2001). Thus, other factors may play a role in the immunogenicity of microparticulate vaccines.

M cells similar to those in the intestine have also been reported in nasal-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT) in a range of species (Fujimura 2000; Spit et al. 1989; Tango et al. 2000). Particulate vaccines delivered to the nasal mucosa are also preferentially taken up by M cells, and eventually reach the cervical lymph nodes where an immune response is induced (Brooking et al. 2001; Fujimura et al. 2006; Heritage et al. 1998). Although studies evaluating the effect of particle size specifically on M cell uptake in vivo following i.n. delivery have not been reported, the size of particles has been shown to influence the general internalization of nasally delivered microparticle-associated antigens and the magnitude of subsequent immune responses. Using an in vivo rat model, Brooking et al. (2001) found that the transport of nasally-applied polystyrene nanoparticles into the bloodstream was size-dependent, with the highest particle uptake observed for 20 nm particles compared to 500 and 1,000 nm beads. Similarly, Vila et al. (2004a) evaluated the effects of PLA-PEG particle size (200–10 μm) on the transport of encapsulated TT across the rat mucosa and found enhanced protein absorption following i.n. administration of smaller nanoparticles. Jung et al. (2001) found that serum IgG and IgA antibody responses to encapsulated TT were greater following i.n. immunization of mice with smaller PVA-graft-PLG nanoparticles (approximately 500 nm) compared to larger-sized particles (>1 μm). However, immunization with even smaller 100 nm particles did not further enhance the antibody titers (Jung et al. 2001). A significant factor in assessing the uptake of microparticle-based vaccines by mucosal tissue and cells is the difficulty in producing PLG/PLA microparticles of a narrow size range using conventional manufacturing techniques. Therefore, the use of polystyrene microparticles, which can be produced with a very narrow size distribution, may be advantageous for these studies.

5.1 Targeting Microparticles to M Cells

While it is well-established that M cells can take up particulates, such as PLG microparticles, it is now becoming clear that in many cases only low levels of particle uptake occurs via these cells in vivo. Improved confocal microscopy techniques and detection methods and enhanced models for assessing particle uptake by M cells have suggested that the levels of uptake are lower than previously thought (Brayden 2001). McClean et al. (1998) reported that only approximately 10% of the total number of PLG particles administered to intestinal loops of rabbits and rats actually bound to the gastrointestinal tract wall. This suggests

that the majority of orally-delivered particles may become trapped in mucus, and that only a small fraction adsorb to epithelial cells, and an even smaller percentage will be translocated across M cells. This low-absorption efficiency may be the main reason why oral vaccines based on polymeric particles have not induced potent immunity. It is therefore possible that enhanced M cell targeting of particulate vaccines could enhance their efficacy. Particle uptake by lymphoid tissue may be enhanced by altering a range of parameters including particle size, surface charge and hydrophobicity (O'Hagan 1996) or by modifying the particle surface so as to allow specific interactions with mucosal cells (Fig. 1). This may be achieved by coating particles with molecules that selectively bind to M cell apical surfaces; including lectins, microbial adhesins and monoclonal antibodies.

5.1.1 Lectins for M Cell Targeting

Lectins are proteins or glycoproteins that recognize and bind reversibly to specific carbohydrates, some of which are expressed on the surface of M cells. Both *Ulex europaeus* agglutinin I (UEA-I), a lectin from the gorse plant *Ulex europaeus*, and *Aleuria aurantia* lectin (AAL) from the edible orange peel fungus have specificity for fucose residues expressed on apical membranes of murine M cells (Giannasca et al. 1994), and thus, have the potential to target microparticles to these cells in vivo. Covalent attachment of UEA-1 to 0.5 μm polystyrene microspheres resulted in specific targeting to M cells in PP of mice and rapid endocytosis of the particles after oral gavage or injection into ligated gut loops of mice (Foster et al. 1998). Similarly, targeting to murine M cells using a gut loop model or to human M-like cells in vitro has been reported after conjugation of UEA-1 to the surface of polymerized liposomes (Clark et al. 2001) or AAL coating of PLG particles (Roth-Walter et al. 2005), respectively.

A number of studies have assessed the ability of UEA-I to enhance immune responses to both soluble antigens and particle-associated antigens following mucosal administration in mice. Foster and Hirst (2005) reported enhanced levels of serum IgG and IgM antibodies after oral immunization of mice with latex particles coated with OVA and UEA-I compared to particles coated with OVA alone. Higher antigen-specific serum IgG and mucosal IgA antibody titers were also reported following oral immunization of mice with PLGA nanoparticles loaded with HBsAg and anchored with UEA-I compared to non-targeted particles (Gupta et al. 2007). Interestingly, in this study, mice orally immunized with particulate lectinized vaccine on three consecutive days and boosted 3 weeks later with the same formulation generated titers of antigen-specific serum IgG which were comparable to those in mice immunized twice by the intramuscular (i.m.) route with antigen and alum (Gupta et al. 2007).

The plant lectin, concavalin A (Con A), has also been investigated as an M cell targeting agent. Coating PLGA microparticles with this lectin enhanced particle uptake following oral administration in rats (Russell-Jones 2001). The induction of antiviral mucosal IgA responses has also been reported following i.vag. or i.n.

immunization of mice with Con A-conjugated polystyrene methacrylate particles coated with inactivated HIV-1 (Akagi et al. 2003; Kawamura et al. 2002). Moreover, i.n. immunization of macaques with similar carriers bearing inactivated chimeric simian/human immunodeficiency virus (SHIV) generated antigen-specific IgA and IgG antibodies in the genital tract and some protection after intravenous challenge with SHIV (Miyake et al. 2004). However, the non-biodegradability of these particles as well as the toxicity of Con A would prevent the clinical application of this delivery system.

The main limitations for the clinical use of lectins as targeting agents are concerns over toxicity and doubts regarding expression of their ligands on human M cells. It may be possible to avert toxicity issues by selecting lectins from edible fruits or plants, such as tomato lectin or AAL. However, edible lectins in mucosal vaccines could induce allergic responses due to their inherent immunogenicity. Alternatively, the component of the lectin directly responsible for M cell binding may be isolated. In a competitive binding assay, stable low molecular weight fragments of the UEA-1 lectin were identified that mimic the binding of UEA-I and may have potential to target drugs and vaccines to M cells (Hamashin et al. 2003). Lambkin et al. (2003) showed that polystyrene particles coated with one of these UEA-I mimetics adhered to murine M cells in vivo. Recently, Misumi et al. (2009) demonstrated by immunofluorescence that a peptide mimetic of UEA-I, called tetragalloyl lysine dendrimer (TGDK), was transported efficiently into rhesus macaque M cells in PP following intestinal inoculation. Furthermore, three oral immunizations of non-human primates with enteric-coated capsules containing a rhesus CCR5 cyclic peptide conjugated to TGDK induced antigen-specific IgA in feces, which inhibited SIV infection of a simian lymphocytic cell line in vitro. The TGDK dendrimer also binds to human M cell-like intestinal epithelial cells in an in vitro M cell culture model (Misumi et al. 2009).

5.1.2 M cell Targeting with Antibodies

Another strategy which may be used to enhance M cell absorption of particles is to coat them with antibodies that selectively recognize specific M cell surface antigens. The uptake of polystyrene particles (1 μm) by rabbit M cells was enhanced by coating with an antibody directed against an antigen expressed on M cells. In contrast, coating the particles with an isotype-matched monoclonal antibody of irrelevant specificity had no effect on particle uptake (Pappo et al. 1991).

A novel, particulate oral cholera vaccine has been developed by Nochi et al. (2007) using protein organelles (1–2 μm) of rice seeds expressing the B subunit of cholera toxin (MucoRice-CTB). MucoRice-CTB is stable at room temperature for over 18 months, obviating the requirement for cold-storage of the vaccine, and it is also resistant to the harsh environment of the gastrointestinal lumen. Oral immunization of mice with MucoRice-CTB induced antigen-specific serum IgG and mucosal IgA responses and protected against an oral challenge with cholera

toxin as assessed by a decrease in intestinal fluid levels (Nochi et al. 2007; Yuki et al. 2009). MucoRice-CTB was taken up by M cells after oral administration to mice (Nochi et al. 2007) and Kiyono et al. are now exploiting this by further developing a vaccine variant expressing a monoclonal antibody that specifically targets murine M cells (Cranage and Manoussaka 2009).

5.1.3 M cell Targeting using Microbial Adhesins

Enteric pathogens such as *Listeria monocytogenes* and *Yersinia pseudotuberculosis* naturally target M cells during invasion. The bacteria express adhesins at their surface which allow binding to and uptake by M cells (Kerr 1999). These adhesins are naturally resistant to proteolytic degradation and several studies have investigated these natural M cell pathways for the targeting of particulate delivery systems. Coating inert, carboxylated microparticles with the protein invasin from *Y. pseudotuberculosis* was reported to augment particle binding and uptake by canine epithelial kidney cells in vitro (Haltner et al. 1997). In addition, enhanced absorption of orally administered, latex nanoparticles across rat epithelium was shown after coating with the cell-binding fragment of the Yersinia invasin protein (Hussain and Florence 1998). Further studies are required to determine the safest and most effective M cell targeting agents to use in order to reliably enhance protective immunity to microparticle-associated antigens, particularly in larger animal models and in humans.

5.2 Targeting to Antigen-Presenting Cells

While numerous studies have demonstrated the importance of M cells in particle uptake following mucosal delivery, other cells such as enterocytes, macrophages and DC are also involved. Ligands suitable for targeting microparticles to enterocytes include tomato lectin (Florence et al. 1995; Naisbett and Woodley 1994) and microbial adhesins. The uptake of microparticles by phagocytic antigen-presenting cells (APC) has been reported on several occasions and it is likely that these cells are crucial for the induction of immunity to particle-associated antigens. Dendritic cells are critical in the activation of na T cells, and they have been shown to take up biodegradable PLGA particles directly both in vitro (Elamanchili et al. 2004) and in vivo following systemic delivery to mice (Lunsford et al. 2000; Newman et al. 2002). Latex particles were also shown to be taken up by immature DC after intravenous administration to rats (Matsuno et al. 1996). Furthermore, $CD11c^+$ DC in PP were reported to phagocytose latex beads (0.28 μm) after oral administration to mice (Shreedhar et al. 2003). An inverse relationship between particle size and the levels of uptake by DC has been reported (Reece et al. 2001). Foged et al. (2005) showed that human DC could internalize polystyrene particles between 0.04 and 15 μm in diameter, although larger beads (1–15 μm) were

phagocytosed by a smaller percentage of cells. Another study demonstrated enhanced IL-1β production, which was dependent on particle uptake, by bone marrow-derived DC incubated with smaller polystyrene particles (430 nm and 1 μm) compared to larger-sized particles (Sharp et al. 2009). Macrophages can also readily internalize PLG microparticles (Luzardo-Alvarez et al. 2005) and, as with DC, maximal uptake has been reported for smaller particles (<2 μm) (Tabata and Ikada 1988). In addition to particle size, the surface charge is also important for uptake. Cationic, polyamine-coated microparticles have been reported to be more efficiently internalized by APC than anionic, hydrophilic microparticles (Thiele et al. 2003). Targeting particles to APC may therefore be achieved by modifying their characteristics and also by coating the surface with specific biological moieties that can interact with these cells. In particular, a number of groups have reported enhanced immunogenicity of particulate vaccines that target DC following systemic delivery using monoclonal antibodies directed to DC surface receptors such as DEC205 (Kwon et al. 2005; van Broekhoven et al. 2004). It remains to be seen if targeting microparticulate vaccines to APC may also be beneficial for mucosal delivery.

6 Inclusion of Immunmostimulatory Agents in Microparticle Vaccines

Recent studies have shown that, contrary to what was previously thought, microparticles do not simply function as antigen delivery systems. They may also activate innate immune responses (Sharp et al. 2009). Both PLG and inert, polystyrene microparticles synergize with adjuvants such as lipopolysaccharide (LPS) to promote the secretion of the pro-inflammatory cytokine IL-1β by murine DC in vitro. This occurs through activation of a protein complex called the NLRP3 inflammasome (Sharp et al. 2009). Although microparticles can therefore have direct effects on the immune system, adjuvants may additionally be included with particulate delivery systems to further activate innate responses, and thus, enhance the magnitude of the adaptive immune response (Fig. 1). In particular, adjuvants derived from microbial compounds may stimulate APC directly, resulting in the secretion of inflammatory cytokines and the upregulation of co-stimulatory molecules on the cell surface. Addition of these adjuvants may also induce the migration of APC to the T cell area of the draining lymph node (O'Hagan and De Gregorio 2009). Microbial adjuvants or pathogen-associated molecular patterns (PAMP) engage receptors known as pathogen recognition receptors (PRR) expressed on cells such as DC and macrophages (Janeway and Medzhitov 2002). Toll-like receptors (TLR) are the best characterized group of PRR, and many microbial compounds which have demonstrated potential as vaccine adjuvants are TLR agonists (van Duin et al. 2006).

The beneficial effect of formulating TLR agonists in microparticles for systemic vaccine delivery has been demonstrated on numerous occasions. A potent immunostimulatory effect was reported by formulating anionic PLG microparticles, containing meningococcal or HIV antigens, with monophosphoryl lipid A (MPL) or synthetic forms (RC-529) of LPS. In particular, mice immunized i.p. with HIV gp120 protein adsorbed onto PLG microparticles in combination with microparticle-entrapped MPL or RC-529 generated higher IgG antibody titers than those immunized with particulate vaccines alone or with soluble forms of the adjuvants (Kazzaz et al. 2006). Strong CTL responses were generated by i.m. immunization of mice with anionic PLG microparticles containing adsorbed HIV-1 antigens in combination with CpG on the surface of cationic particles (Kazzaz et al. 2006). Expression of TLR has been reported on nasal (Dong et al. 2005) and lung epithelial cells (Muir et al. 2004) and also on gut epithelial cells (Cario et al. 2000; Chabot et al. 2006), M cells (Shimosato et al. 2005) and DC (Monteleone et al. 2008). Furthermore, TLR agonists have shown potential as adjuvants for the mucosal delivery of soluble antigens (Gallichan et al. 2001; McCluskie et al. 2000). However, to date, there is little evidence that co-entrapping TLR agonists in microparticles is beneficial for mucosal vaccine administration. Co-encapsulation of CpG with TT in alginate microparticles did not potentiate serum IgG or anti-toxin titers following i.n. administration in rabbits. However, the antigen-specific IgA in nasal lavage was higher in rabbits immunized with microparticles formulated with CpG compared to particles with TT alone (Tafaghodi et al. 2006). A recent study by Pun et al. (2009) did show some enhancement of antigen-specific IgG and IgA antibodies in sera (3–4 fold increases) and IgA in mucosal secretions following a single i.n. immunization of mice with CpG co-encapsulated in microparticles with HIV peptides compared to microparticles with peptides alone (Pun et al. 2009). It is likely that a second i.n. immunization would have increased antibody responses to an even greater extent.

In addition to TLR agonists, ligands for other receptors which are expressed on mucosal tissues and cells may have potential as mucosal adjuvants. One such ligand is mannose, which has high affinity for mannose-binding lectins expressed by lymphoid and non-lymphoid cells of various organs including the intestine (Wagner et al. 2003). Recently, it was shown that oral immunization of mice with 300–400 nm OVA-loaded poly(anhydride) particles coated with mannose or the TLR5 agonist flagellin elicited a stronger and more balanced IgG1 and IgG2a response than non-coated OVA-loaded particles (Salman et al. 2009). Furthermore, higher levels of OVA-specific intestinal IgA were detected in mice immunized with coated particles. Interestingly, in this study a single oral dose of OVA-loaded particles was sufficient for induction of an immune response (Salman et al. 2009).

In another study, lymphotactin, which has chemotactic activity for lymphocytes, was encapsulated in chitosan particles and co-administered intranasally to mice with chitosan microparticles loaded with DNA encoding a coxsackievirus B3 (CVB3) protein. In comparison to particulate DNA vaccine alone, immunization

with lymphotactin in chitosan particles significantly enhanced serum IgG and mucosal IgA antibody titers and promoted CVB3-specific CTL activity and Th1 type immunity as well as enhanced resistance to viral myocarditis after challenge (Yue et al. 2009).

7 Concluding Remarks

Recent clinical studies have demonstrated the feasibility and enhanced immunogenicity of systemic vaccines based on a combination of particulate adjuvants, such as alum, with TLR agonists or other immunostimulatory reagents. A recently licensed vaccine against human papillomavirus (Cervarix) and an improved hepatitis B vaccine (Fendrix™) contain alum and MPL as key components (Boland et al. 2004; Monie et al. 2008). Moreover, a candidate malaria vaccine, which was reported to provide protection against malaria in a Phase II clinical trial, is based on a combination of liposomes with both MPL and QS21 saponin (Bejon et al. 2008). It is likely that new generation subunit vaccines for mucosal delivery will also require combinations of particulates such as micro/nanoparticles with immunostimulatory compounds. Furthermore, targeting agents will probably be required to direct microparticle vaccines to mucosal inductive sites and to enhance their uptake. In recent years, modifications in the design of polymeric microparticle vaccines have enhanced immunogenicity in rodents and small animals. There is now a need to increase our understanding of the role of mucosal M cells, DC and other APC in particle uptake and in promoting mucosal innate responses in order to develop more efficacious microparticle-based vaccines for mucosal delivery to humans. This information will be vital to inform targeted approaches for particulate mucosal vaccines. The challenge remains to identify the best combination of adjuvants and/or targeting agents with particulates to promote optimal immune activation without the induction of adverse reactions.

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