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Exploiting receptor biology for oral vaccination with biodegradable particulates

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Abstract

The effective delivery of antigens via the oral route is an extremely desirable goal. Mucosal delivery of antigens stimulates mucosal and systemic immunity without affecting maternal antibodies and reduces the need for sterile needles or trained personnel. To date, there are very few commercially available oral vaccines and despite numerous reports in the scientific literature to show the success of biodegradable antigen carriers, none of these have achieved commercial status. Nevertheless, many studies have shown the great potential of biodegradable antigen carriers for oral vaccination in preclinical studies, but a more rational approach may be to specifically target antigen-loaded biodegradable microspheres to cells in the mucosal immune system which transport and process antigens for T cell recognition. Modern cell and molecular biology techniques have unearthed a wealth of information regarding important receptors involved in the capture of luminal antigens by microfold or membranous (M) cells and receptors on dendritic cells (DCs) which may allow future targeting of antigens to specific DC phenotypes, thus directing the immune response appropriately.

In this review, we consider the use of currently available biodegradable antigen carriers and speculate on how these may be improved to more efficiently target mucosal effector sites.
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Keywords: M cell; Oral vaccination; Dendritic cell; Biodegradable carriers; Mucosal immunity; Targeted delivery

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1. Introduction

The advent of mass vaccination programmes proved to be one of the greatest medical achievements of the 20th century. Most currently available vaccines require intravenous (i.v.) or subcutaneous (s.c.) delivery of antigens. However, over the past 15 years, numerous studies have assessed the potential of orally delivered antigens on the induction of mucosal and systemic immune responses. Mucosal delivery is the only vaccination route to induce effective B cell class switching and the development of secretory IgA-producing plasma cells. This is evident when comparing oral or s.c. administered poliomyelitis vaccines [1]. However, although the oral polio vaccine has advantages, such as a lack of a need for sterile needles, a reduced need for trained personnel and the possible induction of herd immunity in unvaccinated individuals, it also has disadvantages, such as the possible reversion to virulence and the possible induction of disease in immune-compromised populations reviewed [2]. Subunit vaccines which can be efficiently delivered to mucosal tissues, therefore, have great potential. However, subunit vaccines are less efficacious than live attenuated vaccines and they require adjuvants.

The gastrointestinal (GI) tract is a very hostile environment, with regions of low and high pH, high degradative (enzymic) activity and lipid solubilising ability (biliary phospholipids and bile salts). There is a tendency also for soluble antigens to induce tolerance rather than immunity. For these reasons, many laboratories have investigated the use of biodegradable antigen carriers which protects antigens in transit through the GI tract whilst delivering antigens in a particulate, rather than a soluble, form. Unfortunately, the vast majority of these studies have employed a “dope and hope” strategy whereby antigens encapsulated in biodegradable carriers are administered to laboratory animals by oral gavage, in the hope that a proportion of these will be transported through the intestinal epithelium and to immune effector sites. Few of these studies have reported that this method effectively protects animals against subsequent pathogen challenge and none of these studies have transferred to human volunteers.

In this review, we discuss the potential of biodegradable delivery systems for oral vaccination and speculate on how future studies may be improved by direct targeting of encapsulated antigen to cells involved in intestinal antigen sampling (M cells) and antigen-presenting cells (APCs), which are required for the development of mucosal immunity, in particular dendritic cells (DCs) residing in the subepithelial dome (SED) of Peyer’s patch (PP) tissue.

2. Mucosal antigen sampling and effector sites

Intestinal epithelium provides a barrier function which prevents easy access of pathogens to underlying tissue [3,4]. Absorptive enterocytes do not readily transport biodegradable microspheres but can take up and present soluble antigens, in conjunction

with MHCI and MHCII, to intraepithelial lymphocytes (IEL) [5–7]. However, since enterocytes do not express the costimulatory molecules needed to activate naive T cells [8], antigen processing by these enterocytes may result in suppression of T cell response.

2.1. Organized mucosal-associated lymphoid tissue (O-MALT)

O-MALT is critical to the induction of mucosal immunity in the intestine. The system is represented by diffuse and aggregated PP lymphoid follicles throughout the small and large intestines. These follicles are covered by a specialized follicle-associated epithelium (FAE) characterized by the presence of M cells which utilize pinocytosis or receptor-mediated endocytosis to transport particulate luminal antigens to underlying immunostimulating cells [9–12]. Having sparse microvilli and a thin glycocalyx, [4,13], M cells are anatomically suited to preferentially interact with antigens and recent studies have characterized a number of M cell apical surface receptors in various mammalian species. These include carbohydrate epitopes recognizing specific lectins in rabbits and mice [14,15], and rabbit mucin-like (muc2) epitopes [16]. The lectin *Ulex europeaus*-1 (UEA1) has proved particularly useful in identifying and targeting murine M cells [14,15]. In humans, carbohydrate lectin-binding receptors specific for M cells have yet to be identified, although Peyer's patch FAE (M cells and enterocytes) is identified by the specific binding of some lectins compared with adjacent villous tissues [14,15] (see Fig. 1). The sialyl-Lewis A antigen is expressed on human M cells [17], while other studies indicate the expression of intercellular adhesion molecule 1 (ICAM-1) by colonic M cells [18]. M cell surface receptors, as well as expression of cytoskeletal proteins, differ between species [14], and in mice, receptors even differ between different regions within the GI tract [19]. Thus, M cell receptors within a given species have probably evolved to ensure maximum antigen coverage.

Microbial pathogens have, however, evolved to exploit M cell receptors as a means of transport through the epithelial barrier [20,21], and in some case, this interaction has been identified as receptor/ligand binding. Studies by Baumler et al. [22] showed that *Salmonella typhimurium* utilize an adhesin (long polar fimbriae) to target murine M cells, whilst a *Yersinia* adhesin (invasin) also targets M cells [23] by interacting with M cell β_1 integrin localised to the apical membrane [24]. Recently, Helander et al. [25] have reported that type I reovirus specifically interacts with murine M cells via ligation of σ1 protein with α-2,-3 sialic acid glycoconjugate receptors. Polio virus appears to utilize CD155 receptors to interact with M cells [26], while the receptors on M cells recognized by human immunodeficiency virus type 1 (HIV-1) are yet to be determined, although studies by Fotopoulos et al. [27] using the Caco-2/B cell coculture M cell model have suggested that HIV-1 transport requires both lactosyl cerebroside and CXCR4 receptors but whether these are expressed in vivo by M cells awaits demonstration.

Characterization of M cell surface receptors, therefore, would be predicted to lead to the development of more rational oral vaccines, whereby the surface of biodegradable antigen carriers could be coated with ligands specific for M cell receptors. More detailed discussions of the surface features of M cells relevant to the targeting of oral vaccines are provided elsewhere [15,28,29].

2.2. The fate of M cell transported antigens

To date, there is no evidence to suggest that human M cells can present antigens to lymphoid cells. Studies by Brandtzaeg and Bjerke [30] showed that although human FAE expressed MHC II, M cells within the FAE were negative. In contrast, weak MHC II determinants and lysosomal compartments have been detected in rat M cells [31]. B cells can be activated by native antigen but T cells can only recognize processed antigen in conjunction with MHC I or II on the surface of antigen-presenting cells (APC). Macrophages and DCs are APCs which are present in PP domes. DCs are the most professional of all APCs with the ability to present antigens orders of magnitude greater than macrophages [32,33] and are also the most abundant APC in SED of PP tissue [34,35]. Further evidence to indicate that DCs are the first APC to encounter antigen in the SED can be seen by infection studies which have shown that *S. typhimurium* [36,37] and *Listeria monocytogenes*

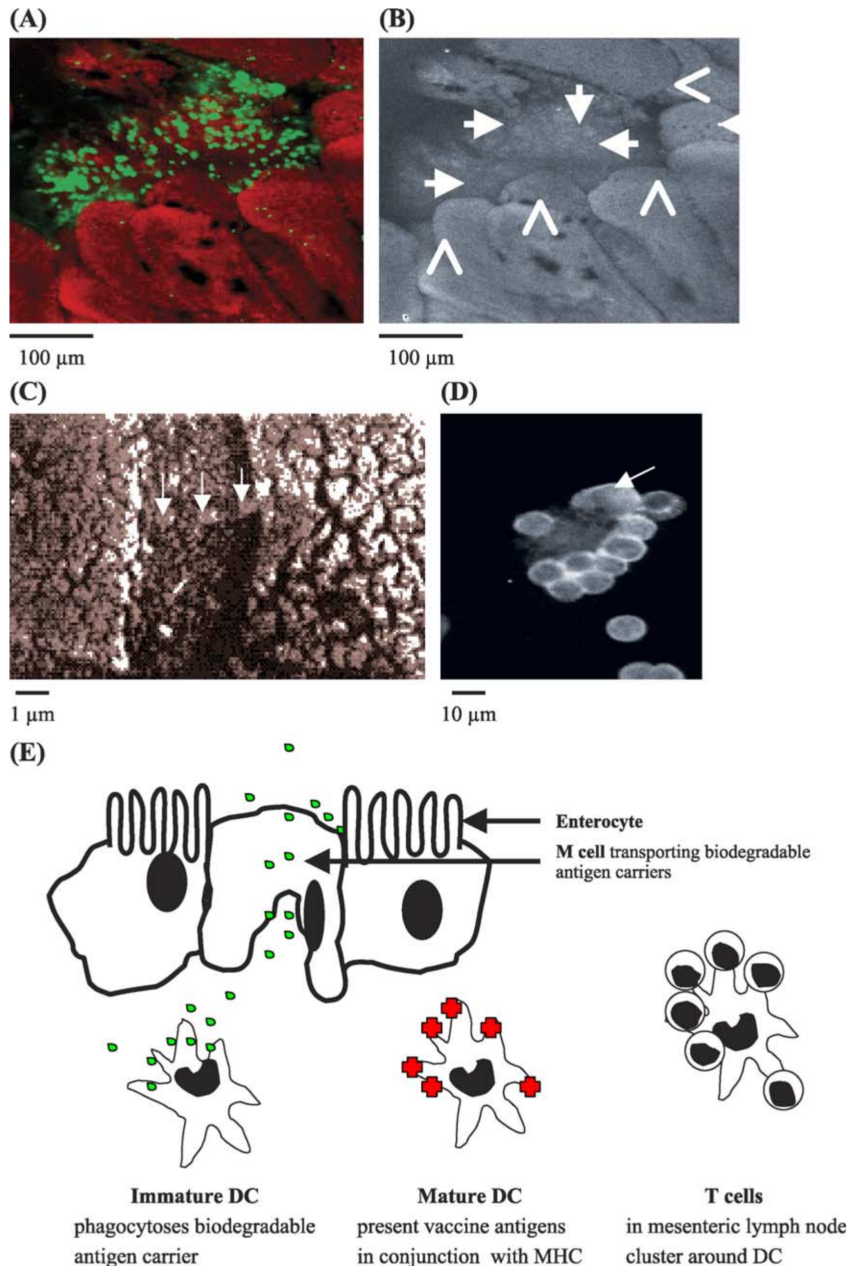

Fig. 1. The possible outcome of specific targeting of M cells by M cell-specific biodegradable antigen carriers. (A) Fluorescein conjugated lectin from Ulex europaeus 1 (UEA1) targeting Peyer's patch M cells in the murine terminal ileum. (B) Black and white image of (A) showing anatomical features of Peyer's patch follicle-associated epithelium (FAE) (arrows) and surrounding villi (arrow heads). (C) Scanning electron micrograph of UEA1-coated latex microspheres on the surface of an M cell which has characteristic short and sparse microvilli compared to the microvilli of the adjacent FAE enterocytes. (D) CD4+ T cells form large clusters around DCs in response to antigen presentation or in allogeneic reactions, large activated T blast cell arrowed. (E) Schematic showing possible scenario when M cell targeted biodegradable antigen carriers are delivered by oral inoculation. Targeting and transport of biodegradable antigen carriers through M cells delivers antigens directly to DCs in the SED region of the patch which present antigen while migrating to the draining lymph node, stimulating T cell clustering and activation.

[38] accumulate in SED DCs. An interesting study by Rescigno et al. [39] has also shown that DCs can acquire antigen directly from the intestinal lumen by pushing out dendrites through the epithelial tight junctions.

DCs in peripheral tissue are regarded as being immature antigen acquisition cells rather than immunocompetent cells; however, upon activation by antigen and/or cytokines (e.g., TNF-α), DCs begin to mature during their migration to the draining lymph node [40–43]. PPs also contain T cell areas and so the possibility remains that DCs may present antigen to T cells in either the PP or the draining lymph node. Studies by Kunkel et al. [44] have shown that whether DCs in PP or mesenteric lymph node (MLN) present antigen depends on antigen concentration. Following oral administration of FITC-labelled ovalbumin (OVA) in mice, these authors reported that low antigen dose (<1 mg) antigen was detected exclusively in PP and MLN DCs. At high dose (>1 mg), all types of APC presented antigen and were not retained in the PP or MLN, although presentation was still maximal in PP DCS. Interestingly, they also showed that PP-deficient mice (generated by IL-7 antibody) could still respond to antigen and DCs in MLN were still capable of presenting antigens to naïve T cells; however, what compensatory pathways operate in the model absence of PPs are unknown. DCs are the only APC capable of stimulating primed [45] and naïve [46] T cells. Interaction of DCs with naïve T cells may also polarize the T cell response, since human myeloid DCs (mDCs) drive T0 cells to become Th1 cells whilst plasmacytoid DCs (pDCs) drive T0 cells to a Th2 phenotype [47,48] and dendritic cells are regarded as key to controlling T cell responses in the gut wall [49]. Furthermore, a report by Mora et al. [50] indicates that antigen-loaded DCs mediate T cell homing back to the intestine by inducing high expression of $\alpha4\beta7$ integrin on T cells they have engaged.

DCs also have a profound effect on humoral immunity since CD40 mature DCs induce T cells to migrate to follicular B cell areas [51]. Production of IL-4 and TGF-β by these T cells are important in the induction B cell isotype switching to produce IgA [52,53]. However, DCs also interact with B cells to induce class switching and, unlike macrophages, they are able to retain native antigen which can then be released for B cell recognition up to 48 h later [54], whilst also providing additional survival and differentiation signals [55].

The importance of DCs in mucosal immunity therefore cannot be overestimated and mechanisms which promote uptake of biodegradable antigen carriers by immune-inducing DC phenotypes would further enhance the efficiency of oral vaccines. Recent observations indicate that there may be a novel receptor for IgA in the apical surface of murine (and possibly human) M cells [56] which may mediate the transport of sIgA from the gut lumen to the underlying lymphoid tissue. Following this transport, the IgA associates with, and is then internalised by, DCs in the subepithelium dome tissue [57]. Thus, simultaneous targeting of M cells and DCs may be considered a realistic proposition.

2.3. Toll-like receptors and dendritic cells

Phagocytic cells respond to 'foreign' inert particles as well as pathogens. Phagocytosis of microparticles, e.g., fluorescent microspheres, is the basis for the phagocytic index, a laboratory test to assess the efficiency of phagocytic cells. However, phagocytic cells recognize Pathogen Associated Molecular Patterns (PAMPS) via specialized pattern recognition receptors [58,59]. One group, the Toll-like receptors (TLRs), has received a great deal of attention over the past few years (reviewed in Ref. [60]). The relevant TLRs which respond to bacterial LPS are the best characterized and, in humans, TLR2, TLR4 and MD-2 (a TLR accessory protein) respond to bacterial LPS [61–63], whereas in mice, only TLR4/MD-2 is required for LPS responsiveness [64,65]. Recently, we have shown that murine TLR4 is required for controlled TLR2 activation in mice, and influences both the nature and number of granuloma produced in response to *Salmonella* [66]. Human DCs express a broad range of TLRs, but plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) differ in the TLRs they express and this would have a bearing on targeted delivery of antigen to TLRs. Human mDCs (CD11+ cells) express TLR4 as well as TLR3, which engage double-stranded RNA (dsRNA) [67], and TLR7, which respond to imidazoquinolines by producing IL-12 [68,69]. Human pDCs (CD123+ cells) express TLR7 and TLR9, the latter being ligated by unmethy-

lated bacterial DNA with an immunogenic CG motif (CpG) producing IFN-α [69–71]. Therefore, engagement of TLR by biodegradable carriers could possibly direct the immune response and further enhance the immune response to the antigen being carried to the DC.

Visintin et al. [72] have reported that although immature DCs (which would be present in steady state afferent lymph prior to vaccination) express TLR1–5, they do so at very low levels (possibly as low as a few hundred molecules per cell). This low-level expression may make targeting in vivo difficult. However, Lore et al. [69] have shown that CD4+ and CD8+ T cell responses to cytomegalovirus (CMV) or human immunodeficiency virus 1 (HIV-1) are significantly enhanced when DCs are exposed to antigen and TLR ligands. In these studies, the TLR ligands were being used as adjuvant, but an exciting possibility exists whereby relevant TLR ligands could be used to coat biodegradable microspheres which are carrying antigen. Therefore, a possible scenario could be the induction of pDCs by antigens entrapped in a biodegradable carrier which is coated with CpG. In theory, this may further enhance the immune response to the antigen by the induction of IFN-α via TLR9 ligation. To date, we are not aware of any such studies, although a number of laboratories have reported studies in which TLR ligand and antigen have been entrapped in biodegradable microparticles. These will be discussed in the relevant sections later in the review.

2.4. Immunity versus tolerance

Systemic unresponsiveness to orally delivered antigens [oral tolerance (OT)] may adversely affect oral vaccination or, conversely, could be used as a possible therapy in patients who hyperrespond to antigens or respond to normally innocuous environmental antigens (e.g., food allergens or commensal bacteria). The complexity of OT has been expertly reviewed elsewhere [73,74] and will not be repeated in this review. Briefly, orally delivered soluble antigens, without adjuvant, induce systemic tolerance due to T cell deletion, T cell anergy or the induction of suppressor T cells, depending on the concentration of the fed antigen [75].

Unfortunately, clinical trials attempting to use OT as a therapeutic in rheumatoid arthritis [76] and Type 1 diabetes [77] have reported no clinical benefit. Although the importance of thymic DCs in the induction of central tolerance during T cell development has been known for a number of years [78], there is increasing evidence that specific DC phenotypes may be involved in OT in the peripheral tissue [79,80]. Rat pseudoafferent lymph contains two distinct phenotypic DC populations [42,43]. DCs which express the signal regulatory protein-alpha (SIRP-α), which is recognized by OX41 antibody (OX41+ DCs) do not enter the T cell areas in MLN unless stimulated by antigen. However, OX41− cells constitutively transit between the villi and the MLN and transport apoptotic material to the T cell areas, thus indicating a role for OX41− DCs in peripheral tolerance [81].

Similarly, studies in mice have also indicated that a specific DC phenotype is involved in peripheral tolerance. Murine CD8+ DCs inhibit T cell IL-2 [82] and induce T cell apoptosis via FAS/FAS-ligand interaction [83]. Studies by Iyoda et al. [84] have also indicated an important role for CD8+ DCs in peripheral tolerance, with the observation that CD8+ DCs in murine spleen, lymph nodes and liver selectively phagocytose apoptotic material and present these in conjunction with MHCI and MHCII. Murine splenic CD8+ DCs specifically coexpress a type 4 lectin (Decalectin) receptor (DEC 205). Ligation of DEC 205 by antibody (α-DEC 205) conjugated to OVA induces an initial CD8+ T cell proliferation, but this is quickly followed by clonal deletion and unresponsiveness to subsequent OVA challenge [85]. This effect has been studied further by Mahnke et al. [86] who reported the induction of regulatory T cell (Treg) phenotypes (CD4+CD25+) by α-DEC 205/OVA-ligated DCs, which suppress IL-2 and subsequent CD4+ induced OVA hypersensitivity reactions. Normal CD4+ T cell responses were restored by removal of CD25+ T cells. However, if α-DEC 205/OVA was administered at the same time as CD40 antibody, CD8+ T cells produced large amounts of IL-2 and IFN-γ and responded to OVA rechallenge [85]. These results highlight the importance of a maturation signal for the activity of steady state DCs and the importance of CD40 in T cell polarization and CD8+ activation

[87], and are broadly in agreement with those of Vosters et al. [88], who have shown that immature DCs activate Treg while mature DCs suppress Treg. DEC 205 may, therefore, be a target for the induction of tolerance, in the absence of a further activation signal. Conversely, studies by Guermonprez et al. [89,90] have reported significant elevation of CTL responses to OVA if the antigen is conjugated to an adenylate cyclase toxin (cyaA) from *Bordetella pertussis.* This study also showed that $CD11b^{+}CD8\alpha^{-}CD11b^{high}$ DCs were the predominant APC phenotype activated by the antigen and is another example of DC targeting since CyaA toxin binds $\alpha M\beta 2$ integrin, which is selectively expressed on $CD11b^{+}CD8\alpha^{-}CD11b^{high}$ DCs, and it suggests that targeted delivery of antigens to DC receptors can be used to manipulate the immune response. It is mere speculation whether such studies will enable directed immune manipulation by antigen-loaded biodegradable carriers, coated with DC ligands which specifically target tolerogenic or immunogenic DC phenotypes. Two studies have suggested that uncoated biodegradable antigen carriers induce OT without further processing. Masuda et al. [91] reported the induction of OT in mice fed liposome entrapped OVA. However, in this study, the best liposome preparations were stable for only 30 min in model gastric juice. This probably suggests that the OVA used in this study was delivered in a soluble, rather than particulate, form. Pecquet et al. [92] also suggested that OT to β-lactoglobulin could be induced by oral administration in poly(lactide-co-glycolide) (PLG) microspheres and, somewhat anomalously, that this was more effective than soluble β-lactoglobulin.

Future studies, therefore, need to be done to ascertain whether DCs can be targeted in vivo to induce either OT or immunity. This need has been recognized by other reviewers [93].

3. Biodegradable antigen carriers in oral inoculation studies

Currently, a variety of different biodegradable antigen-carrier systems are being studied, in many laboratories throughout the world, for their ability to deliver efficiently oral antigens. These include: chitosan particles [94], polyacrylamide starch particles [95] and virosomes [96]. However, for the purpose of this review, we will only consider biodegradable PLG microspheres, liposomes and ISCOMS since these three groups have been extensively studied over a number of years.

3.1. Studies using model latex microspheres or empty poly (DL-lactide-co-glycolide) (PLG) microspheres and liposomes

Many studies have been performed to assess the kinetics of microsphere binding and uptake in the intestine. These studies indicate that microsphere size, polymer composition and surface chemistry are important factors for efficient M cell delivery and transcytosis. We have previously shown that hydrophobic, latex microspheres with a diameter of 0.5 μm selectively target rabbit M cells and are efficiently transcytosed in a gut loop model [96–98], but in a murine gut loop model, the same microspheres are not M cell specific and are transcytosed much less efficiently (M.A. Clark and B.H. Hirst, unpublished data). PLG microspheres also selectively bind to FAE M cells in gut loop models but it has been estimated that as few as 0.01% of the total applied bind to the gut wall [99]. Nevertheless, Shreedhar et al. [37] have shown that latex microspheres (0.28 μm) orally administered to mice are phagocytosed by CD11c+/CD11b−/CD8− DCs residing in the SED of PP tissue. Although this study was designed to use fluorescent microspheres to track DC migration following subsequent exposure to antigen, it shows that microspheres transported through M cells are phagocytosed by immunogenic DC populations (CD8−) but do not stimulate migration per se, unless an antigen is present. In vitro studies have shown that human DCs phagocytose PLG microspheres [100]. However, phagocytic efficiency is size dependent, with microspheres larger than 0.28 μm being less efficiently phagocytosed than smaller microspheres, although larger microspheres are still efficiently phagocytosed [101].

In summary, DCs can efficiently phagocytose microspheres both in vitro and, following M cell transport, in vivo, but the efficiency is size dependent.

3.2. Improving M cell targeting for more efficient uptake in the intestine

We have previously shown that latex microspheres (0.5 μm diameter) coated with UEA1, target murine ileal M cells in vivo with orders of magnitude greater efficiency than noncoated microspheres or microspheres coated with irrelevant proteins [102,28]. This leads to enhanced systemic distribution to organs, such as the liver [103]. Using a complimentary approach, Lambkin et al. [104] demonstrated similar targeting of latex microspheres with synthetic UEA1 analogues identified from combinatorial library screening for high-affinity fucose (the ligand for UEA1) binding. We subsequently showed that if microspheres were coated with both UEA1 and OVA (to target OVA to intestinal M cells), systemic humoral responses were elevated 10-fold in comparison with nontargeted OVA (Fig. 2). Furthermore, we found that high UEA1 coating/low OVA coating ratios still induced higher IgG titres than microspheres coated only with OVA (data not shown). Taken together, our studies and those of Shreedhar et al. [37] allow us to speculate that M cell targeted microspheres are subsequently phagocytosed by immune-inducing DC phenotypes in the SED of PP tissue. We have also shown that UEA1-coated liposomes, which are capable of entrapping antigen, also target murine M cells in vivo [28]. Although we have not, as yet, examined the effect of M cell targeting by antigen-loaded microspheres, two studies have shown that M cell targeting enhances antigen-specific immune responses. Wu et al. [105] used M cell specific reovirus protein (σ1) to target plasmid/reporter DNA (pCMV-luciferase and pCMV-galactosidase) to nasal M cells, although they did not show direct interaction with M cells, inoculation enhanced levels of sIgA, antigen-specific IgG and CTL responses. Recently, this group also showed that a significantly enhanced immune response (antibody and CTL) is achieved when mice are intranasally inoculated with HIV1 gp160 DNA conjugated to σ1 protein [106], although it should be added that the existence of M cells in nasal-associated lymphoid tissue is still controversial. The *Yersinia* adhesin, invasin, has also been utilized to improve intestinal uptake of latex microparticles [107], presumably through invasin-

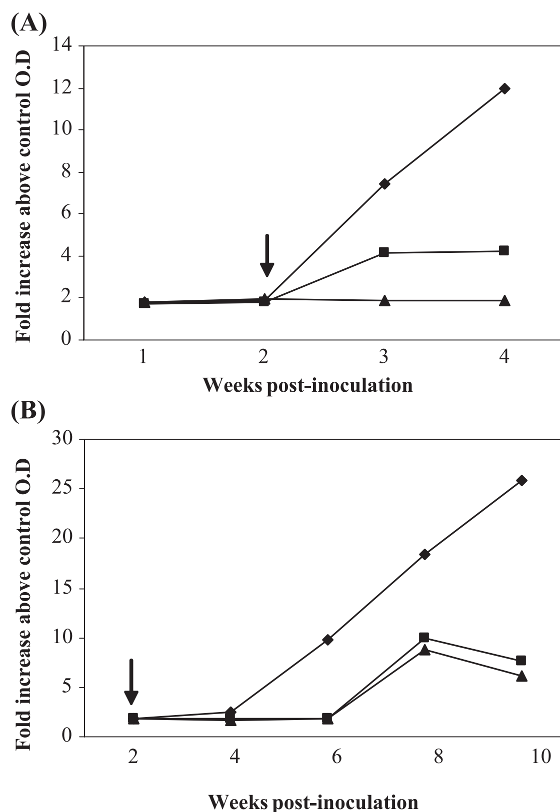

Fig. 2. Serum Ig responses in Balb/C SPF mice following oral inoculation with M cell targeted antigen or nontargeted antigen in particulate or soluble form. ■=Latex microspheres (0.5 μm) coated with 1 mg OVA only. ◆=Latex microspheres coated with 500 μg OVA+500 μg UEA1. ▲=1 mg soluble OVA. (A)=IgM titre and (B)=IgG titre measured as a fold increase in O.D. units above controls (serum taken from uninoculated mice). Two weeks after first inoculation, a booster inoculation was given (arrow). Five mice from each group were sacrificed at each time point and blood was pooled prior to measurement of antibody titres by ELISA analysis.

mediated interaction with β_1-integrin expressed on the apical membrane of M cells [24].

These studies are important if efficient, orally delivered, human vaccines are to be developed since human GALT FAE probably contains as few as 10% [9] or 5% [108] M cells.

3.3. PLG microspheres as antigen delivery vehicles

It is now more than 10 years since Challacombe et al. [109] reported significant elevation of antigen-

specific sIgA titres following oral delivery of antigen entrapped in biodegradable PLG microspheres. Contemporary studies by O'Hagan et al. [110] and Eldridge et al. [111] reported that adjuvanticity, due to PLG microspheres, was comparable to that induced by Freund's adjuvant. Subsequent studies, using OVA as a model antigen, entrapped in PLG microspheres reported elevated cellular and humoral immune responses, and the induction of immunological memory in mice following s.c. [112] or oral inoculation [113]. More studies followed which highlighted the potential of PLG microspheres in oral vaccination, using pathogenic antigens encountered in nature. Kim et al. [114] reported significantly increased sIgA and systemic IgG titres in mice, following oral inoculation with *Helicobacter pylori* whole cell lysate entrapped in PLG microspheres. Studies by Esparza and Kissel [115] showed that tetanus toxoid (TT) entrapped in PLG microspheres could also stimulate long-lasting immune responses. While Nellore et al. [116] and Singh et al. [117] both showed that Hepatitis B surface antigen (HBsAg) entrapped in PLG microspheres also elevated immunogenicity, although in these studies, inoculation was performed via the i.p. or i.m. routes, respectively. The latter study also indicated that one oral dose of entrapped HBsAg gave long-term protection equivalent to three doses of injected antigen, thus indicating that slow release of antigen from PLG microspheres may overcome the need for booster vaccinations. In vitro studies have also shown that cultured DCs take up antigen [100,118], and when human or murine DCs were cultured with Plasmodium or Influenza antigens entrapped in PLG microspheres, they stimulated significant CTL responses and continued to present antigen for up to 9 days [119]. This single dose booster effect was also reported when tetanus toxoid (TT) [120] and diphtheria toxoid (DT) [121,122] were incorporated into PLG microspheres prior to oral administration in laboratory rodents.

As discussed previously, delivery of an immunogenic dose of antigen to intestinal, mucosal inductive sites is more difficult to achieve than delivery by other routes. In 1997, Anton et al. [123] were able to show that endogenous gene products enhance MHC/peptide expression and following this study, many laboratories began to investigate the use of DNA vaccines. Luciferase and galactosidase expressing plasmids conjugated to σ1 rotavirus protein have been used to target M cells in nasal-associated lymphoid tissue, leading to antigen-specific humoral and cellular immune responses [98]. Using the same technique, Wang et al. [106] have recently reported increased, antigen-specific CTL and IFN-ã responses to HIV1 gp160 antigen compared to delivery of naked DNA to mice. These studies highlight two possible features of more efficient vaccines, M cell targeting and the ability to express multiple antigen copies in sub-M cell tissue. Delivery of antigen DNA via the oral route is particularly desirable since antigen DNA, together with a eukaryotic promoter, should induce expression of multiple antigen copies in APCs, whilst reducing or eliminating the need for booster vaccination. The potential of this method has been shown by many studies.

Jones et al. [124] first reported the successful induction of an antigen-specific mucosal immune response (sIgA) following oral delivery of PLG-encapsulated plasmid encoding luciferase DNA. When PLG entrapped rotavirus antigen VP6 [125] and VP7 DNA [126] were delivered orally to mice, both induced antigen-specific sIgA. The latter study went on to show that faecal rotavirus was also reduced following postvaccine rotavirus challenge. Studies have also shown that orally administered HIV1 antigen DNA, adsorbed onto PLG microspheres, stimulated murine sIgA and CTL responses [127] and that orally administered PLG encapsulated HIV1 DNA stimulated CTL activity and IFN-γ production in murine PP tissue and lamina propria [128]. These studies, therefore, indicate that very efficient oral vaccines could be developed if plasmids, encoding antigen DNA, are entrapped in PLG microspheres which have been coated with an M cell specific ligand.

However, very few studies have actually shown that antigens delivered orally in PLG microspheres induce a protective immune response if animals are subsequently challenged with pathogen. Studies of note are those by Reddin et al. [129], who reported protective immunity to *Y. pestis* following oral delivery of F1 antigen entrapped in PLG microspheres, and Allaoui-Attarki et al. [130], who

reported protection to *S. typhimurium* following oral delivery of *S. typhimurium* phosphorylcholine antigen to mice. No reported study has as yet shown that PLG-entrapped antigens induce protective immunity in humans. Tacket et al. [131] used orally administered *E. coli* colonization factor antigen II (CFA II) entrapped in PLG microspheres to study human immune responses. However, in the 10 volunteers examined, vaccine efficacy was only about 30%. A recent study by Katz et al. [132] has reported a significant elevation of IgA, IgG and antibody secreting cells in human volunteers, following orally administered CS6 (a common *E. coli* CFA) and pH buffer entrapped in PLG microspheres. Although, in this study, only 4–5 volunteers per group were tested (in a nonplacebo controlled trial) and were not subsequently challenged with pathogen, the study shows some potential and at least attempts to overcome internal pH lowering, one of the inherent difficulties when using PLG microspheres.

3.4. Designing rational PLG-encapsulated antigen delivery systems

Studies using bovine serum albumin entrapped in PLG microspheres have shown that microsphere degradation causes catalytic degradation of the entrapped protein when released into the media [133]. Yang and Cleland [134] also showed that although excellent levels of incorporation of IFN-ã could be achieved in PLG microspheres, only about 38% activity remained after 7 days. Degradation of PLG microspheres generates oligomers and monomers with carboxylic acid end groups which decrease pH at the polymer surface and in the pores and channels created during degradation [135–137]. This problem has been discussed in more detail in this journal [138]. Not all pathogenic antigens are acid sensitive. O'Hagan et al. [139] have shown that cholera toxin B subunit (CTB) is released intact from microspheres in vivo; however, as other antigens may show pH sensitivity, steps should be taken to either decrease oligomer/polymer buildup or inhibit pH decrease. These are reviewed in more depth by Gupta et al. [140], but include the use of flow-through systems to prevent oligomer/monomer buildup, the use of polymer ratios that allow for fast release of antigens or the incorporation of pH-buffering compounds.

Incorporation of adjuvants with antigen into PLG microspheres may also improve immunity and a number of laboratories have now begun to study the effect of these in animal models. Although studies have previously reported that PLG microspheres may themselves stimulate adjuvanticity comparable with that measured for Freund's adjuvant [110,112], Brayden [108] has argued that antigen entrapped in PLG microspheres may not induce elevated antibody responses in comparison with antigen alone. However, some studies have indicated that entrapped antigen/adjuvant may increase immunity. O'Hagan et al. [141] have shown that incorporation of MF59 adjuvant with HIV1 antigens (p24 gag) into PLG microspheres elevated humoral immune responses in baboons and both humoral and CTL responses in mice compared with MF59 and p24 gag alone. Similarly, Hunter et al. [142] reported elevated immune responses in mice following oral administration of Streptococcal polysaccharide antigen and CpG adjuvant entrapped in PLG microspheres, compared with nonentrapped antigen/adjuvant or PLG entrapped antigen without adjuvant. The use of TLR ligands as adjuvants (e.g., CpG ligates TLR9) may allow targeting of DC TLRs and potentially may significantly improve immunity. However, it may prove more beneficial to coat antigen-loaded PLG microspheres with TLR ligand rather than to entrap the ligand with the antigen since TLRs are found on the surface of DCs (as discussed in Section 2.3).

In conclusion, rational design of PLG microspheres which prevent acidic build up, the addition of buffered antigens (possibly plasmids incorporating antigen DNA) and TLR ligands (possibly adsorbed to the PLG surface) may all prove to induce more successful protective responses in future orally administered PLG vaccine studies.

3.5. Liposomes as antigen delivery vehicles

Polymerized liposomes have many characteristics which make them attractive antigen carriers. Firstly, they are in the nanometer size range which means that they can be transported by M cells. Secondly, liposomes formed by polymerization of 1, 2-bis

[(2E,4E)-Octadecadienoyl]-sn-glycero-3-phospho choline (DODPC) can be manipulated by incorporation of different lipid groups, thus adjusting surface charge and rigidity. DODPC liposomes also have carboxylate groups on their surface which could facilitate cell receptor targeting (reviewed in Ref. [143]). One problem of using liposomes, as opposed to PLG microspheres, is that lower amounts of antigen are incorporated into the liposomes and the incorporation is inherently more difficult.

However, studies have reported the induction of immune responses following oral administration of DT or TT entrapped in liposomes [144,145]. Tana et al. [146] have also reported *E. coli*-specific humoral immune responses in mice inoculated orally with *E. coli* 0157:H7 antigens entrapped in PS-liposomes formed from dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylserine and cholesterol. The sIgA produced by mice in these studies prevented *E. coli* adherence to Caco-2 cells. Incorporation of antigen DNA into lipodine liposomes has also been shown to induce antigen-specific CTL responses following subcutaneous inoculation [147] and sIgA responses via the oral route [148], indicating that DNA vaccination via liposomes may be as effective as DNA vaccination after incorporation into PLG microspheres, as discussed in the previous section of this review.

Specific cell targeting, using liposome encapsulated antigens, has also been investigated. Sugimoto et al. [149] and Fukasawa et al. [150] first showed that liposomes coated with mannopentose and dipalmitoylhosphatidylethanolamine (Man5-DPPE) could elicit strong cellular immune responses. The use of Man-DPPE in vaccination has more recently been investigated by Shimizu et al. [151], who showed that intraperitoneal injection of whole antigen from *Leishmania major* entrapped in Man-DPPE liposomes protected mice from subsequent (normally lethal) challenge with the parasite. TT and class 1 porin protein (PorA) from *Neisseria meningitides* were used by Copland et al. [152] and Arigita et al. [153], respectively, as antigens entrapped in mannosylated liposomes. In these studies, the ability of human and murine DCs to take up and present antigen were examined. Both studies showed that mannosylated liposomes effectively target C-type lectin receptors on the surface of DCs ex vivo and these DCs more effectively stimulated antigen-specific immune responses in comparison with nontargeted liposomes or soluble antigen. In light of these studies and our own studies [28] which show that UEA1-coated liposomes can be efficiently targeted to murine M cells in vivo, we may hypothesise that decorating mannosylated liposomes with M cell specific lectins (such that the targeting agents could be available to both M cell and DCs) may efficiently target orally delivered antigens to M cells and possibly DCs residing in the FAE.

3.6. Immune stimulating complexes (ISCOMS) as vaccine delivery vehicles

ISCOMS are small particles (~40 nm) made of Quil A (a potent adjuvant), cholesterol and phospholipids which can entrap multiple antigens (for a more in-depth review of ISCOM structure, see Ref. [143]). ISCOMS have previously been shown to be very effective antigen delivery vehicles by both oral and parenteral routes, stimulating Th1 and Th2 CD4+ activity, MHC I restricted CD8+ activity and sIgA [154–158]. Mowat et al. [159] constructed a gene fusion protein (CTA1-DD) formed from an enzymatically active cholera toxin subunit (CTA1) fused with a *Staphylococcus aureus* protein A subunit (D) (as adjuvant) and fused this to an antigenic OVA sequence. Mice were fed six times with the fusion protein entrapped in ISCOMS (each dose being equivalent to 750 ng OVA). Elevated serum IgG together with T cell proliferation and elevated production of IFN-γ was detected in the draining lymph nodes 7 days after inoculation indicating that ISCOMS are very efficient antigen delivery vehicles. Furrie et al. [160] have also shown that mice fed OVA in ISCOMS prior to feeding with tolerogenic doses of OVA had elevated OVA-specific antibody responses, indicating that ISCOMS may deviate the immune response to favour immunity rather than tolerance. In the latter study, the authors also showed that OVA incorporated into ISCOMS induced recruitment of DCs and macrophages into MLN and recruitment of macrophages and B cells into PP tissue. DC populations can be expanded in vivo by preinjection of mice with the cytokine flt-3 ligand (flt-3L) [161] and studies by Beacock-Sharp et al. [162] showed that when mice were injected with flt-3L prior to oral

vaccination with ISCOMS containing OVA, there was enhanced CD4+ and CD8+ T cell priming and migration of T cells into B cell areas of draining lymph nodes, thus indicating the importance of uptake of orally delivered ISCOM by DCs in vivo. This group also showed that in the spleens of animals inoculated with OVA entrapped in ISCOMS, only DCs had the capacity to stimulate resting, antigen-specific CD4+ T cells; neither macrophages nor B cells were able to do so [163]. As with most of the studies reported for PLG microspheres or liposomes, very few studies using ISCOMS have investigated their ability to protect against pathogens. Sixty percent protection was reported against *Eimeria falconis* in mice orally inoculated with p27 antigen prior to challenge [164]. Oral inoculation with influenza antigen entrapped in ISCOMS protected mice against subsequent challenge with homologous virus [165,166]. Mohamedi et al. [167] reported complete protection against Herpes simplex virus (HSV) in groups of mice orally inoculated with ISCOMS containing HSV-2 antigen. Protection against rotavirus, via orally delivered antigens in ISCOMs has also been reported in gnotobiotic lambs [168] and gnotobiotic pigs [169].

More studies, using ISCOMS loaded with pathogen antigens, need to be performed to fully assess their potential. However, it seems that ISCOMS may already be ideally suited to be efficiently phagocytosed by DCs. They already contain a potent adjuvant (Quil A) and protein antigens incorporated into ISCOMS probably do not suffer from the same exposure to low pH as occurs in PLG microspheres. The use of ISCOMS in vaccination may, therefore, be more advantageous than either PLG microspheres or liposomes. Whether targeting of ISCOMS, to M cells and/or DCs, is realistic and yields valuable immunisation benefits has yet to be explored. The use of ISCOMS as biodegradable carriers is more fully reviewed in this issue [170].

4. Commercial production of biodegradable microspheres for oral vaccination

Before any commercial production of antigen-loaded biodegradable microspheres for use in human vaccines is undertaken, more studies, using pathogen antigen-loaded biodegradable microspheres, need to be performed on animals and in human clinical trials. PLG microspheres, for use in drug delivery, are already produced commercially but to make use of receptor targeting these microspheres would have to be manufactured with at least one targeting agent incorporated (e.g., coated with an M cell targeting lectin) and each targeting agent would increase the production cost of the vaccine. Some characteristics of oral vaccines (e.g., no need for sterile needles and a reduced need for trained personnel to administer them) make them particularly attractive for use by developing countries. However, the addition of targeting agents may make them too expensive for such countries to use. Conversely, specific targeting of M cells and DCs may reduce the antigenic dose needed to induce protective immunity; which may reduce the overall cost of production of the vaccine. Whether targeted vaccines will prove to be too expensive or more cost effective will, of course, depend upon the cost of the antigen versus the cost of the cell targeting substance(s).

Studies indicate that the chemical make-up of ISCOMS make them natural adjuvants which are preferentially phagocytosed by DCs. ISCOMS may therefore prove to be a cheaper alternative to PLG microspheres or liposomes coated with cell targeting substances, although the addition of targeting moieties to ISCOMS may increase their efficiency further.

5. Conclusions

The increasing development of antibiotic resistance leads to the logical conclusion that vaccination may be paramount to the protection of human populations against infectious disease in the future. Development of oral vaccines is a very desirable goal which has many benefits over conventional parenteral vaccines. Thus, the utilization of biodegradable antigen carriers to deliver oral antigens to mucosal immune inductive sites has great potential. However, many more studies need to be performed to assess the ability of antigen-loaded biodegradable carriers not simply to lead to immune responses but

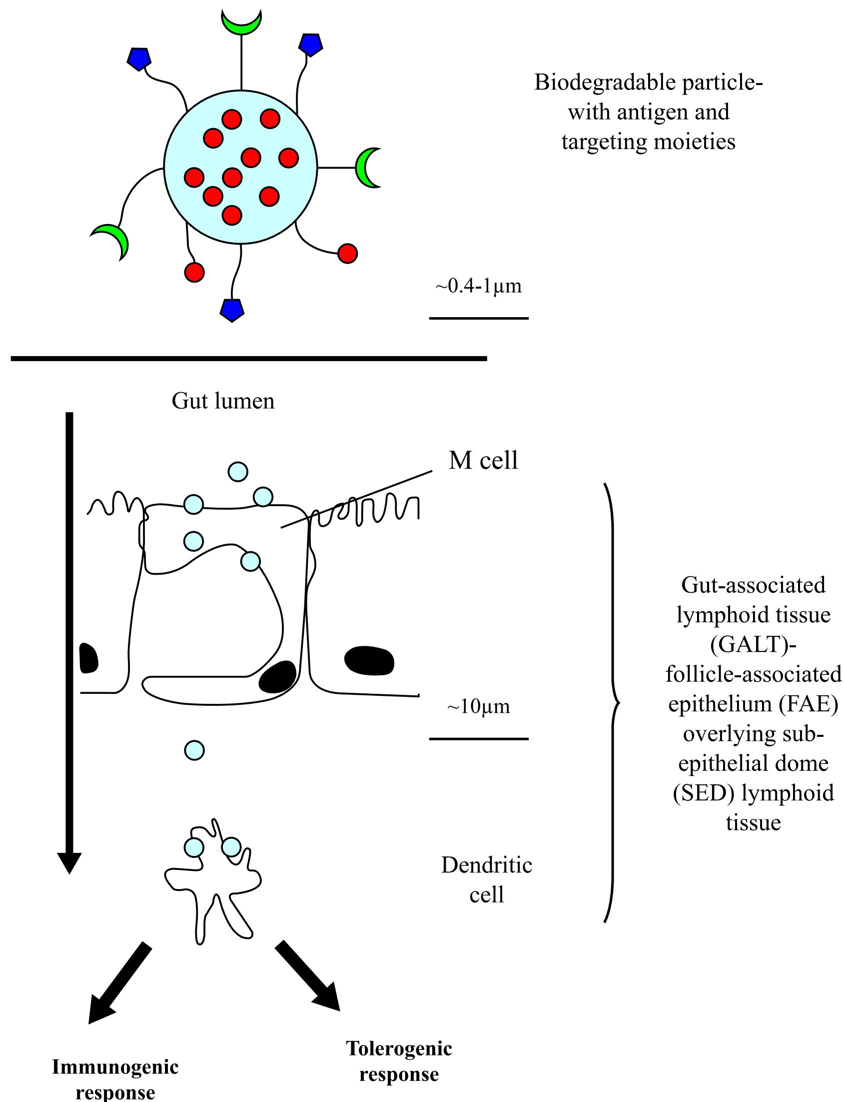

Fig. 3. Schematic illustrating basics of biodegradable particulate targeting for oral vaccination. Biodegradable particulates (light blue), loaded with antigen or DNA antigen (red symbols), may be targeted to M cells, within the FAE, and DCs with specific ligands. Ligands might be distinct for M cells (blue symbols) or DCs (green symbols), or the same ligand might target both M cells and DCs (e.g., sIgA). Antigen might be contained within the internal matrix of the particle, or additionally on the external surface (if resistant to degradation). Subsequent response might produce immunogenic vaccination, or therapeutic tolerogenic responses.

specifically to protect against disease challenges, before biodegradable carriers could be commercially considered. The developments of modern cell and molecular biology techniques have elucidated the role of many important cell surface receptors (Fig. 3). Gene array technologies may identify yet further novel cell surface receptors for such targeting [171,172] and a recent study has used phage display to characterize novel M cell receptors [173]. Targeting antigen-loaded biodegradable carriers to intestinal M cells, or at least FAE tissue, with the possibility of subsequent targeting to DC pheno-

types, could increase the efficiency of oral vaccines or alternatively may stimulate therapeutic oral tolerance (Fig. 3).

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