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Review

Generation of improved mucosal vaccines by induction of innate immunity

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Abstract. Vaccination is a highly effective means of disease prevention and has saved countless lives worldwide over the past 200 years. Traditional vaccines based on killed and attenuated organisms and inactivated toxins have constituted the majority of clinically used vaccines to date, but novel vaccines based on subunits of these organisms will be increasingly represented in future. In contrast to attenuated and whole cell vaccines, subunit vaccines do not generally contain immune-stimulatory components and are poorly immunogenic. As a result, new, potent and safe adjuvants and delivery systems are needed to enhance the immunogenicity of these vaccines. Furthermore, there is a drive to replace injected vaccines with those that can be administered by mucosal routes. Since the induction of innate immunity is crucial for vaccines to elicit potent antigen specific immune responses, a greater understanding of innate immunity at mucosal surfaces and the mechanism of adjuvants and delivery systems is required.

Key words. Vaccine; adjuvant; mucosal; dendritic cell; toll like receptor; delivery system.

Introduction

Aside from measures such as the provision of clean water and sanitation, vaccination represents the most powerful public health intervention to alleviate the impact of infectious diseases. However, despite the elimination of smallpox, the near eradication of polio and major reductions in the incidence of diseases including diphtheria, tetanus, whooping cough and measles, infectious diseases continue to impose tremendous mortality and morbidity across much of the globe [1]. Indeed it is estimated that infectious diseases account for almost 25% of deaths worldwide with a particularly high impact in developing countries [2]. Furthermore, outbreaks of diseases caused by organisms such as human immunodeficiency virus (HIV) , severe acute respiratory syndrome, Ebola and Nipah viruses illustrate that the threat from infectious diseases is difficult to anticipate while the risk of an avian influenza pandemic is ever-present [3–5].

The failure to date to produce effective vaccines against the chronic infections, tuberculosis (TB), malaria and HIV using the empirical approaches successful in the case of diseases including tetanus, polio and hepatitis B [6] has exposed a need to improve our understanding of the immune system and to produce 'rational' vaccines tailored to generate a specific immune response. While effective vaccines exist for a number of other common diseases, poor coverage in developing countries leads to significant mortality. The requirement for refrigeration to support a cold chain for heat-labile injectable vaccines is a major problem in poorer countries. Strategies are thus required to enhance the delivery and immunogenicity of vaccines in order to reduce the number of vaccinations required to elicit production, improve vaccine stability

(obviating a need for the cold chain) or enable mucosal vaccination. The principal focus of this review is to describe the mechanism of action of mucosal adjuvants and delivery systems in the context of their ability to induce innate immunity. In addition, the nature of traditional and subunit vaccines and the adaptive immune responses induced by mucosal administration of specific vaccines and adjuvants will be outlined. Although in many cases the mechanism of action of adjuvants and delivery systems is poorly understood, recent evidence suggests that effective systems must activate innate immunity either by themselves or in the presence of additional immunostimulatory factors.

From traditional to subunit vaccines

As a result of the significant reactogenicity problems associated with some 'traditional' vaccines (killed or live attenuated organisms and toxoids), there has been a shift away from this approach towards vaccines composed of purified subunits. The rationale is that the vaccine should comprise only those factors against which a protective immune response should ideally be elicited, while other factors responsible for side effects are eliminated. The subunits may be peptide or protein antigens, polysaccharides or DNA. This field has been revolutionised recently by the concept of 'reverse vaccinology' by which algorithms are run on the genome sequences of microbes in order to identify vaccine candidates [7]. This approach has led to the identification of vaccine candidates for serogroup B *Neisseria meningitidis* [8], and this genome-based approach is being applied to many other pathogens, including *Plasmodium falciparum* and *Yersinia pestis* [9]. These strategies will generate novel vaccine candidates for a number of diseases, but it is likely that as with most current subunit vaccines, their immunogenicity will be lower than that of traditional vaccines. This is a result of the presence in traditional vaccines of immunostimulatory pathogen-associated molecular patterns (PAMPS) such as lipopolysaccharide (LPS), peptidoglycan, flagellin and bacterial or viral nucleic acids, or to the live and/or particulate nature of the vaccines [10]. The absence of these immunostimulatory components in subunit vaccines has created a demand for factors that can safely enhance and direct vaccine-specific immune responses.

The nature of the immunostimulatory factors and delivery systems required for particular vaccines will vary depending on the route of delivery and nature of protective immunity required. Thus, adjuvants that induce strong cell mediated immunity will be required in vaccines for HIV, TB and malaria [11]. A principal objective of effective T cell vaccines is to induce long-lived memory $CD8^+$ T cells that can act rapidly to recognise, expand and eliminate infection [12]. Many currently used adjuvants are poor inducers of cell mediated immunity and cytotoric I lymphocyte (CTL) responses, so novel adjuvants and immunisation strategies are under study. Thus the shift away from traditional vaccines towards subunit vaccines necessitates the generation of safe, potent adjuvants and delivery systems that can enhance and direct vaccine-specific immunity. While adjuvant research has historically progressed on an empirical basis, there is now a greater understanding of the relationship between adjuvant properties and their immunostimulatory effects.

The importance of the vaccination route employed to induce immune responses

The majority of currently used vaccines are administered by intramuscular injection. This route of delivery ensures that the entire dose is delivered into the body and the vaccine formulation is administered intact into the muscle tissue. Furthermore, the damage induced by injection and deposition of the vaccine in the muscle may impact on the efficacy of the vaccine [13]. In contrast, in the case of mucosal vaccination, only a percentage of the dose will be transported across epithelial cells, particularly in the case of oral delivery. Furthermore, as a result of physical, chemical and enzymatic barriers the formulation may be significantly modified by the time it crosses the epithelium. Thus in addition to producing an effective protective vaccine, these delivery issues must be considered when contemplating a shift from injectable to mucosally administered vaccines.

Immune responses at mucosal surfaces

The majority of pathogens that infect humans do so via mucosal sites, principally the digestive, respiratory and genitourinary tracts. Thus effective vaccination strategies that induce immunity at these sites may prevent disease by interfering with pathogen colonisation and invasion before infection is established [14]. In the case of many bacterial, viral and parasitic diseases the primary site of infection is the gastrointestinal tract (GIT). The most effective means of protection against a disease initiated at a mucosal surface is the induction of a specific immune response at that site [14]. This is achieved most effectively by local delivery of the vaccine, since administration of vaccines by injection generally stimulates poor mucosal immune responses. The most convenient means to achieve this is via the oral route. A live attenuated oral polio vaccine has been successfully and safely used to immunise millions of children, demonstrating the feasibility of oral vaccination. Furthermore, oral vaccination would avoid the pain and discomfort associated with injections and eliminates the possibility of infections caused by

inadequately sterilised needles, or needle re-use, which is responsible for the transmission of infectious diseases. Oral vaccines are cheaper to administer since trained personnel are not required, and vaccine production is less expensive due to less stringent manufacturing conditions for orally administered products.

Despite the many advantages of oral vaccines, few are used and they are extremely difficult to develop [15]. The barriers in the gastrointestinal tract include proteolytic enzymes, bile salts, enterocyte tight junctions, microvilli, a thick layer of glycocalyx with digestive enzymes and carbohydrates and a layer of mucus incorporating vast numbers of bacteria and immunoglobulin (Ig) A [16]. The absorptive epithelium constitutes a single layer of gut epithelial cells joined by tight junctions, with a large surface area due to the presence of microvilli. Under normal circumstances epithelial cells, joined apically and basolaterally by tight junctions, are impervious to large macromolecules and even peptides [17].

The gut and indeed the respiratory immune system have to respond to antigen challenge while being largely unresponsive to food antigens, the microflora or inhaled environmental antigens [18]. As a result of the extreme difficulty in producing nonliving oral vaccines, much work has focused on the development of nasal vaccines which retain the advantages of oral vaccination in terms of inducing mucosal immunity but are not hampered to the same extent by physical and chemical barriers [14].

Mucosa-associated lymphoid tissue (MALT)

The lymphoid tissues associated with mucosal surfaces may be subdivided into inductive sites where antigens are encountered and responses are initiated, and effector sites where local immune responses occur. In the case of the digestive tract, the gut-associated lymphoid tissue (GALT) comprises individual cells and structures in the intestinal epithelium, Peyer's patches (PPs), appendix, lamina propria (LP) and mesenteric lymph nodes. PPs are lymphoid aggregates in the intestine and the principal mucosal inductive sites following oral vaccination. In addition, there are large numbers of smaller individual lymphoid follicles throughout the small intestine and colon. Beneath the PP dome epithelium is a network of immune cells including dendritic cells (DCs), macrophages and lymphocytes [19]. In the respiratory tract of rodents, the nasal-associated lymphoid tissue (NALT) in the nasopharynx is a principal inductive site [14]. The NALT comprises paired lymphoid tissue located at the floor of the nasal cavity and is lined by ciliated respiratory epithelium. The cellular composition of NALT is similar to that of the PP, although the relative numbers of B and T cells and T cell subtypes differ [20]. Lymphoid structures associated with the oronasal mucosa in humans are termed the Waldeyer's ring and consist of tonsils, lymphoid bands and the adenoids [21]. However, there is recent evidence to suggest that NALT is also present in children, which may support the use of nasal vaccination strategies for young children [22].

Uptake of antigens across mucosal surfaces

There are multiple routes for antigen uptake across the epithelia depending on factors including the size and physiochemical properties of the antigen and the mucosal site in question. Most work in this area has focused on the intestine, but similar processes operate in the respiratory tract. The intestinal PP lumenal epithelium has unique properties and is termed the follicle-associated epithelium (FAE), containing enterocytes, goblet cells and microfold (M) cells. Fewer mucus-secreting goblet cells are present at this site than over the villus epithelium [23], and the relatively sparse M cell glycocalyx facilitates interaction with vaccines and delivery systems [24]. Combined, these factors may allow greater contact between oral vaccines/delivery systems and the PP FAE than with the villus epithelium.

M cells contain small cytoplasmic vesicles and few lysosomes [25], the apical membrane expresses reduced levels of hydrolase activity than enterocytes [26] and proteolytic activity may be lower in intestinal PPs than in patch-free zones [27]. Uptake of delivery systems via M cells into the PP is likely to preserve antigenic and immunomodulator integrity since it may be released into the M cell 'pocket' [28], and antigens are thus unlikely to enter phagolysosomes [23]. The pocket is an invagination of the M cell basolateral membrane containing lymphocytes and other lymphoid cells. This capacity of M cells to endocytose and transport protein antigens and inert particles from the lumen into the pocket [29] where macrophages, DCs and lymphocytes are located provides an opportunity for mucosal vaccine delivery if effective systems can be designed.

In addition to their presence in the small intestine, lymphoid follicles have also been described in the caecum [30] and the distal colonic and rectal mucosa of humans [31, 32]. There are differences in M cell surface characteristics at these GIT sites so there may be potential to specifically target particular gut regions with appropriate ligands. The surface properties of enterocytes can also vary in different gut regions (e.g. surface properties of rectal and colonic epithelial cells differ from small intestinal cells). This may be exploited by delivering vaccines rectally, which avoids the low pH and highly proteolytic conditions in the upper digestive tract, and encouraging results have been reported after rectal delivery of antigen associated with lipsosomes [33] or cholera toxin [34]. Recent evidence indicates that individual intestinal villous M cells are present throughout the intestine [35], suggesting that M cell-mediated sampling may not occur exclusively in PPs or isolated lymphoid follicles. However, the overall significance and

immunological consequences of such uptake are unknown as yet.

The role of mucosal DC in vaccine uptake

In addition to the well-described role of M cells in antigen uptake, it is now clear that intraepithelial or lamina propria DC can directly sample lumenal contents by extending dendrites across the epithelium [36, 37]. DCs were shown to take up *Escherichia coli* and transport them to mesenteric lymph nodes by a process dependent on the chemokine receptor CX3CR1. Non-invasive bacteria were unable to cross the epithelial barrier in the absence of CX3CR1 [37], which may suggest a role for these cells in the uptake of killed bacterial vaccines or indeed inert delivery systems. It was further proposed that the CX3CR1-dependent and M cell-dependent systems could be associated with different subsets of DC. In addition to the concept of targeting M cells for vaccine delivery, it was recently suggested that if specific markers were identified, CX3CR1-positive lamina propria DC that sample materials from the lumen could be targeted to achieve specific vaccine delivery [37].

Induction of specific immune responses following mucosal vaccination

Antigen uptake, followed by processing and presentation by antigen presenting cells in the PP leads to activation of antigen-specific B and T cells. Under the PP dome area are follicles containing germinal centres where B cell division takes place and affinity maturation and B cell isotype switching from IgM to IgA occurs [38]. T cell-dependent zones adjacent to the B cell areas contain multiple T cell subsets but are principally $\alpha\beta$ TCR^+. Approximately 65% of these cells are $CD4^+CD8^-$ T helper cells and 30% are $CD4^-CD8^+$ cells containing CTL precursors [39]. Thus, all the cells required for the induction of cellular and humoral immune responses are present at the PP inductive site.

The inductive and effector regions are linked by a 'homing system' whereby antigen-specific cells activated in the MALT inductive sites migrate via the lymphatics and thoracic ducts to the circulation and subsequently 'seed' the mucosae [40, 41]. As a result, oral vaccination can induce humoral and cellular immune responses locally in the gut and at distant mucosal sites. However, there is significant compartmentalisation of effector responses at mucosal sites. Oral vaccination can induce strong antibody responses in the small intestine, ascending colon, mammary and salivary glands while nasal or tonsillar immunisation induces antibody responses in the upper airway mucosa and saliva and in the cervico-vaginal mucosal tissue [42]. The latter finding may be exploited to elicit immunity against sexually transmitted diseases. Effector responses include the induction of antibody (principally IgA)-secreting cells, T helper cell responses and specific cytotoxic T cells [14]. Indeed CTL induction has frequently been demonstrated after mucosal vaccination [43, 44].

Activation of lymphocytes in the PP leads to expression of $\alpha_4\beta_7$ integrin and migration to the blood via mesenteric lymph nodes and the thoracic ducts [45]. Specific homing of these cells to the gut is achieved by expression of the ligand MADCAM-1 on gut endothelial cells, allowing these cells to migrate from the blood into the lamina propria [45]. This process is strongly influenced by the local production of chemokines, particularly thymus-expressed chemokine (TECK) ligand expressed by small intestinal epithelial cells and expression of the CCR9 chemokine receptor [46]. While DC from PPs, peripheral lymph nodes and the spleen induced equivalent activation markers and effector activity in $CD8^+$ T cells, only PP DCs induced high levels of $\alpha_4\beta_7$ integrin expression, increased responsiveness to TECK and the ability to home to the small intestine [47]. DCs from the mesenteric lymph node but not the spleen induced $\alpha_4\beta_7$ integrin and CCR9 expression on T cells [40, 48] but for this to occur in vivo adjuvants were required [40]. DCs play a central role in responding to pathogens and their products and in the induction of antigen-specific immunity (fig. 1). It may be possible to exploit the finding that toll-like receptor (TLR)-mediated signalling by DCs can recruit T cells to the intestine by including appropriate ligands in mucosal vaccines.

Mucosal effector sites include the surfaces of the intestinal, respiratory and genitourinary tracts. At these sites the IgA^+ B cells differentiate into IgA plasma cells following interaction with antigen-specific T helper cells [49]. Dimeric and polymeric IgA is transported across epithelial cells into the lumen after binding to the secretory component [50]. Secretory IgA plays a major role in mucosal defence [51], and the presence of specific antibody in the gut (principally IgA) may prevent infection by gut pathogens. Indeed, it was recently shown that during its transcytosis through epithelial cells, HIV-specific IgA can prevent the replication of the HIV virus [52]. Mucosal but not peripheral B cells are driven into germinal centres through interaction of innate immune receptors with microbial antigens independent of B cell receptor specificity. This process requires T cells and recruitment depends on innate immune mechanisms [53]. Myeloid DCs in the Peyer's patch enhance IgA production by B cells via the production of interleukin (IL)-6 [54]. Additionally, there is recent evidence for the induction of functional mucosal IgA responses independent of germinal centres or T cell help and DC are implicated in these effects [55].

The nature of the immune response induced following mucosal vaccination depends on the nature of the antigen, the type of antigen-presenting cell involved and the local microenvironment [42]. In general mucosal immunisation

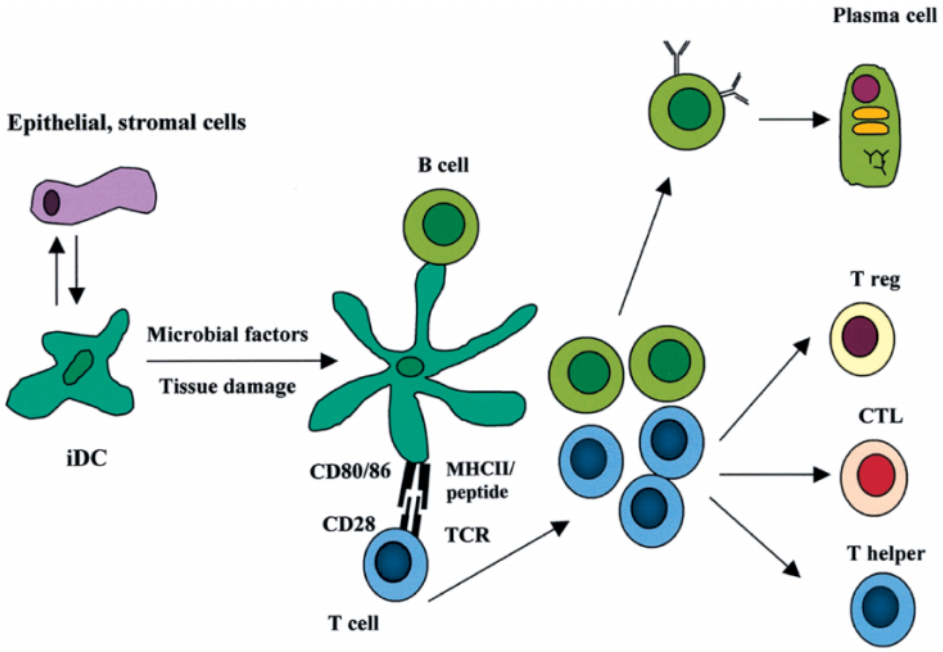

Figure 1. Activation of immature DCs (iDCs) by pathogen-derived and inflammatory factors results in maturation and secretion of T cell polarising cytokines. Interaction with B cells and subsequent T cell help can also activate B cell responses. As a result of these interactions, DCs can induce T helper cell responses in addition to cytotoxic T cells, regulatory T cells and B cell responses. The type of response induced depends on the DC, its interactions with neighboring cells and the nature of the pathogen derived or damage-associated signal.

with protein antigens leads to the induction of regulatory and/or Th2-type responses [56]. In contrast, pathogens such as *Salmonella* or TLR ligands can induce strong cellular and humoral immune responses and prevent the induction of mucosal tolerance to co-administered antigens [56, 57].

Mucosal DCs appear to preferentially induce Th2 cell differentiation [57] and induce B cells to secrete IgA [54]. Lung DCs found in both the parenchymal tissues and the epithelium of the conducting airways [58] express inducible costimulator ligand (ICOSL), secrete IL-10 and induce regulatory T cells in the absence of infection [59], but Th1 responses can be induced following viral infection [60]. Recent evidence also indicates that human gut DCs are predisposed to inducing Th2 responses [61]. Peyer's patches contain a unique population of $CD8\alpha^{-}CD11b^{-}$ DCs that principally produce IL-10 [56, 62]. Mucosal antigen delivery induced the secretion of IL-10 and transforming growth factor (TGF-β) by mesenteric lymph node and pulmonary DCs and elicited antigen-specific T cells producing IL-10 and TGF-β [63, 64]. Thus adjuvants that can induce Th1 responses following injection may not necessarily induce the same responses following mucosal delivery. There is now strong evidence that epithelial cells 'condition' DCs, resulting in cells that produce IL-6 and IL-10 but not IL-12 even in response to Th1-inducing pathogens [61]. While this indicates that Th1 responses may be difficult to induce following the interaction of mucosal vaccines with mucosal DCs, if adjuvants can recruit 'unconditioned' DCs from other sites, these may respond to the adjuvant/vaccine and induce protective Th1 responses [61] (fig. 2). Increased understanding of the properties of DCs at mucosal surfaces is shedding new light on the type of immunity induced by mucosal vaccination and offers the opportunity to devise novel strategies to exploit mucosal DCs in order to activate potent vaccine-specific immunity.

Mucosal tolerance

A frequent consequence of mucosal administration of antigens is the induction of mucosal tolerance [57]. This can be mediated via activation-induced cell death, anergy or the induction of regulatory T cells [42]. In recent years, the central role of regulatory T cells in the suppression of immunity at mucosal surfaces has become apparent. In addition to the induction of antigen-specific regulatory T cells that can suppress proliferation and cytokine production by Th1 cells [65], a naturally occurring population of $CD4^{+}CD25^{+}$ natural regulatory cells exerts a strong suppressive effect on mucosal T cell responses [66]. These cells may also confer suppressive activity on other $CD4^{+}$ T cells, a process termed infectious tolerance [67]. Regulatory T cells limit the strength of effector responses but also the attendant damage resulting from potent anti-microbial (or potentially vaccine-induced) responses [66]. There have also been suggestions that natural regulatory T cells can affect long-term memory cell responses [66]. Clearly the induction of a dominant regulatory or suppressive response is deleterious to the efficacy of vaccines, so adjuvants and delivery systems should elicit a strong effector:regulatory cell balance. The uptake of antigen by immature DCs from the lumen

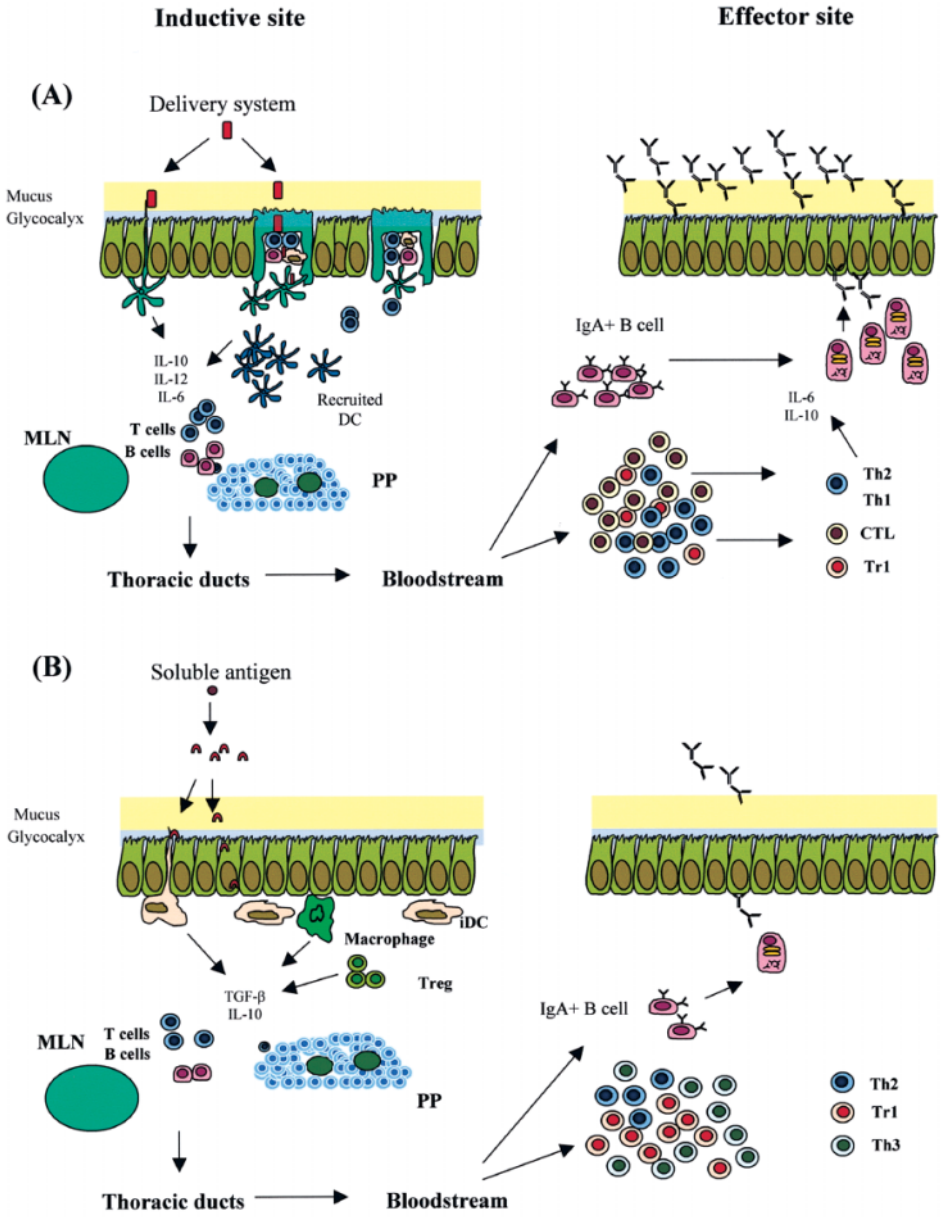

Figure 2. Proposed model for discrimination between the immunostimulatory properties of effective oral vaccine delivery systems/adjuvants and soluble antigens. (A) A hypothetical effective mucosal delivery system is presented that protects the antigen from destruction in the gut, targets it to M cells and DCs and contains immunostimulatory factors. This leads to the maturation and activation of local DCs and the induction of a local innate inflammatory response that recruits additional 'unconditioned' DCs to the site. These cells are also induced to mature and produce polarising cytokines, inducing an effector T and B cell response. This is manifested as strong Th1 and/or Th2 type cellular immunity together with the induction of vaccine-specific IgA that is secreted across the epithelium. It is also likely that specific regulatory T cells will be induced to a potent vaccine to regulate the response. (B) A soluble antigen is administered that is degraded by intestinal enzymes and taken up by enterocytes or by DCs. Presentation by local immature DCs and macrophages producing IL-10 and TGF-β leads to the induction of IL-10-producing Tr1 cells, TGF-β-producing Th3 cells and possibly a weak Th2 type response. In some cases a specific IgA response to the antigen may also be induced.

may lead to antigen presentation to T cells in the absence of co-stimulation, leading to T cell anergy, deletion or induction of regulatory T cells [68]. Indeed, targeting of antigens to immature DCs induces antigen-specific tolerance [69] while simultaneous activation with anti-CD40 antibody leads to specific immunity due to induction of co-stimulatory molecules on DCs [70]. Understanding the nature of immune homeostasis with regard to the effector: regulatory cell balance and the induction of local versus systemic immunity is crucial. For example, the commensal microflora is not ignored or tolerised by the immune system but induces IgA responses mediated by mucosal DCs [71].

The induction of tolerance by oral administration of antigen may potentially be exploited to treat allergies, inflammation and other conditions resulting from immune responses against food antigens or the gut flora. Protection was induced against experimental autoimmune encephalomyelitis (EAE) by feeding low doses of myelin basic protein [72]. This may be enhanced using carrier molecules such as cholera toxin B subunit (CTB). Mucosal delivery of antigen linked to CTB induced both strong mucosal secretory IgA responses and peripheral T cell hypo-reactivity. Mucosally induced uveitis was prevented by administration of a HSP60-derived peptide linked to CTB [73]. Furthermore, intranasal delivery of

a CTB-*Schistosoma mansoni* glutathione S-transferase conjugate protected animals from schistosomiasis. The results suggested that it may be possible to design a therapeutic vaccine against schistosomiasis that both limits infection and suppresses parasite-induced pathology [74]. While the it is conceivable that mucosal subunit vaccines may be used to treat autoimmune and inflammatory conditions by inducing mucosal tolerance, effective vaccines against infectious diseases must overcome mucosal tolerance that might be induced to a subunit vaccine when administered alone and elicit strong effector responses.

Receptor-mediated recognition of pathogens and adjuvants at mucosal surfaces

It has been suggested that recognition of conserved pathogen-derived factors by epithelial, endothelial and haematopoietic cells via TLRs is integral to the innate immune response against pathogens at sites of infection [75]. Pathogen recognition receptors on epithelial cells may directly interact with bacteria or toll-like receptor agonists [76]. Nucleotide-binding oligomerisation domain molecules (Nod1, Nod2) are present in the cytosol of epithelial and immune cells. Signalling through Nods or TLRs can activate transcription factors including NFκB, leading to pro-inflammatory gene expression [77]. Nod1 is expressed in intestinal epithelial cells and is required for recognition of a ligand present in Gram-negative bacterial peptidoglycan [78], while Nod2 recognises muramyl dipeptide, a component of Gram-negative and Gram-positive bacterial peptidoglycan [79]. In addition, epithelial cells express a wide range of TLRs [80], but the importance of this in response to vaccines and adjuvants is presently unclear. In contrast to DCs, TLRs may be compartmentalised on the basolateral surface or within epithelial cells [81]. Colonic epithelial cells respond poorly to PAMPS including LPS and TLR2 ligands; and it has been suggested that the cells may be cross-tolerised to multiple PAMPs due to constant exposure [82]. However, colonic epithelial cells express TLR5 and respond to pathogen-derived flagellin by producing inflammatory chemokines [83, 84] and there are suggestions that small intestinal epithelial cells are responsive to LPS [81]. Significantly, it was recently shown that the TLR adaptor molecule MyD88 was essential for the induction of LPS-dependent Th2 responses to intranasal antigen [85]. Results with pulmonary DCs were strikingly different from bone marrow-derived DCs in terms of their MyD88 dependence, again accentuating the importance of understanding the nature of innate immune responses at the mucosae for the design of improved vaccines.

In addition to regulated expression of these pathogen recognition receptors on epithelial cells, there is evidence that expression of TLRs may be low on lamina propria macrophages [86]. Certain inhibitors of TLR signalling are also expressed at a high level in epithelial cells that may serve to limit intestinal inflammatory responses [82]. A greater understanding of the responsiveness of epithelial cells and mucosal APC cells to various TLR ligands would aid in the design of appropriate adjuvants to enhance mucosal immune responses.

Mechanism of action of vaccine adjuvants and delivery systems

The appreciation in recent years that innate immunity is central to protection against infectious diseases and the induction of adaptive immune responses [87] has cast new light on the mechanism of action of adjuvants, based on their ability to elicit innate immunity and subsequently adaptive immune responses to associated vaccine antigens. Traditional vaccines such as the pertussis whole cell vaccine, BCG (Bacille Calmette-Guérin) and attenuated viral vaccines express TLR agonists and other factors that specifically active innate immune responses [88]. In particular, LPS present in whole cell Gram-negative bacteria and present at high doses in killed whole cell vaccines is a potent inducer of innate and adaptive immunity.

The DC is a principal mediator of these responses to TLR agonists and other pathogen-derived factors and adjuvants (fig. 1). An example of the potency of DCs in antigen presentation is the finding that HIV antigens loaded on DCs are immunogenic in patients with chronic HIV infection [89]. Immature DCs are highly phagocytic but on interaction with pathogen-derived and damage-associated factors undergo a process of ‘maturation’ involving the loss of endocytic and phagocytic receptors, increased expression of co-stimulatory molecules, morphological changes and alterations in lysosomal and major histocompatibility complex (MHC) class II compartments [90]. As a result of these changes, mature DCs are powerful initiators of adaptive immunity and are believed to be the only cells capable of activating naïve T cells.

Factors such as LPS and microbial nucleic acids associated with traditional vaccines lead to the maturation of DCs, upregulating the expression of MHC class II antigens and co-stimulatory molecules such as CD80, CD86 and CD40 on the DC cell surface [91]. Most immunostimulatory adjuvants also induce the secretion of pro-inflammatory and T helper cell polarising cytokines [75]. Indeed, it appears that both DC maturation and the production of pro-inflammatory cytokines are required for T cell activation in vivo [92]. Thus three signals are required to induce and polarise T cell responses: interaction between the MHC-peptide complex and the T cell receptor, engagement of co-stimulatory molecules with their cognate ligands and polarising cytokines. The

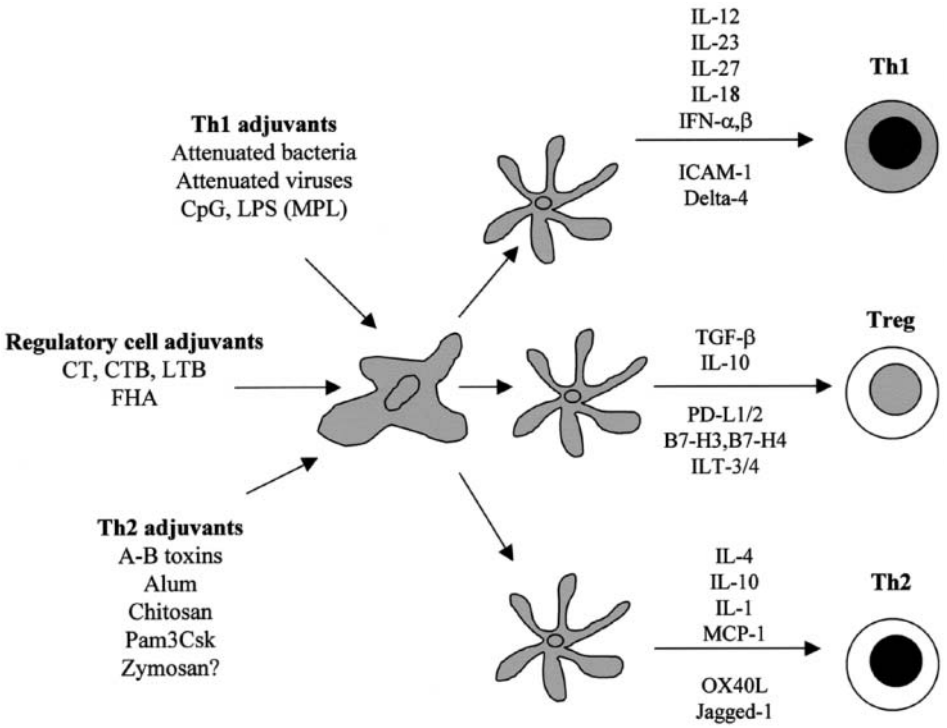

Figure 3. Different adjuvants and delivery systems can selectively modulate DC maturation and cytokine production and thereby influence the nature of the T cell response induced. The information is compiled from a number of references and reviews cited in the text and demonstrates that control may be exerted over the nature of the vaccine-specific T cell response induced by choosing the adjuvants and delivery systems used. Some molecules expressed on the DC surface and cytokine/chemokines proposed to play roles in the ability of DCs to induce particular T cell subsets are listed.

engagement of TLRs by microbial ligands can activate DCs and induce pathogen-specific Th1 type responses [91]. In addition to the well-defined role of IL-12 in the induction of Th1 responses, it is now clear that IL-27 and IL-23 also play important roles, indicating that the three cytokines play complementary roles in the induction and maintenance of Th1 responses [93]. Indeed, it was shown that peptidoglycan or commensal Gram-negative gut flora bacteria primed Th1-promoting DCs with a low capacity to produce IL-12 but a high capacity to produce IL-23 or IL-23 and IL-27 [94].

Induction of Th1 cell polarising factors including IL-12, IL-23 and IL-27 by DCs exposed to LPS and other TLR agonists such as unmethylated CpG oligonucleotides licenses these DCs to promote interferon (IFN)-γ-producing Th1 cells that activate macrophages and support B cell antibody production. Likewise, attenuated viral vaccines activate DCs and induce Th1 and CTL responses via the activation of type 1 interferons and IL-12. It has been proposed that the lack of IL-12 production by DCs in response to Th2 driving factors may result in Th2 responses [95]. However, it appears from recent studies that as with the induction of Th1 immunity by DCs, the induction of Th2 responses results from specific DC-T cell interactions rather than simply the absence of IL-12 or other Th1 polarising factors [96]. It is important to note that not all TLR agonists induce polarised Th1 responses. It has been proposed that TLR2 agonists can induce Th2 type responses [95, 97], but this does not appear to be a universal finding [98].

Thus the three signals required for activation and polarisation of T cell responses by DCs are induced by factors associated by traditional vaccines. It is likely that traditional vaccine efficacy relies on the presence of innate immune cell activators in addition to the vaccine antigen in a single package. Clearly, a PAMP-free recombinant antigen produced as a subunit vaccine will therefore be a very poor immunogen and innate immune cell activator in comparison.

The uptake of antigens lacking immunostimulatory activity, including a majority of subunit vaccines, will generally not result in DC maturation, and these DCs will present antigen to T cells without co-stimulation resulting in the generation of regulatory T cells or leading to T cell hypo-responsiveness or deletion [99, 100]. Recent data indicate that tolerance induction by un-stimulated/resting DCs depends on engagement of the T cell inhibitory receptors PD-1 and CTLA-4 [101], suggesting that these suppressive effects result from specific receptor-ligand interactions. In contrast, the inclusion of appropriate immunostimulatory adjuvants in vaccines will result in DC maturation with high levels of cell surface MHC-antigen complex and co-stimulatory molecule expression.

In addition to the role of immunostimulatory factors in dictating the nature of the ensuing immune response by activating DCs, there is also evidence that different subsets of DCs and DCs at different anatomical locations have particular capacities to drive T cell responses [102]. A comparative study found that liver DCs were relatively immature, captured less antigen and were less

effective stimulators of T cell responses than splenic DCs. It was suggested that this was mainly due to the presence of large populations of two subtypes of DCs in the liver that were not found in the spleen [103]. It has been proposed that splenic murine CD8α$^+$ DC prime naïve CD4$^+$ T cells towards Th1, while splenic CD8α$^-$ DCs prime for Th2 responses [104]. However, other data indicate that it is principally the microbial stimulus and not the DC subtype that dictates the type of T cell response induced [105]. Notwithstanding, there are differences in the expression of pathogen recognition receptors on DC subsets; for example, there is high expression of TLR9 on splenic plasmacytoid DCs, while TLR4 and -2 are expressed at a higher level on monocyte-derived DCs [106]. At present the relative degree to which the type/location of DCs and the nature of the activating stimulus dictates the ensuing adaptive immune response is uncertain. It is now clear, however, that DCs play a central role in the induction of vaccine-specific immunity and thus that effective adjuvants should target these cells. The route of vaccine administration can have a significant effect on the outcome of vaccination, so the nature of innate immunity at the various sites of vaccine delivery must be understood to enable induction of appropriate responses. The challenge is greatest in mucosal vaccination, in which case the induction of sufficiently potent and appropriate immunity is more difficult than with injected vaccines

Mucosal vaccine delivery systems and adjuvants

The difficulty of inducing strong immunity by mucosal and particularly oral vaccination is due to two properties of the mucosal immune system: the destruction and limited uptake of intact proteins and the mechanisms to limit local inflammatory responses [42]. Thus, adjuvants and delivery systems must protect antigens from destruction, promote their uptake across the mucosal epithelia and enhance their immunogenicity.

Multiple strategies have been used on a largely empirical basis for mucosal vaccine delivery with varying degrees of success. Depending on the mucosal route chosen, the precise requirements differ. For example, protection of labile antigens from proteolysis is essential in the case of oral delivery but less important for nasal administration. Imparting antigens with immunostimulatory characteristics and enhancing the extent of antigen uptake is important regardless of the route. A second promising area is the targeting of vaccines to particular mucosal regions or cells to enhance interaction with epithelial cells and to promote uptake. Many of the tested systems have been particulate in nature due to perceived immune-stimulatory properties and to the ability to target the FAE and protect entrapped antigens against degradation.

Mucosal adjuvants

A wide range of TLRs and other pathogen recognition receptor ligands are under study as mucosal adjuvants either alone or combined with delivery systems (table 1). The following sections briefly summarise findings on the use of TLR agonists and bacterial toxins as mucosal adjuvants.

LPS and MPL

LPS is a potent adjuvant when administered by parenteral and mucosal routes [85] but is too toxic for clinical use. To exploit the immunostimulatory properties of LPS and reduce its toxicity, monophosphoryl lipid A (MPL), a derivative of lipid A from *Salmonella enterica* serovar Minnesota, was produced. The adjuvant is widely used experimentally as a vaccine adjuvant [107], and clinical trials have been carried out with encouraging results [108]. Oral or nasal co-administration of MPL with vaccines enhanced the induction of mucosal and systemic antibody responses [109, 110], but the adjuvant is more

Table 1. Overview of the range of receptors on antigen-presenting cells and epithelial cells that can interact with microbial derived adjuvants.

Pattern-recognition receptor	Adjuvant
TLR1 (TLR1/2,TLR1/6) [115, 116]	bacterial lipoproteins
TLR2 [102, 209]	Pam3Csk, zymosan
TLR3 [210]	Poly: IC
TLR4 [81]	LPS, MPL
TLR5 [83, 84]	flagellin
TLR6 [115, 116]	lipotechoic acid, MALP-2
TLR7 [211]	Single-stranded RNA, R-837, R-848
TLR 8 [211]	single-stranded RNA, R-848
TLR 9 [112]	CpG oligonucleotides
NOD 1 [78]	peptidoglycan-derived GM-tri-DAP muropeptide
NOD 2 [79]	muramyl dipeptide
Macrophage mannose receptor [212]	mannan
Dectin 1 [209]	-1,3- and -1,6-linked glucans
DC-SIGN [213]	mycobacterial mannosylated lipoarabinomannan (ManLAM)
L-SIGN [213]	ManLAM
SIGN-R1 [212, 213]	zymosan, ManLAM
Complement receptors [214]	Bordetella pertussis filamentous haemagglutinin, adenylate cyclase toxin

In many cases two or more receptors are involved in recognition and cellular activation by microbial factors, e.g. zymosan. References cited include original papers and reviews describing the use of the molecules as adjuvants and their interactions with receptors.

effective when combined with liposomes [111] or used in parenteral prime-mucosal boost regimes [109].

CpG oligonucleotides

Synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs are ligands for TLR9, stimulate human B cells and plasmacytoid DCs (pDCs) and promote Th1 responses and pro-inflammatory cytokines [112]. CpG is a strong adjuvant for vaccines administered by various mucosal routes [113]. Recent work on novel immunomodulatory oligonucleotides with greater stability in the digestive tract demonstrated enhanced efficacy compared with standard CpG oligonucloetides following oral delivery [114].

Other TLR agonists

The explosion in information on TLRs and their agonists and particularly the generation of synthetic TLR agonists is providing a range of novel adjuvants with potential in mucosal vaccines. A number of these molecules are under study, and some are showing significant potential either when used alone or together with other adjuvants or delivery systems. Mycoplasma-derived lipopeptide MALP-2, a ligand for TLR2/6 heterodimers, enhanced mucosal and systemic humoral and cellular responses to antigens when nasally co-administered [115, 116]. It is likely that the efficacy of these factors can be significantly enhanced if combined with delivery systems such as microparticles and liposomes.

Cholera toxin and Escherichia coli heat-labile enterotoxin

The AB toxins cholera toxin (CT) from *Vibrio cholerae* and heat-labile enterotoxin (LT) from enterotoxigenic strains of *E. coli* are potent mucosal immunogens. Both toxins are composed of an enzymatically active A subunit with adenosine-diphosphate (ADP)-ribosyltransferase activity, which is responsible for their toxicity, and a pentameric B oligomer that binds to receptors on the eukaryotic cell surface [117]. CTB and LTB bind with high affinity to the glycosphingolipid GM1-ganglioside that is present on the surfaces of cells [118]. There is evidence that LTB also binds to other glycosphingolipids (asialo-GM1), glycoprotein receptors, polyglycosilceramides and paragloboside [117]. The induction of cyclic AMP (cAMP) by these toxins has been proposed as a dominant factor in their immunomodulatory effects, although the finding that non-toxic derivatives and isolated B subunits of the toxins retain modulatory effects indicates that both cAMP-dependent and independent factors play a role in the potent effects of these molecules [119]. Co-administration of CT or LT with antigen via the nasal, oral or other mucosal routes potently enhances antigen-specific mucosal and serum antibody and cellular immune responses. Most studies indicate that CT induces a Th2-biased response to itself and to bystander antigens, while LT enhances the induction of Th1 and Th2 cells to antigens delivered by mucosal routes, but unlike CT does not enhance IgE responses [120]. It was recently shown that in addition to the induction of typical Th2-type cells, CT induced a population of IL-10-secreting T cells that did not secrete IL-4 and exhibited suppressive effects on Th1 cell proliferation and cytokine production [121]. CT and LT can also potentiate antigen-specific class I restricted CTL responses to nasally co-administered antigens [122].

The B subunits of these toxins can enhance immune responses to antigen mixed with or directly conjugated to the subunits and delivered by mucosal routes [123], but most evidence indicates that the B subunits are significantly less potent than the active toxins. However, the B subunits may have significant potential as carrier molecules, and in this context have been shown to enhance immune responses to conjugated antigens in both experimental animals [124] and in humans [125].

To address the significant toxicity issues associated with the use of the native toxins, mutants have been generated, principally by eliminating or reducing the enzyme activity of the A subunit [119]. Site-directed mutants generated by replacing amino acids at the active site (LTR72) or in the proteolytically sensitive region of the biologically active domain (LTR192G) that retain partial activity are significantly less toxic than the wild type but exhibit comparable or even superior adjuvant activity. Although weaker than the wild type and partially active toxins, mutants lacking ADP-ribosyltransferase activity (e. g. LTK63) also retain strong mucosal adjuvant properties [126]. An alternative approach was the use of a fusion of the intact CT A1 subunit with a dimer of an Ig-binding fragment D from *Staphylococcus aureus* protein A (CTA1-DD) [127]. CTA1-DD is an effective mucosal adjuvant and in contrast to wild-type CT or CTB did not bind to or accumulate in the nervous tissues of the olfactory bulb [128]. However, the documented interaction of nasally administered GM1-binding toxins with neuronal tissues [129, 130] is a serious concern and must be fully addressed. This concern should also be addressed in the case of other adjuvants administered via the nasal route.

Mucosal immunisation with vaccines formulated with LTK63 as adjuvant induced protective immunity against infection with *Helicobacter pylori* following oral immunisation together with recombinant VacA, urease and CagA antigens [131]. Intranasal immunisation with polysaccharide or protein subunit vaccines formulated with LTR72 or LTK63 also induced protection against invasive pneumococcal [132] and *Bordetella pertussis* [133] infections, respectively. Mucosal CTLs specific for simian immunodeficiency virus were induced by intrarectal immunisation of macaques with a synthetic-peptide vaccine incorporating LT (R192G) as adjuvant [134]. Mucosal delivery of cytokines can also strongly

enhance local vaccine–specific CTL responses [135]. Furthermore, combining adjuvants with cytokines may enhance their efficacy. For example, rectal immunisation with an HIV peptide together with an combination of IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF) and CT induced a more potent mucosal CTL response and protection against viral transmission than administration of CT alone [136]. The increased efficacy of CT in the presence of IL-12 may reflect the ability of the toxin to inhibit IL-12 production [121, 137]. In the same study it was found that the mutant LT, LT (R192G), was as effective at inducing CTL alone as CT was when combined with IL-12. This is likely a result of the less Th2-biased response induced by LT(R192G), and indeed wild-type LT compared with CT [136, 138].

The efficacy of these bacterial toxins as mucosal adjuvants is likely a result of multiple factors, including increased epithelial permeability, activation of antigen-presenting cells and modulation of T cell cytokine production [117]. Oral delivery of CT in mice increased the number of DCs in the follicle-associated epithelium and redirected DCs from the sub-epithelial dome of the PP to the B cell follicles and parafollicular T cell zones [28]. Of the mucosal adjuvants tested to date, derivatives of CT and LT appear to hold most promise. This is likely a result of their relative stability, targeted interaction with epithelial and DCs and immune-stimulatory properties. If recent concerns regarding potential neurotoxicity can be addressed, these molecules may still have significant potential in mucosal vaccination.

Mucosal delivery systems

Alum

Although alum is not used for mucosal delivery, it is the archetypal vaccine delivery system and remains the gold standard due to its universal clinical application. Alum is the most widely used vaccine adjuvant and comprises aluminium salts. Alum has been used as a vaccine adjuvant for more than 70 years and has a history of efficacy and safety with vaccines, including tetanus, hepatitis B and hepatitis A. Alum is an effective inducer of humoral immunity and Th2-biased $CD4^+$ T cell responses [139]. The mechanism by which alum acts is not yet fully understood. The provision of a 'depot effect' allowing sustained presentation of antigen to immune cells and the conversion of soluble antigens into a particulate form is clearly a factor, but the induction of Th2-biased T cell responses indicates that alum exerts a selective immunomodulatory effect. A deposit is formed on injection of alum into tissue, leading to tissue damage, phagocytosis by antigen-presenting cells and possibly the release of danger signals [140]. Furthermore, it has been shown that phagocytosed alum increases the survival of macrophages [141]. Similar findings were found with a number of other particulate adjuvants, including oil-in-water emulsions and calcium phosphate, suggesting that this process may contribute to the efficacy of particulate adjuvants. Exposure to human PBMC increased expression of MHC class II, CD40, CD54, CD58, CD83 and CD86 on the monocyte cell surface and the appearance of cells in the cultures with dendritic morphology [142]. It was further suggested that alum elicited the production of cytokines that led to IL-4 production by T cells. Injection of alum led to priming of splenic B cells for MHC class II signalling and the accumulation of a population of IL-4-producing Gr1+ cells required for in vivo priming and expansion of antigen-specific B cells and optimal antibody production [143]. This priming effect was not noted with Freund's complete adjuvant, although it did elicit recruitment of the Gr1+ cells to the spleen, indicating that a subset of these cells selectively produces IL-4 after alum administration. These studies clearly demonstrate that alum exhibits very specific immunomodulatory properties that underlie its ability to promote B cell and Th2 type responses.

Despite the long history and widespread use of alu, there are a number of safety and immunological issues that necessitate the development of alternative vaccine adjuvants. An association has been proposed between vaccination with alum-adsorbed vaccines and macrophagic myofasciitis (MMF), a recently described inflammatory myopathy [144]. It was suggested that MMF lesions secondary to intramuscular injection of alum-containing vaccines should be regarded as a post-vaccinal immunogenic granuloma [145]. A tentative association among patients with chronic fatigue syndrome has also been proposed [146]. However, at present there are no suggestions that the use of alum should be discontinued (WHO Vaccine Safety Advisory Committee, 1999). From an immunological perspective the use of alum is limited since it principally elicits antibody responses and Th2 type T cell responses and is thus not suitable for vaccines where CMI and Th1 type responses are required.

Particulate delivery systems with potential in mucosal vaccine delivery

Biodegradable microparticles have been widely used for the delivery of mucosal vaccines. Roles for the villus tips, intestinal macrophages, villus enterocytes and the PP epithelium have been claimed in the uptake of particles from the intestine [15, 147]. The extent of particle uptake following mucosal administration is influenced by size, hydrophobicity, dose, the delivery vehicle, animal species and age. It was proposed that the effectiveness of orally delivered particulate antigens resulted mainly from their greater uptake into intestinal PPs [147]. However, there is increasing evidence, particularly in the case of nanoparticles, that the villus enterocytes also play a sig-

nificant role in uptake. Because microparticles are taken up across the GIT, these may be exploited for the oral delivery of labile molecules. The microparticles can be prepared from a range of different polymers and can be designed to protect entrapped vaccines against degradation in the gut, to delay gastric transit and/or to target vaccines for uptake into the PP. However, the low efficiency of microparticle uptake across the gut is a major limiting factor [147].

Polymeric microparticles can induce enhanced antibody [148, 149] and CTL responses [150] to associated antigens. Microparticles have also been used for the mucosal delivery of DNA vaccines [151]. Most work on the use of polymers in the formulation of microencapsulated vaccines has focused on poly(lactide-co-glycolides) (PLGs). These polymers are biodegradable and biocompatible, and have been used clinically in sutures and as controlled release drug delivery systems [147]. A problem with PLG microencapsulation of vaccines is the possibility of antigen denaturation during the encapsulation process. As a result, recent work has focused on the adsorption of antigens onto cationic/anionic microparticles as a means to avoid denaturation as a result of the encapsulation process and to retain particulate presentation. It was found that the structural integrity of the HIV antigen Env gp120dV2 SF162 was retained after adsorption on anionic particles but not if encapsulated [152].

Oral immunisation with microparticle-encapsulated antigens induced protective immunity against *Bordetella pertussis,* ricin, influenza virus and simian immunodeficiency virus (reviewed in [151]). However, the finding that antigens associated with PLG microparticles may be susceptible to protease degradation in the GIT [153] indicates that they are not optimal for oral delivery. A strategy designed to address this issue was the use of enteric coating polymers to stabilize PLG microparticles [153]. These novel particles protected antigen from proteolytic degradation in simulated gastric and intestinal conditions to a greater degree than in the poly(vinyl alcohol) (PVA)-stabilised formulation. Following oral immunisation, an enhanced specific salivary IgA response was induced when OVA was delivered in the novel particles compared with standard PVA-stabilised particles.

Intranasal priming with mutant LT adjuvant alone or combinations of parenteral vaccination with HIV antigens adsorbed on microparticles and IN immunisations with antigen together with LT mutant followed by parenteral booster immunisations enhanced mucosal and systemic memory-type immune responses against HIV-1 antigens [154]. Biodegradable microparticles have significant advantages as mucosal vaccine delivery systems, but their potency when used alone is still uncertain. However, the ease with which other adjuvants and targeting agents may be incorporated into the microparticles suggests these may provide a valuable platform for the construction of potent immunostimulatory mucosal delivery systems.

Lipid-based delivery systems

Liposomes are membranous systems comprising amphipathic molecules such as phospholipids forming multi- or unilayered vesicles [155]. A major advantage of liposomes is that they are composed of natural cell wall components such as phospholipids and cholesterol. Liposome formulations can be produced using either lipid bilayers separated by aqueous phases (multilamellar vesicles) or a single bilayer membrane with an aqueous core (unilamellar vesicles). Additionally, the membrane components can be altered to produce liposomes with particular characteristics. As a result of this flexibility, molecules with different physiochemical properties (e.g. both hydrophobic and hydrophilic molecules) can be incorporated [155]. Liposomes adsorb to cell membranes and can release their contents following uptake. The incorporation of antigens into liposomes can render them more immunogenic than when they are delivered alone. Efficient uptake and processing of liposomal vaccines by antigen-presenting cells is likely to be one of the principal factors responsible for the enhanced immune responses induced. Regarding oral delivery, liposomes have been shown to be taken up by PP M cells [156]. Accumulated evidence suggests that liposomes are not very stable in the digestive tract, although various methods are being used to improve efficacy [157]. In contrast, liposomes do appear to have significant potential as nasal vaccine delivery systems. Intranasal administration of a liposome-encapsulated DNA vaccine increased humoral and cellular mucosal responses in mice [158]. Nasal delivery of *Neisseria meningitidis* proteins in liposomes induced mucosal and systemic antibody responses, including bactericidal antibodies, but only if additional adjuvants were incorporated [159]. Indeed, the association of CpG oligodeoxynucleotides with cationic liposomes was found to be a potent adjuvant for type 1 innate immune responses [160]. Variants on the liposome concept, including archaeosomes incorporating glycerolipids from archaebacteria [161], virosomes incorporating viral envelope proteins [155] and cochleates, which are spiral-shaped lipid bilayer sheets with no internal aqueous composed of phosphatidylserine, cholesterol and calcium [162], are showing significant promise as delivery systems. As in the case of other particulate systems, additional immunomodulators and targeting agents can be associated with these systems to increase efficacy.

ISCOMS

Immune-stimulating complexes (ISCOMs), are highly stable cage-like structures of 30–40 nm in diameter composed of Quil A saponin, lipids and the vaccine antigen [163]. Quil A, derived from the tree *Quillaja saponaria*

Molina, is a strong adjuvant and is important for the efficacy of ISCOMs. To circumvent the problem of Quil A toxicity, a pure fraction of lower toxicity (QS21) was purified which is also an effective mucosal adjuvant [164]. Very small amounts of antigen in ISCOMs are immunogenic, and both humoral and cellular immune responses can be elicited. Hydrophobicity and the ability of saponins to intercalate into cholesterol-containing membranes may explain the ability of ISCOMs to facilitate antigen uptake and entry into the cell cytosol. It is likely that the efficacy of ISCOMs results partly from efficient targeting to antigen-presenting cells. Delivery of vaccines in these systems can facilitate antigen processing and presentation via the endogenous and exogenous pathways leading to stimulation of $CD4^+$ and $CD8^+$ T cells [165]. Antigens associated with ISCOMS can induce immune responses following oral or nasal delivery, but results have been relatively poor following oral delivery [166]. A striking feature of ISCOMs as delivery systems is their ability to elicit MHC class I restricted CTL responses to associated antigens [167]. Recently, it was shown that the induction of IL-12 is important in the adjuvant effect of ISCOMs [168] . A drawback with the use of this system is that incorporation of many antigens into the structure is difficult and extensive modification is often required. To address this problem, the ISCOMATRIX system was developed which is essentially the ISCOM without incorporated antigen [169]. Antigen is simply mixed with the system and the potent immune-stimulatory properties are retained. However, because antigens are then exposed, these systems are unlikely to be effective when used orally unless protected from degradation.

Bioadhesive mucosal delivery systems

Small intestinal transit in humans is usually 3–4 h, which is generally too short to allow complete absorption of vaccines from the gastrointestinal tract [170]. Bioadhesive systems may be designed to increase the time available for vaccine interaction with the GIT epithelium. In addition to the small intestine, vaccines may also be taken up from other parts of the digestive tract, particularly the colon. Strategies may be developed to facilitate enhanced interaction of vaccines with particular regions of the GIT or indeed with other mucosal surfaces, including the respiratory tract. Bioadhesive strategies may be subdivided into non-specific and specific systems depending on whether the interaction with the mucosal is principally mediated by physio-chemical forces or by receptor-ligand interactions [15].

Non-specific bioadhesive delivery systems

Non-specific bioadhesive delivery systems based on polymers and microspheres have been widely investigated for drug delivery [171]. In addition to increasing interaction with mucus and/or the epithelial layer, certain bioadhesive systems may also increase polypeptide or protein uptake across epithelia by passive paracellular diffusion after disrupting intercellular junctions.

Chitosan

Chitosan is a mucoadhesive polysaccharide of marine origin and is attracting increased attention as an agent for mucosal vaccine delivery, since it is regarded as safe and has been shown to promote transmucosal absorption [172, 173]. The polymer may be used in solution or as powders or particles. Nasal or pulmonary delivery of chitosan has been shown to enhance systemic and mucosal antibody responses to a number of antigens in mice [174, 175], and there is also evidence that chitosan may be effective for oral delivery [176]. Promising results have also been reported following nasal delivery of chitosan with DNA vaccines [177, 178]. More significantly, nasal delivery of chitosan with an influenza vaccine strongly enhanced serum haemagglutination inhibition titres in human subjects [179]. Furthermore, nasal delivery of a diphtheria vaccine with chitosan effectively boosted toxin-neutralising antibodies in humans [180]. Murine studies indicate that combination of chitosan with the non-toxic LT mutant LTK63 further enhanced its efficacy [181], indicating that combinations of chitosan and other mucosal adjuvants may be beneficial. The mechanism of action of chitosan is poorly understood at present, although roles for bioadhesion and increased mucosal permeability have been proposed [173]. Recent evidence suggests that DCs were involved in the uptake of orally administered chitosan and that the polysaccharide induced local production of the cytokines IL-4, IL-10 and TGF-β [182].

Specific bioadhesive vaccine delivery systems

Targeting molecules of microbial or plant origin may be use to achieve specific bioadhesion between vaccines and epithelial cells. Targeting strategies may be designed to direct vaccines to a specific tissue, cell type or sub-cellular compartment [15]. For example, the antigen-sampling M cells may be targeted using particulate systems or lectins [183]. Microparticle uptake from the intestine was increased following the adsorption of an M cell-specific monoclonal antibody [184].

The most widely studied specific bioadhesives are lectin-like molecules of plant or bacterial origin. Lectins are proteins or glycoproteins of non-immunological origin that bind to sugar structures specifically and with relatively high affinities [185]. A number of these molecules are stable in the digestive tract and can bind specifically to epithelial cells. The mucosal surfaces are highly glycosylated and thus represent targets for specific ligands. Furthermore, there is evidence that lectins may be translocated across the epithelium and induce immune responses to conjugated or co-administered antigens. Lectins that bind

specifically to the epithelial cells may be used to target conjugated antigens or may be attached to microparticles and other delivery systems such as liposomes to enhance interaction with epithelia. Uptake of orally delivered nanoparticles linked to LTB or ConA was demonstrated following and oral delivery to rats [186]. M cells and enterocytes in different gut regions vary in terms of lectin-binding properties, and this may be exploited for specific vaccine targeting. Investigations into lectin binding to the mouse gut found that a number of fucose-specific lectins [e. g. *Ulex europaeus* agglutinin 1 (UEA-1)] bound specifically to PP M cells. Polystyrene microspheres covalently attached to UEA-1 bound to and were rapidly absorbed by PP M cells following oral delivery [184]. Similarly, the association of UEA-1 or wheatgerm agglutinin with polymerised liposomes promoted liposome uptake from the GIT in mice. While both systems were taken up to a greater extent than the lectin-free liposomes, UEA-1 exhibited the most effective PP targeting [187]. The association of either molecule with liposomes led to increased interaction with PPs and enhanced uptake into organs including the liver. Tomato lectin-modified nanoparticles were more effectively absorbed from the gut than unmodified particles [188]. It was reported that uptake was mainly associated with non-lymphoid intestinal tissue. Since enterocytes constitute the largest cell population in the intestinal mucosa, it may be easier and more effective to target enterocytes for vaccine delivery. However, the potential for induction of innate or specific adaptive immune responses following uptake via enterocytes is unclear.

Oral delivery of a hapten complexed to plant lectins induced a significantly enhanced specific serum antibody response in mice compared with a non-lectin carrier [189]. However, all plant lectins are not strongly immunogenic and vary widely in immunogenicity following mucosal delivery to mice [190]. Lectins such as WGA, UEA-1 and PHA were relatively poorly immunogenic, while Mistletoe lectin 1 (ML1) was a potent immunogen, inducing systemic and mucosal responses comparable to those induced by cholera toxin. In addition, some plant lectins are powerful adjuvants when co-administered with antigens by the intranasal route [191]. Molecules involved in bacterial adherence and invasion may also have application in vaccine targeting to epithelial cells. Coating of microspheres with factors associated with invasion led to enhanced uptake by epithelial cells [192]. The delivery of polystyrene microparticles conjugated to the *Yersinia pseudotuberculosis* invasin resulted in enhanced particle uptake from the mouse gut [193]. The *Vibrio cholerae zonula occludens* toxin (zot) can reversibly open tight junctions in the intestinal mucosa, and the toxin is an effective adjuvant when co-administered with an antigen by the nasal route [194]. Specific targeting agents may have significant potential particularly in combination with mucosal delivery systems. However, this field is still in its infancy, and issues such as interspecies variation in receptor expression, toxicity, the interaction of lectins with mucus versus epithelial cells and the capacity of lectins to direct the uptake of particulates must be addressed.

Edible vaccines

A recent development with considerable potential for oral vaccine production and delivery is the generation of plants expressing antigenic proteins [195]. Advantages of antigen production in plants include avoidance of the risk of contamination with animal pathogens and the potential for high production yields. A number of antigens, including LTB, *Streptococcus mutans* surface protein antigen and hepatitis B surface antigen, have been expressed in various plants [195]. These studies clearly demonstrated that plants can express, fold, assemble and process foreign antigens and represent a simple and effective vaccine-manufacturing process [196]. It has been suggested that antigens may be protected from gastrointestinal degradation by the plant cell (bioencapsulation) [197]. Hepatitis B surface antigen expressed in potatoes were fed to previously vaccinated volunteers in a double-blind placebo-controlled clinical trial [196]. After eating three doses of potatoes, 62.5 % of volunteers exhibited elevated antigen-specific serum antibody titres that were boosted up to 56-fold. However, the potency of plant-expressed vaccines may not be sufficient to induce protective immunity when orally administered alone, and the inclusion of antacids or mucosal adjuvants may be necessary to improve responses [197]. Indeed, a fusion protein consisting of LTB and ESAT6, a candidate TB vaccine antigen, was recently produced in *Arabidopsis thaliana* in a form where both antigenicity and receptor binding capacity were retained [198]. Plants appear to have great potential for low-cost vaccine production and potentially the production vaccine-carrier or adjuvant constructs, but whether the concept of edible vaccines is feasible is uncertain.

Live vaccine carriers

Live vaccines encompass the ideal features of an effective mucosal delivery system: protection of antigens from degradation, targeted delivery to M cells and dendritic cells, and the presence of potent immunostimulatory molecules. Many of the currently used parenteral vaccines, including measles [199] and BCG [200], are live attenuated organisms. In addition, the Sabin live attenuated oral polio vaccine is highly effective [201]. Live attenuated oral vaccines have also been licensed against *Vibrio cholerae* and *Salmonella typhi* [202]. The efficacy of the available live vectors for oral vaccine delivery

(e. g. *Salmonella* species or polio viruses) in expressing antigens from other pathogens is variable. However, live attenuated vaccines are likely to play an important role in the development of mucosal and particularly oral vaccines because of their potency. In the case of influenza it is known that live attenuated vaccines induce more rapid and potent immunity than inactivated vaccines and can be more cross-protective against related strains of the same virus [203].

Live vectors are particularly effective in the induction of $CD8^+$ T cell responses. The strategies used to induce such responses include live attenuated, replication competent live vectored, replication defective live vectored, DNA vaccines and heterologous prime-boost approaches [11]. However, these strategies carry the risk of reversion to virulence and induction of disease in immuno-compromised patients. Heterologous prime boost vaccination regimes have shown significant potential and may be a way forward in mucosal vaccination for a number of diseases. These may comprise a DNA vaccine prime followed by a live vector boost [204] or two different live vectors [205]. Priming with BCG and boosting with a vaccinia virus expressing an antigen from *Mycobacterium tuberculosis* induced strong $CD4^+$ T cell responses. A mucosal CTL response was induced by intrarectal immunisation with a replication-deficient recombinant vaccinia virus expressing an HIV envelope protein [206]. Intranasal immunisation with BCG elicited specific T cell responses in the lung lymph nodes that were enhanced by nasal delivery of recombinant modified vaccinia virus Ankara, expressing *Mycobacterium tuberculosis* Ag 85A [207]. Powerful protective immunity was induced against aerosol challenge with *M. tuberculosis* by nasal boosting with either BCG or the recombinant vector that correlated with the induction of antigen-specific, IFN-γ-secreting T cells in lung lymph nodes. These very encouraging findings suggest that mucosal prime-boost vaccination approaches may have significant potential.

Conclusions and perspectives

Despite the tremendous benefits of and interest in mucosal vaccines there are still only a small number of internationally licensed vaccines against the mucosal diseases polio, cholera, typhoid, rotavirus and influenza. Furthermore, despite decades of research into adjuvants only aluminium salts, the oil-in-water emulsion MF59 and virosomes are licensed for human use. The multiplicity of adjuvants and delivery systems and adjuvants tested experimentally has not yielded many effective candidates. Mucosal vaccination can be highly effective; for example, aerosol delivery of measles and rubella vaccines elicits comparable immunity to injection [208]. Therefore, identification of the optimal combinations of adjuvants and delivery systems is essential to enhance the response to mucosal vaccines. The recent production of synthetic TLR agonists offers new opportunities, and testing of the molecules as mucosal adjuvants may reveal strong candidates. In some cases mucosal and particularly oral vaccination may not be feasible alone but may be an efficient strategy to boost the response following parenteral priming.

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