International Journal of Pharmaceutics, 75 (1991) 1–24

ADONIS 037851739100296Q

IJP 02493

Invited Review

Oral vaccines: Design and delivery

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(Received 22 March 1991)
(Accepted 18 April 1991)

Key words: Oral vaccine; Delivery system; Formulation; Liposome

Summary

The formulation of oral vaccines is reviewed with emphasis on methods used for optimising their immunogenic effects against pathogenic bacteria and virus delivery. Systems considered include microparticles, nanoparticles and liposomes. The use of adjuvants and hybrid vaccines is also described.

Introduction

Presently most vaccines are administered parenterally for protection against both superficial and systemic pathogens. This is due to the fact that the antigens present in vaccines are particles or large peptides which are poorly delivered to the sites of specific immunity. The poor delivery is commonly due to spontaneous or enzymic breakdown and/or poor absorption. This has not deterred continued investigations into oral vaccines as alternatives to currently used parenteral vaccines. In a recent review (Gilligan and Li Wan Po, 1991), we surveyed clinical trials of such oral enteric vaccines. In the present review, we consider the design and delivery of oral vaccines.

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Gut-Associated Lymphoid Tissue

The gastrointestinal tract is constantly confronted by potentially harmful antigens which are usually destroyed by the mucosal barrier: a combination of nonimmunological barriers such as gastric acidity, proteolytic enzymes, peristalsis, commensal microflora and mucus and the immunological barrier (Holmgren and Lycke, 1986; Walker and Owen, 1990). The immune response is stimulated when antigens gain access to lymphoid tissue within the gastrointestinal tract.

The gut-associated lymphoid tissue (GALT) is distributed in four anatomical regions (O'Hagan et al., 1987):

(a) the lamina propria which contains large numbers of plasma cells as well as macrophages, neutrophils, eosinophils and mast cells;
(b) the intraepithelial lymphocytes which are dispersed between the epithelial cells of the mucosal membrane;
(c) isolated lymphoid follicles, present throughout the intestine and colon; and

(d) the Peyer's patches (PP) which are clusters of lymphoid follicles found along the wall of the small intestine.

Lymphoid tissue of the lamina propria and intraepithelial lymphocytes are collectively known as the diffuse lymphoid tissue. An immune response is elicited through lymphoid tissue of the PP and isolated lymphoid follicles.

Antigen Uptake at Mucosal Surfaces

At present, there are thought to be two main pathways for antigen uptake from the intestine. The first involves specialised cells, known as follicle-associated epithelial (FAE) or microfold (M) cells, which overlie the lymphoid follicles of the gastrointestinal tract. These cells are commonly referred to as M cells and will be discussed in greater detail in the following section. The other method of antigen uptake involves the normal epithelium overlying the diffuse lymphoid tissue (Bienenstock and Befus, 1980). Within these cells, unlike M cells which contain a reduced number of lysosomes, antigen intracellular digestion occurs, although small quantities of antigen are able to escape this process to be exocytosed into the interstitial space. Limited studies have been carried out to investigate antigen uptake and presentation by the normal epithelium covering the intestine. Two studies have shown that normal mucosal epithelial cells are capable of presenting antigen and triggering T cell proliferation in vitro (Bland and Warren, 1986; Mayer and Shlien, 1987). A further study has shown that reovirus adheres selectively to and is endocytosed along the basal surface of absorptive cells in isolated sheets of intestinal epithelium (Bass et al., 1988). Another study has shown that reovirus type 3 adhered to and was endocytosed not only by M cells but also by absorptive cells of suckling mice (Wolf et al., 1983).

The two main mechanisms for antigen uptake, described above, are distinct from the process of 'persorption' as first described by Volkheimer (Volkheimer and Schulz, 1968; Volkheimer, 1977). This process involves the extracellular penetration of large particles (in the size range of 5–150 μm) between intestinal cells, preferentially in areas of desquamation, into subadjacent tissue. Mechanical factors are thought to be responsible for the persorption process. Intestinal motility and vascular pulsations transmitted to the mucosa play an important part in the transepithelial passage of particles by this method (Volkheimer, 1977).

Antigen sampling cells similar in character to M cells have also been found in the epithelium overlying the bronchus-associated lymphoid tissue (BALT) and in the reticular epithelium in tonsillar crypts (Bienenstock and Befus, 1980). As it is known that the BALT and GALT have similar characteristics and are both major sites of secretory immunoglobulin A (SIgA) production, they are often termed collectively as the mucosal-associated lymphoid tissue (MALT). As most studies have suggested that the lymphoid aggregates of the PP are the main site for antigen uptake from the gastrointestinal tract we shall limit our discussion to this type of GALT.

Peyer's Patches

The structure of the PP (Fig. 1) includes a dome region, overlying the follicle, consisting of one to two germinal centers (B cell zone) situated towards the base of the follicle and interfollicular areas (T cell zone). The adult PP consist of 40–70% B lymphocytes found primarily in the germinal center and 11–40% T cells predominantly found in the dome and interfollicular areas. The dome region is also populated by macrophages, plasma cells and B lymphocytes. The epithelium covering the dome consists of cuboidal epithelial cells containing only a few goblet cells, thus limiting mucus secretion to facilitate antigen uptake, and M cells (Owen and Jones, 1974; Sneller and Strober, 1986). M cells are both functionally and morphologically different from cuboidal or columnar epithelial cells. Morphologically, they are characterised by short irregular microvilli, numerous cytoplasmic vesicles and close association with intraepithelial cells by tight junctions and desmosomes. M cells contain the same type and number of organelles as the surrounding

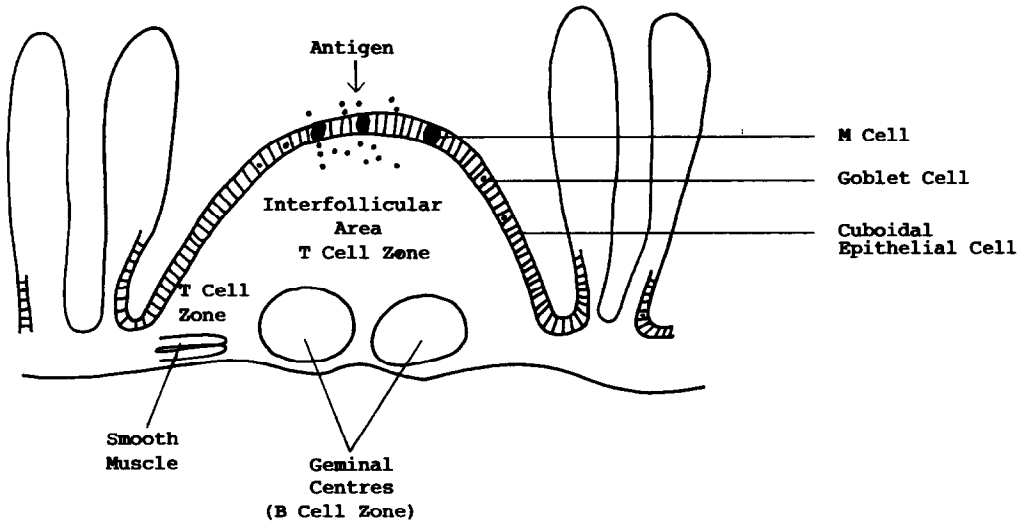

Fig. 1. Schematic diagram of the dome region of the Peyer's patches. (Adapted with permission from McGhee and Mestecky (1990).)

absorptive cells with the exception of lysosomes, which are fewer in number in M cells. The cytoplasm of M cells engulfs one or more intrusive cells located in the extracellular space which indents the M cell; thus they are not situated within the M cell but, as stated by Wolf and Bye (1984), are contained within a "central hollow" (Fig. 2). The intrusive cells are usually lymphocytes, lymphoblasts or macrophages but occasionally are plasma cells or, rarely, polymorphonuclear leukocytes.

Functionally, M cells have been shown to transport macromolecules (Bockman and Cooper, 1973; Owen, 1977; Owen and Bhalla, 1983; Neutra et al., 1987), particles (Sass et al., 1990) and micro-organisms (Tokunaga et al., 1979; Wolf et al., 1981; 1983; Fujimura, 1986; Owen et al., 1986; Bass et al., 1988; Walker et al., 1988; Sicinski et al., 1990) from the gastrointestinal lumen to the underlying lymphoid tissue. Examples of macromolecules, particles and micro-organisms which have been shown to be transported by M cells are listed in Table 1. The study by Owen et al. (1986) describing the M cell transport of *Vibrio cholerae* showed that bacterial viability was required for efficient uptake by M cells, since *V. cholerae* killed by several methods showed no detectable uptake.

Several workers have investigated the transport mechanism of macromolecules and microorganisms across M cells. Owen (1977) studied

TABLE 1

Macromolecular, microbial and particulate transport by M cells

	Reference
Macromolecular	
Native ferritin	Bockman and Cooper (1973)
Cationized ferritin	Neutra et al. (1987)
Horseradish peroxidase	Owen (1977)
Ricinus communis agglutinin (1 and 11)	Neutra et al. (1987)
Wheatgerm agglutinin	Neutra et al. (1987)
Viruses	
Reovirus (types 1 and 3)	Wolf et al. (1981) Wolf et al. (1983) Bass et al. (1988)
Poliovirus (type 1)	Sicinski et al. (1990)
Bacteria	
Mycobacteria	Tokunaga et al. (1979) Fujimura (1986)
V. cholerae	Owen et al. (1986)
C. jejuni	Walker et al. (1988)
Particulate	
Latex particles	Sass et al. (1990)

the sequential uptake of horseradish peroxidase (HRP) by M cells in the normal unobstructed mouse intestine. Uptake was preceded by HRP adhering to the surface of columnar cells and M cells. 5 min after injection of HRP into the unligated intestine, HRP was found in vesicles of M cells indicating that HRP was taken up by a process of endocytosis; no HRP was found in the columnar cells. After 1 h, HRP was detected in the extracellular space between M cells and their enfolded lymphocytes as well as in vesicles within these lymphocytes (Fig. 2). Neutra et al. (1987) showed that lectins (*Ricinus communis* agglutinin 1 and 11 and wheatgerm agglutinin) and polycations (cationized ferritin) which adhere to glycoconjugates on the luminal membranes of M cells are endocytosed from M cell surfaces of rabbits and are transported through the cell in a complex system of tubulocisternae and clear vesicles. Wolf et al. (1981, 1983) and Bass et al. (1988) have shown that reovirus type 1 adhere to the surface of M cells and are taken into the cell by endocytosis. The viruses were shown to traverse the cell in vesicles which liberated their content into the extracellular space enveloped by the M cell and in close proximity to the mononuclear cells which occupy that space. Viruses were also found in coated pits on the surface of these mononuclear cells. Sicinski et al. (1990) showed that poliovirus type 1 adhere specifically to and are endocytosed by M cells, although no viruses were detected in the extracellular space or under the basement membrane of the epithelium.

Owen et al. (1986) investigated the uptake and transport of *V. cholerae* through M cells. They showed that the vibrios were endocytosed by M cells, carried in vesicles through the cytoplasm and were discharged into the extracellular space containing lymphocytes and macrophages. Fujimura (1986) showed that mycobacteria adhering specifically to M cells are endocytosed by them and transported across the cytoplasm in large vesicles. The bacteria were also found in the extracellular space between M cells and columnar

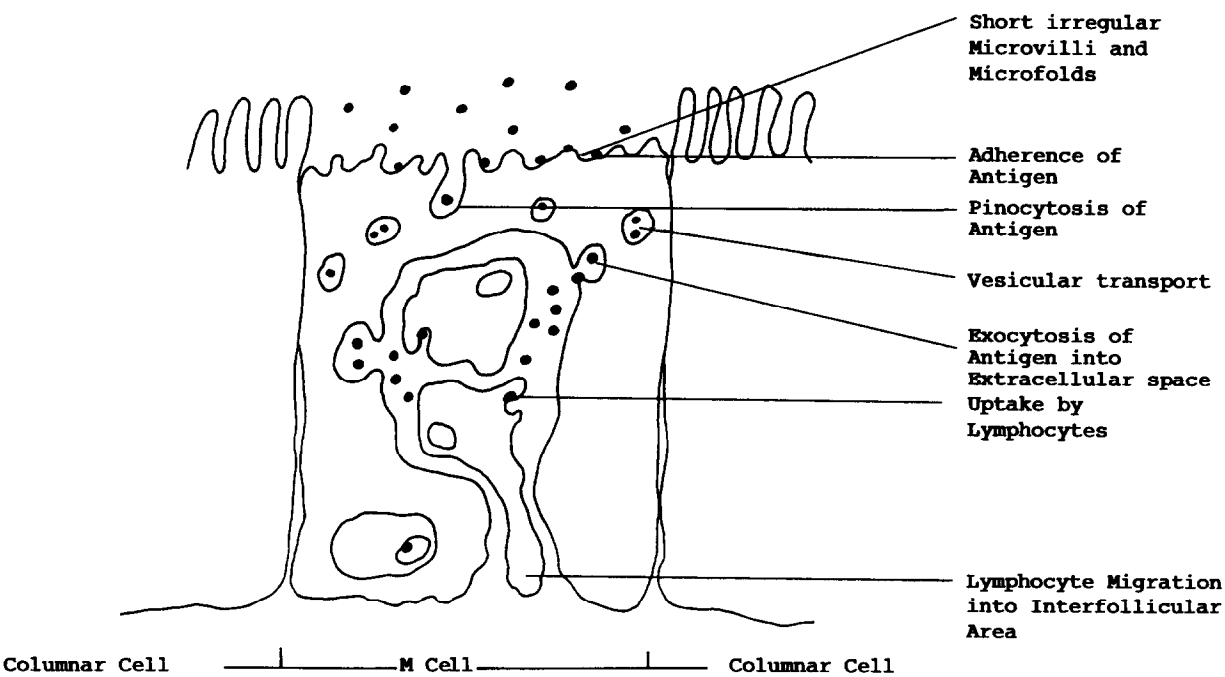

Fig. 2. Schematic diagram of the stages involved in antigen uptake and transport by an M cell. (Adapted with permission from Owen (1977).)

cells and were seen in macrophages enfolded by M cells.

From studies on uptake and transport of micro-organisms and macromolecules, by M cells, it can be concluded that M cells can take up antigens by endocytosis and transport them across the cytoplasm in vesicles to be exocytosed into the extracellular space and processed by cells on the antiluminal side of the M cell. Further studies are required to investigate the actual adherence mechanism of antigens to the apical surface of M cells as understanding of this process could enhance the delivery of antigens by this route.

The primary function of M cells is thought to be to serve as antigen-sampling cells for uptake of antigens from the gastrointestinal lumen and presentation of these to lymphoid tissue to initiate an immune response (negative virulence factor). It is unlikely that M cells actually present antigens to the helper T (Th) cells (see next section) as they lack major histocompatibility complex (MHC) class II molecules which are necessary to form a complex with the antigen before it can be presented to the Th cell population. It is still unknown which cells act as the primary antigen presenting cells in PP, but it is possible that either macrophages, dendritic cells, or even B cells could fulfil this role (Spalding et al., 1983; Kapsenberg et al., 1986; Janeway et al., 1987). M cells can also have a positive virulence factor as they provide an easy route of ingress for invasive organisms, i.e., salmonella and reovirus to produce systemic infection (Sneller and Strober, 1986). M cells, by their ability to transport both soluble and particulate antigens (Seifert and Sass, 1990) from the gastrointestinal lumen to lymphoid tissue, may provide a means by which vaccines may be administered to induce an appropriate immune response for the presenting antigen. This possibility will be discussed further in a subsequent section.

Several other workers have demonstrated the uptake of particles from the gastrointestinal tract, and suggested that the PP aggregates are the site of uptake, although no evidence was presented for the involvement of M cells in the uptake process. In several studies, LeFevre and coworkers investigated the uptake of particles by the PP. In two studies (LeFevre and Joel, 1984; LeFevre et al., 1985a) carbon and iron oxide were observed in PP after chronic feeding of mice with these substances. Asbestos, quartz and carmine did not accumulate in PP. This observation led to the possibility that the physiochemical properties of particles influence their uptake. Carbon and iron oxide are poorly wettable and hydrophobic while asbestos, silica and carmine are readily wettable and therefore possess hydrophilic surfaces. Another observation was that the particles which accumulated in the PP were the smallest of the five particles tested. Penetration of iron oxide (approx. 0.1 μm in diameter) and carbon (approx. 0.05 μm in diameter) into the PP is therefore probably also related to the size of the particle. However, in two other studies (LeFevre et al., 1978; 1985b) chronic feeding of mice with 2 μm latex particles resulted in accumulation of these in the PP. Particulate uptake is probably a function of particle size, physicochemical properties of the particle and experimental protocol. In another study, Eldridge et al. (1990) observed that the efficiency of absorption of microspheres into PP was related to the relative hydrophobicity of the microsphere wall material. The most hydrophobic compounds [poly(styrene), poly(methylmethacrylate) and poly(hydroxybutyrate)] were well distributed in the PP (up to 1500 microspheres were observed in 3 PP of mice) while the cellulosics were poorly absorbed (less than 10 observed with cellulose triacetate). The exception to this was ethylcellulose which, although hydrophobic in nature, was not found in the PP. Jani et al. (1989, 1990) investigated the gastrointestinal uptake of non-ionic and carboxylated fluorescent polystyrene microspheres ranging in size from 100 nm to 3 μm. PP, villi, liver, lymph nodes and spleen of rats fed the non-ionic microspheres from 100 nm to 1 μm showed evidence of uptake and translocation of particles. The carboxylated microspheres were taken up to a lesser degree than non-ionised particles. The particles were shown not to be randomly distributed in the tissues, but accumulated at the serosal side of the PP (Jani et al., 1989). A further study (Jani et al., 1990) attempted to quantify the actual uptake of the polystyrene microspheres. Under their experi-

mental conditions, 500 nm particles were absorbed to the extent of 34% and 100 nm particles to 26%. No 3 μm microspheres were shown to be taken up in the gastrointestinal tract or found in the serosal layer of the PP. The discrepancy in the size of particles taken up in the studies of LeFevre and co-workers and Jani and co-workers may be due to different experimental protocols. It is evident, however, that only very small particles will be taken up by the PP and transported to the underlying tissue.

The Oral Immune Response

The oral immune response is stimulated when antigens gain access, often through M cells but not exclusively, to lymphoid tissue within the gut (Fig. 1). The intestinal immune system has been described in detail (Russell and Mestecky, 1988; Doe, 1989; Kawanishi, 1989; McGhee and Mestecky, 1990). The main determinant of humoral mucosal immunity is secretory immunoglobulin A (Mestecky and McGhee, 1987). Secretory immunoglobulin A (SIgA) response is induced when an antigen is taken up (by M cells) in the GALT (Fig. 3). Th cells (of the helper-induced subset of T cells) and B cells committed to specific IgA synthesis are rapidly generated in PP and migrate to mesenteric lymph nodes, the thoracic duct and systemic circulation. Partly matured lymphocytes return to the mucosal membranes, i.e., the lamina propria of the GALT, to become IgA plasma cells or effector T cells. Within plasma cells two IgA monomers complex with the J chain, a 15 kDa protein having one *N*-glycosylation site to form polymeric IgA (Fig. 4). Polymeric, J chain-containing IgA is released by subepithelial plasma cells and interacts with a secretory component (SC) on the surfaces of epithelial cells. This binding results in endocytosis and subsequent movement of the membrane SC-polymer IgA complex through the epithelial cell. The complex is then extruded on the luminal surface by reverse endocytosis. The secretory IgA (SIgA) is protected from proteolysis by the secretory component. T cell regulation of SIgA production has been discussed by several workers

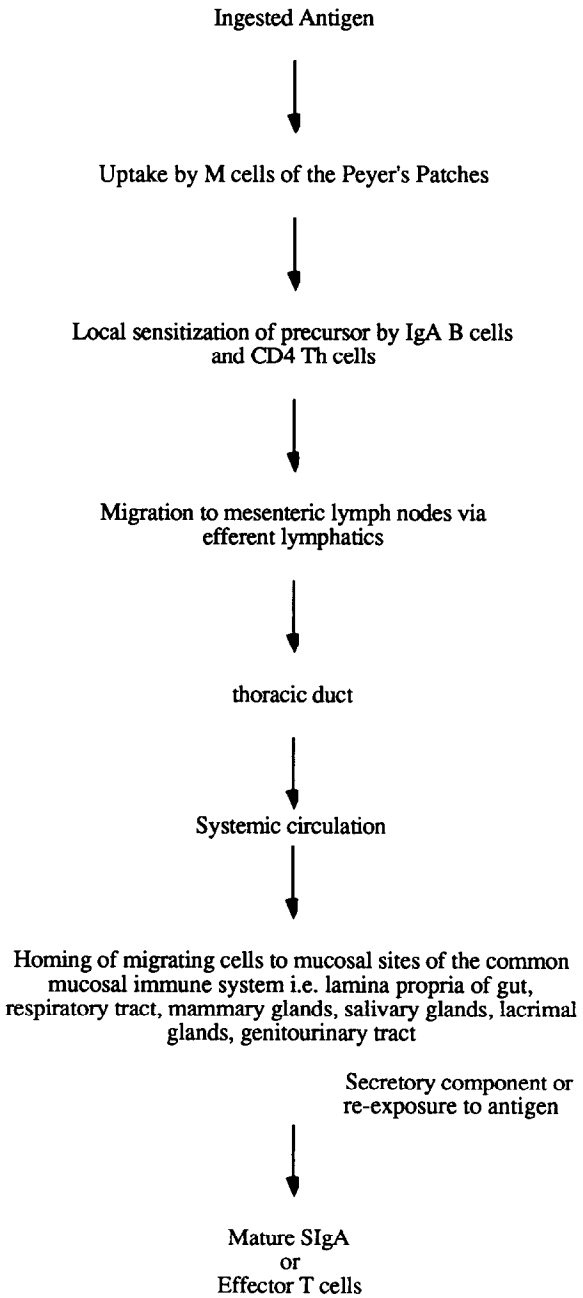

Fig. 3. Events following oral administration of an antigen (vaccine)

(Doe, 1989; Kawanishi, 1989; Tomasi, 1989). Proposed protective properties of SIgA are given in Table 2. Experiments have shown that oral administration of a variety of antigens evokes a

local mucosal SIgA response not only in the intestinal tract, where the antigen was first encountered, but also in the bronchial-associated lymphoid tissue (BALT) and remote secretory glands such as mammary, parotid and lachrymal and cervical glands of the uterus (Bergmann et al., 1986; Jertborn et al., 1986; Briese et al., 1987; Mestecky, 1987; Waldman and Bergmann, 1987; Watanabe, 1989). This is referred to as the common mucosal immune response.

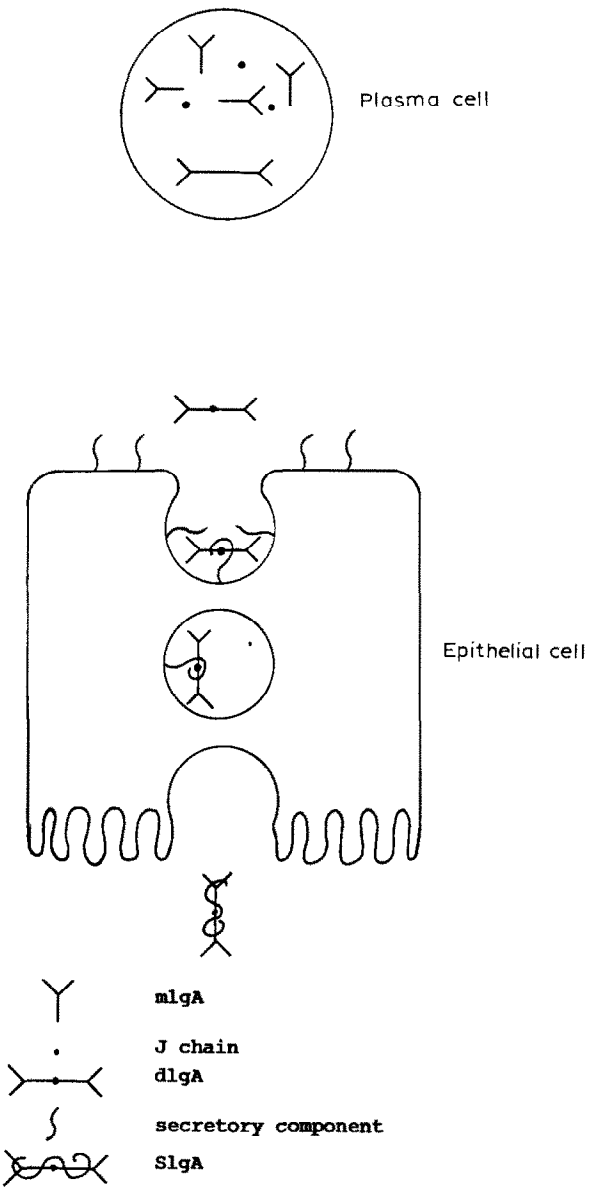

Fig. 4. Formation of secretory immunoglobulin A from monomeric IgA. (Adapted with permission from McGhee and Mestecky (1990).)

TABLE 2

Protective properties of SIgA

Inhibit microbial adherence
Prevent absorption of antigens from mucosal surface
Resistance to proteolysis and avidity for mucous membranes
Enhance the antibacterial efficiency of other immune effector systems
Neutralise biologically active antigens, such as toxins
May interfere with the utilization of various growth factors in the intestinal environment, such as iron

From the above overview of the oral immune system, it would seem that the oral route of administration could be utilized not only to provide protection against enteric pathogens, but also to immunize against a wide range of pathogens which infect other mucosal membranes due to the existence of the common immune response.

Oral Vaccine Formulation Development

There are two main possible ways for oral immunization, firstly by the use of live attenuated organisms and secondly, by using peptides which have the capacity to bind and be absorbed at the intestinal level and generate both a local mucosal response and, if necessary, a systemic immune response.

Most of the oral vaccines available so far are based on live attenuated organisms as these have the capacity to colonize the intestine and elicit an immune response in a manner analogous to the natural infection. Use of modern DNA techniques to produce strains of a defined nature negates the fear of the possibility of reversion to virulence.

The most commonly used oral attenuated vaccine is the poliomyelitis vaccine. The oral polio vaccine is trivalent, being composed of type 1, type 2 and type 3 suitable live attenuated strains of the poliomyelitis virus. Current controversy

exists as to whether oral polio vaccine (OPV) should be superseded by the high-potency intramuscular killed polio vaccine (IPV) due to the relative importance of paralytic poliomyelitis associated with oral polio vaccine (Wassilak et al., 1987; Hinman et al., 1988a,b; Melnick, 1988; Salk, 1988; Beale, 1990). Poliomyelitis after vaccination is generally associated with type 2 and type 3 (Almond et al., 1987; Westrop et al., 1989) vaccine strains and rarely with type 1 (Beale, 1990). The World Health Organisation organised a study to evaluate the safety and efficacy of oral poliomyelitis vaccine (Cockburn, 1988; Esteves, 1988). The main conclusion from this 15 year study was that oral polio vaccine was one of the safest vaccines in use. The risk of vaccine-associated poliomyelitis, in all but one country, was less than one per million vaccinees, cases due to type 3 virus being the most common in both vaccine recipients and contacts. It remains to be seen whether the low incidence of vaccine associated poliomyelitis will change immunization policy to the use of IPV. Another alternative policy to the exclusive use of either vaccine is the sequential use of IPV and OPV (McBean and Modlin, 1987; Modlin et al., 1990). Finally, the use of OPV has been shown, in several studies, to be much less effective in developing countries than industrial, developed countries (John, 1984; Hanlon et al., 1987; Patriarca et al., 1988). Suggested reasons for this reduced protection include interference from other enteric viruses in the gastrointestinal tract, malnutrition or cross-immunity.

The other approach to oral vaccination is by the use of synthetic peptide vaccines to stimulate a mucosal immune response and a systemic immune response, if required (Liew, 1988; Maskell and Dougan, 1988). Synthetic peptide vaccines consist of synthetic peptides with the same structure as the protective antigens isolated from the whole micro-organism, for example, the B subunit of the oral inactivated cholera vaccine (Clemens et al., 1986, 1990). Advantages of synthetic peptide vaccines include (Liew, 1988): (i) chemically defined, generally indefinitely stable product; (ii) absence of infectious agent; (iii) usually less requirement for large production culture plant; and (iv) relatively easy, but still difficult, to formulate into controlled release systems without damage to the protective antigen. The major disadvantage is that synthetic peptides are not usually as immunogenic as the proteins from which they were derived and thus require the presence of adjuvants to elicit an appropriate immune response.

Through studies on vaccination by the oral route several points have emerged: higher doses and more frequent administration, when compared to systemic immunization, are required for orally administered antigens, due to enzymatic degradation, acid sensitivity or poor bioavailability (Hemings, 1978). Particulate antigens, rather than soluble antigens, provide a more effective stimulus for the induction of a local, generalised secretory and systemic immune response (Ebersole and Molinari, 1978; Cox and Muench, 1984; Cox and Taubman, 1984). Attenuated oral vaccines are usually not transmissible to contacts, produce very few side effects and are suitable for mass application without the need for highly trained staff.

Oral vaccination may fail due to several factors: failure to swallow the vaccine; inactivation by gastric acid and intestinal enzymes; poor bioavailability; interference from other bacteria and viruses in the gastrointestinal tract; mutual interference if more than one type of live vaccine is administered concurrently and too rapid transit of the vaccine through the intestine limiting its binding to mucosal cell receptors and hence stimulation of an adequate immune response. In the attempt to overcome the need for higher and more frequent dosing required by oral administration and to minimise vaccine failure, several strategies have been followed. These include the use of buffers, acid-resistant polymers, particulate drug delivery systems, oral adjuvants and hybrid vaccines. These will be discussed in turn.

Buffers and Enteric-coated Tablets

The first attempts to prevent degradation of antigens by acid involved the administration of antacid solutions prior to vaccination. This method has been used in clinical trials on the

development of an oral cholera vaccine (Clemens et al., 1986; Levine et al., 1988) and typhoid vaccine (Gilman et al., 1977). This procedure seemed to be efficient but may be difficult to execute on a large scale. The next stage was the use of polymers for enteric coating to supersede the use of buffer solutions. Enteric protective polymers have been used for the Ty21a oral typhoid vaccine (Levine et al., 1987) and have also been used in vaccines against *Haemophilus influenzae* (Clancy et al., 1985, 1989, 1990), hepatitis B (Lubeck et al., 1989) and tuberculosis (Ishihara et al., 1986). Enteric coating is an efficient means of protecting antigens against inactivation by gastric acid. This type of coating, however, does not protect against proteolytic degradation in the intestine or promote retention in the gastrointestinal tract to aid absorption.

Particulate Delivery Systems

From the observation that oral particulate antigens provide a more effective stimulus, for the induction of an immune response, than orally ingested soluble antigens (Ebersole and Molinari, 1978; Cox and Muench, 1984; Cox and Taubman, 1984) recent research has included development of particulate systems for the oral delivery of antigens. Particulate antigens, as discussed previously, are most likely delivered from the gut lumen to the underlying lymphoid tissue by M cells in Peyer's patches. Several methods have been explored to formulate particulate antigens.

Microparticles and Nanoparticles

Eldridge et al. (1989) synthesized biodegradable and biocompatible microparticles composed of poly(DL-lactide-co-glycolide) containing a toxoid vaccine of staphylococcal enterotoxin B. These workers showed that oral administration of microparticles < 10 μm in diameter were specifically taken up by the PP of the GALT. Particles < 5 μm were shown to leave the PP and enter the mesenteric lymph nodes, the peak number observed therein occurring at day 7. Particles > 5 μm did not enter this tissue but remained in the PP until digested, thereby stimulating a purely mucosal response. Circulating toxin-specific antibodies and a concurrent SIgA antitoxin response in saliva, gut wash fluid and bronchial-alveolar wash fluid were measured as opposed to no response from the orally administered soluble antigen. Thus, in the absence of oral adjuvants, a significant immune response was generated to the toxoid-containing microspheres. The release of drug from this type of system has been found to be related primarily to the concentration of polymer in the coating procedure. Other factors which are thought to be important are the coating procedure and the type viscosity and average molecular weight (M_v) of the polymer employed in the coating (Marcotte and Goosen, 1989). It is interesting to note that the particle size of the microparticles taken up by the PP in this study is greater than that in the studies by LeFevre et al. (1978, 1985b) and Jani et al. (1989, 1990). This may be due to physiochemical differences in the administered particles, the experimental design or method of analysis of particulate uptake.

O'Hagan et al. (1989a) developed a microparticle system composed of ovalbumin (OVA) adsorbed onto poly(butyl-2-cyanoacrylate) particles. Polyalkylcyanoacrylate nanoparticles have been shown to enhance the oral absorption of vincamine (Maincent et al., 1986), iodized oil (Aprahamian et al., 1987) and insulin (Damge et al., 1988; Michel et al. 1991). Particles with adsorbed antigen and a mean particle size of 100 nm and 3 μm, administered to rats by gastric intubation, were shown to significantly enhance the secretory immune responses to OVA after 14 days. The rats had been primed 14 days earlier by intraperitoneal injection with OVA in physiological saline. The 100 nm particles containing adsorbed antigen were also found to enhance the secretory immune response after 50 days, following a booster dose of soluble antigen at 46 days. These authors suggested that the particles may gain access to lymphoid tissue through the M cells of the PP. A further study by O'Hagan et al. (1989b) confirmed the observation of an enhanced immune response to a microparticle system containing antigen and studied the protec-

TABLE 3

Some studies with liposomal antigens: experimental protocol and main observations

Antigen [a]	Liposome composition	Co-adjuvant [a]	Animal model	Administration route	Observations	Reference
Crude wall-extracted antigens (WEA) of *Streptococcus mutans*	large negative liposomes	–	rat	intragastric	A strong salivary IgA anti-WEA response was elicited in animals immunized with WEA liposomes, compared to no IgA response from rats primed with WEA in phosphate-buffered saline or with empty liposomes. A secondary immune response was also observed in animals boosted 66 days later with a single dose of WEA liposomes.	Gregoriadis (1988) Chap. 19
Purified polysaccharide antigen of *Streptococcus mutans*	not stated	cell wall protein which interacts with saliva (74K-SR)	rat	intragastric	Intragastric administration of liposome-associated poly-74K-SR conjugate produced a local IgA response against the polysaccharide and cell surface protein, whereas liposome-associated polysaccharide did not. Thus, the immunogenicity of *S. mutans* polysaccharide was shown to be improved by chemical coupling with a carrier cell surface protein (74K-SR).	Wachsmann et al. (1986)
Streptococcus sobrinus ribosomal protein	dipalmitoyl-DL-α-phosphatidyl-choline, cholesterol, dicetyl phosphate	–	rat	intragastric	Immunized animals exhibited higher levels of salivary IgA antibodies to *S. sobrinus* whole cells and ribosomes than did the control group. As little as 12.5 μg of *S. sobrinus* ribosomal protein incorporated in liposomes protected rats from caries formation after experimental challenge with the virulent organism.	Gregory et al. (1986)

Streptococcus mutans antigens (serotype-specific carbohydrate, CHO)	dipalmitoyl-phosphatidylcholine, cholesterol, dicetyl phosphate	muramyl dipeptide (MDP)	rat	intragastric	Antigen alone did not elicit a salivary IgA response nor was it protective against *S. mutans* infection. CHO incorporated into liposomes produced a good salivary IgA response while incorporation of MDP further enhanced the response.	Michalek et al. (1989)
Cholera toxin (CT) or its heat-aggregated derivative procholer-agenoid (PCG) (not incorporated into liposome)	dimyristoyl-phosphatidylcholine	aviridine	rat	intraduodenal	Aviridine, incorporated into liposomes enhanced the development of memory for a specific mucosal IgA response to a coadministered antigen.	Pierce and Sacci (1984)
Cholera toxin (CT)	dipalmitoylphospha-tidylcholine, cholesterol, dicetyl phosphate	lipid A – from the lipopolysaccharide (LPS) of gram-negative bacteria	rat	intraduodenal	Lipid A enhances enteric priming of liposome-associated CT, but only when it is incorporated into CT-bearing liposomes.	Pierce et al. (1984)
Anti-idiotype (anti-id) vaccine against *S. mutans*	dipalmitoyl-phosphatidylcholine, cholesterol, dicetyl phosphate	–	rat	intragastric	Anti-id incorporated into liposomes resulted in a significant reduction in dental caries as well as *S. mutans* colonization of the oral cavity.	Jackson et al. (1990)

[a] Unless otherwise stated, the antigen and co-adjuvant are incorporated into the liposomes.

tion of antigen to enzymatic degradation by using particles, of diameter 2.55 μm, composed of polyacrylamide. Polyacrylamide microparticles have been shown to protect protein against proteolytic degradation (Ekman and Sjoholm, 1978). In the work of O'Hagan et al. (1989b), the memory SIgA response of rats (at 65 days) was significantly raised relative to a soluble antigen control group. Failure to elicit a primary immune response was thought to be due to degradation of the antigens on the surface of the microspheres during passage through the gut. The secondary immune response was thought to be elicited by release of centrally located antigen after uptake of the microparticles.

There are several advantages in using microspheres for the oral delivery of antigens. By using biodegradable polymers, the release properties of the microspheres can be tailored for the antigen to produce the required immune response. The development of a multi-dose pulsatile release system could be possible. The microparticles provide some protection from the proteolytic enzymes of the small intestine and this may be enhanced by coating with pH sensitive polymers to prevent gastric acid or enzymatic degradation in the stomach and small intestine respectively. The system provides a means of targeted delivery of antigen to the immune system by means of the PP. Several antigens may be delivered simultaneously and adjuvants may be incorporated. Although studies on the use of microparticles and nanoparticles for the oral delivery of antigens are limited at the present time it is believed that this type of system will substantially influence the development of both oral and parenteral vaccines in the next decade, with particular emphasis being placed on the use of biodegradable and biocompatible polymers.

Liposomes

Liposomes have been studied widely as drug carriers (Gregoriadis, 1988). Such studies have included much work on the immunoadjuvant action of liposomes (Gregoriadis, 1990). The majority of studies, to date, have been concerned with systemic administration of liposomes and their ability to act as drug carriers and/or immunoadjuvants (Gregoriadis, 1988 (chapters 8–21); Shahum and Therien, 1988; Therien and Shahum, 1989). It is only in recent years that liposomes have been considered as delivery systems and immunoadjuvants for the oral administration of antigens. Table 3 lists some immunization studies with liposomal antigens administered orally. Several conclusions may be drawn from these studies: liposomes allow the inclusion of adjuvants, are only weakly toxic and immunogenic by themselves, are able to entrap a variety of antigens and may help to protect the antigen from gastric acid and enzymatic degradation in the gastrointestinal tract. One possibility to explain the immunoadjuvant properties of liposomes is through their ability to render soluble antigens particulate and hence facilitate absorption by the M cells of the PP. Wachsmann et al. (1985) observed the presence of antigens in the PP after oral administration of liposome-entrapped antigens but not after administration of soluble antigens. In a review by Kimura (1988) of the absorption of drugs entrapped within liposomes, it was concluded that a small but significant quantity of liposomes can be taken up intact by small-intestinal mucosa, the method probably involving endocytosis.

Thus, liposomes, in preliminary investigations, have been shown to improve the immune response to orally administered antigens. Further work is necessary, however, to characterise the properties of liposomes, particularly concerning stability in the gastrointestinal tract and absorption therein, with a view to producing liposomes to optimize oral administration of antigens. One disadvantage which may limit the usefulness of liposomes as oral immunoadjuvants is the necessity for high temperatures and shear pressures in the synthesis of liposomes, thus possibly inactivating some antigens.

Oral Adjuvants

An adjuvant is a substance administered concurrently with an antigen to potentiate the immune response. As stated previously, one ap-

proach to the development of an oral vaccine is the use of synthetic peptides. As they are usually only weakly immunogenic it is necessary to administer them with adjuvants to elicit a beneficial immune response. Adjuvants would also be necessary with oral inactivated microbial vaccines as, in general, these do not stimulate as strong an immune response as live attenuated vaccines. A diverse group of compounds have been studied as adjuvants including aluminium hydroxide, saponins complexed to membrane protein antigens, pluronic polymers with mineral oil, killed mycobacteria in mineral oil, bacterial products such as lipopolysaccharide and muramyl dipeptide and liposomes (Gregoriadis, 1990).

Some adjuvants which have been shown to be active by the parenteral route are ineffective by the oral route; for example, orally administered iota-carrageenan was ineffective as an adjuvant compared to enhancement of the immune response when given intraperitoneally (Coste et al., 1989). Most studies, to date, have investigated the effect of oral adjuvants on antigen administration by the systemic route. For example, Quillaia saponins administered orally potentiated the immune response to inactivated rabies vaccine administered intraperitoneally to mice (Chavali et al., 1988) and taurine, parotin and lithium demonstrated potential as oral adjuvants to hepatitis B vaccine administered intraperitoneally to mice (Kuriyama et al., 1988). Few studies have been conducted on the ability of oral adjuvants to enhance the immune response to orally administered antigens. One adjuvant which has been shown to enhance the secretory immune response when coadministered with an antigen by the oral route is muramyl dipeptide (MDP). MDP, as stated by Mestecky (1987), is non-immunogenic, by itself, exhibits low toxicity and has none of the side-effects inherent to other adjuvants. MDP when incorporated into liposomes with an antigen has been shown to enhance the immune response compared to that elicited by the antigen alone (Fujimura, 1986). Incorporation of MDP into liposomes or indeed any delivery system may prevent it from enhancing the immune response to a large number of antigens present in the gastrointestinal tract. Avridine, a synthetic lipoidal amine, incorporated into liposomes and administered concurrently with oral killed influenza virus vaccine to mice has been shown to enhance the SIgA antibody response in the respiratory tract without concomitant serum antibody response or side effects (Bergmann and Waldman, 1988).

Through studies on the development of a new oral cholera vaccine it has been found that cholera toxin is an exceptionally potent oral immunogen capable of stimulating strong mucosal SIgA and plasma IgG antitoxin responses as well as immunologic memory in the intestine (Holmgren and Lycke, 1986). Cholera toxin has also been shown to be a strong mucosal adjuvant which does not seem to induce oral tolerance and which can markedly potentiate the immune responses to both immunologically related and unrelated protein antigens given orally (Elson, 1989). The adjuvant activity has been seen with the toxin composed of both A and B subunits, CT (Elson and Ealding, 1984; Lycke and Holmgren, 1986; Liang et al., 1988; Czerkinsky et al., 1989; Wilson et al., 1989; Chen and Strober, 1990) as well as the purified B subunit (BS) of cholera toxin (McKenzie and Halsey, 1984; Tamura et al., 1989a,b; Dertzbaugh et al., 1990). Since only the A subunit leads to activation of adenylate cyclase and elevation of intracellular cAMP levels, the current view is that the adjuvant activity does not depend on this second messenger pathway. Several mechanisms have been proposed to explain the adjuvant properties of CT and BS. Recently, Lycke and Strober (1989) observed that cholera toxin induces immune B cell isotype switching, from IgM to IgA expression, in a polyclonal activation system. This suggests that the adjuvant activity of cholera toxin may not only increase responses in all Ig classes, but also preferentially increase responses of certain Ig classes, such as the IgA antibody response predominantly in the mucosal system. Tamura et al. (1989a) suggested that BS acts on the induction phase of the immune response which consists of uptake, processing and presentation of the antigen by antigen-presenting cells and activation of T cells, while Elson and Ealding (1984) hypothesized that CT stimulates T cell help for both IgA and IgG responses after

TABLE 4

Outcome of the adjuvant effect of either cholera toxin or the B subunit to the immune response of certain antigens

References	Model	Rate of administration	Adjuvant	Antigen	Summary of main findings	Conclusion
Elson and Ealding (1984)	mouse	intragastric	cholera toxin (CT)	keyhole limpet haemocyanin (KLH)	Administration of CT and concurrent administration of KLH and CT produced both a systemic IgG and mucosal IgA response contrasting with a marked lack of an antibody response to KLH given alone by the intestinal route. Antibody response stimulated by CT was synthesised in multiple different lymphoid tissue within and outside the gut-associated lymphoid tissue.	Use of CT to understand the regulation of both immunity and tolerance to intestinal antigens may help to obtain the required mucosal and/or systemic response to antigens in the intestine in the future.
Lycke and Holmgren (1986)	mouse	intragastric	cholera toxin (CT) or B sub-unit (BS)	keyhole limpet haemocyanin (KLH)	Dose-dependent adjuvant effect of CT on gut mucosal immunity in response to oral KLH when administered concurrently. This effect was not evident when CT was given by the i.v. route. BS failed to potentiate the immune response to KLH and was also a poor immunogen compared to CT.	CT may substantially increase the mucosal immunogenicity and efficacy of non-replicating oral vaccines.
Czerkinsky et al. (1989)	mouse	intragastric	cholera toxin (CT) or B sub-unit (BS)	conjugate of streptococcal antigen 1/11 (Ag1/11) covalently coupled to BS	The conjugate and free CT both elicited strong IgA and IgG antibody responses in mucosal tissues, including salivary glands as well as serum antibody responses; the conjugate and free BS could not elicit such response indicating that the integrity of the native toxin is essential.	This immunization strategy, i.e., of an antigen coupled to BS, may find an application in the construction of oral vaccines for the control of infections caused by pathogens encountered at mucosal and extramucosal sites.
Chen and Strober (1990)	mouse	intragastric	cholera toxin (CT) or B sub-unit (BS)	inactivated influenza virus (H3 NZ)	Concurrent administration of either CT or BS with the influenza virus produced higher mucosal antibody responses of both IgM and IgA isotypes than immunization with vaccine alone. IgA response was greater relative to IgM response.	Results indicated that CT preparations enhance the memory B cells response to Peyer's patches. This was found to occur in the presence of appropriate lymphokines.

Liang et al. (1988)	mouse	intragastric	cholera toxin (CT) or B sub-unit (BS)	CT or BS chemically cross-linked to Sendai virus, using a two-stage glutaraldehyde coupling method	The conjugate CT with Sendai virus produced the highest gut immune response compared to virus alone or a mixture of the two. No significant difference was found in the gut immune response to conjugate BS and virus, mixture of the two or virus alone. Respiratory immunity was also investigated.	Mucosal adjuvant effect of CT for orally administered Sendai virus could be enhanced by covalently coupling CT to the virus.
Wilson et al. (1989)	mouse	intragastric	cholera toxin (CT)	keyhole limpet haemocyanin (KLH) or ovalbumin (OVA)	Cholera toxin exerted an adjuvant effect on the mucosal response of all three mouse strains to KLH and OVA, although the responses to CT and the second protein were not correlated.	The findings indicated that while CT had an effect on the mucosal immune system which enhances the immune response to itself and other protein antigens, the final outcome of the response to the second antigen is dependent on differences in the ability of the strains to process, recognize, and respond to a particular antigen.
Tamura et al. (1989a)	mouse	intranasal	B subunit (BS)	influenza vaccine (PR-8HA)	BS produced an augmented and persistent antibody response to PR-8HA vaccine.	Results suggested that the cells that are located in the nasal site and that participate at early stages of immune responses are most affected by BS.
McKenzie and Halsey (1984)	mouse	intraduodenally	B sub-unit (BS)	horseradish peroxidase (HRP) covalently coupled with BS via a 2-step glutaraldehyde procedure (HRP-BS)	The IgA responses in the intestinal washes were 33–120-fold higher when the conjugate was used as the immunogen in comparison with immunization with a mixture of BS + HRP or HRP alone.	BS functioned as an effective carrier to stimulate mucosal immunity to a protein that is not normally immunogenic at mucosal surfaces. Protein conjugates coupled to BS may be useful as mucosal vaccines.
Tamura et al. (1989b)	mouse, pretreated up to 6 weeks before nasal vaccination	intranasal	B sub-unit (BS)	influenza vaccine (PR-8HA)	Levels of nasal IgA antibodies to BS increased with the increase of dose of BS and the frequency of BS inoculation. Pre-existing immunity to BS, did not significantly reduce the levels of both nasal IgA and serum HI antibodies to influenza virus and did not change the ability of the vaccinated mice to resist viral challenge.	The results suggest that a relatively low dose of BS could be inoculated repeatedly into animals as an adjuvant for nasal vaccination.

TABLE 4 (continued)

References	Model	Rate of administration	Adjuvant	Antigen	Summary of main findings	Conclusion
Dertzbaugh et al. (1990)	mouse	intragastric	B subunit (BS)	A synthetic peptide, encoding amino acid residues 345–359 of the glucosyltransferase B enzyme of *Streptococcus mutans* GS-5, was genetically fused to the N-terminal end of the B-subunit gene of cholera toxin.	Addition of a short amino acid sequence has little effect on the structure or function of BS. Mice developed antipeptide antibodies in their serum indicating the chimera was successfully delivered to the gut-associated lymphoid tissue although a relatively large amount of chimera had to be given to elicit this response.	The size of the peptide should be kept to a minimum to minimize any structural changes in BS. Further work required to see if the chimera has retained any of the adjuvant properties associated with BS.

concomitant administration of CT and antigen. Table 4 lists examples of several compounds for which cholera toxin has been used as an adjuvant. Several observations have been made from studies on the adjuvant properties of cholera toxin:

(1) To exhibit mucosal adjuvant activity CT should be administered concomitantly with and by the same route as the antigen (Lycke and Holmgren, 1986; Tamura et al., 1989a,b; Wilson et al., 1989; Chen and Strober, 1990); indeed several investigators have shown that to potentiate the immune response CT must be conjugated to the antigen (McKenzie and Halsey, 1984; Liang et al., 1988; Czerkinsky et al., 1989). One study (Dertzbough et al., 1990) showed that genetic fusion of a small peptide to the N-terminal of BS had a minimal effect on the structure and function of BS.
(2) Some discrepancy exists as to whether BS is an effective adjuvant; some authors observed BS to be a poor adjuvant for oral administration (Liang et al., 1988; Czerkinsky et al., 1989; Wilson et al., 1989) while others found BS to possess adjuvant properties (McKenzie and Halsey, 1984; Tamura et al., 1989a,b; Dertzbough et al., 1990).
(3) In general, both mucosal SIgA and serum IgG responses are potentiated by the adjuvant mixture/conjugate when compared to the antigen administered alone (Elson and Ealding, 1984; McKenzie and Halsey, 1984; Liang et al., 1988; Czerkinsky et al., 1989; Wilson et al., 1989).
(4) Repeated inoculations of small amounts of BS influenced neither the levels of antiviral antibodies nor protection against viral challenge in mice (Tamura et al., 1989b). Pre-immunization with a low dose of BS produced only a small amount of anti-BS antibodies which did not limit the adjuvant effect of BS. It has yet to be investigated whether higher doses of BS would induce the specific antibodies in sufficient amounts to limit the adjuvant effect of BS.

With the advent of biosynthetic and recombinant DNA technology, it is foreseen that more subunit and peptide vaccines will be developed for both systemic and oral administration. This has been accompanied by an upsurge of interest to develop new, safe and effective adjuvants which are biodegradable, non-toxic, non-immunogenic and able to elicit a wide range of immune responses to antigens delivered by various routes. This will indeed prove to be a challenge in the forthcoming years.

Hybrid Vaccines

Recently, there has been much interest in the use of attenuated mutants of *Salmonella* as live delivery systems for other human pathogens with a view to producing protective immunity against both the carrier organism and the pathogen which it carries (Clements, 1987; Curtiss et al., 1989). This interest was stimulated from the knowledge that *S. typhi* in primates and *S. typhimurium* in mice are able to colonise the intestinal tract and proliferate in the gut-associated lymphoid tissue (GALT) before systemic infection occurs. Thus, it was thought that these organisms could deliver specific antigens to the GALT and therein elicit an immune response. Advantages of avirulent *S. typhi* strains as carriers are possible oral administration, a broad immune response to provide protection against a wide range of pathogens and the availability of techniques for stabilising foreign genes in the chromosomes or plasmids of *S. typhi*.

Ty21a has been illustrated here as an example of an avirulent mutant which has been used as a carrier for several other antigens. Other avirulent mutants of *Salmonella* have also been investigated as suitable carriers (Chatfield et al., 1989). These however, will not be mentioned further in this discussion. Table 5 shows some of the Ty21a conjugate vaccines which have been investigated. These include antigens from *V. cholerae*, *S. sonnei*, *E. coli* and *S. flexneri*. From Table 5 it can be seen that a conjugate vaccine shows potential to stimulate immune responses to both the carrier organism and the pathogen for which it codes both in animal models and human volunteers. These vaccines, as they have been conjugated with the *S. typhi* strain Ty21a, portray the inher-

TABLE 5

Use of Ty21a and an S. typhimurium strain as carriers for other antigens

Carrier	Introduced antigen	Model/ volunteer	Route of administration (no. of doses)	Outcome	Reference
Ty21a	genes encoding the protective 0 antigen of *Vibrio cholerae*	human volunteers	oral (3)	Well tolerated and moderately immunogenic.	Forrest et al. (1989)
Ty21a	genes encoding the cell surface antigen, termed Form 1, of *Shigella sonnei*	mouse	intraperitoneal	The conjugated vaccine protected mice against lethal challenge dose of either *S. typhi* or *S. sonnei*.	Formal et al. (1981)
Ty21a	120-MDa plasmid of *S. sonnei*	human	oral	Following vaccination an IgA response to 0 antigens of *S. typhi* and *S. sonnei* was observed in 10 of 13 and 13 of 13 vaccine recipients, respectively. However, the level of antigen-specific antibody-secreting cells generated by the vaccine did not protect vaccinees against challenge.	Van de Verg et al. (1990)
Ty21a	an enterotoxigenic *E. coli* plasmid encoding colonization factor antigen (CFA) 1 fimbriae and heat stable toxin – two known antigens of *E. coli*	–	–	The plasmid carrying conjugate produced CFA1 fimbriae and heat-stable toxin suggesting that this was a viable conjugate.	Yamamoto et al. (1985)
Ty21a	recombinant plasmid containing the gene for the production of the nontoxic B subunit of the heat-labile enterotoxin of *E. coli*	mouse and guinea-pig	oral or intraperitoneal	The bivalent vaccine was shown to be safe when given orally and was capable of inducing significant antitoxic antibody response when injected intraperitoneally. The vaccine retained the galactose sensitivity characteristics of the parent strain Ty21a.	Clements and El-Morshichy (1984)
Ty21a	recombinant plasmid with genes encoding the *Shigella flexneri 2a* type and group antigens	mouse	intraperitoneal	Mice immunized with this vaccine strain were found to be protected against challenge with virulent *S. flexneri 2a*, but not significantly against *S. typhi* challenge.	Baron et al. (1987)
Ty21a	recombinant plasmid pMM-CTB which contains the gene for production of the non toxic B-subunit of *V. cholerae*	mouse	intraperitoneal	The resulting conjugate could produce B subunit with the same immunogenicity of B subunit produced by *V. cholerae*. The conjugate protected against challenge with virulent strains of *S. typhi* and *V. cholerae*.	Ma et al. (1990)
S. typhimurium (WR4017)	*Plasmodium berghei* circumsporozite (CS) protein gene	mouse	oral or subcutaneous	The conjugate vaccine induced antigen-specific cell-mediated immunity and protected mice against sporozite challenge in the absence of antisporozite antibodies.	Sadoff et al. (1988)

ent problems associated with this strain, such as only moderate efficacy and undue vaccine formulation sensitivity. It is hoped that future conjugate vaccines may be produced with a more defined strain of *S. typhi* such as those now being constructed by modern DNA techniques, as it can be seen clearly that conjugate vaccines would greatly ease immunization campaigns by essentially protecting against two diseases with one vaccine and hopefully in one dose. One potential disadvantage of all hybrid vaccines is the possibility that immunity may develop to the carrier organism precluding its use as a carrier for other antigens or indeed as a booster of the original antigen. This type of response would be manifested through secretory antibodies, induced by repeated exposures to a carrier strain which prevents attachment and penetration into the Peyer's patch and thereby reducing the immune response produced.

Conclusions

Immunization by the oral route has many advantages over parenteral administration which is currently the most commonly employed route for vaccination. In recent years, several different methods have been employed to stimulate both local secretory and systemic immune responses by oral antigen administration. These have been reviewed in this paper. The first attempts to prevent antigen degradation in the stomach involved the concurrent administration of antigen with antacid solution or enteric coating of the antigenic material. These methods protected against inactivation by gastric acid but did not protect against proteolytic degradation in the intestine or promote retention in the gastrointestinal tract. One promising area of research involves the formulation of antigens into particulate material and the subsequent delivery mainly through the M cells of the Peyer's patches. Studies on this type of system are limited as yet, but it is hoped that a combination of better understanding of the adherence mechanisms of antigens to M cells and indeed mucosal epithelial cells, transport of antigens across these cells and the mechanism and type of immune responses elicited by oral antigens will enable researchers to formulate particulate delivery systems which are effective by the oral route. Much interest has been shown in the use of liposomes not only as delivery systems but also as immunoadjuvants although their use may be limited in this application due to the necessity of high temperatures and shear pressures in the synthesis of liposomes which may inactivate some antigens.

Immunoadjuvants will play an important role in oral vaccine administration with synthetic vaccines as these are often only weakly immunogenic by themselves. Cholera toxin and its B subunit have been shown to be potent oral immunogens capable of stimulating both a secretory and systemic immune response. It is evident, however, that much work is still necessary to develop safe and effective oral immunoadjuvants to potentiate the immune response to orally administered antigens.

The last area of research into oral vaccination delivery involves the use of carrier organisms as a delivery system for other human pathogens with a view to producing protective immunity against both the carrier organism and the pathogen for which it codes. The most common carrier used to date is Ty21a. Modern DNA techniques will hopefully allow new carriers to be developed which have defined characteristics as hybrid vaccines have the potential ability to protect aganist two diseases simultaneously thereby greatly easing immunization campaigns.

With the recent upsurge of interest in the oral delivery and absorption of antigenic material it is hoped that oral vaccines will be developed which are safe and effective. They should have the ability to stimulate a local immune response and if necessary also a systemic response to a wide range of pathogens which either infect or enter the human host through mucosal surfaces.

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