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Advances in Oral Vaccine Delivery Options

What is on the Horizon?

Diane E. Webster,[1] *Michelle E. Gahan*,[1,2] *Richard A. Strugnell*[3] and *Steve L. Wesselingh*[1,2]

1 Children's Vaccines Group, Macfarlane Burnet Institute for Medical Research and Public Health, Melbourne, Victoria, Australia
2 Department of Medicine, Monash University, Alfred Hospital, Prahran, Victoria, Australia
3 Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria, Australia

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Abstract

Vaccines have been one of the most far-reaching and important public health initiatives of the 20th century. Yet as we move into the 21st century, millions of people still die from vaccine-preventable diseases such as measles and tetanus, and complex diseases, such as malaria and HIV, for which we have no vaccines. New vaccines that can be administered orally, are stable at ambient temperature, and can be produced cheaply, have the potential to transform health policy and practice in both developed and developing countries.

Although oral delivery is the preferred route of administration, it is inefficient for the delivery of 'naked' antigens. Delivery of a vaccine via the oral route in a sufficient dose to induce a protective immune response depends on overcoming the loss of antigen integrity that occurs during intestinal passage. Several strategies have been employed to prevent this loss of antigen(icity) and improve the delivery of vaccinogens by the oral route.

These include live vectors, transgenic plants, and particulate formulations such as microparticles, liposomes, and virus-like particles. Although many of these systems have progressed to clinical trials, the most promising results are seen where strategies are combined. Prime-boost schedules and combined technologies are likely to be a critical component of future oral vaccination schedules. Further technological breakthroughs may also be required before strong protective immune responses can be consistently induced in humans. Nonetheless, the potential of future oral vaccination strategies is readily apparent.

The global eradication of smallpox stands as a major health outcome of the 20th century. A second disease, polio, should be eradicated by the year 2010 as the result of a vaccination program targeting children, especially newborns, in developing countries.[1] The availability of an orally administered, cheap and reliable vaccine has made this program possible. However, diseases such as measles will continue to be difficult to eradicate as the current vaccine is administered by injection, requires refrigeration, and is less effective in children under 12 months of age.[2] The eradication of diseases such as measles will require the development of safe, effective vaccines that are cheap to produce and can be administered via nonparenteral routes. These include oral, intranasal, intrarectal and intradermal routes of administration. This review focuses on current research in the field of oral vaccine delivery, with particular reference to the challenges facing vaccine development in the new millennium.

Oral delivery offers several advantages over other routes of vaccine administration (table I). Oral administration is less invasive and less expensive than injection. Oral vaccines can be administered outside formal clinical settings without the need for highly skilled personnel. Eliminating needles from the vaccination process also reduces the risks associated with needle reuse and disposal, such as the transmission of blood-borne viruses. The ease of oral administration should improve vaccine coverage in remote areas and enhance compliance, particularly in children and when multiple doses of vaccines are required. In addition to systemic immunity, oral delivery can induce mucosal immunity at a number of sites via the common mucosal immune response.

Table I. Potential advantages of oral vaccine delivery

Less expensive than injectable vaccines
Needle-free administration
reduced need for highly-trained medical personnel
eliminates risks associated with needle reuse and disposal
Enhanced availability and vaccine coverage
Enhanced compliance, especially for children
Potential to induce both systemic and mucosal immunity
Compatible with the delivery of multiple antigens and/or vaccines
May be supplemented with adjuvants

1. Methods of Oral Delivery

Although oral delivery is the preferred route of administration, it is inefficient for the delivery of 'naked' antigens. To stimulate an effective immune response, sufficient antigen must be presented to microfold (M) cells, and subsequently be transported into lymphoid tissue located immediately under the ileal Peyer patches and more distally in the colon.[3] This requires antigens to retain their immunogenicity despite the denaturing effects of stomach acid and enzymatic degradation in the intestine. In addition, dilution of the antigen in the gut reduces the chances of interaction between the antigens and the mucosal immune system. As a result, higher doses of antigen are required for successful oral vaccination compared with nasal or parenteral routes.[4,5] Several strategies have been employed to prevent this loss of antigen(icity) and improve the delivery of vaccinogens by the oral route. These include live vectors, transgenic plants, and particulate formulations such as liposomes and virus-like particles (VLPs).

1.1 Live Vectors

The use of recombinant vectors to deliver vaccine antigens is a natural extension of the successful oral delivery of a range of live bacterial and viral vaccines. Live vectors may induce cellular, humoral, and mucosal immune responses against both the vector and the heterologous antigen. Furthermore, the use of live vectors, with the ability to replicate in the host, results in the presentation of antigen to the immune system over an extended period of time. The main limitation of this technology remains the ability to achieve a strong immune response using vectors that are sufficiently attenuated to be considered safe, particularly in immunocompromised individuals.[6] Recombinant bacterial and viral vectors against a range of diseases are currently being studied.

1.1.1 Recombinant Bacterial Vectors

Both attenuated and commensal micro-organisms (table II) have been used to deliver a variety of different viral, bacterial, and parasitic antigens with varying degrees of success.[7-10]

To ensure immunity, not disease, is induced in vaccinated hosts, the choice of attenuating mutation is important. Attenuation should be sufficient to ensure bacteria have an adequate safety profile and are unable to revert to virulence, whilst still enabling the establishment of a limited infection. Carriage of foreign antigens by attenuated bacteria has the additional advantage of generating simultaneous immune responses against both the carrier and foreign antigen, hence acting as a dual vaccine. Of concern with the use of attenuated carriers, which are highly immunogenic, is the role of pre-existing immunity to the carrier, the impact of which is uncertain and debated in the literature.[36-40] If pre-existing immunity significantly compromises the ability of vectors to deliver antigens to the immune system, greater consideration must be given to the choice of vector, particularly in developing countries. For example, *Salmonella typhi* may be ineffective as a vector in areas where typhoid is endemic or mass vaccination campaigns are in place.

Commensal bacteria form part of the normal flora of oral, gut, and vaginal mucosal surfaces. They are generally considered as 'safe' organisms through their long-time use in food processing and preservation. They can be delivered as food products, have additional probiotic properties, and have the ability to colonize specific mucosal regions inducing both mucosal and systemic immune responses.[17,23,24] In contrast to attenuated pathogens, the reduced pathogenicity of commensal bacteria renders them more suitable for use in vulnerable groups such as infants and the elderly.[10]

Recombinant bacteria have been employed to express vaccine antigens either through insertion into a plasmid, or integration into the host chromosome. More recently, the potential of recombinant bacterial spores to deliver vaccine antigens has been explored.[33,41] Bacterial vectors have also been used to deliver DNA vaccines.[42-44] In this system, the carrier organism is used to transfer the gene encoding the vaccine antigen into the host cell. This approach has the advantages associated with DNA vaccination, including increased stability, optimal protein folding, long-lasting immunity, low development costs, and post-translational modifications. It also facilitates the combination of antigens and the addition of immune stimulatory sequences.

Table II. Bacterial vaccine vectors used for oral delivery

Attenuated strains	Commensal strains
Salmonella spp.[11-16]	*Lactobacillus* spp.[17]
Shigella spp.[18]	*Lactococcus lactis*[19,20]
Vibrio cholerae[21,22]	*Streptococcus gordonii*[23-25]
Listeria monocytogenes[26,27]	*Staphylococcus* spp.[28,29]
Mycobacterium bovis BCG[30-32]	*Bacillus subtilis*[33]
Yersinia enterocolitica[34,35]	

BCG = Bacille Calmette-Guérin

One requirement for vaccine effectiveness is the retention and stability of the plasmid. *In vitro* and *in vivo* plasmid stability correlates with vaccinogen-specific antibody production[45] and bacterial colonization[46] in mice. As the immunized mammalian host lacks selective pressures (i.e. antibiotic) it is essential the plasmid used exhibits an inherent capacity for retention. Low copy-number plasmids should preferentially be used as multicopy-number plasmids lack stabilizing loci and undergo random partitioning.[47] Other solutions include the use of *in vivo* regulated inducible promoters,[48] integration of the foreign gene into the chromosome of the bacterial vector,[49] and the incorporation of balanced-lethal host-vector systems.[50] In the case of the latter, a gene encoding an essential enzyme, missing in the mutant, is inserted into the same plasmid as the foreign antigen, ensuring that all of the surviving population retains the plasmid.

While bacterial vectors have been shown to be efficacious in rodent models, results from clinical studies have been limited. For example, *S. typhi* has been used to express *Helicobacter pylori* urease and hepatitis B antigens.[51,52] In both cases, strong immune responses were described in mice;[53,54] however, no immune responses to the recombinant antigen were detected in human volunteers, while *S. typhi*-specific immune responses were detected in study participants. Tacket et al.[55] have shown that delivery of the C fragment of tetanus toxin by *S. typhi* can stimulate a protective level of serum antibodies in individuals whose pre-immunization levels were not protective (<0.01 IU/mL).[55] However, this result should be interpreted with caution as it was observed in only one individual and may represent an oral boosting response as the individual may have previously received the commercial tetanus vaccine. On a more promising note, *S. typhi* vaccine strain CVD 908 expressing the circumsporozoite protein (CSP) of *Plasmodium falciparum* fed to ten volunteers, resulted in the induction of antibody responses in two volunteers,

and a CSP-specific CD8+ cytotoxic T lymphocyte (CTL) response in a third volunteer.[56]

A number of issues remain to be addressed, including the choice of an appropriate carrier and attenuating mutation for the disease in question, the effects of pre-existing immunity, increasing stability, and localization of the antigen. It is not known how many doses will be necessary to induce an immune response or whether bacterial vectors are more suited to priming mucosal and systemic immune responses (see section 1.4). Although a promising strategy for the delivery of oral vaccines, further work is necessary to translate the success of bacterial vectors in animal models into the clinical setting.

1.1.2 Recombinant Viral Vectors

Recombinant viral vectors have been engineered for the delivery of heterologous vaccine antigens to the immune system for a diverse range of diseases, including cholera, influenza virus, HIV, hepatitis B virus, human papillomavirus, and herpes simplex virus.[57-61] While a number of vectors have been used for the production and delivery of antigens, only a few have been successfully employed for oral delivery; namely, adenovirus, vaccinia virus, and poliovirus.[57] These viruses have been extensively tested in humans and are known to induce long-lasting immunity to both the vector and the recombinant antigen. However, the potential of live vectors to establish clinical illness should not be discounted, particularly in immunocompromised individuals.[62]

Recombinant adenoviruses are one of the most promising strategies for oral vaccine delivery. They are considered to be only mildly pathogenic in humans and have been extensively used by the US military to prevent epidemics among recruits.[61,63] The production and use of recombinant adenovirus vaccines has been reviewed by Imler.[61] Both replication-competent and replication-deficient vectors have been trialled using a number of antigens in a range of models. Animal adenoviruses have been used successfully in a number of oral veterinary vaccines.[64] Furthermore, using a mouse model for human disease, Sharpe et al.[65] recently demonstrated a dose-response relationship in mice vaccinated with replication-deficient adenovirus expressing the measles nucleocapsid protein. They found that a single high-titer oral dose was sufficient to induce long-lasting antigen-specific cellular and humoral immune responses, and suggested that the success of replication-deficient vectors may be a function of antigen dose. This may explain primate study results, where partial protection was observed in chimpanzees vaccinated twice with replication-deficient adenoviral vectors expressing the hepatitis B surface antigen (adeno-hepatitis B), but not in the chimpanzee that received a single vaccine dose.[66] Similarly, volunteers in a phase I clinical trial who received a single dose of the adeno-hepatitis B vaccine did not develop a hepatitis B-specific response.[67]

It is worth noting that the cloning capacity of human adenoviruses is limited. E1 and E3 deletion mutants can only package 6–7 kbp of heterologous DNA.[61] While most antigens for subunit-based vaccines are likely to be smaller than this, capacity may be limiting for multi-valent vaccine strategies.

Although the original route of immunization with vaccinia virus was intradermal, studies have shown that recombinant vaccinia virus (rVV) is also immunogenic when delivered orally.[59,68] This approach has a number of advantages; for example, the cloning capacity of rVV is >25kb.[69] In addition, effective oral delivery of rVV has been demonstrated in a range of species, including primates.[68,70] The most successful example of oral immunization used an attenuated rVV expressing the rabies glycoprotein (V-RG).[68] Large-scale field release of the V-RG **:** bait formulations in Europe has resulted in a significant reduction in the number of reported cases of rabies in foxes in the surrounding areas. Despite the success of the V-RG vaccine, development of orally delivered rVV-based vaccines for use in humans has been slow. Only a small number of studies have been published detailing oral immunization of rVV-encoding antigens from influenza virus and HIV.[59,60,71] Collectively, these papers demonstrate that humoral- and cellular-immune responses may be successfully induced systemically and at mucosal surfaces.

Despite the success of the oral polio vaccine there is very little published data with respect to oral delivery of recombinant poliovirus. Recombinant poliovirus research has been hampered by the instability of recombinant vaccine constructs, and limitations of the vector's cloning capacity.[57,58] A single study in mice gavaged with poliovirus replicons encoding the HIV gag gene in place of the capsid protein reports the induction of an HIV-specific mucosal response.[72] Although there are no clinical studies, there are a number of theoretical advantages for using polio as a recombinant vector, including established experience and infrastructure for vaccine delivery.

1.2 Transgenic Plants

Vaccine antigens may also be produced and delivered using plants. This may be achieved through a variety of methods, includ-

ing stable transformation of nuclear (figure 1) or chloroplast genomes and antigen expression using plant viral vectors.[73,74] As a means of oral vaccine delivery, plant-based vaccines offer a range of advantages over traditional vaccine production and delivery systems.[75,76] Production of recombinant proteins in plants is economic, with minimal manufacture and processing requirements. Crops may be grown and processed using existing agricultural knowledge and experience. Appropriate transgenic crops could be grown locally, reducing transport requirements and dependence on foreign supply. There is also evidence to suggest that antigens expressed in plant storage organs, such as seeds, are stable at room temperature, thereby eliminating the need for refrigeration during transport and storage.[77,78] Furthermore, plants have been widely used in traditional medicine for centuries,

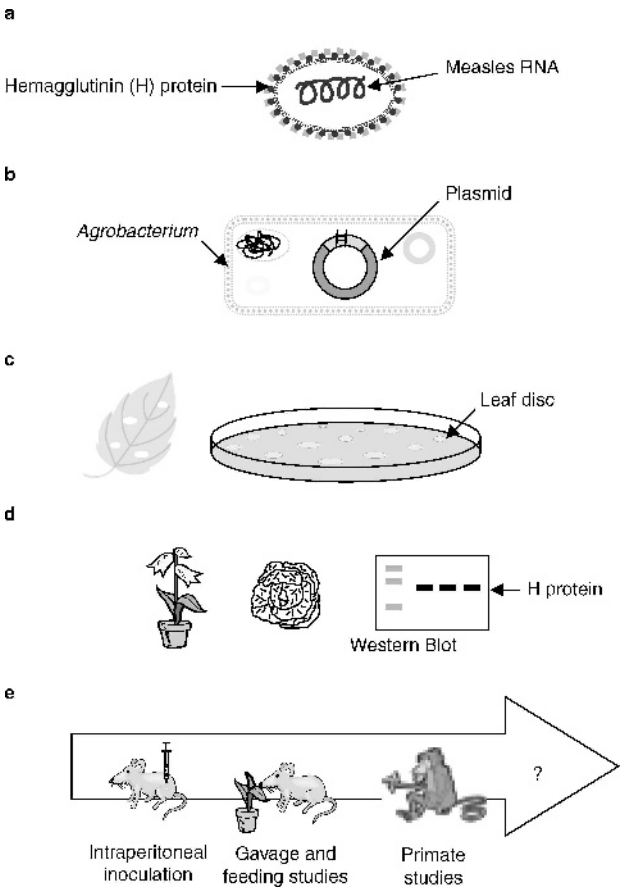

Fig. 1. Development of a plant-based vaccine: (**a**) select a vaccine target (antigen) and identify the coding sequence; (**b**) transfer the coding sequence to a gene vector (e.g. plasmid) and insert into *Agrobacterium* spp.; (**c**) incubate *Agrobacterium* spp. with plant tissue; (**d**) generate whole plants from transformed cells and characterize antigen expression by Western blot; and (**e**) define antigen immunogenicity in animal studies (reproduced from Webster et al.[75] [© Copyright 2002, the Medical Journal of Australia], with permission).

potentially enhancing the acceptance of new vaccines in developing nations and indigenous cultures. Plant-based vaccines are currently being developed for a number of human and animal diseases, including measles, enterotoxic *Escherichia coli* (ETEC), cholera, foot and mouth disease, rabies, malaria, hepatitis B and C viruses, and HIV.[79]

Much of the early work on plant-based vaccines was performed in species such as tobacco and potato. Both of these species contain a number of toxic compounds that make them unsuitable for unprocessed oral delivery. Many laboratories are now moving to species such as rice and corn, which have excellent potential for vaccine production and delivery.[75,80] Seeds may be ground into flour for easy delivery to infants. Rice is also commonly used in baby food due to its low allogenicity, making it a particularly good choice for vaccines intended for delivery to young infants. In corn, the milling process further enriches the antigen content with the separation of the antigen-rich embryo from the endosperm.[78]

An alternative approach is freeze-drying, which significantly increases the antigen dose on a per gram basis and facilitates storage of perishable crops for long periods of time.[76] The use of freeze-drying technology increases the range of species that may be utilized for plant-based vaccines. Powdered formulations also make it easier to ensure consistency of vaccine dose and may be readily supplemented with mucosal adjuvants, vitamins, and/or other vaccines, thereby maximizing the use of infrastructure and reducing the number of contacts necessary with remote communities.

Although it is envisaged that these vaccine crops could be grown locally, consideration needs to be given to the acceptability of genetically modified (GM) crops. There continue to be a number of social, ethical, and environmental concerns surrounding GM technologies. However, current data suggest that the environmental impact of GM crops will be similar to nonmodified crop species.[81] In addition, the health risks from plant-derived vaccines are unlikely to be greater than those associated with subunit vaccines derived from other sources. It will be important to further develop technologies to ensure that plant-derived vaccines do not mix with food supplies. However, the health consequences of any accidental contamination of the food chain will vary and should be assessed on a case-by-case basis. The ability to supply the vaccine as a powdered formulation may represent an acceptable compromise for countries that wish to remain GM-free.

The ability to supply sufficient antigen to induce protective immunity remains a critical issue. Considerable work has been

done to optimize the expression of recombinant proteins in plants.[73] The 3′ untranslated region has been shown to influence RNA stability and antigen accumulation, and subcellular targeting can have a significant effect on antigen survival.[80,82,83] However, there remain many variables and few reliable predictors of transgene expression levels. High-level antigen expression is dependent on the inherent properties of the protein being expressed. It is likely that a combination of improvements in expression levels and advances in manufacturing technologies, such as freeze-drying, will ultimately be required to achieve the dose needed for effective vaccination.

The ability of plant-based antigens to induce protective immune responses has been examined for a wide range of antigens,[79] and at least four of these have progressed to successful phase I clinical trials.[84-87] The success of oral vaccination trials can be linked to the type of antigen being expressed. Antigens that are strong mucosal immunogens or adjuvants, such as cholera toxin B (CTB) and the *E. coli* heat-labile enterotoxin B subunit (LTB), appear to be the most effective vaccines.[84,88] This feature has been exploited by a number of groups through the use of CTB and LTB as carriers for epitopes and small proteins.[89,90] Antigens that form VLPs also result in successful vaccination. This is thought to be due to their inherently stable structure (see section 1.3.4). Successful clinical trials using plant-derived hepatitis B virus surface antigen (HBsAg) VLPs and Norwalk virus VLPs are examples of this type of antigen.[85,86] The final type of antigen is soluble protein. Only four soluble antigens have been shown to induce immune responses following oral vaccination – two of veterinary interest[78,91] and two of medical interest.[92,93] Immune responses to soluble antigens are more variable than responses to strong mucosal immunogens or VLPs, and are often dependent on the addition of mucosal adjuvants and/or high vaccine doses.

1.3 Particulate Antigen Delivery Systems

A number of particulate systems have been developed for the oral delivery of vaccines. These include microparticles, liposomes, immune-stimulating complexes (ISCOMS) and VLPs. In general, particulate formulation acts to maintain antigen integrity and facilitate uptake by ileal M cells. Particulate systems also have comparable sizes to natural pathogens, which may assist their uptake.[94] Furthermore, immunostimulatory and targeting molecules can be included to enhance or focus the immune response.[95,96]

1.3.1 Microparticles

In the microparticle strategy, protein antigens or DNA are encapsulated into microparticles using a range of different polymers. The most commonly used polymers are poly(lactide-co-glycolides) [PLGs]. PLGs are biodegradable and biocompatible with a variety of medical uses, including sutures and controlled-release drug delivery. Other polymers, such as polylactide (PLA) and polylactide-co-poly(ethylene glycol) [PELA], have also been employed.[97,98] In addition, pH-sensitive enteric coatings, such as Eudragit®[1] L30 D-55 and carboxymethylethylcellulose, have been used to microencapsulate vaccines[99] and to coat polymer microparticles.[100]

Encapsulated antigen has been shown to induce stronger immune responses following oral delivery than soluble antigens.[101-103] It is thought that the polymer microparticles provide protection against intestinal degradation and promote uptake by M cells.[101,104] While premature release of vaccines may occur due to microparticle degradation, varying polymer composition and particle size, and pH-sensitive coatings, have been shown to overcome this.[100,101] There remains some debate surrounding the extent to which microparticles are absorbed following oral delivery. Some studies have suggested that fewer that 0.01% of administered microparticles bind to the mucosal wall.[105] In addition, microparticle vaccine efficacy is a trade-off between optimal particle size for uptake by M cells, and antigen loading. Consideration should also be given to the solvents and processes required to generate the microparticle, and the impact they will have on antigen conformation.

Microparticles containing antigens from a variety of infections, including *Bordetella pertussis*,[106,107] *Salmonella typhimurium*,[108] *Chlamydia trachomatis*,[109] *Vibrio cholerae*,[110] malaria,[111] and influenza virus,[112] have been shown to induce immunity in mice when administered orally. Both mucosal and systemic antibody responses have been reported. Orally administered inactivated simian immunodeficiency virus encapsulated in microparticles has been found to induce protective immunity in macaques that had been primed intramuscularly.[113] Microparticles have also been used for the delivery of DNA vaccines for viruses such as HIV and rotavirus.[114,115] Three phase I oral vaccine studies using microparticles have been published.[116-118] No significant immune

1 The use of tradenames is for product identification purposes only and does not imply endorsement.

responses were detected in volunteers fed microparticles containing HIV peptides from the V3 loop of gp120.[118] By contrast, microparticles containing the colony-forming antigen from ETEC conferred protection in 30% of volunteers.[117] In a more recent study, PLG-encapsulated ETEC-CS6 was also found to induce immune responses,[116] however, these microparticles are yet to be tested in a challenge experiment. Some concerns regarding the feasibility of microparticles for oral vaccine delivery remain, particularly given the small number of published phase I trials and the poor responses in trials with pigs.[105,119] Nonetheless, the ETEC phase I trials do provide proof-of-concept for the use of orally delivered microparticle vaccines.

1.3.2 Liposomes

Liposomes are layered membranous vesicles consisting primarily of phospholipids and lipopolysaccharides into which a vaccinogen can be incorporated. Like microparticles, this confers protection from the intestinal mileu and facilitates uptake by ileal M cells. Liposomes can be designed with varying properties, including size, permeability, and charge.[120] Modification of the liposomal surface with natural or hydrophobized polysaccharides (so-called polysaccharide anchored liposomes) improves the stability and targeting of liposomes.[121] In addition, modified and polymerized liposomes have been developed to improve particle integrity and provide for controlled protein release.[122,123] Liposomes can incorporate combinations of antigen, adjuvants, and targeting molecules, allowing them to bind specifically to tissues and cell types.[95,120] Liposomes are currently used in medical practice, primarily for intravenous drug delivery. Vaccine studies in rodents have generated protective immune responses to ETEC strains using liposomal vaccines containing trapped antigen.[124] A few small studies have also demonstrated the potential for a liposomal vaccine in humans. Childers et al.[125] first described salivary IgA responses using liposomes containing antigenic polysaccharides from *Streptococcus mutans*. More recently, Chaicumpa et al.[126] also demonstrated an enhanced mucosal immune response of liposomes containing cholera antigens compared with naked antigen.

Virosomes are an extension of liposome technology, generated by combining phospholipids with viral glycoproteins. This enhances delivery efficiency by mimicking virus-mediated targeting, and facilitates antigen uptake.[127,128] Systemically administered virosome vaccines for hepatitis A virus (Epaxal®, Berna Biotech, Bern, Switzerland) and influenza virus (InflexalV®, Berna Biotech) are currently in clinical use, and a virosome-based intranasal influenza virus vaccine is also performing well in clinical trials (Nasal-Flu®, Berna Biotech).[129,130] Virosomes have theoretical potential as an oral delivery system, however this is yet to be demonstrated.

1.3.3 Immune Stimulating Complexes

ISCOMS consist of antigen incorporated into a lipid and adjuvant (Quil A) matrix. ISCOMS are nontoxic and highly stable structures, which form three-dimensional cages 30–70nm in diameter.[131] When given orally, ISCOMS enhance the absorption and immunogenicity of antigens and are able to enhance humoral, mucosal, and cell-mediated immune responses.[132-134] ISCOMS also facilitate the recruitment of antigen-presenting cells into the mesenteric lymph nodes and Peyer patches.[135] ISCOMS may be able to stimulate immune responses in the presence of pre-existing neutralizing antibodies.[136] This will be particularly important for diseases such as measles where the presence of maternal antibodies is a significant limitation of current vaccine strategies.

1.3.4 Virus-Like Particles

Many viral proteins are able to assemble into noninfectious particles in the absence of nucleic acid. The formation of these VLPs has been reported for viruses with single[137] and multiple capsid proteins.[138] Vaccines which form VLPs are better able to resist the stomach acid and gut proteases encountered during oral delivery than soluble antigens.[139,140] VLPs have been produced using many expression systems, including baculoviruses, insect cell culture, viral vectors, yeast, and transgenic plants.[137,141-143] The increased stability of VLPs may also contribute to the increased antigen accumulation in these systems. VLPs have now been used successfully in a number of clinical oral vaccine trials, including recombinant Norwalk virus and hepatitis B virus.[85,86,144] In both studies, immune responses could be induced without adjuvant.

VLPs have also been used as carrier proteins. This is achieved through the integration of antigen sequences into VLP genes. This approach has many advantages, including copy number, stability, and the presence of helper T cell epitopes in the VLP carrier.[145] Fusion proteins are generally limited to small epitopes that do not interfere with particle formation. However, larger antigens may also be successfully incorporated into chimeric VLPs. For example, a recent publication demonstrated that the HBsAg could be fused with the first 395 amino acids of the envelope protein of Dengue virus and still form immunogenic VLPs.[146] Immunity

may be induced to both the carrier and the fused epitope following oral vaccination with recombinant VLPs.[145,147] However, some VLP carriers only serve a helper function. For example, coat proteins from bacteriophage[148] and a range of plant viruses, such as alfalfa mosaic virus, have been used to present heterologous peptides to the immune system.[149,150]

Recently Shi et al.[151] also demonstrated the potential of VLPs for the delivery of DNA vaccines. They showed that papillomavirus capsid protein could package unrelated DNA, encoding an epitope from the lymphocytic choriomeningitis virus (LCMV). Oral immunization with this 'pseudovirus' resulted in the induction of LCMV-specific mucosal and systemic CTL responses.

A number of oral VLP vaccination studies have demonstrated the induction of antibody responses in the absence of a mucosal adjuvant in mice.[137,145,150,152] Three clinical studies have confirmed this concept. A boost in IgG titers to Norwalk virus from insect cell culture[144] or plants[85] has been described in patients following oral vaccination with VLPs. Furthermore, Kapusta et al.[86] described a *de novo* response in volunteers fed lettuce expressing HBsAg VLPs.

1.4 Prime-Boost Vaccination Strategies

Vaccination schedules that combine different approaches frequently result in improved immune responses. The most common strategy has been to prime the immune system with one vaccine and boost with another vaccine targeting the same antigen.[153] For example, a sequential vaccination strategy for measles, combining priming with an intramuscular DNA vaccine and boosting with an oral plant-derived vaccine, is more effective than either vaccine alone.[154] The strength of these strategies lies in their ability to combine the advantages of each type of vaccine, resulting in both neutralizing antibodies and cell-mediated immunity.[18,71,154,155] Prime-boost vaccination strategies have also been shown to reduce the quantity of antigen that is required to induce a strong immune response. Although prime-boost vaccination strategies are common in the literature, most include systemic routes of administration and do not report the induction of a mucosal immune response.[153] Recent work published by Wierzbicki et al.[71] demonstrates the potential of combining oral delivery methods in a sequential oral-prime, oral-boost vaccination strategy against the gp160 protein of HIV. In studies using mice primed with PLG-encapsulated DNA and subsequently boosted with liposome-associated rVV, strong cellular, humoral and mucosal immune responses were detected.

2. Mucosal Adjuvants

The effectiveness of oral vaccines can be directly augmented through the co-administration of mucosal adjuvants. Adjuvants represent a broad range of molecules and structures, acting through a variety of mechanisms to enhance the immune response to a vaccinogen. Many of the vaccine delivery strategies discussed above may be broadly considered as having adjuvant properties. However, this section will only consider immunostimulatory adjuvants, which activate cells of the innate immune system and potentiate a mucosal immune response.[94,130]

The most commonly used mucosal adjuvants are cholera toxin (CT) and the heat labile enterotoxin of ETEC (LT). In their native form, both are unsafe for human use, however, a number of genetically detoxified mutants have been developed in an attempt to overcome this toxicity while retaining the adjuvant properties. For example, mutations in the active site, which result in reduced adenosine 5′-diphosphate ribosyltransferase activity, and protease site mutations, which result in resistance to proteolytic activation.[156] Oral vaccines containing detoxified mutants have been shown to enhance immune responses in mice and confer protection against challenge, compared with vaccine alone.[157-159] Clinical trials are currently underway to evaluate the safety of an LTK63 mutant delivered intranasally.[130]

Another class of adjuvant now being explored is cytosine-phosphate-guanosine (CpG) DNA. This contains unmethylated CpG dinucleotides in a particular sequence context and developed from the observation that bacterial DNA exerts direct immunostimulatory effects on immune cells *in vitro*.[160,161] In mice, orally administered antigen with CpG has been shown to stimulate both T helper (Th)1 and Th2 responses, in addition to enhancing antigen-specific T cell proliferation, IgG and secretory IgA responses. Antibody titers were equally good, if not better, than results obtained using CT as the adjuvant.[162] CpG DNA appears to have potential as an oral adjuvant; however, as issues regarding toxicities in some animal models have been raised, safety will need to be carefully examined in the clinical setting.[163]

Saponins, derived from the bark of the Chilean tree, *Quillaja saponaria*, have been used as adjuvants, but because of their toxicity, their use must be carefully monitored.[94] A pure fraction of Quil A saponin with low toxicity (QS-21) has been isolated, and

when orally administered to mice, was found to exhibit adjuvant activity and enhance both systemic and mucosal immunity to coadministered antigens.[164-166] The mucosal adjuvanticity was dose-dependent and required early interleukin (IL)-4 help.[165] Although saponins show promise as mucosal adjuvants, further work will be necessary to examine the safety of QS-21, as significant toxicity was observed in a phase I clinical study which delivered QS-21 intramuscularly with an HIV-1 envelope subunit vaccine.[167] Recent reports suggest that mixtures of saponins will be safer.[130]

3. Will Oral Vaccines Be Feasible?

3.1 Translating Successful Model System Data Into Clinical Trials

While a number of oral vaccines work well in murine models, the potential of some of these approaches has failed to be realized in clinical studies. There are a number of possible reasons for this, including variation in antigen dose and stability, vaccination schedules, and the inherent difference between murine and human immune systems.[104] Most proof-of-concept studies employed stable model antigens such as ovalbumin and CTB. Clearly, oral delivery may not work for every antigen. The complexity and lower stability of many vaccine antigens makes them less likely to be successful in some of the oral delivery strategies described above. In addition, the extent to which vaccine delivery by gavage in model systems can simulate the practical requirement of direct antigen feeding is debated. Studies have demonstrated that the efficacy of oral vaccine strategies can be both improved and reduced by direct feeding, depending on the method of oral delivery and the antigen formulation.[150,168] Despite all these limitations, it should be noted that a number of experimental models have been translated into successful clinical studies. Immune responses have been generated in volunteers using orally delivered vaccines.[56,86,117,126,144] At the very least, the success of these trials highlights the clinical potential of oral delivery strategies.

3.2 Oral Tolerance

Much consideration has been given to the potential for the induction of tolerance by orally delivered vaccines. There are many factors that influence whether the immune system will become sensitized or tolerant to an antigen.[169] These include the nature of the antigen, the size and number of vaccine doses, and host-specific factors such as age and digestive flora. Although tolerance can be induced in animal models,[170,171] studies in humans suggest that it is relatively difficult to induce tolerance to idiotypic (self) antigens.[172,173] It may be equally or even more difficult to induce tolerance to the nonself antigens used in oral vaccines, either by design (e.g. to reduce allergy) or by accident. Conventional vaccines have not been associated with oral tolerance and studies in mice have shown that novel oral vaccines can be used without inducing tolerance.[151] The antigen dose necessary to induce protection is likely to be smaller than that required to produce tolerance,[174] and repeated or continuous exposure is usually necessary to induce tolerance.[169] Nonetheless, it will be necessary to assess each oral vaccine on a case-by-case basis, particularly if there is a risk of prolonged vaccine exposure.

3.3 Vaccine Safety – Is it Better to Use a Live Vector or a Subunit-Based Delivery System?

We have presented two major classes of vaccines in this review, live vaccines and subunit vaccines. Live vectors are a very efficient means of immunization. They mimic natural infection and facilitate ongoing presentation of antigen to the immune system following a single dose. However, a live vector that is sufficiently attenuated so as not to cause disease is likely to be of only limited effectiveness, particularly in the presence of maternal antibodies. This results in a significant risk of adverse events following vaccination, as was seen during the recent smallpox vaccinations in the US, where 242 adverse events were reported between January 24 and March 30 2003, including three deaths.[175] The ability of live vectors to replicate can also lead to life-threatening illness in immunocompromised individuals. In addition, the possibility of transfer of vaccine strains from household members in close contact with immunocompromised individuals has significant implications.[62] This is particularly relevant given the high prevalence of childhood HIV/AIDS and malnutrition in developing countries.

Due to their inability to replicate, subunit vaccines are considered to be a safer alternative. Subunit vaccines may also be effective in the presence of maternal antibodies.[2] However, many subunit vaccines require an adjuvant to establish protective immunity. In addition, multiple doses may be required.

3.4 Will it Be Possible to Achieve the Required Vaccine Dose?

A major limitation of oral delivery strategies remains the inability to supply sufficient antigen for immunization in a single dose. Although this is partly overcome by live vaccines, most oral vaccines ultimately depend upon the administration of repeated doses to ensure protective immunity. While the oral route is convenient for the delivery of multiple doses, the ultimate goal must be to develop single dose vaccines. Strategies to increase the effectiveness of oral vaccines, by protecting the antigen against degradation or potentiating the immune response, frequently result in protective immunity following a reduced number of vaccine doses. An additional strategy that shows promise in this area is the improved binding of antigens to M cells. This has been achieved through direct fusions with molecules such as CTB, which binds to G_{M1} gangliosides,[89,90] or incorporation of molecules, such as lectins, into particulate delivery vehicles.[95] Combining vaccine strategies may also prove effective. For example, liposome-associated rVV has been shown to be an effective means of increasing immunogenicity,[71] as have sequential prime-boost strategies.

3.5 Does Oral Delivery Facilitate Multivalent Strategies?

Vaccination schedules now incorporate many diseases, and in the future it is likely that vaccine development will outstrip our ability to eradicate disease. This will increase the already heavy vaccination load that is carried by children. Currently, a fully vaccinated child may receive between 19 and 23 doses of vaccine before the age of 18 months, most of these administered by injection. With the increasing number of vaccinations come increasing costs and increasing risks. Multi-valent strategies reduce contact with healthcare providers, increase compliance, and improve vaccine coverage. Many of the oral delivery systems detailed in this review are not specific to a given antigen. This means that multiple vaccines can be delivered using the same vector, potentially at the same time. For example, Yu and Langridge[89] describe a multi-component plant-based vaccine that induced systemic and mucosal responses against cholera, ETEC, and rotavirus in mice.

4. Conclusions

Vaccines have been one of the most far-reaching and important public health initiatives of the 20th century, yet diseases such as measles and tetanus continue to affect millions of children worldwide, despite the existence of effective vaccines. Disease prevention generally requires vaccine coverage rates of >90–95%. This may be unattainable given the limitations in the production, distribution, and delivery of current vaccines. Vaccines that can be administered orally could improve vaccine coverage in remote areas, reduce costs, and enhance compliance, particularly in children and when multiple doses are required. Furthermore, oral systems can be adapted for the delivery of multivalent vaccines. These strategies are not confined to diseases with established vaccines or vaccinogens. Under development are strategies to combat more complex diseases, such as malaria and HIV. It is likely that successful vaccines for these diseases will employ a combination of approaches. Multiple antigens will be required to acutely focus the immune system, and repeated administration will be needed to maintain chronic protection, both of which may be achieved though oral delivery. In addition, oral prime-boost regimens have the potential to better induce and preserve protective responses.

There remain significant limitations to translating success in experimental models into clinical reality. Further technological breakthroughs may be required before strong protective immune responses can be consistently induced in humans. Nonetheless, the potential of future oral vaccination strategies is readily apparent.

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Correspondence and offprints: Dr *Diane E. Webster*, Children's Vaccines Group, Macfarlane Burnet Institute for Medical Research and Public Health, GPO Box 2284, Melbourne, VIC 3001, Australia.
E-mail: diane@burnet.edu.au