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Mucosal immunization
A realistic alternative

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Most infections occur at or through mucosal surfaces. Despite this knowledge, current vaccination practices rely predominantly on parenteral administration with only a few vaccines being registered for adminstion by the mucosal route. Whilst mucosal immunization brings many advantages, the lack of reliable delivery systems has been a major drawback to date. With the recent advances in delivery system technology and the improved understanding of site specific mucosal immune mechanisms, mucosal immunization offers an exciting alternative vaccination strategy.

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Introduction

Increased immunization coverage, along with improved sanitization and safe water, is the most important public health intervention to improve child health in documented human history, resulting in a significant decrease in child mortality during the 20th century. Immunization is considered by the World Health Organization (WHO)[1] as one of the most cost-effective of all health interventions, having the capacity to not only save but also to transform lives from the misery of infectious diseases. Since 1988, polio has been eradicated in 121 countries and is today endemic in only four countries. Despite the many achievements of vaccines in reducing the burden of disease, many infectious diseases remain endemic in large parts of the world, in particular, in developing countries as well as in many indigenous communities and areas of low socio-economic status within developed nations.

There are two key aspects that need to be addressed for any vaccination to be successful: first, to increase the potential to generate a potent defence against diseases that evade the immune system, and secondly, to induce long lasting protective immunity following a single administration. Consequently, the development of vaccines has always been focused on effective induction of both humoral and cell-mediated immunity (CMI). Most of the vaccines that are used today are delivered by intramuscular or subcutaneous injection with an adjuvant. Whilst this strategy has proved useful for stimulating systemic immune responses, these approaches generally induce poor responses at mucosal surfaces. Given that over 90% of infections occur at or through mucosal surfaces, it is logical to pursue an immunization approach that elicits significant immune responses at the sites where causative pathogens invade. Despite the long-standing recognition that mucosal delivery of vaccines offers many tangible advantages, both immunologic and economic, mucosal vaccination faces numerous challenges which currently inhibit the ability to successfully develop new mucosal vaccines. While significant progress has been made in basic immunological knowledge regarding the regulation of tissue-specific lymphocyte trafficking, the optimal strategies for eliciting potent and protective mucosal immunity remain a significant challenge. As such, the search for effective and safe vaccine formulations and delivery systems to overcome the numerous potential obstacles encountered with mucosal vaccines is of paramount importance. Bacterial toxins and their derivatives are commonly used as potent mucosal adjuvants in experimental models, however, their toxicity has limited their use for human vaccination. Many products have been proposed as vaccine adjuvants but have been rejected because of safety concerns. Devices that use needles may be largely replaced with new approaches such as aerosol formulations sprayed in the nose or lungs, enteric coated oral tablets and suspensions. The combination of the known shortfalls of current vaccines has been the impetus driving research and development resulting in the first decade of the 21st century as being the most productive in the history of mucosal vaccine development.

This review will examine the potential and challenges of the development of mucosal vaccines and how these have been addressed in the design of vaccines that lead to effective mucosal immune responses in human subjects. Whether or not mucosal immunization is a realistic alternative to parenternal immunization is critically assessed.

The Need for Mucosal Immunization

Mucous membranes line the gastrointestinal, respiratory and urogenital tract and represent the most important portal of entry for infectious agents. Most of the human pathogens that cause serious health problems today either replicate and promote disease at the initial mucosal site and then invade tissues and the

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blood stream inducing disease at distant systemic localities. Today, over 1 million children are immunized every year before their first birthday[1] against pathogens that breach the mucosal barrier. Infants and children can expect to receive multiple immunizations for an increasing number of diseases: measles, mumps, rubella, diphtheria, tetanus, pertussis, polio, hepatitis B, *Haemophilus influenzae* B, varicella, *Streptococcus pneumoniae* and often hepatitis A. There have also been efforts to ensure the benefits of immunization are increasingly extended to adolescents and adults to protect against diseases such as influenza, meningitis and vaccine-preventable cancers that occur in adulthood. This increasing number of injections has fuelled the drive to develop alternate delivery systems designed to reduce the number of injections, to maintain or increase the potency of responses and to improve compliance of the community for immunization.

Diarrhoeal diseases represent a major health problem in developing countries, ranked by WHO as third among all causes of infectious disease deaths worldwide.[1] The global death toll due to diarrhoeal diseases is estimated at approximately two million deaths per year—predominantly in children under 5 years of age. Among the principal bacterial pathogens of diarrhoeal disease are *Vibrio cholerae* (cholera), *Salmonella enterica* serovar Typhi (*S. Typhi*, the aetiological agent of typhoid fever), *Shigella* species that cause shigellosis (bacterial dystentery), and a variety of enteropathogenic *Escherichia coli* strains, including the enterotoxigenic *E. coli* strains (ETEC) that are the causative agents of traveller's diarrhoea. Rotavirus gastroenteritis is a significant cause of morbidity in young children. Acute respiratory infections continue to be a leading cause of acute illnesses worldwide and contribute significantly to childhood mortality, responsible for about two million deaths each year.[1] The main aetiological agents responsible for acute respiratory infections in children include: *S. pneumoniae*, *H. influenzae* type b, respiratory syncytial virus (RSV), measles virus, human parainfluenza viruses, influenza virus, *Mycoplasma pneumoniae* and *Mycobacterium tuberculosis. Pseudomonas aeruginosa* and nontypable *H. influenzae* (NTHi) are opportunistic pathogens, establishing chronic infections of the lungs of subjects with compromised airway function such as cystic fibrosis, chronic bronchitis and chronic obstructive pulmonary disease (COPD). NTHi is also becoming recognized as a significant cause of invasive disease. In the middle ear *S. pneumoniae,* NTHi and *Moraxella catarrhalis* are the predominant cause of otitis media and subsequent complications. In the urinary tract, strains of *E. coli* are the responsible pathogens in both complicated and uncomplicated urinary tract infections while sexually transmitted infections caused by Chlamydia, *Neisseria gonorrhoeae* and herpes simplex virus all place a large burden on both the patient and healthcare resources. Since there is significant transmission of human immunodeficiency virus (HIV) by mucosal routes, urogenital and rectal, and that parenteral vaccine approaches have failed, there is substantial interest in the development of mucosal immunization strategies.[2]

Most of the vaccines currently in use—including those against diphtheria-tetanus-pertussis, polio, measles-mumps-rubella (MMR), varicella, tuberculosis, hepatitis and yellow fever—are administered by injection, either intramuscularly or subcutaneously. While parenteral immunization is generally effective in preventing systemic infections, it is limited by the fact that it fails to prevent the initial interaction of pathogen and host and chronic colonization at the mucosal surface. For example, the highly controversial tuberculosis vaccine, is administered by injection, yet must resolve an infection where the primary portal of entry is at the nasopharynx/lungs. It's reported efficacy in the lung have been widely disparate, from excellent protection against tuberculosis to no protection,[3] although, it has been effective at protecting children against extrapulmonary forms of the disease, such as tuberculosis meningitis[4] and leprosy.[5] There is the distinct practical advantage of being able to administer vaccines without the need for needles. Non-invasive technologies are attractive for several reasons. They may be more acceptable to the general population and therefore, may improve patient compliance with vaccination schedules. For example, the fear of needles is an important issue for both adults and children,[6] particularly in regions where there is little endemic disease. Trained medical personnel are not required in most instances for the delivery of mucosal vaccines which makes them highly suitable for mass-vaccination programs, mostly in developing nations.[7] Furthermore, and perhaps more importantly, mucosal immunization alleviates a potential source of infection due to accidental needle-stick injuries to health-care workers or as a result of unsafe injection practices. Alarmingly, the WHO has estimated that a significant number of hepatitis B and hepatitis C virus infections and HIV infections might occur in developing countries because of needle misuse.[8] A consideration with existing vaccination strategies is that current injectable vaccines must be stored at 4ºC and carry relatively short half-lives. Removal of the cold chain with heat-stable vaccines would offer enormous advantages with respect to cost globally and potentially increase access to vaccines in developing countries.

Ideally, vaccines would be best presented to immune induction sites at the mucosa where immune responses can either prevent the pathogen from initial attachment and subsequent colonization of the mucosal epithelium, or from penetrating the epithelium and replicating in the mucosa, and/or that can prevent bacterial toxins from binding to and damaging cells. The most attractive routes for mucosal immunization are oral and intranasal. Pulmonary, vaginal and rectal routes are less attractive. The relative advantages and disadvantages of different mucosal routes are summarized in **Table 1**.

Mucosal Immunity

The first lines of defence against invading microorganisms at mucosal surfaces are innate mechanisms which do not require any prolonged period of induction. These innate mechanisms of immunity include fever, mucosal secretions which form a mucosal barrier and chemical mediators and phagocytic cells which kill pathogens or inhibit their replication. It is only when the pathogen is able to breach this first line of defence, that an adaptive immune response will be initiated. Mucosal routes of immunization have the potential to induce both humoral and cellular responses. Significantly, secretory IgA (SIgA) antibodies

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Table 1. Routes of mucosal immunization, formulations, advantages and disadvantages*

Route	Form	Advantages	Disadvantages
All		• Needle free administration and avoidance of needle stick infections • Easily administered • Less training of health care workers and can often be self administered	• Adjuvant agents are normally required. • Limited availability of adjuvants suitable for human use
Oral	• Liquid formulations with and without buffers • Particles such as liposomes, microspheres and bacterial ghosts administered as a capsule or suspension • Enteric coated tablets • Usually formulated with adjuvant or active component can act as immune enhancing agent	• Good subject compliance • Good induction of mucosal immune responses to other sites through the mucosal immune network • Primary colonization and invasive site of many pathogens • With live attenuated vaccines shedding may contribute to herd immunity.	• Degradation of antigen by gut digestive process and bacterial proteases • Adjuvants are normally required • Access to immune induction sites highly variable • High doses required • Immune responses and vaccine efficacy can be variable • Duration of the immune response may be of short duration • Immune responses and vaccine efficacy can be variable • Live attenuated vaccine and live vectors can cause serious adverse events • Live attenuated vaccine shedding may lead to safety concerns
Nasal	• Spray or liquid drop formulations • Particles such as liposomes and microspheres administered in a suspension by drop • Usually formulated with adjuvant or active component can act as immune enhancing agent	• Good subject compliance • Avoids degradation of the antigen by gut digestive processes and bacterial proteases • Primary colonization and invasive site for airborne pathogens • Easy access to mucosal immune induction sites permits less antigen compared with oral administration. Results in more consistent vaccine responses resulting in lower cost	• Degradation of antigen by host and bacterial proteases • Adjuvants are required • Controlling access time of particulate and soluble antigens to mucosal induction sites is difficult • Administration to infants may be difficult • Safety concerns for live attenuated vaccines and live vectors accessing the central nervous system through the cribiform plate
Inhalation Pulmonary	• Liquid aerosols or powders normally with an adjuvant	• Good subject compliance • Avoids degradation of the antigen • Primary colonization and invasive site for airborne pathogens • Can induce systemic responses	• Need to ensure antigen is delivered to the upper airways • The bronchus is poor mucosal immune induction site • Adjuvants are required but potential lung exposure to these agents raises safety concerns • Sophisticated inhalation devices are required which increase the cost
Vaginal or rectal	• Creams containing adjuvants	• May be advantageous for HIV and other sexually transmitted diseases.	• Poor patient compliance for both routes of immunization • Poor induction of both systemic and vaginal mucosal immune responses are observed with vaginal immunization • Strong adjuvants are required.

*Adapted from references 274 and 275.

directed against the specific pathogen at the site of entry and systemic IgG responses may be generated.[9] SIgA is the predominant immunoglobulin class in human external secretions. Of the two SIgA subclasses, IgA_2 is prevalent in the large intestine and is more resistant to digestion by bacteria than IgA_1 which dominates both in the nasal and bronchial mucosa. Nonetheless, the resistance of SIgA to proteases makes these antibodies uniquely suited for functioning in mucosal secretions, while serum-derived IgG can also contribute significantly to immune defense in the lower respiratory tract and the genitourinary mucosa. In addition, cellular responses, including responses from $CD8^+$ major histocompatability complex-restricted cytotoxic T lymphocytes

(CTLs), as well as induction of CD4+ T helper (Th) lymphocyte responses may be induced to provide an effective means for preventing infection.[10,11] Along with antibody and CMI responses against a specific pathogen, the induction of memory cells is the ultimate goal of a successful vaccination.

The Mucosal Immune System

The mucosal immune system is essentially divided into two compartments known as inductive and effector sites which work together to maintain the mucosal barrier. Antigen sampling occurs at the inductive sites and leads to initial activation of naïve T and B cells, while at effector sites, following extravasation and differentiation, antigen specific antibodies such as SIgA and immune cells can perform their specific function.[10-12]

Region specific lymphoid tissues, known as the mucosa-associated lymphoid tissue (MALT), as well as their surrounding regional mucosa draining lymph nodes are the principle inductive sites for mucosal immune responses. MALT is sub-divided according to anatomical regions: gut-associated lymphoid tissue (GALT); bronchus-associated tissue (BALT); nasopharynx-associated lymphoid tissue (NALT) and the less well characterized mammary and salivary glands and the urogenital organs.[13] In rodents, NALT is located on both basal sides of the nasal cavity while in humans the adenoids, the paired palatine tonsils and other small structures of Waldeyer's ring is considered the anatomical equivalent.[14]

MALT structures resemble lymph nodes with B-cell follicles, intervening T-cell zones, plasma cells, and a variety of antigen presenting cells (APCs) such as dendritic cells (DCs), B-lymphocytes and macrophages. Lymphoid follicles can be located singularly or in organized clusters such as the Peyer's patch (PP) regions of the small intestine.[15] The PPs contain lymphoid follicles which extend into the gut lumen between the normal intestinal epithelium. The MALT is covered by a characteristic follicle associated epithelium (FAE).[16] Highly specialized epithelial cells or "membrane" (M) cells that reside in the FAE promote selective uptake of antigens and deliver samples of foreign material by vesicular transport effectively from the lumen of the gastrointestinal tract into the lymphoid follicle where it is processed by APCs.[17] While M cells constitute only about 10% of the FAE, their high efficiency, in terms of antigen sampling, is due to their characteristic features of short irregular micro-villi and transcytotic capabilities. Therefore, antigenic stimulation must occur directly from the epithelial surfaces, predominantly via M cells and to a lessor extent by DCs which may penetrate the surface epithelium with their processes.[18] Chemokines secreted by the FAE result in an additional attraction of DCs to the FAE with an ensuing accumulation of phagocytic cells at the sites of entry of foreign antigens.[19] Upon capture of antigen, phenotypically immature DCs migrate to adjacent interfolicullar T-cell areas located between B cell follicles where they upregulate the expression of maturation markers and MHC molecules.[20,21] In the germinal centre of the lymphoid follicle of mucosal inductive sites, antigen processing and presentation occurs with subsequent IgA class switching. Both naïve and IgA+ B cells rapidly move from PPs and NALT through the efferent lymphatics to the mesenteric lymph nodes or cervical lymph nodes, respectively, where they further mature. Finally, these antigen-specific CD4+ T cells and IgA+ B cells migrate by preferential homing mechanisms into distant effector sites, such as the intestinal lamina propria, nasal mucosa, lung and so on via the thoracic ducts and blood circulation.[9] Mucosal IgA production is strictly regulated by signals between B cells, T cells, DCs and epithelial cells. Cytokines such as transforming growth factor-β (TGFβ), interleukin (IL)-4, IL-6 and IL-10 are known to be important for the development IgA-producing B cells.[22-24] In GALT, activated CD4+ helper T cells (Th2) release cytokines such as TGFβ and IL-10 and promote class switching and differentiation of mucosal B cells to predominantly IgA-committed B cells (plasmablasts).[25,26] PP DCs have been identified as being integrally associated in producing an environment conducive to the generation of IgA responses in the intestine, although the mechanisms of induction of IgA production by PP DCs have not been identified. A subset of PP DCs has been shown to preferentially secrete IL-6,[27] which induces the production of Th2 cytokines while another subset expresses inducible nitrogen oxide synthase (iNOS)[28] which, in the latter case, promotes the expression of the TGFβ receptor on naïve PP B cells. While GALT must be considered the major inductive site for T cell dependent production of mucosal IgA, recent observations suggest that mucosal IgA responses may also be induced independent of T cells in the diffused effector sites such as the intestinal lamina propria. In response to Toll-like receptor stimulation by intestinal microbes, B-cell-stimulating factors, including IL-6, TGFβ, APRIL (a proliferation inducing ligand) and BAFF (a B-cell activation factor of the tumor necrosis factor family) may be released and induce differentiation of IgA+ B cells to IgA+ plasma cells[29] and protease resistant IgA_2 class switching[30] at the mucosal epithelial surface.

The final differentiation to immunoglobulin-secreting active plasma cells occurs in the presence of IgA-enhancing cytokines IL-5 and IL-6.[31,32] Once produced, the IgA binds to a polymeric Ig receptor on the basolateral surface of epithelial cells and is actively transported through the epithelial cell and released on the apical side of the cell into the intestinal lumen as SIgA.[9]

The Mucosal Immune Network

The migration of lymphocytes from their sites of antigen-driven differentiation in mucosal lymphoid tissues to target effector sites is largely determined by site-specific integrins or 'homing receptors' on their surface and complementary mucosal tissue-specific adhesion molecules or 'addressins' on vascular endothelial cells.[33] Cell migration is controlled by chemokines produced in the local microenvironment which enhance the homing of lymphocytes toward mucosal tissues and regulate integrin expression.[34] The activated T and B cells upregulate the expression of tissue-specific adhesion molecules and chemokine receptors that control lymphocyte homing. The mucosal immune system is integrated with regards to differentiation and homing properties of lymphocytes, however, there is accumulating evidence to suggest that

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there is significant regionalism linking specific mucosal inductive sites with particular effector sites. This sub-networking appears to depend on differences in the chemokines, integrins and cytokines that are differentially expressed between the mucosal tissues. The concept of the 'common mucosal immune system' may be explained by a broad recognition system of receptors and chemokines whereby most mucosal IgA$^+$ B cells express chemokine receptor-10 (CCR10) and α4β1 integrin, which mediate attraction to chemokine ligand 28 (CCL28/MEC) and vascular cell-adhesion molecule (VCAM-1), respectively.[33] CCL28 is expressed by most mucosal epithelia throughout the large intestine, salivary glands, tonsils, respiratory tract, cervix and lactating mammary glands[10,33,35,36] and as such, the lymphocytes can be attracted to all these tissues. The main difference between GALT, NALT and BALT relies on their abilities to induce immune responses with different intensities on distal regions of the mucosal immune system. The original site of antigen activation is known to determine the homing receptor profile of the activated lymphocyte. IgA$^+$ B cells home to the intestinal mucosa because the gut-homing integrin α4β7 interacts with its cognate ligand mucosal vascular addressin cell adhesion molecule 1 (MadCAM-1) which is expressed on intestinal post-capillary venules.[37,38] It has been shown that oral vaccination selectively upregulates expression of the gut-homing integrin α4β7 on B cells and IgA$^+$ B cells compared with parenteral administration of the same antigen.[39-41] Moreover, differentiation of IgA plasmablasts within the small intestinal lymphoid tissues additionally induces upregulation of CCR9 and directs homing to the small intestine which expresses CCL25/TECK.[42-44] In parallel, CCL25 binds to CCR9 on T lymphocytes and promotes activation of α4β7 and αEβ7 integrins, thereby preferencing effector and memory CD8$^+$ T cells to selectively home to both the lamina propria and intestinal epithelium via interactions with E-cadherin.[44-46] It is now known that intestinal DCs, but not DCs from other lymphoid organs, constitutively secrete retinoic acid which synergizes with IL-5 and IL-6 to induce IgA production in B cells and programs B and T lymphocytes to express the gut-imprinting molecules α4β7 and CCR9 required for homing to the small intestine.[47,48] This process of lymphocyte priming in the PPs is significant for vaccination against intestinal infections whereby oral immunization, unlike parenteral immunization, requires the involvement of mucosal DCs with the correct imprinting properties.

In contrast to oral immunization where α4β7 is important for homing of lymphocytes to the intestinal mucosa, it does not appear to be an important homing receptor in the airways[49,50] or genitourinary tract.[51,52] IgA$^+$ B cells that are activated in NALT express α4β1 and CCR10, allowing them to traffic efficiently to the respiratory tract and urogenital tract where their ligands VCAM-1 and CCL28 are strongly expressed.[53] Noteably, and significantly for nasal vaccines, NALT-derived circulating memory and effector cells, in contrast to GALT derived cells, express abundantly CD62L (L-selectin) and CCR7 which may explain their joint tropism for organized lymphoid tissue and the upper aerodigestive tract.[54] Similar to NALT, lymphocyte homing from BALT appears to be mediated by a combination of several molecules, CD62L, α4β1 integrin and LFA-1 adhesion pathways.[50] The upregulated expression of the peripheral homing receptors α4β1 and CD62L explains why mucosal immunization is able to induce the production of serum IgA and IgG and provides a mechanism for integration of respiratory and systemic immunity. While mucosal activated CD8$^+$ T cells might initially have appeared to have a less restricted migration pattern compared to B cells, memory CD8$^+$ T cells have also been shown to preferentially migrate to the tissue in which antigen was originally encountered.[55] Clearly what is currently known about homing mechanisms is relatively limited and at this time there are no mechanistic explanations of the cross-talk that occurs within the various networks of the mucosal immune system. **Figure 1** provides a schematic diagram of the mucosal immune system and the sub-networks.

How can Mucosal Immune Networks be Accessed for Immunization?

Due to the observed regionalism within the mucosal immune system, the choice of vaccination route may be dictated by the requirement for an effective immune response at a desired mucosal site. In general, oral immunization in humans may induce substantial antibody responses in the small intestine, colon, mammary glands and components of the respiratory tract including the salivary glands, but generally relatively low levels of antigen-specific IgA antibody responses in the large intestine, tonsils and female genital tract.[56-58] Thus, immunization by the oral route induces mucosal immunity in the upper part of the intestine, upper airways and the mammary glands. On the other hand, rectal immunization evokes high levels of IgA and IgG in the rectum but very little, if any, in the small intestine or colon,[59] while in mice, CTL immunity may be induced systemically.[60] In animal models and humans, nasal immunization has shown particular success in inducing specific mucosal IgA and systemic IgG antibody responses in the salivary glands and upper and lower respiratory tracts[58,61-64] without evoking an immune response in the human gut.[65] In addition, nasal and to a lessor extent vaginal immunization has been found to induce significant antibody responses in human cervical and vaginal secretions,[62,66] while in mice, the nasal route can induce CTLs and provide longterm immunity and protection in the female genital tract.[67] The genital tract itself in both males and females is a poor mucosal induction site due to the paucity of organized epithelial associated lymphoid tissue.

The Path to Mucosal Immunization

Historically, as reviewed by Mestecky et al.[68] the foundations for prevention of diseases by vaccines were laid in the experiences of ancient peoples who believed that those who survived certain diseases became resistant to repeated attacks. The roots of mucosal immunity can be traced back to about 132-63 BC during the rule of the despotic king Mithridates VI-Euptor. In an attempt to avoid probable attempts on his life, he routinely ingested the blood of ducks that had been fed a formula of poisonous weeds in an effort to enhance resistance to a commonly

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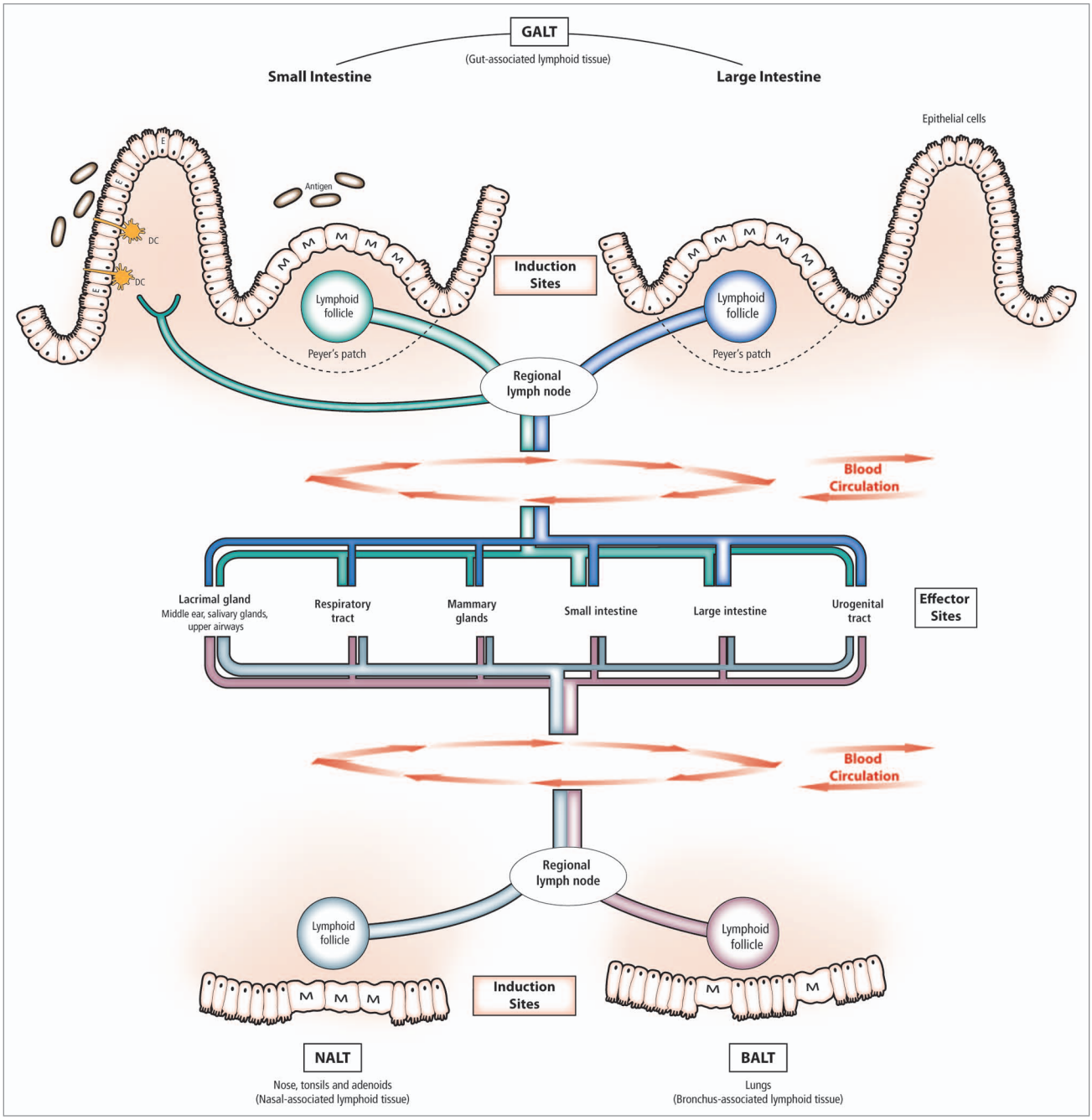

Figure 1. A schematic diagram of the mucosal immune system showing the predominant sub-networks. Cell traffic from mucosal immune induction sites to effector sites is proportionally shown by the thickness of the colored bars to each effector site. E, epithelial cell; M, M cell; DC, dendritic cell. Adapted from references 276 and 277.

used plant-derived poison. Interestingly, 2,000 years later oral immunization with chicken red blood cells adsorbed with killed influenza A virus was shown to induce broad-based immunity in a mouse model of influenza.[69] This and other oral immunization studies in an animal model,[70] suggest that mucosal immunization with whole cells or viruses may be able to induce protection across serotype and strain barriers. The earliest form of nasal immunization was practiced in China more than 1,000 years ago by inhaling powdered smallpox pustules. The practice spread to other parts of the world and with a few modifications the procedure of variolation was brought to England in the 18th century. Vaccinology as a science was created with the proof of concept studies undertaken by Dr. Edward Jenner when he performed the first vaccination in 1796. By injecting one boy with the milder disease, cowpox, he found that the boy became immune to smallpox. A great expansion of knowledge in the area of vaccinology occurred in the 19th century. Following on Robert Koch's technologies for microbial purification and

cultivation, a number of human microbial pathogens were discovered and the first attempts at vaccination through the intestinal tract were carried out in Pasteur's laboratory. There were a series of attempts at oral immunization with bacteria such as *V. cholerae*, *S. dysenteriae*, *M. tuberculosis*, *Yersinia multocida*, *Y. pestis* and *Corynebacterium diphtheriae* with varying degrees of success. While serum antibodies were found to be induced by oral immunization, there was a degree of scepticism among scientists of that time as to whether they could be considered the markers of protection. As such, it was not till the early 1890s that the discipline of mucosal immunology was born when Alexandre Besredka discovered antibodies in external secretions, or more specifically, gut mucosal antibodies. It was at this time that the concept of oral or intestinal immunization was brought to prominence. The validity of results obtained in animal models was soon tested in humans. After extensive tests in animal models by Calmette, a physician and Guérin, a veterinarian, an attenuated variant of *M. bovis*, Bacille Calmette-Guérin (BCG) was created. BCG was first tested in infants as an oral vaccine in 1921. Also during this time, the first studies that demonstrated that protection could be achieved by nasal immunization were conducted by Bull and McKee when rabbits were immunized with a killed suspension of *S. pneumoniae*. Interestingly, this approach has recently been rediscovered by Malley and his colleagues with promising results.[71] Mucosal immunization with novel *S. pneumoniae* protein antigens has also been shown to be effective in an acute lung infection model.[72] However, no human studies have been reported to date.

Despite the early scientific advances in the area of mucosal immunization, there was soon to be shift in the knowledge base of the biomedical sciences and a focus on parenteral immunization with the appearance of many modern day vaccines. Since the development of the Sabin oral polio vaccine (OPV) nearly 50 years ago and even with the recent progress in our understanding of the mucosal immune system, few mucosal vaccines are available for human use today. Current clinically approved oral vaccines include: OPV, which is predominantly used in developing countries; four against cholera; a typhoid vaccine that is restricted to the military, and two rotovirus vaccines. An intranasal influenza vaccine recently had its licensure extended to prevent influenza in healthy children under 5 years of age.[73] The successes in science and industry, however, have not been without their failures with the removal of two mucosal vaccines due to adverse events: an oral live-attenuated rotovirus vaccine (RotaShield®) that produced intussusceptions (where part of the intestine has telescoped into another section of intestine creating an obstruction)[74] and a nasal inactivated enterotoxin-adjuvanted influenza vaccine (NasaFlu™) that resulted in facial paresis of some subjects.[75] OPV was discontinued in the United States (US) because of rare cases of vaccine-associated paralytic polio[76] and was replaced by an inactivated poliovirus vaccine (IPV) that is administered by injection. All of these vaccines use whole cell pathogens, killed or attenuated, and in some cases detoxified bacterial toxin-based adjuvants, which has raised concerns about their safety.

Challenges of Mucosal Immunization

Evaluating immune responses. Vaccines administered mucosally are confronted with various challenges including: dilution in mucosal secretions; entrapment in mucus gel layers, degradation by enzymes; competition with other environmental food and microbial antigens and the epithelial cell barrier. Furthermore many of these factors are highly variable. Accordingly, poor antigen absorption often necessitates administration of large amounts of antigen to supply a sufficient quantity for induction of immune responses. Consequently, it is difficult to determine exactly what dose actually accesses the MALT resulting in variable immune responses. Other challenges include difficulties related with the lack of standardized collection and processing procedures of mucosal secretions following mucosal vaccination and the functional assessment of vaccine-induced mucosal immune responses.[19] Currently, the collection and processing of intestinal lavage fluids for measuring immune responses to oral enteric vaccines continues to remain a challenge in large field trials or with younger age groups. The measurement of antibody levels in body fluids is quite often variable and misleading. Of the mucosal secretions, saliva is the most accessible to collect and has been used widely to characterize the ontogeny of mucosal immunity[77] and responses to vaccination[78] and environmental factors.[79] Standardization of the salivary collection procedures and immunoassay methodology has led to more reproducible results. The confounding affect of variation in the salivary flow rates can be greatly offset by expressing the level of antibody measured in sample as a ratio of total immunoglobulin level of the same immunoglobulin class. Measurement of vaccine specific antibody secreting cells (ASCs) may also be useful in determining induced mucosal immune responses.[80] While the enzyme-linked immunosorbent spot (ELISPOT) test has been the method of choice for measuring ASC responses to date, it too has not been without its limitations. As such, there has been a focus on the development of innovative, analytical techniques for measuring both antibody and T-cell responses that are more practical for use in large-scale mucosal vaccine trials.[81] The efficacy and immunogenicity of enteric vaccines including cholera, polio and rotavirus vaccines have been found to be lower in children in developing countries.[82-85] The determinants of the observed hypo-responsiveness to oral enteric vaccines have been identified as being multifactorial related to vaccine, host and environment. Factors that have been shown to negatively influence the response to the mucosal vaccines include breastfeeding, high levels of maternal antibody, nutritional status, as well as zinc deficiency. There is a greater need to investigate further factors that are endemic to developing countries, particularly high exposure to antigenic challenge through environmental conditions and infection and their possible impact on vaccines. This will facilitate the identification of strategies that might improve the immunogenicity of vaccination in young children. For example, the disease burden due to gastrointestinal parasites is an important health problem in densely populated areas of less developed countries and little is known about the effect of concurrent parasitic infestations on immune responses.[86]

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Safe and effective delivery systems. Integral to mucosal vaccine development and licensure is an internationally accepted system of testing vaccines for their safety, efficacy and quality. There are three main testing phases that evaluated the candidate vaccines in humans. Phase I studies evaluate the safety and immunogenicity of vaccines in a small number of healthy volunteers. Phase II studies address safety and effectiveness of vaccines in patients suffering from the condition. Issues such as vaccine shedding, in particular with respect to gastrointestinal disease vaccines and potential transmissibility of live vaccines may also need to be assessed. Phase III studies are undertaken in typically large numbers of patients for the purpose of determining whether the medicine confers clinical benefit in the disease for which effectiveness was demonstrated in Phase II studies. These studies also monitor the likelihood of any side effects occurring. By this stage, the vaccine is usually presented to patients in a form resembling its final formulation. Phase IV studies may be conducted following regulatory approval of a vaccine for post marketing surveillance; to compare the new vaccine to an already existing vaccine for the disease; or to identify any potential adverse events, vaccine quality assurance and further efficacy. Clinical trials today must conform to International Good Clinical Practice guidelines. Licensure is the objective endpoint of vaccine development, and while many vaccine candidates look promising in preclinical trials, few reach this critical stage of development because of the failure to demonstrate adequate safety and/or efficacy in human clinical trials. Preclinical laboratory testing of vaccine components should be conducted in robust and reproducible animal model/s to assess for criteria such as optimal antigen uptake and immune mechanisms which may then progress to proof of concept studies. Quite often, the correlates of immune protection are not well defined for mucosal vaccines, as exemplified by the licensed enteric vaccines for *S. Typhi* and rotavirus. This makes it difficult to assess the efficacy of any new vaccines in development where immune correlates would be important in selecting the most promising vaccine candidates. Furthermore, the lack of relevant animal models has proven to be a stumbling block to the development of some vaccines, as in the case of replicating human bacillary dysentery in shigellosis. The checks and balances of vaccine efficacy, safety and quality continues following licensure when national regulatory authorities must monitor a given licensed vaccine to ensure that it meets the international standards. While all industrialized countries have a reliable, functional vaccine regulatory system, only one quarter of developing countries do and of those, not all necessarily apply the same regulatory standards of vaccine licensure. This lack of common standards between countries poses further challenges for assuring the quality of vaccines when, in an effort to reduce vaccine costs and increase uptake, more than half of the vaccines are now being manufactured in developing countries.[1]

In order to improve the efficiency of mucosal vaccines, formulations and delivery strategies have to be carefully designed to: target the appropriate mucosal site; protect the vaccine antigen from degradation during delivery, and provide access to APCs at the relevant induction site. Often the antigen itself is only weakly immunogenic, therefore, it may be necessary for the vaccine formulation to include an immunopotentiator or adjuvant to enhance its immunogenicity. However, the potency of these adjuvants needs to be carefully balanced with their potential to be toxic and induce tissue damage. The induction of an appropriate immune response against a specific pathogen can dictate the success of a vaccine and can be highly dependant on the adjuvant and the type of Th cells induced (Th1 or Th2). In brief, Th1 cells mediate cellular immunity against intracellular bacteria and viruses through secretion of IL-2, interferon-γ (IFNγ) and tumor necrosis factor-β (TNFβ); Th2 cells provide protection against extracellular infectious organisms through the secretion of cytokines IL-4, IL-5, IL-10 and IL-13. Th2 cells also assist B cells in the production of IgA and neutralising IgG antibodies against bacterial toxins. Therefore, it is fundamental that the vaccine formulation design considers which type of immune response is required for the specific vaccine pathogen. In this context, a mucosal vaccine requires direction to the relevant mucosal induction site via the appropriate administration route for that site with the incorporation of an APC targeting component also relevant for the induction site and type of immune response required.

Avoiding mucosal immune tolerance. By strict definition mucosal immune tolerance occurs when exposure to antigen mucosally induces a state of unresponsiveness systemically but an antibody immune response at the respective mucosal immune effector site. However, this definition requires extension to include immune hypo-responsiveness at mucosal effector sites and/or systemically that occurs following mucosal antigen exposure through microbial colonization[87] or perhaps vaccination. This observed hypo-responsiveness is most likely to be related to development of a state of immune homeostasis which limits inflammatory responses particularly mucosally but also systemically. Mucosal tolerance has been extensively studied in animal models with respect to both oral[88] and nasal vaccines.[89] Much of this research has been undertaken with antigens fused to cholera toxin B subunit (CTB)[90] or keyhole limpet hemocyanin.[91] These animal studies have been helpful in defining the immune mechanisms responsible for mucosal tolerance. In brief, antigen specific regulatory T cells from immune induction sites in the presence of IL-10 and TGFβ, through processes of deletion, anergy and regulation, can suppress effector T cells, as well as induce antigen specific apoptosis and deletion of these effector T cells.[90,92] Whilst no studies have been undertaken to explore the mechanisms of hypo-responsiveness, it is reasonable to propose that this state reflects suppression of immune responses through regulatory processes rather than anergy or deletion. Factors which may contribute to the development of immune tolerance and hypo-responsiveness are similar to those which are considered in optimization of vaccination formulations and regimes such as frequency and amount of antigen exposure and co-exposure to immune enhancing agents. In addition, previous antigen exposure may also have a significant influence on immune response outcomes. In this context, the tight regulation of mucosal immune responses to environmental and microbial antigens presents a major challenge for inducing effective immunity through immunization via mucosal routes. This is particularly so for oral immunization in the gastrointestinal tract where there is significant and constant

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exposure to microbial and food antigens. Human studies have demonstrated that both nasal and oral immunization can lead to mucosal tolerance to a novel antigen as reviewed by Mestecky et al.[93] However, it is concluded by Mestecky et al.[93] that the observation may not be extrapolated further as mucosal antigen presentation may actually prime for subsequent mucosal and systemic antibody responses. Little is known about the impact of mucosal exposure to high doses of antigen on, particularly CTL responses. Further research is required to examine the possibility that mucosal antigen exposure, either naturally or through vaccination, may induce systemic tolerance.[94] However, there is evidence to suggest that the temporal sequence of antigen exposure (systemic priming and mucosal boosting), as well as the use of adjuvants to target antigens to APCs determine the quality of the ensuing immune response, and as such there is the potential to manipulate the systemic and mucosal immune responses to optimize mucosal immunization without compromising cell mediated responses.[95]

To induce acquired immunity relevant to infection. Infections at mucosal sites may be the result of a pathogen attaching to and colonizing the mucosal epithelium causing disease such as gastrointestinal infections or chronic colonization of the mucosal space with a commensal microbe. This requires different vaccine strategies to be undertaken. A preventative vaccine against an acute infection needs to induce an immune response at the mucosa that prevents initial attachment of the pathogen to the mucosal epithelium and subsequent colonization or invasion. Cholera and typhoid fever are caused by the enteric pathogens, *V. cholerae* and *S. Typhi*, respectively. However, these pathogens possess distinctly different infection mechanisms that result in dissimilar symptoms and immune responses in the host. *V. cholerae* is a non-invasive pathogen and typically attaches itself to the epithelium of the small intestine where it is able to colonize the mucosa and cause disease. *S. Typhi* is an intracellular pathogen characterized by invasion and inflammation in the submucosal lymphoid tissues with further systemic spreading. Accordingly, vaccine-induced protection against *V. cholerae* is predominantly mediated by locally produced SIgA antibodies.[96] In comparison, both antibody (SIgA and serum IgG) and CMI responses against *S. Typhi* are thought to be the relevant immune mechanisms to confer protection against typhoid fever.[97] On the other hand, a therapeutic vaccine against a commensal pathogen needs not only to induce an immune response capable of clearing an established infection, it may also need to alter an already established but ineffective response that permits the microbe to maintain chronic colonization of the host. It must also do this without exacerbating the inflammatory response that accompanies the chronic colonization state. In COPD, oral immunization with a killed whole cell NTHi vaccine re-stimulates a predominantly Th1 T cell response and through Th1 cytokines recruits neutrophils to the intrabronchial space and activates them to clear the established infection in the bronchus.[98]

Funding new initiatives. Until recently, progress in mucosal vaccine development has been hindered by the lack of sufficiently funded and focused efforts. To address this shortcoming, WHO Strategic Advisory Group of Experts (SAGE) on immunization, and the Program for Appropriate Technologies in Health (PATH) have highlighted the need for research into mucosal antigen delivery systems and mucosal vaccine development, including ETEC, rotavirus and Shigella vaccines, as a matter of priority. Accordingly, PATH has initiated collaborations with private- and public-sector partners to accelerate the development of the best vaccine candidates against diarrhoeal disease and have been responsible for the surge in new mucosal vaccine development against the responsible pathogens. Much of the work of the PATH vaccine development program is funded by the Bill and Melinda Gates Foundation. However, if mucosal vaccination is to become a major vaccine delivery platform sustained investment and focus will be required.

Delivery Systems and Technology

Despite the significant advances in vaccine technology and implementation of many public health strategies including mass vaccination programs, infectious diseases still cause substantial morbidity and mortality across the world. There remain many diseases for which new vaccines are required and others for which suboptimal vaccines currently available, need to be replaced. With recent advances in delivery systems and the improving understanding of site specific mucosal immune mechanisms, mucosal immunization offers an exciting alternative vaccination strategy. Whilst the major types of mucosal vaccines in development include live-attenuated bacteria and viruses, as well as killed whole cell vaccines, these delivery systems continue to be expanded upon with the addition of heterologous antigen expression systems,[99,100] new technologies such as nanotechnology that promote antigen uptake at mucosal induction sites[101] and approaches that specifically target M cell surface receptors.[102] What is evident, is that a number of different strategies will be required depending on the mucosal site of infection and/or mucosal portal through which the infecting microbe invades systemically, as well as the type of immune response required both mucosally and systemically.

A major hurdle in mucosal vaccine development is an appropriate adjuvant to enhance the immunogenicity of vaccines. *E. coli* heat labile toxin (LT) and enterotoxic cholera toxin (CT) are the two most potent mucosal adjuvants. CT consists of a central active A subunit responsible for the toxicity of the subunit resulting in hypersecretion of fluids and the B subunit responsible for the binding of the toxin to the epithelial surface. LT consists of five copies of the B subunit and one copy of the A subunit. Both CT and LT help induce T cell responses and have been successfully used for mucosal immunization of animals, but they remain too toxic to be used as such in human vaccines. To alleviate the toxicity issues, less toxic forms of LT and CT have been engineered. The best known mucosal adjuvants are CT, B and A1 subunit (CTB and CTA1). These genetically modified forms have been engineered to reduce toxicity of chimeric proteins and are co-administered or chemically linked to protein antigens to improve cell targeting and safe use in humans.[103] Of the genetically detoxified mutants of LT, three mutants of the enzymatic A subunit, LTK63, LTR72 and LTR192G, maintain a high degree

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of adjuvanicity. LTK63 and LTR192G are potent mucosal, as well as, systemic adjuvants and are being assessed in human vaccine trials.[104-106]

In the following sections, the development of human mucosal vaccines for *V. cholerae*, ETEC, Shigella, NTHi, *E. coli*, *Helicobacter pylori*, poliovirus, adenovirus, rotavirus, human parainfluenza virus, RSV influenza virus, *M. tuberculosis*, *S. Typhi*, Norwalk virus, hepatitis B virus, *S. mutans*, *P. aeruginosa* measles virus, human papillomavirus (HPV) and HIV are reviewed in detail as examples of technological and formulation strategies to produce new and effective mucosal vaccines. Where available, clinical trial registration details are provided in parentheses throughout the text.

Oral Delivery Systems

Oral immunization remains the most attractive vaccination route. It is an acceptable route of delivery across all human cultures which greatly assist subject compliance. Administration of an oral vaccine can be accomplished readily by a less skilled health-care worker without the risk of needle stick infection to either the subject or health care worker. Finally, oral immunization is able to induce specific immune responses both systemically and mucosally.

Killed whole cell oral vaccines. Killed or inactivated oral vaccines, comprising of the whole pathogenic organism, offer the advantage of preserving the cellular integrity of the pathogen and therefore, all of the antigens are available to the immune system. The organism itself is rendered harmless by either treatment with heat, formalin or ethanol. A drawback of killed vaccines is that quite often multiple doses with large numbers of cells and/or the inclusion of an adjuvant is required for the induction of protective immunity.

The licensed killed whole cell oral vaccine Dukoral®, produced by Crucell (Netherlands) is a monovalent vaccine consisting of recombinant CTB (rCTB) and 10,[11] formalin and heat-inactivated *V. cholerae* O1 whole cells representing both serotypes (Inaba and Ogawa) and both biotypes (classical and El Tor). Dosage recommendations include: two doses to individuals 6 years and older and three doses for children aged 2 to less than 6 years old. Doses should be given ≥7 days apart (but <6 weeks apart). If there is continued risk of *V. cholerae* infection, a booster dose is recommended by the manufacturer after 2 years for individuals 6 years of age and every 6 months for children aged from 2 to 6 years. Dukoral® is not licensed for children younger than 2 years of age. A buffer must be used with the liquid formulation to protect the B subunit against stomach acidity. The vaccine has a shelf life of 3 years at 2–8°C and remains stable for 1 month at 37°C. Dukoral® is licensed in more than 60 countries and although available since 1991, it has not been used extensively in populations with endemic disease but rather as a vaccine for travellers to cholera endemic areas. Dukoral® induces a Th1 T cell response in young children;[107] both antibacterial and antitoxic (i.e., CT toxicity is blocked) SIgA mucosal immunity is induced in the intestinal tract, as well as serum antigen-specific IgG immune responses.[96] However, because of the limitations of assessing mucosal immunity in large scale clinical trials, the best immunologic correlate of protection for O1 cholera is serum vibrocidal antibody and is used in clinical trials as a marker of vibrocidal immune responses to *V. cholerae* O1. Clinical trials in Bangladesh, Mozambique and Peru found the vaccine to be safe and immunogenic.[108-111] Three oral doses of Dukoral® gave 80–95% protection for the first 6 months in all age groups greater than 2 years of age but declined rapidly in young children after 6 months, however, protection was still afforded in about 60% of older children and adults after 3 years.[110,111] Both CTB and LT are structurally and functionally similar and able to induce cross-reactive immune responses. Therefore, vaccines stimulating immunity against CTB should be able to confer some protection against ETEC infections. As predicted, Dukoral® has been demonstrated to cross-protect against ETEC disease and reduce the risk of traveller's diarrhoea.[112] In addition, mass vaccination with Dukoral® has the potential to provide herd protection to non-vaccinated individuals.[113] However, the vaccine is not without its limitations. First, it requires at least two doses administered 1–6 weeks apart to confer protection which poses a logistical disadvantage for the control of both endemic and epidemic cholera. Second, the vaccine costs approximately US$5 per dose and as such, is too expensive to be deployed in programs in either endemic or epidemic cholera regions. Third, it requires not only a cold-chain but also safe drinking water because it must be ingested with a bicarbonate buffer to stabilize the acid labile B subunit. Finally, it is effective only against the *V. cholerae* O1 strain. With the emergence of *V. cholerae* O139 in 1992 as the cause of outbreaks in Bangladesh, India and subsequently Asia,[1] *V. cholerae* O139 has been added to recent formulations. The addition of the *V. cholerae* O139 component to the *V. cholerae* O1 vaccine components together with rCTB was found to be as immunogenic as Dukoral® eliciting strong mucosal and vibriocidal antibodies,[114] The Vietnamese government also took the initiative to produce a variant of the Dukoral® vaccine—a bivalent (O1 and O139) killed whole cell oral cholera vaccine that contained no rCTB subunit (ORC-VAX™, Vabiotech, Vietnam). The vaccine has been found to be safe, elicited five-fold rises in serum anti-O1 vibriocidal antibodies[115] and conferred 50% over-all protection against El Tor cholera.[116] In 2004, with funding from the Gates Foundation, the vaccine was reformulated to meet international and WHO production guidelines. Following field trials in India and Vietnam showing the new vaccine to be safe and immunogenic in adults and children and providing 68% protection over-all for 2 years,[117-119] Vabiotech transferred the technology for the vaccine to Shantha Biotechics, India. In 2009, this vaccine was licensed as mORC-VAX™ in Vietnam and Shanchol™ in India. mORC-VAX™ is currently intended for domestic use, whereas Shanchol™ will be produced for use in public health programs in India and countries in Asia and Africa to control endemic and epidemic cholera. This new low cost vaccine ($1.00 US per dose) is administered orally in two liquid doses, 14 days apart for individuals aged ≥1 year. A booster dose is recommended after 2 years. Unlike Dukoral®, children younger than 6 years do not require booster doses every 6 months and no buffer is required since it does not contain the CTB subunit. The vaccine has a

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shelf life of 2 years at 2–8°C. The vaccine is currently undergoing Phase III trials with the primary objective of obtaining WHO prequalification and therefore, assuring its acceptability for worldwide purchase. Surveillance is ongoing to assess longer term protection and potential herd protection. The parenteral vaccine containing phenol-inactivated strains of *V. cholerae* is still manufactured in a few countries, however, its use is not recommended by WHO because of its limited efficacy and short duration of protection.[120]

A new approach in cholera vaccine development is the *V. cholerae* ghost platform which has shown promising results as an effective antigen delivery system for oral immunization.[121] Bacterial ghosts (BGs) are nonliving cell envelope preparations from Gram-negative cells that do not contain cytoplasmic granules while their cellular morphology and surface antigenic structures remain preserved.[122] As such, BGs have strong, intrinsic adjuvant properties. BGs can be freeze-dried, significantly increasing shelf life and stability. The bacterial ghost vector (BGV) system can be used as a potent, flexible and safe delivery platform for the delivery of both recombinant protein and DNA. Owing to its particulate nature, the BGV-vaccine platform technology has the potential to deliver antigens to APCs and to enhance immune responses. Bacteria that have been used for producing BGs include enterohemorrhagic *E. coli*, ETEC, *S. flexneri* and *H. pylori*, among others.[122]

There is currently no licensed vaccine available for diarrhoeal diseases caused by ETEC and progress has been hindered by the lack of appropriate animal models for human disease. The major pathogenic mechanisms that contribute to the pathogenesis of ETEC are the production of colonization factor antigens (CFAs), the hair-like fimbriae that attach to the intestinal epithelium, as well as LT and heat-stable (ST) toxins, which induce diarrhoea. Some CFAs are subdivided into coli surface (CS) antigens. A killed whole cell oral ETEC vaccine containing rCTB together with 5 strains of formalin-killed ETEC cells that collectively express the CFAs of greatest importance in developing countries (CFA/1 and CS1-CS5, inclusive) (University Götenborg, Sweden) has proved to be the most successful in clinical studies.[123] Phase II studies found the vaccine to be safe and immunogenic in Bangladeshi adults and children[124] and infants down to 6 months of age, at a reduced dose[125] inducing mucosal SIgA antibody responses to CTB and to CFA components of the vaccine.[125] A phase III trial in Egyptian children and infants found vaccine efficacy against diarrhoea in young children to be low[126] despite being relatively immunogenic.[127] The lack of a published report from this study makes interpretation of these findings difficult. However, 77% protection was reported against severe forms of ETEC diarrhoea of travellers to Guatemala and Mexico.[128] The vaccine stimulated IgA responses to both LT and several CFAs. Interestingly, the ETEC vaccine was not as efficient at protection against mild symptoms associated with ETEC, the authors[128] suggesting that the vaccine may induce enough immunity to limit the disease process at the mucosal surface without being able to prevent it completely. The failure of the killed oral ETEC vaccine to confer protection in ETEC endemic areas has raised questions about the current vaccine strategy. There is accumulating evidence to suggest that the choice of vaccine strain and CFA expression may be a contributing factor to its failure with the newly recognized diversity of CFAs of ETEC and the observation that protection against re-infection appears to be CFA/toxin phenotype specific.[126] As such, new strategies to enhance immunogenicity of critical antigens are presently being explored. For example, oral immunization with formalin-killed recombinant *E. coli* bacteria K12 overexpressing CFA/1 has been shown to induce a significant antibody response[129] and Phase I clinical trials are planned for the highly promising new vaccine/adjuvant, double mutant heat-labile (dmLT), that PATH in-licensed from Tulane University, US (www.path.org). The new adjuvant is also an ETEC antigen that may offer protection against the disease itself.

Research into the development of a safe and efficacious vaccine for widespread use for the prevention of shigellosis has been ongoing for more than half a century. Up until the last few years, the major roadblock to the development of a successful Shigella vaccine has been the lack of a relevant animal model that replicates human bacillary dysentery. However, following testing in a new guinea pig model, a formalin-inactivated *S. sonnei* vaccine (Ss-WC) has been developed as a killed whole cell oral vaccine (Johns Hopkins University, US) and tested in a Phase I trial where it was found to be well tolerated and induced mucosal IgA and serum IgA and IgG antibodies.[130] Although there has been some success with candidate vaccines, there remain significant impediments to achieving a beneficial Shigella vaccine. One such factor which has limited vaccine advancement is the need to identify the immune correlates of protection against shigellosis. While protection is mediated, at least in part, by mucosal SIgA or serum antibodies to the lipopolysaccharide (LPS) and/or O-polysaccharide antigen of Shigella, other effector mechanisms have also been described, including Th-1 type cytokines IFNγ and IL-10.[131] Additionally, protective immunity is type-specific, therefore, a Shigella vaccine must protect against the broad spectrum of Shigella serotypes namely *S. dysenteriae* type 1 (Sd1), *S. sonnei*, and all 14 classical *S. flexneri* types and serotypes.[131] Population-based surveillance studies have also shown that the serotypes can also vary geographically.[131] To produce a vaccine representing all the species and serotypes would be unrealistic and cost prohibitive and as such there is a need to formulate a multivalent vaccine which includes determinants that offer cross-protection against the prevalent species and serotypes.

NTHi colonizes the mucosal epithelium of the upper respiratory tract. Whilst this microbe is part of the normal commensal flora, infections do occur when the host-commensal balance is disturbed in predisposed individuals. A killed monovalent whole cell oral NTHi vaccine has been extensively trialled in patients prone to recurrent acute exacerbations of chronic bronchitis.[132-138] A Cochrane review of the six trials of 440 participants reported vaccine efficacy of 20–30% in reducing the incidence and severity of acute episodes of chronic bronchitis with a 58% reduction in prescription of antibiotics.[139] Oral immunization reduced bacterial loads in patients who were chronically colonized and NTHi-specific cellular responses were detected in peripheral blood lymphocytes. The protection conferred by the vaccine, however, was not long lasting and its use may be limited to

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bronchitics with recurrent acute exacerbations for temporary reprieve from exacerbations during winter months. Results from in vitro studies have suggested that the killed whole cell NTHi strain used in the vaccine may bind to M cells through pattern recognition receptors (PRRs) and α5β1 integrin.[102] This vaccine is significant in that it demonstrates conclusively, that mucosal immunization is able to be used in the therapeutic context to enhance or modify an immune response to improve the outcomes of an established chronic mucosal infection. The results of a larger multicentre trial currently being planned by the Hunter Immunology Ltd., Australia, will be important to confirm the earlier observations (ACTRN12606000076572). A similar killed whole cell approach for *P. aeruginosa* has also been explored by this group. This developmental oral vaccine has been shown to be safe and immunogenic in healthy volunteers.[140]

A killed whole cell vaccine approach has also been explored for *E. coli* urinary tract infections and *H. pylori* infection of the gastric mucosa. OM-89 (Uro-Vaxom®; OM PHARMA, Switzerland) administered orally as a capsule containing 6 mg of lyophilized lysate of selected *E. coli* strains, has been reported to significantly reduce recurrent urinary tract infections.[141] The immunization schedule is arduous and little is known about the type or immune responses induced or its longevity. A formalin-inactivated whole cell oral *H. pylori* vaccine containing 2.5 x 10^{10} CFU and co-administered with the adjuvant, LTR192G, induced both specific mucosal IgA antibody (fecal and salivary) and cell mediated reponses.[105] Whilst there were some side-effects noted and the vaccine did not eradicate *H. pylori* from infected subjects, this study does demonstrate that with further development and a better understanding of the immune responses to clear the infection, an oral vaccine against *H. pylori* infection is a real possibility.

Live attenuated oral vaccines. Following the success of mass vaccination with the live smallpox vaccine over 200 years ago, the concept of using live organisms to elicit protective immunity has been subsequently adopted for the currently licensed mucosal vaccines against poliovirus, rotavirus, *V. cholerae* and *S. Typhi*. The effectiveness of live pathogens as mucosal vaccines and vaccine vectors is largely attributed to their ability to mimic natural infection. In effect, the immune system treats live attenuated vaccines as it would an infectious pathogen and therefore, innate and adaptive mucosal immune responses may be induced. To ensure the safety of live vaccines, attenuation of the pathogenic organism generally involves the induction and selection of mutants to reduce or eliminate critical virulence attributes. The trivalent Sabin OPV, which was licensed in 1963, is a modified attenuated, live vaccine. OPV is administered as a series of three doses, usually concurrently with other vaccines according to the schedules of national immunization programs. The vaccine is highly heat sensitive and must be kept frozen or, following thawing, stored between 2–8°C for no longer than 6 months. Similar to the parenteral IPV, OPV has proved to be highly effective in inducing virus-specific IgG antibodies. However, OPV has claimed its superiority over IPV by also inducing mucosal IgA responses in the intestinal mucosa, the primary site for poliovirus multiplication. The presence of neutralizing antibody against polioviruses is considered the correlate of protection against poliomyelitis whereas total antibody levels are less reliable. Although antibody concentrations decline over time, immunity against paralytic disease is life-long. The local immunity induced by OPV is probably responsible for the extraordinary effect of OPV mass campaigns in interrupting wild virus transmission. Both vaccines comprise of the three serotypes of poliovirus (types 1, 2 and 3) that are responsible for causing poliomyelitis. Despite the advantages of the oral vaccine with respect to efficacy, ease of use and cost (8 US cents per dose), like all replicating-immunogens, a significant drawback of OPV is the inherent risk of reversion of the attenuated organism toward the virulent wild-type. In rare cases (approximately 4 cases in every 1 million birth cohort per year), OPV can cause paralytic poliomyelitis.[142] Thus, most developed countries use IPV in preference to OPV. OPV has been the vaccine of choice for the Global Polio Eradication Initiative (GPEI) and while there has been substantial progress toward the eradication of poliomyelitis worldwide (wild-strain polio virus type-2 has not been detected globally since 1999), the resurgence in the circulation of wild-type poliovirus serotypes and outbreaks due to circulating vaccine-derived polioviruses in recent years has forced the GPEI to re-evaluate their disease eradication strategies.[1] Among measures, since 2005, monovalent OPVs against type-1 (mOPV1) and against type-3 (mOPV3) and more recently, 2 bivalent OPVs (type-1 and type-3) (bOPV), have been licensed for use in some developing countries to compensate for the low immunogenicity of the trivalent vaccine in these countries. In a head-to-head comparator trial conducted in Egypt, a newly licensed mOPV1 (Sanofi Pasteur, France), convincingly proved to be significantly more immunogenic than the trivalent OPV.[143] Field trials in Taiwan[144] and Oman[145] estimated the three-dose efficacy of OPV as more than 90%, whereas other geographical regions such as India have shown the efficacy of the vaccine to be disappointingly low.[85] A two-dose regimen of IPV was 89% efficacious in preventing paralysis in a case-control study of vaccinated infants in the developing country, Senegal.[146] However, despite the successes of the OPV, in an effort to manage the long-term risks associated with the use of the vaccine, a decision was made by the GPEI in 2008 to eventually remove it from routine immunization.[1] Although considerably more expensive (over five times that of OPV), the IPV will be made available to those countries that warrant its continued inclusion in the immunization schedule. At present, the priority of GPEI is to investigate strategies that would make the use of the IPV as affordable as the efficacious OPV.

Adenoviruses, a group of nonenveloped icosahedral viruses, are a significant cause of repiratory infections and diarrhoea particularly in children. Until the mid 1990s military recruits were immunized with a live oral vaccine against adenovirus serotypes 4 (ADV4) and 7 (ADV7) (Wyeth, US). The vaccine was administered as an enteric tablet containing between $10^{4.6}$ to $10^{4.7}$ tissue culture median infectious doses per tablet ($TCID_{50}$). The vaccine was safe and effective in reducing the incidence of adenovirus acute respiratory infections in this subject cohort.[147] The re-emergence of disease has renewed interest in re-introduction of vaccination against ADV4 and ADV 7 serotypes. A recent Phase

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I trial using strains from the original master seed bank and an enteric coated tablet formulation (Duramed Research, US) has, as might be predicted, demonstrated acceptable safety and good immungenicty.[148]

Unlike, *V. cholerae*, *S. Typhi* and ETEC gastrointestinal disease which are predominantly diseases of developing countries, nearly every child, regardless of socioeconomic level or geographic location, will contract rotavirus at least once before the age of three.[149] The significance of rotavirus disease on child morbidity and mortality prompted the WHO SAGE on immunization, to announce in 2009, a global recommendation that rotavirus vaccines be included in national immunization programs.[150] Targets for vaccine development are the outer capsid proteins of rotavirus, VP4 and VP7, which determine the serotype-specific neutralizing antigens P and G, respectively. P genotypes are denoted in brackets. At present, 19 G types and 27 P types have been described but only 5 G serotypes (G1, G2, G3, G4 and G9) and 3 P serotypes (P[8], P[4] and P[6]) account for more than 90% of rotavirus gastroenteritis worldwide.[151,152] However, the relative prevalence and distribution of rotavirus strains change with regard to location and time and have important implications for vaccine development. The high diversity of rotavirus strains is possible because of the segmented nature of the rotavirus genome which allows for gene reassortment when it is co-infected with other different virus strains, either human or animal. Reassortant vaccines are based on the strategy of mixtures of the most common circulating G types. Nearly a decade after the removal of the failed Rotashield from the market, two oral live attenuated rotavirus vaccines, Rotarix® (GlaxoSmithKline Biologicals, Belgium) and RotaTeq® (Merck & Co., Inc., US) are available internationally in more than 100 and 88 countries, respectively. The two vaccines were developed using different principles to achieve broad protection against a diverse range of common rotavirus strains. Rotarix® is an attenuated human rotavirus vaccine that is made from a tissue-culture adapted human isolate, 89-12, a G1P[8] strain which is the most common serotype worldwide. As a human derived vaccine, it has the theoretical advantage that it could stimulate broad antibody responses without the need to include a complex mixture of strains. Rotarix® was first licensed in Mexico in 2004. The vaccine is a lyophilized powder, stored at 2–8°C, requiring reconstitution in buffer. Rotarix® was developed as a two dose oral vaccine with the first dose administered at 6 to 14 weeks of age followed by the second dose administered after a 4- to 8-week interval. Rotarix® demonstrated 70–85% protective efficacy in healthy infants between 6 and 14 weeks of age against severe rotavirus gastroenteritis in Latin America and Finland and was not associated with an increased risk of intussusceptions.[153] Protection was provided for the first 2 years of life against; wildtype G_1 (81%), pooled non G1 strains (78%) and pooled non-G1 P[8] strains (81%).[154] The vaccine has been prequalified by WHO for procurement by UNICEF and the UN Vaccine Fund. Additional large Phase IIb and Phase III trials have been completed in developing countries of Asia and Africa where reduced immunogenicity of oral vaccines remains a significant challenge. After a 1 year follow-up, the efficacy of Rotarix® in preventing severe rotavirus gastroenteritis was 77% in South Africa, 50% in Malawi (higher incidence of severe disease was reflected in the lower efficacy rate) and 61% in the combined study populations.[150] In Asian countries with low or intermediate mortality rates, Rotarix® had a combined efficacy of 97% in protecting against severe rotavirus gastroenteritis.[150] RotaTeq® is a pentavalent human-bovine reassortant vaccine derived from a bovine (WC3; G6P[5]) rotavirus strain which was reassorted with human rotavirus G types 1, 2, 3, 4 and P1[8]. The animal rotavirus strain is naturally attenuated for humans and depends on serotype-specific immunity to deliver a high level of protection. This strategy relies on multiple strains being incorporated in a vaccine. RotaTeq® was licensed in the US in 2006. The first dose, of a three-dose regimen, is given at 6 to12 weeks of age with subsequent doses administered at 4- to 10-week intervals. The vaccine comes in a ready to use oral liquid that must be stored at 2–8°C. The safety and efficacy of RotoTeq® was assessed in a Phase III trial in Finland and US in healthy infants aged 6 to 12 weeks and found to be safe and to elicit 74% protection against G1–G4 rotavirus gastroenteritis and 98% against severe gastroenteritis through the first rotavirus season post vaccination.[155] Preliminary efficacy results from a Phase III trial completed in 2009, demonstrated a three-dose regimen of Rotateq® to be efficacious against severe rotavirus gastroenteritis during the first year of follow-up in 64% and 51% of infants in Africa and Asia, respectively.[150] On-going post marketing safety monitoring in the US showed vaccine effectiveness against severe rotavirus gastroenteritis to be 85–95%.[150] The observation that the rotavirus vaccines are more efficacious against the severe form of the disease is similar to the experience with another mucosal infection; ETEC gastroenteritis.[128] Little is known about the immune mechanisms by which Rotarix® and RotaTeq® vaccines induce protection against human rotavirus strains not represented in these vaccines. The correlates of immunity to rotavirus infection are incompletely defined and pose a challenge for developing alternate vaccines. It is generally accepted that the immune responses to the VP4 and VP7 proteins are important; however, there is controversy as to whether neutralizing antibody directed at either protein actually correlates with protection. Serum and intestinal rotavirus IgA, serum IgG have been shown in human and animal studies to correlate with protection and the resolution of infection[156] and consequently, serum IgA antibody responses have been used as measures of vaccine immunogenicity of candidate live attenuated oral rotavirus vaccines.[150] Furthermore, studies in animals, suggest that cellular immunity may have a potential role in protection.[156] PATH's Advancing Rotavirus Vaccine Development project is working with emerging-country manufacturers to develop promising new candidates to improve affordability and ensuring a sustainable supply of rotavirus vaccines for widespread use in developing countries.[157] Three live attenuated oral vaccine candidates; the bovine (UK strain)/human reassortant vaccine, the human neonatal RV3 strain, and the bovine/human neonatal 116E strain have been under development for a number of years and have the most potential for broader use. The bovine (UK)/human rotavirus reassortant vaccine candidate is licensed by Wyeth and seven emerging-country manufacturers. The reassortant vaccine contains single VP7 gene

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substitutions from G1, G2, G3, G4 or G8 (present in Africa) or G9 (present in India) human rotaviruses. An earlier formulation of the bovine (UK)/human rotavirus reassortant vaccine containing the G1 to G4 strains was co-administered with routine childhood vaccines and evaluated in a Phase I trial.[158] The tetravalent reassortant vaccine was found to be safe eliciting a strong neutralizing antibody response against the bovine rotavirus strain (95%) compared to the human rotavirus VP7 serotypes 1 (37%), 2 (32%), 3 (32%) and 4 (32%) and non-interfering with the other vaccines. The vaccine was reformulated for a two dose Phase IIb efficacy study in Finland and demonstrated 99% seroresponse rate and good efficacy against severe rotavirus gastroenteritis (88%) during the first epidemic season.[159] Two manufacturers, Shantha Biotechnics Ltd. in India and China National Biotec Group's Wuhan Institute of Biological products will further the development of the vaccine candidate, with Shantha Biotechnics Ltd., sponsoring a Phase I/II, three-dose regimen, dosage selection ($10^{5.5}$ or $10^{6.25}$ FFU), safety and immunogenicity study planned to start in India shortly (NCT01061658). The 116E G9P[11] (Bharat Biotech International, India) human monovalent vaccine candidate is a naturally occurring reassortant human strain with one gene (VP4) derived from a bovine rotavirus. The 116E rotavirus strain was isolated from an asymptomatic rotavirus infected neonate in India. A Phase I/II study evaluated 116E in Indian infants and found that it was well tolerated with seroconversion observed in 62% and 90% of infants after three doses of 10^4 and 10^5 FFU dosages, respectively.[160] Given the high immunogenicity of 116E vaccine, a Phase III field trial is planned to commence this year to evaluate the protective efficacy of the vaccine candidate in India. RV3 G3P[6] is a naturally attenuated, human rotavirus that was originally isolated in an newborn nursery in Melbourne, Australia. Neonates who were naturally infected with the RV3 virus were 100% protected against severe rotavirus disease for the first 3 years of life. A Phase II trial administering a three-dose regimen of 6.5 x 10^5 fluorescent cell forming units/ml (fcfu/ml) of RV3 showed the vaccine candidate to be moderately immunogenic in 46% of infants; protection in the responders against rotavirus disease during the subsequent winter epidemic was 54%.[161] The poor immunogenicity of RV3 has limited its development and strategies to increase infectivity, including increasing the dose of RV3 above 6.5 x 10^5 fcfu are progressing. In 2008, PATH formed a partnership with Murdoch Children's Research Institute, Australia, to assist in the production of clinical trial lots of RV3 at Meridian Life Science in Memphis, US, in preparation for clinical trials to be conducted in Australia. The human neonatal strains, RV3 and 116E, may prove to be beneficial for administration in newborn babies, especially in developing countries where the younger age of onset of gastroenteritis may require vaccines to be administered immediately after birth to provide protection during the critical period of the first few months of life. It is anticipated that 116E would become part of the Indian universal immunization programme. The question remains, however, as to whether the polyvalent bovine (UK)/human reassortant vaccine and the RV3 and 116E monovalent human rotavirus vaccines will offer significant improvements over the current licensed vaccines with respect to protection against emerging serotypes. Although, it is anticipated that future bovine (UK)/human reassortant formulations will carry additional reassortants to improve serotype-specific coverage. While the efficacy data on Rotarix® and RotaTeq® in Asia and Africa generally looks encouraging, the observed poor efficacy of Rotarix® in Malawi, a substantially poorer population with high rates of serious rotavirus gastroenteritis, suggests that the immune response elicited by rotavirus vaccines may be poor in these settings. Continued surveillance will be required with respect to rotavirus disease burden, rates of infant intussusception and types of circulating rotavirus strains, be they unusual or evolving, remain to be evaluated to confirm vaccine efficacy. Furthermore, little is known about the impact of live attenuated vaccines in immunosuppressed children in whom rotavirus may cause chronic infections resulting in the long-term shedding of virus. Significantly, the move to develop and manufacture alternate rotavirus vaccines in emerging countries may have the anticipated advantage of promoting competition and drive down the prices of the expensive rotavirus vaccines ensuring their affordability to developing nations.

The principal virulence factor of *V. cholerae* is CT itself and as such, the licensed live attenuated oral cholera vaccine, CVD 103HgR or Orochol™, produced by Crucell, contains a genetically manipulated classical *V. cholerae* O1 Inaba strain with a deletion in the gene encoding the A-subunit. The lyophilized vaccine is administered as a single dose along with a buffer. Orochol™ is available in two different potencies: a low dose formulation for developed countries for use by travellers and a more concentrated dose formulation for cholera endemic regions countries. Placebo-controlled trials in several South American[162] and Asian[163,164] countries have shown the vaccine to be safe and immunogenic, however, it failed to provide protection in a large-field trial performed in cholera endemic Indonesia.[165] Protection efficacy against experimental challenge in adult volunteers in the US provided the basis for licensure of the vaccine for adult travellers to cholera-endemic countries.[166] Although an estimated 79% protective efficacy was detected retrospectively in a cholera outbreak in Micronesia,[167] production of Orochol™ was discontinued in 2004 due to the limited market potential.

The seventh cholera pandemic in the 1960's saw the El Tor biotype strains gradually replace the classical strains as the cause of cholera. However, efforts to develop an El Tor biotype live attenuated vaccine were hindered, first, due to reactogenicity and associated adverse effects and secondly, the more recent emergence of new pathogenic variants of *V. cholerae* O1 throughout Asian and African countries. These new variants share phenotypic and genotypic traits of both classical and El Tor biotypes[168] and represent a significant challenge in cholera vaccine development. Among the furthest advanced, Peru 15 (Cholera Garde®), is a live attenuated oral *V. cholerae* O1 vaccine candidate containing the El Tor biotype and Inaba serotype which was developed at Harvard University and is being manufactured by AVANT Immunotherapeutics, US. Peru 15 is a spontaneous non-motile mutant that has undergone a series of genetic attenuations with deletions of the CTXΦ cholera prophage encoding the CT gene to render it non-toxigenic and introducing the gene encoding

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CTB while also modifying and deleting genes to make it non-recombinational.[169,170] In a significant advance for vaccine formulation, the manufacturer has pioneered a method of turning the live bacterial vaccine into a glasslike solid that stays stable for months at high temperatures. The vaccine is administered as a single liquid dose of approximately 10^8 CFU and has been found to be safe, immunogenic[171,172] and protective in the US,[172] as well as safe and immunogenic in Bangladeshi adults and children as young as 9 months of age.[173,174] Vibriocidal antibody was induced in approximately 75% of adults and children, while seroconversion for LPS-specific IgA antibodies was seen in 88% and 40% of adults and children, respectively. Phase II studies of Peru 15 are ongoing in cholera-endemic countries to concomitantly administer it to infants with the parenteral measles vaccine (NCT00624975). Another promising live genetically-attenuated cholera vaccine candidate is *V. cholerae* strain 638 from a *V. cholerae* O1 El Tor Ogawa strain. The cholera vaccine strain was attenuated by deletion of the CTXΦ cholera prophage and by insertion of the *Clostridium thermocellum* endonuclease A gene, thereby disrupting the hemagglutinin/protease coding sequence. Human volunteer studies with a single oral dose of 10^9 CFU showed that 96% of volunteers had a significant increase in vibriocidal antibody titres and 50% showed at least a doubling of LPS-specific IgA titres in serum with protection conferred in a challenge study.[175]

Efforts to improve the cost-effectiveness of cholera vaccines have fostered interest in developing a genetically engineered live attenuated bivalent oral *V. cholerae*/ETEC vaccine, Peru-15pCTB (AVANT Immunotherapeutics). The rationale being that CTB secreted from a live attenuated vaccine at a sufficient level would provide cross-protection against LT-producing ETEC strains, as in the case of Dukoral™ which is recommended for protection against traveller's diarrhoea caused by ETEC.[112] The Peru 15 vaccine has provided the attenuation blueprint that has been replicated in the new cholera vaccine. Peru-15pCTB has been genetically engineered to express and secrete high levels of CTB, approximately 30 fold more than Peru-15.[176] A Phase I trial using a single dose of Peru-15pCTB is presently being assessed in healthy volunteers for safety and immunogenicity (NCT00654108). Other live attenuated oral ETEC strains that have completed Phase I clinical studies include: ACE527, PTL-003 and ACAM2010. PATH is supporting the development of ACE527 (TD Vaccines, Denmark), a live whole cell oral vaccine which comprises of three attenuated ETEC strains expressing 6 of the most common CFAs and high levels of the protective B subunit of the LT toxin. The vaccine was designed to provide broad coverage for traveller's diarrhoea. ACE527 has been reported by the manufacturer as being safe and immunogenic; two doses (3 x 10^9 CFU/dose) inducing a strong immune response to colonization factors on all three strains with a strong mucosal and systemic anti-toxin response as well. A Phase II challenge study (NCT01060748) is scheduled to start this year. TD Vaccines has ongoing research to develop temperature stable, oral ETEC vaccine formulations that can be spray- or freeze-dried and therefore, not requiring refrigerated storage. Both PTL-003 and ACAM2010 are spontaneous nontoxigenic (LT-, ST-) ETEC mutant strains developed by the Acambis Corporation, UK. PTL-003 has been attenuated by mutations ΔaroC, ΔompC and ΔompF (genes that code for outer membrane proteins) expressing CFA/II (CS1 and CS3). The vaccine candidate proved to be safe, eliciting a strong immune response as measured by an IgA ASC response rate of 90%,[177] however, a two-dose schedule of 2 x 10^9 CFU failed to protect subjects from a high-dose, virulent ETEC challenge.[178] Another Acambis construct ACAM2010 expressing other prevalent CFAs including CFA/1, $CS1^+CS2^+CS3$ and $CS4^+CS5^+CS6$ and LTB was found to be well tolerated, inducing IgA and IgG-ASCs specific for CFA/1 in 73% of subjects following oral immunization.[179] New attenuated strains which express additional colonization factors and LTB have been constructed and it is anticipated that a multivalent ETEC vaccine will be available for clinical testing in the not too distant future.

Tuberculosis remains a leading couse of morbidity and mortality worldwide. The first BCG immunizations were orally administered and it essentially remained that way until the early 1930s when after the "Lubeck incident" in Germany, intradermal immunization became the standard route with a vaccine efficacy of around 80%. The incident was following the accidental contamination of the attenuated vaccine strain with a wild type strain and a number of children became infected and died following oral immunization. However, an oral formulation of the *M. bovis* Moreau Rio de Janeiro (BCG_{MoWT}) strain is still commercially available.[180] In recent years there has been renewed interest in oral immunization against tuberculosis in order to improve vaccination control of the tuberculosis pandemic. A number of very promising results have been reported in animal models using a range of different mucosal delivery systems.[181-184] Furthermore, oral immunization clinical trials in humans of attenuated BCG are now occurring. A single oral immunization with 10^7 CFU BCG_{MoWT} in a buffer solution significantly boosted a primary intradermal immunization as evidenced by increased IFNγ ELISPOTs to a number of mycobacterial antigens.[185] Recently a clinical trial was registered in the US (NCT00396370) to compare intradermal, oral and combined intradermal/oral routes using the Bacillus Calette-Guerin strain. One or two doses will be administered with the oral dose being 1.2 x 10^8 CFU suspended in bufferd saline.

Numerous live attenuated oral Shigella vaccine candidates have been evaluated, however, the balance between moderate attenuation responsible for excessive reactogenicity and over attenuation leading to insufficient immunogenicity has proved to be a significant obstacle to the advancement of many. Such has been the case for a more recent promising live attenuated oral Shigella vaccine candidate CVD 1208S which has been under development at the Centre for Vaccine Development (CVD), US. Attenuation of *S. flexneri* 2a was achieved by progressive targeted deletion of the virulence genes *guaBA*, *sen* and *set*, culminating in the strain CVD 1208S. CVD 1208S proved to be well tolerated eliciting anti-LPS responses in subjects, measured as IgA ASCs, serum IgG or fecal IgA following administration of 10^9 CFU.[186] Serum INFγ responses to at least one antigen developed in 57% of subjects. However, in early 2010, a Phase II clinical trial evaluating CVD 1208S (NCT00866476) was halted after a small

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number of study volunteers experienced adverse events including fever, abdominal cramping and loose stools. It was suspected that the use of a new medium for growing the vaccine inoculum used in the study may have affected the clinical tolerability of the vaccine. Pending results from investigative studies with respect to the impact of growth mediums on the construct, a decision will be made regarding the next steps forward for CVD 1208S. Certain attenuating mutations introduced into wild-type Shigella strains in recent years have generated promising vaccine candidates. Live attenuated Shigella vaccine candidates, such as *S. dysenteriae* type 1 strain SC599, *S. flexneri* 2a strain SC602, *S. sonnei* vaccine candidate WRSS1 and S. *dysenteriae* type 1 strain WRSd1, are principally attenuated by the loss of the *virG/icsA* protein. As such, these mutants have lost their ability to invade intestinal epithelial cells. These candidates have proven to be safe and immunogenic in volunteer trials. Both SC599 and SC602 were developed at the Pasteur Institute, France. SC602 was tested in adult volunteers in the US[187] and in adults and children in Bangladesh.[188] In US study, a low dosage (10^4 CFU) of SC602 was reactogenic, moderately immunogenic, as measured by serum IgA and IgA ASCs and heavily excreted in volunteers who had received dosages as low as 10^3 CFU. By contrast, at a substantially increased dose of 10^6 CFU, no Bangladeshi child manifested an adverse reaction, neither did any child excrete the vaccine strain, however, immunogenicity was disappointingly low. SC599 is attenuated by deletion of an invasion [*virG/icsA*] gene, as well as the iron chelation [*ent, fep*] and shiga toxin A subunit [*stx*] genes. When SC599 was evaluated in a Phase II study it was found that a single oral dose of 10^5 or 10^7 CFU was well tolerated and immunogenic, inducing significant anti-LPS IgA and IgG ASCs and serum anti-LPS IgG and IgA responses.[189] The magnitude of the response elicited by SC599 was comparable to that of SC602 that prevented illness following experimental challenge, which suggests that this attenuated vaccine may confer protection against shigellosis but further studies are required. WRSS1 was developed at Walter Reed Army Institute Research (WRAIR), US. In a Phase I study, a single oral dose 10^6 CFU was found to be mildly reactogenic and elicited strong O-antigen-specific IgA ASC responses and moderate IFNγ responses in serum.[190] A study performed in Israel in adults who ingested 10^3, 10^4 or 10^5 CFU.[191] Of the three dosages tested, the 10^4 dose of WRSS1 provided the better balance of safety and immunogenicity, since all vaccinees had a significant IgA anti-O-antigen ASC response.[191] Accordingly, WRSS1 is planned to be further assessed at a dosage of 10^4 CFU in a Phase II study for safety, immunogenicity and efficacy in healthy Thai adult volunteers this year (NCT01080716). WRSd1 was also developed at the WRAIR by deletion of its *stx-fnr* genes, as well as *virG*(*icsA*) genes.[192] However, unlike WRSS1 and SC602, WRSd1 was unfortunately found to be modestly immunogenic in US volunteers following ingestion of a 10^4 CFU dose.[193] Investigators hypothesized that the observed poor immune response was most probably due to loss of the *fnr* gene resulting in poor colonization of the gastrointestinal tract. While these *virG/icsA* gene deletion based vaccines have proven to be safe and generally immunogenic at low doses, one drawback has been the reactogenic symptoms of fever and diarrhoea experienced by volunteers. New second generation Shigella vaccine candidates, *S. flexneri* 2a strain WRSFG11,[194] *S. sonnei* strains WRSs2 and WRSs3,[195] have been constructed by deletion of *set* and *sen* enterotoxin genes in addition to the *virG/icsA* gene deletion. These new vaccine candidates have the potential for reduced reactogenicity while retaining the ability to generate protective levels of immunogenicity as seen by their predecessors.

Two typhoid vaccines are currently licensed for the international market: a parenteral purified Vi antigen polysaccharide vaccine and a live attenuated oral vaccine (Ty21a). Vivotif®, currently manufactured by Crucell, was first licensed in Europe in 1983 and in the US in 1989 and is now licensed in 56 countries in Africa, the Americas, Asia and Europe. The vaccine is formulated with the *S. enterica* serovar Typhi strain Ty2 in which multiple genes, including the genes responsible for the production of the capsular Vi (Virulence) antigen, have been chemically mutated. The lyophilized vaccine is available as enteric coated capsules which are swallowed every other day for one week; a three- or four-dose regimen (approximately, 10^9 CFU/dose) is recommended. Vivotif® requires storage at 2–8°C and retains its potency for approximately 2 weeks at 25°C. The vaccine may be given simultaneously with other vaccines. Neither the Vi polysaccharide vaccine nor Vivotif® is licensed for children under two years of age. The Vi polysaccharide and the Vivotif® vaccines have been shown in large-scale clinical trials to be safe and share similar moderate efficacy; 3 year cumulative efficacy was 55% (one dose) and 48% (three doses), respectively.[196] In Chile, Vivotif® provided 49% cross-protection against paratyphoid fever caused by serovar Paratyphi B.[197] The vaccine elicits mucosal IgA and serum IgG antibodies against O, H and other *S. Typhi* antigens, as well as, cell mediated responses, including the production of Type 1 cytokines and $CD8^+$ CTL responses.[198] High levels of gut homing molecules, integrin $\alpha4\beta7$ are expressed on circulating anti-*S. typhi*-specific T cells following oral immunization.[199] However, the immunologic correlates of protection remain largely undefined: elevated serum antibodies to *S. Typhi* antigens do not appear to correlate with protection, whereas, gut-derived IgA ASCs, $CD4^+$ Th and $CT8^+$ CTL cells may play key roles in defense against the pathogen.[198] To date, Vivotif® has been used primarily to protect travellers against typhoid when visiting endemic countries. However, because of its demonstrated safety, efficacy and affordability, WHO have advocated for the vaccine's implementation for controlling endemic disease in developing countries. Nevertheless, given the continuing high burden of typhoid fever, increasing antibiotic resistance of *S. Typhi* and dosing challenges for widespread control of the disease in endemic and epidemic situations, improved vaccines against typhoid fever are desirable. As such, three live attenuated oral vaccines with defined genetic deletions: CVD909 (CVD; Acambis, UK; Crucell), Ty800 (Avant, US), M01ZH09 (Emergent Biosolutions, UK) are currently in stages of clinical development. The aim has been to develop a more highly-immunogenic vaccine than Vivotif® that would provide protection after a single dose. All of these vaccine candidates are derived from the wild-type strain Ty2. Attenuated strain CVD 909 carries deletions in *aroC/aroD* (rendering it incapable to synthesize amino acids

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for bacterial growth), *htrA* (affects survival in macrophages) and engineered to constitutively express the *S. Typhi* Vi antigen. CVD 909 has been found to be well tolerated in Phase I and II clinical trials and immunogenic following ingestion of a single oral dose (10^8 or 10^9 CFU) stimulating anti-Vi ASC responses in 80% of volunteers, although, serum IgG antibody to Vi was induced at a response rate of only 4%.[200] A more recent study has demonstrated CVD 909 oral immunization induces IFNγ secreting $CD4^+$, $CD8^+$ and effector memory T cells which are primarily α4β7 integrin positive. Such cells are able to preferentially home to the intestinal mucosa; the relevant effector site. The Ty800 vaccine was developed using genetic techniques to delete specific genes essential to the virulence of *S. Typhi* (*phoP*/*phoQ*). Data from dose-ranging studies have demonstrated that a single dose of 10^9 CFU of Ty800 to be safe and highly immunogenic,[201] eliciting a 4-fold increase in anti-LPS IgG titres at a response rate of 80% (NCT00498654). The third live oral vaccine candidate, M01ZH09 (Typhella™), is attenuated with two target deletions, *aroC* and *ssaV*. The *ssaV* gene is a component of the type III secretion system encoded by Salmonella Pathogenicity island-2 and its deletion may prevent survival of the vaccine strains in macrophages and systemic spread in humans.[202] M01ZH09 is a single dose, drinkable typhoid vaccine candidate which has been studied in six clinical trials, which included Vietnamese children (ages 4-5 years) and found to be higly immunogenic and well tolerated with an acceptable safety profile.[202] At doses up to 1.7 x 10^{10} CFU, the vaccine stimulated both a systemic and mucosal response: a reported 62–86% of subjects seroconverted to *S. Typhi* -specific LPS IgG and 83–97% to IgA; 92% had a positive *S. Typhi* -LPS IgA ELISPOT. To date, these vaccine candidates have not been evaluated in typhoid-endemic areas. The liquid presentation of the typhoid vaccine candidates would be most advantageous for use by young children.

Another attractive strategy for the delivery of mucosal vaccines by the oral route is to use live attenuated or recombinant strains of various bacteria or viruses as vectors for the expression and delivery of heterologous antigens from other pathogens. Live microbial vectors that have been extensively explored include: Salmonellae, *E. coli*, Lactobacilli, Shigellae, Listeria, Mycobacteria, Streptococci, poxviruses, adenoviruses, polio viruses, herpes viruses and influenza viruses. The advantages of viral vector vaccines is their ability to deliver genes encoding vaccine antigens much more efficiently to the mucosal site of interest and potentiate strong immune responses. Such an approach has been utilized by MicroScience, UK, using their spi-VEC™ oral live attenuated typhoid vaccine as a vector for the delivery of ETEC antigens.[203] The oral vaccine, ETEC Vaccine 1, is based on a Ty2 derivative, TSB7, harboring deletion mutations of *ssaV* (SPI-2) and *aroC* together with a chromosomally integrated copy of the B subunit of ETEC LTB. In a pilot study, two oral doses of 10^8 or 10^9 CFU have been reported to be highly immunogenic, inducing both serum IgG and mucosal IgA responses to LTB and *S. Typhi* LPS in over 67% and 95% of volunteers, respectively.[203] ETEC Vaccine 1 was well tolerated, relatively unreactogenic and induced only transient low level shedding in volunteers. There are significant benefits of the ETEC Vaccine 1 as demonstrated in the pilot study. Firstly, the ETEC Vaccine 1 did not appear to stimulate anti-carrier immunity and thereby preventing boosting of LTB responses. Second, the LTB antigen was sufficiently expressed to mount an immune response, in contrast to the oral administration of another live attenuated *S. Typhi* vectored-vaccine expressing outer membrane epitopes of *P. aeruginosa*, OprF and OprI.[204] The ETEC Vaccine 1 successfully induced mucosal IgA responses detected by ELISPOT which correlated with serum IgG antibody. Finally, an attractive feature of ETEC Vaccine 1 is that it might elicit protection against both ETEC diarrhoea and typhoid fever. The spi-VEC™ system is a novel oral vaccine delivery platform. It is reported by the manufacturer to preferentially target APCs in the gut and stimulate both mucosal and systemic immunity. Spi-VEC™ has broad applicability in that it is designed to package and deliver a wide range of antigens to prevent or treat bacterial and viral infections. It has the added advantage of being formulated as a stable, freeze-dried powder that is reconstituted as a liquid that can be self administered.

Immune responses generated by viral vector vaccines have been shown to increase when a prime-boost regimen is used, as in the case of priming the immune system with an oral administration of the live attenuated *S. Typhi* vector expressing *P. aeruginosa*, OprF and OprI, followed by systemic boosting with an intramuscular injection of OprF-OprI antigens (100 μg).[204] Oral primary vaccination failed to induce an immune response, however, high levels OprF-OprI specific IgA and IgG bronchial antibody was detected in some volunteers following systemic boosting.

Particle-mediated delivery systems. Particle-mediated delivery systems/adjuvants have been developed in order to improve the efficiency of mucosal vaccines. Therefore, much attention has been focused on finding particulate antigen-delivery systems that can be readily taken up at mucosal surfaces to induce better immune responses. The formulation of antigens in various particulate systems offer the advantages of protecting the antigen from degradative enzymes, facilitating their preferential uptake by M cells and promoting the interaction with the APCs including macrophages, DCs and B cells, while facilitating generation of memory B and T cells. The particle shape and size can be manipulated to maximize interactions at the cellular level. Nanoparticles facilitate antigen uptake in the gastrointestinal tract by intestinal epithelial cells in the mucosa, M-cells and cells of the PPs following oral presentation.[205] Vaccine antigens may be incorporated into biodegradable particles that can protect them from enzymatic degradation (e.g., polymeric microparticles, microemulsions; immunostimulating complexes (iscoms), liposomes, proteosomes and choleates) and/or coadministered with particles containing antigens with various immunostimulatory or mucoadhesive properties (e.g., Chitosan, Aliginate lectins, coatings of particles with positive charged peptides or other ligands) to enhance their uptake and immunogenicity at the mucosal site. One of the most popular particulate delivery systems that has been used in clinical studies for the formulation of vaccine antigens has been the biodegradable and biocompatible polymer, poly(lactide-co-glycolide) (PLG). In these formulations, antigen can be entrapped or adsorbed to the surface of the PLG nanoparticle. Furthermore, by adjusting the rate of degradation of the

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particles, they can act as a depot from which the antigen can be gradually released. However, despite considerable effort predominantly in pre-clinical studies, oral immunization with encapsulated antigens is still limited by several issues. In human studies, oral immunization against ETEC using PLG-microencapsulate purified CS6 (meCS6) was found to be safe and well tolerated, inducing immune responses of IgA ASCs and serum IgA and IgG specific antibodies, albeit, at levels that were not that different to that of the non-encapsulated antigen.[206] This study served as the basis for a follow-on Phase I trial in which the mucosal adjuvant LTR192G was added to meCS6 to enhance anti-CS6 immune responses.[106] Oral administration of the vaccine with or without adjuvant by either regimen was safe but once again was not adequately immunogenic and consequently further clinical development of the ETEC vaccine candidate has been suspended. While the particulate system does have potential, there are issues with the scaling up of the results of oral immunization from animals to humans and the doses of antigens delivered remain relatively low.[207] Difficulties in ensuring appropriate and uniform particle size for antigen uptake and the possibility of antigen denaturation on exposure to organic solvents during the process of microencapsulation remain problematic.

Nasal Delivery Systems

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The nasal mucosa has proved to be very attractive for mucosal vaccination compared with the oral route because of the lack of high acidity and plethora of secreted degradative enzymes. In addition, the relatively small surface area of the nasal mucosa requires lower doses of antigen and adjuvant to induce immune responses. Nasal administration of vaccines has the potential to preferentially induce better immune responses at systemic and genital/urinary compartments, although a combination of intranasal and parenteral immunization may be preferable for optimum response.[208,209] However, the development of nonliving nasal vaccines has proved challenging. Notwithstanding, safety concerns in terms of the proximity of the olfactory nerves to the site of nasal application and the use of enterotoxin-based adjuvants[210] has focussed attention on the development of alternate adjuvant and delivery systems that may circumvent toxicity issues.

Live attenuated nasal vaccines. Live attenuated, cold adapted influenza vaccines (LAIVs) were developed in the 1960s but were not licensed in the US until 2003. The seasonal influenza vaccine Flumist®, (MedImmune; US) is an intranasal LAIV. A parenteral trivalent inactivated influenza vaccine against seasonal influenza is also available. The inactivated vaccine is licensed for individuals 6 months of age or older. Both vaccines contain two type A (H1N1 and H3N2) subtypes which circulate widely in the population and one type B attenuated influenza strain. The influenza strains in FluMist® can replicate efficiently at 25°C but the temperature sensitive nature of the cold adapted influenza strain (i.e., it is restricted in replication at 39°C) restricts virus replication to the mucosa of the nasopharynx after intranasal inoculation, thereby largely eliminating reactogenicity. Attenuation of the original cold-adapted influenza virus was achieved by serial passage at 25°C in tissue culture cells. The vaccine must be stored at 2–8°C. Flumist® is approved for use in individuals 2–49 years of age. LAIVs can boost virus specific CTLs, as well as induce both SIgA and systemic antibodies primarily against the major surface glycoproteins haemagglutin (HA) and neuraminidase (NA) and provide broad protection against heterologous influenza A viruses.[211] The immune response peaks within 4 weeks after one dose in individuals who have been previously vaccinated or exposed to circulating types of seasonal flu. To induce protection in children younger than 9 years of age, two doses of influenza vaccine are recommended. Influenza vaccine effectiveness is largely related to the age and immune competence of the vaccinee and how well matched the antigens of the vaccine strains are to circulating strains. Flumist® was demonstrated to be highly efficacious in a two year Phase III trial in a population aged 5 to 49 years, with overall protective efficacy of 91% for matched influenza strains. The vaccine also showed 87% protective efficacy against circulating antigenically drifted influenza virus.[212] FluMist® has been most extensively studied in children and has been shown to be more effective than the injectable inactivated trivalent vaccine. In a pivotal study that included more than 4,000 children aged 6 to 59 months, there was 55% reduction in cases of influenza in children who received FluMist® compared with those who received the traditional parenteral vaccine.[73] The LAIV may offer significant advantages for children with respect to a more acceptable route of administration and superior protection against influenza disease compared to that provided by the parenteral inactivated trivalent vaccine. However, concerns have been raised with the administration of LAIVs to children with a history of recurrent wheezing or reactive airways disease. As such, the vaccine is not recommended for children younger than 2 years old.

The segmented genome of influenza viruses facilitates production of the reassortant vaccine strains for inclusion in vaccines requiring different influenza formulations. For the reassortant influenza virus, six internal gene segments responsible for the desired phenotypes are derived from the attenuated master donor virus, together with the two segments that encode the two surface glycoproteins, HA and NA, derived from corresponding antigenic relevant circulating viruses that are recommended by WHO for inclusion in the vaccine. A potential concern for manufacturing of any live vaccine is genetic instability during production, although, no mutations occurred in nine LAIVs evaluated during the manufacturing process.[213] Conventionally, the production of LAIV takes place in eggs which is a time consuming and labor intensive process, involving millions of eggs and long lead times for vaccine production are required. As a consequence, there is an increased risk of mismatch between the vaccine that has been chosen for production and the circulating virus strains. FluMist® was recently manufactured, in time for the 2008–2009 influenza season, using a proprietary "reverse genetics" process; a method by which RNA genes from circulating viruses are reverse-transcribed into DNA. Plasmid DNAs encoding the influenza virus are then used to transfect cells as a means to generate vaccine viruses,[214] abrogating the need for using live virus to produce the vaccine. The obvious advantage of using this technology is to improve the efficiency of vaccine

production, therefore, shortening lead times required and, hopefully, averting or limiting the chance of a vaccine mismatch occurring. According to the Centre for Disease Control and Prevention (CDC), US, vaccine mismatch has occurred to varying degrees in 7 of the last 13 influenza seasons; unfortunately for influenza vaccine manufacturers, the most recent being the 2008–2009 season.[215] Consequently, prior to the flu season in 2009, MedImmune LLC received approval by the Federal Drug Authority for use of an intranasal Influenza A (H1N1) 2009 Monovalent Vaccine containing the strain A/California/7/2009 (H1N1) in the prevention of influenza caused by the 2009 pandemic influenza A (H1N1). MedImmune is commencing a Phase I/II trial this year (NCT01055184) with the monovalent intranasal vaccine to ascertain vaccine efficacy and immune response in persons older than 60 years of age. The results from this study may be helpful for extending age group licensure of the intranasal vaccine. Parenteral inactivated influenza vaccines approvals have no upper age limit. As with any live attenuated vaccine, an important safety concern of FluMist® is that it can replicate in the upper respiratory tract, resulting in viral shedding for up to 21 days. A novel type of influenza vaccine is being developed by AVIR Greens Biotechnology, Austria, with a replication-deficient influenza virus strain lacking the viral interferon antagonist, NS1 (ΔNS1-H1N1).[216] In a proof of concept study, intranasal vaccination was well tolerated with mild adverse events. A single dose of ΔNS1-H1N1 induced significant levels of strain-specific mucosal and systemic antibodies in a dose dependent manner. Importantly, the vaccine also induced neutralizing antibodies against drift variants.

Human parainfluenza viruses types 1, 2 and 3 (PIV-1, PIV-2, PIV-3, respectively) are second only to RSV in causing significant morbidity and mortality in young children due to acute lower respiratory tract infection, yet there is no licensed vaccine against either pathogen. Over the past 40 years, a number of RSV and parainfluenza virus vaccine candidates have been evaluated yet their development has been complicated. This has been the case with respect to an RSV vaccine, as host immune responses appear to play a significant role in the pathogenesis of the disease. Early attempts at vaccinating children in the 1960s with a formalin-inactivated RSV vaccine experienced enhanced RSV disease (bronchoconstriction and pneumonia) and pulmonary eosinophilia upon subsequent RSV infection,[217,218] resulting in numerous hospitalizations and two deaths. The application of reverse genetic systems to RSV and parainfluenza virus has provided a number of genetically designed vaccine candidates that are attenuated with respect to virus replication via mutations or deletions without compromising immunogenicity.[219] Achieving an appropriate balance between attenuation and immunogenicity has, however, hampered the advancement of these vaccine candidates. Intranasal administration is the preferred administration route because it provides mucosal IgA and serum IgG virus-neutralizing antibodies conferring protection against infection,[219] without the risk of potentiating RSV disease.[220] Neutralizing antibodies are directed at surface glycoproteins haemagglutin-neuraminidase (HN) or fusion (F) protein on PIV and F protein on RSV. There have been extensive efforts directed at developing an intranasal vaccine for PIV-3 and RSV which are the responsible pathogens for causing pneumonia and bronchiolitis in the first 6–12 months of an infant's life. MEDI-534 (MedImmune) is a live attenuated intranasal vaccine containing a chimeric bovine/human (b/h) PIV-3 construct that expresses the hPIV F, the hPIV-3 HN and the RSV F proteins from bPIV-3 which was shown to be safe and well tolerated in dose escalation Phase I clinical studies in the US in adults[221] and seropositive children aged 1 to 9 years.[222] MEDI-534 has restricted replication and therefore, was minimally immunogenic at a single administered dose of 10^6 $TCID_{50}$; no viral shedding was observed.[222] Phase I/IIa clinical studies are planned in seronegative infants, who are the target population for this vaccine, to assess a safe and immunogenic dosage level. A temperature sensitive (*ts*) live attenuated cold passaged (*cp*) intranasally administered human PIV-3 vaccine, designated (HPIV3)*cp*45 (Wyeth), is currently undergoing clinical evaluation. The (HPIV3)*cp*45 mutant was selected after 45 serial passages of a wildtype human PIV-3 strain in cell culture at 20 and 22°C. The HPIV3cp45 candidate vaccine virus was administered at a dose of 10^4 to 10^5 $TCID_{50}$ in PhaseI/II trials in PIV-3 seronegative and seropositive children and in seronegative infants <6 months of age. It was found to be highly infectious, satisfactorily attenuated, phenotypically stable, eliciting an IgA response to PIV-3 HN[223-225] and showed little risk of transmission to unvaccinated children and toddlers.[226] HPIV3cp45 is a promising candidate for PIV-3 infection and is anticipated to progress to efficacy trials. A two year Phase I study is currently in progress to evaluate intranasal immunization against human PIV-1 (NCT00641017), the leading cause of viral croup in children 2 to 6 years old and viral respiratory infections in the elderly and those who are immunosuppressed. The human recombinant live attenuated PIV-1 vaccine, rHPIV1 84/del170/942A has also been developed using a reverse genetics system.[227] The rHPIV1 vaccine candidate has two non-temperature sensistive (non-*ts*) and attenuating (*att*) mutations primarily in the P/C gene. The vaccine will be assessed in adults, seropositive children and seronegative infants and children, 6 to 59 months of age.

Non-replicating nasal delivery. In the past it has been generally accepted that the delivery of non-replicating antigenic material to the NALT resulted in the stimulation of weak immune responses. However, there is now a body of evidence to suggest that it is time to revisit this paradigm. A number of non-replicating delivery systems are being developed and are entering early phase clinical trials. These are discussed below. In addition to these delivery systems there is also a single report showing that a non-living, non-adjuvanted influenza aqueous formulation when instilled as drops (four doses over a four week period) intranasally, induced serum HAI antibody titres at levels considered to be protective in 80% of the vaccinated subjects. Significant increases in $CD4^+$ T cell proliferation was also observed against the vaccine strain.[228]

Virosomes. Virosomes are small spherical vesicles (mean diameter 150 nm) of reconstituted empty influenza virus envelopes which are devoid of the nucleocapsid including the genetic material. As such, virosomes can act as a non-replicating adjuvant and carrier system. Virosomes contain functional viral envelope

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glycoproteins, influenza virus HA and NA. The biologically active influenza HA protein not only confers structural stability but facilitates receptor mediated uptake and intracellular processing of the antigen formulations with the distinct advantage of being able to stimulate both humoral and cellular immunity.[229] The adjuvant effect of virosomes is achieved by the association of the antigen of interest and the virosome. Antigens adsorbed to the virosome surface elicit a humoral response, generated by a predominantly MHCII pathway following antigen degradation; whereas, antigens incorporated into virosomes generate a $CD4^+$ and $CD8^+$, as well as CTL response through the MHC I pathway. Alternatively, antigens can be integrated into the lipid membrane. Mymetics Inc., US, has developed a prophylactic intranasal HIV-1 vaccine called MYM-V101 based on a virosome delivery platform. In a current Phase I study, MYM-V101 is administered intramuscularly in healthy female subjects in combination with intranasal administration to enhance the immune response in the vaginal and rectum mucosa (NCT01084343). Preliminary results suggest that the vaccine is well tolerated but no data are available in the public domain at this time.

Virus-like particles (VLPs). Another virus non-replicating carrier system is the VLP platform. By preserving the authentic conformation of the viral capsid, VLPs mimic the antigen presentation of the live virus with cellular receptors, thereby eliciting a strong humoral and cell-mediated immune response, including CTLs.[230] VLPs are safe because they do not package viral nucleic acids required for replication and infection. The VLP platform has been used for the development of a novel intranasal Norwalk VLP vaccine (Ligocyte Pharmaceuticals, Inc., US) for prevention of acute infectious gastroenteritis or "stomach flu". The Norwalk VLP vaccine also includes the adjuvant Monophosphoryl Lipid A (MPL®, GSK) and chitosan (ChiSys®) (Archimedes Development Ltd., UK), to enhance nasal delivery. MPL® is a potent immunostimulating toll-like-receptor (TLR)-4 agonist composed of detoxified LPS from *S. minnesota*. Chitosan is a mucoadhesive cationic polysaccharide of marine origin that enhances transepithelial transport of antigen to the nasal mucosa. In an intranasally administered dry powder formulation, the vaccine is currently being evaluated in a Phase I/II study designed to assess safety and immunogenicity of the vaccine including potential protection against clinical symptoms of Norwalk infection in a live virus challenge arm of the study (NCT00973284). Interim results, as reported by the manufacturer, have shown a two-dose regimen of Norwalk VLP vaccine to be immunogenic and generally well tolerated in volunteers with local nasal symptoms. Oral administration of a Norwalk VLP vaccine has been shown to induce mucosal, systemic and cellular immune responses.[231] A significant advantage of the VLP platform is the potential for VLPs to be produced in a variety of expression systems, such as mammalian, insect, bacteria and plant cells. This provides flexibility in tailoring manufacturing conditions to the specific needs of the product.

Nanoparticulate systems. The Proteosome™ adjuvant/delivery system consists of hydrophobic, proteinaceous, nanoparticles comprised of purified *N. meningitidis* OMPs. Due to the hydrophobic nature of OMPs, these nanoparticles can non-covalently associate with amphiphilic antigens to form appropriate complexes. The proteosome nasal delivery technology was used to develop a FluINsure™Proteosome™-trivalent subunit influenza vaccine, comprising of three monovalent influenza antigens which was found to be well tolerated in a Phase I/II study in healthy adults.[232] FluINsure™Proteosome™ induced significant mucosal and systemic immune responses against all three-vaccine virus types in a dose responsive manner. These findings were consistent with previous observations using a prototype monovalent, influenza A/H1N1 Proteosome intranasal vaccine in a Phase I study.[233] Additionally, an intranasally administered Proteosome-*S. flexneri* 2a LPS vaccine was found to be immunogenic in healthy volunteers.[234] Another nanoparticulate system is the Synthetic Biometric Supramolecule Biovector (SMBV) consisting of a positively or negatively charged polysaccharide core surrounded by a lipid membrane (Biovector Therapeutics SA, France). SMBV simulates the size and structure of a virus and as such has the potential to be useful as an antigen delivery system. A Phase I clinical study evaluating intranasally administered SMBV for delivery of a trivalent influenza vaccine was found to be well tolerated, however, it was modestly immunogenic in human volunteers.[235] The vaccine candidate NASVAC, consisting of hepatitis B virus surface (HBsAg) and core antigens (HBcAg) formulated to produce nanoparticles, was only moderately immunogenic when administered intranasally to healthy volunteers in a Phase I study.[236] While both particulate vaccine formulations had been found to be highly immunogenic in preclinical animal studies, the clinical experience in humans has been decidedly different. However, a liposomal enriched-GTF antigen preparation from *S. mutans* administered intranasally to subjects previously immunized via the intranasal route, was successful in stimulating antigen specific nasal IgA and systemic IgA responses.[237] A colloidal silica gel, aerosil, was used to formulate an intranasal vaccine consisting of the recombinant OMPs, OPrF-OprI, from *P. aeruginosa* for uniform suspension and retention in the nasal cavity. The vaccine was assessed in a PhaseI/II study in patients with COPD with a three-dose regimen of intranasally administered OPrF-OprI/aerosil/sodium dodecylsulfate followed by a booster vaccination with the systemic vaccine OPrF-OprI adsorbed to alum.[209] The combination of mucocal vaccination with a systemic booster vaccination induced potent immune responses in both the upper and lower airways as measured by specific IgG and IgA antibodies in sera, saliva and sputum of 90% of patients. The induction of such responses in subjects with chronic colonization of *P. aeruginosa* in the airways is a promising outcome. Future studies are required to determine if the approach will reduce the colonization of *P. aeruginosa* in sputum and reduce the frequency of acute exacerbations of bronchitis in such a cohort.

Pulmonary Delivery Systems

Aerosolized vaccines have been delivered through the pulmonary route. This route is a common site for the natural transmission of respiratory pathogens and the lungs provide a large surface area for vaccine exposure. The aerosol route may be better suited for childhood inoculations and for planned mass immunization and

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outbreak control given its ease of administration and good acceptance in the population. However, concerns have been raised with respect to vaccines stimulating unwanted inflammatory responses in the lungs and particularly in children with reactive airways disease. Despite these concerns pulmonary delivery of vaccines have been effectively used for the immunization of humans against measles, using a live attenuated virus. Measles vaccine strains in use today include; the Schwarz, the Edmonston-Zagreb, the AIK-C and the Moraten strains. Aerosolized measles vaccines have been used in mass vaccination campaigns in Mexico since the 1990s using a compressor attached to a nebulizer with reconstituted vaccine placed in a container with crushed ice. Children were exposed to the aerosolized live-attenuated measles vaccine which was reported to be safe, immunogenic and effective, receiving wide population support. Meta-analyses of measle vaccination by the respiratory route[238] or more specifically when administered aerosolized to children aged 10 to 36 months[239] demonstrated immunogenicity comparable to subcutaneous administration of the vaccine. In infants younger than 10 months and children aged 5 to 15 years, the study results were heterogeneous; therefore, the results were unable to be pooled to give any meaningful seroconversion rates.[239] Both studies did not report any substantial serious adverse events between recipients who received the measles vaccine via the respiratory tract and those who were subcutaneously immunized.[238,239] The aerosolized measles vaccine was more immunogenic as a triple viral (MMR) vaccine in young children[240] and adults[241] compared to the subcutaneous vaccine. The addition of aerosolized mumps and rubella vaccines to the triple viral formulation did not interfere with the measles response.[240,241] Aerosolized measles vaccine induces both mucosal and systemic antibodies, as well as measles-specific CD4 T cells.[242] The WHO Measles Aerosol Project, financed through the Gates Foundation, is investigating the potential to develop and license one method (vaccine and delivery device) for respiratory delivery of currently licensed measles vaccines as an alternative to the injectable vaccine.[243] The WHO project is trialling at least three methods, including a device that uses dry-powder vaccine, for the delivery of reconstituted aerosol measles vaccine. The studies are being undertaken in India and Mexico with a plan to seek licensure in India in 2011. The strategy is to implement an aerosolized measles vaccine for routine immunization of children aged 12 to 59 months and for mass immunization of children between the ages of 9 months to 18 years. Despite much progress towards the realization of a safe method to deliver an aerosolized measles vaccine, there is a need for further long-term assessment, including clinical studies, regarding the effectiveness of the respiratory measles vaccine.

Similar to the experience with the aerosolized measles vaccine, human papillomavirus 16 (HPV16) VLPs administered in an aerosol formulation has demonstrated similar immunogenicity compared to parenteral administration of the vaccine and greater immunogenicity compared with nasal administration.[244] A mucosal immune response was induced by aerosol vaccination as demonstrated by the induction of anti-HPV16 VLP IgA ASCs in blood and SIgA in cervical secretions. Aerosol administration of the vaccine may represent an alternative to parenteral vaccination of women for the prevention of HPV associated cervical cancer.

Vaginal and Rectal Delivery Systems

Vaginal and rectal immunization has received much focus recently, in particular, as a strategy for immunization against sexually transmitted diseases such as HIV. However, due to the uniqueness of the respective mucosal immune systems, intravaginal and rectal immunization has proved to induce minimal humoral responses in the absence of potent mucosal adjuvants. Consequently, vaccination strategies using vaccinia and canarypox virus-vectored vaccines have been used in alternate or combination immunization routes, including; systemic, intranasal, oral or rectal immunization, in order to optimize immune responses in the vaginal tract or rectum. As such, a candidate vaccine based on recombinant vaccinia virus expressing the HIV-1 (LAI strain) envelope protein subunit gp160, HIV-1 gp160MN/LAI, has been evaluated with or without DC-CHol, a cationic lipid 3beta-[N-(N',N'-dimethylaminoethane) carbamoyl] cholesterol adjuvant in females who were immunized intranasally or vaginally.[245] There were mild adverse events related to the vaccine. No anti-gp160 IgA antibody was detected in sera, saliva or cervicovaginal and nasal secretions during the 12 month study period. A potential drawback of using the microbial vector system is the possibility of vector-induced immunity which can limit the effectiveness of certain vector vaccines. In this regard, the delivery of HIV-1 antigen in a canarypox virus-vectored system, mucosally and/or systemically, only induced antibodies against the vector, irrespective of the immunization routes used.[246] A therapeutic vaccine HIV-1 p17/p24:ty VLP (p24-VLP) produced by British Biotech Pharmaceuticals LTD., UK, has been assessed in a 48 week Phase II study for long-term immunogenicity effects in HIV-1 infected subjects.[247] Subjects who were primed intramuscularly with the VLP-based vaccine in a three-dose regimen, followed by two rectal boosts, showed no long-term effect on cellular immune response deterioration. Vaginal mucosal immunization with a multivalent vaccine containing inactivated bacteria from 10 human uropathogenic strains in a vaginal suppository was shown to be efficacious in reducing recurrence of *E. coli* urinary tract infections in women.[248] Weak urinary and vaginal anti-*E. coli* SIgA and IgG levels showed no correlation with vaccine treatment. Variabilities in the level of immunoglobulins during the menstrual cycle and the loss of immunologically active tissue in the cervix may all be confounding factors in assessing protective immune responses in the urogenital tract. Though multiple doses of vaccine candidates were administered to various mucosal sites, all mucosal vaccines were well tolerated. There is a logistical challenge in designing an acceptable mucosal vaccine with complicated routes of administration (nasal, vaginal and/or rectal) that require multiple doses. For vaginal and rectal route of immunization to progress, more suitable adjuvants and delivery systems will need to be developed.

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M Cell Targeting

How M cells sample the luminal milieu and present antigen to the underlying lymphoid apparatus is a fundamental gap in knowledge on inducing immune responses at mucosal sites. M cells have short irregular microvilli which permits antigen to come in close proximity to apical surface of the cell. Uptake is known to occur by both non-specific and specific receptor-mediated mechanisms. Uptake of synthetic particles through non-specific mechanisms is dependant on particle size, surface charge and hydrophobicity. Given the variability encountered in uptake between the various synthetic particle technologies and the models in which these technologies have been tested, recent research has focused on targeting M cells through specific receptor-mediated mechanisms. A number of targeting approaches have been explored, such as; lectin-mediated, PRRs and integrins.

Lectin-mediated targeting. The *Ulex eurpaeus* agglutinin (UEA-1) binds preferentially to the apical surface of murine M cells through fucose-specific binding sites.[249] Mucosal immunization (intranasal, oral and rectal) of mice with PLG (5–10 μm) into which four HIV peptides (gp41-LZ; gp41-FD; gp120-C2 and Nef) were entrapped with UEA-1, resulted in enhanced immune responses in all mucosal routes and all peptides compared with particles formulated without UEA-1. Intranasal was the superior route of immunization.[250] It has also been demonstrated that polystyrene microparticles and liposomes coated with UEA-1 are preferentially take up by PP M cells.[15,251] The co-administration of UEA-1 with killed whole cell preparations of *H. pylori* or *Campylobacter jejuni* in a mouse model resulted in both systemic and mucosal immune responses, as well as protection against live *C. jejuni* challenge.[252] The report that the expression of galectin-9 (gal-9) is upregulated on the surface of M cells[253] suggests that gal-9 may also be an important lectin binding moiety for oligosaccharides[254] on the M cell apical surface and certainly warrants further investigation.

PRR targeting. Microorganisms express unique molecular motifs known as pathogen-associated molecular patterns (PAMPs) which are recognized by the innate immune system through PRRs on the apical surface of M cells by either direct receptor-bacterial ligand or endogenous adaptor-bacterial molecule interactions.[255] In a Caco-2 in vitro M cell model, research in our laboratories has demonstrated that TLR-4 and platelet-activating factor receptor (PAFR) on the apical surface of M cells are able to mediate the uptake and transcytosis of whole cell NTHi.[102] This process can be partially blocked by specific receptor inhibition[256] and is presumably dependent on TLR-4, in association with CD14 and MD-2 and PAFR recognising their respective PAMPs, lipooligosaccharide[257] and phosphorylcholine[258] respectively expressed on NTHi.

The development of an invasion-LPS complex from Shigella known as Invaplex[259] raises the possibility of being able to target more than one uptake mechanism using a single delivery agent: TLR-4 through the LPS component and enhanced internalization of the complex, possibly by the binding of the invasion plasmid antigens to integrin receptors. Based on promising results in animal models of shigellosis following intranasal immunization,[260] a Phase I study has been undertaken. Invaplex was administered by intranasal spray over a range of concentrations. Three doses were given over a period of 35 days (NCT0082069). Cited results indicate that this complex was safe and immunogenic in humans.[259] What is of wider interest is that Invaplex has been demonstrated to be an effective mucosal adjuvant when co-administered intranasally with DNA-encoded antigens.[259] Further studies are clearly needed to assess the potential of the complex as an effective, non-toxic mucosal adjuvant.

Integrin targeting. Unlike enterocytes, β1 integrins are expressed on the apical surface of M cells whereas enterocytes express β1 integrins on the basolateral surface.[261-263] Like TLR-4 and PAFR, α5β1 intregrin mediates the uptake and transcytosis of whole cell NTHi and can be specifically inhibited.[256] It is likely that α5β1 intregrin binds to fibronectin expressed endogenously or to fibronectin binding proteins expressed on the bacterial surface.[264] There is also good evidence that enteropathogenic microbes of the Yersinia spp. invade by binding to α5β1 intregrin expressed on the apical surface of M cells.[261,265]

In summary, it has been clearly demonstrated that the targeting of M cells through apical surface binding sites mediates transcytosis irrespective of the nature of the binding site. In addition, limited animal studies suggest that immune responses can be enhanced by targeting M cells directly. Further studies are required to understand the cellular mechanisms initiated by the various receptors following binding, including the induction of immune responses and to establish that the observations made in the animal models can be translated to humans. It is possible that if M cells can be specifically targeted through apical surface receptors then both efficient delivery of antigen to the mucosal immune induction sites and effective immunity could be achieved. This would be a significant breakthrough.

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Plant-based Vaccines

The rationale for an oral vaccine derived from plants is to improve vaccine delivery and production. Plant-based vaccines refer to antigens being expressed in recombinant plant tissues. The major advantage of plant expression systems over other vaccine production systems is reduced manufacturing costs. The production of plant-derived vaccines is, theoretically, limitless with no need for expensive fermentation and reduced downstream costs associated with antigen purification systems, cold storage, cold transportation and delivery via sterile needles by trained medical personnel. Advantages include the ability to scale up production of vaccine antigens from transgenic seed stocks. Vaccine production in plants is attractive in terms of safety because the plant expression system would produce a vaccine free of human or animal pathogens. The earliest research using plants for recombinant expression of vaccine antigens was performed in the 1990s when hepatitis B antigen was introduced into the tobacco plant.[266] Since that time, other transgenic plant systems have been developed, however, few have advanced as oral vaccine candidates.[267] Early clinical studies evaluated oral transgenic plant vaccines against enteric infections. Transgenic potato tubers expressing ETEC LTB toxin and antigens from the hepatitis B

and Norwalk viruses have been shown to be immunogenic as raw, edible vaccines in separate Phase I clinical trials.[268-270] LTB and Norwalk virus capsid antigen induced both serum IgG and fecal SIgA responses in human volunteers, respectively, though the levels were very modest in each case.[268,270] Plant virus-based expression systems demonstrated greater potential as supplementary oral booster immunizations when 63% of previously vaccinated individuals showed significantly higher serum hepatitis B surface antigen titres after eating three doses of raw HBsAg-expressing potatoes.[269] Transgenic corn expressing LTB has also proved to be immunogenic, inducing LTB-specific serum IgG and LTB-specific fecal IgA antibodies in 78% and 44% of volunteers, respectively.[271] Although these studies demonstrated the feasibility of safe and immunogenic oral delivery of plant-based vaccines, they did not progress further in clinical studies. As oral delivery vehicles, potato and corn systems and most other plant organs have a limited shelf life in the fresh state, vaccine dosage may be variable and the harsh environment of the gastrointestinal tract can lead to rapid antigen degradation.[272] The use of seed tissues is more promising and as such, a rice-based vaccine system (MucoRice™) has proved to be physically and chemically stable and capable of inducing antigen-specific mucosal and systemic immune responses. The rice-based oral vaccine expresses CTB. Inherently, rice seeds are efficient at producing and storing proteins. Protein storage organelles accumulate the expressed antigen and naturally confer protection against the degradative enzymes of the gastrointestinal environment. Following oral immunization in animal studies, the rice seeds expressing CTB induced CTB-specific serum IgG, as well as intestinal IgA responses at sufficient levels to protect against oral challenges with cholera toxin.[273] MucoRice™ is stable and immunogenic at room temperature for 24 months.[273] While this rice-based vaccine has been advocated as a possible "new generation" cholera vaccine, this novel vaccine platform also pathes the way for future development of cold-chain-free, needle-free and potentially economically viable oral vaccines against other infectious diseases that will prove beneficial to developing and developed countries alike.

Concluding Remarks

Humankind's quest to avoid illness caused by infectious diseases stems back as far as recorded history. In the absence of suitable injection devices, early attempts to vaccinate against infections were overwhelmingly mucosal. However, the invention of injection devices allowed antigen to be administered beyond dermal abrasion and blood to be sampled to assess the immune responses induced. With increased knowledge of systemic immunity, and the inherent difficulties of delivering antigen to mucosal induction sites and monitoring the responses induced, most efforts during the 20th century focused on the development of parenteral vaccines. There is no doubt that the implementation of mass vaccination programs has been one of history's most significant public health interventions with millions of lives being saved and the burden of co-morbidities associated with infectious disease significantly reduced. Despite these outcomes, infection still remains a major cause of death and suffering. Access to currently available vaccines needs to be improved and new vaccines need to be developed to replace those which induce sub-optimal protection against infection and to address many infectious diseases for which there are no currently available effective vaccines. Despite the focus on parenteral immunization researchers with a commitment to mucosal immunology have amassed an enormous body of knowledge since the time of Alexandre Besredka who is recognized as the founder of the discipline. Mucosal immune networks have been identified and their respective interactions described, immune regulatory mechanisms that exist in the mucosa have been characterized and the basis of antigen sampling from the mucosal space and antigen presentation has been identified. In addition, there is a greater understanding of the interactions between the mucosal immune system, commensal microorganisms and potential pathogens. Alongside this knowledge, are the benefits of modern techniques in molecular biology and chemical synthesis which are now enabling the development of a plethora of potential vaccine delivery systems that can be specifically designed to produce an appropriate and protective mucosal immune response for a given infection. Over the last 10 years, there has been a rapid progression from animal models demonstrating that mucosal immunization is highly immunogenic and effective in protection against live infection challenge, to human clinical trials. It is interesting that one approach being investigated is the combination of a parenteral primary immunization followed by mucosal boosting to enhance the mucosal immune response which is often suboptimal with parenteral immunization alone. There is now substantial financial investment in the development of human mucosal vaccines by granting organizations and industry. In conducting this review, the authors were struck by the number of clinical trials of mucosal vaccines that are being undertaken and the fact that much material had to be sourced from public domain websites in addition to the peer reviewed literature. Given the rapid advances being made, there is little doubt that the 21st century will be the era for mucosal immunization. New and effective mucosal vaccines will soon be an important intervention strategy in the battle against infectious diseases—a realistic alternative.

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