Vaccination against enteric pathogens: from science to vaccine trials

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Recent advances in scientific research and clinical trials have shown promise for vaccine development against enteric pathogens. Identification of new virulence factors, such as the two distinct S*higella* enterotoxins, has allowed the development of new immunogen or new attenuated strains. Improved knowledge facilitated the development of safer attenuated live microorganism and construction of multivalent vaccines. Finally, an important advantance is the use of nonreplicating plasmid DNA vectors to express protective antigens in the host.

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Abbreviations

CF	colonization factor
CFU	colony forming units
CT	cholera toxin
ETEC	enterotoxigenic *E. coli*
LPS	lipopolysaccharide
LT	*E. coli* heat-labile enterotoxin
ShET1	*Shigella* enterotoxin 1
ShET2	*Shigella* enterotoxin 2
ST	*E. coli* heat-stable enterotoxin

Introduction

Vaccines undoubtedly are among the most important tools available to health officials attempting to prevent the appearance and transmission of infectious agents. Since the eradication of smallpox in the 1970s the World Health Organization has targeted polio as the next pathogen to be eradicated, a goal that probably can be achieved in the first years of the next century.

Against a background of other vaccine successes in the control of diphtheria, tetanus, whooping cough, measles, mumps, rubella, polio, hepatitis A and B, and most recently *Haemophilus influenzae* type b stands a poor track record in the control of enteric pathogens. Undoubtedly both poliovirus and hepatitis A are pathogens transmitted via the fecal-oral route, but their control is due to the success of humoral antibodies blocking their spread to the central nervous system and liver, respectively, where the pathophysiological damage takes place. So far no comparable protective efficacy has been seen with antibodies when the target for the pathogen is the intestinal wall. The disease burden caused by enteric pathogens is formidable: each year more than three million children are reported to succumb in the acute phase to infections caused by enterotoxigenic *Escherichia coli*, rotavirus, *Shigella* spp. and *Vibrio cholerae*. Many more may succumb to chronic persistent diarrheas after the acute phase has passed.

Enteric pathogens make contact with the intestinal mucosal lining and exercise their pathogenic effect by one (or more) of several mechanisms. Some stay the gut lumen side after adhering to the cell surface with colonization factors and secreting toxins like the heat-labile (LT) and heat-stable (ST) enterotoxins of *E. coli* and the heat-labile enterotoxin (CT) of *V. cholerae*. Others invade the epithelial cells where they may exercise their tissue damaging effect through induction of apoptosis (e.g. *Shigella* spp.), elaboration of toxins that arrest protein synthesis (e.g. Shiga toxin), disruption of calcium homeostasis (e.g. rotavirus), or elaboration of the cell envelope lipopolysaccharide (LPS), which upregulates proinflammatory cytokines (e.g. *Salmonella* spp., *Shigella* spp., enteroinvasive and enteropathogenic *E. coli*, *Campylobacter* spp. and others). Some even escape, or merely transcytose, the epithelial cell lining and disseminate via the lymphatics and the blood throughout the entire host (e.g. *S. typhi* and some other *Salmonella* spp.).

In making contact with the host the enteric pathogens encounter the mucosal immune system, which is an integrated network of tissues, lymphoid and constitutive cells, and effector molecules. The network is finely regulated and upon encounter with the enteric pathogen the host can react with immune responses ranging from mucosal and serum antibody production to T cell mediated immune reactions and even tolerance. To maximize the immune response, vaccines against enteric pathogens should logically be developed to be delivered via the oral route and preferably as replicating organisms. With this knowledge why have the scientific community and the vaccine manufacturers been so unsuccessful in developing efficacious vaccines against enteric pathogens? A simple answer is that until recently we lacked the knowledge and technologies of how to develop attenuated live microorganisms that were safe at a dose where protective immune responses were elicited. A book containing an exhaustive review of mucosal vaccines against enteric pathogens was recently published [1•].

Can we not use parenteral immunization to induce protective immunity against enteric pathogens? Here we must recognize that in the peripheral immune system the lymphoid cells are not in a network as in the intestine but are confined to individual lymph nodes and the spleen. Communication is dependent on cell transport through the lymphatics and blood. The induction of

peripheral immune responses often does not result in significant, or any, mucosal immune responses, unless the individual has been mucosally primed. High serum IgG concentrations can, however, result in transudation over mucosal membranes with a concomitant inactivation of the pathogen on the luminal surface or in the lumen.

Table 1

Series of steps involved in vaccine development.

Stages	Choices
Immunogen	Killed microorganism Subunit(s) of microorganism Live microorganisms Vector expressing subunit(s) DNA
Formulation	Adjuvants Liposomes Particles
Administration	Systemic or local
Immune responses	Antibodies, systemic and/or local Cell-mediated immune responses Cytokines Chemokines Memory of antigen

The goal for licensing a vaccine against enteric pathogens can be set at one of three levels: the minimal goal is to reduce the severity of disease and thus prevent hospitalization; a higher goal is to prevent disease (i.e. all clinical symptoms); and the ideal is in addition to prevent transmission of the pathogen. It is only when the last criterion is met that we have a fully efficacious vaccine.

Vaccine development can be seen as a series of discrete steps involving the choice of immunogen, its formulation, its administration, and finally the immune response(s) elicited by it and their modulation and regulation (Table 1). This review will discuss the recent significant advances in the priority areas of enteric pathogen vaccine research (Table 2).

Microbial virulence factors

Lipopolysaccharide

Lipopolysaccharide (LPS), a major constituent of the cell envelope of bacterial enteric pathogens, is a potent endotoxin and a stimulator of the production of proinflammatory cytokines. The endotoxic properties reside in the lipid A part, which is embedded in the cell envelope. The polysaccharide, with its O-antigenic properties, extends from the cell surface and is recognized by antibodies. Thus LPS plays a major role as a virulence factor for bacterial enteric pathogens and is an important target for host immune defenses. But how is LPS handled by the epithelial cells?

The apical surface of epithelial cells is continuously exposed to numerous microbial products, particularly in the colon, among them LPS. The basolateral surface interfaces with the mucosal immune system with its resident and migrating immune cells. LPS at the apical surface upregulates proinflammatory cytokines, which signal for an influx of immune cells from the basolateral surface. Studies of cultured epithelial cells show that *S. flexneri* LPS released at the apical surface was internalized (perhaps by an endocytotic process) and rapidly distributed within endosome compartments accessible to basolaterally internalized transferrin (WL Beatty, S Méresse, P Gounon, PJ Sansonetti, JP Gorvel, personal communication). Some of the LPS appeared to be recycled to the apical membrane. LPS transcytosis is interesting in light of the role of IgA antibodies directed against the O-antigen in the protection against *S. flexneri* [2]. IgA binds to immunoglobulin receptors at the basolateral surface and is transcytosed to the apical surface, where it is secreted into the gut lumen. It is plausible that IgA plays a significant role in the intracellular neutralization of *Shigella* LPS, and most likely also of LPS from other intracellular enteric pathogens. It is noteworthy

Table 2

Significant progress in enteric pathogen vaccine research (1995–1997).

Enteric pathogen	Scientific research	Clinical trials
Rotavirus	Identification of NSP4 glycoprotein as a potential enterotoxin [11••] Anti-VP6 IgA protects intraepithelially but not in gut lumen [10•] DNA (encoding VP4, VP6, VP7) protects in animal model [14•]	RRV-TV to be licensed in 1998 (?) Vaccine efficacy against severe diarrhea [8•,9]
Shigella spp.	LPS trafficking in epithelial cells Anti-LPS IgA protects and neutralizes intraepithelially [13] Identification of enterotoxins ShET1 and ShET2 [18,19]	*S. sonnei* O-antigen glycoconjugates elicit protection [23•]
S. typhi	Trivalent *S. typhi* strain protects against typhoid, tetanus and schistosomula in mouse model [35•]	Construction of live strain which appears not to cause clinically 'silent' bacteremia [25]
V. cholerae		Construction of live bivalent (classical and E1 Tor) protects in challenge model [30]

NSP, nonstructural protein; RRV, rhesus rotavirus.

that LPS is not detoxified by the epithelial cells (WL Beatty, S Méresse, P Gounon, PJ Sansonetti, JP Gorvel, personal communication), which makes an antibody-based detoxification even more important for the host. Also, the recycling of LPS to epithelial and macrophage cell surfaces make these cells susceptible to antibody-dependent cell cytotoxic effector mechanisms.

Because of the intrinsic noxious properties endowed to LPS by its lipid A part, LPS cannot be used as a subunit vaccine. Moreover, LPS predominantly elicits an IgM antibody response with no memory function. Several attempts have been made to convert the LPS from a T-cell independent immunogen to a T-cell dependent immunogen by covalently linking the O-antigenic polysaccharide to an immunogenic carrier protein. This has proven promising for conferring immunity to *Shigella* [3] and would be technically simple for LPS from other enteric pathogens. Another recently elaborated method is to link a synthetic antiendotoxin cyclic decapeptide into the lipid A with tailored bifunctional spacers [4]. The completely detoxified complex elicited bactericidal IgG antibodies. Such a conjugate may still act as a Th1 (T helper cells of type 1) type adjuvant, but no longer shows the toxic effect of LPS. A third approach recently being explored is to use mimeotopes. With the aid of phage displayed peptide libraries and selection using anti-O-antigen neutralizing monoclonal antibodies, peptides can be identified which, as haptens, elicit neutralizing antibodies. Such a protein subunit immunogen, selected by a neutralizing IgA antibody against *S. flexneri*, was shown to elicit protective immunity against *Shigella* challenge in an animal model (A Phalipon, personal communication). The validity of the approach is currently being studied, and if successful could result in the development of protein subunit vaccines against LPS from enteric and other pathogens.

Toxins

Toxins secreted from microorganisms are important virulence factors. When detoxified (mostly using formalin), they can be excellent immunogens as demonstrated for diphtheria, tetanus and pertussis toxins. The importance of toxins for enteropathogenic bacteria is evident in their pathogenesis, but has been poorly assessed with the exception of the LT and CT toxins of enterotoxigenic *E. coli* and *V. cholerae*. The isolated subunit of CT, CT-B, has proven to be an important component for protection soon after vaccination with the inactivated oral whole cell cholera vaccine [5]. In the development of live attenuated strains attempts have been directed at deleting toxin genes, but scant attention has been given to the potential benefit of antitoxin immunity.

Genetic inactivation of toxins is a new approach to vaccine development. Using site-directed mutagenesis, genes coding for toxins are modified so that amino acids that are involved in the enzymatic, and therefore toxic, activity are replaced by other amino acids that make the subunit nonfunctional. In this way the toxin can be maintained in its native state, or bound to its receptor, and preserve its B- and T-cell recognition epitopes. Genetic detoxification of the *E. coli* LT, which is composed of five identical B subunits in an oligomer and one enzymatically active A subunit, yielded mutant holoproteins that were shown to be both efficient mucosal immunogens, inducing toxin neutralizing antibodies, and mucosal adjuvants [6]. The LT mutant, and genetically detoxified mutants of *V. cholerae* and *Shigella* toxins, most likely owe their immunogenicity and adjuvanticity to the fact that they bind to cellular receptors on immune cells. Genetically detoxified proteins are currently being explored in clinical trials as adjuvants.

Vaccine candidates

Rotavirus

A live oral vaccine against rotavirus infection is expected to be licensed in early 1998. The vaccine is a heterotypic Jennerian construct based on a rhesus rotavirus (RRV) with the VP7 glycoprotein subunit of human rotavirus [7]. There are four reassortants, each with one of the four common human VP7 subunits (antibodies against which are neutralizing). The RRV-TV vaccine has been given to more than 17,000 children in nine different developed and developing countries and found to protect 48–80% of them against all rotavirus disease and 70–95% against severe disease when given as three doses of 10^5 plaque-forming units (pfus)/dose and each dose separated by a minimum of three weeks [7,8•,9]. The protective efficacy is similar to that seen after natural infection. This 'first generation' rotavirus vaccine will protect against death and severe disease (i.e. hospitalization) but its ability to interfere with rotavirus transmission is probably limited.

The lack of animal models to assess the protective efficacy of rotavirus vaccine candidates has forced the vaccine community to conduct human trials. Today we still lack an understanding of how rotaviruses cause disease and how protective immunity is elicited and maintained. Consequently, the vaccine development approach has been pragmatic, rather than based on identification of target antigens through an understanding of pathophysiological and host reactive mechanisms.

In addition to the VP7 glycoprotein, the outer capsid of the rotavirus contains the VP4 hemagglutinin spike [10•]. Both are key targets for virus neutralization [7]. Greenberg's laboratory [10•] unexpectedly found that in an experimental BALB/c mouse model, where the mice were transplanted with a 'backpack tumor' secreting antibodies, two non-neutralizing IgA antibodies directed against the VP6 inner capsid protein prevented primary, and resolved chronic, murine rotavirus infections. Since these IgA antibodies were nonprotective when given orally, the reasonable conclusion is that the inactivation of rotavirus occurred when the anti-VP6 IgA antibody was transcytosed from the basolateral to the apical surface

rather than when secreted into the gut lumen (see also *Shigella* below). Thus, VP6 has been added as a potential target for vaccine-induced immunity.

Until recently no single gene product of the 11 rotavirus proteins had been associated with virulence. The fortuitous discovery by Estes' laboratory [11••] that the nonstructural glycoprotein NSP4 induced diarrhea in young CDI mice was the first indication that it was a viral enterotoxin. The NSP4 glycoprotein, which plays a role in rotavirus assembly, was found to cause a secretory diarrhea by potentiating chloride secretion via a calcium-dependent signaling pathway. An NSP4 peptide (residues 114–135) likewise induced diarrhea, and is also known to mobilize intracellular calcium in eukaryotic cells. When the tyrosine at position 131 was replaced by a lysine no diarrhea was induced. The protective effect of antibodies to NSP4 was demonstrated by challenge with SA11 rotavirus of pups either born to dams immunized with the NSP4 114–135 peptide, or pups given NSP4 antiserum every four to six hours for 60 hours [11••].

The identification of NSP4 glycoprotein as a potential virulence factor raised the possibility that mutation of the NSP4 gene may result in an attenuated live vaccine strain. Studies are ongoing which may validate or dispute the role of NSP4. It is interesting that in a recent study of the parent and attenuated human rotavirus strain 89–12, in which the attenuated strain was given to 20 previously uninfected infants aged 6–22 weeks, who shed the virus, the NSP4 glycoprotein was unaltered except for a single base pair change [12]. This base pair change caused the substitution of alanine for threonine at position 45, but both amino acids have been found at position 45 in human rotaviruses causing both symptomatic and asymptomatic infections. Thus, both the parent and attenuated rotavirus strains had an apparently functional NSP4 protein and thus the single base pair change in NSP4 was not responsible for the attenuation.

A rotavirus subunit vaccine based on virus-like particles (VLPs) was produced by coinfection of SF9 insect cells with VP2/6/7 or VP2/4/6/7 combinations from bovine (VP2 protein) and monkey SA11 (VP4, VP6 and VP7 proteins) rotaviruses. This rotavirus subunit vaccine was recently tested in a rabbit model and found to elicit, with QS21 as an adjuvant, an immune response comparable to that of live SA11 virus [13].

Virtually all vaccines reported above have been constructed with the aim of eliciting virus neutralizing antibodies. The only exception is the protective effect of the non-neutralizing anti-VP6 IgA antibody as assessed in the BALB/c mouse model [10•]. There are earlier reports, however, that cellular immune responses to VP6 may also be protective (see [14•]). The RRV-TV vaccine most likely generates cell-mediated as well as humoral immune responses.

Direct inoculation of plasmid DNA encoding specific proteins is a new approach to making subunit vaccines against rotavirus infection [14•]. The cDNAs encoding for murine rotavirus proteins VP4, VP6 or VP7 were inserted into the pCMV intron A expression vector (plasmid JW4303) and the plasmids injected into eight week old BALB/c mice by gene gun. Each of the three DNA vaccines elicited protective immunity as estimated by reduction in viral shedding. It is noteworthy that an active anti-VP6 immunity was observed using the DNA vector, whereas VP6 encoded in an adenovirus or vaccinia virus vector failed to elicit protective immunity. Again, no anti-VP6 neutralizing antibodies were elicited, but cellular immunity or IgA-mediated inactivation [10•] may be the effector mechanism(s). Against VP4 and VP7 the DNA plasmids elicited virus-neutralizing antibodies as well as virus-specific cytotoxic T lymphocyte responses. The DNA approach holds promise for the future and the recent observation that short DNA sequences are immunostimulatory [15•,16] may allow us to tailor the immune response.

Presently we can expect the human VP7 RRV-TV vaccine to benefit infants from 1998 onwards. Then follows an array of promising candidates whose supposed virtues today rely on only immunogenicity studies and protective data in nonpredictive animal models. Only testing in humans, from toddlers to infants, will tell us if any of the vaccine candidates will realize their scientific potential.

Shigella

Among the *Shigella* species the dominating serotypes are *Shigella dysenteriae* type 1, *Shigella flexneri* types 2a, 1a, 3a and 6 (local differences occur) and *Shigella sonnei*. An efficacious *Shigella* vaccine most likely has to contain *S. dysenteriae* 1, *S. sonnei* and two or more serotypes of *S. flexneri*.

The need for a *Shigella* vaccine is obvious. A large number of candidate vaccines has been developed over the past 50 years, but to date there is no licensed vaccine on the market. One major reason for this is the absence of correlates of protection which makes the assessment of candidate *Shigella* vaccines difficult. Early attempts to immunize with parenteral vaccines consisting of live or inactivated organisms failed to protect against homologous challenge, despite high titers against the serotype-determining LPS. Therefore the focus has shifted to the construction of attenuated vaccines to be given orally [17].

Live attenuated vaccines

Originally, attenuated strains were developed without any knowledge of how *Shigella* spp. invaded the gut mucosal cells, how it multiplied intracellularly and how it spread to adjacent cells. Consequently the vaccine candidates were less defined and set-backs occurred in human trials. Recent discoveries of specific genes associated with

defined virulence properties make it possible to attenuate microorganisms via modulation of selected pathogenic processes.

Attenuation of *S. flexneri* via deletions in the aromatic metabolic pathway yields mutant strains with a severely limited ability to grow intracellularly. Mutant strains, attenuated about a millionfold, when given to volunteers illustrated a delicate balance between reactogenicity and immunogenicity (summarized in [17]). A dose of 10^6 to 10^7 CFU/dose of *S. flexneri* serotype 2a strain SFL1070 (Δ*aro*A) was safe, inducing transient mild gastrointestinal symptoms, but elicited relatively low immune responses. At 10^8 CFU/dose, symptoms were still mild and the volunteers developed significant increases in anti-LPS and anti-invasive protein antigen serum and fecal antibody, and increases in numbers of antibody-secreting cells. A dose of 10^9 CFU caused more severe, although transient, symptoms in the gastrointestinal tract and also fever. The inherent virulence of the parent wild-type *Shigella* strain was shown to dictate the clinical tolerance to vaccine candidate strains with identical attenuated properties. The conclusion was that aromatic auxotrophy alone was probably insufficient for a safe and immunogenic live *Shigella* vaccine. Additional or alternative mutations/deletions of genes are required for increased attenuation with retained immunogenicity.

Recently two distinct enterotoxins were detected in *Shigella*: enterotoxin 1 (ShET1), which is a 55 kDa protein complex with an A_1B_5 subunit configuration and which is iron-regulated [18], and enterotoxin 2 (ShET2), which is a 62.8 kDa single moiety protein [19]. ShET1 is a chromosomally encoded protein found almost exclusively in *S. flexneri* serotype 2a, whereas ShET2 is encoded by the invasion plasmids of all *Shigella* spp. It is likely that the reactogenicity with watery diarrhea seen with *S. flexneri* serotype 2a strains 1070 [17] and CVD1203 (Δ*aroA*, Δ*virG*) (see [19]) was a consequence of expression of either, or both, of the enterotoxins ShET1 and ShET2. Deletion of these genes will most likely result in further attenuation of *Shigella* vaccine candidates.

Two attenuated strains with deletions of the gene for *Shigella* enterotoxin have been constructed. Strain CVD1207 is an *S. flexneri* serotype 2a strain which has, as the primary attenuating mutation, a deletion of a chromosomal operon (Δ*guaB-A*) which interrupts the biosynthesis of guanine nucleotides and constitutes the primary attenuating mutation, a deletion of a plasmid-encoded virulence gene which reduces intracellular and intercellular spread (Δ*virG/icsA*) and also deletions of *set* (coding for ShET1) and *sen* (coding for ShET2). Strain CVD1253 is an *S. dysenteriae* serotype 1 strain with Δ*guaB-A*, Δ*virG/icsA*, Δ*sen* and a deletion in the gene coding for the A subunit of the Shiga toxin (Δ*stxA*) [17]. Strain CVD1207 is in phase I trials.

Recent unpublished data from the collaboration between the Institut Pasteur and the Walter Reed Army Institute of Research concern the *S. flexneri* serotype 2a vaccine candidate strain SC602 (Δ*virG/icsA* and with a deletion in the chromosomal *iuc/iut* genes which encode the biosynthetic and transport genes in iron-binding siderophore affecting the survival of SC602 in the tissues). So far, strain SC602 appears to be the most promising live attenuated oral *Shigella* vaccine candidate. Although the study was small, and has to be validated and expanded, the data suggest that a single dose of a live attenuated strain can either protect against all clinical signs of shigellosis or limit it to a benign, self-limiting diarrheal infection. Although safe in healthy adults a key test is to see if it is safe in infants the primary target population in developing countries where shigellosis is endemic.

The continued testing of strain SC602 as well as strains CVD1207 and CVD1253 allows me to think that efficacious and safe live *Shigella* vaccines are a distinct possibility. These vaccine strains can be lyophilized, and are as such stable, and easily reconstituted for administration. In addition the continued dissection of the pathophysiological mechanisms behind *Shigella*-induced lesions in the intestine promises future improvements of the vaccine candidates [20,21•,22].

Shigella *subunit vaccines*

The use of a parenteral vaccine to elicit protective immunity against *Shigella* has recently received support [3,23•]. The O-antigenic polysaccharide chain of the LPS of *S. flexneri* serotype 2a and *S. sonnei* were covalently linked to exoprotein A of *Pseudomonas aeruginosa* as a carrier. When given intramuscularly to Israeli soldiers in a single dose, 90% of recipients of the *S. sonnei* conjugate and 73–77% of recipients of the *S. flexneri* conjugate had fourfold or greater IgG and IgA serum antibody titer increases against the homologous LPS [3]. Even two years after vaccination the titers were still significantly higher ($p < 0.01$) than before vaccination. A booster dose, however, given six weeks after the first vaccination failed to increase the antibody titers.

The efficacy of the *S. sonnei* conjugate was then assessed in a double-blind efficacy trial in Israeli soldiers [23•]. In vaccinees the protective efficacy was found to range from 43% in a group where cases of *S. sonnei* dysentery started appearing at the time of vaccination (cases observed on days 1–17) to 74% in groups where the *S. sonnei* dysentery cases were seen 70–155 days after the inoculation. The duration of protection, assumed to be dependent on IgG and/or IgA antibodies, has so far only been assessed for seven months. This protective efficacy is obviously significant but the question remains whether the conjugate merely boosted antibody levels in individuals already *S. sonnei* O-antigen primed or if the vaccine itself primed the volunteers. The fact that the conjugate apparently

intervened in the group where dysentery started the day after vaccination and gained a 43% protective efficacy suggests that many individuals already were primed.

Glycoconjugates have an important advantage over attenuated live organisms in their safety, as demonstrated for the various *H. influenzae* type b capsular polysaccharide vaccines. Are the serum antibody responses elicited in a previously unprimed individual, such as infants in a developing country (who would be the primary beneficiaries of *Shigella* vaccines) or travelers from a developed country, sufficiently high to transudate into the intestinal lumen and opsonise the *Shigella* bacteria? A glycoconjugate vaccine is more likely to succeed than a killed whole cell bacterial vaccine because IgG (and IgA) antibodies elicited by the conjugate transudate over the mucosal membranes much better than the IgM antibodies, which are preferentially elicited by the killed bacteria (i.e. by the LPS in the cell envelope).

Salmonella typhi

Attenuated vaccine

S. typhi is the most virulent of all salmonellae: it does not cause a gastrointestinal illness in man because the bacteria rapidly transcytose the mucosal layer and disseminate, causing bacteremia (i.e. enteric fever). *S. typhi* is one of the few salmonellae that carry a capsular polysaccharide, the Vi-antigen. The intestinal and systemic phases of the bacterium make it a target for mucosal as well as systemic immune defenses, as seen in the attempts to develop vaccines. It is interesting that both serum IgG antibody titers and the number of cells secreting IgA antibody against O-antigen of the envelope LPS correlate with protection [24].

The licensed live attenuated *S. typhi* strain Ty21a is given in three or four spaced doses every other day. This schedule has been reported to give a protective efficacy of 67 and 63%, respectively, over three and seven years follow-up [24]. The weak immunogenicity of Ty21a, calling for several doses, has resulted in numerous attempts over the past 20 years to develop an attenuated vaccine to be given in a single dose. A large number of mutant *S. typhi* strains have been generated and in the absence of suitable animal models tested in human volunteers [24]. (*S. typhimurium* causing murine typhoid as a surrogate model has proven unreliable). But either these *S. typhi* vaccine candidate strains have been overattenuated and therefore poorly immunogenic, or they have caused too many serious adverse events. Several candidate strains have been immunogenic, and clinically safe, but 'silent bacteremias' have been detected between days 3 and 10 after vaccination. The bacteremias have been transient and cleared without antimicrobial treatment. Nevertheless, the presence of bacteremias in healthy adult volunteers has made the candidate strains unsuitable for licensing.

Strain *S. typhi* CVD908 (Δ*aroC*, Δ*aroD*) is immunogenic but produces 'silent bacteremias'. Subsequent deletion from strains CVD908 of gene *htr*A, which encodes a heat-shock protein also serving as a serine protease, resulted in strain CVD908(Δ*htr*A) [25]. A single oral dose of the CVD908(Δ*htr*A) strain given to 22 volunteers resulted in serum IgG antibody titers (as evidence of a systemic immune response) and IgA-secreting cells (as evidence of a mucosal response) which were the same as for strain CVD908. None of the 22 volunteers, given from 5×10^7 to 5×10^9 CFU of CVD908(Δ*htr*A), however, had bacteremia ($p<0.001$). It is evident that in spite of the low number of volunteers tested so far strain CVD908(Δ*htr*A) holds promise of being developed into a live oral vaccine that could be given in a single oral dose, with potentially a better protective efficacy than that seen for the licensed Ty21a vaccine.

Attenuated Salmonella typhi *as vector*

The ability of *S. typhi* vaccines to elicit systemic as well as mucosal immune responses has made them ideal candidates for carriers of heterologous antigens to create multivalent vaccines. The attempts have met with more disappointment than success. The problems have been, firstly, to obtain stable expression of the antigen in quantities sufficient for an adequate immune response, secondly, that the heterologous antigen has compromized the vector strain, and, finally, that the expression of the heterologous antigen is not in the relevant host compartment.

Significant progress was reported based on experiments using an *S. typhi aro* mutant strain expressing the genes for fragment C of tetanus toxin and the full length 28 kDa glutathione *S*-transferase (P28) of *Schistosoma mansoni*. The *S. typhi* mutant strain given to mice in a single dose was protective against challenge with virulent *S. typhi*, tetanus toxin, and with schistosomula (as measured by a reduction in worm burden in vaccinated mice) [26]. Expression of these antigens in *S. typhi* CVD908-*htr*A could result in a trivalent oral vaccine protecting against typhoid, tetanus and schistosomiasis. Success in this area will undoubtedly lead to the expression of other antigens, already tested, such as *E. coli* colonization factor antigens, modified enterotoxins, outer membrane components and O-antigenic polysaccharide chains from enteric pathogens, be they bacterial, viral (if nonglycosylated protein) or parasitic.

Vibrio cholerae

Inactivated oral Vibrio cholerae *vaccines*

Cholera is caused by *V. cholerae* 01. There are two biotypes, classical and El Tor, and two serotypes, Inaba and Ogawa. The ongoing seventh pandemic is caused by the El Tor biotype of *V. cholerae* 01. The recent spread of *V. cholerae* 0139 may be the early part of an eight pandemic.

After three doses, the inactivated cholera B subunit whole cell vaccine had a protective efficacy of 85% during the first six months post vaccination, compared to 58%

protective efficacy seen in vaccinees given whole cells only [27]. Thereafter the influence of the CT-B waned and both types of vaccine gave the same protective efficacy after three years, approximately 60%. After five years follow-up the protective efficacy was just below 50% [5].

A whole cell cholera vaccine developed in Vietnam, and modeled on the vaccine created by Holmgren and coworkers [5,27], showed a protective efficacy of 65% against El Tor cholera during an epidemic that occurred more than six months after immunization of 130,000 participants in an open trial [28].

A new epidemic *V. cholerae* strain, designated as serogroup 0139 and coexisting with traditional *V. cholerae* 01, appeared in India in 1992 and spread rapidly in Asia. The strain mainly affected adults and it appeared as if there was no, or only little, preexisting immunity in the population previously naturally exposed to *V. cholerae* 01. Therefore an inactivated *V. cholerae* 0139 whole cell vaccine has been developed for the creation of a bivalent CT-B subunit plus 01/0139 whole cell oral inactivated vaccine [29]. The vaccine elicited significant intestinal and systemic antibacterial immune responses in volunteers, and addition of *V. cholerae* 0139 whole cells did not interfere with the immunogenicity of either the CT-B subunit or the *V. cholerae* 01 whole cells [29].

Attenuated live oral Vibrio cholerae *vaccine*

The modest protective efficacy of the oral inactivated whole cell vaccine and the need for two or more doses prompted the development of a single-dose attenuated live vaccine. Vaccine strain *V. cholerae* Inaba (classical 01) CVD103 HgR was safe and immunogenic and afforded protection in up to 100% of volunteers against challenge with classical *V. cholerae* and up to 60% against challenge with El Tor cholera. The efficacy is presently being assessed in a large scale, randomized, double-blind, placebo-controlled phase III study in Indonesia. The study will be unblinded and analyzed in late 1997 [28].

The moderate protective efficacy seen in individuals with classical *V. cholerae* and challenge studies with biotype El Tor started development of an attenuated El Tor vaccine. The construction of a safe and immunogenic El Tor Ogawa strain CVD111 which elicited vibriocidal antibody titers, and could be combined with strain CVD103 HgR, has just been reported [30]. Co-administration of the two strains, which are complementary in both biotype and serotype, induced fourfold rises in vibriocidal titers against Inaba in 69–76% of volunteers, and in 53–75% of the volunteers against Ogawa. This bivalent vaccine may possibly induce in a single dose a broadly protective immunity against all strains of 01 cholera.

Enterotoxigenic *Escherichia coli*

Colonization factors (CFs), fimbrial and other adhesive surfaces, mediate attachment of the bacteria to the intestinal epithelial cell lining. The LT and heat-stable (ST) enterotoxins induce a net secretion of electrolytes and water into the gut lumen. An efficacious enterotoxigenic *E. coli* (ETEC) vaccine must elicit effector mechanisms (antibodies) that operate on the gut mucosal surface, and are targeted towards surface appendages of the ETEC strains and/or its toxins.

Antibodies against CFs are protective [1•,27] but there are several different antigenic structures: CFA/I is a single fimbrial structure, CFA/II and CFA/IV each consist of three different antigens in various combinations, CFA/III is one entity, and there are several new, as of now, putative CFs [28]. In a recent study of 111 ETEC strains from North Indian children a quarter of the isolates were negative for known and putative CFs [28]. An efficacious vaccine either may have to contain up to ten or more different CF antigens or subcomponents.

ETEC isolates are either LT only, LT–ST or ST only producers. The CT-B subunit, closely related to the B subunit of LT was earlier shown to elicit a substantial but short-lived (months) efficacy against LT–ST producing ETEC stains [5]. A prototype oral inactivated CT-B subunit-colonization factor antigen ETEC (five strains) vaccine from the laboratory of AM Svennerholm and J Holmgren is currently in phase 1 and 2 clinical trials. Data so far demonstrate that the vaccine is safe and ASC (antibody secreting cells) responses to most vaccine antigens in ≥70% of vaccinees, and that two doses elicited responses similar to those seen after naturally occurring ETEC diarrhea in Bangladeshi adults [31]. ST is in itself non-immunogenic; it is a 19 amino acid peptide with three disulfidebonds. Attempts to mutate ST and to create immunogenic fusion proteins have been largely unsuccessful. Attempts to couple them to appropriate carrier molecules have provided immunogens giving promising animal results [27] but proof of protective immune responses in man are still lacking.

DNA vaccines

The use of nonreplicating plasmid DNA vectors as vaccines offers the advantage of expressing the antigens in the host instead of delivering the vaccine as a manufactured inactivated microorganism, subunit or peptide [32]. The potential success of mimeotope vaccines (A Phalipon, personal communication) will allow DNA sequences coding for O-antigen determinants to be introduced into the plasmid vectors. The DNA vector competes in efficiency with a replicating attenuated microorganism, but it has the advantage of selection of only relevant antigenic epitopes and therefore is potentially safer (assuming that the DNA vector does not integrate, activate oncogenes or inactivate oncogene suppressors etc.). The introduction of DNA vaccines at the experimental model level has met with much enthusiasm since excellent protection has been achieved. Proof of concept is, however, still missing in man.

So far most of the data has been collected after intramuscular and intradermal inoculation with various DNA vectors, and results can be summarized as follows: high antibody titers can be achieved; intracellular antigen-processing and presentation by MHC class I molecules leads to induction of cytotoxic T lymphocyte (CTL) responses; MHC determinant selection can be achieved; nucleotide sequences coding for several peptide epitopes can be strung together, resulting in a polyepitope; and immunostimulatory DNA motifs can be introduced into the vector [5].

The utility of mucosal immunization was demonstrated in a study in mice with a DNA plasmid carrying the measles virus hemagglutinin gene (Pv1j-HA) [33]. A single 100 μg dose was given either intranasally, transepithelially in the buccal mucosa, orally by gastric intubation, or enterically by intrajejunal injection. Hemagglutinin-specific CTL responses elicited by nasal and buccal immunization could not be boosted. Administering the DNA orally or intrajejunally resulted in lower CTL responses which, however, could be potentiated by co-administration of CT or cationic lipids as adjuvants.

The success of gene gun inoculation of mice with vectors expressing rotavirus proteins VP4, VP6 or VP7 [14•] is discussed in the rotavirus vaccine section above.

The potential of DNA vaccines is evident. Should the proof of concept studies in man prove successful we will see a rapid and potentially revolutionizing development in the vaccine field.

Conclusions

The past couple of years have seen significant advances in both science and clinical trials which hold promise for vaccine development against enteric pathogens. The scientific community identifies virulence factors with increasing velocity, to a large extent thanks to genomics, and develops means either to delete them or to disarm them and to turn them into immunogens. Likewise the improved knowledge facilitates the development of safer attenuated microorganims, but the rare presence of individuals with immune defects, such as lack of interferon (IFN)-γ [34] and interleukin (IL)-12 receptors, may make them too virulent for some. A potentially important advance is the ability to string gene sequences coding for protective epitopes with immune modulatory gene sequences into naked DNA vaccines for systemic as well as mucosal delivery. The next decade could turn into a golden age for enteric vaccines.

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