Research priorities for diarrhoeal disease vaccines: Memorandum from a WHO meeting*

Diarrhoeas caused by rotaviruses, Shigella, Vibrio cholerae, and enterotoxigenic Escherichia coli (ETEC) represent a major health burden in developing countries, and have stimulated much effort towards vaccine development in order to protect against these four disease agents. This Memorandum describes the state of the art and points the way to future research and test trials in this area.

Introduction

Diarrhoeal diseases constitute a very important public health problem in most developing countries, with over 1000 million episodes and over 4 million deaths annually in children under 5 years of age. For each child, these episodes of diarrhoea occur more than 5 times a year during the first 5 years of life, and then decrease with age. This age-related decrease in diarrhoeal disease incidence provides hope that successful immunization will be possible. Epidemiological studies also point out the partial protection provided by natural infection. Multiple infection with the same pathogen is common. Immune protection is therefore not solid, and there are suggestions that protection may be better related to specific virulence factors, such as colonization factors or serotype of the pathogen.

The WHO Programme for Control of Diarrhoeal Diseases (CDD) has been heavily involved in promoting and supporting research aimed at the development and evaluation of vaccines against diarrhoeal diseases. A major effort has been made to set up a number of field trials of candidate vaccines in developing countries. Recently, CDD decided to focus on disease control activities which resulted in a decreased involvement in vaccine research. It was, therefore, decided that from 1991 the WHO/UNDP Programme for Vaccine Development (PVD) would coordinate research efforts in the area of diarrhoeal diseases, whereas vaccine trials would be carried out with the active involvement of CDD. In view of the awareness that new approaches were needed for the development of several of these vaccines, a joint meeting was organized by CDD and PVD on 6–8 March 1991 to define new research priorities towards diarrhoeal disease vaccines against rotavirus, Shigella, cholera, and enterotoxigenic Escherichia coli (ETEC) infections.

Rotaviruses

Vaccine development

Rotaviruses are the major cause of severe dehydrating diarrhoea among children (most commonly between 6 and 24 months of age) in developed countries, where they can account for 40–60% of the cases requiring hospitalization. Rotavirus diarrhea is equally common in developing countries, but the proportion of diarrhoeal episodes is smaller because of the higher incidence of bacterial and parasitic diarrhoeas. However, rotavirus diarrhoeas are usually more severe than bacterial diarrhoeas and rotaviruses account for a significant proportion (20–40%) of severe dehydrating diarrhoeas among young children in developing countries. An effective vaccine might prevent between half and one million deaths of young children annually.

The aim is to strengthen research for the development of vaccine(s) able to protect children in the first years of life against severe dehydrating rotavirus diarrhoea. Protection against severe disease (as opposed to protection against mild illness and asymptomatic infection) should be close to 100%.
Vaccination must provide protection against diarrhoea caused by the most common human rotavirus serotypes even in very young infants.

**Previous efforts**

**Candidate vaccines.** Attempts to develop live, attenuated oral vaccines for human rotavirus gastroenteritis have used the following approaches:

—Heterologous (animal) rotaviruses that are adapted to tissue cultures. These include bovine rotavirus (vaccines RIT4237 and WC3) and rhesus rotavirus (vaccine RRV-1).

—Human–animal “reassortant” rotaviruses in which the VP7 surface protein of human rotaviruses, corresponding to serotypes 1–4, is incorporated into an animal host virus. They include bovine–human vaccine viruses and rhesus–human vaccine viruses.

—Naturally attenuated (“nursery strains”). These differ from virulent rotaviruses by a modification in the VP4 surface protein.

**Efficacy trials.** (i) *Bovine rotavirus vaccines.* The RIT4237 strain of bovine rotavirus was tested in several clinical trials in developed and developing countries, but was later withdrawn by its manufacturer because it showed insufficient immunogenicity and efficacy. This vaccine, after 1 or 2 oral doses, induced 50–60% protection against all, and 80–90% protection against severe rotavirus diarrhoea in infants aged 6–12 months in Finland. In a WHO-supported trial in Peru, the vaccine (given in 3 doses) was 40% effective against all, and up to 75% effective (depending on the criteria) against severe rotavirus diarrhoea. However, in the Gambia, vaccine efficacy after 3 doses was only 33%, whereas in trials involving a single dose of vaccine in Rwanda and among Apache Indians in Arizona, USA, no efficacy was found.

The WC3 vaccine strain is derived from a different bovine rotavirus and may be less attenuated than RIT4237. The vaccine dose is 10⁷ particles as compared to 10⁸ for RIT4237. In a trial in infants aged 3–11 months in Philadelphia, USA, this vaccine (after a single dose) induced 76% protection against all, and 100% protection against severe rotavirus diarrhoea. Further evaluations using two doses of the WC3 vaccine have, however, resulted in disappointing results. In a trial in Cincinnati, USA, the vaccine showed only 20% efficacy, and in the Central African Republic no efficacy against all and 38% efficacy against severe rotavirus diarrhoea. The WC3 vaccine has also been withdrawn by its manufacturer.

(ii) *Rhesus rotavirus vaccine.* The RRV-1 vaccine has been tested in several efficacy trials in developed countries, with varying results. In the first of these, in Sweden, a vaccine dose of 10⁵ PFU conferred 48% protection against all, and up to 100% protection against severe rotavirus diarrhoea. This dose, however, caused fever in 79% of Swedish children. In other studies, a vaccine dose of 10⁴ PFU showed 38% protection in Finland, 29% protection in Baltimore, USA, 0% in Rochester, USA, and 0% among Apache Indians in Arizona, USA.

**Live attenuated vaccine candidates under investigation**

*Rhesus, rhesus–human rotavirus reassortant tetravalent vaccine.* Rhesus rotavirus-based tetravalent vaccines (RRV) are currently under investigation at 4 × 10⁵ PFU in an efficacy trial in Myanmar (supported by the CDD programme). Phase I trials of 4 × 10⁵ and 4 × 10⁶ PFU and an efficacy trial of 4 × 10⁵ PFU are planned in the USA (supported by Wyeth-Ayerst Research Laboratories). A phase I study of 4 × 10⁵ PFU, 4 × 10⁶ PFU and placebo is planned in Venezuela, in order to determine the best dose to be used in a study to evaluate efficacy against cases requiring treatment at a hospital or clinic. These efficacy trials will be sufficient for final evaluation of the usefulness of this vaccine. If efficacy is proved in the Myanmar or Venezuela trials, more detailed studies on possible interference of this vaccine with oral poliovirus vaccine (OPV) should be considered, aimed at assessing rotavirus vaccine delivery at the highest dose within the EPI schedule of immunization. Studies on interference with OPV should be performed in developing countries. A vaccine to be used in different doses in different countries does not seem to be ideal.

**W179-9 vaccine.** This serotype 1 bovine–human reassortant vaccine was reported to be 100% protective in a small efficacy trial in Philadelphia, USA. It would be important to confirm these findings in a larger and more heterogeneous population of children in the USA. Without this confirmation, further efficacy trials in developing countries are not recommended.

**M37 (“nursery strain”) vaccine.** This vaccine (asymptomatic human strain, serotype 1) showed less than optimal immunogenicity when used at 10⁴ PFU (47% responders) and better immunogenicity at 10⁵ PFU (76% responders). Although it was safe and immunogenic at 10⁵ or higher PFU when tested in developed countries, this vaccine did not induce a good heterotypic antibody response. If the vaccine cannot be produced at higher titres, alternative methods of delivery to increase immunogenicity might be considered.
**Cold-adapted human strains.** These are under development for safety testing. Once vaccine lots for human studies are made available, phase I trials in developing countries should be considered.

**Other live attenuated strains.** Considering the interest in novel approaches for the development of effective live, attenuated human rotavirus or reassortant vaccine strains, studies on new strains to be developed should be encouraged, provided that they can be shown to be sufficiently distinct from previous candidate strains, e.g., strains eliciting cross-reactive neutralizing antibodies.

**Needs for vaccine development and assessment**

Based on the experience of testing live, attenuated rotavirus vaccines for children in the developing countries during the last ten years, several problems related to vaccine development can be identified:

1. The efficacy of a vaccine in a developed country cannot predict similar efficacy in a developing country. This problem might be overcome by testing rotavirus vaccine in developed countries in more heterogeneous populations, where rotavirus serotype variation is documented, before field trials in developing countries.

2. No clear immunologic correlates of protection have been identified as yet to permit definitive prediction of the success of a vaccine in a developing country. There is, therefore, a need for research on basic mechanisms for protective immunity against rotavirus illness and infection. Serotype-specific antibodies in human sera should be measured using assays that differentiate and measure separately anti-VP7 and anti-VP4 antibodies. Basic research on the immune response after rotavirus infection is required. In this respect, valuable information could be derived from detailed analyses of faecal and serum specimens from previous trials, which have still to be completed. Measurements and correlations between titres of serum IgG/IgA and faecal IgA antibodies and their relation to protection should be encouraged. It is desirable that future trials include the analyses of these immune parameters.

3. Phase I trials should examine safety and immunogenicity of different doses and dose schedules for vaccine optimization prior to proceeding to phase II trials.

**Novel approaches**

Based on data available, it is unclear whether the live, attenuated rotavirus vaccines investigated so far will be able to provide protective immunity in children in developing countries. While final tests of the RRV-based tetravalent vaccines are underway, there is a clear need to pursue novel alternative approaches for vaccine development.

Approaches foreseeing immunization with inactivated whole virus vaccines or with subunit vaccines should be considered. In this respect, an increased knowledge of the antigenic structure of rotavirus particles would facilitate this task. There are numerous approaches for the development of subunit vaccines, which could contain:

1. proteins isolated from purified virus;
2. double-shelled empty particles (lacking RNA) produced in vitro;
3. proteins and particles from cloned genes produced in prokaryotic or eukaryotic expression systems;
4. synthetic peptides.

Inactivated whole virion or subunit vaccines may either be effective alone or useful to prime the immune response. Various approaches for their presentation (parenteral, oral, etc.) to enhance their immunogenicity should be considered, as well as their administration in combination with live attenuated vaccines. The practicability of these novel approaches should be first demonstrated in suitable animal models. These studies should include the optimization of the immune response and the evaluation of the mucosal immunity induced.

Direct delivery of rotavirus antigens to the intestine via live bacterial or viral delivery systems (adenoviruses, polioviruses, *Salmonella*, *E. Coli*, BCG) are very attractive; however, special attention should be given to the expression of conformational determinants. Research on novel methods of presentation of non-replicating antigens to the intestine (e.g., via microcapsules, liposomes, “mucosal adjuvants”, etc.) should receive high priority.

Connections between investigators with potential new vaccine candidates and industry should be established to ensure appropriate development of promising vaccines.

**Recommendations**

1. An optimal strategy of procedures for vaccine evaluation should be pursued. Candidate vaccines should be evaluated in different populations in developed and developing countries.
2. Vaccine trials for efficacy of RRV-based vaccines should be monitored and plans prepared for an implementation study in the EPI schedule, also considering possible interferences with OPV.
3. Vaccine trials with other live attenuated vaccines (such as bovine–human reassortant rotavirus and human rotavirus vaccines) should be monitored and plans prepared for efficacy trials if candidates are deemed promising.
(4) Novel approaches of live vaccines should be encouraged, if deemed attractive and different from those used for previous candidate vaccines (e.g., a specific natural strain or reassortant strains inducing broad heterotypic antibodies).

(5) Continued long-term surveillance and extensive genetic and antigenic characterization of group A rotavirus strains, isolated in longitudinal studies at different times and places, should be considered in order to understand the basis and the extent of antigenic variation. Consideration should also be given to more detailed studies of wild-type rotavirus isolates collected from previous unsuccessful trials to determine if the lack of success resulted from antigenic variation of VP7 or VP4 epitopes.

(6) Studies on the immune responses after natural infection and/or active immunization should be encouraged, in order to generate more information on immune parameters correlating with protection. Immunological studies using serum and faecal samples from previous trials should be planned, in the attempt to determine whether levels of specific neutralizing antibodies can predict vaccine efficacy.

(7) Infants who receive candidate parenteral rotavirus vaccine(s) should be studied for their mucosal immune response upon re-infection with wild-type rotavirus.

(8) Support should be given to novel approaches for vaccine development not based on live attenuated vaccines. Such approaches could include the use of inactivated non-living virus particles or viral subunit antigens produced by a variety of recombinant DNA or synthetic means.

(9) Research on novel delivery systems (microcapsules, liposomes) and adjuvants to enhance immunogenicity and efficacy of live or non-replicating vaccine candidates should be strengthened.

**Shigella**

**Vaccine development**

Shigella annually causes at least 200 million cases of diarrhoea, of which 650,000 are fatal. Almost two thirds of those falling ill are under 5 years of age and approximately 90% of the severe cases and deaths are seen in this group. The emergence of *Shigella* strains which are resistant to the commonly used antibiotics and the rapid spread of resistance against the newly introduced antimicrobial agents makes the development of safe and efficacious shigella vaccines extremely important.

The aim is to support the development of shigella vaccines to protect children and adults against diarrhoea and dysentery caused by *Shigella dysenteriae* type 1, *S. flexneri*, and *S. sonnei*.

**Previous efforts**

Parenterally given inactivated and live vaccines showed no efficacy in humans. A live oral non-invasive vaccine (*S. flexneri*) was safe and efficacious in field trials, but required multiple doses. Live oral streptomycin-dependent vaccines were protective in volunteer trials in the USA and in well-controlled field trials in Yugoslavia, where 80–100% protection was seen, but frequent booster doses were required to obtain protection. The failure of the small Willowbrook custodial trial was possibly due to the overwhelming challenge doses. This vaccine gave vomiting in a small percentage after the first dose and showed some genetic instabilities in terms of occasional reversion to streptomycin independence and virulence. Neither of these vaccines are currently considered for further investigation and production.

**Current research**

Three approaches to live oral vaccination, several approaches to immunization with oral encapsulated antigens, and a glycoconjugate approach to systemic immunization are under investigation.

**Hybrid constructs: Shigella and E. coll.** (i) An *E. coli* K12 received the insertion of *S. flexneri* invasion antigen, coding for invasion protein antigens (IPA) and chromosomal genes coding for the O-antigen in the *his* and *pro* regions, as well as an *arg* region segment to help stabilize the plasmid. An *aroD* deletion was then added. This construct invaded epithelial cells, bound congo red, and conferred protection in the guinea-pig eye and monkey challenge models. Approximately one in 1000 organisms showed intercellular invasion, as evidenced by plaque formation, indicating that the *E. coli* *kcp* gene had been activated and turned on the *virG* region responsible for invasion. In humans, at $1 \times 10^7$ and $1 \times 10^9$ this vaccine was safe with only mild cramping in several volunteers; at $2 \times 10^9$ it showed reactivity in 20% of volunteers after the first, but none after subsequent doses; and at $2 \times 10^{10}$ a shigellosis-like disease occurred in 50% of volunteers. This vaccine (ECSf2a-2) will be tested at $10^8$ in a 4-dose regimen. In challenge trials this vaccine conferred 40% protection in one study and 0% in a second study. Modifications of this vaccine currently being constructed are: a) deletion of *kcp* or *virG* genes or continuous activation of *kcp*, to examine the role of these genes involved in the intercellular invasion; b) deletion of the *arg* region.

(ii) Other constructs consist of the insertion into *E. coli* K12 of the plasmid coding for *S. sonnei* LPS and *ipa* genes, or of the insertion into *E. coli* construct for *S. flexneri* of plasmid coding for *S. sonnei* LPS.
**Hybrid constructs: Shigella and Salmonella.** Ty21a has been made Vi positive. A plasmid complementing the gal mutation of Ty21a and which contains S. sonnei genes has been constructed for insertion into Ty21a.

Improvements to be made to these hybrids include insertion of the particular Shigella rfa locus, so that the Shigella O-antigen polysaccharide chains can be properly linked to the core LPS.

**Deletions and mutations: auxotrophic Shigella vaccines.** Attenuation (at least 100,000-fold) is based on the auxotrophy for metabolites not available intracellularly in mammalian cells. One strain, S. flexneri SFL124, serotype Y, had a deleted aroD gene, making it dependent on para-aminobenzoic acid (PABA), and therefore unable to multiply for more than a few generations in mammalian cells. The same aroD deletion was introduced into S. flexneri strain 2457T, serotype 2a: this has a reported ID_{so} dose of around 180 bacteria. In monkeys both strains conferred 100% protection against challenge with the wild-type parent strain.

SFL124 strain (2 x 10^9 bacteria given either as freshly grown live bacteria or as a lyophilized reconstituted live vaccine) was reactogenic in 2/21 (9.6%) adult Swedish volunteers (watery diarrhoea and 38–39°C fever lasting for 1 and 2 days) and in 0/30 adult Vietnamese volunteers. In the Swedes seroconversion against LPS was seen in 10/11 (91%) receiving 3 doses on alternate days and in 5/10 (50%) receiving 1 dose. All volunteers produced anti-S. flexneri LPS SIgA in the gut with higher responses in those receiving 3 doses than in those receiving only one. Anti-LPS IgA antibody-secreting cells were shown in peripheral blood by the ELISPOT assay. Proliferative responses to the saccharide portion of the S. flexneri LPS was also shown. After a booster dose of 2 x 10^9 S. flexneri SFL124 given 9–10 months later, ten volunteers showed a significant increase of the faecal anti-LPS SIgA antibody titres with a concomitant decrease of the faecal excretion of SFL124 bacteria, suggesting an immune memory induced by the primary immunization. Investigation of the immune response in the Vietnamese volunteers is in progress.

Early vaccine studies suggested that protection against S. flexneri is serotype-specific (based on O-antigen modification in LPS). Therefore, the genes encoding the group-antigen 6 (O-acetyl-transferase) and 7,8(glucosyl-transferase) were cloned and expressed in the S. flexneri SFL124 strain. Attempts are underway to clone glucosyl-transferase genes specifying type-specific epitopes.

A 14-mer peptide sequence of the shiga-toxin-binding (B) subunit, produced by S. dysenteriae type 1 and some S. flexneri, was inserted into the lamB outer membrane protein of E. coli and expressed in the S. flexneri SFL124 strain. However, the need for elicitation of anti-shiga-toxin immunity with Shigella vaccines remains unclear.

An cassette containing the structural genes for the repeating tetrasaccharide unit of the O-antigen polysaccharide chain of S. dysenteriae type 1 LPS was constructed and expressed in E. coli K12 and S. flexneri 4BR strain. The cassette will be moved into S. flexneri SFL124 strain and the expression there will be studied before phase I trials are undertaken.

**Deletions and mutations: Shigella vaccines with deletions of genes encoding virulence factors.** Based on current knowledge of the molecular basis for Shigella virulence, deletions were introduced in genes which define steps in the extra and/or intracellular infectious process.

(i) S. flexneri serotype 2a strain with deleted genes for: iuc, iut (coding for iron-scavenging proteins); icsA (coding for intra- and intercellular spread); and thyA. The vaccine candidate was immunogenic and protective in monkeys.

(ii) S. dysenteriae type 1 with deleted genes for: stxA^{-}B^{+} (coding for the cytotoxic A subunit of the shiga toxin); fepA, fes, entF (coding for iron-scavenging and internalizing proteins); icsA; and thyA. This construct has not yet been tested in monkeys.

(iii) S. flexneri serotype 5 with deleted genes for ompB (coding for double component osmoregulatory system). This construct was immunogenic and induced protective immunity in a monkey model.

Phase I trials are scheduled for these constructs.

**Non-living oral vaccines.** Covalent conjugation of O-antigenic chain to cholera toxin B subunit, with or without encapsulation in microcarriers, is currently underway. Microcapsule presentation of LPS with or without adjuvant is also envisaged. Local presentation of O-antigenic chain to the immune system using microcarrier technology is promising because of the potential ability to affect antigen trafficking and reduce toxicity. Cost issues will probably be overcome if efficacy can be demonstrated.

**Glycoconjugate vaccines.** Conjugates between the O-antigenic saccharides of Shigella and carrier proteins were produced and shown to elicit high levels of antibodies in animals. Such vaccines rely on a spill-over effect or on unknown immune mechanisms. Epidemiological studies in Israel indicated a correlation between serum antibodies and protection. Guinea-pig eye models also demonstrated protection...
with glycoconjugate vaccines when followed by E. coli hybrid constructs. Phase I studies are imminent.

Evaluation of vaccine candidates

This large number of candidate vaccines is promising in the field of Shigella vaccine development, and also challenging. The procedure for deciding which of these candidates will be evaluated for eventual human trials is then of critical importance. Elements involved in this decision process include animal model studies, manufacturing issues, phase-I safety and immunogenicity studies, and human challenge studies.

(i) The guinea-pig eye model may be as predictive as the monkey challenge system. However, neither of these models is predictive of safety in humans for live oral vaccines. A candidate vaccine should protect in one or both of these models. It should have stable phenotypic properties to allow manufacturing under GMP.

(ii) Phase I safety trials are required. If these studies were performed in outpatients, costs would be dramatically reduced. These vaccines present no threat to the environment since they are attenuated far below the organisms found in nature and because Shigella is host-restricted in nature.

(iii) The absolute requirement for human challenge trials prior to the initiation of field trials is somewhat problematic. Infection with Shigella protects against subsequent challenge in the model. Vaccines (streptomycin-dependent) that protect in the challenge model also protect in field trials. It is not known if vaccines that are only partially protective or failed in the challenge would be effective in field studies.

(iv) Shigella efficacy trials can be performed on relatively small numbers of volunteers. Efficacy studies in adults would be desirable prior to safety and efficacy studies in children. Obviously, there are not enough sites to perform field trials on 10–15 candidate vaccines. If several promising candidates for field trials arose simultaneously, they would be evaluated on manufacturing, reproducibility, cost and other technical considerations including immunogenicity. Development of immunologic assays in vitro that predict protection would be extremely important in order to facilitate the decision process. The availability of standard serum samples and methodologies would be most useful.

Recommendations

(1) Development of the new vaccine constructs discussed above should continue.

(2) Sites for phase-I human testing in developed and developing countries should be greatly expanded. The ability to perform these studies safely in outpatients should be emphasized.

(3) Emphasis should be put on in vitro immunologic correlates of protective immunity along with the standardization of such assays.

(4) The capability of intercellular spread of live candidate vaccines should be evaluated as a determinant of reactogenicity and immunogenicity in humans.

(5) The potential role of combinations of systematically and locally administered vaccines to reduce reactogenicity and enhance immunogenicity, as well as variation of immunization schedules to reduce reactogenicity, should be emphasized.

(6) The molecular and immunological basis of the balance between immunogenicity and reactogenicity of live oral vaccines needs to be explored in detail.

(7) The establishment of appropriate coordinating structures for the development and early evaluation of Shigella vaccines should be considered.

Cholera

Vaccine development

Cholera remains an important cause of morbidity and mortality in many developing countries, mainly in south-east Asia, but also in Africa and most recently in South American countries. It is estimated that more than 150,000 people, both children and adults, die each year from cholera. Because of its limited efficacy, the old parenteral cholera vaccine is no longer regarded as useful from a public health standpoint. Attention has instead turned to development of oral vaccines that could more efficiently stimulate local immunity. Both killed and live oral vaccines have been developed.

The aim is to develop cholera vaccines which would provide long-lasting protection against cholera in both young children and adults.

Killed whole cell/B subunit and whole cell cholera vaccines

Killed whole cell/B subunit (B-WC) and whole cell (WC) vaccines developed in Sweden have been tested in a large field trial in Bangladesh. A total of 63,000 persons (children aged 2–15 years and adult women) received three oral doses, at six-week intervals, of B-WC vaccine, WC vaccine or placebo; another 26,000 participants took one or two doses of the same. Vaccines or placebo were given with a bicarbonate-citrate buffer to protect the B subunit component during the passage through the stomach. Each dose of vaccine contained $1 \times 10^{11}$ killed whole vibrios, representing three different strains.
belonging to the Inaba and Ogawa serotypes and the classical and El Tor biotypes; the B-WC vaccine also contained 1 mg of purified B subunit from the cholera toxin. Previous phase-I and phase-II studies had shown that both vaccines did not cause any detectable side-effect and that, after either two or three doses, they stimulated a gut mucosal antibacterial IgA antibody response and, for the B-WC vaccine, also an antitoxic response (including memory) comparable to that induced by cholera disease itself. After a 3-year follow-up, the main results of the field trials can be summarized as follows:

(i) For the initial 4–6 months the B-WC vaccine showed a higher efficacy (85%) than the WC vaccine (58%); however, after the first 8–12 months, the efficacy of the two vaccines was similar. For the entire 3-year follow-up the B-WC and the WC vaccine conferred, respectively, 50% and 52% protection against culture-proven cholera. Protection appeared similar after two or three doses of the vaccines. For both vaccines the protective efficacy was lower and of shorter duration in children vaccinated at the age of 2–5 years: no protection was observed in this age group in the third year of follow-up. In contrast, the level of protection in individuals aged 6 years or more exceeded 60% for both vaccines and was sustained during the entire follow-up. Protection was slightly higher for classical than El Tor cholera, but did not differ with serotype (Inaba or Ogawa). Assuming that protection in adult males would have equalled that seen in adult females, the overall protective efficacy of two or three doses over the three years of follow-up can be estimated at 62% for the B-WC and 60% for the WC vaccine.

(ii) Through its B subunit component, the B-WC vaccine also protected (about 70%) for a few months against diarrhoea caused by ETEC producing either heat-labile toxin alone (LT, immunologically cross-reacting with the cholera toxin) or LT together with heat-stable toxin (ST). Furthermore, both the B-WC and WC vaccines substantially and in a sustained manner reduced the overall diarrhoea morbidity among the vaccinates: over the 3-year follow-up period there was a 25% reduction in hospitalizations for “all diarrhoeas” and a 50% reduction in hospitalizations for life-threatening diarrhoeas in the vaccinated versus the placebo group.

(iii) These results represent marked improvements compared to those previously achieved with parenteral cholera vaccines. In a cholera-endemic area, immunization with these oral vaccines could decrease the episodes of life-threatening cholera by more than 50% over three years. In an area with adequate intravenous and oral rehydration treat-

ment facilities (rarely found in rural communities in developing countries), the number of admissions to hospitals could decrease and, in areas without adequate facilities, it could provide a proportionate decrease in cholera morbidity.

**Live V. cholerae O1 vaccine strain CVD-103HgR**

Significant progress has also been made recently towards the development of a live attenuated cholera vaccine by preparing, through recombinant DNA techniques, *V. cholerae* O1 mutant strains in which the genes encoding cholera toxin had been deleted. The candidate vaccine currently being evaluated is the strain CVD-103HgR, developed in the USA and which (a) lacks the genes encoding the A (toxic) subunit of cholera toxin, and (b) carries a gene encoding resistance to mercury to aid with the identification of the vaccine organism in the environment. The CVD-103HgR strain is a derivative of classical Inaba 569B, a strain that produces no detectable shiga-like toxin and also has reduced colonization properties relative to other classical and El Tor strains of *V. cholerae*. A vaccine formulation has been prepared which consists of two sachets: one for the lyophilized organisms and one for buffer salts. The vaccine is prepared by adding the buffer salts to water, stirring, and adding the lyophilized bacteria.

In volunteer studies in the USA the vaccine (5 x 10^8 organisms/dose) was almost not reactogenic (only 1.9% of the adult volunteers developed very mild diarrhoea), yet conferred 83% protection against disease, with complete (100%) protection against moderate or severe diarrhoea (equal or greater than 3 litres). The same dose was tested in adult Thai civilian volunteers. No side-effects were observed and 92% of the vaccinees showed a significant vibriocidal antibody response. However, in a subsequent study in Thai military personnel a much lower serological response rate (20%) to the same vaccine was observed. A significant difference was again documented between Thai civilians (university students) and military personnel in a second phase-I trial (63% versus 39%, respectively). These differences in seroconversion rates may be due to the socioeconomic backgrounds of these two groups of volunteers and, therefore, their previous exposure to *V. cholerae* or alternatively a difference in the content of their normal proximal intestinal flora.

A study was also carried out to evaluate safety and immunogenicity of a single-dose of the vaccine in Indonesian children (aged 5 to 9), and different doses were examined. No adverse reactions were observed at any dose. Doses less than or equal to 5 x 10^8 induced 5–16% seroconversion, while doses of 5 x 10^9 and 1 x 10^10 induced seroconversion...
Further lines of research

Future improvements of the killed oral vaccines. Several biological issues will require consideration in future generations of killed oral cholera vaccines. These include efforts to improve the overall levels of protection, particularly long-term protection in young children and to augment protection against El Tor cholera.

(i) Future preparation of vaccine as a heat-stable powder or tablet formulation (in combination with an alkaline buffer) would facilitate storage and distribution of the vaccines and further increase their stability for use in cholera-endemic areas.

(ii) If cholera toxin B subunit is to be included (this would augment short-term protection against cholera and cross-protect against LT-producing ETEC), it will be necessary to devise techniques that do not rely upon expensive and technically demanding biochemical procedures for extraction and purification of this component. Using recombinant DNA technology, it now seems possible to produce the B and WD components in a single step; this would greatly simplify the procedures for inclusion of the B subunit that could be accomplished at low cost.

(iii) It should be tested whether booster doses for young children, in whom protection was initially comparable with that of older persons but rapidly declined after the first year, would also provide long-lasting protection to this highly susceptible age group.

(iv) Moreover, a more balanced formulation of El Tor and classical organisms (three-quarters of the current formulation consist of classical organisms) might increase protection against El Tor cholera.

(v) Better clinical protection may come from growth and inactivation of organisms in a way that permits expression of TCP pilus (which may be a protective antigen) and of El Tor-specific colonization factor antigens, such as MSHA.

(vi) Development of adjuvants and mucosal delivery systems augmenting mucosal immunity remains a basic research priority for cholera as well as for other oral vaccines.

Future research on live vaccines. Several biological and epidemiological issues need to be addressed.

(i) There is a need for further research to develop vaccine formulation(s) easy to reconstitute and resistant to adverse conditions (including avoiding the need for a cold chain) in developing countries. This research should also include the definition of dosage margins between the minimal effective dose for safe immunogenicity and that causing increased rates of side-reactions.

(ii) The influence of deleting genes from other known V. cholerae virulence factors (such as zot, a new toxin affecting the zonula occludens; irgA, the outer-membrane receptor protein of vibrio siderophores; and acfD, a lipoprotein involved in serum resistance and colonization) should be defined. These new deletion mutations should be introduced into ctxA-deleted, V. cholerae O1 strains with high colonizing potency in the human intestine (e.g., Ogawa 395), with the aim of obtaining a maximally immunogenic, yet possibly completely non-reactogenic live candidate vaccine strain.

Recommendations

(1) A coordinated effort is recommended, under the auspices of PVD, with the object of defining a group of immunological correlates of protection serving as baseline information for the licensing of currently available live and killed oral cholera vaccines. This information would also serve as data with which to accurately predict improvements in the immunogenicity or formulation of new future vaccines. The establishment of appropriate coordinating structures for the evaluation of currently available vaccines in real epidemic and endemic situations should also be considered.

(2) Studies are required using techniques of the social sciences on the perceptions among the users and policy-makers in developing countries, concerning the use of oral enteric vaccines.

(3) Continued phase-I trials of new live attenuated V. cholerae vaccine candidates, including those derived from El Tor strains, should be encouraged.

Enterotoxigenic E. coli (ETEC)

Vaccine development

Strains of enterotoxigenic E. coli (ETEC) cause disease worldwide, but are especially common in developing countries. In hospital- and clinic-based studies of acute diarrhoea in developing countries, the proportion of cases in which ETEC was identified ranged from 10% to 50%, with an average of about 20% in children under 5 years of age. Prospective community-based studies in Asia and South America indicate that the incidence of ETEC diarrhoea is highest among children under 2 years of age.
age. Overall, during the first 5 years of life, children in areas with high rates of diarrhoea experience 1–2 episodes caused by ETEC per year.

Analysis of the antigenic structure of ETEC strains from endemic areas shows many different O : H serotypes, at least 7 important types of fimbrial colonization factors (CFA/I and CS1–CS6 fimbriae), and three different toxin phenotypes (LT, ST, and LT/ST). Despite this antigenic heterogeneity, evidence from volunteer epidemiological surveys shows that prior infection with ETEC confers homologous immunity. A review of these studies leads to the conclusion that, in endemic areas, multiple infections with distinct strains bearing different fimbrial colonization factor antigens and of different toxin phenotypes must occur in order that broad-spectrum immunity be elicited. Protection is believed to be mediated by SIgA antibody directed against fimbrial colonization factors, LT, and O antigens. ST, which is a small peptide, does not stimulate neutralizing ST antitoxin following natural infection. Passive administration of milk immunoglobulin concentrate containing antibodies against various colonization factor fimbriae, LT and O antigens, provided 100% passive protection to adult volunteers experimentally challenged with ETEC.

The aim is to develop ETEC vaccines, including non-living and live oral vaccines, that will give broad-spectrum immunity in young children.

Non-living antigen vaccines

LT/ST toxoids. The B-WC oral vaccine against cholera, recently field-tested in Bangladesh, provided significant protection against diarrhoea due to LT-producing E. coli, including both LT and LT/ST strains, in the initial three months following vaccination. This short-lived protection was apparently due to the strong antigenic similarity between LT and cholera toxin. ST is a small peptide (18–19 amino acids) which is non-immunogenic in the course of natural infection. Attempts to prepare safe and immunogenic ST toxoids (as either recombinant fusion proteins or synthetic ST conjugated to carrier proteins) have been problematic, and clinical studies have taken place with only one LT/ST toxoid candidate.

Purified colonization factor (CFA) fimbriae as vaccines. Attempts to administer purified CFA fimbriae as oral vaccines to elicit anti-colonization immunity have been hampered by the adverse effect of gastric juice on fimbrial proteins, despite pre-treatment of vaccines with cimetidine and sodium bicarbonate. Nevertheless, the demonstration that purified CFA/II fimbriae instilled directly into the jejunum can successfully induce anti-CFA/II SIgA antibodies suggests that this approach to immunization is promising, if the antigen could be delivered undamaged by gastric juice. More effective and innovative ways must be devised to deliver soluble protein antigens that are sensitive to the gastric environment into the small intestine. The use of microspheres consisting of biodegradable polymers (such as a mixture of polyactide and polyglycolide) may be an approach to solving this problem.

Oral whole-cell vaccine inactivated by treatment with colicin or formalin. Inactivation of ETEC by treatment with colicin E2 has been utilized to generate non-viable intact bacterial cells with intact surface antigens (such as fimbrial colonization factors). These vaccines elicited intestinal SIgA antibodies against both CFA and LT and were protective in experimental challenge studies. Oral whole-cell vaccines consisting of formalin-inactivated ETEC are also being evaluated.

Inactivated whole bacterial cells plus toxoid. Based on studies in rabbits showing that a combination vaccine, stimulating both antibacterial and antitoxic immunity, confers greater protection against ETEC than vaccines that stimulate only one or the other type of immunity, another candidate vaccine has been prepared consisting of formalin-inactivated CFA-positive bacteria (to stimulate antibacterial immunity) and the B subunit of cholera toxin to elicit anti-LT immunity. Preliminary clinical trials in adult volunteers have shown that this vaccine is well tolerated and elicits gut mucosal SIgA anti-CFA and anti-LT responses.

Live attenuated oral vaccines

E. coli E1392-75-2A, a CFA/II-positive (CS1, CS3) spontaneous mutant lacking genes coding for LT and ST, has been used as a live oral immunogen in volunteers to answer fundamental questions of anti-colonization immunity in the absence of antitoxic immunity. A single 5 × 10^10 organism dose of this strain elicited anti-CS1 and CS3 fimbriae SIgA antibody in the intestinal fluid and conferred significant protection against experimental homologous challenge. However, mild diarrhoea was seen in approximately 15% of the vaccinees. Research is ongoing to prepare a live oral ETEC vaccine that will be acceptably immunogenic and efficacious without causing mild diarrhoea or other adverse reactions.

A similar approach would be to express CFA fimbriae and LT (and possibly ST) in live vectors, such as attenuated Salmonella typhi.
Memorandum

**Recommendations**

1. Further development of both non-living and live candidate ETEC vaccines should be encouraged.
2. More detailed and intensive studies of human immune responses to ETEC antigens, using state-of-the-art methodologies, should be promoted.
3. Innovative antigen delivery systems should be investigated as a means to enhance the immunogenicity of fimbrial and toxoid antigens.
4. Continued attempts to construct an effective ST immunogen should be encouraged.
5. Rapid development of candidate live oral ETEC vaccines should be promoted including vector-based vaccine strains expressing fimbrial antigens and toxoids.
6. Phase-I clinical studies should be initiated to test candidate live vaccine strains for safety and immunogenicity.
7. Clinical trials should be considered using the B subunit/whole inactivated ETEC combination vaccine in adults and children in endemic areas.

**General recommendations**

1. Novel approaches for the development of new vaccines should be encouraged. Such approaches could use subunit antigens produced by a variety of recombinant or synthetic means.
2. Further development of new live vaccines should be encouraged.

3. Efforts should be made a) to encourage the involvement of scientists in developing countries in the different stages of the vaccine development research progress, and b) to support and encourage the development, in developing countries, of means to produce locally and quality control enteric vaccines.
4. A better knowledge of pathogenic and virulence mechanisms should be obtained. This information could contribute greatly to the design of efficacious and “targeted” vaccine constructs.
5. Research on novel delivery systems (microcapsule, liposomes) and adjuvants to enhance systemic and local immunogenicity should be strengthened.
6. Research on *in vitro* immunologic correlates of protective immunity should be encouraged.
7. Particular attention should be given to the immune effector mechanisms (other than antibodies) induced by natural infections versus those induced by vaccine candidates under investigation. Since some of these pathogens are intracellular, immune-mediated protection could be exerted by CD8⁺ (cytotoxic) T lymphocytes. The possible involvement of these cells in immune protection after natural infection and/or vaccination should be investigated.
8. The risk of inducing immune-mediated pathology after vaccination with candidate vaccines should be evaluated.