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DEVELOPMENT OF VACCINES AGAINST CHOLERA AND DIARRHOEA
DUE TO ENTEROTOXIGENIC ESCHERICHIA COLI

Report of a meeting held at
The University of Maryland, Baltimore, Maryland, USA

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INTRODUCTION

Although parenteral cholera vaccines have existed for a century, they have not played a significant role in the control of cholera since they induce only a low level of immunity over a short period. Since the early 1970s, the use of such vaccines has been discouraged by the World Health Organization, both because of their low efficacy and because it was feared that ineffective immunization would distract attention and resources from other, more effective means of treating and controlling the disease. Meanwhile, research to develop effective oral vaccines based on non-living organisms and antigens, or on live, attenuated strains of Vibrio cholerae 01, has continued. The meeting reviewed the results that have recently emerged from such studies, which included a field trial of two formulations of an inactivated oral cholera vaccine in Bangladesh, research on protective antigens of V. cholerae 01 that might lead to improved inactivated vaccines, and studies of the safety, immunogenicity, and efficacy of live cholera vaccines in volunteers. Following its review, the meeting made recommendations for further research in these areas.

Diarrhoea due to enterotoxigenic Escherichia coli (ETEC) is an important problem in most developing countries, but progress towards the development of vaccines for this disease has been slow. However, important recent advances in understanding of the virulence antigens of ETEC have led to the development of possible candidate vaccines, and the first of these has reached the stage of initial evaluation in man. The meeting reviewed current research on the protective antigens of ETEC and made a number of recommendations with the aim of stimulating further efforts towards the development of vaccines against ETEC disease.

CHOLERA VACCINES

RELEVANT EPIDEMIOLOGICAL ASPECTS

Currently, cholera is reported to WHO by about 35 countries. Left untreated, it is still one of the most dangerous infectious diseases, with case-fatality rates of up to 40%. An estimated one million cases of cholera occur in the world each year, causing about 20 000 deaths in Africa and 100 000 in Asia. About one-third of the deaths are in children under 5 years of age, one-quarter are in children aged 5-14, and the remainder are in adults.

Both epidemic and endemic patterns of disease are present. Epidemics that occur in previously uninfected areas with seronegative populations affect all age groups equally and are often associated with a single mode of spread. Such epidemics are characterized by a relatively low rate of asymptomatic infection and there is usually no environmental reservoir of infection. In contrast, with endemic cholera, disease incidence is highest in children 2-15 years of age and declines with increasing age, except in women of child-bearing age in whom higher rates are found. Yearly seasonal outbreaks are prominent and transmission is associated with (i) an environmental, aquatic reservoir, (ii) multiple modes of spread (contaminated water or food, occasionally person-to-person contact), and (iii) frequent asymptomatic infections, leading to a high prevalence of antibody by 20 years of age.

In endemic areas, risk factors for severe cholera include: (i) absence or low titres of vibriocidal antibodies, (ii) close contact with the family of a cholera patient, (iii) low gastric acidity (whether naturally acquired, surgically induced, or resulting from the use of antacids), (iv) absence of breast-feeding (in children under 3 years), and (v) O blood group. The existence of natural immunity to V. cholerae 01 in endemic settings is suggested by the declining attack rate with increasing age, the lower attack rates in persons with elevated levels of vibriocidal antibody, and epidemiological evidence that an episode of cholera evokes substantial protection against a second episode. In Bangladesh, the prevalence of vibriocidal antibody is 40% at 5 years and 80% at 20 years of age. It is

estimated that every person is exposed to V. cholerae 01 at least once each decade, and that some people may be exposed annually; immunity appears to be boosted with each infection, including asymptomatic infections.

Groups that may benefit from effective cholera immunization include children and adults in endemic areas, refugees in unsanitary camps, and populations in non-endemic areas threatened by nearby epidemics. International travellers experience a very low risk of cholera, which would probably not be changed measurably by vaccination.

HISTORY OF CHOLERA VACCINES

Parenteral cholera vaccines began to be used shortly after Koch showed that Vibrio cholerae 01 was the cause of the disease. At the end of the 19th century, large numbers of persons were immunized in Spain, and later in India, with killed V. cholerae. Although early studies in India suggested that the vaccine was protective, controlled clinical trials of the killed whole-cell parenteral vaccine were not carried out until the 1960s. Then, a series of trials in Bangladesh, India, and the Philippines confirmed that the vaccine was efficacious, but also showed that protection was short-lived (3 to 6 months), the vaccine was much less protective in young children than in adults, and vaccination did not reduce the faecal excretion of V. cholerae. It was concluded that the parenteral vaccine was not a cost-effective tool for use in the control of cholera.

During the 1970s, two trials were carried out in India and Indonesia with parenteral vaccines containing aluminium adjuvants. The protection provided by these vaccines was greater and more sustained than that provided by the vaccine given without adjuvant. Toxoid antigens were also tested as parenteral vaccines in Bangladesh and the Philippines, but failed to protect despite the fact that they stimulated serum antitoxin responses.

During the same period, WHO began actively to discourage the use of parenteral cholera vaccine for any purpose since it was not cost-effective in endemic areas, was ineffective in controlling epidemics, and did not stop the international spread of cholera. This lack of enthusiasm for the vaccine was also a consequence of improvements and simplifications in the treatment of the disease. Rehydration therapy, by both the intravenous and the oral route, was shown to reduce case-fatality rates to less than 1% and it was argued that treatment was a more cost-effective intervention for saving lives than immunization.

Even today, many people in Asia and Africa live in areas that experience high rates of cholera and where effective treatment is unavailable. In such areas, mortality due to cholera remains high and epidemics cause panic and severe social and economic problems. Hence, there is continuing need for an effective vaccine against cholera. Based on the concept that natural protection against cholera is conferred by intestinal antibacterial and antitoxic antibodies, research in the 1980s has focused on the development of oral vaccines that would induce protective immunity by stimulating an intestinal immune response against one or more relevant antigens of V. cholerae 01.

IMPORTANT ANTIGENS OF VIBRIO CHOLERA 01

Studies in animals and humans have shown that V. cholerae cell-wall lipopolysaccharide (LPS) and cholera toxin (CT) both evoke protective immune responses. Antibodies to these the gut, protect synergistically. Antibacterial immunity is mainly afforded by antibodies to LPS, but antibodies to other cell-associated protein antigens may also be important. Antitoxic immunity is primarily directed against the B subunit of cholera toxin.

Colonization of the human intestine by V. cholerae 01, a prerequisite for developing diarrhoeal disease, is undoubtedly a complex process, requiring the coordinated expression of chemotactic and motility functions, proteolytic enzymes, haemagglutinins, colonization pili, and finally, production of CT. The prevention of bacterial adherence

would block the pathogenesis of cholera at its earliest stage. Recent studies in infant mice and volunteers have indicated that a pilus elaborated by both the classical and the El Tor biotype of V. cholerae O1 is probably required for colonization. This pilus has been designated TCP (toxin coregulated pilus) because the growth conditions that modulate its level of expression also modulate toxin production. In addition, the major pilus structural gene, TcpA, and the toxin genes are part of a virulence regulon (toxR) which antigens independently protect against experimental cholera and, when present together in controls gene expression. Because of their critical roles in the pathogenesis of cholera, toxR-regulated surface and secreted proteins, in particular TCP and cholera toxin B subunit (CT-B), are potential immunogens for use in combination vaccines, in addition to LPS.

Rabbit polyclonal antiserum directed against TCP has been found to be protective in passive immunization experiments in infant mice challenged with either Ogawa or Inaba strains of V. cholerae O1. The protective activity of anti-TCP serum is lost after it has been absorbed with wild-type V. cholerae O1, but retained after absorption with a pilus-negative mutant, suggesting that protection is associated with the anti-TCP antibodies. The sequences of the TcpA genes from two classical strains of V. cholerae O1, Ogawa 395 and Inaba 569B, have been found to be identical. The sequence of TcpA from an El Tor strain of Ogawa serotype, E7946, shows less homology: about 80% with respect to the predicted amino-acid sequence. However, the predicted secondary structure, distribution of charged residues, and potential antigenic epitopes are conserved to a high degree between the two biotypes, suggesting that functional domains, as well as epitopes inducing protective antibodies, may be shared by the pili of both biotypes.

Genetic analysis has identified several additional gene products besides TcpA that are required for TCP biogenesis and function. One of these, TcpG, may also function as an adhesin, and therefore is another candidate for immunization studies. In addition to the genes involved in TCP synthesis, the genes encoding the outer membrane proteins and the production of an accessory colonization factor (ACF) are toxR-regulated. Thus, TcpA could perhaps be used as an indicator of the presence of other potentially important toxR-regulated antigens during the preparation of whole-cell (WC) vaccines.

The killed oral vaccines recently tested in Bangladesh (see below) contain no detectable TCP by western blot analysis, possibly because the growth conditions of the bacteria when the vaccine was prepared were not optimal for the expression of these antigens. Neither formalin nor heat (as used to inactivate the bacteria in the WC vaccine) seem adversely to affect the immunoreactivity of TCP in western blots. Thus, it might be possible to improve the oral WC vaccine by using bacterial strains that produce TCP and by employing production methods that ensure the expression and preservation of TCP and other toxR-regulated antigens.

CANDIDATE ORAL VACCINES

Non-living vaccines

Killed whole-cell vaccines with or without the B subunit of cholera toxin have recently been developed and tested. The oral whole-cell/B-subunit (WC/BS) vaccine contains purified B subunit from cholera toxin and formalin- or heat-inactivated classical and El Tor cholera vibrios of the Inaba and Ogawa serotypes. The purified B subunit is completely non-toxic and gives rise to levels of neutralizing antibodies comparable to those evoked by holotoxin; the capacity of B subunit to bind to cell membranes may contribute to its immunogenicity. The heat-killed organisms in the vaccine provide Inaba and Ogawa LPS antigens, and the formalin-killed organisms provide heat-labile antigens. Because the B-subunit pentamer is acid-labile, the vaccine is administered with a buffer solution of sodium bicarbonate and citric acid.

The safety of the WC/BS vaccine was first demonstrated in small-scale clinical trials in Bangladesh, Sweden, and the USA. In Bangladesh, an evaluation of the vaccine's ability to stimulate mucosal antibacterial and antitoxic immune responses showed that two or more

oral doses evoked anti-LPS and antitoxin IgA antibody responses in intestinal lavage fluid comparable to those induced by natural disease. These responses were considerably higher and longer-lasting than those induced by two intramuscular doses of the same vaccine. Oral WC/BS vaccine also induced significant antibacterial and antitoxic antibody responses in Swedish volunteers, though they were of lower magnitude than in Bangladeshis of similar age. Furthermore, the B-subunit component of the vaccine given orally to Bangladeshi or Swedish volunteers induced mucosal (IgA) immunological memory which lasted at least 15 months in the Bangladeshis and 5 years in the Swedes.

In volunteers in the USA, three doses of WC/BS vaccine afforded 63% protection, and the WC component alone 56% protection, against a subsequent ED₁₀₀ challenge with virulent V. cholerae O1 of the El Tor biotype. Protection against diarrhoeal illness with a stool output of at least 2 litres was 100% for the WC/BS vaccine and 56% for the WC vaccine.

Encouraged by these results, the International Centre for Diarrhoeal Disease Research in Bangladesh, in collaboration with the Government of Bangladesh and the World Health Organization, initiated in 1985, in its Matlab field area, a randomized, double-blind, placebo-controlled field trial of killed oral cholera vaccines. In this trial, 63 000 persons aged 2-15 years and females over 15 years received three doses, at six-week intervals, of (a) WC/BS vaccine, (b) WC vaccine, or (c) a placebo consisting of killed E. coli strain K12. Each dose of WC/BS contained 1 mg of B subunit plus 1×10^{11} killed V. cholerae O1 whole cells, consisting of heat-killed classical Inaba (Cairo 48), heat-killed classical Ogawa (Cairo 50), formalin-killed El Tor Inaba (Phil 6973), and formalin-killed classical Ogawa (Cairo 50), in equal proportions. The WC vaccine had the same cellular constituents, but lacked B subunit.

Each vaccine elicited an approximately two-fold rise in serum vibriocidal antibody titre and WC/BS vaccine also evoked a four to six-fold rise in serum IgG anti-cholera toxin titre. No side-effects could be attributed to either vaccine during the follow-up of vaccinees.

The data on vaccine efficacy against cholera were derived from surveillance of persons attending the three diarrhoea treatment centres which serve the Matlab population. The major findings of the trial are summarized in Table 1. During three years of follow-up of the recipients of three doses, the WC vaccine conferred 52% protection and the WC/BS vaccine 50% protection against culture-proven cholera for all vaccinees ($P < 0.0001$ for each vaccine), including children and adults.

For both vaccines, the protective efficacy was lower in children vaccinated at the age of 2-5 years: 31% and 24% for WC, and 38% and 47% for WC/BS vaccine in the first and second year of follow-up, respectively. In the third year of follow-up there was no protection among children vaccinated at the age of 2-5 years. In contrast, significant levels of efficacy were observed during each of the three years of follow-up in persons who were 6 years or older when vaccinated. The WC vaccine conferred 68% protection and the WC/BS vaccine 63% protection in this age group during the entire three-year period. In the placebo group, 65% of all cases occurred in persons aged 6 years or older.

During each year of surveillance, disease caused by both the classical and the El Tor biotype of V. cholerae O1 was observed, and most isolates were of the Ogawa serotype. Throughout the period of follow-up, protection was greater against disease caused by the classical biotype (Table 1). Each vaccine appeared to confer a similar degree of protection against cholera episodes associated with severe dehydration or against milder disease. However, the protective efficacy against the former appeared to be lower in persons with O blood group than in persons with blood groups A, B, or AB.

TABLE 1: EFFICACY OF KILLED WHOLE-CELL (WC) AND WHOLE-CELL + B SUBUNIT (WC/BS) CHOLERA VACCINES IN BANGLADESH

Group and period of follow-up	Vaccine efficacy (%)	
	WC	WC/BS
<u>Individual years</u>		
First year	53	62
Second year	57	57
Third year	43	17
<u>All 3 years</u>		
Age 2-5 years	23	26
Age ≥6 years	68	63
All ages	52	50
<u>Etiology</u>		
Classical biotype	60	58
El Tor biotype	40	39

WC/BS was more protective than WC against cholera during the first eight months after vaccination; this was a non-epidemic season during which V. cholerae biotype El Tor was prevalent. WC/BS was also associated with short-term (approximately three months) cross-protection against diarrhoea caused by strains of E. coli that produce heat-labile toxin (LT); this is presumably due to the antigenic similarity of the B subunits of CT and the LT of ETEC.

Although the study had not been designed to examine the efficacy of different doses, two doses of vaccine appeared to be as protective as three. One dose, however, did not provide any protection.

Live, attenuated V. cholerae O1 vaccines

During the past decade, a considerable amount of research has been devoted to developing attenuated mutants of V. cholerae O1 for possible use as live oral vaccines. Particular attention has been given to producing mutant strains that are non-toxicogenic (A⁻B⁻ toxin phenotype) or produce only the B subunit of cholera toxin (A⁻B⁺). Several candidate strains have been developed and shown to be immunogenic and protective in volunteers. However, they are not suitable for use as vaccines because they caused mild diarrhoea in 25-40% of volunteers. Recent research has been seeking to determine how these strains cause diarrhoea and to develop new mutants that lack this side-effect but remain immunogenic.

The live vaccine candidate that is currently of greatest interest is V. cholerae O1 strain CVD 103. This is an A⁻B⁺ mutant prepared by recombinant DNA techniques which deleted the genes encoding the A (toxic) subunit of CT from the pathogenic classical Inaba strain 569B, leaving intact production of the immunogenic, non-toxic B subunit. Neither the parent strain nor CVD 103 produce Shiga-like toxin, which is produced by many strains of V. cholerae O1 and is considered to be a possible contributor to the diarrhoea caused by A⁻B⁺ or A⁻B⁻ strains.

When this vaccine was fed to healthy adult volunteers in the USA in a dose of 10⁸ viable organisms, no serious adverse reactions were observed; however, five of 46 volunteers (11%) developed diarrhoea that was not accompanied by other symptoms such as malaise, nausea, cramps, or anorexia. Forty-five of 46 volunteers (98%) developed significant increases in serum vibriocidal antibody, with a geometric mean reciprocal titre of 1339; and 93% had significant rises in serum antitoxin.

In three separate challenge studies, a total of 26 vaccinees who ingested a single 2×10^8 dose of CVD 103 (prepared from strains that differed from CVD 103 either in biotype or in serotype) was challenged one month later with pathogenic V. cholerae O1 in a dose that caused diarrhoea in 24 of 25 unimmunized controls. In each study, the vaccine provided statistically significant protection against illness. Overall, in the three studies, a single dose of CVD 103 conferred 80% protection against any diarrhoea, and 94% protection against severe diarrhoea (>2 litres of stool).

CVD 103 was subsequently modified by inserting a gene that encodes mercury resistance into the hlyA locus of the chromosome, and renamed CVD 103-HgR. This marker allows the vaccine strain to be differentiated from wild strains. A potentially practical, lyophilized formulation of CVD 103-HgR was given to 90 volunteers in the USA in a dose of 5×10^8 viable organisms, while 15 others received 5×10^7 organisms. These doses were well tolerated, although three of the volunteers experienced loose stools. Significant rises in vibriocidal antibody occurred in 91%, the mean titre being three to four-fold higher than in similar volunteers given three doses of WC/BS vaccine. CVD 103-HgR was recovered from the stool cultures of approximately 30% of vaccinees, in contrast with a recovery rate of 90% from recipients of the parent strain, CVD 103. A single dose of CVD 103-HgR also conferred 65% protection against challenge with pathogenic V. cholerae O1 of the heterologous biotype, El Tor Inaba.

In a subsequent study in Thailand (carried out in a containment facility), 12 healthy adult volunteers who ingested a dose of 5×10^8 CVD 103-HgR organisms experienced no adverse reactions; 11 of the 12 vaccinees had significant rises in serum vibriocidal antibody and 9 had significant rises in serum antitoxin. However, additional out-patient studies in military recruits in Thailand, designed to evaluate the safety and immunogenicity of the vaccine, showed much poorer serological responses. This suggests that there may be technical problems with the freeze-dried vaccine preparation (possibly due to reduced viability on reconstitution), and this question is currently under investigation.

Vaccines using live carrier bacteria

Proceeding from the idea that a bacterial antigen can be more immunogenic when it is produced by a carrier organism within the intestine than when it is given orally in non-living form, a bacterial hybrid has been created which contains genes from V. cholerae O1 that specify the synthesis and assembly of cholera LPS on the surface of the live, attenuated oral typhoid vaccine Salmonella typhi Ty21a. This hybrid strain has been evaluated for immunogenicity and safety in about 500 volunteers. Only very minor side-effects were seen. Following three doses of 2×10^{10} live bacteria, serum antibody responses to V. cholerae LPS occurred in about 50%, and vibriocidal antibody responses in about 35% of the recipients. In contrast, 90-100% of the volunteers showed responses to S. typhi LPS. In a volunteer challenge study, three doses of 10^{10} live organisms of a freeze-dried preparation induced only marginal overall protection (25%) against clinical cholera, but the severity of diarrhoea was significantly reduced in comparison with controls.

These studies support the concept of a hybrid cholera vaccine, but have not yielded a practical vaccine with sufficient protective efficacy. The possible advantages of developing a carrier organism such as an avirulent Salmonella as a vector for vaccine antigens include the following: (1) one suitable bacterial vector could perhaps be used to create separate hybrid vaccines for several mucosal infections, and (2) vaccine safety depends primarily on the bacterial vector and may only need to be established once.

However, a number of questions regarding hybrid bacterial vaccines have still to be answered, in particular: (1) would another vector be more effective than Ty21a, but still non-reactogenic? (2) how can the expression of foreign antigens be optimized? and (3) can a single carrier be used sequentially in the same individual to deliver different antigens? Some of these issues are discussed below in relation to the development of candidate vaccines for enterotoxigenic E. coli.

VACCINES AGAINST DIARRHOEA DUE TO ENTEROTOXIGENIC ESCHERICHIA COLI**EPIDEMIOLOGY AND ACQUISITION OF NATURAL IMMUNITY**

Enterotoxigenic E. coli (ETEC) cause disease worldwide, but are especially common in the developing countries. In hospital- and clinic-based studies of acute diarrhoea in developing countries the percentage of cases in which ETEC were identified has ranged from 10 to 50%, with an average of about 20% in children under 5 years of age. Prospective community-based studies in Bangladesh, Brazil, Peru, and several other countries have found that the peak incidence of ETEC diarrhoea occurs in early childhood, particularly in children under 2. Overall, in the first 5 years of life, children in areas with high rates of diarrhoea have 1-2 episodes caused by ETEC per year.

To cause disease, ETEC must be able to (i) colonize the small intestine, and (ii) elaborate enterotoxins. ETEC may produce heat-stable toxin (ST), heat-labile toxin (LT), or both. The proportion of ETEC isolates that produce only ST, only LT, or both enterotoxins varies somewhat from country to country, but each of these toxin phenotypes usually accounts for at least one-quarter of all ETEC. Several studies suggest that ETEC producing both ST and LT are restricted to relatively few O groups, and within these groups to a few O:H serotypes. Strains producing only ST or only LT occur in a wider range of serotypes. Although all ETEC serotypes are found worldwide, there is geographical variation in the relative importance of specific serotypes, and also some temporal variation in individual locations. The prevalence of other important determinants of virulence, such as colonization factors, may also vary geographically, but there is only limited information on this point.

The illness caused by ETEC ranges from mild diarrhoea without dehydration, which is the most characteristic clinical picture, to cholera-like disease. In addition, in endemic areas, at least two-thirds of infections appear to be asymptomatic. In Bangladesh, the highest infection rates occur in young children and decrease with increasing age. The percentage of ETEC infections that are symptomatic also declines as age increases. Both observations are indicative of naturally acquired immunity. These age-related changes have been seen with ETEC that produce ST/LT or only ST, but have not been sufficiently studied in relation to ETEC that produce only LT. Although high titres of serum antibodies to LT are found in young children in endemic areas, these children remain susceptible to diarrhoea caused by LT or ST/LT-producing ETEC. This suggests that the decline in rates of ETEC infection and illness with age is due to immune mechanisms that are not entirely dependent on antibody to LT.

Seasonal peaks of ETEC diarrhoea are often observed in developing countries, generally in the warmer seasons; in some areas they may also coincide with the rainy season. The available evidence indicates that ETEC are transmitted by faecally contaminated food and water. Food may be more subject to contamination in the hot season, in part because E. coli multiply better at higher environmental temperatures.

Studies of travellers' diarrhoea offer a few additional insights into the epidemiology of ETEC and the development of natural immunity. Travellers from industrialized to developing countries experience a high attack rate for diarrhoea (usually at least 30%) during the first few weeks, which appears to lessen with repeated travel or prolonged residence in the developing country, suggesting acquired protection. ETEC are usually the predominant enteric pathogen in travellers' diarrhoea. In 19 studies in Latin America, a median of 46% (range 28-72%) of diarrhoeal episodes was associated with ETEC. In eight studies in Asia, ETEC were found in 14% (range 0-37%) of episodes, and in three studies in Africa, in 36% (range 31-75%) of episodes. The proportion of isolates producing ST, LT, or both and various colonization factors has varied from study to study.

IMPORTANT ANTIGENS OF ENTEROTOXIGENIC E. COLI

To produce diarrhoea, ETEC must have the ability to adhere to the gut epithelial surfaces by means of colonization factors and to produce enterotoxins. Since ETEC possess these common mechanisms, consideration has been given to the possibility of incorporating the relevant antigens in a vaccine that would be effective against a range of ETEC serogroups.

Colonization factors

The best characterized of the human colonization factor antigens (CFAs) are CFA/I, CFA/II, and CFA/IV (formerly called PCF8775). CFA/I is a single fimbrial antigen, whereas CFA/II and CFA/IV are both antigen complexes. Strains producing CFA/II possess either the CS1 or the CS2 (coli-surface-associated) fimbrial antigens and a fibrillar antigen CS3, or may possess CS3 alone. ETEC producing CFA/IV have either the fimbrial antigens CS4 or CS5, as well as the antigen CS6 which is probably non-fimbrial. ETEC that produce only CS6 have also been identified. These factors are all encoded by plasmids which usually also encode enterotoxins (Table 2).

The CFA/I, CS1, CS2, and CS4 fimbriae are all rigid and rod-shaped with a diameter of 6-7 nm. The terminal amino-acid sequences have been elucidated for the residues 1 through 20 and are very similar.

A number of surveys have been carried out to determine the prevalence of these colonization factors in ETEC in different geographical areas. The reported combined prevalence of CFA/I, CFA/II, and CFA/IV has varied from 29 to 79% of the ETEC isolated in a particular area. CFAs were mainly identified on ST/LT and ST-only strains, as would be expected from the fact that plasmids coding for colonization factors usually encode these enterotoxins.

As a result of the inability to detect colonization factors on many ETEC, efforts have been made to identify new factors. The putative colonization factors CFA/III, PCF0159:H4, and PCF0166 are plasmid-encoded fimbrial antigens found, respectively, on E. coli serotypes 025.H16 and H-; 0159.H4 and H20; and serogroups 071, 078, 098, and 0166. Other possible colonization factors have also been described: 334 was reported on a strain of serotype 015.H11, 2230 on a strain of serotype 025.H16, and CFA/VI on a strain of serotype 09.H-; their occurrence on other serotypes has not been examined. PCF0148 and INT407 are factors that occur on strains of the 0148 or 027 serogroups, respectively (Table 2). The definitive role of these various factors in the pathogenesis of diarrhoea has not been examined in animal experiments.

Studies in animals and volunteers have shown that ETEC strains producing CFA/I, CFA/II, and CFA/IV, given orally or intractestinally, induce protective immunity, which suggests that they should be included in vaccines. In passive protection systems in animals, anti-CFA antibodies protect against challenge with ETEC that express the homologous CFA. Furthermore, anti-CFA is synergistic with anti-LT in affording protection against LT diarrhoea caused by ETEC. In rabbits, prior exposure to organisms expressing CFA/I, or the different CS components of CFA/II or CFA/IV, confers significant protection against disease, intestinal colonization, or both following challenge with E. coli carrying the homologous CFA/CS factor. Such protection was not induced by colonization-factor-negative, toxin-negative mutants.

TABLE 2: COLONIZATION FACTORS OF ETEC

CFA	Coli surface antigen combinations	Associated enterotoxin
CFA/I		ST or ST/LT
CFA/II	CS1+CS3 CS2+CS3 CS3	ST/LT ST/LT ST/LT
CFA/III		ST or LT
CFA/IV	CS4+CS6 CS5+CS6 CS6	ST/LT ST ST or LT
PCF0159:H4	ST/LT	ST/LT
PCF0166	ST or ST/LT	ST or ST/LT

Other possible colonization factors include 334, 2230, PCF0148, INT407, CFA/VI. These have been reported but are not well characterized.

CFA - colonization factor antigen

CS - coli-surface-associated antigen

Enterotoxins

Studies in both man and animals have shown that ETEC infection evokes significant antitoxic as well as antibacterial immune responses in the intestine. Antitoxic immunity is directed only against LT; ST is a small polypeptide which is not immunogenic in its natural form. The anti-LT response is directed mainly against the B-subunit portion of the molecule, which cross-reacts immunologically with the B subunit of cholera toxin.

Although STa (the form of ST produced by ETEC that infect man) is not naturally immunogenic, it can give rise to neutralizing antibodies when coupled to a protein carrier; this approach is being used to develop ST antigens for use in vaccines. Both chemical coupling and recombinant DNA techniques have been used to link STa to a variety of carriers. However, chemical conjugates of STa to the B subunit of CT, bovine serum albumin, or STb (a form of ST produced by ETEC that infect piglets) have remained toxic. Several synthetic full-length, or shorter, STa-peptides have been produced in an attempt to identify non-toxic antibody-binding STa epitopes. By replacing one or two of the cysteines of STa by alanine, a non-toxic STa has been produced which binds monoclonal antibodies that neutralize STa.

Based on these results, research is under way to produce ST-protein conjugates through genetic manipulation of bacteria. A synthetic oligonucleotide has been constructed and fused to the structural gene for CT-B subunit in *V. cholerae* 01. This gene construct directs the expression of high concentrations of an STa-CTB fusion protein with substantially reduced residual toxicity. Immunization of experimental animals with this protein evoked detectable, but non-neutralizing anti-STa antibodies. Work is in progress to develop other non-toxic STa-fusion proteins which, it is hoped, will stimulate anti-STa neutralizing antibodies.

In the past few years there has also been significant progress in creating recombinant STa-LT-B antigens. The design of these chimeric proteins has been based on empirical considerations, because there is still little information on how LT-B and STa fold into

their tertiary and quaternary conformations. Portions of the native STa gene as well as synthetic oligonucleotides encoding 18 or 19 amino acids of STa have been inserted at different sites within the gene encoding LT-B. In some studies, single and tandem copies of STa coding sequences have been inserted in LT-B.

Using this approach, STa-LT-B recombinant hybrids have been generated which possess important structural and immunological properties, including stability, high-affinity binding to GM1-ganglioside (the receptor for LT), immunoreactivity with monoclonal antibodies that neutralize ST and LT, and the capacity to induce anti-LT and anti-ST serum responses in rabbits immunized with a partially purified fusion protein. In evaluating the potential of these recombinant STa-LT-B proteins as oral immunogens for protection against ETEC diarrhoea, it will be necessary to ascertain whether they have any residual toxicity and to determine the best mode of delivery, either as components of a killed vaccine or as the products of live, attenuated bacterial vectors.

Determinants of immunity to ETEC as observed in volunteer studies

Studies in adult volunteers in the USA have shown significant protection against re-challenge with the homologous strain after an initial experimental infection with an ST/LT strain of ETEC. In one set of studies, protected volunteers excreted the challenge organism, but failed to develop diarrhoea. In contrast, volunteers who developed diarrhoea after ingesting an LT/ST strain of serotype O148:H28, and who manifested significant rises in LT antitoxin, were not significantly protected against subsequent challenge with an LT-only strain of a heterologous serotype (O25:NM). In another study, volunteers who were immunized with a single dose of an oral attenuated cholera vaccine (CVD 103-HgR) which stimulated strong cholera antitoxin responses were not protected when challenged one month later with an ST/LT ETEC strain. Although data from the field trial of the WC/BS cholera vaccine in Bangladesh suggested that anti-CT provides short-term protection against diarrhoea caused by LT-producing ETEC, this has not been seen in volunteer studies.

Other volunteer studies have indicated that protective immunity may be evoked by fimbrial colonization factor antigens. Volunteers immunized once with an attenuated strain of ETEC (5×10^{10} live bacteria) that expresses CS1 and CS3 fimbriae, but lacks LT and ST genes, exhibited marked rises in sIgA anti-CS1/CS3 antibody in their jejunal fluids. When the vaccinees were challenged with an ST/LT strain of a heterologous O:H serotype which also expressed CS1 and CS3, highly significant protection against diarrhoea was observed. Although there was no difference in the counts of the challenge strain detected in coprocultures, the vaccinees demonstrated significantly diminished colonization of the proximal small intestine (assessed by counts in duodenal fluid specimens) compared with controls, which suggests that the vaccine induced immune mechanisms in the small bowel which interfered with mucosal colonization.

In related studies, passive administration of a cow's milk immunoglobulin concentrate containing high levels of antibody to CFA/I and LT (as well as to other ETEC antigens) provided 100% protection against challenge with ETEC strain H10407 (O78:H11, ST/LT, CFA/I) in a randomized, placebo-controlled, double-blind trial in volunteers. Although this approach would not be of practical public health value, the demonstration of the efficacy of orally administered anti-ETEC antibodies provides further encouragement to seek ways of achieving active immunity with oral vaccines.

CANDIDATE VACCINES

Inactivated whole-cell/purified antigen vaccine

Research is under way on an oral vaccine against ETEC which would consist of killed *E. coli* bacteria representing the major O-groups associated with ST/LT production and expressing the key CFAs in immunogenic form, combined with the B subunit of LT or CT, or a non-toxic STa-B-subunit conjugate. The treatment of bacteria with mild formalin causes complete killing, but retention of 50-100% of the antigenicity of the different colonization factors. The CFAs of inactivated organisms are stable at 4°C for at least 8 months and when incubated in acid gastric juice. An evaluation of the safety and immunogenicity of this vaccine in volunteers is planned for the near future.

Colicin-E₂-treated whole-cell vaccine

A novel method of killing ETEC bacteria has been developed which does not damage the protein antigens associated with the organisms. This involves treatment with colicin E₂, an endonuclease which enters the cell by means of receptors on sensitive strains of E. coli. Killed bacteria show no change in the antigenicity or concentrations of LT enterotoxin, CFA/I, or flagellar antigens. Colicin-E₂-treated ETEC preparations have been tested as candidate vaccines in animals and volunteers.

Two oral doses of 3×10^{10} freshly prepared colicin-E₂-killed E. coli strain H10407 given one month apart induced both serum IgG and intestinal IgA antibody responses to LT enterotoxin and CFA/I in 29 of 32 adult volunteers. None of the 22 placebo-immunized controls showed increased levels of these antibodies. To evaluate the protection induced by colicin-E₂-killed organisms, groups of nine or 10 subjects consisting of approximately equal numbers of vaccinees and placebo-treated controls were challenged with virulent ETEC six to eight weeks after immunization. The vaccinees showed 75% protection against diarrhoea when challenged with either homologous or heterologous strains of ETEC. The heterologous challenge was with a strain that differed in both serotype and CFA type, which suggests that other protective antigens may be important. In another challenge study, protection was demonstrated six to eight months after vaccination. Methods of preserving colicin-E₂-killed E. coli have not yet been developed.

Avirulent salmonellae as carriers for ETEC antigens

Using strain Ty21a as the vector, a potential multivalent live oral vaccine expressing LT-B subunit has been constructed. The S. typhi derivative, strain SE12, induced a significant serum anti-LT response when injected parenterally into mice and guinea-pigs. The potential of this strain as a live oral vaccine for ETEC has not been tested in animals owing to the limited range of hosts of S. typhi.

More recently, an avirulent aroA⁻ mutant mouse strain of S. dublin (SL1438) has been used as the vector strain. This strain produces an infection in orally inoculated mice analogous to that produced in man with Ty21a. The strain was transformed with a plasmid carrying genes for the production of LT-B; the derivative strain produced LT-B and induced high levels of serum IgG antitoxin and intestinal sIgA antitoxin in orally inoculated mice. These mice also developed progressively increasing mucosal and serum antibody responses to the LPS of the vaccine strain. In further studies in mice, only a strain with a single aroA mutation has been able to colonize significantly, invade, and persist in tissues.

In accordance with the latter results, recent studies in humans have shown that recipients of aroA⁻, purA⁻ S. typhi mutants develop only low serum antibody responses to the O polysaccharide of the vaccine strain. These observations suggest that the purA defect, which creates a requirement for exogenous adenine, reduces the efficacy of the attenuated Salmonella as a live oral vaccine, and would probably limit the effectiveness of salmonellae as vectors for heterologous antigens. As an alternative to the aroA single-deletion mutant, strains with two mutations in the aroA pathway should be considered as potential vectors for ETEC antigens. Two mutations are considered desirable to ensure that the strain does not revert to virulence.

Effect of multiple use on vector efficacy

An important consideration concerning the use of Salmonella or other live bacteria as vectors for heterologous antigens is whether they would remain effective when used repeatedly, especially for the delivery of unrelated antigens. It is possible that pre-existing immunity to the vector arising from its initial use, or from natural exposure, may interfere with the sequence of colonization, replication, and antigen delivery, and thus limit the immunological response to the heterologous antigen produced by the vector. This effect has been demonstrated in rabbits immunized sequentially with A⁻B⁻ and A⁻B⁺ strains of V. cholerae, which showed markedly reduced mucosal IgA antitoxic responses compared with controls given only A⁻B⁺ strains. Similarly, there is preliminary evidence that mice immunized with a vector (Salmonella SL1438) and then re-immunized with the same vector expressing a heterologous antigen (LT-B) develop lower serum IgG and mucosal IgA anti-LT-B responses than mice not previously immunized with the vector.

RESEARCH RECOMMENDATIONS

I. CHOLERA VACCINES

1. Killed oral whole-cell (WC) vaccine

Further research on WC vaccine should seek to increase its efficacy and duration of protection, especially against disease caused by V. cholerae O1 El Tor, and in young children; efforts are also needed to simplify vaccine delivery. Possible approaches to improving vaccine efficacy include (1) incorporating in the vaccine an El Tor strain from the current pandemic, (2) increasing the quantity of bacteria per dose, (3) ensuring full expression of TCP antigen in the vaccine, and (4) including strains that produce or hyper-produce B-subunit. Simplified vaccine delivery might require (1) a capsule or soluble tablet formulation that is stable at ambient temperatures, (2) a more practical immunization schedule, and (3) administration with a buffer.

2. Live oral cholera vaccine

The objective should be to develop a live vaccine strain that colonizes the intestine efficiently, is non-reactogenic, and contains a stable genetic marker to distinguish it from wild-type strains. Prior to field trial, candidate strains should be studied to determine the extent and duration of their efficacy in adults and their safety and immunogenicity in adults and children. The efficacy of killed whole-cell vaccine and live strains should be compared in volunteers. Transmissibility to non-vaccinated persons and dispersion into the environment should be evaluated in respect of any candidate live oral cholera vaccine.

Further research is required to define the mechanism by which non-toxigenic mutants of V. cholerae O1 cause diarrhoea, with the objective of developing candidate vaccine strains that lack this capacity but retain the other qualities required for efficient induction of protective immunity.

3. Alternative designs for cholera vaccine trials

Research is needed to develop novel designs and new sites for future trials of cholera vaccines. If possible, the designs should be more efficient than those of the traditional large-scale prospective trials. Designs are also required that address specific issues, such as vaccine efficacy in epidemic settings, in young children, and in family settings.

4. Cost-effectiveness evaluation of selected vaccines

Cost-effectiveness analyses should be carried out in respect of vaccines that have been evaluated by field trial and found to be at least moderately effective. The analysis should be based on conditions in the country in which the trial was performed, but may also attempt to predict cost-effectiveness in other settings.

II. ENTEROTOXIGENIC E. COLI VACCINES

1. Potential vaccine antigens

(a) Colonization factor antigens: The prevalence of specific colonization factor antigens (CFAs) among ETEC isolated from young children with acute diarrhoea in developing countries needs to be better defined; this is especially true for Africa. The CFAs to be identified should at least include CFA/I, CFA/II, CFA/III, CFA/IV, PCF 0159:H4, and PCF 0166. CFA prevalence should be determined in prospective, population-based epidemiological studies. Research to identify and characterize new CFAs should be continued.

To facilitate epidemiological studies, standardized reagents should be developed for the identification of CFAs. These might include polyclonal or monoclonal antibodies, and DNA probes.

(b) STa-toxoids: Research to develop safe and immunogenic STa-toxoids, based either on STa-protein conjugates or chimeric proteins (e.g., STa-LT-B) synthesized by genetically manipulated bacteria, should continue. Candidate conjugates or chimeric proteins should be evaluated for residual toxicity and immunogenicity. Using such antigens, the potential protective role of anti-ST should be evaluated in animals and, if possible, volunteers. Practical approaches to the inclusion of safe and protective STa-toxoids in candidate oral ETEC vaccines should be explored.

(c) Possible common protective antigens: The possibility that antigens may evoke cross-protection against ETEC strains that differ with regard to serogroup, CFA type, and production of LT requires further study in volunteers and animal models. If such cross-protection is confirmed, its extent should be defined and an attempt made to identify and characterize the responsible antigen(s). Killed whole-cell vaccines should be prepared by methods that are known to preserve such antigens in immunogenic form.

2. Killed oral whole-cell (WC) vaccines

Research to develop a safe and effective killed oral WC vaccine for ETEC diarrhoea should continue. Consideration should be given to including bacteria that represent the most important ETEC serogroups and produce the most prevalent CFAs. The benefit of including a non-toxic STa-protein conjugate (if available) or LT-B, or both, should be evaluated. For antigens of presumed importance, particular care should be taken to use methods of killing and preserving bacteria that retain immunogenic activity. Candidate vaccines should be tested for safety, immunogenicity, and efficacy in adult volunteers. Those that prove promising should also be evaluated for safety and immunogenicity in children.

With regard to oral WC vaccine based on colicin-E₂-treated bacteria, further evidence of protective efficacy against heterologous challenge strains should be sought and the range of such protection determined in volunteer studies. If this approach proves effective, the efficacy of other vaccine strains (different serogroups and CFA types) should be evaluated. This may require a search for appropriate strains that are sensitive to colicin E₂.

3. Live, attenuated ETEC vaccines

Efforts are needed to develop and evaluate the safety and immunogenicity of ETEC strains that express selected CFAs and possibly LT-B or an STa-LT-B conjugate. The safety and efficacy of such strains given singly and in combination should be tested in volunteers.

III. RESEARCH RELEVANT TO BOTH CHOLERA AND ETEC VACCINES

1. Preservation of live bacterial vaccines

Research is required to develop methods of preserving live bacterial vaccines that ensure maximum viability and uniform bacterial characteristics, particularly as regards antigenic composition, with a minimum of variation between production lots. This may require an evaluation of preservation methods other than lyophilization.

2. In-vitro correlates of immunity

Practical measures for predicting the protective value of candidate vaccines or immunizing regimens are required. In some instances, serum antibody responses may prove satisfactory as proxy measures of protective mucosal immune responses. In others, a simple and accurate measure of the local immune response will probably be required.

3. Improved bacterial vectors

Research to identify improved bacterial vectors for the delivery of selected antigens of V. cholerae O1 and ETEC should continue. Ideally, vectors should evoke a vigorous mucosal immune response after a single dose without significant side-effects; this will no doubt require that the vector colonizes or penetrates the small bowel mucosa, at least transiently. The Ty21a strain of S. typhi should be regarded as a prototype vector; further research is needed to optimize the expression of one or several foreign antigens by this vector.

4. Mucosal adjuvants

The development of efficient killed oral vaccines for cholera and ETEC diarrhoea, as well as other oral or topical vaccines, may require the use of adjuvants that enhance the mucosal (sIgA) immune response. Research is required to identify such adjuvants and optimize their efficacy. Mucosal adjuvants for practical use should be safe, relatively inexpensive, and probably have an effect that is "focused" on the vaccine antigens.