crease in enterotoxin production. Based on their findings, toxin-enriched culture filtrates for separation studies have been produced by the Centre for Applied Microbiology and Research, Porton Down, in collaboration with this laboratory, using a 20 litre batch fermentation technique with controlled pH 7.5, aeration of 10 litres/min, agitation at 500 rev./min and incubation at 36°C for 5-6 h. Cells were removed by continuous centrifugation and the enterotoxin extracted. Concentrated culture filtrates using narrow-range preparative isoelectric focusing in dextran gel (Turnbull et al., 1979b).

The diarrhoeal enterotoxin is a thermolabile antigenic protein produced to some degree by most strains of *B. cereus*. As a metabolite it is distinct from phospholipase C and the two haemolysins of *B. cereus*, and constitutes one of the two lethal factors produced by this organism (the other being the thiol-activated haemolysin, cereolysin). The instability of the enterotoxin, its sensitivity to proteolytic enzymes, the partial loss of toxigenicity in strains on repeated sub-culture and the problems encountered in separating it from other metabolites of *B. cereus* have all contributed to the slow progress in purification of the product. Although complete purification and characterization are still awaited there is now good evidence that this enterotoxin is responsible for the diarrhoeal syndrome of illness, and that the dermonecrotic and intestinonecrotic properties are relevant in the pathogenesis of the various non-gastrointestinal *B. cereus* infections (Turnbull & Kramer, 1983).

The emetic toxin produced by *B. cereus* has only been partially purified (Melling & Capel, 1978; Melling et al., 1978). Optimal production occurs in a rice culture slurry incubated at 25-30°C during the stationary growth phase of the organism (Fig. 2) and synthesis may be associated with sporulation. This toxin is a non-antigenic polypeptide of low relative molecular mass possessing remarkable properties in its stability to heat, pH extremes and enzymes. Strains of *B. cereus* that synthesize this toxin are not exceptional with respect to their ability to produce other metabolites including the diarrhoeal toxin, but until a simpler model than monkey feeding is available it is not possible to predict the proportion of strains that might be able to elaborate the toxin. Epidemiological evidence, however, that 75% of strains incriminated in the emetic syndrome outbreaks are serotype 1 suggest that only certain strains may be able to produce this toxin; the spores of serotype 1 strains also exhibit a markedly higher resistance to heat than spores of other serotypes.

A detailed review of the principal toxic metabolites produced by *B. cereus* has been presented by Turnbull (1981).


Present status of cholera vaccines

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Strong impetus for the development of improved cholera vaccines stems from the observations in epidemiologic (Mosley et al., 1968; Glass et al., 1982) and experimental challenge studies in volunteers that an initial clinical cholera infection is followed by potent, long-lasting immunity. In endemic areas such as Bangladesh the incidence of cholera is higher in children of 2-4 years of age and diminishes in older ages, while the prevalence of serum vibriocidal antibody increases with age (Mosley et al., 1968). These epidemiologic observations point to the development of acquired immunity.

Abbreviation used: SIgA, intestinal secretory immunoglobulin A.

In volunteers, prior clinical infection with either Ogawa or Inaba serotype of classical or El Tor biotype induces solid clinical protection against re-challenge with vibrios of the homologous or heterologous serotype (Cash et al., 1974; Levine et al., 1979; Levine, 1980; Levine et al., 1981a, b); significant protection has been shown to endure for at least 3 years (Levine et al., 1981b). The goal of the development of new cholera vaccines is to achieve comparable long-lasting immunity without causing adverse reactions.

Cholera vaccines

Immunizing agents stimulate antigenic immunity, antibacterial immunity or both; antibacterial and antigenic immunity probably work synergistically. In the past, most vaccines tested in man were inactivated parenterally. Since *Vibrio cholerae* does not penetrate the mucosa, immunity at the mucosal surface mediated by SIgA is of paramount importance. Thus current research involves oral immunization to optimize stimulation of intestinal SIgA antibodies.
Killed whole cell vaccines. (a) Parenteral whole cell vaccines. Killed whole *V. cholerae* organisms have been employed as parenteral vaccines since the end of the 19th century (Feeley & Gangarosa, 1980). In field trials they have provided efficacy of 40–80% for a short duration (3–6 months) (Feeley & Gangarosa, 1980). (b) Oral whole cell vaccines. Killed whole *V. cholerae* administered orally stimulate local intestinal antibody (Ganguly et al., 1975) and induce significant protection against experimental challenge (Cash et al., 1974).

Toxoids. Immunizing agents intended to stimulate anti-toxic immunity include formaldehyde-treated cholera toxoid, glutaraldehyde-treated cholera toxoid, purified B subunit and pro-choleraegoid.

Experiences with parenterally-administered toxoids have been generally disappointing. One formalinized toxoid reverted and caused unacceptable adverse reactions at the site of inoculation (Northrup & Chisari, 1972). A non-reactogenic formalinized toxoid and a glutaraldehyde-treated toxoid gave little or no protection when tested as parenteral vaccines in field trials in the Philippines (Nori, 1976) and Bangladesh (Curn et al., 1975). An aluminium-adjuvanted formalin toxoid stimulated increases in blood vibriocidal antibody when given parenterally to lactating Bangladeshi women but was not field tested for efficacy (Merson et al., 1980).

The glutaraldehyde-treated cholera toxoid also failed as an oral vaccine. Neither three 2.0 mg doses nor three 8.0 mg doses given a month apart provided significant protection against experimental challenge (Levine et al., 1979; Levine, 1980).

Purified B subunit has been given as an oral or parenteral vaccine to persons living in both endemic (Bangladesh) and non-endemic areas without adverse reactions and stimulated significant rises in intestinal SfG A antitoxin after two or three oral doses (Svennerholm et al., 1982).

Procholeragenoid is the large molecular mass (c. 1 x 10^8 daltons) toxoid that results when cholera enterotoxin is heated at 65°C for at least 5 min. Procholeragenoid is highly immunogenic while containing less than 5% of the biological toxic activity of the parent toxin. Working with dogs, Pierce et al. (1983) found procholeragenoid to be well tolerated as an oral vaccine and five 500 μg doses spaced over 28 days stimulated significant protection against oral challenge with pathogenic *V. cholerae*.

Doses of 50 μg and 200 μg of procholeragenoid prepared by the Swiss Serum and Vaccine Institute were given (with NaHCO₃ to volunteers (M. M. Levine, R. E. Black, M. L. Clements & R. Germanier, unpublished work) without causing adverse reactions.

Combination vaccines. The major attraction of non-living oral cholera vaccines is safety. Vaccines consisting of a combination of antigens intended to stimulate both antibacterial and antitoxic immunity would be most likely to succeed.

Three studies so far have been carried out in man with combination oral vaccines. In the first, nine volunteers who ingested glutaraldehyde-treated cholera toxoid (2 mg weekly for 4 weeks) plus killed El Tor Inaba vibrios (10^10 vibrios twice weekly for 4 weeks) were challenged after 1 month with 10^6 pathogenic El Tor Inaba vibrios, along with six unimmunized controls. Diarrhoea occurred in only two of nine vaccinees compared with four out of six controls (vaccine efficacy 67%) and illness was clearly attenuated in the two ill vaccinees. *V. cholerae* could be directly cultured from stools of one out of nine vaccinees compared with six out of six controls, suggesting that immunologic mechanisms impeded the proliferation of vibrios.

More recently, three oral doses of B subunit/killed whole cell vaccine (provided by J. Holmgren, Gothenburg, Sweden) were given to adult volunteers who participated in a vaccine efficacy challenge study (R. E. Black, M. M. Levine, M. L. Clements, J. Holmgren & A.-M. Svennerholm, unpublished work). The combination vaccine was given on days 0, 14 and 28. Each of the three doses of vaccine contained 5.0 mg of purified B subunit and 2 x 10^11 killed *V. cholerae* (5 x 10^12 classical Inaba, 5 x 10^10 classical Ogawa and 1 x 10^11 El Tor Inaba). A group of 11 volunteers immunized with this combination vaccine were challenged 1 month after their last dose with 10^6 pathogenic *V. cholerae* El Tor Inaba, along with seven control volunteers. Diarrhoea occurred in seven out of seven controls but in only four out of 11 vaccinees (*P* = 0.01). The illness in the four vaccinees was significantly milder as quantified by total diarrhoeal stool volume and total number of loose stools.

In a third study, a group of 15 volunteers received three oral doses (days 0, 14 and 28) of a procholeragenoid/killed whole cell vaccine combination (prepared by R. Germanier, Swiss Serum and Vaccine Institute, Berne). The whole cell component contained both Ogawa and Inaba serotypes of both biotypes, 5 x 10^10 organisms each. The bacteria were heat-treated in the presence of formalin. Two doses of procholeragenoid contained 50 μg, while the third dose had 200 μg. These 15 immunized volunteers and six controls participated in a challenge study to assess vaccine efficacy. Attack rates were not significantly different between the vaccines (11 out of 15) and controls (six out of six) and by this parameter vaccine efficacy was only 27%. However, by other parameters, such as total diarrhoeal stool volume (1.61 vs 9.4, *P* < 0.05) and total number of loose stools (6.5 vs 22.0, *P* < 0.05), it was apparent that illness was significantly attenuated in vaccines as compared with controls.

Thus studies with oral toxoid/killed whole cell vaccine combinations demonstrate complete safety and a moderate (27–67%) degree of efficacy; however, multiple doses are required to induce protection.

Attenuated *V. cholerae* vaccines. Another highly promising approach toward immunologic control of cholera is by means of attenuated non-enterotoxigenic *V. cholerae* strains employed as oral vaccines. (a) Naturally-occurring strains. Non-toxigenic *V. cholerae* O1 strains isolated from environmental sources in India and Brazil have been evaluated in volunteers as potential vaccine candidates with promising results (Levine et al., 1982). They either failed to colonize the intestine of man or did so minimally; vibrio-cidal antibody responses were meagre; and they failed to provide protection in experimental challenge studies.

(b) Chemically mutagenized attenuated strains. Honda & Finkelstein (1979) mutagenized El Tor Ogawa 3083 with nitrosoguanidine and analysed thousands of colonies to detect one (Texas Star) that continued to produce the immunogenic B subunit while failing to produce detectable A (enzymically active) subunit or holotoxin.

Texas Star-SR has been extensively evaluated in volunteers who ingested 10^5–5 x 10^10 organisms (Levine et al., 1983). Mild diarrhoea was seen in 16 of 68 vaccinees (24%), typically consisting of two or three small loose stools totalling less than 400 ml in volume. Vaccine organisms were recovered from coprocultures of approx. one-half of the vaccine recipients while jejunal fluid cultures were positive in 76%. Hundreds of Texas Star clones were recovered from coprocultures, and jejunal fluid cultures were examined for cholera holotoxin by the sensitive Y-1 adrenal cell assay; none were positive.

Significant rises in serum antitoxin were detected in only 29% of the vaccinees; however, 93% manifested significant rises in serum vibriocidal antibody and the titres mimicked those encountered following infection with pathogenic *V. cholerae*. In experimental challenge studies in volunteers,
one or two doses of Texas Star-SR conferred significant protection against challenge with either El Tor Ogawa or El Tor Inaba vibrios.

Studies with Texas Star have provided invaluable data to support the concept of using attenuated strains to mimic infection-derived immunity to choleragen. However, the Texas Star strain suffers from certain drawbacks. (1) The method of attenuation, mutagenesis with nitrosoguanidine, induces multiple mutations, not all of which are necessarily recognized. (2) The genetic lesion presumed to be responsible for the attenuation of Texas Star is not known. Therefore, until this is clarified there remains the theoretical possibility for reversion to virulence. (c) Vibriophage induced mutants. One method to overcome reversion to toxigenicity was reported by Mekalanos et al. (1982) who employed mutagenic bacteriophages to delete DNA sequences encoding cholera toxin. So far no mutants derived by this method have been evaluated in man. (d) Genetically engineered mutants. Kaper et al. (1981, 1983) proceeded to develop an attenuated V. cholerae strain having none of the drawbacks of a chemically mutagenized strain such as Texas Star by using recombinant DNA techniques. Through the use of these techniques, precise, non-reverting deletions can be introduced into the chromosome which eliminates toxigenicity without affecting the production of other antigens important for immunity. Starting with V. cholerae N16961, a strain that produces both disease and protective immunity in volunteers, a 21 kilobase chromosomal fragment containing the cholera toxin genes was cloned from N16961 and the toxin genes mapped. Restriction enzymes were employed to specifically delete the cholera toxin genes in vitro and genes encoding resistance to mercury cloned in their place. This mutation was then introduced into the chromosome of V. cholerae N16961 by the marker rescue technique and resulted in a derivative of N16961 in which the toxin genes were displaced by the mercury resistance genes. This mutant, designated V. cholerae JBK70, produced neither A nor B subunit of choleragen and, because both structural genes for cholera toxin were deleted, was incapable of reversion.

JBK 70 was fed to 14 volunteers in doses of 10, 10 or 10 organisms. Coprocultures of 13 showed heavy colonization and vibriocidal antibody responses were equal to those following choleragen. As with Texas Star, some individuals developed loose stools. In a challenge study, cholera occurred in seven out of eight controls but in only one of ten vaccinees immunized with a single dose of JBK 70 (P < 0.003). This is the first vaccine to provide efficacy comparable with that due to pathogenic vibrios and does so without stimulation of choleragen antitoxin, stressing the importance of antibacterial immunity.

A plasmid directing production of B, but not A, subunit was introduced into V. cholerae JBK70 to produce an A – B- derivative. In addition, strains of the Ogawa serotype have been similarly treated to attenuate them by deletion of the toxigen genes. Volunteer studies are underway to further evaluate the safety and efficacy of vaccine candidates produced by this method.


Antimotility drugs in the treatment of acute diarrhoea

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Every year more than 18 million people die of diarrhoeal diseases; 5 million of these are children under the age of 5 years. Although most deaths are due to severe acute secretory diarrhoeas in malnourished patients in the third world, acute diarrhoea is still a major cause of death in infants in the developed world. Despite the enormity of this problem surprisingly few antidiarrhoeal drugs are available, and their classification into absorbents, astringents, and antimotility agents has remained unchanged for over a century. Recent advances in gastrointestinal physiology have revealed new potential therapeutic agents and thrown further light on the mode of action of some well-established drugs.

Pathophysiological considerations

A number of mechanisms may contribute to the production of diarrhoea but the importance of intestinal electrolyte secretion in the pathogenesis of acute diarrhoeas

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