with lower affinity than those induced by other protein carriers (9). However, neither of these factors has been problematic in the case of the Hib-OMP vaccine (9, 16).

Several manufacturers are evaluating pneumococcal conjugate vaccines in phase I and phase II studies in children and infants. Initial results with five- and seven-component vaccines indicate that infants respond well to each serotype, but require three doses to achieve antibody responses reliably. Efficacy trials may begin as early as 1995. A variety of studies are planned, including large-scale trials with sufficient power to detect significant reductions in bacteremic pneumococcal infections (a rare outcome for which high efficacy is expected) and in pneumonia and middle ear infections (common outcomes for which lower rates of efficacy are expected). These trials should also seek to establish serologic correlates of protection so that future vaccines can be evaluated on the basis of antibody responses. To achieve this goal, current efforts to standardize antibody assays by the World Health Organization, the Centers for Disease Control, and the Food and Drug Administration are vital.

Alternative approaches to pneumococcal vaccines are also being considered. Immunization with noncapsular antigens that might induce protection against all serotypes would be advantageous either instead of, or as a complement to, polysaccharide-based vaccines. Such a vaccine could enhance protective efficacy of type-specific conjugate vaccines and extend protection to strains not included in the vaccine. The C-polysaccharide antigen, found in the cell wall of all pneumococci, has been proposed as a candidate (17), as have several protein antigens including pneumolysin, pneumococcal surface protein A (PspA), and a 37-kD surface protein (18). Attractive as the noncapsular antigens are, skeptics point out that the "proof of principle" that they can provide broad-based, species-specific protection has not been satisfactorily demonstrated in animals or in humans (7, 19, 20).

It seems likely that the next generation of pneumococcal vaccines will be based on anticapsular immunity induced by protein-polysaccharide conjugates. Trials may be completed as early as 1998, so these vaccines could come into use before the end of the century. The availability of a vaccine that is effective in infants will enable us, for the first time, to integrate pneumococcal vaccine into routine childhood immunization schedules and thus substantially reduce the burden of pneumococcal disease in the United States and globally. An important remaining challenge will be to reduce the cost of manufacturing protein-polysaccharide conjugate vaccines so that they will be accessible to children throughout the world.

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# Cholera Vaccines: Fighting an Ancient Scourge


John J. Mekalanos and Jerald C. Sadoff


Cholera is a potentially lethal diarrheal disease caused by the Gram-negative bacterium *Vibrio cholerae*. This disease has a long history; descriptions of it can be found in Sanskrit, Arabic, and Chinese writings dating back at least two millennia. Since 1871, seven cholera pandemics have occurred (1). The first six were probably caused by the classical biotype of *V. cholerae*, whereas the seventh, which began in 1961 and continues today, is caused by the El Tor biotype. Strains of these two biotypes differ in a variety of properties, including production of intestinal adherence factors and other virulence determinants. There have been nearly a million estimated cases of El Tor disease in Latin America since its introduction in Peru in 1991.

Until recently, epidemic strains of both biotypes characteristically produced a serotype lipopolysaccharide O antigen, termed O1, which is known to be one of the primary targets of a protective immune response to cholera. In October 1992, however, a new serogroup of *V. cholerae* (designated O139 or "Bengal") was recognized as the cause of a massive cholera epidemic in South Asia (see figure) (2). The O139 strains are closely related to El Tor O1 strains but have acquired new genetic information encoding a distinct lipopolysaccharide O antigen and a polysaccharide capsule (3). Epidemiological data suggest that prior immunity to the O1 serogroup of *V. cholerae* offers little protection against the O139 strains. The incidence of cholera in endemic regions of the world, together with the global emergence of new epidemics, emphasize the need for effective cholera vaccines.

Ideally, a cholera vaccine should offer a high degree of safety and should induce long-term immunity against both overt disease and asymptomatic intestinal carriage of *V. cholerae* (the latter being responsible for the majority of infections in endemic areas). The vaccine should also be inexpensive, easy to administer, and afford protection against both the O1 El Tor biotype and the new O139 serogroup. Recent work indicates that the cholera vaccines now under study may approach several of these goals.

Despite 11 decades of research, efforts to develop effective cholera vaccines have produced few successes (4). Parenterally administered vaccines (killed whole-cell, lipopolysaccharide, and toxoid) have been largely abandoned because these vaccines induce only weak or short-term immunity. The limited success of these vaccines is attributed to their inability to induce a local intestinal or "mucosal" immune response. Such mucosal immunity appears to be a critical feature of natural convalescence from cholera, a highly immunizing process that provides long-lasting protection.

The strong mucosal immunity elicited by *V. cholerae* results from direct exposure of intestinal epithelial surfaces to bacterial antigens. In principle, orally administered vaccines could provide analogous exposure. Two different types of oral vaccines are being actively pursued. The first is a mixture containing the nontoxic B subunit of cholera toxin and killed whole bacterial


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cells (BS-WC). A randomized, double-blind, placebo-controlled field trial involving 63,498 individuals in rural Bangladesh established the safety, immunogenicity, and efficacy of the BS-WC vaccine (5). Two or three doses of the BS-WC vaccine conferred 85% protection against cholera for the first 6 months in all age groups tested. This protection was most evident against the classical biotype of *V. cholerae*. However, after 36 months, protection fell to 51% overall, with young children showing the most rapid loss. Remarkably, in the first 3 months of the trial, the BS-WC vaccine was shown to confer 75% protection against diarrhea caused by strains of *Escherichia coli* that produce an enterotoxin immunologically cross-reactive with cholera B subunit. This type of vaccine should be adaptable to new serogroups of *V. cholerae* as they arise, a concept that will be tested by inclusion of an O139 strain into the killed whole-cell component of BS-WC vaccine. Potential limitations of the BS-WC vaccine include its complex manufacturing process (although recombinant DNA technology has already had a positive impact), its reduced efficacy against El Tor strains, its reduced protection of individuals within the O blood group, and its inability to block intestinal carriage of El Tor strains.

Live attenuated *V. cholerae* vaccines, the second type of oral vaccine, may offer some advantages over inactivated vaccines (4). Live vaccines can be inexpensive to manufacture and easy to administer (in a single oral dose) and are likely to induce immune responses that best mimic natural convalescence from cholera because they allow local expression of bacterial antigens that are not readily produced in cell culture. M. M. Levine and colleagues showed this to be true for both an El Tor nonrecombinant vaccine, Texas Star-SR (6), and a recombinant vaccine, JBK70 (7). For example, a single oral dose of the JBK70 vaccine conferred 89% protection against diarrheal disease in volunteers challenged 1 month later with a virulent El Tor Inaba strain. However, both of these vaccine prototypes induced moderate side effects in a significant subset of vaccinees.

Other recombinant vaccines constructed from the classical biotype (8–10) also displayed similar "reactogenicity" in volunteers (manifested as diarrhea, loose stools, abdominal cramps, vomiting, fever, nausea, or malaise). Several hypotheses have been put forth to explain the adverse side effects of live cholera vaccines. One proposal, that a second bacterial toxin might be responsible (4), has been discounted because appropriately deleted strains still display a high degree of reactogenicity in clinical studies (10, 11). An alternative explanation is the "colonization-reactogenicity" hypothesis (4),

**The Grim Reaper.** The O139 strain of *V. cholerae*, first identified in October 1992, has spread rapidly throughout India and Bangladesh. Shown is a scene outside a hospital in Dahka, where a makeshift tent is filled to capacity with cholera cots. [Photograph by J. J. Mekalanos]

which suggests that bacterial colonization (persistence and multiplication of a bacterium within the small intestine) induces symptoms by a mechanism that does not involve a bacterial toxin per se (12). Consistent with this proposal is the observation that *V. cholerae* vaccine strains that display the least reactogenicity are also the most poorly shed by volunteers, suggesting that they colonize less avidly than reactogenic strains (7, 10, 11, 13).

Currently, the only live attenuated O1 vaccine that has advanced beyond phase II clinical studies is strain CVD103-HgR, a derivative of classical biotype strain 569B (14). Deletion of functional *ctxA* genes from CVD103 (the progenitor of CVD103-HgR) and CVD103-HgR is the primary basis for attenuation, although it is unclear why these derivatives are less reactogenic than previous strains with similar deletions. Interestingly, only low numbers of CVD103-HgR are shed by vaccinees, which suggests that reduced colonization may be associated with attenuation. A single oral dose of CVD103 conferred 87% and 82% protection when volunteers were challenged 1 month later with the homologous Inaba and Ogawa strains, respectively (14). However, CVD103-HgR was less effective when volunteers were challenged with the heterologous El Tor Inaba and Ogawa strains, and it did not significantly reduce carriage of an El Tor challenge strain (14). A large field trial is in progress in Indonesia to assess the efficacy of a lyophilized oral formulation of CVD103-HgR.

Safety is a critical issue when dealing with live attenuated vaccines (15). The possibility that attenuated *V. cholerae* vaccine strains could revert to virulence was a legitimate concern until recombinant DNA technology provided the means to delete the genes encoding essential virulence determinants such as cholera toxin (7, 8). However, some recombinant vaccines can reacquire cholera toxin and other virulence genes by recombinational mechanisms. For example, the CVD103-HgR strain has been shown to reacquire the cholera toxin A subunit gene from a *V. cholerae* strain possessing a conjugative sex factor (9). Although it has been argued that such transfer would be rare in nature (9), it is sobering to note that horizontal gene transfer led to the emergence of the O139 serogroup (2, 3).

Another matter of concern with respect to vaccine safety is the finding that the cholera toxin genes reside on a large DNA segment (the CTX genetic element) that is a site-specific transposon (16). This element also encodes the Zot and Ace toxins and an intestinal colonization factor (10, 16). Recently, strains of *V. cholerae* have been engineered so as to remove the entire CTX element together with its requisite chromosomal target site for insertion, *attRS1* (16). Such *attRS1* deletions have been introduced into both El Tor O1 and O139 vaccine candidates together with deletions in *recA* (thereby inactivating site-specific and homologous recombination in the vaccine strain) (11). The resultant strains, Peru-3, Peru-14, Bengal-3, and Bengal-15, offer an extremely high level of safety in terms of recombinational reversion (15).

Clinical studies on these El Tor O1 and O139 vaccines have been encouraging. Two types of motility-deficient vaccine derivatives, one a filamentous mutant (Peru-14) and the other a nonmotile mutant (Bengal-15), have been tested in volunteers and compared to their motile, isogenic counterparts (Peru-3 and Bengal-3, respectively). Peru-14 was well tolerated compared to Peru-3 and conferred 80% protection against the El Tor challenge strain N16961 (11). In another study, Bengal-15, the nonmotile O139 vaccine candidate, was also found to be well tolerated, and conferred 83% protection against challenge by an O139 strain, MO10 (17). Asymptomatic carriage of the El Tor O1 and O139 challenge strains was substantially reduced or delayed in the vaccinees in both studies. Thus, mutagenesis of motility-chemotaxis genes merits further exploration as a way to reduce the reactogenicity of attenuated *V. cholerae* vaccines.

Over the next few years both inactivated and live attenuated cholera vaccines are likely to become available for use as public health tools. The target groups for these vaccines would include entire populations living in endemic areas, refugee camps, and possibly travelers to such locales. One must ask whether the control of cholera justifies

the expense and logistical challenge associated with the wide application of these vaccines (*18*). Cholera and other diarrheal diseases are relatively easy to treat by intravenous and oral rehydration therapies. However, in the grip of explosive epidemics, medical facilities can be overwhelmed and significant mortality can result. For example, early in the Matlab, Bangladesh trial, the BS-WC vaccine dramatically reduced mortality by 45% in women over age 15, demonstrating that even in a community well versed in the treatment of cholera, death can still be a consequence of this disease. Cholera can have a devastating economic impact on countries that is measured not only in treatment costs but also in the deleterious effect that this disease has on food exportation and tourism. Although provision of safer water sources and sewage treatment is no doubt the best way to control cholera, estimates by the World Health Organization indicate that this goal would cost Latin America alone tens of billions of dollars.

*Vibrio cholerae* has often played the role of Grim Reaper (see figure), but it has also been a great educator in the public health arena and a marvelous catalyst for scientific discovery. We have now reached an historic time when the fruits of what we have learned from *V. cholerae* can be applied to effective immunization. In addition, continued studies on the properties that enable *V. cholerae* to be such a potent immunogen may help to clarify the general physiology of mucosal immunity.

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# Rotavirus Vaccines: Success by Reassortment?


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Diarrhea is one of the most common diseases of children and, in developing countries, is responsible for 3.5 to 4 million deaths each year (*1*). Before 1973, infectious agents were identified in so few cases that it was difficult to envisage a strategy for prevention. In that year, R. F. Bishop and colleagues discovered 70-nm wheel-shaped (rota) virus-like particles by electron microscopy in the intestinal mucosa of infants with gastroenteritis (*2*). The identification of rotavirus has led to two decades of energetic research that established this agent as the main cause of severe diarrhea in children and may soon culminate in a vaccine recommended for routine use in children worldwide.

Early research on rotavirus was directed at the development and application of rapid diagnostic tests that allowed researchers to examine the spectrum of disease and the epidemiology of infection. The burden of disease caused by rotavirus is staggering: rotavirus is the most common cause of severe dehydrating diarrhea in children worldwide, infecting nearly every child in the first few years of life (see table) (*3*). First infections are generally associated with acute diarrhea, which in some instances can be severe, leading to dehydration and death. In developing countries, 20 to 40% of hospitalizations for childhood diarrhea and an estimated 870,000 deaths are associated with rotavirus infections each year, making it the most important single cause of diarrheal mortality among children (*4*). In the United States, although mortality is relatively low (75 to 125 deaths per year), rotavirus diarrhea incurs direct medical costs in excess of $500 million and total costs in excess of $1 billion (*5*).

Early epidemiologic studies indicated that rotavirus might best be controlled through vaccination. Longitudinal studies of children followed from birth to 2 years of age provided evidence for natural immunity: Rotavirus diarrhea infrequently occurs more than once, and repeat illnesses are less severe or asymptomatic (*6*, *7*). Furthermore, children infected as newborns are protected from disease later in life (*8*, *9*). Unfortunately, immunity is not fully protective; repeat infection can sometimes lead to disease, and adults with antibodies indicative of previous infection can develop rotavirus diarrhea while caring for sick children or traveling to developing countries where enteric infections are common (*10*).

Rotavirus vaccine development achieved a major breakthrough when human rotavirus was adapted to grow in cell culture (*11*). This advance enabled researchers to prepare vaccine seed lots, set up plaque assays to measure neutralizing antibody titers, and provide replenishable stocks of virus needed to study the molecular biology of the virus.

Rotavirus is classified in the family Reoviridae and its genome consists of 11 segments of double-stranded RNA, each coding for a viral protein. The gene-coding assignments and function of most of these proteins have been determined (*12*). Of particular interest for vaccine development are the two outer capsid proteins, VP7, a glycoprotein on the viral surface, and VP4, a protease-cleaved hemagglutinin (in some strains), which are important in virus neutralization and protection from disease. Cross-neutralization studies have identified four common serotypes of rotavirus on the basis of the VP7 glycoprotein ($G_1$ to $G_4$) and two VP4 serotypes ($P_4$ and $P_8$); all of these serotypes are found in children with diarrhea (*13*, *14*). In theory, an effective vaccine must protect against rotaviruses encoding any of these common neutralization antigens. Because the rotavirus genome is segmented, reassortment of the VP7 and VP4 genes occurs; in nature, this can lead to the development of new strains, and in the laboratory, this can be exploited for preparation of reassortant strains as vaccine candidates or for studying gene function.

In 1983, just 10 years after the discovery of rotavirus, Vesikari conducted the first rotavirus vaccine field trial, in which Finnish infants were administered a live bovine strain of rotavirus, prepared as a vaccine lot by Smith Kline–RIT (*15*). To the surprise


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