Special Issue on S. N. De and
Cholera Enterotoxin

From Cholera Toxin to Subunit Vaccines

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Introduction

With his description of the enterotoxicity of bacteria-free culture filtrate of *Vibrio cholerae* in 1959, S. N. De set in motion a dynamic new era of cholera research. Through the molecular characterization of cholera toxin and its mode of action the pathogenesis of cholera became exceptionally well understood. This in its turn has led to the development of oral vaccines against both cholera and diarrhea caused by enterotoxinogenic *Escherichia coli*.

The possibility that cholera was a toxin-mediated disease had been postulated already in 1884 by Robert Koch. However, this concept soon fell in general disregard, when both Koch himself and others were unable to elicit cholera-like disease by injecting (*parenterally*) culture medium from *Vibrio comma* (*cholerae*) and it was assumed that toxins had to act via the bloodstream. The pathogenesis of cholera remained quite obscure for the next half-century, until in 1959, the situation changed dramatically. From India S. N. De could then report that a cell-free culture filtrate of *V. cholerae* after addition to the *mucosal* side of a ligated loop of the small intestine in a rabbit gave rise to fluid accumulation in the exposed loop[1].

This discovery started a new era in cholera research focussing on the characterization of the putative ''cholera toxin'' that was implied by De's finding. Within a short time, Panse and Dutta[2] described the excretion of toxin in the stool of cholera patients, and Craig[3] both confirmed this and could demonstrate that cholera patients when recovering from disease had started to produce cholera toxin-neutralizing antibodies. These observations set the scene for the subsequent ''modern history'' of cholera toxin research, which in the ten-year period 1969–1978 made the pathogenesis of cholera exceptionally well understood. Furthermore, during the same period, certain strains of *Escherichia coli* and various other bacterial species were found to cause diarrhoeal disease by producing analogous enterotoxins. This made cholera the prototype of a much larger group of ''enterotoxic enteropathies'' that were found to produce several hundred millions of diarrheal disease episodes each year. Based on the new knowledge about the enterotoxins and their mode of action, novel principles were also outlined which gave promise of the possibility to develop more effective diagnostic, therapeutic as well as preventive methods for cholera and the related enterotoxic enteropathies.

In 1978 a Nobel symposium was held in Stockholm to review and discuss the remarkably rapid progress in knowledge about ''Cholera and Related Diarrhoeas'' that had taken place in the past two decades[4]. S. N. De participated in this conference and could then take pleasure in the fact that his early discovery of the rabbit loop-active toxic factor had not only stimulated a new and successful line of research on cholera and other diarrhoeal disorders with great promise for future practical medical applications, but it had as well provided a widely used biological research tool to biomedical scientists in many different disciplines[4].

Structure and Action of Cholera Toxin

As mentioned, many of the discoveries that defined the role of cholera toxin in the molecular pathogenesis of cholera were made in the ten-year period 1969–1978. Among the most important ''chapters'' were the ones listed below in a more or less chronological order:

(i) The identification in 1969–71 by Field, Greenough, Sharp and others, of the strong, practically irreversible stimulatory effect of cholera toxin on the adenylate cyclase/cyclic AMP system and of the link between cyclic AMP and intestinal electrolyte and fluid secretion;

(ii) The purification of cholera toxin by Finkelstein and LoSpalluto and others in 1969–70, and the subsequent elucidation by Holmgren, Cuatrecasas, S. van Heyningen and others in 1972–74 of the A-5B subunit structure of cholera toxin and the different role of the two types of subunit in the toxin function: the "light" B subunits were shown to provide tight, high-affinity binding of the toxin to cells and the "heavy" A subunit to mediate the direct cytotonic action on intestinal adenylate cyclase leading to cyclic AMP formation and electrolyte and fluid secretion;

(iii) The identification in 1973–75 by W. van Heyningen, Holmgren and Svennerholm, Cuatrecasas and others of the GM1 ganglioside as the cell membrane receptor for cholera toxin;

(iv) The elucidation by Gill, Moss and Vaughan, Selinger and others in 1977–78 of the intracellular enzymic mechanism by which the cholera toxin A subunit activates the adenylate cyclase system, involving NAD hydrolysis with ADP-ribosylation of the N_s (G_s) regulatory subunit of adenylate cyclase.

I will not review here in any further detail these and the many additional contributions that led to cholera toxin soon becoming perhaps best defined of all bacterial toxins; several comprehensive reviews with appropriate references exist on this subject (see e.g. 4, 5). For the main topic of this article, which is to describe how the new knowledge about cholera toxin has led to the recent development of oral subunit vaccines against cholera and related diarrhoeal disorders, it is sufficient to recite that by the end of the 1970s a fairly detailed picture of the pathogenesis of cholera had emerged based on the new understanding of cholera toxin (see Figure 1), which came also to guide the new era of vaccine development.

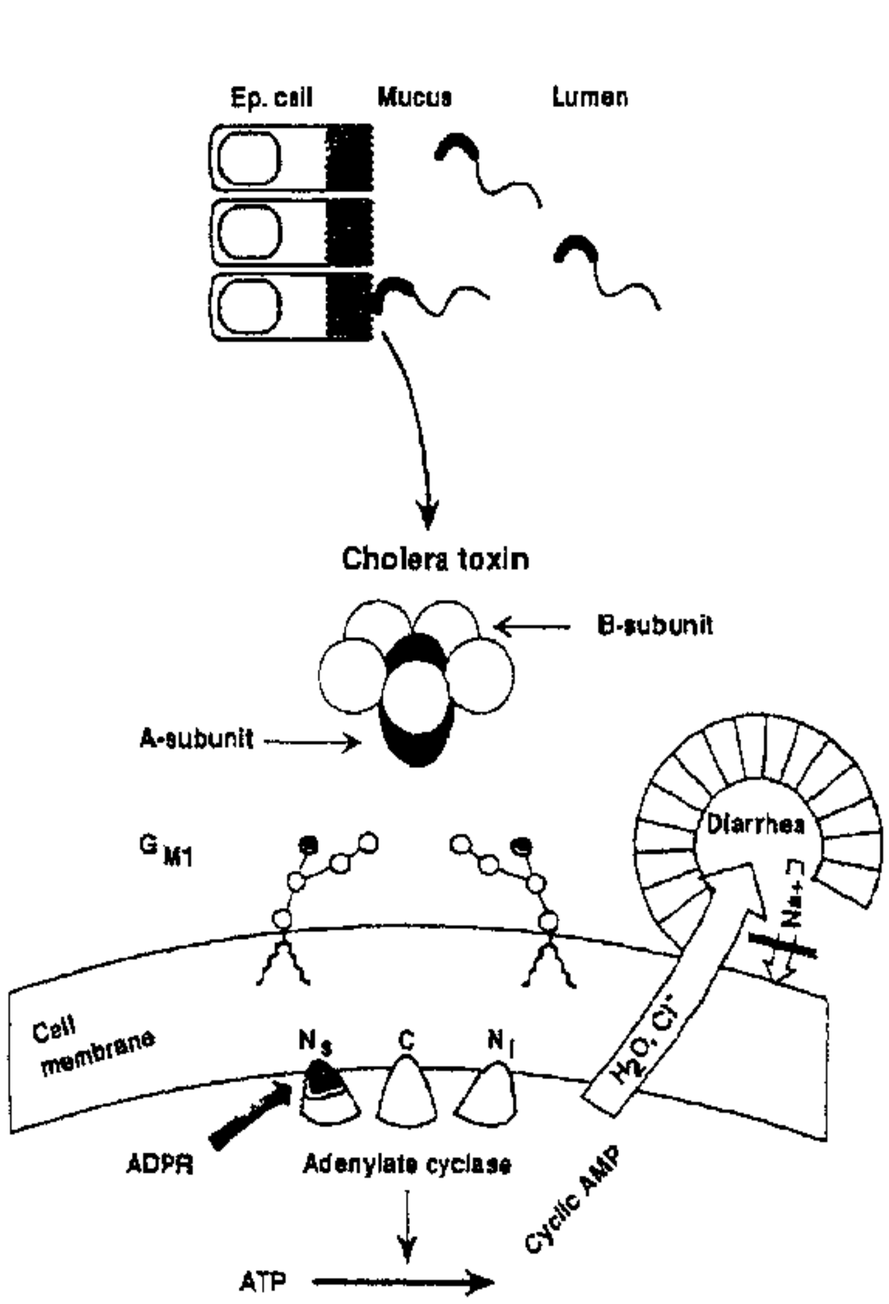

Figure 1. Pathogenesis of cholera and the action of cholera toxin (for explanations, see text).

New Cholera Vaccines

There is a great need for an effective cholera vaccine. Cholera remains an important cause of illness and death in many parts of the world, especially in Asia but also in many parts of Africa and the Middle East. Although cholera can be treated simply by oral and intravenous rehydration, diarrhoea treatment centers are still scarce in areas endemic for cholera, and use of an effective vaccine may be the most promising possibility to control the disease. However, the parenteral whole-cell cholera vaccines that have existed since almost a century to prevent cholera are no longer regarded useful from a public health standpoint, mainly because of the short duration of protection they provide. Several field trials since 1960 have shown that these vaccines usually protect older people for only a few months and fail to protect many young children at all[4].

The identification of the critical role of a toxin in the pathogenesis of cholera led to great expectations that a more effective cholera vaccine could be developed based on use of a toxoid. However, a large field trial with a parenterally administered glutaraldehyde toxoid vaccine in Bangladesh in the mid-1970s failed to give any better protection than that achieved by previous parenteral vaccines. This did not come as a total surprise, however, since during the time it took to organize, execute and evaluate the trial, it had become more clearly apparent from animal studies that protective cholera immunity did not depend on serum antibodies that were mainly stimulated by the parenteral vaccination, but rather on mucosal secretory IgA antibodies produced locally in the gut, which are only inefficiently stimulated by parenteral antigen administration[6]. Therefore, since the late 1970s, attention has turned instead to development of oral vaccines that could stimulate intestinal immunity more efficiently.

The different "modern" approaches towards

development of cholera vaccines all have taken their departure from the new insights into the mechanisms of disease and immunity in cholera achieved during the 1970s and early 1980s.

Firstly, the clarification of the subunit structure of cholera toxin and of the role in different subunits in the toxin action immediately suggested a way to prepare a safe and highly immunogenic "toxoid" consisting of the purified cholera B subunits. Studies showed that the purified B subunit portion of cholera toxin was entirely devoid of toxicity, yet it contained selectively the protective toxin epitopes against which neutralizing antitoxin antibodies were directed. Indeed, it was found that the B subunit is particularly well suited to oral immunization because it retains the ability to bind to the intestinal epithelium, which has been shown to be important for stimulating mucosal immunity in animals, including local immunological memory[6].

Another important line of vaccine research has focused on the gut mucosal immune system and how it can be stimulated by immunogens. These studies have defined the importance of locally produced IgA antibodies and IgA immunologic memory for protection against cholera, and they have shown that the oral route of vaccine administration is usually superior to the parenteral route in both priming and boosting the mucosal immune system[6].

A third important observation guiding the design of the new cholera vaccines has concerned the synergistic cooperation between antitoxic and antibacterial immune mechanisms in cholera. Two main protective antibodies have been identified, one being directed against the *V. cholerae* cell wall lipopolysaccharide (LPS) and the other against the cholera toxin B subunits. Either of these two types of antibody can confer protection against disease by inhibiting bacterial colonization and toxin binding, respectively; when present together in the gut they can have a strongly synergistic protective effect[6].

The intense vaccine development and research efforts during the last 10-year period have now provided several oral vaccine candidates based on either non-living bacteria and purified B subunit antigen or on live attenuated mutants of *Vibrio cholerae* O1 producing the B subunit.

The Oral B Subunit-Whole Cell Vaccine

The composition of this vaccine was designed to safely provide the key antigens for evoking protective antitoxic and antibacterial mucosal immunity against *V. cholerae* O1 of different serotypes (Inaba and Ogawa) and biotypes (classical and *El Tor*). As mentioned, the B subunit component is completely non-toxic and is an exceptionally potent oral immunogen because of its ability to bind to the intestinal mucosa. The whole-cell (WC) component provides protective LPS antigens as well as heat-labile bacterial antigens that may add further to the antibacterial immunogenic effect.

The B-WC vaccine is given orally together with an alkaline buffer (provided by an effervescent tablet) to protect the vaccine during passage through the stomach. The vaccine has been extensively tested in several clinical trials in Swedish, Bangladeshi and American volunteers. In these studies, the vaccine has proved to be completely safe with no adverse reactions, and after either two or three doses, it stimulates a gut mucosal IgA antitoxic and antibacterial immune response (including memory) comparable to that induced by cholera disease itself[7,8]. In American volunteers who received three oral immunizations with either the B-WC vaccine or the WC vaccine component alone, vaccination protected against challenge with a dose of live cholera vibrios that caused disease in 100 percent of concurrently tested unvaccinated controls[9].

Since 1985 the B-WC vaccine and the WC component without any B subunit (both vaccines being produced by Institut Mérieux, France and the National Bacteriological Laboratory of Sweden) have been evaluated in a large, randomized, placebo-controlled field trial involving 89,596 vaccinated adults and children in Bangladesh. Both vaccines have been found to protect against cholera for at least three years. During the initial 4–6 month period after immunization the protective efficacy of BS-WC vaccine was 85%, and was similar for all age groups[10]. Thereafter a protection level of ca 70% has persisted for at least three years in those over the age 5, whereas in younger children protection decreased substantially after the initial 6 months[11]. Two doses of either vaccine were equivalent to three doses with regard to long-term protective efficacy. The B-WC vaccine had the advantages over WC vaccine alone of a greater initial protective efficacy against cholera[10]. In addition, the B-WC vaccine, in contrast to WC, gave substantial short-term protection against diarrhoea caused by LT as well as LT/ST enterotoxigenic *E. coli* (67% protection against all LT *E. coli* diarrhoea and 86% protection against severe disease)[12]. Furthermore, the B-WC vaccine and to a lesser extent the WC vaccine, significantly decreased the over-all incidence of diarrheal disease. This impact was especially pronounced on severe, life-threatening disease which was reduced by nearly 50% in this cholera endemic area[13].

These results with the oral B-WC and WC

vaccines differ favorably from those achieved previously using parenteral cholera vaccines. The short-term efficacy is notably higher for the oral vaccines with high-grade protection afforded also to the young children. In addition, and of greater public health significance, the duration of protection is much superior lasting at least 3 years for the oral vaccines as compared with 3–6 months for most parenteral vaccines tested.

Work to further facilitate the use of these oral vaccines is also in progress, e.g. to prepare a tablet formulation containing vaccine in combination with alkaline buffer. This would facilitate storage and distribution and probably also further increase the stability of these already rather stable vaccines for use in cholera endemic areas. By utilizing recombinant DNA technology, it also seems possible to produce the B and WC components in a single step[14]. This could greatly simplify large-scale production of the B-WC vaccine, reduce costs as well as allow local production of this vaccine in developing countries.

The strong, though relatively short-lasting cross-protection against diarrhea caused by LT-producing *E. coli* achieved with the B-WC vaccine also makes this vaccine a relevant immunoprophylactic agent for travellers to areas where cholera and/or *E. coli* diarrhea are prevalent. Indeed, based on these results, cholera B subunit is one component together with CFA-containing *E. coli* bacteria in a specific vaccine against *E. coli* diarrhea which is now being developed in our laboratory.

Live Attenuated Cholera Vaccines

Attenuated live organisms are appealing as oral cholera vaccine candidates because of their ability to colonize the intestine and to stimulate an immune response in a manner analogous to natural cholera infection. Through sustained delivery of antigens to the mucosal immune system a live vaccine may have a greater probability than a non-living vaccine to evoke a satisfactory immune response after only a single dose.

Recombinant DNA techniques have allowed the preparation of a series of *V. cholerae* 01 mutant strains in which the genes encoding both the A and B subunits of cholera toxin or just the A subunit have been deleted. Studies in human volunteers, have revealed that these strains evoke a protective immune response against challenge. However, even though the mutant strains were markedly attenuated compared with the parent strains they gave rise to diarrhea (usually mild) in more than one-half of the vaccinated volunteers which precluded their further testing as potential vaccines[15]. Another more recently developed such strain, CVD103-HgR, which was prepared by deleting the A subunit gene from *V. cholerae* 01 strain 569B by recombinant DNA techniques, has only rarely caused any diarrheal side-effects in volunteers and yet has elicited good antibacterial and antitoxic immune responses along with significant protection against challenge. This makes CVD103-HgR an interesting candidate for expanded clinical trials now initiated in cholera endemic areas[16].

B Subunits as Antigen Carriers

Recently there has been much interest in the possibility to also use cholera B subunit as carrier for foreign antigens or epitopes for the purpose of achieving a mucosal immune response. The reason for this is that, even with oral immunization the stimulation of gut-associated lymphoid tissue by many antigens is often relatively inefficient, requiring large quantities of the immunogens and yielding only modest antibody responses. Notable exceptions are cholera toxin and (in humans) its B subunit component, which are potent enteric immunogens eliciting strong mucosal immune responses. As mentioned above, this is to a large extent due to the ability of these proteins to bind avidly to GM1 ganglioside receptors on cell surfaces, and also, at least for cholera toxin, to the immunomodulating activity of these proteins. It might even be possible with this approach to achieve protective immunity also in mucosal or glandular tissues distant from the intestinal mucosa itself.

Indeed, in a recent study[17], it was shown that oral administration of microgram amounts of a putative caries-protective antigen of *Streptococcus mutans* called protein antigen I/II when covalently coupled to the B subunit of cholera toxin elicited a vigorous mucosal as well as extramucosal IgA and IgG anti-streptococcal antibody response in mice. These responses, which were not achieved when antigen I/II was given in free form or coupled to bovine serum albumin, were manifested by the presence of large numbers of antibody-secreting cells in salivary glands, intestine and spleen as well as by the development of high levels of circulating antibodies.

Hybrid proteins between cholera B subunit (CTB) and foreign antigens for immuno-protection can be prepared by chemical coupling or by genetic fusion. In the genetically engineered CTB overexpression system mentioned above the possibility of obtaining CTB-derived hybrid proteins by gene fusion was described as an extra bonus[14]. This could allow link-

age of various peptide antigens to CTB for immunoprotection studies against various viral, bacterial and parasitic diseases. The accessibility of this approach has recently been demonstrated by the fusion and expression of a synthetic DNA sequence encoding a nontoxic decapeptide antigen, derived from the heat-stable enterotoxin, to the CTB gene[18].

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Reactions

De's work was not accepted at first, nor its significance realized for example, Jenkin and Rowley, who had already invested heavily in endotoxins, were unable to repeat De's observations on the effect of cholera culture filtrate and claimed, astonishingly, that his results were due to the combined action of the old favourite, endotoxin, plus the new favourite, mucinase, plus a new-comer to the field, lactic acid. Instead of such a complicated idea, they might have been better advised to consider the far simpler idea that their cholera culture filtrates contained no exotoxin. We have seen that it is all too easy to grow a culture of the cholera vibrio producing no exotoxins. In any case, the lactic acid hypothesis was soon demolished by S. B. Formal and his colleagues at the Walter Reed Army Institute of Research in Washington, D.C.

. . . . In time, the reservations about De's ligated intestinal loop diminished as it came in for more and more use in the study of other diarrhoeal diseases as well as cholera. False positives seemed to become rarer as experience was gained in handling the small intestine, and it was found to be feasible, and very convenient, to tie off as many as 24 loops in a single rabbit.

Cholera toxin, or cholera exo-enterotoxin, to be specific both about its nature and about its site of action, had at last found its way out of a maze of confusion, soon to fall into the welcoming arms of a scientific community that was ready and able to make the fullest use of it.

Excerpted from *Cholera: The American Scientific Experience, 1947-1980* by W. E. van Heyningen and J. R. Seal