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Construction of Nontoxic Derivatives of Cholera Toxin and Characterization of the Immunological Response against the A Subunit

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Using computer modelling, we have identified some of the residues of the A subunit of cholera toxin (CT) and heat-labile toxin that are involved in NAD binding, catalysis, and toxicity. Here we describe the site-directed mutagenesis of the CT gene and the construction of CT mutants. Nine mutations of the A subunit gene were generated. Six of them encoded proteins that were fully assembled in the AB_5 structure and were nontoxic; these proteins were CT-D53 (Val-53→Asp), CT-K63 (Ser-63→Lys), CT-K97 (Val-97→Lys), CT-K104 (Tyr-104→Lys), CT-S106 (Pro-106→Ser), and the double mutant CT-D53/K63 (Val-53→Asp, Ser-63→Lys). Two of the mutations encoded proteins that were assembled into the AB_5 structure but were still toxic; these proteins were CT-H54 (Arg-54→His) and CT-N107 (His-107→Asn). Finally, one of the mutant proteins, CT-E114 (Ser-114→Glu), was unable to assemble the A and the B subunits and produced only the B oligomer. The six nontoxic mutants were purified from the culture supernatants of recombinant *Vibrio cholerae* strains and further characterized. The CT-K63 mutant, which was the most efficient in assembly of the AB_5 structure, was used to immunize rabbits and was shown to be able to induce neutralizing antibodies against both the A and B subunits. This molecule may be useful for the construction of improved vaccines against cholera.

Cholera toxin (CT), a protein secreted by *Vibrio cholerae*, is responsible for the watery diarrhea characteristic of cholera, an epidemic and severe disease causing over 150,000 deaths each year (3, 6). Recently, the appearance of new epidemic waves of cholera in South America, Asia, Europe, and Africa has confirmed the importance of this ancient disease (23, 40, 42, 50–52). CT is 80% homologous in amino acid sequence to the heat-labile toxin (LT) produced by enterotoxigenic *Escherichia coli* strains, which is responsible for travellers' diarrhea (38, 46). CT and LT are hexameric proteins with an AB_5 structure. The determination of the three-dimensional structures of LT and of the B subunit of CT (29, 44, 45) has increased our level of understanding of the structures and functions of these molecules (8). Five identical B subunits, of 103 amino acids each, are assembled into a ring-like pentameric structure with a central cavity that houses the carboxy-terminal end of the A subunit. The B subunit and the B pentamer contain the GM1 ganglioside receptor-binding site, while the A subunit is an ADP-ribosyltransferase responsible for the toxicity of the molecule. To become active, the A subunit must be proteolytically cleaved in positions 192 to 194, and a disulfide bond must be reduced to form the A1 and A2 polypeptides. The A1 fragment binds NAD and transfers the ADP-ribose moiety of NAD to $G_{s\alpha}$, a GTP-binding protein which controls the activity of adenylate cyclase in eukaryotic cells (12). The modification of $G_{s\alpha}$ causes an increase in the level of cyclic AMP, induction of prostaglandins, and, possibly, a stimulation of local sensory neurons. These modifications result in the accumulation of salts and water in the intestinal lumen, producing the watery diarrhea characteristic of cholera (10).

Immunization with fully toxic or chemically detoxified CT and LT, usually in the presence of strong adjuvants, has shown that the toxin-neutralizing antibodies are directed almost exclusively against the B subunit (11, 17, 24, 33, 39). On the basis of these findings, recently developed live and killed cholera vaccines are based on strains which contain only the gene coding for the nontoxic CT B subunit (4, 5, 18, 19, 22, 43). The A subunit was not included because it is responsible for the toxicity and is believed to be immunologically not relevant. We have recently readdressed the problem of the immunogenicity of the A subunit of LT by using nontoxic derivatives of this molecule obtained by site-directed mutagenesis of the A subunit (35). Surprisingly, we have found that immunization with a nontoxic derivative of LT, carrying a Ser-63→Lys mutation (LT-K63), in the absence of adjuvant induced neutralizing antibodies against both the A and the B subunits, suggesting that nontoxic mutants of LT could be used for the construction of vaccines against travellers' diarrhea and that similar mutants could be constructed for CT (36). In this paper we report the construction of several nontoxic mutants of CT and show that they also induce neutralizing antibodies against the A subunit.

Figure 1 shows the cavity that contains the NAD-binding and catalytic sites on the A subunits of all ADP-ribosylating toxins (8). This cavity consists of a beta-strand and an alpha-helix, which form the floor and the ceiling of the cavity, respectively. A number of amino acids located within or close to this cavity have previously been changed by site-directed mutagenesis and have been shown to cause loss of enzymatic activity in either CT or LT (2, 13–15, 25, 30, 48, 49). The mutated residues are Arg-7, His-44, Glu-110, Ser-61, and Glu-112 and are shown in Fig. 1 with triangles. We have recently described a number of different amino acid substitutions that make LT nontoxic (35, 36). Here we describe the construction

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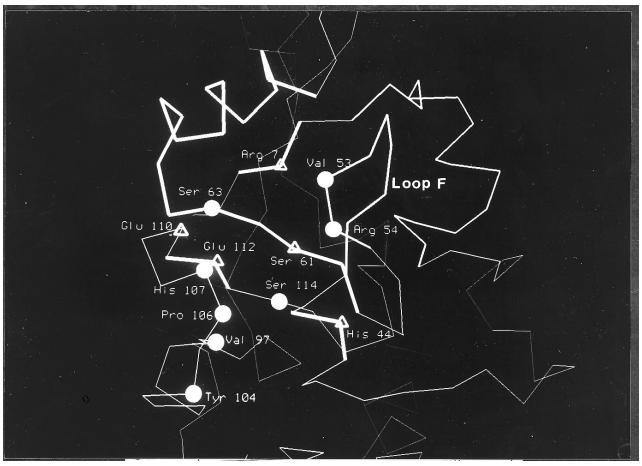

FIG. 1. Model of the α-carbon structure of the active site of CT. The model was constructed by using the coordinates of the LT structure (45). The heavy trace indicates amino acids 58 to 76, which are folded in a beta-strand followed by an alpha-helix, which form the NAD-binding site. In heavy trace also are shown amino acids Arg-7, His-44, Ser-61, Glu-110, and Glu-112, which are residues essential for the catalytic activity (8). The intermediate trace indicates the loop F region and other sequences which form the NAD-binding site. The amino acids that have been previously shown to be important for enzymatic activity (2, 14, 48, 49) are indicated by triangles. The positions of the amino acids modified in this work are indicated by circles.

of several mutants of CT. Some of them, such as CT-D53 (Val-53→Asp), CT-K63 (Ser-63→Lys), CT-K97 (Val-97→Lys), and CT-K104 (Tyr-104→Lys), contained mutations that have been found to be optimal for LT (35). Others, such as CT-H54 (Arg-54→His), CT-S106 (Pro-106→Ser), CT-N107 (His-107→Asn), and CT-E114 (Ser-114→Glu) contained newly designed mutations, and one contained a combination of the above-described mutations. The nine CT mutant proteins obtained are listed in Table 1, and the corresponding mutated amino acids are indicated by circles in Fig. 1. For mutagenesis, the CT A and B subunit genes were amplified by PCR from the pJM17 plasmid (31) by using the oligonucleotides 5′-GGCA GATTCTAGACCTCCTGATGAAATAAA-3′ (ctxA) and 5′-TGAAGTTTGGCGAAGCTTCTTAATTTGCCATACTA ATTGCG-3′ (ctxB). The 1.1-kb *Xba*I-*Hin*dIII fragment was then cloned into the pEMBL19 vector (7) and used for site-directed mutagenesis (53) with the oligonucleotides listed in Table 2. Following mutagenesis, the mutated *Xba*I-*Hin*dIII fragments and the upstream regulatory sequences of wild-type CT were subcloned into the high-copy-number pGEM-3 vector, generating pGEM-CT plasmids that were transformed into *E. coli* or electroporated (41) into the 0395-NT strain of *V. cholerae*, which carries a chromosomal deletion of the CT gene (28). The mutant proteins were detected by immunoblotting in the periplasmic extracts of *E. coli* (34) and in the culture supernatants of *V. cholerae* (Fig. 2a and b, respectively). As expected, the A subunit was present in different forms in the two microorganisms, since *E. coli* was unable to proteolytically cleave the A subunit into the A1 and A2 polypeptides. In one of the mutants, CT-E114, no A subunit was detected in either *E. coli* or *V. cholerae*. Since efficient expression could be obtained in both systems, for convenience all subsequent tests were performed with the culture supernatant of *V. cholerae*. The culture supernatant was first tested for toxicity on Y1 cells (9). Toxicity was detected only with mutants CT-H54 and CT-N107. CT-D53, CT-K63, CT-K97, CT-K104, CT-S106, CT-E114, and the double mutant CT-D53/K63 were nontoxic. In the case of CT-E114, the absence of toxicity was expected, since this mutant does not produce the A subunit. The other nontoxic mutants confirmed the results previously obtained with LT (35). Mutant CT-S106 is a new nontoxic mutant described here for the first time.

The nontoxic CT mutants CT-D53, CT-K63, CT-K97, CT-K104, and CT-S106 and the double mutant CT-D53/K63 were precipitated from the culture supernatants of *V. cholerae* strains, grown in Syncase modified medium (21), by adding 2.5 g of sodium hexametaphosphate per liter of culture supernatant and adjusting the pH to 4.5 with concentrated HCl (37). The precipitate was dissolved in 0.1 M sodium phosphate (pH 8), dialyzed in 10 mM sodium phosphate (pH 7) (27), and loaded on a CM-Sepharose column (Pharmacia LKB, Uppsala, Sweden), which was eluted first with 20 mM sodium phosphate buffer (pH 7.5) and then with 40 mM sodium phosphate buffer (pH 7.5).

As shown in Fig. 3, the CT mutant proteins, which were already present in large amounts in the precipitated material (Fig. 3b, lane 1), were retained completely by the CM-Sepharose column (Fig. 3b, lane 2). Elution of the column not only gave a peak containing the pure CT mutant protein (Fig. 3a, peak I; Fig. 3b, lanes 3 and 4), but also separated it from the B oligomer, devoid of the A subunit (Fig. 3a, peak II; Fig. 3b, lanes 5 and 6). The peak containing the B oligomer, although different in size in the different mutants, was constant in different experiments for the same mutant. One example of this

TABLE 1. CT mutants and their properties

Name	Mutation(s)	A subunit[a]	Toxicity on Y1 cells[b]	ADP-ribosylation in vitro[c]	% Holotoxin[d]
Wild type		+	+++	+	83
CT-D53	Val→Asp	+	−	−	75
CT-H54	Arg→His	+	++	ND[e]	ND
CT-K63	Ser→Lys	+	−	−	82
CT-K97	Val→Lys	+	−	−	47
CT-K104	Tyr→Lys	+	−	−	61
CT-S106	Pro→Ser	+	−	ND	75
CT-N107	His→Asn	+	++	ND	ND
CT-E114	Ser→Glu	−	−	ND	ND
CT-D53/K63	Val→Asp, Ser→Lys	+	−	−	79

[a] Detection of the A subunit in culture medium by Western blot with rabbit anti-CT polyclonal antibodies.
[b] +++, toxic activity comparable with that of wild-type CT; ++, intermediate toxicity; −, absence of toxicity.
[c] ADP-ribosylation reaction was performed with 3 μg of wild-type or mutant CT and polyarginine as a substrate (20). The assay can detect 500 ng of CT.
[d] Amount of holotoxin recovered at the end of the purification process.
[e] ND, not determined.

TABLE 2. Oligonucleotides used for site-directed mutagenesis

Mutant	Oligonucleotide	Mutation
CT-D53	5′-ACGGGATTTGACAGGCACGAT-3′	Val-53→Asp
CT-H54	5′-GGATTTGTTCATCACGATGAT-3′	Arg-54→His
CT-K63	5′-GTTTCCACCAAGATTAGTTTG-3′	Ser-63→Lys
CT-K97	5′-ATGTTTAACAAGAATGATGTA-3′	Val-97→Lys
CT-K104	5′-TTAGGGGCAAAAAGTCCTCAT-3′	Tyr-104→Lys
CT-S106	5′-GGCATACAGTAGCCATCCAGA-3′	Pro-106→Ser
CT-N107	5′-TACAGTCCTAACCCAGATGAA-3′	His-107→Asn
CT-E114	5′-CAAGAAGTTGAAGCTTTAGGT-3′	Ser-114→Glu

variation is shown in Fig. 3a, in which peak II is very small in CT-D53 and much larger in CT-K104. The ratio between the areas of peaks I and II was calculated for each mutant to evaluate the percentage of assembled holotoxin (AB_5) recovered at the end of the purification process. The results reported in Table 1 showed that the amounts of the fully assembled molecules varied from as little as 41% in the case of CT-K104 to 83% in the case of wild-type CT. Mutant CT-K63, which was the most efficient in holotoxin assembly, was purified in large scale and used for subsequent studies. The finding that mutations in the A subunit affect the yield of the assembled molecule had been obtained also for the LT mutants (35) and is likely to reflect the stability of the A subunit and its ability to interact with the B subunit. The results obtained here are consistent with those obtained with LT. However, the CT mutants gave a lower yield of assembled molecule; for instance, mutations K63 and K104 gave 98 and 72%, respectively, yields of fully assembled molecule in LT and 82 and 61%, respectively, in CT.

To test the immunological properties of the CT mutants, we selected the mutant that had shown the greatest yield of assembled molecule. Rabbits were immunized with 25 μg of CT-K63, either fluid or with Freund's adjuvant, and the sera obtained were analyzed by Western blotting. All sera contained antibodies recognizing the A and B subunits, regardless of whether rabbits had been immunized with or without Freund's adjuvant (data not shown). The anti-A antibodies contained in each serum were then separated from the antibodies against the B subunit by passing the sera through a column containing immobilized B oligomer, as we had previously done for LT (36). The different fractions were analyzed for their specificity in a Western blot (Fig. 4). The results showed that, as expected, the flowthrough contained antibodies that recognized only the A subunit (Fig. 4, lane 2), while the fraction eluted from the B column recognized only the B subunit (Fig. 4, lane 3). To verify that the CT-K63 mutant induced neutralizing antibodies against the A subunit, the total sera and the anti-A fractions were analyzed for their abilities to neutralize the toxic activity of the wild-type CT on Y1 cells. The results (Table 3)showed that the total sera obtained by immunizing with Freund's adjuvant had, as expected, a high neutralization titer of 1/16,384, while immunization without adjuvant gave a fourfold-lower neutralizing titer of 1/4,096. However, the toxin-neutralizing titer of the anti-A antibodies was much higher (1/1,024) in the sera obtained by immunizing

TABLE 3. CT-neutralizing titers of anti-CT K63 antibodies.

Antibodies	+Freund's adjuvant	−Freund's adjuvant
Total serum	1/16,384	1/4,096
Anti CT-A	1/256 (1.5%)	1/1,024 (25%)

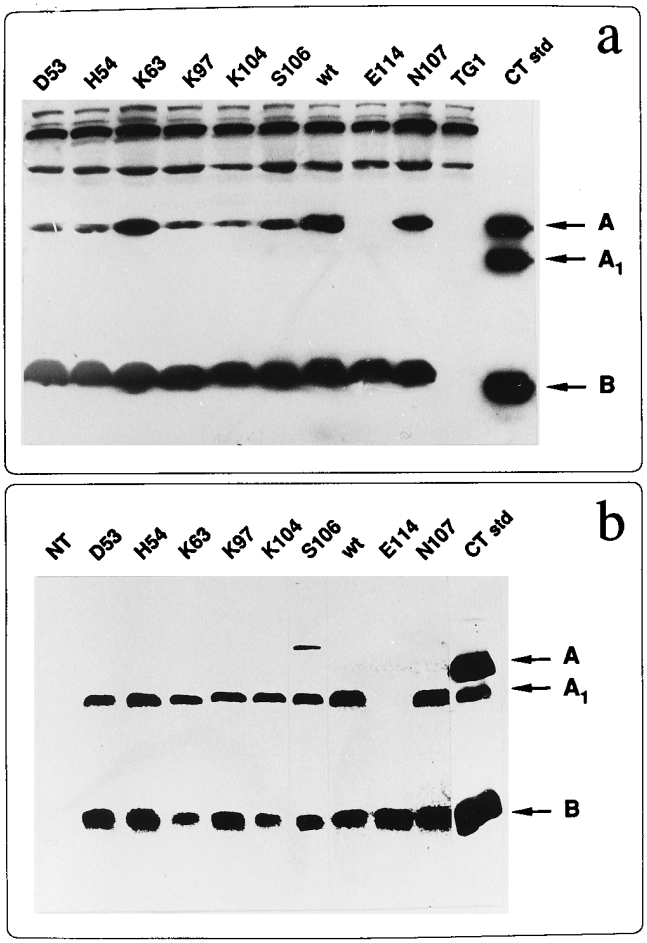

FIG. 2. Western blot showing the expression of CT mutants in the periplasmic fraction of *E. coli* TG1 (a) or in culture supernatants of *V. cholerae* (b). wt, wild type; std, standard purified CT; NT, supernatant of 0395-NT strain of *V. cholerae*, which contains a chromosomal deletion of the CT gene.

without adjuvant than in those obtained by immunizing with adjuvant (1/256). From a quantitative point of view, the anti-A neutralizing activity represented 25 and 1.5%, respectively, of the total, for immunization without and with Freund's adjuvant. These results show that when immunization is performed in the presence of Freund's adjuvant, most of the neutralizing antibodies recognize the B subunit. When the antigen is used without adjuvant, up to 25% of the neutralizing activity is against the A subunit. This finding is in disagreement with most of the literature published during the last 2 decades, which has reported that the A subunit does not induce neutralizing antibodies, but it concords with our recent results for the LT-K63 mutant (36). The effect of Freund's adjuvant may be explained by an interaction with the A subunit that causes a change in conformation or dissociation from the B pentamer.

In spite of the high level of homology between CT and LT, these two proteins are known to induce antibodies with low cross-neutralizing titers. Since the A subunit is more conserved in amino acid sequence than the B subunit, we expected the anti-CT A antibodies to have an increased ability to neutralize LT compared with the anti-CT B fraction. To test this hypothesis, the anti-CT-K63 serum obtained by immunizing rabbits with Freund's adjuvant, which contained primarily neutralizing anti-CT B antibodies, and the affinity-purified anti-CT A anti-

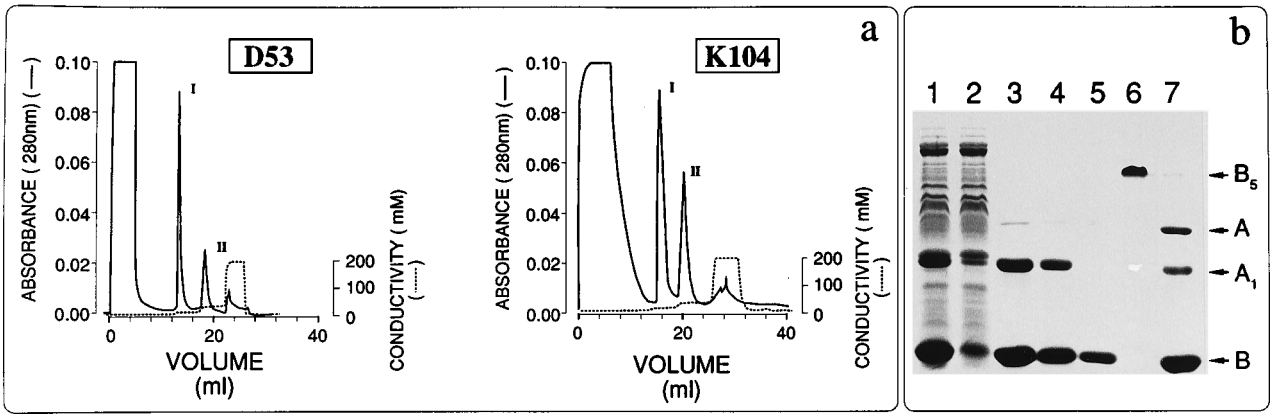

FIG. 3. Purification of CT mutants from *V. cholerae* culture supernatants. (a) Chromatographic plots for two purified mutants (CT-D53 and CT-K104). Peak I, eluted from the column with 20 mM sodium phosphate (pH 7.5), contains the holotoxin, while peak II, eluted with 40 mM sodium phosphate (pH 7.5), contains the B pentamer. (b) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of samples derived from each purification step. Lane 1, concentrated culture supernatant; lane 2, flowthrough material; lanes 3 and 4, peak I; lane 5, denatured (boiled) peak II; lane 6, nondenaturated peak II; lane 7, purified CT from Sigma (St. Louis, Mo.).

bodies were tested in parallel for the neutralization of LT and CT. The concentrations of anti-CT A and anti-CT B antibodies were adjusted so that both of them neutralized 80 pg of CT at a dilution of 1/512. Subsequently, we tested the abilities of the sera to neutralize 80 pg of LT. The results showed that the anti-CT B antibodies neutralized LT only up to a dilution of 1/4, while the anti-CT A antibodies neutralized LT up to a dilution of 1/32. This confirmed that the anti-CT A antibodies neutralized LT approximately eightfold more efficiently than the anti-CT B antibodies.

In conclusion, we have shown that nontoxic mutants of CT can be used for immunization and that they induce neutralizing antibodies against the A subunit. Until recently, this finding was unexpected because the A subunit is generally considered to be unable to induce toxin-neutralizing antibodies. In fact, cholera vaccines have been developed with the assumption that the toxin-neutralizing antibodies reside entirely in the B subunit and that the in vivo protection is mediated mostly by antibacterial antibodies. The new finding reported in this paper and previously reported for LT (36) suggests that nontoxic CT derivatives may be used to improve both live and killed vaccines. In fact, the CT-K63 mutant could be easily used instead of the B subunit in both vaccine formulations. The vaccines developed so far, although very effective against the 01 cholera strains, are unable to protect against strains with different serotypes, such as the newly emerged 0139 strain, that have different somatic antigens (26, 42). By increasing the protective immunity against the antigens that are common to all strains, the efficacy of vaccines may improve. Furthermore, this increase can provide broad protection not only against the strains present today but also against strains of different serotypes that may arise in the future and against diarrhea caused by enterotoxigenic *E. coli*. A role of immunity against the B subunit of CT in protection against disease has been demonstrated in several instances. Anti-CT-B antibodies are known to be synergistic with anti-lipopolysaccharide antibodies in inducing protection against challenge with live *V. cholerae* (47). In a field trial in Bangladesh, during the first 6 months, protection was much higher (85%) in the group that received the BS-WC vaccine, containing the B subunit and bacterial cells, than in the group that received the bacterial cells alone (58%) (1, 4). In the same clinical trial, the BS-WC vaccine conferred good protection against diarrhea caused by enterotoxigenic *E. coli* strains producing LT, which are known to share with *V. cholerae* only the toxin antigen (5). In a subsequent study of travellers to Morocco, the antitoxin immunity induced by the BS-WC vaccine was confirmed to protect against *E. coli*-induced diarrhea (32).

In conclusion, there is enough evidence in the literature to support the idea that improving immunity against CT may improve the efficacy of cholera vaccines. This should be true especially when short-term protection is needed, such as in the cases of military personnel and travellers. Finally, the use of CT-K63 instead of the B subunit in vaccines would provide the immune system with a larger immunological target. This may be very important, because the CT B subunit is a small polypeptide of 103 amino acids that may have not enough T-cell epitopes, and therefore the response to it can be genetically restricted. Such a restriction has been already observed in mice (16). The addition of the A subunit, which is a polypeptide of 239 amino acids, should overcome any genetic restriction.

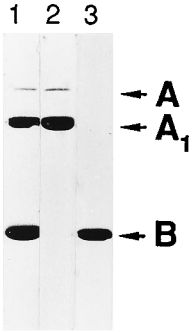

FIG. 4. Western blot of total anti-CT-K63 sera and purified antibody fractions. Lane 1, total sera; lane 2, fraction of sera containing the anti-A antibodies; lane 3, fraction of sera containing the anti-B antibodies.

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REFERENCES

1. **Black, R. E., M. M. Levine, M. L. Clements, C. R. Young, A. M. Svennerholm, and J. Holmgren.** 1987. Protective efficacy in humans of killed whole-vibrio oral cholera vaccine with and without the B subunit of cholera toxin. Infect. Immun. **55:**1116–1120.

2. **Burnette, W. N., V. L. Mar, B. W. Platler, J. D. Schlotterbeck, M. D. McGinley, K. S. Stoney, M. F. Rhode, and H. R. Kaslow.** 1991. Site-specific mutagenesis of the catalytic subunit of cholera toxin: substituting lysine for arginine 7 causes loss of activity. Infect. Immun. **59:**4266–4270.
3. **Cholera—update, end of 1993.** Weekly Epidemiol. Rec. **69:**13–17.
4. **Clemens, J. D., J. R. Harris, D. A. Sack, J. Chakraborty, F. Ahmed, B. F. Stanton, M. U. Khan, B. A. Kay, N. Huda, M. R. Khan, M. Yunus, M. R. Rao, A. M. Svennerholm, and J. Holmgren.** 1988. Field trial of oral cholera vaccines in Bangladesh: results of one year follow-up. J. Infect. Dis. **158:**60–69.
5. **Clemens, J. D., D. Sack, J. R. Harris, J. Chakraborty, P. K. Neogy, B. Stanton, N. Huda, M. U. Khan, B. A. Kay, M. R. Khan, M. Ansaruzzaman, M. Yunus, M. R. Rao, A. M. Svennerholm, and J. Holmgren.** 1988. Cross protection by B subunit-whole cell cholera vaccine against diarrhea associated with heat-labile toxin-producing enterotoxigenic Escherichia coli: results of a large-scale field trial. J. Infect. Dis. **158:**372–377.
6. **Del Giudice, G.** 1991. Research priorities for diarrhoeal disease vaccines: memorandum from a WHO meeting. Bull. W. H. O. **69:**667–676.
7. **Dente, L., G. Cesareni, and R. Cortese.** 1983. pEMBL: a new family of single stranded plasmids. Nucleic Acids Res. **11:**1645–1655.
8. **Domenighini, M., C. Magagnoli, M. Pizza, and R. Rappuoli.** 1994. Common features of the NAD-binding and catalytic site of ADP-ribosylating toxins. Mol. Microbiol. **14:**41–50.
9. **Donta, S. T., H. W. Moon, and S. C. Whipp.** 1973. Detection and use of heat-labile Escherichia coli enterotoxin with the use of adrenal cells in tissue culture. Science **183:**334–335.
10. **Field, M., M. C. Rao, and E. B. Chang.** 1989. Intestinal electrolyte transport and diarrheal disease. N. Engl. J. Med. **321:**800–806.
11. **Finkelstein, R. A., M. F. Burks, A. Zupan, W. S. Dallas, C. O. Jacob, and D. S. Ludwig.** 1987. Epitopes of the cholera family of enterotoxins. Rev. Infect. Dis. **9:**544–561.
12. **Gill, D. M., and M. J. Woolkalis.** 1991. Cholera toxin-catalyzed [32P]ADP-ribosylation of proteins. Methods Enzymol. **195:**267–280.
13. **Glineur, C., and C. Locht.** 1994. Importance of ADP-ribosylation in the morphological changes of PC12 cells induced by cholera toxin. Infect. Immun. **62:**4176–4185.
14. **Harford, S., C. W. Dykes, A. N. Hobden, M. J. Read, and I. J. Halliday.** 1989. Inactivation of the Escherichia coli heat-labile enterotoxin by in vitro mutagenesis of the A-subunit gene. Eur. J. Biochem. **183:**311–316.
15. **Hase, C. C., L. S. Thai, M. Boesmanfinkelstein, V. L. Mar, W. N. Burnette, H. R. Kaslow, L. A. Stevens, J. Moss, and R. A. Finkelstein.** 1994. Construction and characterization of recombinant *Vibrio cholerae* strains producing inactive cholera toxin analogs. Infect. Immun. **62:**3051–3057.
16. **Hirabayashi, Y., S. I. Tamura, Y. Suzuki, T. Nagamine, C. Aizawa, K. Shimada, and T. Kurata.** 1991. H-2-unrestricted adjuvant effect of cholera toxin B subunit on murine antibody responses to influenza virus haemagglutinin. Immunology **72:**329–335.
17. **Holmes, R. K., and E. M. Twiddy.** 1983. Characterization of monoclonal antibodies that react with unique and cross-reacting determinants of cholera toxin and its subunits. Infect. Immun. **42:**914–923.
18. **Holmgren, J., A. M. Svennerholm, J. Clemens, D. Sack, R. Black, and M. Levine.** 1987. An oral B subunit-whole cell vaccine against cholera: from concept to successful field trial. Adv. Exp. Med. Biol. **216B:**1649–1660.
19. **Holmgren, J., A. M. Svennerholm, M. Jertborn, J. Clemens, D. A. Sack, R. Salenstedt, and H. Wigzell.** 1992. An oral B subunit: whole cell vaccine against cholera. Vaccine **10:**911–914.
20. **Lai, C., F. Cancedda, and L. K. Duffy.** 1981. ADP-ribosyltransferase activity of cholera toxin polypeptide A1 and the effect of limited trypsinolysis. Biochem. Biophys. Res. Commun. **102:**1021–1027.
21. **Lebens, M., S. Johansson, J. Osek, M. Lindblad, and J. Holmgren.** 1993. Large-scale production of Vibrio cholerae toxin-B subunit for use in oral vaccines. Biotechnology **11:**1574–1578.
22. **Levine, M. M., D. Herrington, G. Losonsky, B. Tall, J. B. Kaper, J. Ketley, C. O. Tacket, and S. Cryz.** 1988. Safety, immunogenicity and efficacy of recombinant live oral cholera vaccines, CVD 103 and CVD 103-HgR. Lancet **i:**467–470.
23. **Levine, M. M., and O. S. Levine.** 1994. Changes in human ecology and behavior in relation to the emergence of diarrheal diseases, including cholera. Proc. Natl. Acad. Sci. USA **91:**2390–2394.
24. **Lindholm, L., J. Holmgren, M. Wikstrom, U. Karlsson, K. Andersson, and N. Lycke.** 1983. Monoclonal antibodies to cholera toxin with special reference to cross-reactions with *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **40:**570–576.
25. **Lobet, Y., C. W. Cluff, and W. Cieplak, Jr.** 1991. Effect of site-directed mutagenic alterations on ADP-ribosyltransferase activity of the A subunit of *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **59:**2870–2879.
26. **Mandal, B. K.** 1993. Epidemic cholera due to a novel strain of V. cholerae non-01. The beginning of a new pandemic? J. Infect. **27:**115–117.
27. **Mekalanos, J.** 1988. Production and purification of cholera toxin. Methods Enzymol. **165:**169–175.
28. **Mekalanos, J. J., D. J. Swartz, G. D. Pearson, N. Harford, F. Groyne, and M. de Wilde.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature (London) **306:**551–557.
29. **Merritt, E. A., S. Sarfaty, F. Vandenakker, C. Lhoir, J. A. Martial, and W. G. J. Hol.** 1994. Crystal structure of cholera toxin B-pentamer bound to receptor GM1 pentassacharide. Protein Sci. **3:**166–175.
30. **Okamoto, K., A. Miyama, T. Tsuji, T. Honda, and T. Miwatani.** 1988. Effect of substitution of glycine for arginine at position 146 of the A1 subunit on biological activity of *Escherichia coli* heat-labile enterotoxin. J. Bacteriol. **170:**2208–2211.
31. **Pearson, G. D. N., and J. J. Mekalanos.** 1982. Molecular cloning of Vibrio cholerae enterotoxin genes in Escherichia coli K-12. Proc. Natl. Acad. Sci. USA **79:**2976–2980.
32. **Peltola, H., A. Siitonen, H. Kyronseppa, I. Simula, L. Mattila, P. Oksanen, M. J. Kataia, and M. Cadoz.** 1991. Prevention of travellers' diarrhoea by oral B-subunit/whole-cell cholera vaccine. Lancet **338:**1285–1289.
33. **Peterson, A., K. E. Hejtmancik, D. E. Markel, J. P. Craig, and A. Kurosky.** 1979. Antigenic specificity of neutralizing antibody to cholera toxin. Infect. Immun. **24:**774–779.
34. **Pizza, M., A. Covacci, M. Bugnoli, R. Manetti, and R. Rappuoli.** 1990. The S1 subunit is important for pertussis toxin secretion. J. Biol. Chem. **265:** 17759–17763.
35. **Pizza, M., M. Domenighini, W. Hol, V. Giannelli, M. R. Fontana, M. M. Giuliani, C. Magagnoli, S. Peppoloni, R. Manetti, and R. Rappuoli.** 1994. Probing the structure-activity relationship of Escherichia coli LT-A by site-directed mutagenesis. Mol. Microbiol. **14:**51–60.
36. **Pizza, M., M. R. Fontana, M. M. Giuliani, M. Domenighini, C. Magagnoli, V. Giannelli, D. Nucci, W. Hol, R. Manetti, and R. Rappuoli.** 1994. A genetically detoxified derivative of heat-labile E. coli enterotoxin induces neutralizing antibodies against the A subunit. J. Exp. Med. **6:**2147–2153.
37. **Rappaport, R. S., B. A. Rubin, and H. Tint.** 1974. Development of a purified cholera toxoid. I. Purification of toxin. Infect. Immun. **9:**294–303.
38. **Rappuoli, R., and M. Pizza.** 1991. Structure and evolutionary aspects of ADP-ribosylating toxins, p. 1–20. *In* J. Alouf and J. Freer (ed.), Sourcebook of bacterial protein toxins. Academic Press, New York.
39. **Remmers, E. F., R. R. Colwell, and R. A. Goldsby.** 1982. Production and characterization of monoclonal antibodies to cholera toxin. Infect. Immun. **37:**70–76.
40. **Rich, V.** 1994. Cholera in Russian federation. Lancet **344:**607.
41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
42. **Shimada, T., G. Balakrish, B. Deb, M. Albeert, R. Sack, and Y. Takeda.** 1993. Outbreak of Vibrio cholerae non-01 in India and Bangladesh. Lancet **341:** 1346–1347.
43. **Simanjuntak, C. H., P. Ohanley, N. H. Punjabi, F. Noriega, G. Pazzaglia, P. Dykstra, B. Kay, Suharyono, A. Budiarso, A. R. Rifai, S. S. Wasserman, G. Losonsky, J. Kaper, S. Cryz, and M. M. Levine.** 1993. Safety, immunogenicity, and transmissibility of single-dose live oral cholera vaccine strain CVD-103-HgR in 24-month-old to 59-month-old Indonesian children. J. Infect. Dis. **168:**1169–1176.
44. **Sixma, T. K., K. H. Kalk, B. A. M. Vanzanten, Z. Dauter, J. Kingma, B. Witholt, and W. G. J. Hol.** 1993. Refined structure of Escherichia coli heat-labile enterotoxin, a close relative of cholera toxin. J. Mol. Biol. **230:** 890–918.
45. **Sixma, T. K., S. E. Pronk, K. H. Kalk, E. S. Wartna, B. A. van Zanten, B. Witholt, and W. G. Hol.** 1991. Crystal structure of a cholera toxin-related heat-labile enterotoxin from E. coli. Nature (London) **351:**371–377.
46. **Spangler, B. D.** 1992. Structure and function of cholera toxin and related *Escherichia coli* heat-labile enterotoxin. Microbiol. Rev. **56:**622–647.
47. **Svennerholm, A. M., and J. Holmgren.** 1976. Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. Infect. Immun. **13:**735–740.
48. **Tsuji, T., T. Inoue, A. Miyama, and M. Noda.** 1991. Glutamic acid-112 of the A subunit of heat-labile enterotoxin from enterotoxigenic Escherichia coli is important for ADP-ribosyltransferase activity. FEBS Lett. **291:**319–321.
49. **Tsuji, T., T. Inoue, A. Miyama, K. Okamoto, T. Honda, and T. Miwatani.** 1990. A single amino acid substitution in the A subunit of Escherichia coli enterotoxin results in a loss of its toxic activity. J. Biol. Chem. **265:**22520–22525.
50. **Waldor, M. K., and J. J. Mekalanos.** 1994. Emergence of a new cholera pandemic: molecular analysis of virulence determinants in Vibrio cholerae O139 and development of a live vaccine prototype. J. Infect. Dis. **170:**278–283.
51. **Weber, J. T., E. D. Mintz, R. Canizares, A. Semiglia, I. Gomez, R. Sempertegui, A. Davila, K. D. Greene, N. D. Puhr, D. N. Cameron, F. C. Tenover, T. J. Barrett, N. H. Bean, C. Ivey, R. V. Tauxe, and P. A. Blake.** 1994. Epidemic cholera in Ecuador—multidrug-resistance and transmission by water and seafood. Epidemiol. Infect. **112:**1–11.
52. **Wilson, M. M., and C. Chelala.** 1994. Cholera is walking south. JAMA **272:**1226–1227.
53. **Zoller, M. J., and M. Smith.** 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. Nucleic Acids Res. **10:**6487–6500.