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A Nontoxic Cholera Enterotoxin (CT) Analog Is Chimeric with Regard to Both Epitypes of CT-B Subunits, CT-B-1 and CT-B-2

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The gene encoding a nontoxic analog, CT-2*, of cholera enterotoxin (CT) with attenuating codon substitutions in the A subunit was introduced into the attenuated *Vibrio cholerae* classical biotype mutant candidate vaccine strain CVD103, which produces the B subunit (but not the A subunit) of CT-1. The recombinant strain produces a chimeric nontoxic analog holotoxin protein containing both CT-B-1 and CT-B-2 subunits. This offers potential advantages over CVD103 in the induction of immunity against El Tor biotype and *V. cholerae* O139 strains which produce CT-B-2. The recombinant protein may also be useful in polysaccharide-protein conjugate vaccines against both O1 and O139 serovars of *V. cholerae*.

It has previously been demonstrated that *Vibrio cholerae* O1 produces two different epitypes of the immunodominant B subunits of the cholera enterotoxin (CT) that are immunologically related but not identical (5, 6, 14, 17). CT-B-1 is the prototype elaborated by the classical biotype, Inaba serotype strain 569B; by other classical biotype strains; and by the Gulf Coast clone of the El Tor biotype (5, 6, 8, 14, 17). CT-B-2 is produced by the El Tor biotype, Ogawa serotype strain 3083, by other El Tor biotype strains, and by the new choleragenic vibrios, *V. cholerae* O139 (syn. Bengal) (4, 5, 8, 14, 17). The two epitypes are readily differentiated with monoclonal antibodies (6, 14, 17). Quantitative cross-neutralization titrations have revealed the superior neutralizing activity of homologous antisera (15), suggesting that inclusion of both epitypes in cholera vaccines might prove beneficial. All previous field studies involving vaccines containing CT antigens have used CT-B-1 preparations against the prevailing epitype, usually CT-B-2, and the results have been disappointing. Some evidence suggests that the holotoxin, and even some nontoxic holotoxin analogs and derivatives, is a more effective antigen and adjuvant than is the B subunit protein alone (9, 10, 16). For example, a whole-cell vaccine combined with CT-B-1 subunit protein exhibited little, if any, long-term beneficial effects of the B subunit protein, and the vaccine was markedly less effective in children and against the El Tor biotype (3).

Recently, *V. cholerae* strains have been constructed which produce inactive CT analogs with two codon substitutions in the A subunit gene (12). The mutated gene, encoding A subunit Arg-7→Lys and Glu-112→Gln mutations, was introduced in place of the wild type gene in the previously cloned *ctxAB* operon from *V. cholerae* 3083, which produces CT-2. The resultant plasmid construct, pCT-2*, was introduced into the various serotypes and biotypes of *V. cholerae* (12). The CT isolated from engineered strains was nontoxic in infant rabbits and Y-1 adrenal cells and contained no detectable ADP-ribosyltransferase activity. Among the O1 strains used was CVD103, a genetically engineered A^- B^+ mutant of strain 569B that produces CT-B-1, which has been evaluated and found to be acceptably nonreactogenic and immunogenic in volunteers (13). The *ctxA** gene, in combination with *ctxB-2*, was introduced into CVD103 by homologous recombination (12).

The resulting CT analog protein was purified to homogeneity, from syncase medium fermentor cultures of *V. cholerae* CVD103 containing the CT-2* gene, by precipitation with sodium hexametaphosphate (18) followed by affinity chromatography on galactose-agarose (19) and gel filtration on Sephadex G-75 (7). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed nicked and unnicked CT-A subunit and CT-B subunit monomers (Fig. 1). No other bands were present. Checkerboard immunoblotting (14, 17) using mouse monoclonal anti-CT-A antibody and specific mouse monoclonal anti-CT-B-1 and anti-CT-B-2 antibodies revealed that both epitypes, CT-B-1 and CT-B-2, were present in the purified CT analog (Fig. 2). These observations suggest that the CT analog may be chimeric, but they are also consistent with the interpretation that there are two populations of CT*, one consisting of CT-A*-B-1 and one consisting of CT-A*-B-2.

This question was resolved by serologic analysis of fractions eluted from an immunoaffinity chromatography column containing immobilized monoclonal antibody specific for CT-B-1 (Fig. 3). Purified CVD103 CT* was reacted with the anti-CT-B-1 antibody-containing column, and after being washed with 10 mM sodium phosphate buffer, pH 7.0, the CT* antigen was eluted with 0.5 M glycine buffer, pH 3.5, and fractions were neutralized with 0.5 M Tris base. The column fractions were assayed in enzyme-linked immunosorbent assay (ELISA) plates coated with G_{M1} ganglioside (6) and developed with monoclonal antibodies to CT-B-1 and CT-B-2 (Fig. 3). Since only CT* molecules containing CT-B-1 antigen were trapped on the anti-CT-B-1 antibody-containing immunoaffinity chromatography column and both CT-B-1 and CT-B-2 epitypes were eluted from that column, as demonstrated by G_{M1}-ELISA, the data provide conclusive proof that CT* molecules produced by strain CVD103 carrying *ctxA*B-2* are chimeric relative to their CT-B subunits. Thus, the strain now produces

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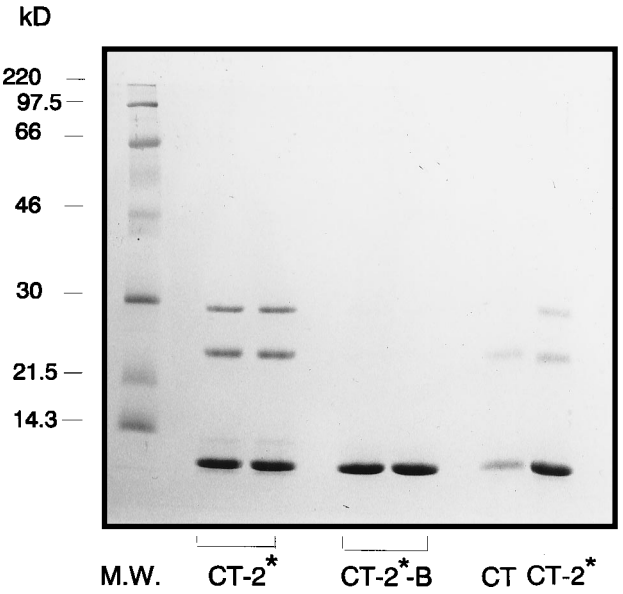

FIG. 1. SDS-PAGE of molecular size markers (M.W.), in kilodaltons, and purified CVD103 CT analog, CT-2*; its purified B subunit protein, CT-2*-B; and CT-1 (CT) and CT-2*. Unlike CT-1, the A subunits of the CT-2* proteins are not completely nicked because the preparations were purified more rapidly (1).

mutant CT-A* subunit and the two antigenic types of CT-B subunit on the same nontoxic holotoxin analog molecule. Western immunoblotting, modified from Burnette (2), confirmed these findings (results not shown).

The recombinant strain, producing the chimeric analog protein, may have advantages over the original construct in induction of immunity against El Tor and O139 strains that produce CT-B-2. The recombinant protein may also be useful in conjugate vaccines (11), including those directed at *V. cholerae*

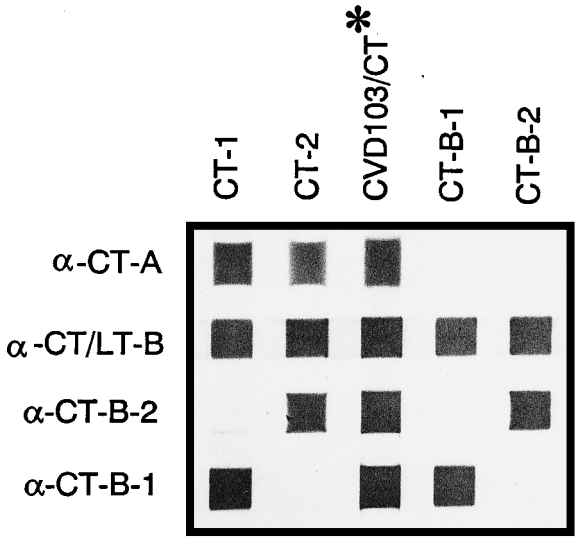

FIG. 2. Checkerboard immunoblot (14, 17) of reactions of (left side, top to bottom) monoclonal mouse anti-CT-A antibody, promiscuous monoclonal mouse anti-CT-B and anti-LT-B antibodies (14, 17), specific mouse monoclonal anti-CT-B-2 antibody, and specific mouse monoclonal anti-CT-B-1 antibody versus (top, left to right) CT-1, CT-2, CVD103 CT*, CT-B-1, and CT-B-2.

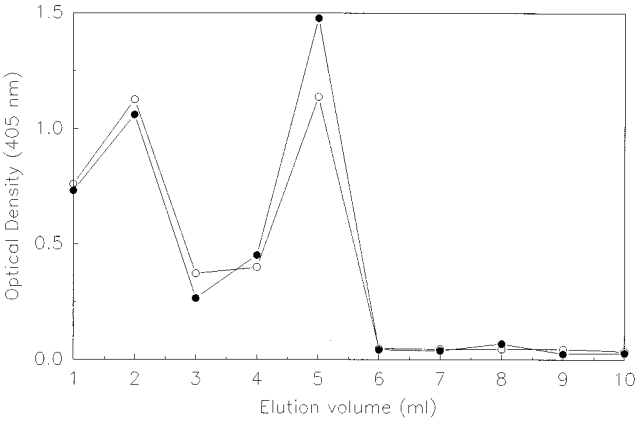

FIG. 3. Elution of purified CVD103 CT* from an anti-CT-B-1-specific monoclonal antibody affinity chromatography column. After being bound to the column and washed, the CT* was eluted from the column with 0.5 M glycine, pH 3.5. The fractions were neutralized with 0.5 M Tris base and assayed for CT-B-1 and CT-B-2 antigens with the specific monoclonal antibodies in a G_{M1}-ELISA (6). ○, anti-CT-B-1 antibody; ●, anti-CT-B-2 antibody.

O139 and in studies on the role of ADP-ribosyltransferase activity in immunogenicity and adjuvanticity of CT.

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REFERENCES

1. **Booth, B. A., M. Boesman-Finkelstein, and R. A. Finkelstein.** 1984. *Vibrio cholerae* hemagglutinin protease nicks cholera enterotoxin. Infect. Immun. **45:**558–560.
2. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112:**195–203.
3. **Clemens, J., D. A. Sack, J. R. Harris, F. van Loon, J. Chakraborty, F. Ahmed, M. R. Rao, M. R. Khan, M. D. Yunus, N. Huda, B. F. Stanton, B. A. Kay, S. Walter, R. Eeckels, A.-M. Svennerholm, and J. Holmgren.** 1990. Field trial of oral cholera vaccines in Bangladesh: results from three-year follow-up. Lancet **335:**270–273.
4. **Cunningham, J., and R. A. Finkelstein.** 1993 and 1994. Unpublished observations.
5. **Finkelstein, R. A., M. L. Vasil, and R. K. Holmes.** 1974. Studies on toxinogenesis in *Vibrio cholerae*. I. Isolation of mutants with altered toxinogenicity. J. Infect. Dis. **129:**117–123.
6. **Finkelstein, R. A., 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.
7. **Finkelstein, R. A., and J. J. LoSpalluto.** 1969. Pathogenesis of experimental cholera: preparation and isolation of choleragen and choleragenoid. J. Exp. Med. **130:**185–202.
8. **Finkelstein, R. A., K. L. Mackey, R. S. Newsham, and M. F. Burks.** 1988. Epitypes of the cholera and related enterotoxins, abstr. B103, p. 46. *In* Abstracts of the 88th Annual Meeting of the American Society for Microbiology 1988. American Society for Microbiology, Washington, D.C.
9. **Fontana, M., R. Manetti, V. Glannelli, C. Magagnoli, A. Marchini, R. Olivieri, M. Domenighini, R. Rappuoli, and M. Pizza.** 1995. Construction of nontoxic derivatives of cholera toxin and characterization of the immunological response against the A subunit. Infect. Immun. **63:**2356–2360.
10. **Fujita, K., and R. A. Finkelstein.** 1972. Antitoxic immunity in experimental cholera: comparison of immunity induced perorally and parenterally in mice. J. Infect. Dis. **125:**647–655.
11. **Gupta, R. K., S. C. Szu, R. A. Finkelstein, and J. B. Robbins.** 1992. Synthesis, characterization, and some immunologic properties of conjugates composed of the detoxified lipopolysaccharide of *Vibrio cholerae* O1 serotype Inaba bound to cholera toxin. Infect. Immun. **60:**3201–3208.
12. **Häse, C. C., L. S. Thai, M. Boesman-Finkelstein, V. L. Mar, W. N. Burnette, H. R. Kaslow, L. A. Stevens, J. Moss, and R. A. Finkelstein.** 1994. Construc-

tion and characterization of recombinant *Vibrio cholerae* strains producing inactive cholera toxin analogs. Infect. Immun. **62:**3051–3057.
13. **Kaper, J. B., J. G. Morris, Jr., and M. M. Levine.** 1995. Cholera. Clin. Microbiol. Rev. **8:**48–86.
14. **Kazemi, M., and R. A. Finkelstein.** 1990. Study of epitopes of cholera enterotoxin-related enterotoxins using checkerboard immunoblotting. Infect. Immun. **58:**2352–2360.
15. **Marchlewicz, B. A., and R. A. Finkelstein.** 1983. Immunologic differences among the cholera/coli family of enterotoxins. Diagn. Microbiol. Infect. Dis. **1:**129–138.
16. **Pierce, N. F., W. C. Cray, Jr., J. B. Sacci, Jr., J. P. Craig, R. Germanier, and E. Fürer.** 1983. Procholeragenoid: a safe and effective antigen for oral immunization against experimental cholera. Infect. Immun. **40:**1112–1118.
17. **Qu, Z.-H., and R. A. Finkelstein.** 1993. Checkerboard immunoblotting recognizes twenty epitopes among the B subunit proteins of the cholera enterotoxin family. Electrophoresis **14:**899–901.
18. **Rappaport, R. S., B. A. Rubin, and H. Tint.** 1974. Development of purified cholera toxoid. I. Purification of toxin. Infect. Immun. **9:**294–303.
19. **Uesaka, Y., Y. Otsuka, Z. Lin, S. Yamasahi, J. Yamaoka, H. Kurazono, and Y. Takeda.** 1994. Simple method of purification of *Escherichia coli* heat-labile enterotoxin and cholera toxin using immobilized galactose. Microbiol. Pathol. **16:**71–76.

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