CURRENT MICROBIOLOGY Vol. 37 (1998), pp. 231–235

Current Microbiology
An International Journal

Overexpression of a Mutant B Subunit in Toxigenic *Vibrio cholerae* Diminishes Production of Active Cholera Toxin In Vivo

Anisia Silva,[1] Rafael Fando,[2] Jorge A. Benitez[2]

[1]Facultad de Biología de la Universidad de La Habana, La Habana, Cuba
[2]Centro Nacional de Investigaciones Cientificas, P.O. Box 6990, La Habana, Cuba

Received: 29 January 1998 / Accepted: 30 April 1998

Abstract. A mutant cholera toxin B subunit containing a G33E substitution was constructed and expressed in *V. cholerae*. The G33E amino acid substitution did not affect the amount of recombinant CTB secreted to the culture medium. The overexpression of the mutant B subunits in wild-type toxigenic cholera vibrios led to an 80% decrease in production of active cholera toxin in vitro and in vivo. Overexpression of B^{G33E} subunits could be instrumental in the increase of the biosafety of live attenuated cholera candidate vaccine strains.

Vibrio cholerae of serogroups O1 and O139, the causative agents of cholera, secrete a potent enterotoxin, cholera toxin (CT), which causes the severe diarrhea of cholera [6]. The active holotoxin is an oligomeric protein composed of one A subunit (CTA; 28 kDa) and five B subunits (CTB; 11.5 kDa) [6]. CTA catalyzes NAD-dependent ADP-ribosylation of the adenylate cyclase stimulatory (Gsα) protein [6]. CTB contains the binding site for ganglioside GM_1, the CT receptor molecule [6]. CTA and CTB are synthesized as precursor polypeptides and secreted to the periplasmic space of the bacterium, where they assemble to form the AB_5 holotoxin [8]. Although CTA can not associate with fully assembled CTB pentamers in vitro, it increases the rate of CTB pentamerization in vivo [8]. Kinetic experiments suggest holotoxin molecules be assembled according to the pathway [8]:

$$nB \rightarrow B_n + A \rightarrow AB_n + (5 - n)B \rightarrow AB_5 \qquad (I)$$

where n (an integer from 1 to 4) has yet to be defined. Crystal structure determination of CT-related, heat-labile enterotoxin from *E. coli* suggested at least two B subunits be required to form the GM_1 binding site [21].

Oligonucleotide-directed mutagenesis has been used to study the contribution of specific residues to enzyme activity, receptor binding, and subunit interaction. An R7K, E112Q double-mutant A subunit lacking ADP-ribosyltransferase activity assembled properly with B subunits of CT-1 and CT-2 immunotypes to produce an inactive holotoxin [3, 9]. Missense mutations G33E, G33D, G33I, G33L in the B subunit of CT produced normal amounts of immunoreactive holotoxin which failed to bind ganglioside GM_1 and were non-toxic in the Y-1 adrenal cell assay [11].

A current strategy to develop cholera vaccines is to delete attributes of virulence to create attenuated strains. Recently, genetically engineered Δ(*ctxA*$^-$, *ctxB*$^+$) candidate vaccine strains have been claimed to be well tolerated and immunogenic in volunteers [13]. However, the recent discovery that CT genes are transduced by a filamentous bacteriophage [22] has raised concerns on the safety of live attenuated vaccine candidates. The possibility that vaccine strains currently evaluated could re-acquire CT genes by the above-mentioned or other mechanisms justifies the introduction of biosafety features in their design.

In the present work we show that overexpression of a mutant B subunit with a G33E substitution in a wild-type EI Tor biotype cholera vibrio significantly diminished production of active cholera holotoxin in vitro and in vivo. Thus, overexpression of CTB^{G33E} can be used for the enhancement of the biosafety of live attenuated cholera vaccines.

Materials and Methods

Strains and media. *V. cholerae* strains C7258 (wild type, EI Tor, Ogawa) [2, 5], 81, a Δ(*cep*, *orfU*, *ace*, *zot*, *ctxA*, *ctxB*) derivative of

Correspondence to: J.A. Benitez

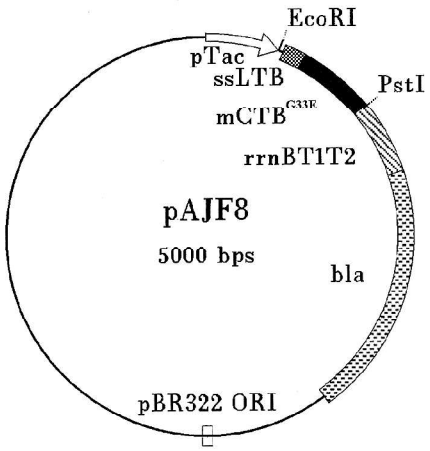

Fig. 1. Physical map of expression plasmid pAJF8. The following abbreviations are used: ssLTB (shadowed box), LTB signal sequence; mCTB (filled box), mature CTB; rrnBT_1T_2 (slanted box), *rmB* ribosomal terminator; bla (dotted box), β-lactamase.

C7258 [2, 5], and JBK70 Δ(*ctxA*, *ctxB*), El Tor, Inaba) [12] were used in this work. *E. coli* ES1301 *mutS* and JM109 [20] were used for site-directed mutagenesis and plasmid construction. All strains were grown in LB medium and conserved at −70°C in the same medium supplemented with 20% glycerol. Antibiotics were added, when necessary, at the following concentrations: ampicillin, (Amp; 100 μg/ml); tetracycline (Tet; 10 μg/ml); and chloramphenicol (Cm; 20 μg/ml). For determination of CT production in vitro, cholera vibrios and their transformants were grown in AKI cultures [10].

Genetic techniques. Plasmid pJS752-3 [18] (Amp^R) containing a recombinant CTB gene as an *Eco*RI-*Hin*dIII fragment was digested with *Hin*dIII, treated with Klenow enzyme, and subsequently digested with *Eco*RI. The fragment encoding CTB was ligated to pALTER-Ex^2 (Tet^R Cm^S; Promega Co., Madison, WI) [18], digested with *Eco*RI and *Hpa*I. The clone pALTCTB2 (Tet^R Cm^S) was purified and used as template for site-directed mutagenesis following the Promega Altered Sites II in vitro Mutagenesis System [18]. The mutagenic oligonucleotide (5′-TCTCTAGCTGAAAAAAGAGAG-3′) was designed to contain the desired GGA ($\overline{G33}$) → *GAA* (E33) amino acid substitution in the B subunit. After completion of the mutagenesis procedure, 12 JM109 transformants were analyzed for CTB production by urea extraction [19], followed by GM_1 ELISA and 4E1G5-ELISA. Clones producing a CTB detectable in the 4E1G5-ELISA but not in GM_1-ELISA were selected, and the mutation was ascertained by DNA sequencing with the T7 Sequenase 7-deaza-dGTP sequencing kit (Amersham, Life Science). The mutated CTB gene was exchanged for the wild-type CTB allele in plasmid pJS752-3 [20] to produce pAJF8 (Fig. 1). Plasmids pJS752-3 and pAJF8 are identical except for the G33E mutation in CTB. In both constructions the sequence coding for the mature CTB polypeptide has been fused to the *E. coli* heat-labile enterotoxin (LT) B subunit (LTB) secretion signal and expressed from the strong Tac promoter (Fig. 1). Plasmids pJS752-3 and pAJF8 were introduced in *V. cholerae* by electroportion [17].

Enzyme-linked immunoassays (ELISA). Production of CT was measured in culture supernatants with monoclonal antibodies (mAbs) 4E1G5 and 1G10G5 directed against CTB and CTA from *V. cholerae* strain 569B (classical, Inaba) respectively [2, 5]. The concentration of wild-type holotoxin was determined with mAb 1G10G5 in a GM_1-ELISA assay with a standard curve constructed with purified CT from *V. cholerae* strains 569B (classical, Inaba) or 3083 (El Tor, Ogawa) kindly provided by Richard A. Finkelstein (University of Missouri–Columbia School of Medicine). In this assay, CT is captured by solid phase-bound ganglioside GM_1 and recognized by mAb 1G10G5. In a second type of ELISA, designated 4E1G5-ELISA, CTB molecules were captured by coating the plates with mAb 4E1G5 and recognized by a polyclonal rabbit CTB antiserum. This ELISA detects both wild-type and GM_1-defective CTB oligomers. A third type of ELISA, designated 1G10G5-ELISA, used mAb 1G10G5 to capture CT molecules and 4E1G5 to recognize them. This assay measures the amount of oligomers containing an A subunit bound to any B subunit (total CT). ELISA plates were developed with either anti-mouse IgG peroxidase or anti-rabbit IgG peroxidase conjugates as required.

Rabbit ileal loops. 10^8 CFU of live vibrios in 0.5 ml of phosphate-buffered saline were injected into ligated ileal segments of adult rabbits as described in [4]. Briefly, New Zealand adult rabbit (2–2.5 kg) were fasted for 24 h, the small intestine withdrawn, ligated approximately 10 cm from the appendix, and subsequently divided into 5- to 6-cm segments by ligatures. After 6 h the animals were sacrificed and loop length and fluid volume measured. Results are expressed as FA = fluid volume (mL)/length (cm).

Results and Discussion

We constructed a G33E amino acid substitution in CTB by site-directed mutagenesis. This mutation was previously reported to abolish binding to GM_1 without affecting the amount of immunoreactive holotoxin as determined by a sandwich solid phase radioimmunoassay [11]. This assay, which used affinity purified equine anti-CT IgG and monospecific rabbit anti-CTB as first and second antibodies, does not provide information on the aggregation state of the A and B subunits.

We hypothesized that, if we introduce in a toxigenic strain a gene construct directing the synthesis of an excess of CTB^{G33E} subunits (B*) capable of competing with a small quantity of wild-type B molecules, the reaction

$$nB^* \rightarrow B_n^* + A \rightarrow AB_n^* + (5 - n)B \rightarrow AB_5^* \quad (II)$$

should be favored over reaction (I), leading to the synthesis of an inactive holotoxin (AB_5^*). The above assertion is based on the premise that the G33E mutation specifically affects GM_1 binding without affecting secretion to periplasm and interaction with A subunits. Consequently, transformation of a toxigenic vibrio with a gene construct directing overexpression of CTB^{G33E} would be expected to decrease the production of active holotoxin.

In order to test this hypothesis, we used the same expression system as in pJS752-3 [20] to overexpress the mutant CTB. Plasmids pJS752-3 (CTB) and pAJF8 (CTB^{G33E}) were introduced in strain JBK70 Δ(*ctxA*, *ctxB*). As shown in Fig. 2, the mutant and wild-type CTB were expressed to the same level when measured by 4E1G5-ELISA. No mutant CTB was detected in the GM_1-ELISA assay (Fig. 2). We conclude that the G33E

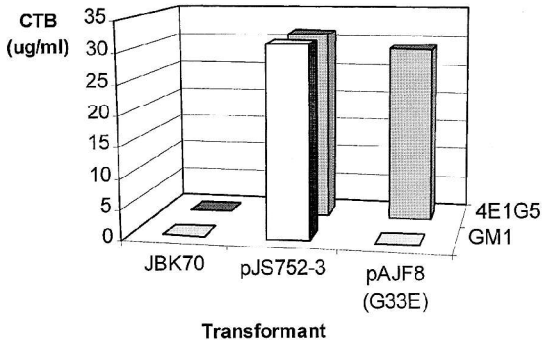

Fig. 2. Expression and secretion of wild-type CTB and mutant CTB^{G33E} in strain JBK70 as detected in two types of assays: GM1-ELISA and 4E1G5-ELISA. Each value is the mean of at least three independent AKI culture supernatants.

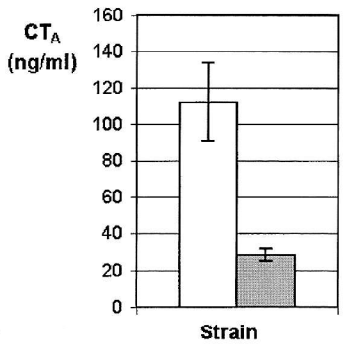

Fig. 3. Production of wild-type cholera holotoxin detected by GM_1-ELISA in *V. cholerae* strain C7258 (open bar) and C7258 transformed with pAJF8 (shaded bar). Each value is the mean of at least four independent AKI culture supernatants.

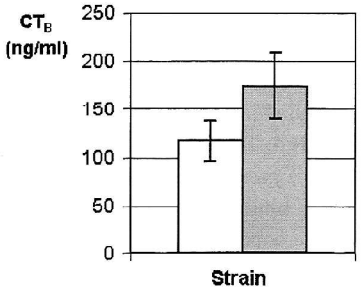

Fig. 4. Production of total cholera toxin detected by 1G10G5-ELISA in *V. cholerae* strain C7258 (open bar) and C7258 transformed with pAJF8 (shaded bar). Each value is the mean of at least four independent AKI culture supernatants.

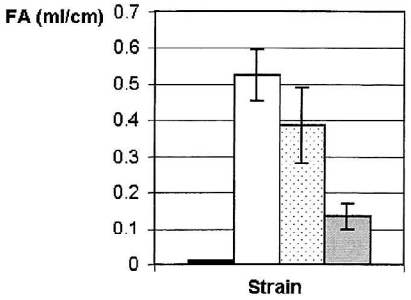

Fig. 5. Production of active cholera holotoxin in vivo detected in the adult rabbit ileal loop model. Strain 81, filled bar; Strain C7258, open bar; C7258 transformed with pJS752-3, dotted bar; C7258 transformed with pAJF8 (shaded bar). Each value is the mean of at least five independent FA determinations.

substitution does not affect the amount of CTB produced when expressed from the Tac promoter and LTB secretion signals.

Next, plasmid pAJF8 was introduced in the choleragenic vibrio C7258 and the amount of CT produced determined by GM_1-ELISA. As shown in Fig. 3, the presence of pAJF8 significantly decreased (80%; $p < 0.05$) the production of wild-type CT. In a control assay we determined the total amount of CT produced in C7258 and its transformant by 1G10G5-ELISA, which measures the amount of CTA molecules bound to any CTB (B or B*). As indicated in Fig. 4, both strains produced similar amounts of CT in this assay, indicating that the decrease in wild-type holotoxin detected in the GM_1-ELISA is due to the overexpression of B* and not an unrelated plasmid effect. Antibody 4E1G5 is more reactive toward the plasmid-encoded CTB (CT-1 immunotype) than the chromosomally encoded subunit of CT-2 immunotype [3]. The data in Fig. 4 were corrected for this difference by comparison of standard curves using purified CT from strain 569B (CT-1) and 3083 (CT-2) [3].

The slight increase in CT production measured by 1G10G5-ELISA in C7258 transformed with pAJF8 could be explained by the observation that samples of CT produced by this strain separated by SDS–PAGE without previous boiling or treatment with 2-mercaptoethanol displayed a higher molecular weight in immunoblots than predicted for an AB_5 holotoxin under identical conditions (data not shown). Each CTA molecule could be bound to a higher number of CTB subunits, enhancing the output signal of the 1G10G5-ELISA. From these studies we conclude that overexpressed CTB^{G33E} can act as a sink for CTA molecules to diminish assembly of wild-type CT.

We used the ileal loop model to examine the production of active CT in strain C7258 and its transformants. As indicated in Fig. 5, transformation of C7258 with expression plasmid pJS752-3 had a small effect on the CT activity produced in vivo. This effect could be due to competition between chromosome-encoded CT and plasmid-encoded, wild-type pentameric CTB for receptor sites. In striking contrast, transformation of C7258 with pAJF8 significantly ($p < 0.05$) diminished active holotoxin production. This result indicates that the mutant

CTB was overexpressed in vivo to diminish assembly of active CT.

It is likely that mixed oligomers containing CTB and CTB^{G33E} molecules are synthesized in C7258 transformed with pAJF8. Two contiguous CTB molecules are required to form a ganglioside GM_1 binding pocket [21]. However, since binding of CT to ganglioside GM_1 exhibits positive cooperativity [6], mixed oligomers with diminished binding sites would be expected to bind GM_1 with much lower affinities.

The re-emergence of cholera in the Americas and the recent outbreak of a new toxigenic vibrio, O139 [1], in India has emphasized the necessity for a safe and effective vaccine. Volunteer studies have shown that orally administered live, genetically attenuated cholera vibrios (*ctxA*; *ctxB*; or $ctxA^-$, $ctxB^+$) are strongly immunogenic and protective [16]. The discovery that CT genes can be transduced to live vaccine strains by a filamentous phage [22] has prompted studies directed to enhance the biosafety of live attenuated vaccines. The CTXΦ phage uses a colonization factor (toxin co-regulated pilus, TCP) as receptor. It has not been possible to inactivate the phage receptor domain of TCP without affecting colonization and immunogenicity [15]. Recently, the *rstR* gene product has been proposed to confer immunity to El Tor biotype CTXΦ [14]. It has not been demonstrated that this factor confers immunity to CTXΦ from classical biotype vibrios [14]. Our results provide a simple approach to increase the biosafety of live cholera vaccines. In summary, given an A-B type toxin, overexpression of a receptor-defective carrier (B*) can be used as a sink for a potentially reacquired toxic A subunit.

A G33D mutation, which rendered LT incapable of binding GM_1, severely reduced its immunogenicity and adjuvanticity [7]. Volunteer studies have demonstrated that live attenuated cholera vaccine strains provide solid protection in the absence of anti-toxin immunity [16]. Nevertheless, experiments are in progress to determine immunogenicity of live cholera vaccines expressing CTB^{G33E}.

Conclusions

1. We constructed a mutant CTB with a G33E amino acid substitution that does not bind ganglioside GM_1.
2. The CTB^{G33E} subunit was efficiently expressed and secreted in *V. cholerae*.
3. Overexpression of CTB^{G33E} in a choleragenic vibrio diminished production of active cholera holotoxin in vitro and in vivo.

ACKNOWLEDGMENTS

We are grateful to R.A. Finkelstein (University of Missouri School of Medicine, Columbia, MO) for encouragement and fruitful discussions. We thank Reynaldo Oliva (Instituto Finlay, La Habana, Cuba) for assistance in the ileal loop experiments.

Literature Cited

1. Albert MJ (1994) *Vibrio cholerae* O139 Bengal. J Clin Microbiol 32:2345
2. Benítez JA, Silva A, Rodríguez BL, Fando R, Campos J, Robert A, García H, García L, Pérez JL, Oliva R, Torres CA, Ledón T (1996) Genetic manipulation of *Vibrio cholerae* for vaccine development: construction of live attenuated El Tor candidate vaccine strains. Arch Med Res 27:275–283
3. Boesman-Finkelstein M, Peterson JW, Thai LS, Finkelstein RA (1996) A nontoxic cholera enterotoxin analog is chimeric with regard to both epitypes of CTB subunits CTB-1 and CTB-2. Infect Immun 64:346–348
4. De SN (1959) Enterotoxicity of bacterial-free culture filtrates of *Vibrio cholerae*. Nature 183:1533–1534
5. Fando R, Pérez JL, Rodriguez BL, Campos J, Robert A, García L, Silva A, Benitez JA (1997) Promoter activities in the CTXΦ prophage. Infect Immun 65:1561–1565
6. Finkelstein RA. (1992) Cholera enterotoxin (choleragen): a historical perspective. In Barua D, Greenough WB (eds) Cholera. New York: Plenum Medical Book Company, pp 155–187
7. Guidry JJ, Cardenas L, Cheng E, Clements JD (1997) Role of receptor binding in toxicity, immunogenicity, and adjuvanticity of *Escherichia coli* heat-labile enterotoxin. Infect Immun 65:4943–4950
8. Hardy SJS, Holmgren J, Johansson S, Sanchez J (1988) Coordinated assembly of multisubunit proteins: oligomerization of bacterial enterotoxins in vivo and in vitro. Proc Natl Acad Sci USA 85:7109–7113
9. Häse CC, Thai LS, Boesman-Finkelstein M, Mar VL, Burnette WN, Kaslow HR, Stevens LA, Moss J, Finkelstein RA (1994) Construction and characterization of recombinant *Vibrio cholerae* strains producing inactive cholera toxin analogs. Infect Immun 62:3051–3057
10. Iwanaga M, Yamamoto K, Higa N, Ichinose Y, Nakasone N, Tanabe M (1986) Culture conditions for stimulating cholera toxin production by *Vibrio cholerae* O1 El Tor. Microbiol Immunol 30:1075–1083
11. Jobling MG, Holmes RK (1991) Analysis of structure and function of the B subunit of cholera toxin by the use of site-directed mutagenesis. Mol Microbiol 5:1755–1767
12. Kaper JB, Lockman H, Balsini, Levine MM (1984) Recombinant nontoxigenic *Vibrio cholerae* strains as attenuated cholera vaccine candidates. Nature 308:655–658
13. Kenner JR, Coster TS, Taylor DN, Trofa AF, Barrera-Oro M, Hyman T, Adams JM, Beattie DT, Killeen KP, Spriggs DR, Mekalanos JJ, Sadoff JC (1995) Peru-15, an improved live attenuated oral vaccine candidate for *Vibrio cholerae* O1. J Infect Dis 172:1129–1129
14. Kimsey H, Waldor M (1997) Heteroimmunity amongst CTXΦ. Proceeding of 33rd US-Japan Cholera and Related Diarrheal Disease Joint Panel Meeting. December 3–5, Clearwater Beach, FL, pp 42–46
15. Kirn TJ, Sandoe CMP, Chiang SL, Mekalanos JJ, Taylor RK (1997) Genetic Analysis of TCPA pilin structural and functional domains. Proceeding of 33rd US-Japan Cholera and Related Diarrhea Disease Joint Panel Meeting. December 3–5, Clearwater Beach, FL, pp 30–32

16. Levine MM, Tacket CO (1994) Recombinant live cholera vaccines. In Kaye Wachsmuth I, Blake PA, Olsvik O (Eds) *Vibrio cholerae* and cholera: molecular to global perspective. Washington DC: American Society for Microbiology Press, pp 395–413
17. Marcus H, Ketley JM, Kaper JB, Holmes RK (1990) Effect of DNase production, plasmid size and restriction barrier on transformation of *Vibrio cholerae* by electroportion and osmotic shock. FEMS Microbiol Lett 68:149–154
18. Promega Technical Manual (1995) Altered Sites II In Vitro Mutagenesis System. Part # TM001
19. Qu Z-H, Boesman-Finkelstein M, Finkelstein RA (1991) Urea-induced release of heat-labile enterotoxin from *Escherichia coli*. J Clin Microbiol 29:773–777
20. Sánchez J, Holmgren J (1989) Recombinant system for overexpression of cholera toxin B subunit in *Vibrio cholerae* as a basis for vaccine development. Proc Natl Acad Sci USA 86:481–485
21. Sixma TK, Pronk SE, Kalk KH, Wartna ES, Van Zanten BAM, Witholt B, Hol WGJ (1991) Crystal structure of a cholera toxin-related heat labile enterotoxin from *E. coli*. Nature 351:371–377
22. Waldor MK, Mckalanos JJ (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. Science 272:1910–1914