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Construction of a *Vibrio cholerae* Vaccine Candidate Strain O395-N1-E1 which Accumulates Cell-associated Cholera Toxin B Subunit

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Abstract

Because of its production and use in Vietnam, the most widely used oral cholera vaccine consists of heat- or formalin-killed *Vibrio cholerae* whole cells (WC). An earlier version of this type of vaccine called whole cell-recombinant B subunit vaccine (BS-WC) produced in Sweden also contained the B subunit of cholera toxin (CTB). Both WC and BS-WC vaccines produced moderate levels of protection in field trials designed to evaluate their cholera efficacy. *V. cholerae* cells in these vaccines induce antibacterial immunity, and CTB contributes to the vaccine's efficacy presumably by stimulating production of anti-toxin neutralizing antibody. Although more effective than the WC vaccine, the BS-WC vaccine has not been adopted for manufacture by developing world countries primarily because the CTB component is difficult to manufacture and include in the vaccine in the doses needed to induce significant immune responses. We reasoned this was technical problem that might be solved simply by engineering strains of *V. cholerae* that express cell-associated CTB that would co-purify with the bacterial cell fraction during manufacture of WC vaccine. Here we report that construction of a *V. cholerae* O1 classical strain, O395-N1-E1, that has been engineered to accumulate CTB in the periplasmic fraction by disrupting the *epsE* gene of type II secretion pathway. O395-N1-E1 induces anti-CTB IgG and vibriocidal antibodies in mice immunized with two doses of formalin killed whole cells. Intraperitoneal immunization of mice with O395-N1-E1 induced a significantly higher anti-CTB antibody response compared to that of the parental strain, O395-N1. Our results suggest that this type of cholera vaccine candidate strain may assist in preparing improved, effective, and inexpensive oral or parenteral cholera vaccine without the need to purify CTB separately.

Keywords

cholera vaccine; cholera toxin B subunit; *Vibrio cholerae*

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1. Introduction

Cholera is an acutely dehydrating, watery diarrheal disease caused by intestinal infection with the bacterium *Vibrio cholerae*. Epidemic cholera is caused by *V. cholerae* serogroups O1 and, more recently, O139 [1]. Among the multiple virulence factors in *V. cholerae*, cholera toxin (CT) plays a major role in the pathogenesis of infection [1, 2]. CT is composed of two types of subunits: a single copy of A (CTA), which is an ADP-ribosylating toxin, and a pentamer of CTB which binds the holotoxin to its ganglioside receptor located on the apical membrane of intestinal epithelial cells [2]. Exposure to CT elevates cAMP levels in intestinal epithelial cells, resulting ultimately in the secretory diarrhea that is characteristic of cholera [2, 3].

CT is secreted extracellularly by a type II secretion system (T2SS) [4, 5]. The T2SS is assembled from at least 12 *eps* encoded gene products and mediates transport of proteins from the periplasm of *V. cholerae* to the extracellular compartment [4, 5]. One such gene product is the cytoplasmic NTPase *EpsE* [6, 7]. Mutations in this protein block extracellular secretion of T2SS substrates such as cholera toxin and lead to its accumulation in the periplasmic compartment [8, 9, 10].

Antibacterial immunity is thought to play a dominant role in protection of cholera [11, 12, 13, 14], but CTB contributes to the efficacy of cholera vaccine. Vaccination with oral BS-WC vaccine provided better protection against cholera than oral WC vaccine alone, although the increased efficacy of the B-subunit whole-cell preparation was evident only in the first 8 to 12 months after immunization [11, 12, 13]. This result indicated the importance of CTB as a component in an oral cholera vaccine in which CTB is combined with killed whole *V. cholerae* cells. One such oral BS-WC is produced by SBL in Sweden and contains 1 mg of the nontoxic CTB and either heat or formalin killed *V. cholerae* O1 strains. However, the cost of producing purified CTB by traditional recombinant methods is considered to be relatively high and prohibitive to developing world countries that might be interested in producing oral cholera vaccines locally such as Vietnam [15, 16, 17, 18]. CTB has also been reported to have mucosal adjuvant and immunomodulating activity [19]. Thus, inclusion of CTB in an oral vaccine formulation might produce unexpected changes in the immune response to bystander bacterial antigens that include variations in magnitude of antibody responses and the types of antibodies made [19, 20]. However, the physical association of CTB with other antigens might be necessary to elicit adjuvant effects [21]. Although, cholera toxin is a potent mucosal adjuvant that can be simply mixed with antigens, its toxicity prohibits its use in orally administered preparations [19].

Over two decades ago, recombinant DNA technology was used to engineer *V. cholerae* strains that produce only the nontoxic CTB subunit of cholera toxin [22, 23]. Such strains have been explored as live attenuated cholera vaccines [22, 24, 25, 26, 27] but not as components of a killed oral cholera vaccine. This is simply because the CTB these strains produce is exported to the supernatant during cell growth; thus, extra purification and processing would be required to include the co-produced CTB with the WC fraction. We reasoned this was technical problem that might be solved by making derivatives of CTB producing vaccine candidates that did not extracellularly secrete CTB. The same processing steps that produce the WC component of killed oral vaccine would be predicted to concentrate the CTB. If formalin or heat inactivation of the whole bacterial cells from such “nonsecretor” strains did not destroy all the immunogenicity of this cell-associated CTB, one might predict that a vaccine made from these cells might be equal to or perhaps even better than the first combination WC+CTB vaccines [11, 12]. In this study, we report the construction of a *V. cholerae* O1 vaccine strain in which disruption of the *epsE* gene [10], has resulted in accumulation of CTB in the periplasmic fraction. The constructed strain was

tested for the suitability as a component of a killed WC cholera vaccine by measuring anti-CTB IgG response and vibriocidal activity in mice.

2. Materials and Methods

2.1. Bacterial strains and plasmids

All strains and plasmids used in this study are described in Table 1. Strains were grown in Luria broth (LB) or on L agar (LA), and stored at -80°C in LB containing 20% glycerol (v/v). Antibiotics were used at the following concentrations unless otherwise noted: Ampicillin (Amp; 100 $\mu\text{g/ml}$), Rifampicin (Rif; 30 $\mu\text{g/ml}$), Kanamycin (Kan; 30 $\mu\text{g/ml}$), Streptomycin (Strep; 100 $\mu\text{g/ml}$).

2.2. Nucleic acid manipulations

All nucleic acid manipulations were carried out according to standard protocols [28]. Cloning of PCR products were accomplished using the TOPO TA Cloning kit (Invitrogen, Carlsbad, Calif.) in accordance with the manufacturer's directions. PCR primers were synthesized either at Quiagen (USA) or Bioneer Co. (Korea). DNA sequencing was performed by the DNA sequencing facility at Korean National Institute of Health. PCR reactions were typically carried out in 50 μl volumes with exTaq polymerase under conditions specified by the manufacturer (Takara).

2.3. Construction of *epsE* mutants of *V. cholerae*

For the construction of pWM91-D3E4-Kan, the coding region of *epsE* and *epsF* was PCR-amplified from the *V. cholerae* 0395-N1 DNA template using oligonucleotides 5'-GAAGGCGCGTCAGATATTCA-3', and 5'-CAAACACCACCAGCACCACA-3' carrying the sequences of the *epsE* and *epsF*, respectively. The PCR product was cloned into pCR II-TOPO vector to produce pCRII-D3E4. For cloning the Kan cassette, the promoter and coding region of kan were PCR amplified from the pKD4 template [29] using oligonucleotides 5'-GTCTGACTGTAGGCTGGAGCTGCTTC-3', and 5'-ACGCGTATCCTCCTTAGTTCCTATT-3' carrying the sequences of the kan containing SaI and MluI sites (underlined), respectively. The Kan cassette generated with these primers was flanked by directly repeated FRT sites from pKD4, which enables deletion of the Kan cassette by FLP-recombination [29]. The PCR product was cloned into pCRII-TOPO (Invitrogen, CA) and digested with *SaI* and *MluI*. The resulting 1.5-kb fragment was inserted between the *SaI* and *MluI* sites of pCRII-D3E4 to generate pCRII-D3E4-kan. By digesting pCRII-D3E4 with *SaI* and *MluI*, 0.3 kb of the coding region of the *epsE* gene was deleted. pCRII-D3E4-kan was then digested with *Bam*HI and *Xho*I and introduced between the *Bam*HI and *Xho*I sites of pWM91 [30] to generate pWM91-D3E4-kan. pWM91 carries the *pir*-dependent R6K replicon, the RP4 origin of transfer, and the counter-selectable *sacB* gene [30]. pWM-D3E4-kan was mobilized from *E. coli* SM10/ λ pir+ [31] into *V. cholerae* 0395-N1 and 569B-N1 by conjugational transfer, and recombinants were selected on Amp and Kan-plates with Strep or Rif, respectively. To allow for positive selection of a second recombinant event, recombinants were grown in LB overnight at 37°C without selection and plated on LB agar (without NaCl) containing 6 % sucrose, Kan, and appropriate selective antibiotics to select for sucrose-resistant, Kan-resistant, Amp-sensitive colonies. The disruption of the *epsE* gene in each strain was confirmed by PCR and sequencing.

2. 4. CTB assay

V. cholerae cells were grown in LB with appropriate antibiotic at 37°C overnight. Then, culture supernatants and cells were collected by centrifugation. Cells were washed with PBS and broken by sonicator. CTB of either supernatant or cell fraction was quantitized with

ganglioside-dependent ELISA described previously with commercial CTB subunit (Sigma) as a standard [32].

2. 5. Mouse Immunization

Groups of female ICR mice at 4 weeks of age (Damul Science, Korea) were immunized by intraperitoneal (i. p.) injection on days 1 and 14. For vaccination, each strain was grown, and washed, and suspended with PBS. Then the cells were inactivated by adding formaldehyde to 0.5% (v/v). Before injection, the cells were washed with PBS to eliminate residual formaldehyde, and injected at 2.0×10^9 cells per dose without adjuvant. Each dose was dissolved in 500 μ l of PBS and PBS was used as negative control. Sera were obtained before immunization (preimmune) and 1 week after the second vaccination.

2. 6. Measurement of anti-CTB IgG

Anti-CTB IgG was measured by the method of Ryan et al. [20] with modifications. In brief, microtiter plates were coated with type III gangliosides (Sigma) in 50 mM sodium carbonate buffer (pH 9.6). Following overnight incubation at room temperature and three washes in PBS-T of each plate, 100 ng of CTB in carbonate buffer was applied to each well and the plates were again incubated overnight at room temperature. Following washing in PBS-T, the plates were blocked with PBS-BSA (1%). Then, to detect anti-CTB IgG antibody in sera, duplicate samples of serial dilutions of sera were placed in wells of microtiter plates previously coated with ganglioside-CTB and blocked with PBS-BSA. The plates were incubated at room temperature overnight and washed in PBS-T. A 1:2,000 dilution in PBS-T of goat anti-mouse IgG conjugated to peroxidase was added each well and the plates were incubated at room temperature for 2 h. Following washing in PBS-T, the plates were developed for peroxidase activity and the optical density at 405 nm was measured. End dilutions were calculated as the highest dilution of test sample producing an optical density greater than three times the mean of control wells to which PBS-T without serum was added.

2. 7. Vibriocidal Activity

Serum vibriocidal antibody titers were measured in a microassay according to the method of Ryan et al [20] with modifications. Briefly, the endogenous complement activity of test sera was inactivated by heating the sera to 56 °C for 1h. Fifty μ l aliquots of serial dilutions of test sera in PBS were placed in the wells of sterile 96-well tissue culture plate; 50 μ l of a 10^8 -CFU/ml culture of *V. cholerae* strain in PBS with 22% guinea pig complement (Gibco BRL Life Technologies, Gaithersburg, Md.) was added to the serum dilutions, and the mixtures were incubated for 1h at 37 °C. One hundred fifty μ l of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) was added to each well, and the plates were incubated for 2 h at 37 °C. The optical density at 600 nm was then measured; the vibriocidal titer was calculated as the dilution of serum causing 50% reduction in optical density compared with that of wells containing no serum.

3. Results

3. 1. Construction of 0395-N1-E1 and 569B-N1-E1

For the construction of CTB-accumulating vaccine strains, we used classical O1 strains in which the *ctxA* genes were deleted, 0395-N1 and 569B-N1 strains, Ogawa and Inaba serotypes, respectively [33, 34]. These strains were known to be good producers of toxin-coregulated pili, which plays a critical role in colonization of *V. cholerae* [35] and is a potent protective antigen [36, 37]. The *epsE* genes of classical strains, 0395-N1 and 569B-N1 were disrupted by deleting the 0.3 kb *SaI*-*MluI* fragment of the *epsE* gene and insertion of the Kan cassette between those sites by using the plasmid, pWM91-D3E4-Kan as

described in the Materials and methods section. Since the presence of antibiotic markers is undesirable in vaccine candidates, pWM91-D3E4-Kan was designed such that its *kan* resistance allele is flanked by directly repeated FRT sites. The FRT-*kan* cassette permits the use of antibiotic selection to isolate *V. cholerae* mutant cells, and subsequent FLP-mediated excision to remove the marker from the chromosome. The constructed mutant strains were named 0395-N1-E1 and 569B-N1-E1, respectively.

Recent reports indicate that either *epsE* or *epsD* deficiency impairs growth of *V. cholerae* [38, 39]. In the case of the *epsD* deficiency mutant, the authors indicated that their *epsD::Kan* mutants are not entirely devoid of functional EpsD and that residual EpsD activity enables bacterial survival [39]. Consistent with the previous studies, 0395-N1-E1 strain constructed in this study grew more slowly than 0395-N1 in LB (data not shown). 569B-N1-E1 grew slowly in LB agar than 569B-N1 but did not grow in LB liquid medium. In our lab, efforts either to delete the complete *epsE* gene or to delete the *kan* gene from 0395-N1-E1 and 569B-N1-E1 by FLP-recombination as described previously [40] were unsuccessful. This may indicate that our *epsE::Kan* mutant strains, in which the *Kan* cassette was inserted 355 amino acids from the NH₂-terminal of 504 amino acid *EpsE* protein, are not entirely devoid of functional *EpsE*, similar to the case of the *epsD::Kan* mutants described in the earlier report [39].

3. 2. Determination of cell-accumulated CTB in 0395-N1 and 0395-N1-E1

We determined whether CTB accumulated inside 0395-N1 and 0395-N1-E1 cells during normal growth in rich media. Cells were grown in LB (pH 6.5) either with Strep or Strep and Kan at 30°C, and culture supernatants and cells were collected by centrifugation. Cells were washed with PBS and broken by sonication. CTB in both the supernatant and cell fractions was measured with ganglioside-dependent ELISA [32]. Approximately, 56% of the CTB produced in 0395-N1-E1 was accumulated intracellularly, compared to only 10% in 0395-N1 and 7% in 569B-N1 (Table 2). The 0395-N1-E1 cells harboring an isopropyl β-D-thiogalactoside-inducible CTB-expressing plasmid, pMal-CTB, produced 18.35 ± 6.15 μg of CTB per ml. Approximately, 89% of the expressed CTB, 16.35 ± 6.40 μg accumulated intracellularly. Due to a growth defect, 569B-N1-E1 cells were not analyzed by ELISA (data not shown).

3. 3. Anti-CTB IgG response and vibriocidal activity of 0395-N1 and 0395-N1-E1

Because animal models for evaluating oral cholera immunogens that are predictive of the likely immune responses expected in humans generally do not exist [26, 27], we chose to study the immunogenicity of 0395-N1-E1 in mice by parenteral immunization. Sera obtained before immunization (pre-immune) and 1 week after the second vaccination were assayed for anti-CTB IgG and vibriocidal antibody titers according to the methods of Ryan et al [20] with modifications. Compared to responses in mice that received 0395-N1, mice that received 0395-N1-E1 showed approximately 40-fold higher anti-CTB IgG responses ($p < 0.05$) (Fig. 1A). Vibriocidal activities were measured against 0395-N1 cells. As expected, sera from mice immunized with either 0395-N1 or 0395-N1-E1 showed high vibriocidal activities; there were no significant differences between these two groups (Fig. 1B). Pre-immune sera and sera from mice injected with PBS did not show any significant anti-CTB IgG response or vibriocidal activity (Figs. 1A and 1B).

4. Discussion

In summary, we have generated a *V. cholerae* O1 classical vaccine candidate strain, 0395-N1-E1, in which disruption of the *epsE* gene of the type II secretion pathway causes expressed CTB to accumulate in the whole cell fraction. Whole cells of 0395-N1-E1

inactivated with formalin induced an anti-CTB-IgG response and serum vibriocidal activity in mice. Although 0395-N1-E1 grew slowly compared to the parental strain 0395-N1, our results indicate that 0395-N1-E1 might be useful as a component of formalin- or heat-killed parenteral or oral cholera vaccines, and this strain eliminates the need for separately purifying CTB for inclusion in such vaccines.

Live attenuated cholera vaccine candidates, deleted in the gene for CTA and other putative virulence genes have also been evaluated in volunteer studies over the last few decades [22, 24, 25, 26, 27, 41, 42]. While vaccines such as O395-N1 [23, 25] have been generally immunogenic and protective even after a single oral dose, they generally suffer from some degree of “reactogenicity” which is defined as the ability to induce adverse symptoms in vaccines that include diarrhea, nausea, cramps and vomiting [22]. The putative toxin responsible for these symptoms remains undiscovered [23]. Indeed, reactogenicity may not be associated with a toxin since at least one strategy for production of non-reactogenic, live attenuated vaccines involves eliminating production of flagella which are known to contain the proinflammatory Toll-like receptor ligand ‘flagellin’ [24, 26, 27, 41]. In this context, it is interesting to note that *V. cholerae* *epsE* mutants have been reported to be altered in other properties, such as translocation of many extracellular enzymes and the production of certain outer membrane proteins [8, 9, 10]. Thus, O395-N1-E1 might also be an interesting strain to evaluate as a live, attenuated vaccine candidate, since its *epsE* mutation might attenuate the relatively mild reactogenicity associated with O395-N1 [25].

Finally, it is important to note that CTB cell-associated phenotype of O395-N1-E1 is quite unique for either a killed or live oral vaccine candidates. The cell-associated CTB of this candidate might produce unexpected changes in the mucosal immune responses to CTB and bacterial antigens by virtual of its ‘packaging’ with other stimulatory bacterial ligands such as lipopolysaccharide and flagella. The uptake of killed bacterial cells by mucosal M cells is likely to be one step in the process that leads to anti-bacterial IgA responses [43]. By packaging CTB together with whole bacterial cells, one might induce better anti-CTB immune responses simply because CTB may traffic through the immune system differently under these conditions than when it utilizes its own high affinity ganglioside receptors on the apical membrane of the epithelium. Cell-associated CTB might also improve the immune response to heterologous antigens expressed in killed or live attenuated carrier vaccines based on *V. cholerae* [3, 19, 20, 21, 44].

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Fig. 1. Geometric mean titers (GMT) of anti-CTB IgG ELISA (A) and vibriocidal antibody responses (B) results from sera obtained one week after the second immunization. Mice were i. p. injected with formalin-inactivated 0395-N1, 0395-N1-E1 or PBS. Values are mean $\pm$ SD. *, $p < 0.05$ anti-CTB IgG response of mice that received 0395-N1 compared to mice that received 0395-N1-E1.

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Table 1

Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<i>E. coli</i> strains		
DH5α λ <i>pir</i>	λ <i>pir</i> -lysogen of DH5α	Laboratory collection
SM10 λ <i>pir</i>	<i>thi thr leu tonA lacy supE recA::RP-4-Tc::Mu</i> (λ <i>pir</i>) R6K	Laboratory collection [31]
TOP10	<i>F-mrcA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) <i>f801 lac</i> Δ M15 Δ <i>lacX74 deoR recA1 araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK tpsL (strR) endA1 nupG</i>	Invitrogen
<i>V. cholerae</i> strains		
0395-N1	0395 Δ <i>ctxA</i> Strep ^r (O1, Classical, Ogawa)	Laboratory collection [34]
569B-N1	569B Δ <i>ctxA</i> Rif ^r (O1, Classical, Inaba)	Laboratory collection [33]
0395-N1-E1	0395 Δ <i>ctxA</i> Strep ^r (O1, Classical, Ogawa) <i>epsE::kan</i>	This study
569B-N1-E1	569B Δ <i>ctxA</i> Rif ^r (O1, Classical, Inaba) <i>epsE::kan</i>	This study
Plasmids		
pWM91	<i>oriR6K mobRP4 sacB Amp^r Suc^s</i>	Laboratory collection [30]
pWM91 - D3E4 – Kan	<i>oriR6 mobRP4 sacB Amp^r Suc^s</i> containing the Kan cassette in <i>epsE</i> gene	This study

Table 2

Expression of CTB in classical *V. cholerae* cells and their *epsE* mutants

Strain	CTB in cell extract (μg/ml) (%)	CTB in medium (μg/ml) (%)
O395 – N1	0.26 ± 0.05 (10 %)	2.18 ± 0.87 (90 %)
O395 – N1 – E1	1.56 ± 0.07 (56 %)	1.21 ± 0.33 (44 %)
569B – N1	0.20 ± 0.10 (7 %)	2.59 ± 0.67 (93 %)
569B – N1 – E1	ND	ND

O395-N1 and O395-N1-E1 strains were grown in LB (pH 6.5) at 30°C overnight before harvest either with Strept or Strept and Kan, respectively. 569B-N1 was grown in LB with Rif. CTB of supernatant or cell fraction was quantified with ganglioside-dependent ELISA with commercial CTB (Sigma) as a standard [32].