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Short communication

Construction of a *Vibrio cholerae* prototype vaccine 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 a technical problem that might be solved by engineering strains of *V. cholerae* that express cell-associated CTB that would co-purify with the bacterial cell fraction during the 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 prototype cholera vaccine candidate strain may assist in preparing improved and inexpensive oral BS-WC cholera vaccine without the need to purify CTB separately.

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* O1 serogroup strains of the classical and El Tor biotypes and more recently, strains of the O139 serogroup [1]. Among the multiple virulence factors in *V. cholerae*, cholera toxin (CT) plays a major role in the pathogenesis of infection [1,2]. The toxin also exists in two closely related forms (classical and El Tor) encoded by two distinct filamentous bacteriophages that are present in different *V. cholerae* strains [3]. Both CTs are 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 a characteristic of cholera [2].

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 [8–10].

Antibacterial immunity is thought to play a dominant role in protection of cholera [11–14], 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 8–12 months after immunization [11–13]. 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

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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 [15–18]. 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].

Formulation of killed WC cholera vaccines can affect their efficacy by unknown mechansims. This may be due in part to the nature of the strains circulating in a given geographical region. For example, *V. cholerae* biotype El Tor entered Vietnam in 1964 and has since become an endemic cause of cholera in the region [18] and thus a recent outbreak of cholera in Hue in 2003 was exclusively caused by El Tor biotype strains [17]. However, in Bangladesh, both classical and El Tor cholera simultaneously circulated into the 1980s and more recently classical-El Tor "hybrid" strains which encode classic-like cholera toxin have been recognized in locales such as East Africa and India [3]. Because classical strains are known to produce other protein antigens distinct from El Tor strains (such as various pilins and outer membrane proteins), investigators have elected to include classical strains in killed vaccine formulations despite the fact that these strains are no longer a significant endemic cause of cholera.

In 1980s, Vietnamese scientists reported the development of a killed WC oral cholera vaccine that was modeled on a similar vaccine originally developed in Sweden [15,17]. The original formulation of the Swedish WC vaccine was changed in part to reflect the prevalence of seogroups of *V. cholerae* causing outbreaks in Vietnam. The WC monovalent serogroup vaccine produced in Vietnam contained 2.5×10^{10} formalin-killed *V. cholerae* Inaba, El Tor (strain Phil 6973); 2.5×10^{10} heat-killed *V. cholerae* Ogawa, classical (strain Cairo50); 2.5×10^{10} heat-killed *V. cholerae* Inaba, classical (strain Cairo48); and 2.5×10^{10} heat-killed *V. cholerae* Inaba, classical (strain 569B) [18]. The original Swedish WC vaccine was different from the Vietnamese monovalent vaccine in the fourth component which in the former was 2.5×10^{10} formalin-killed *V. cholerae* Ogawa, classical (strain Cairo50) [18]. In field trials in Vietnam and Bangladesh, the monovalent serogroup Vietnamese WC vaccine provided better protection compared to the monovalent seropgroup Swedish WC vaccine [11,18]. This was possibly due to the additional presence of classical strain 569B in the Vietnamese vaccine in that this strain expresses copious amounts of toxin-coregulated pili (TCP) [17], a known protective antigen and critical colonization factor of *V. cholerae* [22,23].

Over two decades ago, recombinant DNA technology was used to engineer *V. cholerae* strains that produce only the nontoxic CTB subunit of cholera toxin [24,25]. Such strains have been explored as live attenuated cholera vaccines [24,26–29] but not as components of a killed oral cholera vaccine despite the fact that they could provide a safe nontoxic CTB component. In theory these strains could be included in the killed oral cholera vaccines however extra purification and processing would be still required to include the co-produced CTB with the WC fraction because the CTB these strains would be presumably exported to the supernatant during cell production. 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 μg/ml), Rifampicin (Rif; 30 μg/ml), Kanamycin (Kan; 30 μg/ml), Streptomycin (Strep; 100 μg/ml).

2.2. Nucleic acid manipulations

All nucleic acid manipulations were carried out according to standard protocols [30]. Cloning of PCR products were accomplished using the TOPO TA Cloining kit (Invitrogen, Carlsbad, CA) 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

Table 1
Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
E. coli strains		
DH5α λ *pir*	λ *pir* lysogen of DH5α	Laboratory collection
SM10 λ *pir*	*thi thr leu tonA lacy supE recA*::RP-4-Tc::Mu (λ *pir*) R6K	Laboratory collection [33]
TOP10	*F-mrcA Δ(mrr-hsdRMS-mcrBC) f801lacΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (strR) endA1 nupG*	Invitrogen
V. cholerae strains		
O395-N1	O395 *ΔctxA* Strep^r (O1, Classical, Ogawa)	Laboratory collection [23]
569B-N1	569B *ΔctxA* Rif^r (O1, Classical, Inaba)	Laboratory collection [35]
O395-N1-E1	O395 *ΔctxA* Strep^r (O1, Classical, Ogawa) *epsE::kan*	This study
569B-N1-E1	569B *ΔctxA* Rif^r (O1, Classical, Inaba) *epsE::kan*	This study
Plasmids		
pWM91	*oriR6K mobRP4 sacB* Amp^r Suc^s	Laboratory collection [32]
pWM91-D3E4-Kan	*oriR6 mobRP4 sacB* Amp^r Suc^s containing the Kan cassette in *epsE* gene	This study