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Exploiting cholera vaccines as a versatile antigen delivery platform

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Abstract

The development of safe, immunogenic and protective cholera vaccine candidates makes possible their use as a versatile antigen delivery platform. Foreign antigens can be delivered to the immune system with cholera vaccines by expressing heterologous antigens in live attenuated vectors, as fusion proteins with cholera toxin subunits combined with inactivated *Vibrio cholerae* whole cells or by exposing them on the surface of *V. cholerae* ghosts. Progress in our understanding of the genes expressed by *V. cholerae* during infection creates unprecedented opportunities to develop an improved generation of vaccine vectors to induce immune protection against a broad range of pathogenic organisms.

Keywords

Antigen delivery; Cholera vaccines; Protein expression; Vaccine development

Cholera and cholera vaccines

Cholera is an acute diarrheal disease consisting of the passage of voluminous stools of rice water character caused by serogroup O1 *Vibrio cholerae* strains of classical and El Tor biotypes. The O1 *V. cholerae* serogroup contain a common A antigen and can be subdivided in Ogawa and Inaba serotypes on the basis of possessing type-specific antigens B and C, respectively. Currently, the 7th pandemic El Tor biotype strains have replaced the classical biotype. In 1992, a new serogroup, O139 Bengal, causing epidemic cholera was identified (Albert 1994). The current epidemiology of cholera shows a predominance of the El Tor biotype with periodic emergence of O139 strains, which exhibit a new LPS as well as a capsule. Cholera continues to be a major public health problem in developing countries. In 2006, 52 countries reported 236,896 cases and 6,311 deaths to the World Health Organization (<http://www.who.int/wer/2007/wer8231.pdf>).

The cholera bacterium is a Gram-negative, highly motile bacillus that secretes a potent enterotoxin, cholera toxin (CT), which causes the clinical symptoms of the disease (Kaper et al. 1995). CT is composed of one A subunit (CTA) which catalyzes NAD-dependent ADP-ribosylation of host adenylate cyclase and five B subunits (CTB) that carry the ganglioside GM₁ receptor binding site (Finkelstein 1992). The genes encoding CTA (*ctxA*) and CTB (*ctxB*) are encoded by the genome of the filamentous cholera phage CTXΦ (Waldor and Mekalanos 1996). The second major *V. cholerae* virulence factor is the toxin co-regulated

pilus, a type IV pilus essential for intestinal colonization in humans (Herrington et al. 1988). *V. cholerae* mutants lacking *tcpA* encoding the pilus major subunit are less immunogenic due to the intestinal colonization defect (Herrington et al. 1988). TCP also functions as the receptor of the CTX Φ phage (Waldor and Mekalanos 1996). The CTX Φ genome also encodes the zonula occludens toxin (ZOT) (Fasano et al. 1991) and the accessory cholera enterotoxin (ACE) (Trucksis et al. 1993). In addition, *V. cholerae* strains make additional putative toxic factors such hemagglutinin (HA)/protease (Häse and Finkelstein 1991), hemolysin (HlyA) (Kaper et al. 1995) and the repeat toxin RTX toxin (Lin et al. 1999). We have recently shown that both HA/protease and motility are necessary for full expression of enterotoxicity in the rabbit ileal loop model (Silva et al. 2006).

The fact that a clinical episode of cholera induces long lasting protection has fueled a sustained effort to develop an affordable, safe and effective cholera vaccine. Since mucosal immunity plays a prominent role in protection against cholera, efforts have been directed toward developing oral vaccines capable of stimulating this type of immune response. The properties expected from an ideal cholera vaccine are: (i) clinical safety, (ii) rapid onset of protection, (iii) long-term protection against O1 and O139 cholera, (iv) inexpensive to produce and deploy, and (v) safe to the environment. Significant advances have been made toward these goals during the last 10 years, yet much remains to be done.

Current cholera vaccines undergoing clinical trials are described in Table 1. These vaccines fall into two broad categories: heat or formalin-inactivated whole cells (WC) and genetically attenuated live vaccines. Both vaccine types have advantages and drawbacks. For instance, field trials of the WC/rCTB in Bangladesh and Peru have shown that the vaccine is safe and induced 85–90% protection after a two-dose administration regime (Van Loon et al. 1996). However, protection declined rapidly after six months, particularly in children. A variant of the WC/rCTB lacking rCTB has been tested in Vietnam and shown to have 66% efficacy after eight months among all age groups (Trach et al. 1997). Live genetically-attenuated vaccine candidates date back to the pioneering work of Finkelstein et al. who developed the *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced non-toxigenic and immunogenic El Tor biotype vaccine candidate Texas Star-SR (Levine et al. 1984). Since then, several generations of genetically-attenuated live vaccine candidates have been developed by precisely deleting cholera toxin and other potential toxic factors from the chromosome. The vaccine CVD103-HgR was constructed from the classical biotype strain 569B by deleting *ctxA* and *ctxB* genes from the chromosome and re-insertion of *ctxB* encoding the non-toxic CTB subunit in the *hlyA* (hemolysin) locus (Levine and Tacket 1994). This vaccine has been consistently shown to be safe, immunogenic and protective in healthy volunteers (Levine and Tacket 1994). The protective efficacy of this strain in an endemic area could not be conclusively demonstrated due to low incidence of cholera during a large field trial conducted in Indonesia (Richie et al. 2000).

Efforts to develop an El Tor biotype live attenuated vaccine to protect against 7th pandemic El Tor biotype strains encountered the unexpected hurdle of reactogenicity. El Tor biotype strains lacking *ctxA*, *ctxB*, ZOT and ACE still elicited adverse side effects (mild to moderate diarrhea) in volunteers (Michalski et al. 1993; Tacket et al. 1993). We developed a live attenuated cholera vaccine candidate, *V. cholerae* strain 638 (Robert et al. 1996). *V. cholerae* 638 was obtained by deletion of the CTX Φ cholera phage from the chromosome and inactivation of the *hapA* gene encoding HA/protease (Robert et al. 1996). Strain 638 induced strong local and systemic immune responses against cholera antigens in animal models and humans (Robert et al. 1996; Benítez et al. 1997) without inducing significant side effects (Benítez et al. 1999). These studies identified HA/protease as a reactogenicity factor. A recent volunteer challenge study demonstrated the protective efficacy of strain 638 (García et al. 2005). Another promising live genetically-attenuated cholera vaccine is Peru-15. This strain is a spontaneous non-motile mutant containing a deletion of CTX Φ and the cholera phage

attRS1 attachment sites (Kenner et al. 1995). In addition, the *ctxB* gene was re-inserted within the *recA* locus. Deletion of the attRS1 site and interruption of *recA* prevents re-acquisition of the phage encoded CT genes and homologous recombination. The strain has been demonstrated to be clinically safe, immunogenic and protective in phase I and II clinical trials (Cohen et al. 2002; Qadri et al. 2005, 2007). Live genetically attenuated cholera vaccines are a more potent immunogen than dead vaccines and require a single dose to induce a longer lasting protection.

The cost-effectiveness of cholera vaccines will be sensitive to their price (Table 1). Although these prices could become lower due to massive production, the investment of vaccinating a population against cholera will compete with other public health needs. The rationale to use a cholera vaccine in an endemic area would be stronger if it would afford protection against a second disease with similar socioeconomic and geographic distribution. For instance, it has been reported that cholera vaccination could provide some cross protection to traveler's diarrhea produced by enterotoxigenic *E. coli* (ETEC) (Peltola et al. 1991). An effort to enhance cross protection to ETEC consisted in engineering CVD103-HgR to express several ETEC colonization factors (Favre et al. 2006). Cholera vaccines can also be used as an antigen delivery system to induce cross protection against a broad range of infectious disease.

Antigen delivery systems

An antigen delivery system can be defined as a live or non-living vector capable physically delivering an antigen to cells of the immune system. Delivery of the antigen by a suitable vector is expected to target the antigen to the appropriate tissue and enhance its uptake to yield a more robust immune response. The capacity of *V. cholerae* to colonize and adhere to the mucus blanket and epithelial cells lining the gastrointestinal tract can be exploited to target antigens to the microfold (M) cells overlaying the mucosa-associated lymphoid tissue. An antigen targeted to mucosal cells of the gastrointestinal and upper respiratory tract would be more likely to elicit a strong immune response when administered by oral or intranasal (needle-free) routes. A practical advantage of in vivo delivery is that it avoids the effort and cost associated with antigen purification. Live vectors used as antigen delivery systems include Vaccinia vaccine vectors, genetically-attenuated *Salmonella typhi* vaccine strains and BCG strains currently used in some countries as a tuberculosis vaccine (Curtis III et al. 1989). More recently, genetically-attenuated *Listeria monocytogenes*, *Shigellae* and non-pathogenic commensal bacteria have received increasing attention as antigen carriers (Pouwels et al. 1996,1998; Shata et al. 2000; Spreng et al. 2000). Live vectors can also be used to deliver a DNA vaccine (Sizemore et al. 1997). For instance, splenocytes from mice provided two intranasal inoculations with a *Shigella* carrier transformed with a pCMV plasmid vector showed a proliferative response to the plasmid-encoded β -galactosidase (Sizemore et al. 1997). More recently, an inducible lysis system using lambda phage S and R genes has been described for DNA vaccine delivery from *V. cholerae* and *Salmonella enterica* (Jain and Mekalanos 2000).

An attractive, although unexplored, facet of live attenuated vaccine vectors is the possibility of co-expressing foreign antigens with other immunostimulatory molecules. As an example, genetically detoxified forms of the *E. coli* heat-labile toxin (LT) retaining immunoadjuvant activity have been expressed in *V. cholerae* live attenuated vaccine candidates (Ryan et al. 1999). Similar vaccine vectors have been constructed using a genetically detoxified CT that retains its adjuvant activity (Fontana et al. 2001). It is foreseeable that other immunomodulatory molecules, such as interleukin-12, could be co-expressed with foreign antigens to modulate the strength and type of immune responses.

Antigen delivery systems are not limited to live vectors. For instance, the rCTB moiety of the WC/rCTB vaccine can be used to deliver small epitopes fused to its amino or carboxy terminus

(Table 2). Finally, non-living *V. cholerae* ghosts discussed below have been successfully employed to express and deliver foreign antigens to induce immunity.

Engineering the expression of foreign antigens for delivery by *V. cholerae* vaccines

The rCTB part of the inactivated WC/rCTB vaccine can be potentially exploited to deliver epitopes fused to its *N*-terminus or *C*-terminus (Sanchez et al. 1990) (Table 2). For instance, a decapeptide non-toxic antigen from ETEC heat stable toxin A (STa) was fused to the *N*-terminus of CTB and expressed in a non-toxigenic *V. cholerae* strain. The chimeric protein was secreted to the culture medium, bound ganglioside GM1 and reacted with anti-STa antibodies. In a second example, a dodecapeptide repeat of the serine-rich *Entamoeba histolytica* protein (SREHP) was fused to the *N*-terminus of CTB and expressed in the genetically attenuated strain Peru-2. Oral immunization with Peru-2 expressing this construct induced systemic and mucosal anti-amebic and anti-*V. cholerae* responses (Ryan et al. 1997b). The practical downside of CTB fusions is that they are limited to small epitopes that may not always induce strong protection. It has been shown that fusion proteins containing the A₂ fragment of CTA can assemble to the CTB subunits to form immunoreactive holotoxin-light chimeras that bind to ganglioside GM1 (Jobling and Holmes 1992). Using this approach, Sultan et al. (1998) demonstrated that oral immunization of mice with a holotoxin-like molecule containing the SREHP antigen induced serum IgG and mucosal IgA anti-amebic antibodies. This antigen was expressed and purified from *E. coli* (Sultan et al. 1998). However, these recombinant holotoxin-like molecules could potentially replace the rCTB portion of the WC/rCTB vaccine or be expressed and delivered from any of the current live genetically attenuated vaccine candidates (Table 1).

Efficient expressions of heterologous proteins in bacterial vectors require suitable promoters and secretion signals. Most promoters appropriate for *E. coli* are also functional in *V. cholerae*. A list of some foreign antigens expressed in *V. cholerae* and corresponding expression signals is provided in Table 2. A study was performed to compare the performance of different promoters in the $\Delta ctxA \Delta ctxB$ *V. cholerae* vaccine candidate Peru-2 in vivo. To this end, *ctxB* was expressed from the *V. cholerae* iron-regulated *irgA* promoter, the *htpG* heat shock promoters and the *E. coli* Tac promoter (John et al. 2000). Peru-2 expressing these constructs (on a plasmid or chromosomally integrated) was used to immunize mice and promoter performance was assessed from the production of anti-CTB antibodies. Best results were obtained with the Tac promoter and a multicopy plasmid (John et al. 2000). Interestingly, the Tac and iron-regulated *irgA* were equally effective in vitro (John et al. 2000) suggesting that elevated expression in vitro does not always correlate with elevated expression and antigen delivery in vivo. We have used the Tac promoter and the LT B subunit (LTB) signal peptide on a plasmid vector to express and secrete *Coccidioides immitis* antigen 2 or proline-rich antigen (Ag2/PRA) to the periplasmic space of vaccine strain 638 (Silva et al. 2003). Delivery of Ag2/PRA using vaccine strain 638 induced both Th1- and Th2-type immune responses and splenocytes from immunized mice stimulated with purified Ag2/PRA exhibited a proliferative response with production of interferon λ (Silva and Benitez 2005). Effective antigen delivery and induction of a robust immune response requires the foreign antigen to be stably maintained in the vaccine vector strain in the host. This can be achieved by integrating the antigen into a chromosomal locus without affecting the colonization capacity (and immunogenicity) of the vector strain. Loci used to integrate foreign antigens include the *irgA* and *lacZ* genes (Butterton et al. 1993, 1995). Another suitable locus to integrate genes encoding foreign antigens is *hapA* encoding HA/protease. We have shown that *hapA* mutants colonize better the suckling mouse intestine (Robert et al. 1996; Silva et al. 2006) most likely because they detach less from the mucus blanket lining the gastrointestinal tract (Benitez et al. 1997). Furthermore, we have

shown that foreign antigens such as tetanus C fragment (TetC) are rapidly degraded by HA/protease (Hazra et al. 2007).

Integration could decrease the amount of antigen produced by the vector strain leading to a weaker immune response. An approach to solve this problem is to introduce an additional mutation in the vaccine strain *V. cholerae* 638 conferring thymidine auxotrophy by inactivating the *thyA* gene encoding thymidilate synthase (Valle et al. 2000; Silva and Benitez 2005). Since the *thyA* mutations creates a requirement for elevated thymine or thymidine concentrations not present in vivo, antigens can be expressed from a multicopy plasmid containing a complementing *thyA* gene for positive selection. A similar approach to creating a balanced lethal plasmid system consisted of constructing a $\Delta glnA$ derivative of vaccine strain Peru-2 which is unable to grow in a medium lacking glutamine (Ryan et al. 2000). Multicopy plasmids encoding a foreign antigen can then be maintained by introducing a complementing *glnA* gene leading to enhanced immune response (Ryan et al. 2000).

It is often intuitively expected that secretion of foreign antigens to the extracellular milieu should result in more effective antigen presentation and thus enhanced immunogenicity. However, this has not been demonstrated and antigens released to the extracellular milieu can also be targeted by host and bacterial proteases. Nevertheless, several systems have been used to secrete foreign antigens to the extracellular medium in *V. cholerae*. One system is the *E. coli* hemolysin system (Gentschev et al. 1996, 2001, 2002). In this prototype type I secretion system, proteins covalently linked at their C-terminus with HlyA are recognized by the HlyB–HlyD–TolC translocator and secreted to the culture medium (Gentschev et al. 1996, 2001, 2002). This approach has been used to secrete *Clostridium difficile* toxin A (TcdA) in a live attenuated vaccine (Ryan et al. 1997a). We have used the LTB and HA/protease secretion signals to secrete foreign antigens in *V. cholerae* (Silva et al. 2003; Hazra et al. 2007). In these cases the antigens were targeted to the periplasmic space (Silva et al. 2003; Hazra et al. 2007).

As discussed above, expression of foreign antigens in *V. cholerae* has relied on expression systems imported from *E. coli* and few *V. cholerae* promoters. However, new technologies such as signature-tag mutagenesis (STM) (Chiang and Mekalanos 1998), recombination-based in vivo expression technology (RIVET) (Camilli and Mekalanos 1995; Lee et al. 1999, 2001; Osorio et al. 2005) and gene expression profiling of in vivo grown *V. cholerae* (Xu et al. 2003) has opened unprecedented opportunities to engineer *V. cholerae* vaccine vectors to express foreign antigens in vivo. For instance, analysis of the transcriptome of *V. cholerae* grown in rabbit ileal loops revealed a metabolic shift toward higher expression of genes involved in anaerobic energy metabolisms (Xu et al. 2003). This finding is consistent with the successful use of the *E. coli* anaerobic *nirB* promoter to deliver TetC using a natural non-toxicogenic *V. cholerae* vaccine strain (Chen et al. 1998). Strong in vivo-expressed promoters can be identified by in vivo induced antigen technology (IVIAT). IVIAT has been devised to conduct a proteome-wide screening of in vivo expressed genes on the basis of their antigenicity using sera from patients and volunteers (Rollins et al. 2005). An interesting recent observation is that *V. cholerae* isolated from rice-water stool exhibit a 10-fold reduction of infective dose (Butler et al. 2006). Investigation of the bacterial factors responsible for hyper-infectivity will allow the development of new cholera vaccine vectors that could be used at lower doses to induce immunity to cholera and foreign antigens engineered to be strongly expressed in the human gut.

Antigen delivery using *V. cholerae* ghosts

Vibrio cholerae ghosts (VCG) are non-living, non-toxicogenic empty bacterial cell envelopes that maintain their native surface antigenic structures and cellular morphology. The VCG

platform represents a new approach in vaccine development and has been proposed as an alternative to chemical or heat-killed vaccines (Eko et al. 1999). Ghosts are produced by the controlled expression of cloned bacteriophage PhiX174 lysis gene E (Witte et al. 1990). This causes the formation of a transmembrane tunnel structure through the cell envelope of Gram-negative bacteria leading to the expulsion of cytoplasmic material through the lysis tunnel. This process results in a 99.99% killing of the bacterial population (Eko et al. 2000). The VCG system represents a highly potent, flexible and safe vaccine platform for delivery of heterologous antigens. A membrane targeting system has been developed for the attachment of foreign antigens to the inner side of the cytoplasmic membrane (Szostak and Lubitz 1991). By cloning the foreign DNA sequences into the membrane targeting vector, any gene of interest can be expressed as a hybrid protein with membrane anchors directing and attaching the heterologous protein to the bacterial envelope prior to E-mediated lysis. It has been shown that the enzymatic activities of membrane anchored enzymes are not impaired by the attachment process indicating that the membrane anchors do not interfere with the proper folding of targeted foreign proteins (Eko et al. 1999). In addition, foreign antigens can also be expressed as outer membrane fusions or exported into the periplasmic space.

Vibrio cholerae ghosts prepared from serogroups 01 and 0139 expressing TCP induced elevated serum vibriocidal antibodies in rabbits that passively protected suckling mice against oral challenge (Eko et al. 2000). Oral immunization of rabbits with VCG candidate vaccines protected against challenge in the reversible intestinal tie adult rabbit diarrheal (RITARD) model (Eko et al. 2003). The VCG platform has recently been investigated as a carrier and delivery system for multiple *Chlamydia trachomatis* proteins, eliciting Chlamydia-specific protective immunity (Eko et al. 2004; Ifere et al. 2007). In addition, a rVCG-based combination vaccine comprising chlamydial major outer membrane protein and glycoprotein D of *Herpes simplex virus 2* (HSV-2) elicited adequate Th1-associated immune effectors that simultaneously protected mice from genital challenge with high doses of live *Chlamydia* and HSV-2 (Macmillan et al. 2007). These results clearly place the VCG system as a promising alternative to current cholera vaccines and an effective antigen delivery system.

Conclusions

Cholera vaccines provide several well documented live and inactivated platforms for the expression and delivery of protective antigens. Exploiting cholera vaccines as versatile antigen delivery systems will increase the cost-effectiveness of cholera vaccination and contribute to the prevention of other infectious diseases.

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Table 1

Current cholera vaccines candidates

Vaccine	Price per dose*	Inactivation or genetic attenuation	Status	Licensing
Whole cells (WC) (ORC-Vax)	0.51	Mixture of heat or formalin-killed <i>V. cholerae</i> of classical biotype Ogawa, classical biotype Inaba, El Tor biotype Inaba and O139.	Phase III clinical trials	VaBiotech, Vietnam
Whole cells plus rCTB (WC/rCTB) (Dukoral)	2.00–3.00	Mixture of heat or formalin-killed <i>V. cholerae</i> of classical biotype Inaba, classical biotype Ogawa, El Tor biotype Inaba and El Tor biotype Ogawa supplemented with recombinant CTB.	Phase III clinical trials	SBL Vaccines, Sweden
CVD103-HgR (Orochol)	1.75	Live attenuated classical biotype O1, Inaba <i>V. cholerae</i> containing a deletion of <i>ctxAB</i> and the <i>ctxB</i> gene re-inserted in <i>hlyA</i> locus.	Phase III clinical trials	Berna Biotech, Switzerland
Peru-15 (CholeraGarde)	–	Live attenuated El Tor biotype O1 Inaba <i>V. cholerae</i> containing a deletion of the CTXφ prophage, attRS1 and the <i>ctxB</i> gene re-inserted in the <i>recA</i> locus.	Phase I and II clinical trials	AVANT Immunotherapeutics
<i>V. cholerae</i> 638	–	Live attenuated El Tor biotype O1 Ogawa <i>V. cholerae</i> containing a deletion with a deletion of the CTXφ prophage and a <i>Clostridium thermocellum</i> endoglucanase (<i>celA</i>) gene inserted in the <i>hapA</i> locus	Phase I and II clinical trials	Finlay Institute, Cuba

* Prices in US Dollars obtained from http://www.who.int/topics/cholera/publications/WHO_CDS_CSR_EDC_99_4/en/index.html

Table 2Foreign antigens expressed in *Vibrio cholerae* vaccine strains

Antigen	Expression signals	Immunogenicity	References
ETEC colonization factor antigen CFA/I	Native signals	Not tested	Favre et al. (2006)
Hepatitis B surface antigen epitope	LTB fusion	Not tested	Schödel et al. (1991)
HIV-gp120 epitope	CTB fusion	Serum anti-gp120 IgG	Bäckström et al. (1994)
ETEC heat stable toxin (STa) decapeptide	CTB fusion	Not tested	Sanchez et al. (1988)
Serine-rich <i>Entamoeba histolytica</i> protein (SREHP)	CTB fusion	Serum anti-SREHP IgG, IgA; stool and bile IgA	Ryan et al. (1997b)
ETEC genetically detoxified LT toxin (LTR192G)	<i>lacZ</i> promoter	Serum anti-LT IgG, IgA	Ryan et al. (1999)
<i>E. coli</i> O157:H7 Shiga toxin B subunit (StxB1)	Tac promoter and StxB1 signal peptide	Serum anti-StxB1 IgG	Butterton et al. (1997)
Human growth hormone (hGH)	Tac promoter and LTB signal peptide	Not tested	Ghorpade and Garg (1996)
<i>Coccidioides immitis</i> proline-rich antigen/antigen 2 (PRA/Ag2)	Tac promoter and LTB signal peptide	Serum anti-PRA/Ag2 IgG1, IgG2a	Silva et al. (2003), Silva and Benitez (2005)
<i>Clostridium difficile</i> toxin TcdA	<i>hlyA</i> promoter and HlyBD translocator	Serum anti-TcdA IgG	Ryan et al. (1997a)
Tetanus C fragment (TetC)	<i>nirB</i> promoter	Serum anti-TetC IgG	Chen et al. (1998)
<i>Bordetella pertussis</i> tracheal colonization factor (TCF)	<i>nirB</i> promoter	Not detected	Chen et al. (1998)
<i>E. coli</i> O157:H7 StxB1 and intimin EaeA	<i>hlyG</i> promoter and StxB1 signal peptide	Not detected	Butterton et al. (1995, 1997)
<i>E. coli</i> O157:H7 StxB1	<i>irgA</i> promoter and StxB1 signal peptide	Serum Anti-StxB1 IgG, bile IgA	Butterton et al. (1995)
TCF	<i>ctxA</i> promoter	Not detected	Chen et al. (1998)
TetC	<i>hapA</i> promoter and HA/protease signal peptide	Not tested	Hazra et al. (2007)