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Evaluation in Mice of a Conjugate Vaccine for Cholera Made from *Vibrio cholerae* O1 (Ogawa) O-Specific Polysaccharide

Mohammad Murshid Alam[1,2☯], Megan Kelly Bufano[1☯], Peng Xu[3☯], Anuj Kalsy[1], Y. Yu[1], Y. Wu Freeman[1], Tania Sultana[1,2], Md. Rasheduzzaman Rashu[1,2], Ishaan Desai[1], Grace Eckhoff[1], Daniel T. Leung[1,2,4], Richelle C. Charles[1,4], Regina C. LaRocque[1,4], Jason B. Harris[1,5], John D. Clements[6], Stephen B. Calderwood[1,4,7], Firdausi Qadri[2¶], W. F. Vann[8¶], Pavol Kováč[3¶], Edward T. Ryan[1,4,9¶*]

1 Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts, United States of America, **2** International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B), Dhaka, Bangladesh, **3** NIDDK, LBC, National Institutes of Health, Bethesda, Maryland, United States of America, **4** Department of Medicine, Harvard Medical School, Boston, Massachusetts, United States of America, **5** Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, United States of America, **6** Tulane University School of Medicine, New Orleans, Louisiana, United States of America, **7** Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, United States of America, **8** CBER, FDA, Laboratory of Bacterial Toxins, Bethesda, Maryland, United States of America, **9** Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts, United States of America

Abstract

Background: Protective immunity against cholera is serogroup specific. Serogroup specificity in *Vibrio cholerae* is determined by the O-specific polysaccharide (OSP) of lipopolysaccharide (LPS). Generally, polysaccharides are poorly immunogenic, especially in young children.

Methodology: Here we report the evaluation in mice of a conjugate vaccine for cholera (OSP:TThc) made from *V. cholerae* O1 Ogawa O-Specific Polysaccharide–core (OSP) and recombinant tetanus toxoid heavy chain fragment (TThc). We immunized mice intramuscularly on days 0, 21, and 42 with OSP:TThc or OSP only, with or without dmLT, a non-toxigenic immunoadjuvant derived from heat labile toxin of *Escherichia coli*.

Principal Findings: We detected significant serum IgG antibody responses targeting OSP following a single immunization in mice receiving OSP:TThc with or without adjuvant. Anti-LPS IgG responses were detected following a second immunization in these cohorts. No anti-OSP or anti-LPS IgG responses were detected at any time in animals receiving un-conjugated OSP with or without immunoadjuvant, and in animals receiving immunoadjuvant alone. Responses were highest following immunization with adjuvant. Serum anti-OSP IgM responses were detected in mice receiving OSP:TThc with or without immunoadjuvant, and in mice receiving unconjugated OSP. Serum anti-LPS IgM and vibriocidal responses were detected in all vaccine cohorts except in mice receiving immunoadjuvant alone. No significant IgA anti-OSP or anti-LPS responses developed in any group. Administration of OSP:TThc and adjuvant also induced memory B cell responses targeting OSP and resulted in 95% protective efficacy in a mouse lethality cholera challenge model.

Conclusion: We describe a protectively immunogenic cholera conjugate in mice. Development of a cholera conjugate vaccine could assist in inducing long-term protective immunity, especially in young children who respond poorly to polysaccharide antigens.

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* E-mail: etryan@partners.org

☯ These authors contributed equally to this work.

¶ FQ, WFV, PK and ETR also contributed equally to this work.

Author Summary

Cholera is a severe dehydrating diarrheal illness of humans caused by organisms *Vibrio cholerae* serogroups O1 or O139 serogroup organisms. Protective immunity against cholera is serogroup specific. Serogroup specificity in *V. cholerae* is determined by the O-specific polysaccharide (OSP) of lipopolysaccharide (LPS). Generally, polysaccharides are poorly immunogenic, especially in young children. Unfortunately, children bear a large burden of cholera globally. Here we describe a novel cholera conjugate vaccine and show that it induces immune responses in mice, including memory responses, to OSP, the T cell-independent antigen that probably is the target of protective immunity to cholera. These responses were highest following immunization of the vaccine with a novel immunoadjuvant, dmLT. We also show that immunization of mice with this conjugate vaccine protects against challenge with wild-type *V. cholerae*. A protectively immunogenic cholera conjugate vaccine that induces long-term memory responses could have particular utility in young children who are most at risk of cholera.

Introduction

Cholera is a severe dehydrating diarrheal illness of humans caused by organisms *Vibrio cholerae* O1 or O139 serogroup organisms. *V. cholerae* O139 has largely disappeared and is reported from just a few Asian countries [1]. Cholera affects 3–5 million people each year, killing ~100,000 annually, and cholera is endemic in over 50 countries [2]. *V. cholerae* O1 can be distinguished genotypically and phenotypically into classical and El Tor biotypes [2] and Ogawa and Inaba serotypes. Ogawa differs from Inaba only by the presence of a 2-O-methyl group in the non-reducing terminal sugar of O-specific polysaccharide (OSP) [3–5]. Currently, the global cholera pandemic is caused by organisms *V. cholerae* O1, El Tor, organisms, with the prevalent serotype fluctuating during cholera outbreaks, switching between Ogawa and Inaba [1].

Protection against cholera is serogroup specific. Previous infection with *V. cholerae* O139 provides no cross-protection from cholera caused by *V. cholerae* O1, and vice versa [6–8]. Serogroup specificity is largely determined by the O-specific polysaccharide (OSP) of lipopolysacharide (LPS). OSP is attached to lipid A that is part of the outer membrane of *V. cholerae* [9]. We have previously shown that a synthetic neoglyconjugate cholera vaccine containing a hexasaccharide of *V. cholerae* O1 Ogawa is protectively immunogenic in mice [10–12]. We were therefore interested in evaluating whether a cholera conjugate vaccine containing native OSP recovered from *V. cholerae* O1 would also be immunogenic.

Materials and Methods

Ethics statement

The use of animals complied fully with relevant governmental and institutional requirements, guidelines, and policies. This work was approved by the Massachusetts General Hospital Subcommittee on Research Animal Care (SRAC) – OLAW Assurance # A3596-01; Protocol #2004N000192. The work adheres to the USDA Animal Welfare Act, PHS Policy on Humane Care and Use of Laboratory Animals, and the "ILAR Guide for the Care and Use of Laboratory Animals".

Bacterial strains and media

V. cholerae O1 El Tor Ogawa strain X25049 [13] was used to prepare LPS for use in vaccine preparation and immunological assays, in addition to vibriocidal assays, and wild-type classical *V. cholerae* O1 classical Ogawa strain O395 [10] was used in vibriocidal assays and the neonatal challenge. Strains were grown in Luria-Bertani broth.

Vaccine antigen

LPS was recovered from X25049, and OSP-core (OSPc) was derived from LPS as previously described [9,14]. As a carrier protein, recombinant tetanus toxoid heavy chain fragment (TThc) was used [15,16]. TThc was prepared as a 52,108 Da recombinant protein in *E. coli* BL21 (DE3) Star with a self-cleaving intein tag using affinity and size exclusion chromatography, as previously described [17].

Conjugation was carried out as previously described [14]. Briefly, 3,4-dimethoxy-3-cyclobutene-1, 2-dione (4.0 mg) was added to a solution of Ogawa O-SP–core antigen (8.0 mg) in pH 7 phosphate buffer (0.05 M, 400 μL) contained in a 2 mL V-shaped reaction vessel, and the mixture was gently stirred at room temperature for 48 h. The solution was transferred into an Amicon Ultra (4 mL, 3K cutoff) centrifuge tube and dialyzed against pure water (centrifugation at 4°C, 7,500× g, 8 times, 35 min each time). The retentate was lyophilized to afford the O-SP–core squarate monomethyl ester as white solid (7.4 mg, 91%).

TThc (3.2 mg) and the methyl squarate derivative of the Ogawa O-SP–core antigen described above (7.4 mg) were weighed into a 1 mL V-shaped reaction vessel and 240 μL of 0.5 M pH 9 borate buffer was added (to form ~5 mM solution with respect to the antigen; antigen/carrier = 20:1). A clear solution was formed. The mixture was stirred at room temperature and the progress of the reaction was monitored by SELDI-TOF MS at 24, 48, 72, 96, and 168 h, when no more increase of antigen/carrier ratio could be observed. The mixture was transferred into an Amicon Ultra (4 mL, 30 K cutoff) centrifuge tube and dialyzed (centrifugation at 4°C, 7,500× g, 8 times, 8 min each time) against 10 mM aqueous ammonium carbonate. After lyophilization, 4.6 mg (83%, based on TThc) of conjugate was obtained as a white solid. On the basis of the molecular mass of the carrier (52,108 Da), conjugate (90,000 Da, determined by SELDI TOF MS) and average MW of the OSP antigen of 5,900 Da [14], the antigen/TThc ratio was 6.4:1 (conjugation efficiency, 32%) (figure 1). A corresponding conjugate was made of OSP: bovine serum albumin (BSA; Sigma #A-4503) using the same approach as described above for use in immunologic assays. The OSP:BSA product contained 4.8 moles OSP per BSA.

For these experiments, we used dmLT, a double mutant derivative of *Escherichia coli* heat labile toxin (LT), as an immunoadjuvant. dmLT (R192G/L211A) retains immunoadjuvanticity with markedly reduced enterotoxicity [18]. dmLT was prepared as previously described [18,19].

Immunization of mice and sampling

We immunized cohorts of 10–15, three to five week old Swiss Webster female mice intramuscularly with OSP:TThc or OSP (10 μg sugar per mouse; total 3 doses) with or without dmLT (5 μg). Mice were immunized on days 0, 21, and 42. We collected blood samples via tail bleeds on days 0, 21, 28, 42, 49 and 56. Samples were collected, processed, aliquoted, and stored as previously described [10,11]. For the memory B cell assay, splenocytes were isolated after day 56 and processed for ELISPOT as previously described [20].

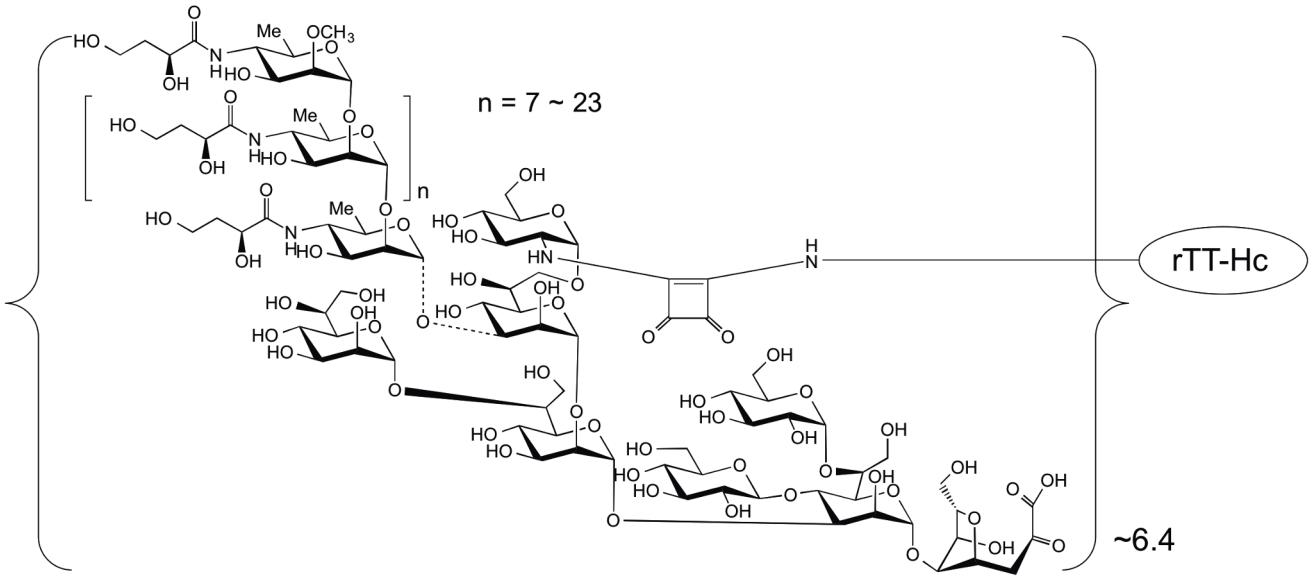

Figure 1. Structure of Ogawa OSP:TThc conjugate. 6.4 moles OSP per mole conjugate. TThc = recombinant tetanus toxoid heavy chain fragment.
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Detection of specific antibody responses in serum

We quantified anti-LPS and OSP IgG, IgM and IgA responses in serum using standard enzyme-linked immunosorbent assay (ELISA) protocols [10,11]. To assess anti-LPS antibody responses, we coated ELISA plates with *V. cholerae* O1 Ogawa LPS (2.5 μg/mL) in PBS [10,11]. To assess anti-OSP antibody responses, we coated ELISA plates with OSP:BSA (1 μg/mL) in PBS. To each well, we added 100 μL of serum (diluted 1:25 in 0.1% BSA in phosphate buffered saline-Tween) and detected the presence of antigen-specific antibodies using horseradish peroxidase-conjugated anti-mouse IgG, IgM or IgA antibody (diluted 1:1000 in 0.1% BSA in phosphate buffered saline-Tween) (Southern Biotech, Birmingham, AL). After 1.5 h incubation at 37°C, we developed the plates with a 0.55 mg/mL solution of 2,2′ 0-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma) with 0.03% H_2O_2 (Sigma), and determined the optical density at 405 nm with a Vmax microplate kinetic reader (Molecular Devices Corp. Sunnyvale, CA). Plates were read for 5 min at 30 s intervals, and the maximum slope for an optical density change of 0.2 U was reported as millioptical density units per minute (mOD/min). We normalized ELISA units (EU) by calculating the ratio of the optical density of the test sample to that of a standard of pooled sera from mice vaccinated with cholera vaccine from a previous study run on the same plate. We characterized a responder as a ≥2-fold increase in anti-LPS and OSP EU kinetic responses.

Measurement of serum vibriocidal responses

We assessed serum vibriocidal antibody titers against *V. cholerae* X25049 in a micro-assay as previously described [21,22]. We inactivated endogenous complement activity of mouse serum by heating it for 1 hr at 56°C. We then added 50 μl aliquots of two-fold serial dilutions of heat-inactivated sera in 0.15M saline (1:25 to 1:25,600) to wells of sterile 96-well tissue culture plates containing 50 μl/well of *V. cholerae* X25049 (OD 0.1) in 0.15M saline and 22% guinea pig complement (EMD Biosciences, San Diego, CA). The plates were then incubated for 1 hr at 37°C. 150 μl of brain heart infusion broth (Becton Dickinson, Sparks, MD) was added to each well, and plates were incubated for an additional 1.5 h at 37°C, when optical density at 600 nm was assessed. We calculated the vibriocidal titer as the dilution of serum causing 50% reduction in optical density compared with that of wells containing no serum [23,24]. We characterized a responder as a ≥4-fold increase in vibriocidal titer.

Memory B cell responses

We assessed memory B-cell assays after the third round of immunization based on previously described methods [20]. Briefly, we treated splenocytes from mice with 1 ml erythrocyte lysis buffer (Sigma) and resuspended them in RPMI supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), beta-mercaptoethanol (Sigma, St. Louis, MO), R595 LPS (Alexis), ConA stimulated supernatant and antibiotics (penicillin, streptomycin). The ConA stimulated supernatant was made from naïve mice splenocytes cultured with 2.5 ug/ml ConA and 20 ng/ml PMA for 48 hours at 37°C in a humid atmosphere with 5% CO_2. We then cultured spleen cells in 96 well round-bottom plates containing 1×10^7 cells/mL irradiated syngeneic spleen cell feeders (1200 rad) from naïve mice, and 1×10^5 cells/well from immunized mice in a total volume of 200 μl. Plates were then incubated at 37°C in a humid atmosphere with 5% CO_2. After 6 days in culture, cells were harvested and antigen-specific memory B cell responses were measured by conventional ELISPOT method. We assessed antigen-specific OSP and total IgG ELISPOT assays on these cultured cells. Specifically, nitrocellulose bottom plates (MAHAS4510, Millipore, Bedford, MA) were coated with OSP:BSA (100 ng/well) or with goat anti-mouse IgG (Southern Biotech, Birmingham, AL) or with keyhole limpet hemocyanin (KLH; Pierce Biotechnology, Rockford, IL) (2.5 μg/mL, negative control). After we blocked the plates with RPMI supplemented with 10% FBS, we added the cultured cells to the wells and incubated the plates for 5 h at 37°C in a humid atmosphere with 5% CO_2. We then added biotinylated anti mouse IgGγ (Southern Biotech, Birmingham, AL) antibody at 1:1000, detected IgG antibody expressing cells using horseradish peroxidase-conjugated avidin-D (5 mg/ml, Vector Labs), and developed plates with AEC (3 amino-9-ethyl-carbozole; Sigma). We used unstimulated

samples as negative controls and assessed responses to KLH. We characterized a responder as having >2 times total IgG cells with stimulation versus no stimulation and >3 anti-OSP spots.

Neonatal challenge experiments

To assess protection afforded by immunization, we used a cholera neonatal mouse challenge assay, as previously described [10,11], using wild-type O1 Ogawa *V. cholerae* O395. In brief, we removed three to five days old un-immunized CD-1 suckling mice ($n = 20$ mice/cohort) from dams two hours prior to inoculation. We then administered to pups a 50 µl inoculum comprised of 2.3×10^9 CFU of *V. cholerae* O395 mixed with a 1:250 dilution of pooled day 56 serum from mice intramuscularly immunized with the conjugate vaccine OSP:TThc with dmLT, or immunized with dmLT alone. Following oral challenge, we kept neonates separate from dams at 30°C and monitored animals every 3 hr for 36 hr, after which surviving animals were euthanized.

Statistics and graphs

We compared data from different groups using Mann-Whitney U tests. Within each group, comparisons of data from different time points to baseline data (day 0) were carried out using Wilcoxon Signed-Rank tests. Kaplan-Meier and log rank analysis were carried out to compare survival curves in the neonatal challenge study. All reported P values were two-tailed, with a cutoff of $P<0.05$ considered a threshold for statistical significance. We performed statistical analyses using GraphPad Prism 4 (GraphPad Software, Inc., La Jolla, CA).

Results

Analysis of OSP:TThc

We determined progress of conjugation and average carbohydrate content/carbohydrate–protein ratio of OSP:TThc by Surface-Enhanced Laser Desorption–Ionization Mass Spectrometry (SELDI) [25]. Similar to the matrix assisted variant (MALDI) [26], this technique determines average degree of incorporation of carbohydrate onto protein, as well as molecular weight distribution in glycoconjugates. The SELDI analysis showed that the average molecular mass of the conjugate was 90,150 Da. Subtracting from that value the molecular mass of the recombinant protein TThc carrier, 52,108 Da [17][27] the conjugate product molecular mass increased by 38,042 Da. Based on the difference between m/z values of subpeaks within the SELDI peak [14] ; also [28] the molecular mass of the polymolecular OSP–core was determined to average ~5,900 Da, representingattachment of various lengths of OSP to core. The molecular mass of the conjugate determined by SELDI, 91,150 Da, then indicated the molar ratio of OSP–core:TThc to be ~6.4:1.

OSP-specific antibody responses

Following the first injection, we detected significant anti-OSP serum IgG antibody responses in mice receiving OSP:TThc with or without adjuvant (figure 2). Higher magnitude and response rates ($P<0.01$) were observed in the cohort of animals receiving conjugate vaccine with dmLT (response rate after two doses: 100%). No anti-OSP IgG responses were detected at any time in animals receiving un-conjugated OSP only, with or without immunoadjuvant, or in animals receiving immunoadjuvant alone. Mice receiving OSP:TThc with or without immunoadjuvant and mice receiving OSP alone developed anti-OSP IgM responses (figure 3). IgM responses were only detected following a minimum of two immunizations, and response frequency and magnitude were highest in animals receiving OSP:TThc with adjuvant. No

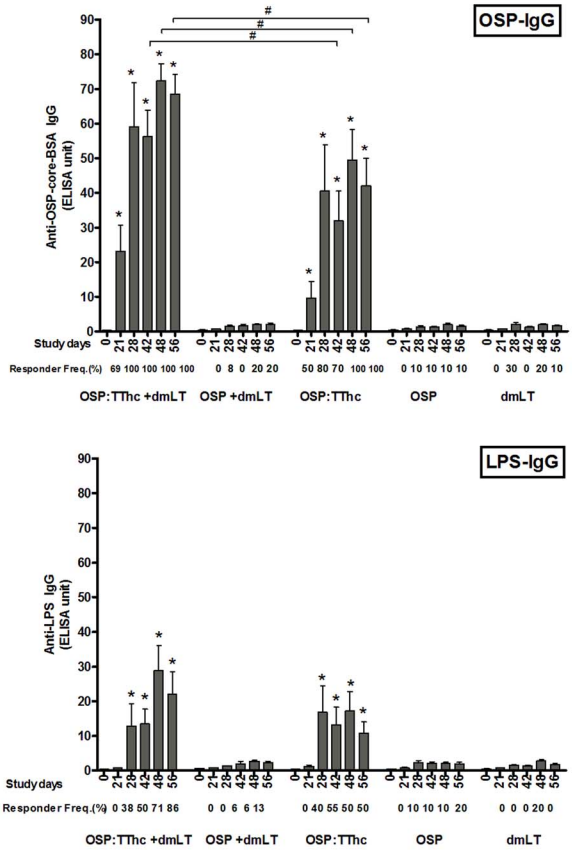

Figure 2. Serum anti-OSP-BSA and anti-LPS IgG antibody responses in mice intramuscularly immunized with OSP:TThc (with dmLT), OSP (with dmLT), OSP:TThc (no dmLT), OSP (no dmLT), or dmLT alone. Mean and standard error of the mean are reported for each group. An asterisk denotes a statistically significant difference ($P<0.05$) from baseline (day 0) titer. Responder frequencies are also listed. #, statistically significant difference among the compared cohorts ($P<0.05$).
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significant IgA anti-OSP antibody was detected in any group (not shown).

LPS-specific antibody responses

Significant serum anti-LPS IgG responses developed following a second immunization in mice receiving conjugate with or without adjuvant (figure 2). Anti-LPS IgM responses were detected in all vaccine cohorts except in mice receiving immunoadjuvant alone (figure 3). No significant anti-LPS IgA responses developed in any group (not shown).

Vibriocidal responses

Low-level vibriocidal responses (magnitude and response frequency) were detected in animals receiving unconjugated OSP with or without adjuvant (figure 4). Administration of the immunoadjuvant alone did not elicit any vibriocidal response in animals.

Antigen-specific memory B cell responses

Antigen-specific IgG memory B-cell responses are shown in table 1. OSP IgG specific memory B cell responses were detected in 65% of mice immunized with conjugate vaccine and adjuvant. 18% and 22% of mice immunized with OSP in the presence of

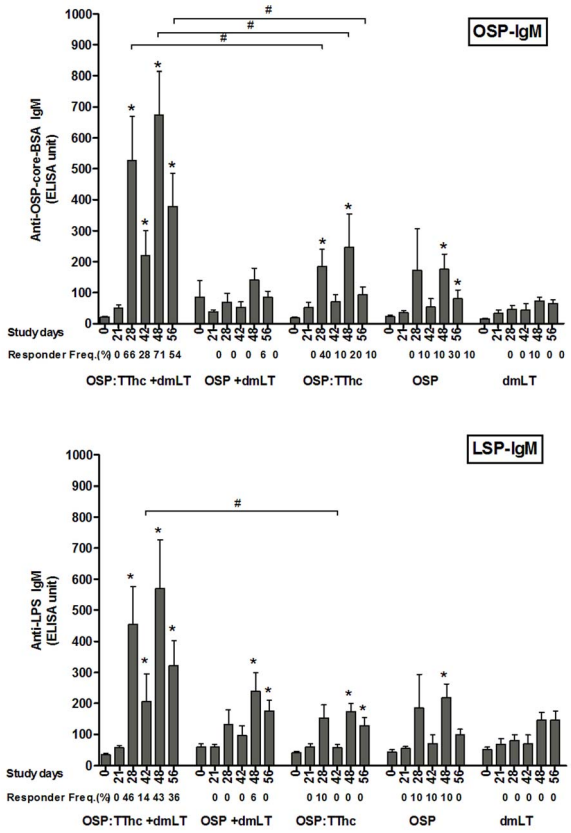

Figure 3. Serum anti-OSP-BSA and anti-LPS IgM antibody responses in mice intramuscularly immunized with OSP:TThc (with dmLT), OSP (with dmLT), OSP:TThc (no dmLT), OSP (no dmLT), or dmLT alone. Mean and standard error of the mean are reported for each group. An asterisk denotes a statistically significant difference ($P<0.05$) from baseline (day 0) titer. Responder frequencies are also listed. #, statistically significant difference among the compared cohorts ($P<0.05$).
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dmLT or OSP:TThc alone developed detectable OSP specific memory B cell responses, respectively. No OSP memory response was detected in mice receiving dmLT alone.

Neonatal mouse challenge assay

We found a significant difference in survival between mice challenged with wild-type *V. cholerae* O1 Ogawa O395 mixed with sera collected from mice immunized with conjugate and adjuvant (95% survival at 36 hours), compared to mice challenged using sera from mice immunized with adjuvant alone (0% survival at 30 hours; 95% protection; $P<0.0001$) (figure 5).

Discussion

In this study, we demonstrate that a cholera conjugate vaccine containing OSP recovered from *V. cholerae* is protectively immunogenic and induces anti-OSP memory B cell responses in mice. There is a growing body of evidence that anti-OSP responses may be a prime mediator of protective immunity against cholera. Protective immunity to cholera is serogroup specific. Previous infection with *V. cholerae* O1 provides no protection against O139 and vice versa. This is despite the fact that O1 and O139 express essentially identical cholera toxins (CT) and that O139 is thought to be a derivative of an O1 El Tor strain

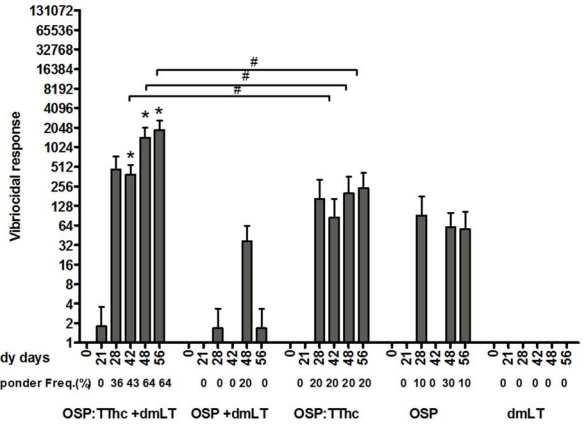

Figure 4. Vibriocidal responses in mice intramuscularly immunized with OSP:TThc (with dmLT), OSP (with dmLT), OSP:TThc (no dmLT), OSP (no dmLT), or dmLT alone. The columns indicate mean reciprocal end titers, and error bars represent the standard errors of the mean. An asterisk denotes a statistically significant difference ($P<0.05$) from baseline (day 0) titer. Responder frequencies are also listed. #, statistically significant difference among the vaccine cohorts ($P<0.05$).
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with high-level homology of most genes in O1 El Tor and O139 [29,30]. O139 differs from O1 in its genes encoding OSP and in the presence of capsule. The capsule of O139 is comprised of a polysaccharide whose repeating unit is identical to the O139 OSP [15]. The core moieties of O139 and O1 are identical [31]. These data suggest that protection from cholera may be mediated by the serogroup OSP of LPS.

Analysis of anti-OSP responses in cholera patients and their potential role in protection has only recently been initiated [9,32]. There is however significant evidence that anti-LPS responses correlate with protection from cholera [33,34]. The vibriocidal response correlates with protection [35] and is largely comprised of anti-LPS IgM responses [36]. We have recently shown that the vibriocidal response can be largely adsorbed away by OSP [9]. Anti-LPS IgA responses in serum and stool have also been associated with protection against cholera among household contacts of cholera patients in Bangladesh [34]. Anti-LPS memory B cell responses similarly correlate with protection against cholera [33].

Currently, two oral killed cholera vaccines are WHO-prequalified and commercially available [37]. One contains approximately 10^{11} killed *V. cholerae* O1 classical and El Tor strain organisms (Ogawa and Inaba) and is supplemented with

Table 1. OSP-BSA specific IgG memory B cell responses in mice immunized with different vaccine antigens.

Vaccine cohort	Anti-OSP (%)*	Anti-KLH (%)*
OSP:TThc+dmLT	7/11 (65)	0/11 (0)
OSP+dmLT	2/11 (18)	0/11 (0)
OSP:TThc	2/9 (22)	0/9 (0)
OSP	1/9 (11)	0/9 (0)
dmLT	0/8 (0)	0/8 (0)

*Data are expressed as responder frequencies (see text).
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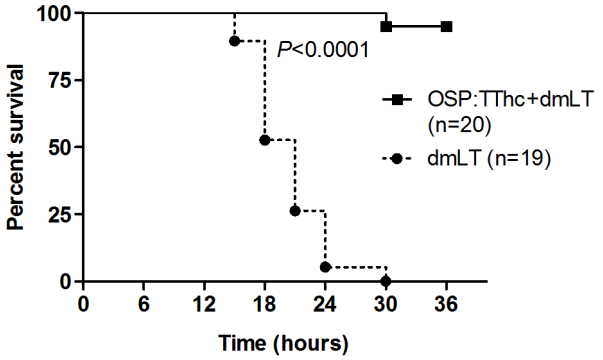

Figure 5. Survival likelihood of neonatal CD-1 mice following oral challenge with wild-type O1 Ogawa *V. cholerae* O395. Three- to five-day-old pups (cohort size 20) were orally gavaged with 50 µl of a preparation containing 2.3×10^9 CFU of wild type *V. cholerae* O395 mixed with a 1:250 dilution of pooled day 56 serum from mice intramuscularly immunized with conjugate vaccine (OSP:TThc) and immunoadjuvantative dmLT, or dmLT alone. Survival curves were compared by log rank testing.
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1 mg of recombinant non toxic B subunit of cholera toxin (WC:rBS; Dukoral, Crucell, Sweden). The other is bivalent, containing killed classical and El Tor O1 organisms as well as an O139 strain, and it does not contain supplemental cholera toxin subunit (Shanchol, Shantabiotechnic-Sanofi, India). Following two doses, these vaccines are 40–85% effective for 6–60 months [13,37–39]. The level of response and duration of protection is particularly decreased in children younger than 5 years of age, compared to older children and adults [38,40,41], with booster doses of Dukoral being recommended every 6 months for children under 5 years of age [42]. In comparison, wild-type cholera is associated with high level (90–100%) of protective immunity for at least 3 years in volunteer challenge studies [43] and 3–10 year protection in population-based studies [44]. The level and duration of protection afforded by previous wild-type cholera appears to be the same in young children and in older individuals [45,46].

We have previously shown that wild-type cholera is associated with a pro-inflammatory response even in young children in Bangladesh, but that vaccination of Bangladeshi children with WC-rBS induces a T-regulatory response [47]. We have also shown that wild-type cholera induces anti-LPS memory B cell responses, even in young children [45], but that children and adult recipients of WC-rBS do not develop such responses [13,41]. In addition, induction of memory B cell responses correlates with the magnitude of early T cell responses in older recipients of WC-rBS [47], but younger child recipients do not develop T cell responses [47]. These observations may in part explain the lower level and shorter duration of protection afforded by WC-rBS in young children compared to that induced by wild-type disease. Unfortunately, children bear a very large burden of cholera, especially in endemic areas [48,49]. For instance, 40–80% of children in Bangladesh develop serologic evidence of previous exposure to *V. cholerae* by the age of 15 years [35,50], and in areas of India, there is an estimated cholera incidence of 7 per 1000 for children less than 5 years of age, compared to 2.19 in older children and 0.93 in adults (>14 years age) [51]. There is thus a need for improved cholera vaccines or immunization strategies capable of inducing high-level and long-term immunity, especially in young children.

Immune responses targeting OSP may be critical in determining protective immunity from cholera. Since OSP is a T cell-independent antigen, and because young children do not develop prominent responses to polysaccharide antigens administered alone, we are particularly interested in developing a cholera conjugate vaccine. Here we show that a cholera conjugate vaccine is protectively immunogenic in mice and induces memory B cell responses against OSP. Previous prototype cholera conjugates have been developed [52][53][54] Our work contains a number of innovative features. The conjugation process is carried out using squaric acid chemistry, linking the glucosamine present in core oligosaccharide to carrier protein via single point attachment [14]. This takes advantage of the core oligosaccharide, effectively using it as a linker and resulting in a sun-burst display of OSP in a manner that may mimic that present on the surface of *V. cholerae*. Recent data suggest that the way LPS antigen is presented can impact subsequent immune responses [55]. The fact that the resulting conjugate in our analysis is not cross-linked and, therefore, easier to characterize, together with conjugation methodology that produces conjugates in a predictable manner [56], maximizes the likelihood that vaccine generated in this way and its immunological properties would be reproducible, which is not the case with a number of conjugate vaccines for cholera reported to date. Of note, we do not think that core oligosaccharide contributes significantly to the protective immunity that we observed since previous infection of humans with *V. cholerae* O1 does not provide protection from *V. cholerae* O139 and vice versa, despite the presence of identical core oligosaccharides. We also employed as carrier a recombinant immunogenic fragment of tetanus toxoid that could be used as carrier in other vaccines as well. Individuals at risk of cholera are often the most globally disenfranchised and impoverished and may not have received all recommended immunizations, including tetanus vaccine. In addition, we used a novel immunoadjuvant, dmLT [18]. A number of derivatives of the ADP-ribosylating LT molecule of *E. coli* have been developed and evaluated in humans [18,57,58]. These molecules have in common their retained immunoadjuvanticity but markedly diminished enterotoxicity [18]. We have previously shown that transcutaneously applied CT or LT can act as an immunoadjuvant [10,11]; here we show that low-dose dmLT can also be safely administered parenterally in mice.

Our study is encouraging, but many questions remain. Would an Inaba-based vaccine result in comparable results? Would a response targeting Ogawa OSP cross protect against Inaba? Previous human suggests it may not [59] Would an Inaba-based vaccine protect against Ogawa-associated disease? Could a bi/multi-valent conjugate vaccine be developed? Our vaccine induced vibriocidal responses. Is this a reflection of the fact that a significant component of the vibriocidal response can be adsorbed with OSP [9] or are additional purification steps required? How do conjugates using purified OSP compare to glycoconjugate vaccines prepared from synthetic carbohydrates, which are also under development [10,11,60,61]? Could other immunoadjuvants be used? Is it possible to induce mucosal responses, or would a parenteral cholera vaccine be sufficient when most humans at risk of cholera are also at high risk of tropical or environmental enteropathy with attendant leaking of serum antibodies into the intestinal lumen?

Despite these questions, it is notable that previously produced killed LPS-based whole cell parenteral cholera vaccines were associated with up to 80% protection against disease in humans [62]. Our data suggest that an improved parenteral cholera conjugate vaccine can be developed, one that induces immune responses, including memory B cell responses, to a normally T cell independent antigen (OPS) that is the major target of protective immunity to cholera. Furthermore, this conjugate vaccine can

protect against wild-type challenge in animals. Such a conjugate vaccine could have particular utility in young children who are most at risk of cholera.

Author Contributions

Conceived and designed the experiments: MMA RCL JBH JDC SBC FQ WFV PK ETR. Performed the experiments: MMA MKB PX AK YY YWF TS MRR ID GE. Analyzed the data: MMA MKB PX DTL RCC RCL JBH JDC SBC FQ WFV PK ETR. Contributed reagents/materials/analysis tools: DTL RCC RCL JBH JDC SBC FQ WFV PK ETR. Wrote the paper: MMA MKB PX AK YY YWF TS MRR ID GE DTL RCC RCL JBH JDC SBC FQ WFV PK ETR.

References

1. Harris AM, Chowdhury F, Begum YA, Khan AI, Faruque AS, et al. (2008) Shifting prevalence of major diarrheal pathogens in patients seeking hospital care during floods in 1998, 2004, and 2007 in Dhaka, Bangladesh. Am J Trop Med Hyg 79: 708–714.
2. Harris JB, LaRocque RC, Qadri F, Ryan ET, Calderwood SB (2012) Cholera. Lancet 379: 2466–2476.
3. Hisatsune K, Kondo S, Isshiki Y, Iguchi T, Kawamata Y, et al. (1993) O-antigenic lipopolysaccharide of Vibrio cholerae O139 Bengal, a new epidemic strain for recent cholera in the Indian subcontinent. Biochem Biophys Res Commun 196: 1309–1315.
4. Ito T, Higuchi T, Hirobe M, Hiramatsu K, Yokota T (1994) Identification of a novel sugar, 4-amino-4,6-dideoxy-2-O-methylmannose in the lipopolysaccharide of Vibrio cholerae O1 serotype Ogawa. Carbohydr Res 256: 113–128.
5. Wang J, Villeneuve S, Zhang J, Lei P, Miller CE, et al. (1998) On the antigenic determinants of the lipopolysaccharides of Vibrio cholerae O:1, serotypes Ogawa and Inaba. J Biol Chem 273: 2777–2783.
6. Albert MJ, Alam K, Rahman AS, Huda S, Sack RB (1994) Lack of cross-protection against diarrhea due to Vibrio cholerae O1 after oral immunization of rabbits with V. cholerae O139 Bengal. J Infect Dis 169: 709–710.
7. Waldor MK, Colwell R, Mekalanos JJ (1994) The Vibrio cholerae O139 serogroup antigen includes an O-antigen capsule and lipopolysaccharide virulence determinants. Proc Natl Acad Sci U S A 91: 11388–11392.
8. Qadri F, Wenneras C, Albert MJ, Hossain J, Mannoor K, et al. (1997) Comparison of immune responses in patients infected with Vibrio cholerae O139 and O1. Infect Immun 65: 3571–3576.
9. Johnson RA, Uddin T, Aktar A, Mohasin M, Alam MM, et al. (2012) Comparison of immune responses to the O-specific polysaccharide and lipopolysaccharide of Vibrio cholerae O1 in Bangladeshi adult patients with cholera. Clin Vaccine Immunol 19: 1712–1721.
10. Tarique AA, Kalsy A, Arifuzzaman M, Rollins SM, Charles RC, et al. (2012) Transcutaneous immunization with a Vibrio cholerae O1 Ogawa synthetic hexasaccharide conjugate following oral whole-cell cholera vaccination boosts vibriocidal responses and induces protective immunity in mice. Clin Vaccine Immunol 19: 594–602.
11. Rollenhagen JE, Kalsy A, Saksena R, Sheikh A, Alam MM, et al. (2009) Transcutaneous immunization with a synthetic hexasaccharide-protein conjugate induces anti-Vibrio cholerae lipopolysaccharide responses in mice. Vaccine 27: 4917–4922.
12. Wade TK, Saksena R, Shiloach J, Kovac P, Wade WF (2006) Immunogenicity of synthetic saccharide fragments of Vibrio cholerae O1 (Ogawa and Inaba) bound to Exotoxin A. FEMS Immunol Med Microbiol 48: 237–251.
13. Alam MM, Riyadh MA, Fatema K, Rahman MA, Akhtar N, et al. (2011) Antigen-specific memory B-cell responses in Bangladeshi adults after one- or two-dose oral killed cholera vaccination and comparison with responses in patients with naturally acquired cholera. Clin Vaccine Immunol 18: 844–850.
14. Xu P, Alam MM, Kalsy A, Charles RC, Calderwood SB, et al. (2011) Simple, direct conjugation of bacterial O-SP-core antigens to proteins: development of cholera conjugate vaccines. Bioconjug Chem 22: 2179–2185.
15. Boutonnier A, Villeneuve S, Nato F, Dassy B, Fournier JM (2001) Preparation, immunogenicity, and protective efficacy, in a murine model, of a conjugate vaccine composed of the polysaccharide moiety of the lipopolysaccharide of Vibrio cholerae O139 bound to tetanus toxoid. Infect Immun 69: 3488–3493.
16. Schneerson R, Robbins JB, Chu C, Sutton A, Vann W, et al. (1984) Serum antibody responses of juvenile and infant rhesus monkeys injected with Haemophilus influenzae type b and pneumococcus type 6A capsular polysaccharide-protein conjugates. Infect Immun 45: 582–591.
17. Bongat AF, Saksena R, Adamo R, Fujimoto Y, Shiokawa Z, et al. (2010) Multimeric bivalent immunogens from recombinant tetanus toxin HC fragment, synthetic hexasaccharides, and a glycopeptide adjuvant. Glycoconj J 27: 69–77.
18. Norton EB, Lawson LB, Freytag LC, Clements JD (2011) Characterization of a mutant Escherichia coli heat-labile toxin, LT(R192G/L211A), as a safe and effective oral adjuvant. Clin Vaccine Immunol 18: 546–551.
19. Summerton NA, Welch RW, Bondoc L, Yang HH, Pleune B, et al. (2010) Toward the development of a stable, freeze-dried formulation of Helicobacter pylori killed whole cell vaccine adjuvanted with a novel mutant of Escherichia coli heat-labile toxin. Vaccine 28: 1404–1411.
20. Slifka MK, Ahmed R (1996) Limiting dilution analysis of virus-specific memory B cells by an ELISPOT assay. J Immunol Methods 199: 37–46.
21. Crean TI, John M, Calderwood SB, Ryan ET (2000) Optimizing the germfree mouse model for in vivo evaluation of oral Vibrio cholerae vaccine and vector strains. Infect Immun 68: 977–981.
22. Butterton JR, Ryan ET, Shahin RA, Calderwood SB (1996) Development of a germfree mouse model of Vibrio cholerae infection. Infect Immun 64: 4373–4377.
23. Ryan ET, Butterton JR, Smith RN, Carroll PA, Crean TI, et al. (1997) Protective immunity against Clostridium difficile toxin A induced by oral immunization with a live, attenuated Vibrio cholerae vector strain. Infect Immun 65: 2941–2949.
24. Ryan ET, Butterton JR, Zhang T, Baker MA, Stanley SL, Jr., et al. (1997) Oral immunization with attenuated vaccine strains of Vibrio cholerae expressing a dodecapeptide repeat of the serine-rich Entamoeba histolytica protein fused to the cholera toxin B subunit induces systemic and mucosal antiamebic and anti-V. cholerae antibody responses in mice. Infect Immun 65: 3118–3125.
25. Chernyak A, Karavanov A, Ogawa Y, Kovac P (2001) Conjugating oligosaccharides to proteins by squaric acid diester chemistry: rapid monitoring of the progress of conjugation, and recovery of the unused ligand. Carbohydr Res 330: 479–486.
26. Kamath VP, Diedrich P, Hindsgaul O (1996) Use of diethyl squarate for the coupling of oligosaccharide amines to carrier proteins and characterization of the resulting neoglycoproteins by MALDI-TOF mass spectrometry. Glycoconj J 13: 315–319.
27. McCarthy PC, Saksena R, Peterson DC, Lee CH, An Y, et al. (2013) Chemoenzymatic synthesis of immunogenic meningococcal group C polysialic acid-tetanus Hc fragment glycoconjugates. Glycoconj J 30: 857–870.
28. Jahouh F, Xu P, Vann WF, Kovac P, Banoub JH (2013) Mapping the glycation sites in the neoglycoconjugate from hexasaccharide antigen of Vibrio cholerae, serotype Ogawa and the recombinant tetanus toxin C-fragment carrier. J Mass Spectrom 48: 1083–1090.
29. Comstock LE, Maneval D, Jr., Panigrahi P, Joseph A, Levine MM, et al. (1995) The capsule and O antigen in Vibrio cholerae O139 Bengal are associated with a genetic region not present in Vibrio cholerae O1. Infect Immun 63: 317–323.
30. Bik EM, Bunschoten AE, Gouw RD, Mooi FR (1995) Genesis of the novel epidemic Vibrio cholerae O139 strain: evidence for horizontal transfer of genes involved in polysaccharide synthesis. EMBO J 14: 209–216.
31. Cox AD, Perry MB (1996) Structural analysis of the O-antigen-core region of the lipopolysaccharide from Vibrio cholerae O139. Carbohydr Res 290: 59–65.
32. Leung DT, Uddin T, Xu P, Aktar A, Johnson RA, et al. (2013) Immune responses to the O-specific polysaccharide antigen in children who received a killed oral cholera vaccine compared to responses following natural cholera infection in Bangladesh. Clin Vaccine Immunol 20: 780–788.
33. Patel SM, Rahman MA, Mohasin M, Riyadh MA, Leung DT, et al. (2012) Memory B cell responses to Vibrio cholerae O1 lipopolysaccharide are associated with protection against infection from household contacts of patients with cholera in Bangladesh. Clin Vaccine Immunol 19: 842–848.
34. Harris JB, LaRocque RC, Chowdhury F, Khan AI, Logvinenko T, et al. (2008) Susceptibility to Vibrio cholerae infection in a cohort of household contacts of patients with cholera in Bangladesh. PLoS Negl Trop Dis 2: e221.
35. Glass RI, Svennerholm AM, Khan MR, Huda S, Huq MI, et al. (1985) Seroepidemiological studies of El Tor cholera in Bangladesh: association of serum antibody levels with protection. J Infect Dis 151: 236–242.
36. Losonsky GA, Yunyongying J, Lim V, Reymann M, Lim YL, et al. (1996) Factors influencing secondary vibriocidal immune responses: relevance for understanding immunity to cholera. Infect Immun 64: 10–15.
37. WHO (2012) Cholera, 2011. Wkly Epidemiol Rec 87: 289–304.
38. Sur D, Kanungo S, Sah B, Manna B, Ali M, et al. (2011) Efficacy of a low-cost, inactivated whole-cell oral cholera vaccine: results from 3 years of follow-up of a randomized, controlled trial. PLoS Negl Trop Dis 5: e1289.
39. Sur D, Lopez AL, Kanungo S, Paisley A, Manna B, et al. (2009) Efficacy and safety of a modified killed-whole-cell oral cholera vaccine in India: an interim analysis of a cluster-randomised, double-blind, placebo-controlled trial. Lancet 374: 1694–1702.
40. Sinclair D, Abba K, Zaman K, Qadri F, Graves PM (2011) Oral vaccines for preventing cholera. Cochrane Database Syst Rev: CD008603.
41. Leung DT, Rahman MA, Mohasin M, Patel SM, Aktar A, et al. (2012) Memory B cell and other immune responses in children receiving two doses of an oral killed cholera vaccine compared to responses following natural cholera infection in Bangladesh. Clin Vaccine Immunol 19: 690–698.
42. Anonymous (2013) World Health Organization. International travel and health. Vaccines.
43. Levine MM, Black RE, Clements ML, Cisneros L, Nalin DR, et al. (1981) Duration of infection-derived immunity to cholera. J Infect Dis 143: 818–820.

44. Koelle K, Rodo X, Pascual M, Yunus M, Mostafa G (2005) Refractory periods and climate forcing in cholera dynamics. Nature 436: 696–700.
45. Leung DT, Rahman MA, Mohasin M, Riyadh MA, Patel SM, et al. (2011) Comparison of memory B cell, antibody-secreting cell, and plasma antibody responses in young children, older children, and adults with infection caused by Vibrio cholerae O1 El Tor Ogawa in Bangladesh. Clin Vaccine Immunol 18: 1317–1325.
46. Ali M, Emch M, Park JK, Yunus M, Clemens J (2011) Natural cholera infection-derived immunity in an endemic setting. J Infect Dis 204: 912–918.
47. Arifuzzaman M, Rashu R, Leung DT, Hosen MI, Bhuiyan TR, et al. (2012) Antigen-specific memory T cell responses after vaccination with an oral killed cholera vaccine in Bangladeshi children and comparison to responses in patients with naturally acquired cholera. Clin Vaccine Immunol 19: 1304–1311.
48. Leung DT, Chowdhury F, Calderwood SB, Qadri F, Ryan ET (2012) Immune responses to cholera in children. Expert Rev Anti Infect Ther 10: 435–444.
49. Deen JL, von Seidlein L, Sur D, Agtini M, Lucas ME, et al. (2008) The high burden of cholera in children: comparison of incidence from endemic areas in Asia and Africa. PLoS Negl Trop Dis 2: e173.
50. Mosley WH, Benenson AS, Barui R (1968) A serological survey for cholear antibodies in rural east Pakistan. 1. The distribution of antibody in the control population of a cholera-vaccine field-trial area and the relation of antibody titre to the pattern of endemic cholera. Bull World Health Organ 38: 327–334.
51. Ali M, Lopez AL, You YA, Kim YE, Sah B, et al. (2012) The global burden of cholera. Bull World Health Organ 90: 209–218A.
52. Gupta RK, Szu SC, Finkelstein RA, Robbins JB (1992) Synthesis, characterization, and some immunological properties of conjugates composed of the detoxified lipopolysaccharide of Vibrio cholerae O1 serotype Inaba bound to cholera toxin. Infect Immun 60: 3201–3208.
53. Gupta RK, Taylor DN, Bryla DA, Robbins JB, Szu SC (1998) Phase 1 evaluation of Vibrio cholerae O1, serotype Inaba, polysaccharide-cholera toxin conjugates in adult volunteers. Infect Immun 66: 3095–3099.
54. Kossaczka Z, Shiloach J, Johnson V, Taylor DN, Finkelstein RA, et al. (2000) Vibrio cholerae O139 conjugate vaccines: synthesis and immunogenicity of V. cholerae O139 capsular polysaccharide conjugates with recombinant diphtheria toxin mutant in mice. Infect Immun 68: 5037–5043.
55. Wade WF, King RG, Grandjean C, Wade TK, Justement LB (2013) Murine marginal zone B cells play a role in Vibrio cholerae LPS antibody responses. Pathog Dis.
56. Hou SJ, Saksena R, Kovac P (2008) Preparation of glycoconjugates by dialkyl squarate chemistry revisited. Carbohydr Res 343: 196–210.
57. Leach S, Clements JD, Kaim J, Lundgren A (2012) The adjuvant double mutant Escherichia coli heat labile toxin enhances IL-17A production in human T cells specific for bacterial vaccine antigens. PLoS One 7: e51718.
58. Kotloff KL, Sztein MB, Wasserman SS, Losonsky GA, DiLorenzo SC, et al. (2001) Safety and immunogenicity of oral inactivated whole-cell Helicobacter pylori vaccine with adjuvant among volunteers with or without subclinical infection. Infect Immun 69: 3581–3590.
59. Mosley WH, Woodward WE, Aziz KM, Rahman AS, Chowdhury AK, et al. (1970) The 1968–1969 cholera-vaccine field trial in rural East Pakistan. Effectiveness of monovalent Ogawa and Inaba vaccines and a purified Inaba antigen, with comparative results of serological and animal protection tests. J Infect Dis 121: Suppl 121:121–129.
60. Ftacek P, Nelson V, Szu SC (2013) Immunochemical characterization of synthetic hexa-, octa- and decasaccharide conjugate vaccines for Vibrio cholerae O:1 Serotype Ogawa with emphasis on antigenic density and chain length. Glycoconj J 30: 871–880.
61. Grandjean C, Wade TK, Ropartz D, Ernst L, Wade WF (2013) Acid-detoxified Inaba lipopolysaccharide (pmLPS) is a superior cholera conjugate vaccine immunogen than hydrazine-detoxified lipopolysaccharide and induces vibriocidal and protective antibodies. Pathog Dis 67: 136–158.
62. Graves PM, Deeks JJ, Demicheli V, Jefferson T (2010) Vaccines for preventing cholera: killed whole cell or other subunit vaccines (injected). Cochrane Database Syst Rev: CD000974.