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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

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Abstract Cholera remains to be a global health problem without suitable vaccines for endemic control or outbreak relief. Here we describe a new parenteral vaccine based on neoglyco-conjugate of synthetic fragments of O-specific polysaccharide (O-SP) of *Vibrio cholerae* O1, serotype Ogawa. Hexa-, octa- and decasaccharides of the O-SP with carboxylic acid at the reducing end were chemically synthesized and conjugated to tetanus toxoid (TT). The conjugates prepared by a novel linking scheme consisted of 17-atom linker of hydrazide and alkyl bonds elicited robust serum IgG anti-LPS responses with vibriocidal activities in mice. There is a length dependence in immune response with decasaccharide conjugates elicited the highest anti-LPS IgG. There seems to be an indication that regardless of the carbohydrate chain length, a molar ratio of 230±10 monosaccharide units per TT induced high antibody response. The conjugates also elicited cross-reactive antibodies to serotype Inaba. The formulation of the proposed cholera conjugate vaccine, similar to other licensed polysaccharide vaccine, is suitable for children immunization. A parenteral cholera vaccine could overcome the diminishing immunogenicity in most of oral vaccines due to the gastrointestinal complexity and environmental enteropathy in children living in impoverished environment and could be considered for global cholera immunization.

Keywords Neoglyco conjugate · Cholera vaccine · Immunogenicity · Vibriocidal

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Introduction

Cholera, despite improvements of living and health standards globally, remains a serious health threat especially when health infrastructures are disrupted by natural disasters or war [1–3]. Several African countries have been plagued by cholera outbreaks with seasonal mortality as high as 15 % [3–5]. The highest attack rate of cholera is in children [6–10]. The latest cholera outbreak in Haiti, after the devastating earthquake in 2010, had over 600,000 cases in 18 months and there was no sign of diminishing of the spread [11–13]. The shortage of safe drinking water was worsen during the epidemic. Cholera vaccination, one of the most efficient measure for cholera control, was not implemented during the 2 years of sustained infliction of cholera in Haiti [11–15].

The first cholera vaccine was a killed whole cell parental vaccine with low efficacy (~60 %), short duration, high adverse reactions and was removed from licensing in the 70's [10, 15–18]. A live attenuated oral vaccine, CVD103-Hg, demonstrated protections in volunteers in the US but showed much lower efficacy in Indonesia [19, 20]. Two killed *Vibrio cholerae* oral vaccines, with or without B-subunit cholera toxin, were developed in the 80's and licensed in certain countries. Both vaccines offer suboptimal efficacy, required multiple doses, difficult to ramp up in production and were not available in stockpile when needed in Haiti outbreak [12, 14, 21, 22]. None of the oral vaccines are suitable for routine immunization in young children [8–10, 15]. Our aim is to develop a cholera vaccine that is safe, efficacious, long lasting and suitable for children immunization.

Immunity to *V. cholerae* O:1 is mediated by serum IgG antibody against the surface polysaccharide [23–28]. Based on Mosley's landmark observation of decade-long field trials of

inactivated whole cell vaccine and serologic epidemiology data in the high endemic regions, the best correlation between immunity to cholera is the serum vibriocidal antibodies [26–30]. Vibriocidal antibodies are mostly mediated by the LPS for serotype O:1 and the capsule for O139 [28–32]. Absorption of convalescent sera with these polysaccharides, not the cholera toxin, removed the activities [23, 25, 32]. Based on these observations, we evaluated the safety and immunogenicity of hydrazine-treated LPS (DeALPS) of *V. cholerae* O1, serotype Inaba conjugated to cholera toxin in healthy adults. In our phase 1 trial, the conjugates elicited IgG anti-LPS with vibriocidal activities [25, 33]. The study demonstrated that vaccine consists of the O-specific polysaccharide (OSP) on LPS was sufficient to elicit vibriocidal antibodies against the organism. Unfortunately the OSP extracted from *V. cholerae* O:1 is short and linked with the non-vibriocidal core saccharide, and therefore is not ideal for vaccine preparation [25, 33]. Synthetic OSP overcomes these problems with additional advantages, such as linking schemes can be designed to suit specific purposes.

V. cholerae 01 has two distinct but cross-reactive antigenic variants: Ogawa and Inaba. The O-specific polysaccharide (OSP) of *V. cholerae* O1 LPS is composed of the repeating units of monosaccharide *N*-(3-deoxy-L-glycero-tetronyl)-D-perosamine [34]. The difference in the antigenic epitope between the two LPS is conferred by a methoxy group at the non-reducing end of Ogawa OSP [35, 36]. Exasaccharides composed of the cholera OSP repeating unit have been chemically synthesized and studied in mice [37–40]. There are advantages to using synthetic oligosaccharide as the carbohydrate portion of the cholera conjugate [40–43]. The synthetic antigen is purer than the material harvested from bacteria and affords better control of the conjugation reaction and standardization [37, 43–45]. We introduced several different linking functional groups at the reducing terminal of synthetic OSP to accommodate different conjugation schemes [manuscript in preparation]. A carboxylic acid at the reducing terminal and a linking arm of 17 methylene units showed to be most efficient and effective. Here with this scheme, synthetic Ogawa OSP were conjugated to tetanus toxoid and the effect of chain length, loading density on immunogenicity and vibriocidal activity were evaluated in mice.

Materials and methods

Saccharides

LPS of *V. cholerae* O1, serotype Ogawa (strain 3083) and Inaba (strain 569B) were purified from acetone-dried cells (gift from Dr. Richard Finkelstein, University of Missouri) following published procedures [46, 47]. Ogawa LPS was detoxified by anhydrous hydrazine at 37 °C for 1 h to produce de-O-acylated polysaccharide (DeALPS) [25]. The final polysaccharides contained <2 % protein and nucleic acid and <10 endotoxin unit/μg.

Synthetic hexasaccharide fragment of the O-SP was prepared following published methods with modifications to include the new linker methyl carboxylate at the reducing end and to increase the polymerization from hexaccharide to octa- and deca-saccharides [38, 39, 48–52].

After Zemplen' de-acetylation of the fully protected hexamer-linker-methyl carboxylate construct, the benzyl groups were removed by catalytic dehydrogenation as described [52]. To achieve complete de-benzylation, we added ca. 5 % acetic acid to the methanol solution, used 10 % palladium on charcoal instead of 5 %, and allowed the reaction under 400 psi of hydrogen gas for five days. The NMR spectra (H and C, Varian 500 MHz) of the final hexaccharide, agrees with published data and the MALDI-TOF MS (1665.8 [M.I.+ Na+], Voyager DE) agree with predicted molecular weight [34]. Octasaccharide and decasaccharide were synthesized in similar fashion with modifications as described in separate manuscript [in preparation].

Derivatization of tetanus toxoid with adipic acid dihydrazide (ADH)

Tetanus toxoid (TT, a gift from Chengdu Institute of Biologic, Chengdu, China) was derivatized with adipic acid dihydrazide (ADH) (Sigma-Aldrich) (0.3 M in 70 mM MES buffer, pH 5.6) in the presence of EDC (Sigma-Aldrich) at room temperature for 2 h. The concentration of EDC ranged from 10 to 170 mM to achieve various degrees of derivatization. The reaction mixture was dialyzed against phosphate buffered saline (PBS, pH 7.2) overnight and passed through Bio-Gel P10 column (1.6×38 cm, in PBS). The void volume peak was collected, concentrated, and designated as TT_{AH}. The level of AH derivatization was determined by 2,4,6-trinitrobenzenesulfonic acid assay (TNBS, Thermo Scientific) using ADH as the reference [25]. The antigenicity of TT_{AH} was verified by immunodiffusion with equine hyperimmune TT serum. Molecular weight of TT_{AH} was estimated by MALDI-TOF (vide infra).

Conjugation

All conjugation reactions were performed at RT and the reaction was terminated after 2 h by adjusting the pH to 7.2 with 1 N NaOH [25, 33]. Oligosaccharide (20 mg/ml) was dissolved in MES buffer (70 mM, pH 5.6) and mixed with EDC (50 mM) for 5 min to activate oligosaccharide carboxyl. TT_{AH} was added to a final concentration 10 mg/ml. For octa- and decasaccharide the amount used in conjugation was adjusted according to their molecular weight. The final oligosaccharide to protein molar

ratio in the reaction mixture was ~200:1. The pH of the reaction mixture was keep between 5.6 and 5.8 for 2 h. The reaction mixture was centrifuged (2800×g, Sorval Legend RT) and supernatant was loaded to Sephacryl S-300 column (1.6×90 cm in PBS, pH7.2,). Chromatography was performed by ÄKTA purifier system (GE Healthcare; UNICORN software). Fractions were analyzed for carbohydrate by phenol-sulfuric acid assay using synthetic monosaccharide of Ogawa as the reference and for protein by Coomassie blue assay (Thermo Scientific) using tetanus toxoid as a reference [53]. Fractions containing both carbohydrate and protein were pooled, concentrated and analyzed by immunodiffusion, SDS-PAGE electrophoresis and westernblot. The loading density of saccharide per TT in the final conjugate was determined colorimetrically, expressed as carbohydrate/protein in molar ratio and confirmed by Matrix-Assisted Laser Desorption/ionization (MALDI)-TOF (vide infra) in selected samples [54].

To achieve the desired saccharide loading density in the conjugates, we utilized TT_{AH} of various levels of derivatization. The conjugates were designated as Hex/n for conjugates prepared with hexasaccharide, and Oct/n for octasaccharide, Dec/n for decasaccharide conjugates respectively, with n denotes the number of oligosaccharide chain per protein.

For comparison, a conjugate based on the native Ogawa O-antigen was prepared by using hydrazine detoxified LPS (DeALPS) linked to TT with double EDC scheme as described [25].

Mouse immunization

Female NIH general purpose mice, 6 weeks old, were injected 3 times 2 weeks apart subcutaneously (sc) with 5 or 10 μg carbohydrate as a conjugate in PBS or with 2.5 μg of TTd in control groups. Adjuvant alum (Alhydrogel 2 %, Brenntag, Denmark) was diluted with sterile saline to the final concentration 1.8 mg Aluminum/ml and mixed with equal volume of conjugate in PBS for 1 h at 4 °C prior to injection. In the injection volume of 0.1 ml contained 90 μg alum. The corresponding amount of TT_{AH} injected differs in accordance with the saccharide/protein ratio in each conjugate. Sera were collected 1 week after the 3rd injection and stored at −40 °C until analyzed for LPS and TT antibodies by ELISA and for vibriocidal activities in selected serum samples.

Serology and molecular weight analysis

Double immunodiffusion of sera from mice injected with conjugates were reacted with Ogawa LPS (10 μl of 100 μg/ml) or TT (25 μg/mL) in 1 % agarose gel.

The conjugates were analyzed by SDS-PAGE electrophoresis for molecular size distribution in 4-12 % NuPAGE Bis-Tris/MOPS system or NuPAGE Tris-Acetate system along with HiMark Pre-Stained HMW Protein Standard (Invitrogen). Gels were stained by Simply blue Safestain (Invitrogen).

Western blot of electrophoresed gels were blotted with PVDF membrane in NuPAGE transfer buffer (both Invitrogen), then blocked by 1 % BSA in PBS for 1 h and incubated with mouse anti-LPS *V. cholerae* IgG (1/2000) as described [53].

ELISA

IgG anti-LPS level against *V. cholera* Ogawa and Inaba was determined by ELISA using plates coated with respective LPS (1 μg/well in PBS) as described [25, 33]. Pooled murine hyperimmune *V. cholera* Ogawa and Inaba antisera, produced by hyperimmune mice with heat killed organism as described, were used as reference standards and assigned 100 ELISA units (EU) for each serotype [33].

A similar ELISA procedure was used to measured anti-TT IgG, plates were coated with TT (0.5 μg/well) and a pooled reference serum from mice injected with TT (2.5 ug/injection, 3 injections at 2 weeks apart), assigned 100 EU.

ELISA results were computed with an ELISA data processing program provided by the Biostatistics and Information Management Branch, CDC, based upon four parameters of logistic-log function using the Taylor Series Linearization Algorithm [25]. For statistical analysis the unpaired Student's *t*-test was applied.

Vibriocidal assay

Mouse sera elicited by synthetic Ogawa conjugates were examined by the vibriocidal assay [55–57]. The target strains were: *V. cholerae* O1 serotype Ogawa (ATCC 9458) and Inaba CHO076, a clinical isolate from a patient infected with cholera in Peru, 1991. The seed lot of each strain was stored in 10 % glycerol at −70 °C (Microbank beads, Fisher Scientific). Bacteria were retrieved, cultured on alkaline peptone agar plate, then a single colony from the plate was transferred to 10 ml alkaline peptone broth. The culture was incubated at 37 °C with gentle shaking to reach OD=1.0. Guinea pig complement serum (Sigma-Aldrich) mediated vibriocidal assay was performed on microtiter plates as described [55–57]. Briefly, the bacteria culture was diluted with chilled PBS to 1/10 dilution and incubated with 10 % guinea pig complement in PBS (final concentration 5 %) for 30 min in ice bath. Complement culture mixture was incubated with serum dilutions at 37 °C for 1 h on microtiterplates, followed by addition of alkaline peptone broth to each well and incubated at 37 °C for 2 h. Residual live *V. cholerae* cells were detected by addition of redox indicator (2.7 % sodium succinate, 0.1 % 2,3,5-Triphenyltetrazolium chloride redox indicator, all from Sigma-Aldrich) to each well and incubated for 1 h at RT. The end points were again confirmed by colony counting on alkaline peptone plates the following day. Representative sera

were assayed before and after treatment with 0.1 M 2-mercarptoethenol (2-ME) at 37 °C for 30 min to inactivate IgM [25, 32, 33]. Controls were complement treated or untreated *V. cholera* culture and sera from mice injected with PBS. The vibriocidal titer was defined as the reciprocal of the highest serum dilution showing>70 % reduction of the bacteria growth compared to the saline injected serum control.

MALDI TOF

MALDI-TOF mass spectra of the derivatized carrier proteins and of the conjugates were obtained with an OmniFlex MALDI-TOF instrument (Bruker Daltonics, Billerica, MA) operated in the linear mode. Samples for analysis were first desalted by dialysis and to each 1 μl, mixed with 20 μl of sinnapinic acid matrix made in 30 % CH_3CN and 0.1 % trifluroacetic acid.

Results

Synthetic oligosaccharide of Ogawa OSP with carboxylic acid at the reducing terminal

Hexa-, octa- and deca-saccharide fragments of Ogawa O-SP were synthesized with a linker containing carboxylate ester at the reducing end and confirmed by NMR. This semifinal product was de-esterified to free carboxylic acid construct to facilitate conjugation with the carrier protein. The final purification by chromatography elution with H_2O:MeOH 9:1=>6:4 from reverse-phase C-18 silica gel showed H^1 and C^{13} NMR spectra were identical to those published [51, 52]. Molecular weight of oligosaccharides measured by MALDI MS [MI+Na+] agrees with predicted value (Fig. 1). TLC on Whatman C-18 SiO_2 in H_2O:MeOH:NaCl 6:4:0.1 showed one spot at Rf 0.67. Other UV: max 216.5 nm, MeOH; abs. 2.47. The amount of impurity estimated by comparison of MALDI MS major and secondary peaks (1651 MU and 1721 MU) are 99.5 %, 99.1 % and 97 % for hexasaccharide, octasaccharide and decasaccharide respectively. All the oligosaccharides contained<0.0001 endotoxin units/μg measured by Limulus assay.

ADH derivatization of TT (TT_{AH})

The level of adipic acid in the ADH derivatized TT (TT_{AH}) increases with the increasing EDC concentration (Fig. 2). The level of molar ratio of AH to TT ranges from the lowest 7 mol/mol to the maximum 75 mol/mol. The molecular weight of TT_{AH}, estimated by MALDI-TOF, range from 162.5 kDa to 173.7 kDa (data not shown), agrees with the results derived from TNBS assay. Limited by the instrumentation, molecular weight higher than 200 kDa could not be resolved by MALDI-TOF. All TT_{AH} formed line of identity with TT when reacting with anti-TT serum in immunodiffusion, an indication of retention of TT antigenicity after derivitization with ADH (data not shown).

Physical-chemical characteristics of conjugates

A representative Sephacryl S300 gel filtration profile of the conjugate reaction mixture was shown in Fig. 3a. The fractions containing both protein and saccharides showed 2 separated regions, designated as FI and FII, both peaks have similar PS/PR ratios but distinct molecular weight distributions in SDS-Page Coomassie blue stain (Fig. 3b). Immunogenicity analysis in mice consistently showed FI elicited higher IgG anti-LPS than those by FII (data not shown). Therefore for simplicity, in the rest immunogenicity analysis only FI from each conjugate was used for comparison.

All conjugates reacted with Ogawa hyperimmune sera in Western blot (exemplified by Fig. 3c) and in immunodiffusion (data not shown). Hexaccharide conjugates at various loading density were achieved by using TT_{AH} with different levels of

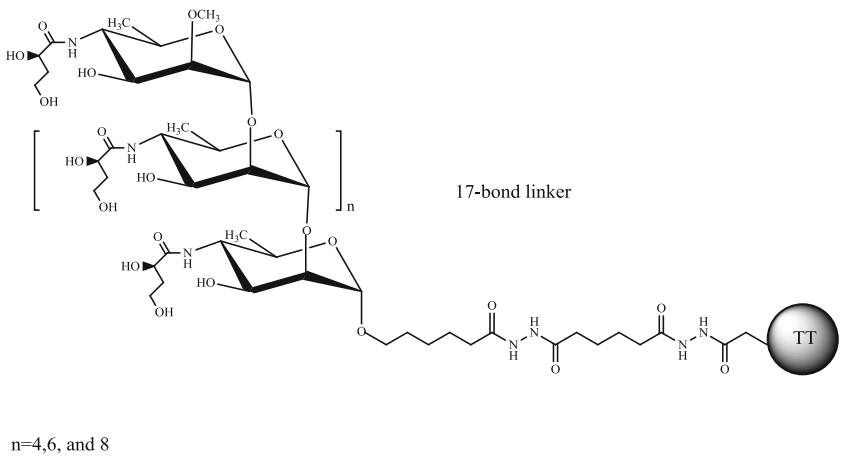

Fig. 1 Synthetic oligosaccharide of the O-specific polysaccharide (OSP) of *V. cholerae* O1, serotype Ogawa with attached 6 methylene spacer and a carboxylic acid as the linking site at the reducing end; *n*=4, 6, 8

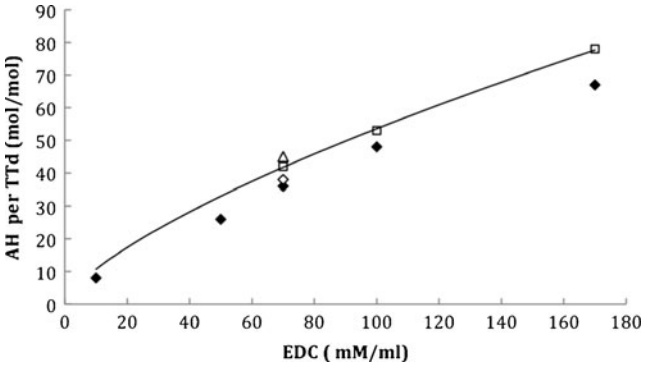

Fig. 2 Level of adipic hydrazide (AH) derivatization of tetanus toxoid as a function of EDC concentration in reaction mixture

AH. The saccharide loading density (molar ratio of hexamer chain/TT) ranged from the lowest 6 (conjugate HexC/6) to the highest 53 (conjugate HexC/53) (Table 1). The weight ratio of sugar to protein are also listed for comparison.

The conjugates synthesized with octa- and decasaccharides showed similar characteristics as the hexaccharide conjugates. However, even using TT_{AH} at the same level of derivatization, the maximum loading density could be reached by decasaccharide was lower than those achieved by hexaccharide or octasacchairde. The loading density of octasaccharide conjugates (OctC) ranged from 27 to 40 mol/mol, and for decasaccharide (DecC) from 10 to 23 mol/mol (Table 1).

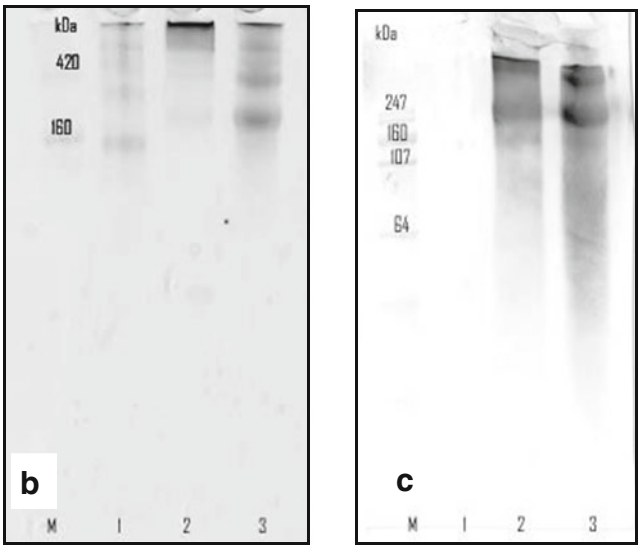

Fig. 3 (continued)

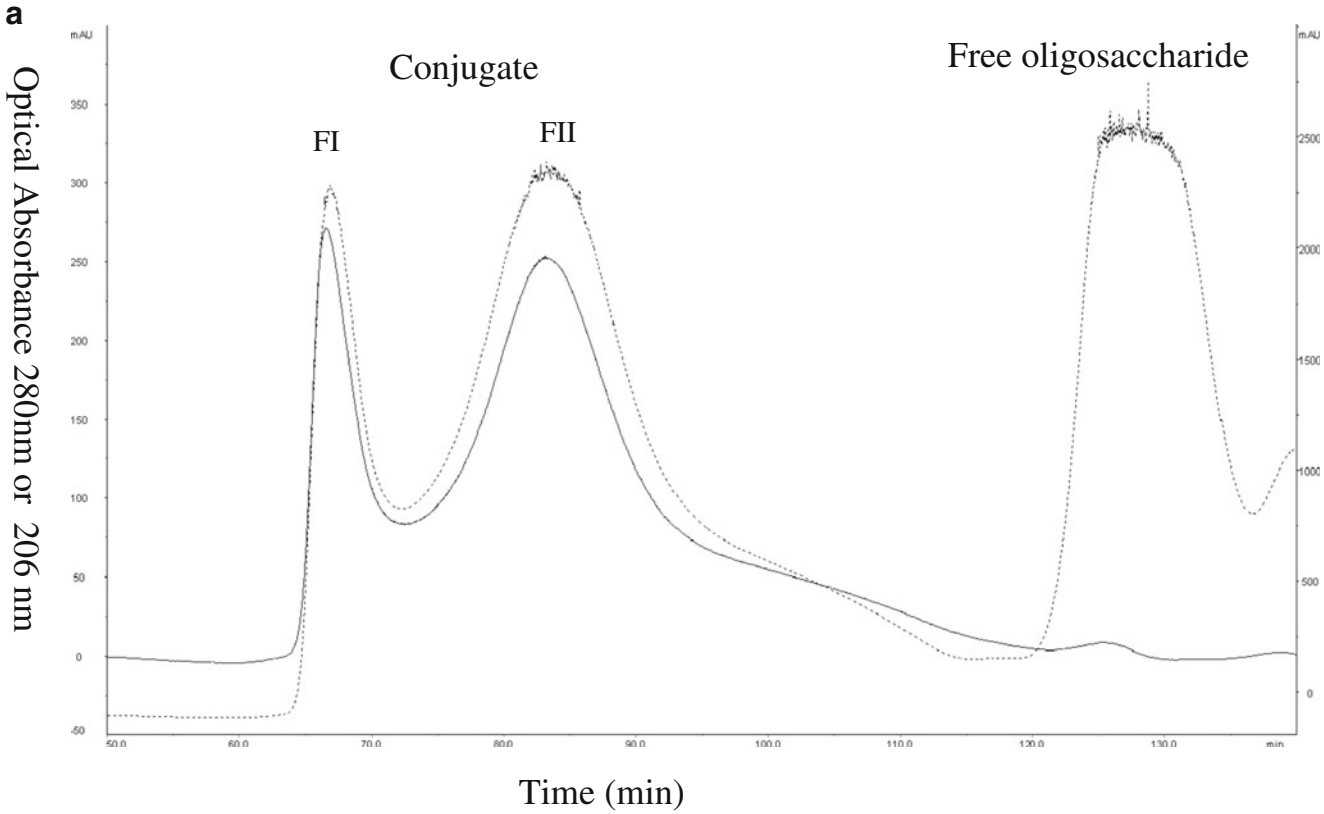

Fig. 3 **a** Representative gel filtration profile of synthetic O-SP Ogawa/TT conjugates. The reaction mixture of Dec/23 was loaded through Sephacryl S-300 column in phosphate buffered saline (pH=7.4, monitored by UV absorptions: 280 nm in solid line, 206 nm in dash line). The two front peaks, FI and FII, were pooled separately. FI was designated as Dec/23. **b** gel analysis of Dec/23 by NuPAGE 4–12 % Bis-Tris gel/w MOPS buffer, stained with Commassie blue; **c** western blot of Dec/23 with mouse anti-LPS Ogawa mAb IgG. Lane 1, $TT_{AH,}$ lane 2, fraction FI, lane 3, fraction FII

Table 1 Serum IgG anti-LPS Ogawa elicited in mice injected 3 times 2 weeks apart with synthetic hexasaccharide, octasaccharide and decasaccharide conjugates of different loading density with or without alum

Oligo saccharide	Conjugate (/loading denstity)+	PS/PR (%wt/wt)	Anti-LPS IgG (EU, 25–75 %)	
			No Alum	Alum
Hexamer	Hex/6	0.09	0.38 (0.1–2.5)	0.39 (0.1–1.3)
	Hex/18	0.20	0.18 (0.1–0.3)	0.48 (0.1–2.3)
	Hex/22	0.23	0.66* (0.1–1.0)	4.85* (0.9–36.1)
	Hex/26	0.27	1.11 (0.2–8.5)	1.61 (0.2–28)
	Hex/30	0.33	1.28 (0.1–9.4)	0.84 (0.1–6.4)
			1.44* (0.1–74.7)	8.16* (0.3–89.2)
	Hex/38	0.40	6.37 (1.4–101)	35.32 (14.4–239)
			12.90* (1.8–60.7)	11.86* (8.7–54.2)
	Hex/43	0.44	0.40 (0.1–2.1)	6.52 (0.1–107)
	Hex/49	0.52	0.39 (0.1–2.1)	5.45 (0.1–203)
	Hex/53	0.55	0.18 (0.1–0.8)	6.53 (0.9–60)
Octamer	Oct/27	0.39	18.54* (1.8–400)	51.41* (5.8–421)
	Oct/40	0.51	6.74* (0.2–73)	17.07* (20–49)
Decamer	Dec/10	0.14	0.82* (0.2–2.7)	2.20* (0.9–22.1)
	Dec/15	0.22	1.05* (0.48–1.17)	3.41* (0.7–27)
	Dec/23	0.36	10.25* (1.1–131)	71.24* (22–410)
DeALPS	~8 DeALPS per TT	0.35	0.78 (0.1–5.33)	0.71 (0.1–1.98)

+ loading density denotes number of saccharide chain per TT

*10 μg of saccharide per injection, others 5 μg per injection

N=10 in all groups except N=20 in Hex/38

Inter group comparison of the highest IgG levels within groups of hexamer, octomer or decamer conjugates:

51.41 vs 11.86, P=0.02; 51.41 vs 18.54, 17.07, P=0.05; 71.24 vs 11.86, 10.25, 17.07, 18.54, 12.90, P=0.01; 71.24 vs 51.41, 35.32, P=NS;

Immunogenicity as a function of loading density and chain length

All synthetic OSP conjugates elicited significantly higher levels of anti-LPS IgG than those prepared with native polysaccharide DeALPS (Table 1) [25, 44]. On the average, conjugates prepared with longer OSP chains such as octa- or decasaccharide, elicited higher levels of anti-LPS IgG than the hexaccharide conjugates (Table 1).

The level of IgG anti-LPS Ogawa elicited by the synthetic OSP conjugates is related to the number of saccharide chains in the conjugate, similar to those observed in synthetic Shigella studies (Table 1) [41]. There is a loading density dependent immune response. Exemplified by hexaccharide conjugate, there is a progressive increase in immune response as the number of loading increased from n=6 to n=38 (R^2=0.88) (Table 1) and the highest antibody level peaked at HexC/38=6.4EU. However, the antibody response started to decline sharply as the loading density increased beyond this optimal point: for HexC/49 and HexC/53, the IgG anti-LPS were 0.4 and 0.2 EU respectively (6.4 vs 0.4 or 0.2; P<0.1). Similar peaking pattern was observed in longer saccharide conjugates too, the optimal loading for octasaccharide conjugate is n=27. There is insufficient data for decasaccharide conjugates to derive the optimal loading.

We also investigated the effect of total molar ratio of monosaccharide to protein (instead of the number of chains). There appears to have a preferred molar ratio (~ 220–240 [monosaccharide]/[TT]), regardless of the degree of polymerization of the oligosaccharide. Similar trend was also observed in groups injected with alum.

As observed in many polysaccharide conjugates, we also noticed that addition of alum enhanced antibody response with the exception of HexC/38 where injection with alum lowered the antibody response (35.32 vs 11.86, P=0.13) (Table 1) [58]. The effect of dosage on immune response is insignificant: for HexC/38 the anti-LPS levels were 6.37 EU at 5 μg/injection and 12.90 EU at 10 μg/injection (12.91 vs 6.37, P>0.5).

Vibriocidal activities against *V. cholerae* Ogawa

Sera from mice injected with PBS or unconjugated oligosaccharides did not exhibit detectable vibriocidal activity. Sera before 2ME reducing treatment showed higher titers than those after the treatment as expected (data not shown). After 2ME treatment to inactive IgM function, the vibriocidal titers ranged from 160 to >960 (Table 2). There is a direct correlation between the level of anti-LPS IgG with the vibriocidal titers: the correlation coefficients R^2=0.72, 0.41 and 0.45 for Hex/38, Oct/27 and Dec/23, respectively (Fig. 4). Conjugates of longer oligosaccharides elicited higher vibriocidal titers than those from hexaccharide conjugates: there is a statistically significant difference comparing the titers elicited by Hex/38 with Oct/27 or Dec/23, (P=0.07) but the difference between Oct/27 and Dec/23 is not significant.

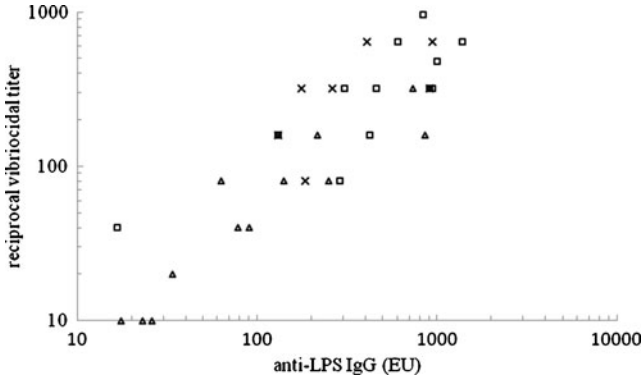

Fig. 4 Serum IgG anti-LPS levels in mice injected with Hex/38, Oct/27 or Dec/23 plotted against the corresponding vibriocidal titers after treatment with 2-mercarptoethanol. Serum anti-LPS IgG levels are expressed in EU/ml. The challenge strain was *V. cholera* Ogawa ATCC 9458: Δ for Hex/38, □ for Oct/27 and X for Dec/23. The correlation coefficients are: R^2=0.85 for Hex/38, R^2=0.62 for Oct/27 and R^2=0.54 for DecC/23. All 3 conjugate combined R^2=0.68

Immune response to LPS Inaba

The cross-reaction with serotype Inaba was evaluated by ELISA and vibriocidal in 18 samples. Mice injected with conjugates elicited anti-LPS IgG to both Ogawa and Inaba (Table 2). There is little correlation between the levels of anti-LPS IgG of the two serotypes (R^2=0.11)

Vibriocidal activity of selected sera from mice immunized with Oct/27 and Dec/23 conjugates were tested against *V. cholerae* 01 Inaba and the correlation between Inaba and Ogawa vibriocidal titers is low, R^2=0.36 (Table 2). Interestingly, a large fraction of samples (10 out of 18), despite of having detectable antibodies to Inaba LPS, did not demonstrate vibriocidal activities against the Inaba bacteria.

Antibody to carrier protein TT

All neoglyco conjugates elicited similar or higher levels of IgG anti-TT than TT alone (data not shown). Since the dosage in the mice injection scheme was based on the carbohydrate content, the corresponding amount of TT injected was higher in the conjugate groups than those in TT control group (13 to18 μg vs 2.5 μg), which may explain the higher TT responses in the conjugate groups. Similar to the IgG anti-LPS responses, groups immunized with adjuvant alum also elicited higher levels of TT antibodies but the difference is not significant.

Discussion

Native OSP extracted from *V. cholerae* O:1 are short, attached to the non-vibriocidal core polysaccharides of similar length and are not ideal for polysaccharide conjugate vaccine [25]. Here neoglyco conjugates against serotype Ogawa were

Table 2 Anti-LPS IgG and corresponding vibriocidal titers of of representative sera from mice injected with synthetic O-SP Ogawa -TT conjugates against *V. cholerae* O1 serotype Ogawa and Inaba

Conjugate	Alum	Anti-LPS IgG (EU) +		Vibriocidal titer*	
		Ogawa	Inaba	Ogawa	Inaba
Hex/38	No	90	NA	40	NA
	No	63	NA	80	NA
	No	854	NA	160	NA
	No	23	NA	20	NA
	No	140	NA	80	NA
	No	17	NA	40	NA
	Yes	734	NA	320	NA
	Yes	216	NA	160	NA
	Yes	34	NA	20	NA
	Yes	26	NA	< 20	NA
	Yes	22	NA	20	NA
	Yes	251	NA	80	NA
Oct/27	No	938	2.3	640	<20
	No	460	67	640	80
	No	290	185	320	40
	No	35	17	160	<20
	Yes	912	71	320	80
	Yes	838	32	960	<20
	Yes	605	281	640	160
	Yes	421	131	160	160
	Yes	306	2.1	320	<20
Dec/23	No	261	214	320	<20
	No	176	32	80	<20
	No	131	175	160	<20
	No	24	38	40	<20
	Yes	939	317	640	320
	Yes	908	154	320	80
	Yes	861	83	320	<20
	Yes	696	44	160	<20
	Yes	410	222	640	160

[+] Serum anti-LPS IgG levels are expressed in ELISA units with hyperimmune mouse reference serum assigned 100EU for each of Ogawa and Inaba serotypes respectively

*Sera were treated with 0.1 M 2-mercaptolethanol for 30 min (2ME) to remove IgM function before vibriocidal assay. The challenge strains were Ogawa ATCC 9458 and Inaba CHO076, a clinical isolate from Peru

Correlation coefficients of all conjugates (combining data from both with and without alum in injections):

IgG Ogawa and IgG Inaba, R^2 =0.11;

VC Ogawa and VC Inaba, R^2 =0.36;

Ogawa IgG vs VC, R^2 =0.68;

Inaba IgG vs VC, R^2 =0.73

VC *t*-test comparison (combining data from with and without alum in injections)

Oct/27 vs Dec/23, *P*=NS, Oct/27 or Dec/23 vs Hex/38, *P*=0.07

prepared with OPS chemically synthesized in various length and covalently linked to tetanus toxoid. The conjugates elicited high levels of serum IgG anti-LPS with vibriocidal activities. Based on knowledge compiled from licensed vaccines, we predict that serum IgG vibriocidal antibody is sufficient to confer protection against cholera infection by inactivating the innoculum on mucosal surfaces and to provide immunity against the disease [58–59].

We have improved the conjugation scheme by extending the polymerization from hexasacchride to decasaccharide and by introducing a novel carboxylic acid linking site at the reducing terminal to facilitate conjugation. In addition, we introduced a longer linker between the saccharide and the carrier protein TT to compensate the short molecular span of the monosaccharide repeat of *V. cholerae* O:1 OSP. The current conjugates consist of a linker of 17-atom with linear hydrazide and alkyl bonds, and do not involve non-physiological cyclic compounds in any of the intermediate steps or in the final product [38–40, 62, 63].

We have found several factors affecting the immunogenicity of synthetic conjugates. There appears to be a progressive increase of antibody response as the carbohydrate chain length increases, similar to findings in other polysaccharide conjugates [41, 64, 65]. Correspondingly, the vibriocidal titers were higher for the conjugates prepared with longer oligosaccharides.

Using aluminium hydroxide as adjuvant showed moderate enhancement in anti-LPS IgG response and vibriocidal titer in most of the conjugates, which could be an important consideration in formulation for future clinical trials.

All conjugates elicited similar levels of antibodies to the carrier protein TT, indicating that treatment of TT with carbodiimide at both the derivatization and conjugate steps did not impede TT immunogenicity.

There are two serotypes of *V. cholerae* O:1, Inaba and Ogawa. We detected low levels of cross-reactive Inaba LPS antibodies in mice injected with the synthetic Ogawa conjugates [33]. Both Inaba and Ogawa shared the identical core region and O-SP, except that at the non-reducing end the C2 atom of Ogawa is methylated [35, 36, 66, 67]. Since there is no core region in the synthetic antigens and the correlation between Ogawa-Inaba antibody responses is low, we postulate that the observed cross-reactive antibodies were elicited from the common epitopes along the internal region of the OSP, or as commonly addressed as the factor C [66, 67]. According to the molecular modeling proposed in dextran studies the antibody binding site to factor C may have groove conformation. In contrast, the stronger antibody response specific to Ogawa is likely elicited by the sugar unit at the non-reducing terminal, or the so called factor B and has a cavity conformation at the binding site [66–68]. Despite of having detectable Inaba antibodies, 55 % of the sera tested did not show corresponding vibriocidal activity against Inaba. It is probably because the epitopes responsible for vibriocidal activities are near the non-reducing end (Factor B) which is the most outreached region of LPS when encountering host cells [69].

Currently there is no cholera vaccine licensed in the U.S. Oral vaccines licensed in some countries have shortcomings that are inadequate for immunization in high endemic regions or used as cholera epidemic relief in outbreaks [11, 12, 14, 15, 22, 70, 71]. In general, most oral vaccines suffer from diminishing immunogenicity due to the gastrointestinal complexity and environmental enteropathy that is often present in children living in fecally contaminated, impoverished environment [20, 71, 72]. A parenteral vaccine such as the polysaccharide conjugate could overcome these deficiencies and should be considered in global cholera control strategy.

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