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Evaluation of synthetic schemes to prepare immunogenic conjugates of *Vibrio cholerae* O139 capsular polysaccharide with chicken serum albumin

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Vibrio cholerae **serotype O139 is a new etiologic agent of epidemic cholera. There is no vaccine available against cholera caused by this serotype.** ***V. cholerae*** **O139 is an encapsulated bacterium, and its polysaccharide capsule is an essential virulent factor and likely protective antigen.**

This study evaluated several synthetic schemes for preparation of conjugates of ***V. cholerae*** **O139 capsular polysaccharide (CPS) with chicken serum albumin as the carrier protein (CSA) using 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) or 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) as activating agents. Four conjugates described here as representative of many experiments were synthesized in 2 steps: 1) preparation of adipic acid hydrazide derivative of CPS (CPS_{AH}) or of CSA (CSA_{AH}), and 2) binding of CPS_{AH} to CSA or of CPS to CSA_{AH}. Although all conjugates induced CPS antibodies, the conjugate prepared by EDC-mediated binding of CPS and CSA_{AH} (EDC:CPS-CSA_{AH}) was statistically significantly less immunogenic than the other three conjugates. Representative sera from mice injected with these three conjugates contained antibodies that mediated the lysis of** ***V. cholerae*** **O139 inoculum.**

Evaluation of the different synthetic schemes and reaction conditions in relation to the immunogenicity of the resultant conjugates provided the basis for the preparation of a ***V. cholerae*** **O139 conjugate vaccine with a medically useful carrier protein such as diphtheria toxin mutant.**

Keywords: Vibrio cholerae **O139, O139 capsular polysaccharide, cholera O139 vaccine, vibriocidal activity to serotype O139**

Abbreviations: **CPS, Capsular polysaccharide; CSA, chicken serum albumin; EDC, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide; CDAP, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate; ADH, adipic acid dihydrazide; AH, adipic acid hydrazide; CPS_{AH}, derivative of CPS; CSA_{AH}, AH derivative of CSA; EDC:CPS-CSA_{AH}, conjugate prepared by EDC-mediated coupling of CPS with CSA_{AH}; CDAP:CPS-CSA_{AH}, conjugate prepared by CDAP-mediated coupling of CPS with CSA_{AH}; CPS_{AH1}, AH derivative of CPS prepared by EDC-mediated activation of hydroxyl groups; EDC:CPS_{AH1}-CSA, conjugate prepared by EDC-mediated coupling of CPS_{AH1} with CSA; CPS_{AH2}, AH derivative of CPS prepared by CDAP-mediated activation of carboxyl groups; EDC:CPS_{AH2}-CSA, conjugate prepared by EDC-mediated coupling of CPS_{AH2} with CSA.**

Introduction

Vibrio cholerae O139, the second etiologic agent of epidemic cholera, emerged in the early nineties [1–3]. *V. cholerae* O139 has a capsule on its surface that is composed of polysaccharide (CPS) [4–6]. The O139 CPS is a complex branched copolymer of a hexasaccharide-repeating unit containing 3 saccharides (N-acetyl-glucosamine, galactouronic acid and N-acetyl-quinovosamine) within a backbone and 3 other saccharides (two colitoses and galactose) in 2 branches [7,8]. It carries 2 negatively charged groups per repeating unit: 1) a carboxyl of galacturonic acid; 2) a cyclic phosphate bound to C4 and C6 of galactose.

Epidemiologic and clinical studies indicate that the capsule of *V. cholerae* O139 is an essential virulence factor and the antibodies to CPS are associated with immunity to this bacterium [3,5,6,9–11].

In this study, we describe the preparation and immunogenicity of four *V. cholerae* O139 CPS conjugates with chicken

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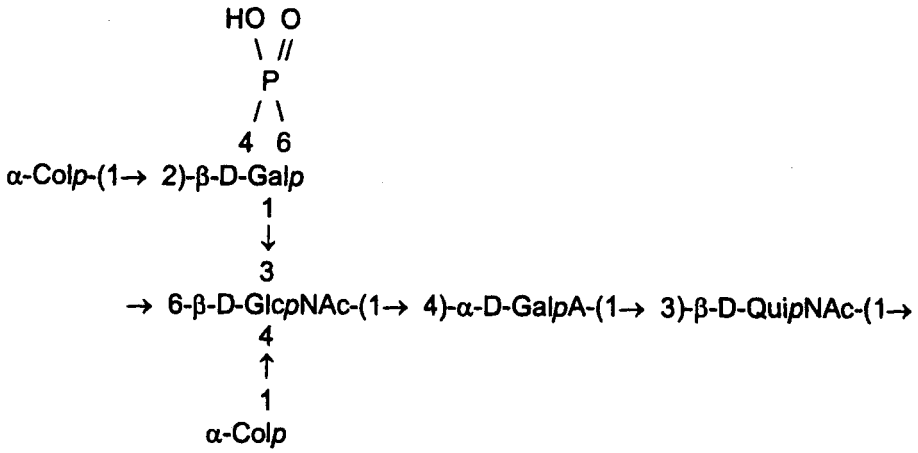


serum albumin (CSA). CSA is a stable, immunogenic, and readily-available protein useful as a model carrier protein [12]. Two sets of conjugates CPS-CSA_{AH} and CPS_{AH}-CSA were prepared, each by 2-step synthesis: 1. the preparation of adipic acid hydrazide (AH) derivative of CPS (CPS_{AH1} and CPS_{AH2}) [Scheme 1A and 1B] or of CSA (CSA_{AH}) [Scheme 1C], and 2. coupling CPS with CSA_{AH} [Scheme 2A and 2B] or CPS_{AH1} or CPS_{AH2} with CSA [Scheme 2C]. Two activating agents EDC [13] and CDAP [14] were used to facilitate either step.

Materials and methods

Substances

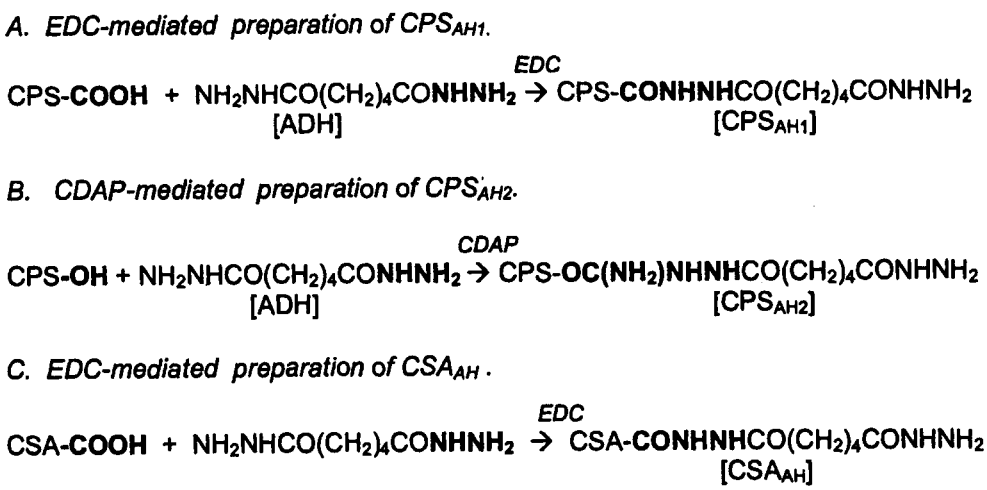
Chicken serum albumin Fraction V (CSA), rabbit CSA antiserum, adipic acid dihydrazide (ADH), 1-ethyl-3(3-dimethylamino-propyl) carbodiimide (EDC), 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate (CDAP), 2-[N-morpholino]ethanesulfonic acid (MES-acid) and (2-[N-morpholino]ethanesulfonic acid) sodium salt (MES-Na), and agarose were from Sigma, St Louis, MO; Sephadex G-25 and Sepharose CL-2B were from Pharmacia AB, Uppsala, Sweden; Bio-Gel P-10 from Bio-Rad Laboratories, Richmond, CA; BSA standard solution, Coomassie blue protein assay reagent, triethylamine (TEA) was from Pierce, Rockford, IL; 2,4,6-trinitrobenzenesulfonic acid (TNBS) solution were from Fluka (Switzerland); dialysis membranes (molecular weight cut off 6–8000) were from Spectra-Por, Laguna Hills, CA. Deionized water was used in all experiments.

Bacteria

A heavily capsulated variant of *V. cholerae* O139 (MDO12C), provided by Richard A. Finkelstein [15], was used for purification of CPS. Another *V. cholerae* O139 clinical isolate from a Thai patient, SPH1168, provided by David N. Taylor (Walter Reed Army Institute of Research, Washington, DC), was used as a target strain in vibriocidal assay.

V. cholerae O139 capsular polysaccharide (CPS)

CPS was purified as described [16] except that the CPS was not diafiltrated through Amicon membrane YM100. The CPS



Scheme 1. Derivatization of CPS or CSA with ADH.

A. EDC-mediated coupling of CPS with CSA_{AH}

$$\text{CPS-COOH} + \text{CSA}_{AH} \xrightarrow{EDC} \text{CPS-CONHNHCO(CH}_2)_4\text{CONHNH-CSA}$$
$$[\text{EDC:CPS-CSA}_{AH}]$$

B. CDAP-mediated coupling of CPS with CSA_{AH}

$$\text{CPS-OH} + \text{CSA}_{AH} \xrightarrow{CDAP} \text{CPS-OC(NH}_2)\text{NHNHCO(CH}_2)_4\text{CONHNH-CSA}$$
$$[\text{CDAP:CPS-CSA}_{AH}]$$

C. EDC-mediated coupling of CPS_{AH1} or CPS_{AH2} with CSA

$$\text{CPS}_{AH1} + \text{CSA-COOH} \xrightarrow{EDC} \text{CPS-NHNHCO(CH}_2)_4\text{CONHNHCO-CSA}$$
$$[\text{EDC:CPS}_{AH1}\text{-CSA}]$$

or

$$\text{CPS}_{AH2} + \text{CSA-COOH} \xrightarrow{EDC} \text{CPS-OC(NH}_2)\text{NHNHCO(CH}_2)_4\text{CONHNHCO-CSA}$$
$$[\text{EDC:CPS}_{AH2}\text{-CSA}]$$

Scheme 2. Conjugation reactions.

contained <0.5% protein, <1% nucleic acids and <5 endotoxin units/μg. The distribution coefficient (Kd) of CPS on Sepharose CL-2B was 0.52.

The ^{13}C NMR spectrum of CPS (100 mg/mL) was measured using a Varian XL300 spectrometer by averaging 50,000 scans with a 10-s decay between acquisition and 10-μs 90° pulse. Prior to Fourier transformation, a 5-Hz line broadening was applied and zero-filled to 32,000 datum points. The ^{13}C NMR spectrum of CPS (Figure 1) agrees with the published reports [7,8].

Adipic acid hydrazide (AH) derivatives of CPS

Adipic acid dihydrazide (ADH, a linker) was bound to the CPS in the presence of EDC (CPS_{AH1}) or CDAP (CPS_{AH2}).

CPS_{AH1} [Scheme 1A]. Concentrations of the reactants in the reaction mixture were 20 mg/mL of CPS, 0.05 M ADH, and 0.05 M EDC. MES buffer (pH 5.5) was added to the CPS in water to adjust the pH to 5.5. EDC and ADH (both in powder form) were then added. The reaction was carried out for 2 h at room temperature and pH 5.5–5.6 was maintained with 0.5 M MES-acid. The pH was then brought to 7.0 with 0.1 M sodium phosphate buffer (pH 8.0), dialyzed overnight against water, and passed through a 1.5- by 24-cm column of Bio-gel P-10 in water. The void volume fractions were freeze-dried and designated as CPS_{AH1}.

CPS_{AH2} [Scheme 1B]. The reaction was performed at a CDAP/CPS ratio of 3:10 (w/w) [17]. 1 mL of CPS (30 mg/mL water), pH 5.0, was mixed with 90 μL of CDAP in acetonitrile (100 mg/mL). After 30 s, 90 μL of 0.2 M TEA was added. During the next 2 min the pH dropped from 8.1 to

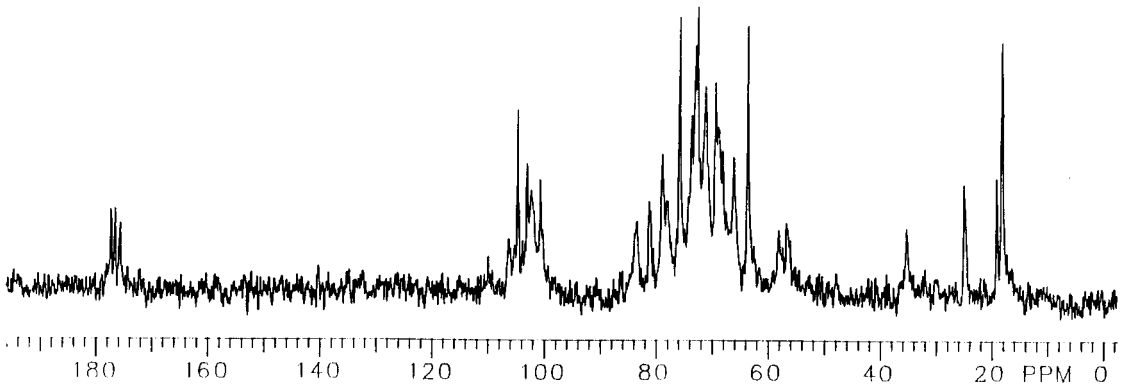


Figure 1. ^{13}C NMR spectrum of *Vibrio cholerae* O139 capsular polysaccharide (CPS).

7.2, and 1 mL of 0.8 M ADH in 0.5 M $NaHCO_3$ was added. The reaction was carried out for 2 h at room temperature and a pH 8.3–8.6 maintained with 0.1 M HCl. The mixture was dialyzed overnight against water and passed through a 2.5- by 40-cm column of Sephadex G-25 in water. The void volume fractions were freeze-dried and denoted as CPS_{AH2}.

Adipic acid hydrazide (AH) derivative of CSA [Scheme 1C]

AH derivative of CSA (CSA_{AH}) was prepared by EDC-mediated condensation of ADH and CSA [18].

Concentrations of the reactants in the reaction mixture were 10 mg CSA/mL, 0.2 M ADH, and 0.015 M EDC. ADH (powder) was added to the CSA, the pH adjusted to 5.5 with 0.1 M MES buffer (pH 5.5), and EDC (powder) was added. The reaction was carried out at room temperature, pH 5.5 to 5.7 for 1 h. The mixture was dialyzed overnight at 4°C against saline and passed through a 2.5- by 40-cm column of Sephadex G-25 in saline. The void volume fractions were pooled, concentrated by ultrafiltration, stored at 4°C, and designated as CSA_{AH}.

Conjugates

Two sets of conjugates CPS-CSA_{AH} and CPS_{AH}-CSA were prepared using EDC [18] and CDAP [17] as the activating agents.

EDC-mediated synthesis of CPS-CSA_{AH} [Scheme 2A]. Concentrations of the reactants in the reaction mixture were 10 mg/mL of CPS and 10 mg/mL of CSA_{AH}, and 0.02 M EDC. CPS was mixed with CSA_{AH}, and the pH was adjusted to 5.5 with 0.5 M MES buffer (pH 5.5). The mixture was brought to the final volume with saline, and EDC was added as powder. The reaction was carried out at room temperature for 3 h, during which the pH rose from 5.5 to 5.7. The conjugation was accompanied by a formation of precipitate that became gradually heavier. The mixture was dialyzed overnight against saline and centrifuged ($7000 \times g$, 5 min) before passing through a 1.5- by 90-cm column of Sepharose CL-2B in saline. Fractions were assayed for polysaccharide and protein. The fractions 21 to 29 of Vo peak were pooled and denoted as EDC:CPS-CSA_{AH}.

CDAP-mediated synthesis of CPS-CSA_{AH} [Scheme 2B]. CPS was activated with CDAP and bound to CSA_{AH} at the CDAP/CPS ratio of 3:10 (w/w). 10 mg of CPS in water (100 mg/mL) was mixed with 30 µL of the CDAP in acetonitrile (100 mg/mL). The mixture (pH 5.2) was stirred for 30 s, and 30 µL of 0.2 M TEA was added. After 2 min, 0.1 M NaOH was added to bring the pH from 7.0 to 8.2. 10 mg of CSA_{AH} was added, and the volume adjusted with saline to 2 mL. The reaction was carried out for 3 h at room temperature, and the pH of 8.0 to 8.3, maintained with 0.1 M NaOH. The mixture was passed through a 1.5- by 90-cm column of Sepharose CL-2B in saline. Fractions were assayed for polysaccharide and protein. The fractions 30 to 46 were pooled and denoted as CDAP:CPS-CSA_{AH}.

EDC-mediated synthesis of CPS_{AH1}-CSA and CPS_{AH2}-CSA [Scheme 2C]. CPS_{AH1}-CSA. Concentrations of the reactants in the reaction mixture were 10 mg/mL of CPS_{AH1}, 10 mg/mL of CSA, and 0.02 M EDC. CPS_{AH1} was mixed with CSA, and the pH was adjusted to 5.5 with 0.5 M MES buffer (pH 5.5). EDC was added as powder, and the mixture was brought to the final volume with saline. The reaction was carried out at room temperature for 3 h during which the pH rose from 5.5 to 5.6. The reaction mixture was passed through a 1.5- by 90-cm column of Sepharose CL-2B in saline. Fractions were assayed for polysaccharide and protein. The fractions 36 to 52 were pooled and denoted as EDC:CPS_{AH1}-CSA.

CPS_{AH2}-CSA. Concentrations of the reactants in the reaction mixture were 5 mg/mL of CPS_{AH2}, 5 mg/mL of CSA, and 0.05 M EDC. The procedure was performed as described above. Fractions were assayed for polysaccharide and protein. The fractions 36 to 52 were pooled and denoted as EDC:CPS_{AH2}-CSA.

Immunization of mice

Six-week-old Swiss albino female mice (10/group) were injected s.c. 3 times at 2-week intervals, with 100 µL of the immunogen in saline containing 2.5 µg of CPS as a conjugate or polysaccharide alone. A control group of mice received 100 µL of saline. Mice were exsanguinated 7 days after the third injection, and sera were stored at −20°C.

Enzyme-linked immunosorbent assay (ELISA)

Serum CPS antibodies were measured by ELISA as described [16]. Murine hyperimmune *V. Cholerae* O139 serum, used as the reference, was assigned a value of 1000 ELISA units/mL (EU) for IgG and 100 EU for IgM (arbitrarily assigned upon the observation that 1/20,000 dilution of anti-IgG and 1/100 dilution of anti-IgM gave approximately the same magnitude of A_{405} in this assay).

Results were computed with the ELISA data processing program, based on a four-parameter logistic-log function using a Taylor series linearization algorithm [19].

Statistics

Comparisons of the geometric mean values of anti-CPS IgG (EU) were performed using the two-sided *t* test or Wilcoxon analysis.

Serum vibriocidal assay

Twelve representative sera from groups of mice injected with the three most immunogenic conjugates, CDAP:CPS-CSA_{AH}, EDC:CPS_{AH1}-CSA, or EDC:CPS_{AH2}-CSA, were examined for vibriocidal activity using vibriocidal assay as described [16]. Vibriocidal titer of murine hyperimmune *V. cholerae* O139 serum before and after absorption with CPS were also determined.

Double-immunodiffusion assay

Double-immunodiffusion of the CPS and CPS_{AH} with murine hyperimmune *V. cholerae* O139 serum [16] or CSA and CSA_{AH} with rabbit CSA antiserum was performed in 1% agarose gel in saline.

Chemical assays

Polysaccharide concentration in the conjugates was measured by the 3,6-dideoxyhexose assay, using CPS as the standard [20]. Protein concentration was measured by Coomassie Blue assay using BSA as the standard [21]. Free hydrazide groups in AH derivatives of CPS and CSA were measured by TNBS with ADH as the standard [22].

Limulus amebocyte lysate test

CPS was assayed for endotoxin by limulus amebocyte lysate test [23]. The FDA Reference Standard Endotoxin (Lot EC-5) was used as the reference for the assay.

Results

AH derivatives of CSA and of CPS (Table 1)

CSA_{AH} [Scheme 1C] contained ~ 9 moles of AH per mole CSA. CSA_{AH} formed a line of identity with CSA when reacted with rabbit anti-CSA serum by double-immunodiffusion (Figure 2A).

CPS_{AH1}, prepared by EDC-mediated reaction [Scheme 1A], contained 0.08 moles of hydrazide per mole of CPS-repeating unit. CPS_{AH2}, prepared by CDAP-method [Scheme 1B], contained 0.12 moles of hydrazide per mole of CPS-repeating unit. Both derivatives formed a line of identity with CPS when reacted with murine hyperimmune *V. cholerae* O139 serum by double immunodiffusion (Figure 2B).

Conjugates

All conjugates were characterized by gel filtration through Sepharose CL-2B followed by analysis of their fractions for polysaccharide (PS) and protein (PR) [Table 2, Figure 3]. The results were evaluated in comparison with Sepharose CL-2B profiles of CPS and of CSA before conjugation: CPS was eluted between the fractions 34 to 60 with the peak at Kd 0.52, and CSA between the fractions 50 to 60 with the peak at Kd 0.78.

EDC:CPS-CSA_{AH} [Scheme 2A]. Gel filtration profile of this conjugate, prepared by EDC-mediated binding of CPS to CSA_{AH}, showed 2 peaks (Vo and Kd 0.52) that contained both PS and PR (Figure 3A). The Vo material consisted mostly of PR (PS/PR 0.15). The majority of PS was detected in the second peak (Kd 0.52). Formation of this conjugate was accompanied by the development of a protein precipitate that accounted for about 30% of total PR. Only Vo material was included in the final pool of the conjugate, and the yield (by the recovery of PS) was 2.7%.

CDAP:CPS-CSA_{AH} [Scheme 2B]. This conjugate was prepared from the same components as EDC:CPS-CSA_{AH} but using CDAP as the activating agent. Gel filtration of this conjugate (Figure 3B) showed that PS was present in fractions 30–65, similar to the elution range of CPS alone (34–60). PR was detected within Fr 30–70, that is 20 fractions before the elution range of CSA alone. Only fractions 30–47 of the PS and PR overlapping region were included in the final pool of the conjugate. The PS/PR ratio of the conjugate was 2.6, and the yield based on the recovery of PS was 51%.

Table 1. Adipic acid hydrazide (AH) derivatives of *Vibrio cholerae* O139 capsular polysaccharide (CPS) and chicken serum albumin (CSA).

		AH/CPS		*AH/CSA*	
Derivative	*Method*	*mg/mg*	*mol/mol*	*mg/mg*	*mol/mol*
CSA_{AH}	EDC	NA	NA	0.024	9.10
CPS_{AH1}	EDC	0.014	0.084	NA	NA
CPS_{AH2}	CDAP	0.020	0.121	NA	NA

NA: not applicable.

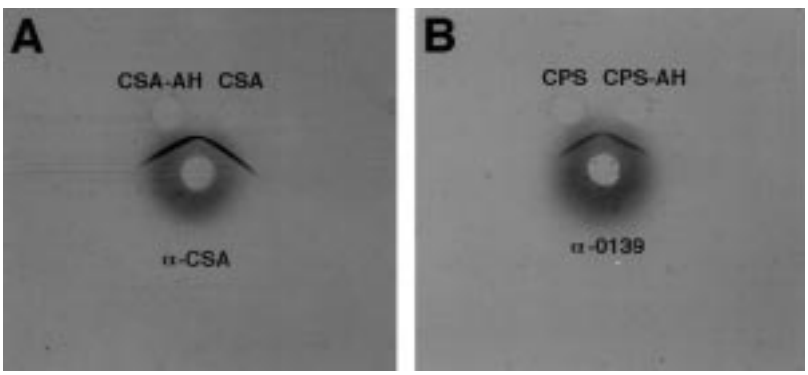


Figure 2. Double immunodiffusion **(A)** of CSA and CSA_{AH} with rabbit CSA antiserum and **(B)** of CPS and CPS_{AH} with murine hyperimmune *V. cholerae* O139 (representative picture of both derivatives).

Table 2. Composition and immunogenicity of *Vibrio cholerae* O139 capsular polysaccharide (CPS) conjugates with chicken serum albumin (CSA).

Conjugate	*Synthetic scheme*	*PS/PR (w/w)*	*Yield (%)*	*Anti-CPS IgG (GM Elisa units/ml*)*
EDC:CPS-CSA_{AH}	EDC	0.15	2.70	2.61[a]
CDAP:CPS-CSA_{AH}	CDAP	2.60	50.7	52.2[b]
EDC:CPS_{AH1}-CSA	EDC	2.21	77.4	12.9[c]
EDC:CPS_{AH2}-CSA	EDC	1.10	54.3	15.7[d]

*GM Elisa units/ml: geometric mean Elisa units.
The level of antibodies (class IgG) specific to CPS (anti-CPS IgG) induced by CPS alone was 0.19 Elisa units/ml, which was not statistically different from 0.12 obtained in the control group of mice injected with saline; a vs b ($P = 0.0005$); c vs d NS; b vs c or d NS; a vs c or d, $P < 0.03$.

EDC:CPS_{AH1}-CSA and EDC:CPS_{AH2}-CSA [Scheme 2C]. Gel filtration profiles of this conjugates are shown on Figure 3C and 3D, respectively. Both conjugates were prepared by EDC-mediated conjugation of the AH derivative of CPS with CSA, but the w/w ratio of EDC/PR was 5-fold higher in the synthesis of EDC:CPS_{AH2}-CSA. It should be also noted that

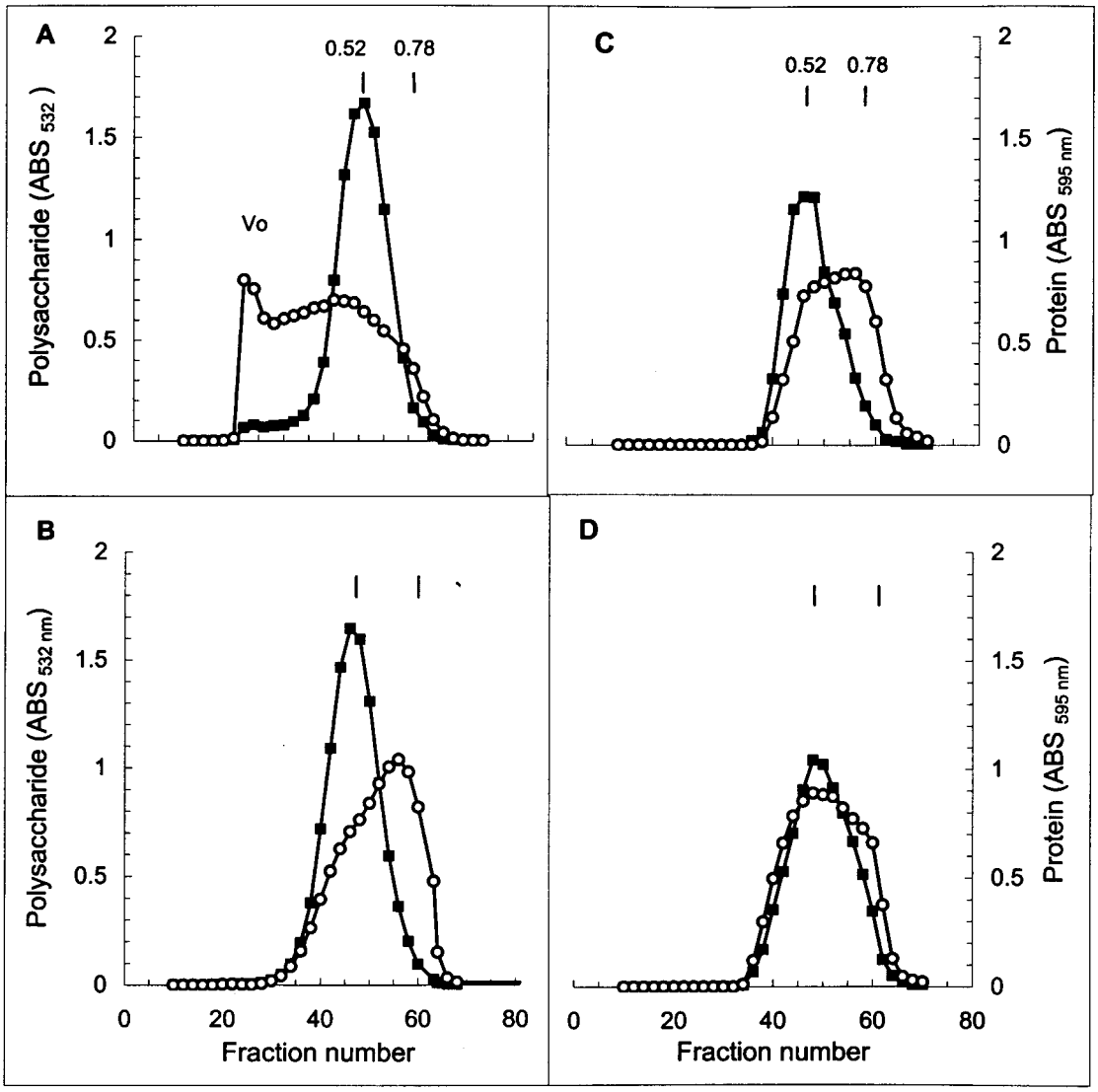


Figure 3. Sepharose CL-2B profiles of *Vibrio cholerae* O139 capsular polysaccharide (CPS) conjugates with chicken serum albumin (CSA): (A) EDC:CPS-CSA_{AH}, (B) CDAP:CPS-CSA_{AH}, (C) EDC:CPS_{AH1}-CSA, (D) EDC:CPS_{AH2}-CSA. Polysaccharide (solid symbol); protein (open symbol); distribution coefficients of CPS alone (Kd 0.52) and CSA alone (Kd 0.78) are depicted.

Table 3. Vibriocidal activity of representative sera from mice injected 3 times with *Vibrio cholerae* O139 capsular polysaccharide (CPS) conjugates with chicken serum albumin (CSA).

Conjugate	Anti-CPS (EU/mL) IgG	Anti-CPS (EU/mL) IgM	Vibriocidal titer*
CDAP:CPS-CSA_{AH}	18.4	1.68	1000
	73.9	1.67	2000
	102.3	1.50	1000
	325.0	11.53	8000
EDC:CPS_{AH1}-CSA	14.5	2.51	2000
	58.9	2.38	4000
	71.9	0.91	2000
	118.1	2.50	2000
EDC:CPS_{AH2}-CSA	18.1	2.38	1000
	62.4	2.21	2000
	70.3	2.32	4000
	103.5	9.92	4000

*Vibriocidal titer is defined as a reciprocal of the highest serum dilution that caused the $\geqslant$ 60% reduction in the number of bacteria compared to complement control (no serum). Sera from mice injected with CPS alone or with saline were not vibriocidal (VT $<$ 50) (not shown). Anti-CPS IgG or IgM : antibodies specific to CPS of IgG or IgM class.

CPS_{AH1} and CPS_{AH2} had a different content of hydrazide and were prepared by different derivatization reactions. Gel filtration of the conjugates showed that PS and PR peaks overlapped in the range of 36–52 (EDC:CPS_{AH1}-CSA) and 36–60 (EDC:CPS_{AH2}-CSA). There was more PR eluted at Kd 0.78 (identical to Kd of CSA alone) in the first conjugate. Only fractions 36–52 were included in the final pools of each conjugate. The PS/PR (w/w) ratio and yield, by the recovery of PS, were higher for EDC:CPS_{AH1}-CSA than for EDC:CPS_{AH2}-CSA.

Serum IgG response to CPS induced by conjugates

CPS alone elicited 0.19 EU of anti-CPS IgG that was not significantly different from the level 0.12 EU in mice injected with saline. All conjugates induced statistically significant levels of anti-CPS IgG ranging from 2.61 to 52.2 EU (Table 2).

EDC:CPS-CSA_{AH} elicited the lowest level of anti-CPS IgG (2.61 EU) of all conjugates. In contrast, CDAP:CPS-CSA_{AH}, composed of the same components as EDC:CPS-CSA_{AH}, but prepared by CDAP-mediated synthesis, elicited the strongest anti-CPS response (52.2 EU vs. 2.61, $P = 0.0005$).

The last two conjugates prepared by EDC-mediated conjugation, EDC:CPS_{AH1}-CSA and EDC:CPS_{AH2}-CSA, elicited 12.9 EU and 15.7 EU, respectively, and the difference between these levels was not statistically significant. Neither of these levels was significantly different from 52.2 EU elicited by CDAP:CPS-CSA_{AH}.

Serum vibriocidal activity to *V. cholerae* O139

The murine hyperimmune *V. cholerae* O139 serum [16], raised by multiple injections of formalin-killed bacteria (MDO12C), had a vibriocidal titer of 16,000. Absorption of this polyvalent serum with CPS reduced the titer by 80%.

Sera from mice injected with saline or CPS had no vibriocidal activity. Sera from mice injected with the 3 most immunogenic conjugates (CDAP:CPS-CSA_{AH}, EDC:CPS_{AH1}-CSA, EDC:CPS_{AH2}-CSA), were vibriocidal and their titers ranged from 1000 to 8000. It is important to note that the target strain in vibriocidal assay (SPH 1168) was different clinical isolate than that used for purification of CPS (MDO12C).

Discussion

Several synthetic schemes and different reaction conditions for preparation of *V. cholerae* O139 CPS conjugates with CSA were investigated. Here we describe synthesis, physico-chemical characterization and immunogenicity of four representative conjugates. Two of them were prepared by coupling CPS to CSA_{AH} facilitated with either EDC or CDAP. The other two conjugates were prepared by EDC-mediated binding of CPS_{AH} to CSA.

Conjugates such as EDC:CPS-CSA_{AH} and CDAP:CPS-CSA_{AH}, although prepared from the same components but using different activating agents, are structurally different molecules. EDC activates carboxyls, while CDAP activates hydroxyls for the reaction with nucleophilic groups [13,14]. In addition, the chemistry of both conjugations is complex because the potentially activated groups (carboxyls or hydroxyls) are present on both CPS as well as CSA_{AH}, and they can react with both hydrazides and amines (ε amine group of lysine) on the protein. It should be pointed out that hydrazides are stronger nucleophilic groups than amines. Therefore, carboxyls or hydroxyls will react preferentially with hydrazides.

Synthesis of EDC:CPS-CSA_{AH} is representative of several conjugation experiments: all were accompanied with precipitation of protein, and the resultant conjugates were large in molecular size, had low w/w PS/PR ratios ($\leqslant 0.15$), and were poor immunogens. Together these findings indicate that during EDC-mediated conjugation of CPS and CSA_{AH} the protein became self-cross-linked, and such structural alteration of carrier protein could explain the low immunogenicity of this conjugate. Self-cross-linking of protein could be a direct result of the comparatively higher reactivity of CSA-carboxyls than CPS-carboxyls.

In contrast, no protein precipitation was observed during EDC-meditated binding CPS_{AH} with CSA. In this synthesis the higher reactivity of protein-carboxyls than CPS-carboxyls favors the binding of protein-carboxyls with hydrazides on CPS_{AH}, which results in the formation of conjugate. The lower reactivity of CPS-carboxyls reduces the possibility for self-

crosslinking of CPS-molecules. Both resultant conjugates, EDC:CPS_{AH1}-CSA and EDC:CPS_{AH2}-CSA, had high PS/PR ratios and were significantly better immunogens than EDC:CPS-CSA_{AH}

In contrast to the EDC-mediated coupling of CPS to CSA_{AH}, the CDAP-mediated coupling of the same components resulted in the formation of highly immunogenic conjugate CDAP:CPS-CSA_{AH}. It is also of interest, that although CSA_{AH} was prepared by EDC-mediated derivatization reaction, this exposure to relatively mild conditions (10 mM EDC for 1 h) had no apparent negative effect on the carrier protein and subsequently on the immunogenicity of the resultant conjugate.

In summary, this study demonstrated that immunogenicity of CPS could be stimulated by conjugating CPS to CSA and that the conjugate-elicited sera contained antibodies that neutralized *V. cholerae* O139. Evaluation of different synthetic schemes and reaction conditions in relation to immunogenic properties of the resultant conjugates provided the basis for selecting effective ways for synthesis of *V. cholerae* O139 CPS conjugates with a clinically acceptable protein carrier, such as diphtheria toxin mutant [16].

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