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Synthetic Fragments of *Vibrio cholerae* O1 Inaba O-Specific Polysaccharide Bound to a Protein Carrier Are Immunogenic in Mice but Do Not Induce Protective Antibodies

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Development of *Vibrio cholerae* lipopolysaccharide (LPS) as a cholera vaccine immunogen is justified by the correlation of vibriocidal anti-LPS response with immunity. Two *V. cholerae* O1 LPS serotypes, Inaba and Ogawa, are associated with endemic and pandemic cholera. Both serotypes induce protective antibody following infection or vaccination. Structurally, the LPSs that define the serotypes are identical except for the terminal perosamine moiety, which has a methoxyl group at position 2 in Ogawa but a hydroxyl group in Inaba. The terminal sugar of the Ogawa LPS is a protective B-cell epitope. We chemically synthesized the terminal hexasaccharides of *V. cholerae* serotype Ogawa, which comprises in part the O-specific polysaccharide component of the native LPS, and coupled the oligosaccharide at different molar ratios to bovine serum albumin (BSA). Our initial studies with Ogawa immunogens showed that the conjugates induced protective antibody. We hypothesized that antibodies specific for the terminal sugar of Inaba LPS would also be protective. Neoglycoconjugates were prepared from synthetic Inaba oligosaccharides (disaccharide, tetrasaccharide, and hexasaccharide) and BSA at different levels of substitution. BALB/c mice responded to the Inaba carbohydrate (CHO)-BSA conjugates with levels of serum antibodies of comparable magnitude to those of mice immunized with Ogawa CHO-BSA conjugates, but the Inaba-specific antibodies (immunoglobulin M [IgM] and IgG1) were neither vibriocidal nor protective in the infant mouse cholera model. We hypothesize that the anti-Inaba antibodies induced by the Inaba CHO-BSA conjugates have enough affinity to be screened via enzyme-linked immunosorbent assay but not enough to be protective in vivo.

Cholera, an enteric diarrheal disease caused by the gram-negative bacterium *Vibrio cholerae*, continues to be a worldwide health concern. *V. cholerae* lipopolysaccharide (LPS), a critical component of the outer membrane that is required for virulence, is a known target for immune responses following infection or immunization. Antibodies specific for *V. cholerae* LPS are correlated with protection against cholera (31, 32). The importance of preexisting anti-LPS antibody was highlighted by a change in the susceptible population of a *V. cholerae* O139 outbreak, where disease was seen in adults that are normally thought to have some immunity because of previous exposure to cholera LPS antigens, but in this circumstance, previous exposure did not cross-protect against the new LPS antigens of O139 (2). Multiple serologic reagents (1, 12, 14-16, 29) have been developed against *V. cholerae* LPS and used to define three O-antigen-associated B-cell epitopes (epitopes A, B, and C). The A epitope is expressed equally well by *V. cholerae* O1 serotypes Inaba and Ogawa LPS. Structurally, epitope A was postulated to be either the perosamine residues or the *N*-tetronic acid (*N*-3-deoxy-L-*glycero*-tetronic acid) side chain, elements common to both LPS serotypes (29). The serologic designation B is found only in the Ogawa O-specific polysaccharide (O-SP) (29, 39). Several groups showed that anti-C reactivity was associated with only the Inaba LPS, although some groups described anti-C monoclonal antibody (MAb) reactivity to the Ogawa strain (1, 12, 14–16). The nature of the C epitope has not been experimentally identified (29). The O-SP terminal sugar of *V. cholerae* LPS is now known to differentiate Ogawa and Inaba serotypes. *V. cholerae* O-SP consists of (1→2)-α-linked 4-amino-4,6-dideoxy-D-mannose (perosamine) whose amino group is acylated with 3-deoxy-L-*glycero*-tetronic acid (17, 21). In the Ogawa O-SP, the terminal sugar is characterized by a 2-*O*-methyl group, while the terminal sugar in the Inaba O-SP has a hydroxyl at the 2 position (17, 18, 40, 41).

V. cholerae serotypes can undergo serotype conversion in both directions during epidemics or in areas where cholera is endemic (9, 11). For example, the initial serotype in South America in 1991 was 95% Inaba, whereas 1992 to 1995 saw Ogawa as the predominant (90%) serotype (9). Others have noted seroconversion in response to immune selective pressure in vitro where anti-serotype-specific antibodies can select for the nonreactive serotype (reviewed in reference 2). *V. cholerae* O1 LPS induces protective immune responses in humans and experimental animals (13, 19, 27, 35) and thus is an immunogen of choice for cholera vaccine development. Therefore, it is important to develop O-SP-based cholera vaccines that can protect against Inaba as well as Ogawa serotypes. These vaccines could be based on the common A epitope or both the unique B and C epitopes.

It has recently been reported that synthetic hexasaccharide-

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protein conjugate immunogens that mimic in part the terminus of Ogawa LPS induced vibriocidal antibodies as well as protective antibodies, as measured by an infant mouse protection assay (7). We reasoned that if antibodies specific for the terminal sugar of Ogawa O-SP were protective, then antibodies to the analogous structure in Inaba LPS would also be protective. We now report that a series of conjugates made from Inaba di-, tetra-, and hexasaccharide and bovine serum albumin (BSA) are immunogenic in mice, inducing immunoglobulin M (IgM) and the T-dependent IgG1 subclass. For the majority of the conjugates, the length of oligosaccharide and the degree of carbohydrate (CHO) substitution (CHO/BSA, mole/mole) did not affect the serologic response in the tertiary sera. In contrast to the protective antibody induced by the Ogawa O-SP protein conjugates, the Inaba O-SP protein conjugates failed to induce antibodies which were vibriocidal in vitro that were protective in the infant mouse assay or that bound *V. cholerae* LPS in situ.

MATERIALS AND METHODS

Animals. Six-week-old female BALB/c mice were purchased from the National Cancer Institute (Bethesda, Md.). Pregnant, female CD-1 mice were purchased from Charles River (Raleigh, N.C.) for the infant mouse protection studies. All mice were housed under standard conditions in the Animal Resources Center located at the Dartmouth-Hitchcock Medical Center, Lebanon, N.H.

Inaba CHO-BSA constructs. Immunogens 1a to 3c were prepared by linking the chemically synthesized di-, tetra-, and hexasaccharide fragments of the O-SP of *V. cholerae* O1 serotype Inaba to BSA by using squaric acid chemistry (20, 36, 38). The oligosaccharides were assembled in a stepwise manner (28) (Fig. 1) from the monosaccharide glycosyl donor 4 and the monosaccharide glycosyl acceptor 5. Note that in contrast to our previous syntheses of oligosaccharides related to the O-SP of *V. cholerae* O1 from intermediates containing the azido group at position 4, the building blocks 4 and 5 have the 4-(3-deoxy-L-*glycero*-tetronic acid) side chain already in place. Also, the glycosyl acceptor 5 is a glycoside of methyl 6-hydroxyhexanoate. Thus, the coupling of these intermediates afforded, after deprotection, haptens in the form of glycosides whose aglycon made them amenable to conjugation to proteins. The advantage of the use of fully equipped intermediates such as 4 and 5 is that the assembled oligosaccharides have to undergo a lesser number of chemical manipulations. Briefly (Fig. 1A), condensation (23) of the glycosyl acceptor 5 and glycosyl donor 4, prepared from the corresponding amine (7) and 2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronic acid (24, 28), gave the fully protected disaccharide 6. Partial deprotection by Zemplén deacetylation afforded alcohol 7, which was used as a glycosyl acceptor in the next coupling with 4 to extend the oligosaccharide chain and obtain the trisaccharide 8. Complete deprotection of 7 was achieved by hydrogenolysis, which simultaneously removed the benzyl and benzylidene protecting groups, to give the disaccharide hapten 10. Repeating the sequence of reactions with 8 that effected the conversions 6→7→8 and then repeating the sequence with the higher oligosaccharides provided the linker-equipped tetrasaccharide (11) and the hexasaccharide hapten (12). Next (Fig. 1B), haptens 10 through 12 were subjected to aminolysis to afford ethylenediamine derivatives 13 through 15, which were treated with squaric acid diethyl ester. The squaric acid monoesters 16 through 18, thus obtained, were treated with BSA to give neoglycoconjugates 1a through 3c, whose molar hapten-BSA ratio was determined by surface-enhanced laser desorption ionization–time of flight (mass spectrometry) (6). The chemical composition of the synthetic components used to construct the Inaba CHO-BSA immunogens were confirmed by nuclear magnetic resonance and mass spectrometry after synthesis and then assembled to produce the Inaba CHO-BSA.

Immunization and serum collection. Eight groups of five mice each were used to test the immunogenicity of the Inaba O-SP epitope conjugates according to the regimen shown in Fig. 2. Ten micrograms (based on carbohydrate weight) of Inaba CHO-BSA conjugate resuspended in 150 mM NaCl and mixed 1:1 in RIBI adjuvant (Sigma, St. Louis, Mo.) was injected intraperitoneally (i.p.) on days 0, 14, and 28. Blood collection via retro-orbital sinus or plexus was done on days 0, 10, 17, and 35, which represent preimmune, primary, secondary, and tertiary sera, respectively (Fig. 3). Retro-orbital plexus bleeding yielded between 80 to 120 μl of blood, which can provide 50% of that volume as serum after processing. Resulting sera from individual mice within a group were pooled and stored at 4 or −20°C until use.

BALB/c female mice used to generate control sera for protection and vibriocidal-antibody assays were immunized with purified LPS from either *V. cholerae* O1 Ogawa strain P1418 (a generous gift from S. Kondo, Josai University, Josai, Japan) or Inaba strain 569B LPS (Sigma). Mice were immunized i.p. with 9 μg of Ogawa or 9 μg of Inaba LPS on days 0, 15, 60, and 71. Tertiary and quaternary sera were used as positive controls for these studies.

Serology. The presence of anti-O-SP Inaba-specific antibodies in individual serum samples was measured by enzyme-linked immunosorbent assay (ELISA). High-binding, flat-bottomed 96-well microtiter plates (Corning Life Sciences, Acton, Mass.) were coated with 100 μl of an Inaba solution (5 μg of Inaba LPS/ml) in 0.1 M carbonate-bicarbonate buffer (pH 9.5) per well and incubated overnight at 4°C. Plates were washed three times by using a Molecular Devices (Sunnyvale, Calif.) Skan plate washer with 250 μl of phosphate-buffered saline (PBS)–0.05% Tween 20 (Fisher Scientific, Pittsburgh, Pa.) per well. Nonspecific binding was blocked by using 200 μl of blocking buffer consisting of PBS–1.0% fish gelatin (BioFX, Owings Mills, Md.)–1.0% normal goat serum (Jackson ImmunoResearch, West Grove, Pa.)–0.05% Tween 20 per well for 1 h at room temperature. Plates were washed three times more, after which 50 μl of antiserum (serially diluted twofold in blocking buffer) was added to each well and incubated overnight at 4°C. The initial dilution of 1:25 was used for all sera for the initial ELISA, and then a dilution of 1:100 was used for the tertiary serum sample in a follow-up ELISA to determine an endpoint titer for these sera. Following incubation with primary sera, plates were washed three times, and 50 μl of horseradish peroxidase-labeled goat anti-mouse IgM (μ-chain-specific) or anti-IgG1 (γ1-specific) (Southern Biotechnology Associates, Birmingham, Ala.) detector antibodies (diluted 1:6,000) was added to each well and incubated at room temperature for 1 h in the dark. Plates were washed three times and then developed with 100 μl of *O*-phenylenediamine dihydrochloride (OPD) peroxidase substrate (Sigma) per well for 5 min at room temperature. OPD peroxidase substrate was prepared by diluting 10-mg tablets into 0.05 M phosphate-citrate buffer, pH 5.0 (Sigma), to a final concentration of 0.4 mg/ml. Fresh 30% H_2O_2 was added to the OPD substrate solution immediately before use to a final concentration of 0.02%. The reaction was stopped with an equal volume of 3 M HCl. Optical densities (OD) were read at 490 nm (OD_{490}) by using a Dynex Technologies MRX microtiter plate reader (Thermo Labsystems, Helsinki, Finland) with Dynex Revelation 3.04 software.

We compared the tertiary serum sample titers, as they were the source of sera for the functional assays. Endpoint titers for ELISA were defined as the reciprocal of the antibody dilution for the last well in a column with a positive OD for each sample after subtracting the background. Background values were determined with preimmunization sera. Preimmunization serum samples for each treatment group were analyzed on multiple 96-well plates. The OD values of the preimmunization sera were averaged and then doubled. This value was subtracted from the OD of all the wells containing the titration of the pooled serum samples.

Vibriocidal antibody. (i) Spread plate method. Titers of vibriocidal antibody against *V. cholerae* (classical Ogawa strain O395, classical Inaba strain 569B, and El Tor Inaba strain N16961) were assessed in vitro (10, 22). Bacteria were grown in Luria-Bertani (LB) broth at 37°C for 18 h. The culture was centrifuged for 10 min, resuspended into an equal volume of PBS plus 0.1% peptone, and diluted $1{:}1.0 \times 10^4$ in PBS. Pooled preimmune and tertiary sera from each treatment group were diluted in 50 μl of ice-cold PBS containing 20% guinea pig complement (Sigma) with the dilutions of $1{:}(1.0 \times 10^2)$, $1{:}(1.0 \times 10^3)$, $1{:}(1.0 \times 10^4)$, $1{:}(5.0 \times 10^4)$, and $1{:}(1.0 \times 10^5)$ and kept in an ice-water bath until needed. Bacteria (3.2×10^4 CFU) were mixed with diluted antiserum (1:1), incubated for 1 h on a platform shaker at 37°C (125 rpm), and then returned to the ice-water bath. Each sample (100-μl total volume) was then spread on LB agar plates and allowed to dry at room temperature before overnight incubation at 37°C. CFU were recorded for each plate. Inhibition of bacterial growth (endpoint titer) was considered significant if 50% or more of the bacteria were killed compared to CFU from plates containing preimmune serum and complement.

(ii) Microtiter method. The recently developed microtiter test protocol (5) was generously provided by Fournier's group (Pasteur Institute, Paris, France). *V. cholerae* O1 El Tor Inaba strain N16961 was inoculated into 2.0 ml of alkaline peptone water (1.0% peptone and 1.0% NaCl [pH 8.6]) and grown overnight at 37°C. The culture was transferred to a prewarmed nutrient agar plate and incubated for 90 min at 37°C. Five milliliters of cold PBS was applied to the plate and swirled gently to resuspend the bacteria and then was transferred to a 15-ml conical tube. The OD_{600} of the bacterial suspension was adjusted to 0.80 with PBS to approximate the bacteria to 10^9 CFU/ml. Seven volumes of cold PBS, 2 volumes of guinea pig complement, and 1 volume of bacterial suspension were mixed in a chilled tube and kept on ice for 20 min. Fifty microliters of heat-inactivated mouse serum from the various treatment groups was placed in a

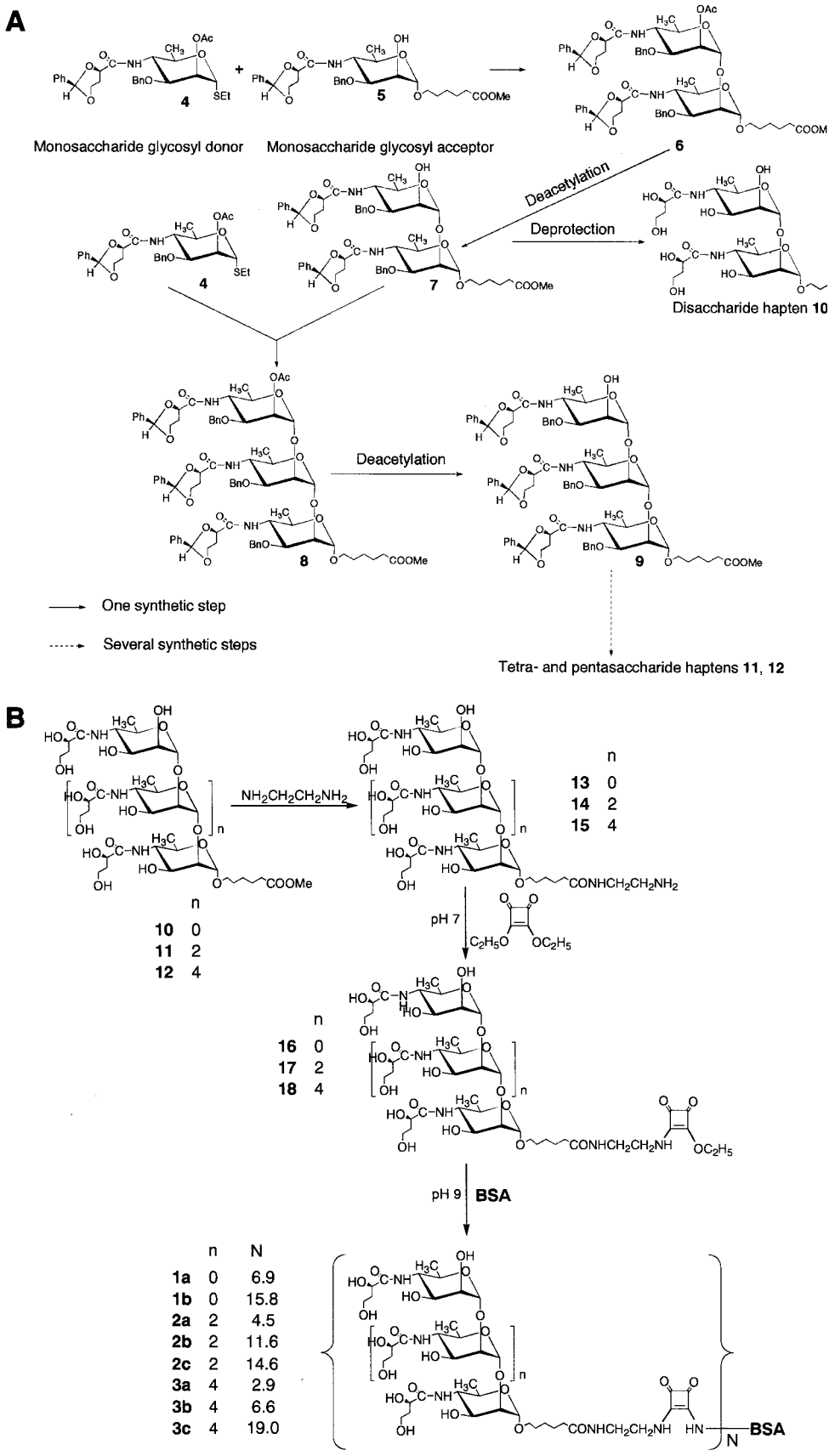


FIG. 1. Schema for generation of Inaba neoglycoconjugate immunogens and Inaba CHO-BSA.

A.

Construct	O-SP Substitution Ratio	O-SP length	Conjugate MW	μg CHO/mg conjugate	μg BSA per 10 μg CHO
Group 1	6.9	Di	71,589	49.0	194.1
Group 2	15.8	Di	78,283	103.0	87.1
Group 3	4.5	Tetra	72,028	62.0	151.3
Group 4	11.6	Tetra	80,854	144.0	59.4
Group 5	14.6	Tetra	84,588	173.0	47.8
Group 6	2.9	Hexa	71,342	61.0	153.9
Group 7	6.6	Hexa	78,007	126.0	69.4
Group 8	19.0	Hexa	99,737	285.0	25.1

B.

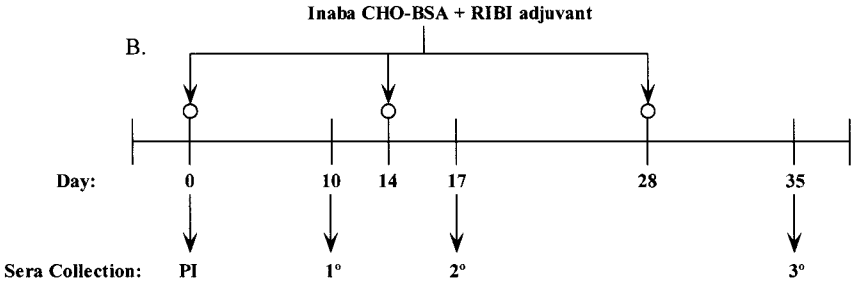


FIG. 2. Description of Inaba CHO-BSA immunogens (A) and timeline of immunization and serum sampling (B). Each treatment group consisted of five female BALB/c mice, aged 7 weeks at the start of the treatment regiment. Mice received injections of Inaba CHO-BSA neoglycoconjugate immunogen mixed 1:1 in RIBI adjuvant in 150 mM NaCl on days 0, 14, and 28. Immunogens were dosed at 10 μg per mouse per immunization based upon weight of CHO in each conjugate and administered via i.p. injection. Individual serum samples were collected on days 0 (preimmune control sample [PI]), 10 (primary sample), 17 (secondary sample), and 35 (tertiary sample) via the retro-orbital sinus and subsequently pooled by treatment group and stored at either 4 or −20°C until assessed.

round-bottomed sterile microtiter plate with a lid and serially diluted 1:2 in PBS. Twenty-five microliters of the complement-treated bacteria was added to each well and covered and incubated for 1 h at 37°C. One hundred fifty microliters of LB broth was then added to each well, and the plate was incubated uncovered in a humidified chamber for 2 h at 37°C. An aqueous solution containing 1 volume of 1.0% neotetrazolium chloride (ICN Biomedicals, Irvine, Calif.) and 9 volumes of 2.7% sodium succinate (ICN Biomedicals) was made. Twenty-five microliters of this solution was added to each well and incubated uncovered for 15 min at room temperature before the OD_{570} was recorded. The plate was then placed in a humidified chamber at 4°C overnight, and the optical density was recorded again the next day. A violet color in the well indicated the presence of live vibrios. Inhibition of bacterial growth (endpoint titer) was reported as the reciprocal of the antibody dilution for the negative well containing the lowest concentration of antibody for each sample tested.

Infant mouse challenge. The suckling mouse challenge model for cholera was used for assessing the protective quality of anti-O-SP Inaba-specific antibodies in vivo (33, 37). Cultures of *V. cholerae* (El Tor Inaba strain N16961) were grown for 16 h in LB broth, pH 6.5, at 30°C. Twenty-five microliters of bacterial suspension, representing 25 to 44 50% lethal doses (LD_{50}) (44 LD_{50} were used to test the positive control sera), was combined with 25 μl of either preimmune sera (negative control), sera from BALB/c mice previously immunized with Inaba LPS (positive control), or tertiary anti-O-SP Inaba-specific sera immediately before administration intragastrically to 4- to 5-day-old CD-1 mice. Challenged mice were kept at 30°C and monitored every 4 h starting 24 h postchallenge.

In situ analysis of anti-LPS antibody binding. Live *V. cholerae* O1 El Tor Inaba bacteria were used to assess the binding ability of anti-O-SP Inaba-specific antibodies in situ. Cultures of *V. cholerae* strain N16961 were grown for 16 h in standard LB medium at 37°C. One hundred microliters of bacterial suspension, representing approximately 10^9 CFU, was pelleted in a microcentrifuge and washed in PBS (pH 7.2) three times to remove all culture medium. The bacteria were then resuspended in an equal volume of tertiary anti-O-SP Inaba-specific or anti-Inaba whole-LPS serum which was diluted 1:10 in PBS. The resulting mixture was incubated at room temperature for 2 h followed by overnight incubation at 4°C. Samples were then washed three times with PBS (pH 7.2) to remove unbound antibodies. Pelleted bacteria were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein sample buffer containing 2-mercaptoethanol. Two micrograms of mouse IgM was used as a positive control for the IgM heavy chain. Each sample was boiled for 5 min and then centrifuged for 5 min to remove precipitates. Twenty microliters of each sample was loaded onto a 12% Tris-HCl polyacrylamide gel (Bio-Rad Laboratories, Inc., Hercules, Calif.) and electrophoresed for 45 min at 150 V. The samples were then transferred to a nitrocellulose membrane by using a semidry transfer cell (Bio-Rad Laboratories, Inc.) at 23 V for 30 min. The membrane was blocked at room temperature for 2 h in PBS, 0.05% Tween 20, and 5.0% nonfat dry milk. Horseradish peroxidase-conjugated goat anti-mouse IgM antibody (Southern Biotechnology Associates) was added at a dilution of 1:5,000 and allowed to incubate at room temperature for another 2 h. The membrane was washed for 5 min in PBS with 6 buffer changes and then exposed to enhanced chemiluminescent Western blot detection reagent (Amersham Biosciences, Piscataway, N.J.) for 1 h. Data were recorded with Kodak BioMax MR scientific imaging film. The developed film was scanned with Adobe Photoshop 7.0 by using a UMax PowerLook 1120 overhead flatbed scanner at 1,200 dpi and converted into JPEG format.

Statistical analyses. The ELISA titers of the tertiary anti-Inaba IgM and anti-Inaba IgG1 were compared for significant differences by using an established parameter that requires a fourfold or greater difference between endpoint titers of pooled individual sera for significance (42). The Prism GraphPad program was used to evaluate the statistical significance of the cross-reactivity analysis and the infant mouse protection assay data. The analysis of significance for the cross-reactivity curves was based on assessing the null hypothesis that the slopes of the lines (anti-Inaba versus anti-Ogawa) are not different. A P value of less than 0.050 indicates that the slopes are significantly different. If the slopes are statistically the same, a second test (with a P value of >0.050 indicating significance) determines whether the lines are identical or parallel. The analysis of significance for the protection data was based on the log rank test, which is equivalent to the Mantel-Haneszel test. The null hypothesis that was tested was that the survival curves are identical in the overall population; i.e., treatment did not change survival.

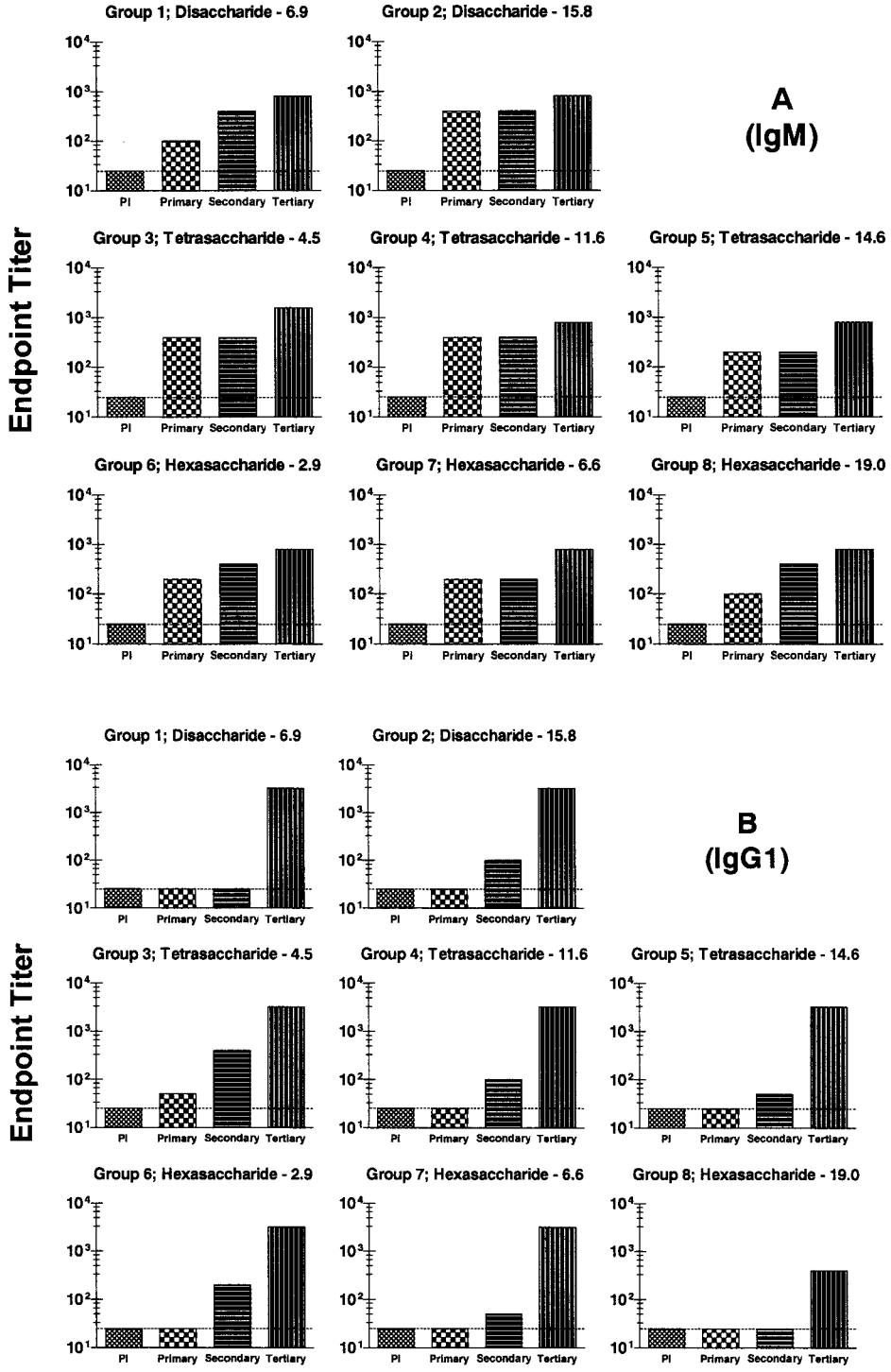


FIG. 3. *V. cholerae* Inaba LPS-specific IgM (A) and IgG1 (B) responses following immunization with synthetic Inaba CHO-BSA neoglycoconjugates. For a description of Inaba constructs, see Materials and Methods. Horizontal dashed lines indicate the starting dilution (1:25) of antiserum used in the ELISA. Serum samples were collected as described in the legend to Fig. 2.

RESULTS

Inaba CHO-BSA conjugates. It was recently reported that conjugates of the hexasaccharide fragment of the O-SP of *V. cholerae* (Ogawa) and BSA were both immunogenic and able to induce protective antibody responses in mice (7). To determine if a related structural epitope on the Inaba O-SP was a target for B-cell responses, we synthesized Inaba CHO-BSA conjugates that varied in the length (di-, tetra-, and hexasaccharide) of the O-SP fragment (Fig. 1B). Note that the linker (spacer) described here, which provides a connection between

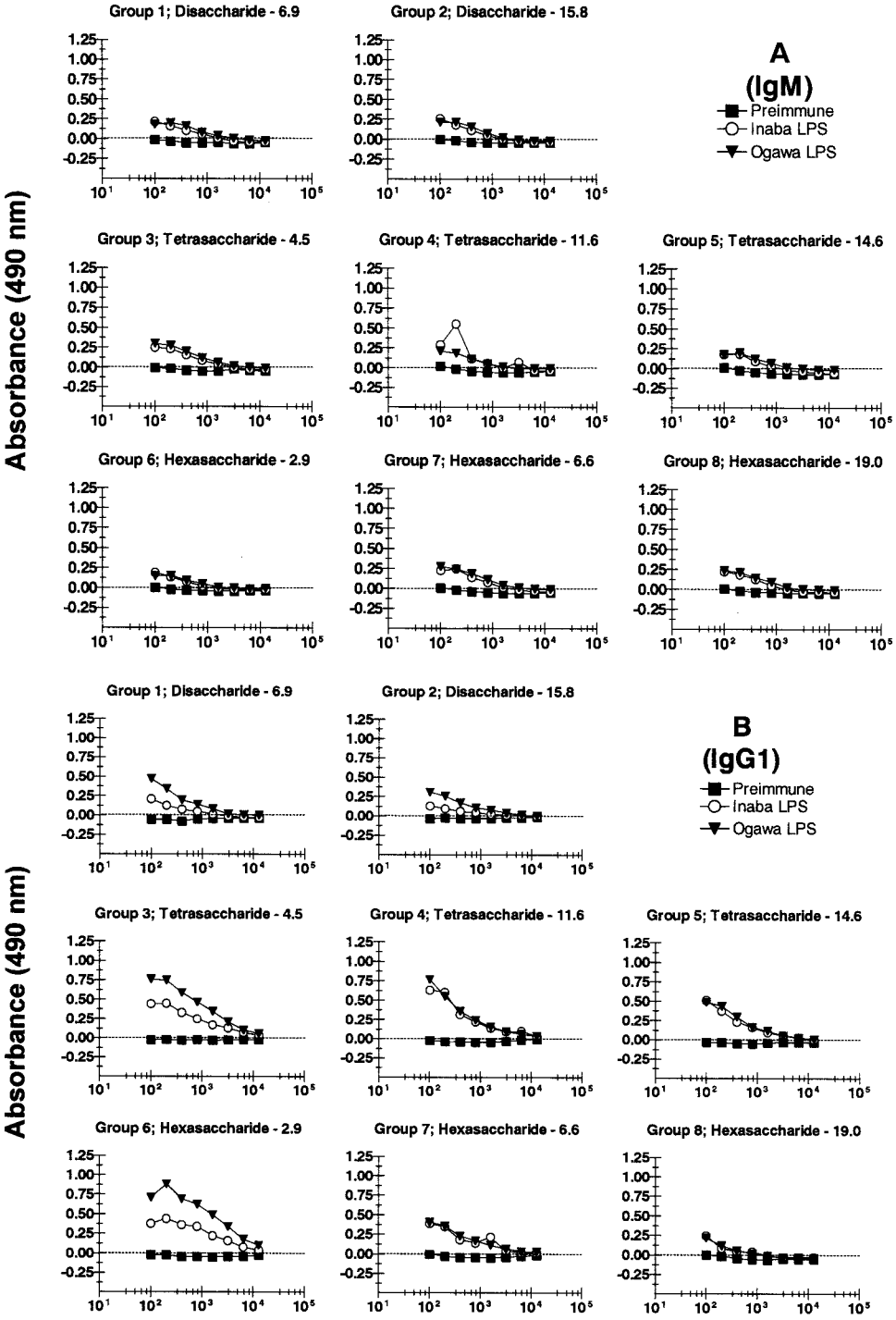


FIG. 4. Cross-reactivity of tertiary Inaba CHO-BSA antisera to *V. cholerae* Ogawa and Inaba LPS reported as the OD_{490}. The preimmune serum control sample is represented by the closed squares. Pooled tertiary sera reacted against Inaba LPS (open circles) and against Ogawa LPS (closed inverted triangles). Initial serum dilutions were 1:100 for the ELISA for IgM (A) and IgG1 (B). A comparison of the slopes of the lines for the anti-Inaba and anti-Ogawa responses indicated that within any particular group, the IgM ELISA and the IgG1 ELISA results did not differ. Only the lines for the IgG1 ELISA describing groups 2 ($P = 0.043$), 3 ($P = 0.032$), and 6 ($P = 0.001$) were not identical but parallel; for all other comparisons, the lines were identical.

the antigen and the carrier protein in the Inaba CHO-BSA conjugates, differs from the one used previously in the similar Ogawa hexasaccharide-BSA constructs (7). With the Ogawa hexasaccharide (7), the amino group required by the squaric acid chemistry of conjugation was generated in the spacer-equipped hexasaccharide by hydrazinolysis of the methyl ester with hydrazine. In the present study, the amino group was introduced by aminolysis of the methyl ester with ethylenedi-

TABLE 1. Antivibriocidal activity of pooled sera of mice immunized with Inaba CHO-BSA[a]

Inaba CHO-BSA conjugate groups (mol of CHO per mol of BSA)	Vibriocidal titers of pooled sera determined by[f]:							
	Spread plate method for strain:						Microtiter method for strain N16961 (Inaba)[e]	
	569B (Inaba)[b]		O395 (Ogawa)[c]		N16961 (Inaba)[d]			
	Preimmune sera	Tertiary sera	Preimmune sera	Tertiary sera	Preimmune sera	Tertiary sera	Preimmune sera	Tertiary sera
Group 1, disaccharide (6.9)	<100	<100	<100	<100	<20	<20	<50	<50
Group 2, disaccharide (15.8)	<100	<100	<100	<100	<20	<20	<50	<50
Group 3, tetrasaccharide (4.5)	ND	ND	ND	ND	<20	<20	<50	<50
Group 4, tetrasaccharide (11.6)	ND	ND	ND	ND	<20	<20	<50	<50
Group 5, tetrasaccharide (14.6)	<100	<100	<100	1,000	<20	<20	<50	<50
Group 6, hexasaccharide (2.9)	<100	<100	<100	100	<20	<20	<50	<50
Group 7, hexasaccharide (6.6)	ND	ND	ND	ND	<20	<20	<50	<50
Group 8, hexasaccharide (19.0)	<100	<100	<100	<100	<20	<20	<50	<50
Anti-Inaba LPS control	<100	≥50,000	<100	≥50,000	ND	>8,000	ND	≥100,000
Anti-Ogawa LPS control	<100	<100	<100	≥100,000	ND	ND	ND	ND

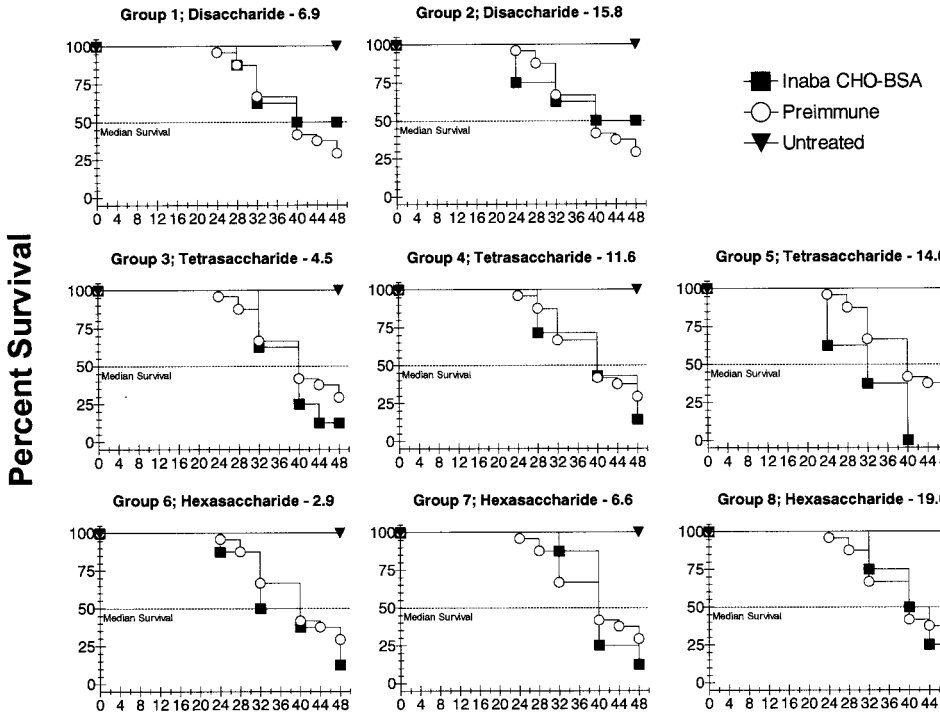
[a] Synthetic Inaba LPS epitope.
[b] Input, 7.15×10^3 CFU/sample.
[c] Input, 8.9×10^3 CFU/sample.
[d] Input, 4.5×10^3 CFU/sample.
[e] Input, 9.3×10^5 CFU/sample.
[f] ND, not determined.

amine (Fig. 1B). Consequently, the linker used for the Inaba conjugates is longer than the linker used in Ogawa conjugates by two methylene groups (7). Preliminary data (data not shown) and the data presented here indicate that the change in linker length does not substantially affect the immunogenicity of the CHO-BSA conjugates.

Serologic response to Inaba CHO-BSA conjugates. Eight groups of BALB/c mice were immunized three times i.p. with 10 μg (based on the CHO weight) of the various Inaba BSA-CHO constructs (Fig. 2A). Serum was collected over a 35-day period as shown in the immunization and serum collection schema (Fig. 2B). Purified Inaba LPS was used to assess pooled serum from individual mice of the various groups for Inaba-specific IgM and IgG1 antibodies by ELISA. The IgM anti-Inaba response can be detected in the primary sera, which were collected 10 days after the primary immunization (Fig. 2B and 3A). Most groups of mice showed increased levels of IgM-specific Inaba antibody in serum over the next 25 days, with the exception of groups 2 and 8, whose levels of antibody in serum did not increase after the first immunization. In contrast, while the IgG1 response was universal at day 35 after initiation of the immunization schedule, only select groups of mice had IgG1-specific Inaba antibody at day 17, a point at which mice were immunized twice with the Inaba CHO-BSA conjugate (Fig. 3B). Groups 2, 3, 4, and 6, which were immunized with disaccharide (15.8 mol of CHO/mol of BSA [group 2]), tetrasaccharide (4.5 mol of CHO/mol of BSA [group 3] and 11.6 CHO/mol of BSA [group 4]), and hexasaccharide (2.9 mol CHO/mol of BSA [group 6]) conjugates, had different kinetics of accumulation of IgG1 specific for Inaba O-SP in serum. There was, however, no correlation with length of saccharides or level of substitution for these earlier responses. With the exception of group 8, the tertiary ELISA titers for either Inaba-specific IgM or Inaba-specific IgG1 in serum were not considered different among the groups, as the endpoint titers did not differ by fourfold or more (42). The responses to Inaba CHO-BSA conjugates in serum were similar in magnitude for some of the groups immunized with Ogawa CHO-BSA conjugates (7).

Cross-reactivity of Inaba CHO-BSA conjugate antisera with Ogawa LPS. We assessed the cross-reactivity of pooled tertiary sera from the groups immunized with the various Inaba CHO-BSA conjugates against Ogawa LPS. In general, the anti-Inaba CHO-BSA sera reacted equivalently with Ogawa and Inaba LPS in an ELISA (Fig. 4) that assessed either IgM or IgG1 antibodies. Sera induced by the Inaba CHO-BSA disaccharides (groups 1 and 2), as well as those induced by the low-level substitution tetrasaccharide (group 3) and hexasaccharide (group 6) conjugates were more reactive to the Ogawa LPS epitopes. A comparison of the slopes of the lines for the anti-Inaba and anti-Ogawa responses was performed. The slopes of the lines within any particular group for the IgM ELISA were the same, as were those for the IgG1 ELISA. Further analysis to determine if the lines were parallel or identical revealed that only the curves for the IgG1 ELISA for groups 2 ($P = 0.043$), 3 ($P = 0.032$), and 6 ($P = 0.001$) were not identical yet were parallel; the lines for all other groups were identical. The results for group 1, mice immunized with the disaccharide (6.9 mol of CHO/mol of BSA), had a P value of 0.071 and thus were not considered significant. With the exception of group 4, mice immunized with the Inaba CHO-BSA tetrasaccharide (11.6 mol of CHO/mol of BSA), these groups (2, 3, and 6) were also the groups that had faster accumulations of anti-Inaba IgG1.

Vibriocidal activity of antisera specific for Inaba. A well-accepted assay for assessing the functional significance of *V. cholerae* anti-LPS antibodies is the in vitro vibriocidal-antibody assay that measures complement-mediated killing. Using an agar plate-based complement fixation assay, we tested pooled sera from mice immunized with the various Inaba CHO-BSA conjugates to determine if immunization induced vibriocidal antibody (Table 1). Control antisera generated to either Inaba or Ogawa whole-LPS induced vibriocidal antibody of high titer (≥50,000). This result was in sharp contrast to the results obtained with sera from tertiary bleeds of mice immunized

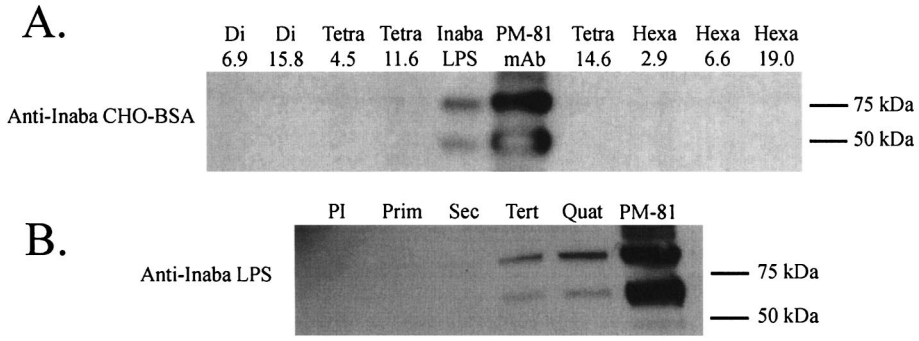


% Survival		Inaba CHO-BSA, *V. cholerae* O1 El Tor Inaba (Strain N16961)									
		6.9 Di	15.8 Di	4.5 Tetra	11.6 Tetra	14.6 Tetra	2.9 Hexa	6.6 Hexa	19.0 Hexa	Preimmune	Untreated
50%	**6.9 Di**										
50%	**15.8 Di**	0.8886									
12.50%	**4.5 Tetra**	0.2922	0.3417								
14.30%	**11.6 Tetra**	0.3276	0.4225	0.6460							
0%	**14.6 Tetra**	**0.0433**	0.0654	0.0863	0.0588						
12.50%	**2.9 Hexa**	0.2287	0.2846	0.9070	0.7988	0.1252					
12.50%	**6.6 Hexa**	0.4399	0.4619	0.5536	0.9796	**0.0204**	0.6520				
12.50%	**19.0 Hexa**	0.3547	0.3890	0.5541	0.9078	**0.0173**	0.8438	0.9656			
29.20%	**Preimmune**	0.4551	0.5494	0.4465	0.6744	**0.0133**	0.4407	0.7755	0.7045		
100%	**Untreated**	**0.0005**	**0.0005**	**P<0.0001**	**P<0.0001**	**P<0.0001**	**P<0.0001**	**P<0.0001**	**P<0.0001**	**P<0.0001**	

FIG. 5. (Top) Percent survival of neonatal mice following oral challenge with live *V. cholerae*. Four- to five-day-old CD-1 neonatal mice were orally administered by gavage 25×10^6 CFU of virulent (25 LD_{50}) *V. cholerae* O1 El Tor Inaba strain N16961, which was cultured overnight in LB medium at 30°C for 16 h and then mixed 1:1 with tertiary antisera or preimmune antisera. The untreated group did not receive challenge. Tertiary and preimmune antisera were diluted into normal mouse sera for a final dilution of 1:5. Eight mice were used per treatment group. The data from the preimmune group are a collective result from three randomly chosen preimmune sera which were individually evaluated. Mice were kept at 30°C and monitored every 4 h starting 24 h after oral challenge until termination of the experiment at 48 h. Groups 1 and 2 showed a potential for protection, and the tests for these groups were repeated in the analysis with 44 LD_{50} of bacteria and found not to be protective (data not shown). (Bottom) The tabulated values show the results of a log rank comparison test for significance between the survival curves. Multiple comparisons were made. The top row of the table defines the vaccine modality used to generate serum for a particular Inaba CHO-BSA conjugate. The columns under the individual vaccine modality headings show the *P* values for the various comparisons to the groups listed in the first column. The second to the last and the last row show the *P* value generated from the comparison of the survival curve for mice treated with tertiary sera of a particular vaccine modality to the survival curves of either mice treated with preimmune sera or untreated mice, respectively. The *P* values shown in boldface type are less than 0.050, which is considered significant.

with Inaba CHO-BSA immunogens. The latter sera were uniformly negative in two different assessments of the plate vibriocidal-antibody assay. In general, for the vibriocidal-antibody assay, there was no cross-reaction of anti-Inaba sera with Ogawa LPS, with the exception of two groups that had low titer responses (2.9 mol of hexasaccharide, 1:100; 14.6 mol of tetrasaccharide, 1:1,000). This was in contrast to the anti-Inaba whole-LPS sera, which bound purified LPS from either serotype of *V. cholerae* O1.

Because the plate vibriocidal-antibody assay uses large volumes of reagents and requires a large input of bacteria for the enumeration of colonies, it is difficult to test lower dilutions of antiserum or to vary the number of target bacteria. To confirm the results, we used a microtiter test (5) developed by Fournier's group (Pasteur Institute). This test measures the metabolic activity of the bacteria following treatment with antisera and complement. As with the plate method, the vibriocidal activity of the anti-Inaba CHO-BSA antiserum was negative (Table 1). Similar to its effectiveness in the plate vibriocidal-antibody assay, positive-control anti-Inaba LPS serum made to whole Inaba LPS was effective in inhibiting bacterial growth and thus metabolic activity as measured by the microtiter assay.



C.

	Inaba CHO-BSA Tertiary Antisera							
	Di 6.9	Di 15.8	Tetra 4.5	Tetra 11.6	Tetra 14.6	Hexa 2.9	Hexa 6.6	Hexa 19.0
ELISA Titer (IgM)	800	800	1,600	800	800	800	800	800
Vibriocidal Titer	< 25	< 25	< 25	< 25	< 25	< 25	< 25	< 25

D.

	Inaba Whole LPS Antisera				
	Preimmune	Primary	Secondary	Tertiary	Quaternary
ELISA Titer (IgM)	< 100	1,600	1,600	6,400	6,400
Vibriocidal Titer	< 40	80	1,280	2,560	5,120

FIG. 6. Adsorption of anti-O-SP Inaba-specific and anti-Inaba whole-LPS antibodies with *V. cholerae* O1 El Tor Inaba bacteria. Bacteria (10^9 CFU) were incubated with 1:10 dilutions of antisera overnight, washed to remove unbound antibody, and then run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer followed by transfer to nitrocellulose membranes. The nitrocellulose membranes were probed with horseradish peroxidase-labeled goat anti-mouse IgM. (A) Adsorbed IgM from anti-whole-LPS Inaba quaternary antiserum and the positive-control mouse IgM samples are in the two center lanes, flanked on either side by anti-Inaba CHO-BSA tertiary antiserum. (B) Adsorbed Ig assessed by using anti-whole-LPS Inaba preimmune, primary, secondary, tertiary, and quaternary antiserum samples is shown with the positive IgM control. Corresponding IgM ELISA and vibriocidal titers (previously described in the legend to Fig. 3 and in Table 1, respectively) are shown.

Anticolonization capacity of antisera specific for Inaba. Another means of assessing the functional activity of antisera directed against *V. cholerae* surface antigens is the infant mouse protection assay. Similar to the results with the vibriocidal-antibody assays, the antisera from the various groups did not provide protection to infant mice (Fig. 5). A log rank analysis of the survival curves did not reveal any significant difference (P of <0.050 is significant) in survival of mice treated with either preimmune sera or tertiary sera of mice immunized with the various Inaba CHO-BSA conjugates. There was a hint of protection in groups 1 and 2, namely, mice immunized with the disaccharide Inaba CHO-BSA, but repeated analysis of those tertiary sera compared to the corresponding prebleed sera did not support this contention (data not shown).

In situ LPS binding. The lack of vibriocidal or protective capacity of the anti-Inaba sera raised against the Inaba CHO-BSA conjugates is interesting given the relatively high titers of anti-Inaba LPS IgM and IgG1 present in the tertiary sera. In addition, similar and even lower IgM-IgG ELISA titers induced in response to Ogawa CHO-BSA conjugates were protective for mice (7). Sera from mice immunized i.p. with whole, purified Inaba LPS have ELISA (secondary [IgM]) titers similar to those measured by using pooled sera from mice immunized with the Inaba CHO-BSA conjugates (data not shown). This result suggests similar concentrations and/or affinities of Inaba-specific antibodies in the antisera regardless of the immunogen used. Perhaps the LPS concentration used in the ELISA is not reflective of the LPS environment (density and spatial distribution) of in situ LPS on *V. cholerae* bacteria.

We tested whether the inability of the sera elicited by the Inaba CHO-BSA conjugates to protect resulted from the lack of binding LPS due to in situ considerations or from binding LPS being in a position that was not protective. We used anti-whole-Inaba LPS and anti-Inaba CHO-BSA sera at dilutions similar to those used for the protection assay, with amounts of bacteria that were the same as that of the challenge dose for the infant mouse protection assay. After an overnight incubation with sera, bacterial pellets were washed extensively, and the binding capacities of the two sera were compared by assessing the bacterium-bound IgM in a Western blot (Fig. 6). The tertiary sera from the various groups of mice immunized with the Inaba CHO-BSA conjugates did not bind to the bacteria at detectable levels. The quaternary and tertiary anti-whole-LPS sera had a titer that was higher ($\log_{10}$) than that of the secondary sera. Similarly, the quaternary and tertiary sera derived from mice immunized with Inaba LPS efficiently associated with LPS in situ. The secondary anti-Inaba whole-LPS sera had an ELISA titer similar to that of the sera of mice immunized with the Inaba CHO-BSA; it also did not effectively

associate with LPS in situ. However, in contrast to the anti-Inaba CHO-BSA sera, the secondary, anti-whole-Inaba LPS sera were vibriocidal. These results suggest that the antibodies induced by the Inaba CHO-BSA conjugates do not bind with enough affinity or specificity to native LPS when expressed on the bacterial surface. Alternatively, the anti-whole-Inaba LPS sera may be specific for more epitopes other than the Inaba terminal sugar which enhances the association with LPS in situ.

DISCUSSION

The search for a universally effective cholera vaccine is ongoing. Subunit vaccines have the potential to combine multiple defined targets for optimal immunogenicity. A consensus component for any cholera vaccine is LPS or its derivatives. Anti-*V. cholerae* LPS antibodies correlate with protection against virulent *V. cholerae* challenge of vaccinated volunteers and those infected naturally (19, 27, 32, 35). It is important to develop a *V. cholerae* LPS-based vaccine that can induce neutralizing antibodies to both Inaba and Ogawa serotypes, as both serotypes can initiate infection. Individuals need to be immune to both serotypes for optimal protection, as immune pressure can drive serotype conversion (9, 11). Currently, there are two well-defined antibody targets on *V. cholerae* LPS associated with the Inaba and Ogawa serotypes. Several protective MAbs that bind the terminal sugar of Ogawa (S-20-4), and are thus Ogawa specific (39, 40), have been described, while an epitope found on both Inaba and Ogawa LPS recognized by MAb I-24-2 is defined by the core and O-SP regions of *V. cholerae* LPS, suggesting an epitope found at the boundary of the core and O-SP (39). Inaba-specific MAbs, while reported, are not presently in general use for experimental manipulation (12, 14-16, 29).

The two O1 serotypes of *V. cholerae* associated with endemic and epidemic cholera, Ogawa and Inaba, are defined by the composition of the terminal sugar of their respective O-SP. The Inaba O-SP differs from that of Ogawa in that the terminal perosamine moiety has a hydroxyl group, rather than a methoxyl group, at the 2 position. The methoxyl group has been shown to be critical for protective anti-Ogawa MAb binding (40, 41). To test whether the terminal sugar of Inaba LPS was also a protective B-cell epitope, we constructed Inaba CHO-BSA conjugates similar to the Ogawa hexasaccharide conjugates described previously (7). The solution binding studies with Ogawa and Inaba oligosaccharides by Wang and colleagues (41) did not reveal observable binding of the Inaba oligosaccharides by anti-Ogawa-specific MAbs, suggesting substantially different epitopes based on Inaba and Ogawa structural differences. Thus, anti-O1 serotype-specific sera would be predicted to bind unique structural elements which should provide protection unless the Inaba LPS is differently distributed or expressed in situ compared to Ogawa LPS.

The studies we report herein tested the hypothesis that Inaba CHO-BSA conjugates would induce protective immunity. Analogous to the results reported for the Ogawa CHO-BSA conjugates (7), the Inaba conjugates induced antibody responses in serum that were of similar magnitude or higher than those of some of the anti-Ogawa CHO-BSA conjugates but, in contrast, failed to induce antivibriocidal antibodies or antibodies that were protective in the infant mouse assay. A new finding for synthetic *V. cholerae* LPS antigens is that anti-Inaba CHO-BSA responses were not modulated by the length of the saccharide attached to the carrier. This finding is consistent with the data for synthetic *Streptococcus pneumoniae* conjugates, whereby the length of the saccharides did not affect the magnitude of the humoral immune responses (3), but is different from results of immunization studies involving synthetic *Shigella dysenteriae* oligosaccharides, which showed that the difference in the length of the antigen, as well as antigen-carrier ratio, affected the magnitude of the murine immune response (34).

The only structural difference between the Ogawa and Inaba serotypes places the serotype-defining epitope at the terminal sugar of O-SP (17, 18, 40, 41). This difference, and the existence of MAbs specific for either Inaba or Ogawa LPS (1, 12, 14–16, 29), supports the notion that unique, serologically defined epitopes exist for the Inaba and Ogawa serotypes. The Inaba MAbs were developed for serologic typing; we are unaware of any protective efficacy that might have been reported (12, 14–16, 29). The anti-Inaba MAbs were screened by ELISA with purified LPS used as the test antigen. Some anti-Inaba MAbs were found to bind LPS in situ but were not tested for protective efficacy. Multiple, anti-Ogawa MAbs have been made and, importantly, some have been reported to be protective (7, 12, 14–16, 29, 39). One MAb, S-20-4, was cocrystallized with the terminal Ogawa monosaccharide or disaccharide (40). Recently, the analysis of the cross-binding of S-20-4 to the terminal monosaccharide of the Inaba O-SP was reevaluated (25). The difference of a methoxyl group versus a hydroxyl group at position 2 on the terminal perosamine moiety decreased the affinity of S-20-4 binding to Inaba synthetic terminal monosaccharides 840-fold compared to that of the Ogawa terminal O-SP residue. Liao and colleagues (25) postulated that for the Inaba terminal monosaccharide, the loss of the *O*-2 methyl group and its electron-donating effect might alter the negativity of the 3-OH group. Previously, the 3-OH group had been shown to play an important role in hydrogen bonding in the crystal structure of the S-20-4/Ogawa saccharide complex (40, 41).

Perhaps similar to anti-Ogawa MAbs (7), the remodeling of the antigen binding pocket of anti-Inaba might be critical for optimal binding to the Inaba terminal sugar, especially for in situ LPS. Somatic mutations in the anti-Inaba Fab, in VDJ/VJ sequences (antigen binding domains of the IgG heavy and light chains, respectively), or within the Ig fold framework region sequences that alter the shape of the binding pocket are driven by multiple immunizations with reagents that drive B-cell proliferation, such as LPS. The Ig variable segment (V heavy/light) family member(s) that is initially selected for binding Inaba terminal sugars might be different than those that bind Ogawa, as the Inaba antibody Fabs need to accommodate the smaller and electrochemically different Inaba terminal sugar. Anti-idiotype sera (a measure of V-segment uniqueness) specific for the anti-Inaba MAb idiotype did not bind anti-Ogawa (epitope B) MAb, suggesting that the MAbs specific for Inaba and Ogawa do use different variable Ig gene segments to obtain serotype specificity (12). We speculate that because of the limitations imposed by the molecular signature of the Inaba epitope, the anti-Inaba antibodies induced by the Inaba CHO-BSA conjugates are of lower affinity than are anti-Ogawa an-

tibodies induced by Ogawa CHO-BSA conjugates or antibodies induced by native Inaba LPS. This is consistent with the studies of Ghosh and Campbell, who reported that three different Ogawa MAbs could effectively compete for antigen with three different anti-Inaba MAbs but that anti-Inaba MAbs could not compete with anti-Ogawa MAbs (12). Multiple Inaba-specific MAbs (C6, A18, 11A, 14B1, and 14C3) were readily measured by ELISA (12, 14–16, 29), which used solid-phase binding to purified LPS, a physico-chemical state of the antigen which enhances binding because of the enhanced potential (monogamous bivalency) for one of the Fabs of IgG to be bound at any time (4). However, similar to our results, A18 MAb was not reactive when tested against LPS in situ by using a whole-cell ELISA (1). The anti-Inaba antibodies raised against the Inaba CHO-BSA conjugates did not functionally bind LPS in situ, as evidenced by the vibriocidal-antibody assay, Western blot analysis, and the infant mouse protection assay.

A potential complicating issue for protective antibodies is that of the IgG subclass, as subclass-defining structures or sequences can affect anti-carbohydrate binding (8, 30). The early studies to generate anti-Inaba MAb used native LPS as the immunogens, and thus, IgM and the IgG3 subclasses were dominant among the MAbs (12, 14–16, 29). We found low levels of anti-Inaba O-SP IgG3 (W. F. Wade, unpublished data) but high levels of IgG1 in response to Inaba CHO-BSA conjugates.

The data presented herein raise the question as to whether protective, anti-Inaba O-SP-specific antibodies can be easily generated. Our results are seemingly in conflict with some epidemiological evidence that supports the perspective that Inaba LPS is a better immunogen than Ogawa LPS (reference 26 and references therein). The vibriocidal-antibody data we present suggest that the Inaba LPS is a better immunogen than Ogawa LPS, as antisera to Inaba LPS cross-reacts more readily with Ogawa LPS in the vibriocidal-antibody assays (Table 1). Caution must be employed, however, when the results of the vibriocidal-antibody assays we report here are compared to the epidemiologic data and inferences that Inaba is a superior immunogen. The systems that lead to the different data sets are quite diverse with respect to the complexity (whole cell versus purified LPS) of immunogens used and the potential for preexisting immunity to a particular serotype or other undefined antigens (reference 26 and references therein). The Inaba conjugates we investigated do not have the same number of epitopes as that of native *V. cholerae* LPS; thus, it is inappropriate to compare them for efficacy. The common core O-SP epitope is missing from the Inaba CHO-BSA conjugate; this region has been previously shown to be a protective epitope (39). The lack of this epitope may be the reason that the anti-Inaba conjugate serum is less effective than anti-Inaba whole-LPS serum. An alternate explanation we favor, as discussed above, is that native LPS has an advantage that can more efficiently select and drive the proliferation of B cells that express an antibody with a specificity that is protective or can be driven to somatically mutate to a protective antibody.

Others have reported that anti-Inaba polyclonal antibodies induced to purified, detoxified LPS conjugated to cholera toxin are vibriocidal (13). The ELISA titers of these IgG or IgM antibodies from BALB/c mice were substantially lower than those we report in response to immunization with Inaba CHO-BSA conjugates. However, the sera of whole detoxified LPS conjugates were vibriocidal against both Inaba and Ogawa LPS-expressing bacteria, suggesting that a shared epitope was the target for in vitro killing. The B-cell epitope(s) for the antisera described by Gupta and coworkers (13) was not defined, but reactivity was adsorbed by LPS, detoxified LPS, or O-SP.

The failure of the Inaba CHO-BSA conjugates to induce protective antibodies could be due to the fact that the terminal sugars of Inaba LPS are not protective epitopes or that the means by which the Inaba CHO-BSA was delivered have not been optimized. Future studies are needed to conclusively determine if fragments of the Inaba O-SP can be used to induce protective antibody, regardless of the vaccine format in which they are presented. A panel of anti-Inaba-specific MAbs should be generated so that the affinity and concentrations can be carefully controlled and compared to the protective capacity of an anti-Ogawa-specific MAb panel. If the majority of the Inaba MAbs should be found to have, on average, lower affinity for their targets and do not afford in vivo protection, then the hope for the terminal fragments of the Inaba O-SP as a vaccine antigen would be diminished. However, if a proportion of the MAbs that are anti-Inaba terminal sugar specific or even cross-reactive with the Ogawa epitope but that are protective can be found, it will motivate the search for immunization schemas or alterations in the Inaba CHO structure and/or conjugate architecture to maximally induce the B cells that express the protective antibody (25).

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