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 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 (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-
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) were not affected 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-glycerol-tetraetonic 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, hapten 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 24-o-pyridine-3,4-dihydroxy-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), hapten 10 through 12 were subjected to aminolysis to afford ethynylated-amine derivatives 13 through 15, which were treated with squaric acid diethyl ester. The squaric acid monooesters 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 could provide 97% of that volume of bacterial suspension. 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-buffeter buffer (pH 9.6) coated overnight at 4°C. Plates were washed three times with a using a Molecular Devices (Sunnyvale, Calif.) Skan plate wash 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% 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 sample (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-mouse IgG1 (δ-specific) (Southern Biotechnology Associates, Birmingham, Ala.) detector antibodies (diluted 1:6000) 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 diluted by preparing 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% H2O2 was added to the OPD substrate solution immediately before use to final a concentration of 0.02%. The reaction was stopped with an equal volume of 3 M HCl. Optical densities (OD) were read at 490 nm (OD490) 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 assay. 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). 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 1:10 10 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 1:10, 1:100, 1:1000, 1:10,000, 1:50,000, and 1:1000 and kept in an ice-water bath until needed. Bacteria (3.2 × 10^4 CFU/ml) were mixed with diluted serum (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 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 Fourrier’s group (Pasteur Institute, Paris, France). V. cholerae O1 Ogawa and El Tor Inaba strains N16961 was inoculated in 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 OD560 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 (30%), 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 were placed in a
FIG. 1. Schema for generation of Inaba neoglycoconjugate immunogens and Inaba CHO-BSA.
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_{560} 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 antibodies in situ. Cultures of V. cholerae 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 LB broth, pH 6.5, at 30°C. Twenty-five microliters of bacterial suspension, representing 25 to 44 50% lethal doses (LD_{50}) were used to test the viability of the bacteria. Cultures of V. cholerae 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}) were used to test...
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

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**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.
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*  

<table>
<thead>
<tr>
<th>Inaba CHO-BSA conjugate groups (mol of CHO per mol of BSA)</th>
<th>Spread plate method for strain:</th>
<th>Microtiter method for strain N16961 (Inaba)*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Preimmune sera</td>
<td>Tertiary sera</td>
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<td></td>
<td>Preimmune sera</td>
<td>Tertiary sera</td>
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<tr>
<td>Group 1, disaccharide (6.9)</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Group 2, disaccharide (15.8)</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Group 3, tetrasaccharide (4.5)</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Group 4, tetrasaccharide (11.6)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Group 5, tetrasaccharide (14.6)</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Group 6, hexasaccharide (2.9)</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Group 7, hexasaccharide (6.6)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Group 8, hexasaccharide (19.0)</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Anti-Inaba LPS control</td>
<td>&lt;100</td>
<td>≥50,000</td>
</tr>
<tr>
<td>Anti-Ogawa LPS control</td>
<td>&lt;100</td>
<td>&lt;100</td>
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*Synthetic Inaba LPS epitope.  
  b Input, 7.15 × 10^3 CFU/sample.  
  e Input, 8.9 × 10^3 CFU/sample.  
  d Input, 4.5 × 10^3 CFU/sample.  
  f Input, 9.3 × 10^3 CFU/sample.  
  g ND, not determined.  

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). 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 bleed of mice immunized
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.
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 $\leq 0.050$ 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-LPS Inaba preimmune, primary, secondary, tertiary, and quaternary antisera 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.

![Image](https://via.placeholder.com/150)

**FIG. 6.** Adsorption of anti-O-SP Inaba-specific and anti-Inaba whole-LPS antibodies with V. cholerae O1 El Tor Inaba bacteria. Bacteria (10⁹ 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 antisera 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.
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 1-24-2 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 differentially 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 antibibriocidal 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 \(S.\) 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 \(S.\) 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 cocystal-
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 detected 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 Fab's 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 pre-existing 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 vibrioidal (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 vibrioidal 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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