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 vaccination. Antibodies specific for *V. cholerae* LPS are correlated with protection against cholera. 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-\(^\text{racta},\) 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-\(^\text{racta},\) 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-\(3\text{-deoxy-1\text{-glycerol-tetra} \text{tionic} \text{acid}}\) 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 2,4-\(\text{o} \text{a} \text{zy} \text{l} \text{e} \text{n} \text{e} \text{d} \text{n} \text{e} \text{t} \text{i} \text{a} \text{c} \text{y} \text{d} \text{e} \text{y} \text{n} \text{o} \text{g} \text{l} \text{b} \text{u} \text{d} \text{i} \text{d} \text{i} \text{i} \text{n} \text{e} \text{s} \text{e} \text{r} \text{a} \text{c} \text{h} \text{e} \text{n} \text{i} \text{c} \text{h} \text{e} \text{e} \text{r} \text{a} \text{h} \text{e} \text{n} \text{t} \text{a} \text{s} \text{i} \text{c} \text{h} \text{e} \text{r} \text{a} \text{n} \

### 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 sinuses 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 l of blood, which can provide 50% of that volume after processing.

### 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 l of an Inaba solution (5 l of Inaba LPS) in 0.1 M carbonate-bicarbonate buffer (pH 9.9) at 37°C overnight at 4°C. Plates were washed three times with a Molecular Devices (Sunnyvale, Calif.) Scan plate washer with 250 l of phosphate-buffered saline (PBS) per well. Nonspecific binding was blocked by using 200 l of blocking buffer consisting of PBS--0.05% Tween 20 (Fisher Scientific, Pittsburgh, Pa.) per well. Specific binding was determined by using 200 l of each 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 l of horseradish peroxidase-labeled goat anti-mouse IgM (\(\mu\)-chain-specific) or anti-mouse IgG1 (\(\gamma\)-chain-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 l 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\(\text{nm}\)) 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 1:10 in PBS. Pooled preimmune and tertiary sera from each treatment group were diluted in 50 l of ice-cold PBS containing 20% guinea pig complement (Sigma) with the dilutions of 1:10, 1:10\(^{2}\), 1:10\(^{3}\), and 1:10\(^{4}\) and kept in an ice-water bath until needed. Bacteria (3\(\times\)10\(^{5}\) CFU) were mixed with diluted antiseraum (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-l 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 Fourrier’s group (Pasteur Institute, Paris, France). \(V.\) cholerae O1 El Tor Inaba strain N16961 was inoculated in broth medium at a concentration of 5\(\times\)10\(^{5}\) colony-forming units (CFU) per ml. After 24 h at 37°C, 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\(\text{nm}\) of the bacterial suspension was adjusted to 0.80 with PBS to approximate the bacteria to 10\(^{4}\) CFU/ml. Seven volumes of cold PBS, 2 volumes of guinea pig complement, and 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.
representing 25 to 44 50% lethal doses (LD$_{50}$) (44 LD$_{50}$ were used to test the in vivo (33, 37). Cultures of V. cholerae strain N16961 were grown for 16 h in LB broth, pH 6.5, at 30°C. One hundred fifty microfilters 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 microfilters 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 microfilters 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 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 microfilters 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 microfilters 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 Bio-technology 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.

### Table 1

<table>
<thead>
<tr>
<th>Construct</th>
<th>O-SP Substitution Ratio</th>
<th>O-SP length</th>
<th>Conjugate MW</th>
<th>μg CHO/mg conjugate</th>
<th>μg BSA per 10 μg CHO</th>
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<tbody>
<tr>
<td>Group 1</td>
<td>6.9</td>
<td>Di</td>
<td>71,589</td>
<td>49.0</td>
<td>194.1</td>
</tr>
<tr>
<td>Group 2</td>
<td>15.8</td>
<td>Di</td>
<td>78,283</td>
<td>103.0</td>
<td>87.1</td>
</tr>
<tr>
<td>Group 3</td>
<td>4.5</td>
<td>Tetra</td>
<td>72,028</td>
<td>62.0</td>
<td>151.3</td>
</tr>
<tr>
<td>Group 4</td>
<td>11.6</td>
<td>Tetra</td>
<td>80,854</td>
<td>144.0</td>
<td>59.4</td>
</tr>
<tr>
<td>Group 5</td>
<td>14.6</td>
<td>Tetra</td>
<td>84,588</td>
<td>173.0</td>
<td>47.8</td>
</tr>
<tr>
<td>Group 6</td>
<td>2.9</td>
<td>Hexa</td>
<td>71,342</td>
<td>61.0</td>
<td>153.9</td>
</tr>
<tr>
<td>Group 7</td>
<td>6.6</td>
<td>Hexa</td>
<td>78,007</td>
<td>126.0</td>
<td>69.4</td>
</tr>
<tr>
<td>Group 8</td>
<td>19.0</td>
<td>Hexa</td>
<td>99,737</td>
<td>285.0</td>
<td>25.1</td>
</tr>
</tbody>
</table>
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
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-
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 CHO-BSA 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]), tetrascarsharide (4.5 mol of CHO/mol of BSA [group 3] and 11.6 CHO/mol of BSA [group 4]), and hexascarsharide (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 tetrascarsharide (group 3) and hexascarsharide (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 tetrascarsharide (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

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**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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preimmune sera</td>
<td>Tertiary sera</td>
</tr>
<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, tetrascarsharide (4.5)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Group 4, tetrascarsharide (11.6)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Group 5, tetrascarsharide (14.6)</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Group 6, hexascarsharide (2.9)</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Group 7, hexascarsharide (6.6)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Group 8, hexascarsharide (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>
</tr>
</tbody>
</table>

* Synthentic Inaba LPS epitope.
* Input, 7.15 × 10³ CFU/sample.
* Input, 8.9 × 10³ CFU/sample.
* Input, 4.5 × 10³ CFU/sample.
* Input, 9.3 × 10³ CFU/sample.
* ND, not determined.
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 antisera 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.

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 LD50) 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 LD50 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.
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 (log10) 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

![Fig. 6](http://iai.asm.org/) Adsorption of anti-O-SP Inaba-specific and anti-Inaba whole-LPS antibodies with *V. cholerae* O1 El Tor Inaba bacteria. Bacteria (10⁶ 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.
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-\textit{V. cholerae} LPS antibodies correlate with protection against virulent \textit{V. cholerae} challenge of vaccinated volunteers and those infected naturally (19, 27, 32, 35). It is important to develop a \textit{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 \textit{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 \textit{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 \textit{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 binding study solutions with Ogawa and Inaba oligosaccharides by Wang and colleagues (41) did not reveal observable binding of the Inaba oligosaccharides by anti-Ogawa-specific MAbs, indicating 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 antibibriocidal antibodies or antibodies that were protective in the infant mouse assay. A new finding for synthetic \textit{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 \textit{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 \textit{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 cocystalized 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/NJ 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. Antidiotypic sera (a measure of V-segment uniqueness) specific for the anti-Inaba MAB idiotypic did not bind anti-Ogawa (epitope B) MAbs, 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). The MAbs (C6, A18, 11A, 14B1, and 14C3) were tested against 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 vibrioidal-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 antibodies induced by native Inaba LPS could effectively compete for antigen with antibodies induced by Ogawa CHO-BSA conjugates or antibodies 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).

ACKNOWLEDGMENTS

This work was supported by NIH grants to R.K.T. (AI25096) and W.F.W. (AI47373) and by intramural NIH support to P.K.

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