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Effect of saccharide length on the immunogenicity of neoglycoconjugates from synthetic fragments of the O-SP of *Vibrio cholerae* O1, serotype Ogawa

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Abstract—A synthetic hexasaccharide, identical to the terminal hexasaccharide of Ogawa LPS, coupled to bovine serum albumin induced protective antibodies in mice. To determine if there was a minimum saccharide length required for immunogenicity and efficacy, shorter (mono- to pentasaccharide) neoglycoconjugates (CHO–BSA) were tested in mice. The Ogawa CHO–BSA was inoculated at either a constant mass but differing moles, or equal moles but differing masses. Humoral responses were essentially the same when mice received 9 μg of the carbohydrate (0.007 mM with the pentasaccharide) in each of the neoglycoconjugates prepared from mono- through the pentasaccharide, or the same molar amount (0.007 mM), proportionally less by weight when going from the penta- to the monosaccharide. These data show that, within this dose range, the responses occurred virtually independently of the amount of immunogen. Humoral antibodies induced by these immunogens were generally not vibriocidal. Selected antisera induced by CHO–BSA immunogens were protective, but the ELISA titers of the sera were not predictive of the protective capacity. Purified, Ogawa LPS induced anti-Ogawa LPS IgM antibody titers similar to those induced by the Ogawa CHO–BSA conjugates. The anti-whole LPS sera were strongly vibriocidal, as were the previously reported sera induced by hexasaccharide conjugates. This suggests either that the shorter oligosaccharides lack a conformational epitope provided by the hexasaccharide or that the LPS has additional B cell epitopes or selects different B cells in the primary response.

Keywords: *Vibrio cholerae* O1; Cholera vaccine; Ogawa; LPS; Conjugate; Neoglycoconjugate

1. Introduction

Cholera is a diarrheal disease caused by the gram-negative bacterium *Vibrio cholerae*. LPS from *V. cholerae* O1 serogroup (a serogroup is a collection of serotypes with different structures within the LPS defined by antibodies) induces protective immune responses in humans and experimental animals.[1–3] *V. cholerae*-specific, anti-LPS antibodies are correlated with protection against cholera[2] and, thus, LPS is an immunogen of choice for cholera vaccine development. An immunogen is a macromolecule capable of inducing an immune response. The immunogenicity of a particular immunogen, does not necessarily correlate with its ability to induce protective antibodies and thus immunogenicity must be associated with efficacy.

Multiple monoclonal antibodies[4–8] developed against *V. cholerae* LPS to define the serotypes (structural identity of the LPS) of clinical isolates suggests that there are multiple B cell epitopes (antibody binding targets, protective or not) expressed by the two most prevalent serotypes of *V. cholerae* LPS, Ogawa and Inaba. Three

Abbreviations: ELISA, enzyme-linked immunosorbant assay; ANOVA, analysis of variance; IgM, immunoglobulin M; IgG1, immunoglobulin G1; LPS, lipopolysaccharide; O-SP, O-specific polysaccharide; CHO–BSA, carbohydrate–bovine serum albumin conjugate.

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O1-antigen-associated B cell epitopes (A, B, and C) have been identified. The A epitope, expressed equally by Inaba and Ogawa LPS, is postulated to be one of two common structural elements, which include a linear sequence of perosamine residues and the *N*-3-deoxy-L-*glycero*-tetronic acid side chain associated with the perosamines.[8] The B serologic epitope is present only on Ogawa O-SP (O-specific polysaccharide, representing the terminal sugars of LPS).[8–10] The serologic data describing the C epitope are conflicting. Some indicate only Inaba reactivity and, in other cases, cross-reactivity to Ogawa LPS.[4–8] Anti-Ogawa and anti-Inaba antibodies or antibodies that cross-react are needed for protection against cholera as both LPS serotypes are commonly present in endemic areas.

Serologic data suggests that there are multiple antibody targets on *V. cholerae* LPS that could be effective in a cholera subunit vaccine. Toward that end, several groups have determined the structure of Ogawa and Inaba LPS. *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.[11,12] The upstream terminal sugar of the O-SP of *V. cholerae* LPS differentiates the Ogawa and Inaba serotypes. Protective Ogawa-specific mAbs bind the Ogawa O-SP upstream terminal sugar.[10] A 2-*O*-methyl group[9,10,12,13] defines Ogawa LPS while the Inaba terminal sugar has a hydroxyl group in that position.[14]

Synthetic hexasaccharide neoglycoconjugates whose carbohydrate component mimics the upstream elements of Ogawa O-SP induced vibriocidal and protective antisera.[1] The length of the saccharide chain, as well as the antigen–carrier ratio of *Shigella dysenteriae* based immunogens influenced the magnitude of the murine immune response.[15] In contrast, the length of the saccharides in *Streptococcus pneumoniae* conjugates, did not affect the magnitude of the humoral immune responses.[16]

The current studies we report were motivated by the need to develop a cholera subunit vaccine that was effective, easy to construct, and relatively inexpensive to prepare. Thus we needed to understand if the length of the saccharide was critical for the efficacy of the cholera vaccine. We also examined if equal moles of immunogens were as effective at vaccinating mice as an equal mass. Equal mass of the carbohydrate component of the conjugates is defined as the same weight of carbohydrate present in administered immunogen (the whole conjugate) and is independent of the saccharide length. Equal moles of immunogen refers to inoculations with a conjugate mass that contains the same molar amount of the carbohydrate. Previous studies of the Ogawa hexasaccharide used 10 μg of carbohydrate in the immunogen, which was equal to 0.0065 mM of carbohydrate.[1] Immunogens made from a shorter saccharide and having the same molar carbohydrate–protein ratio, used at 10 μg would contain more moles of carbohydrate. We wanted to test how the immunogenicity would be affected by equal molar amounts of different size oligosaccharide components of the glycoconjugate. This information is important because if a smaller weight amount of a monosaccharide conjugate and a larger weight of a hexasaccharide conjugate, both containing the same molar amount of the B cell epitope are equally effective, then a vaccine based on a shorter saccharide would be easier and less expensive to produce and allow more individuals to be immunized with the same amount of saccharide produced.

This study evaluated the immunogenicity and efficacy of Ogawa mono–pentasaccharide neoglycoconjugates synthesized[24] as shown, in abbreviated form, in Figure 1. As in previous studies, the effect of the CHO substitution ratio (≈5, ≈10, or ≈15 mol of CHO/mol of BSA carrier) was investigated. The difference in the humoral responses between mice immunized intraperitoneally (ip) with differing molar amounts (equal mass, two doses, 9 μg) of immunogen versus equal molar (0.0065 mM) amounts but different masses (four doses ranging from 1.8 to 8.2 μg, with mono- and pentasaccharide, respectively) of immunogen was evaluated. Mice immunized ip with 9 μg of purified Ogawa LPS served as a positive control. The equal mole immunization protocol had two additional immunizations to ensure the efficacy of the immunization process as we did not know the efficacy of neoglycoconjugates delivered at such low doses. Using equal moles of CHO epitopes results in a lower mass of protein in the conjugates (Table 1) and thus it was reasonable to increase the number of immunizations to ensure that a sufficient amount of antigen was present to induce a measurable humoral response.

2. Results

2.1. IgM serum responses to Ogawa neoglycoconjugates

2.1.1. Equal mass (two doses, 9 μg). Mice immunized with equal mass (Fig. 2A) of Ogawa neoglycoconjugates produced anti-Ogawa LPS IgM and IgG1 antibodies. Ten days after the primary immunization, IgM titers for the various groups, ranged from <1:100 to 1:1600 (Fig. 3A). The mode (7/15 groups) titer, regardless of saccharide length or degree of CHO substitution was 1:400. On day 17, the anti-Ogawa LPS IgM titers ranged from 1:400 to 1:6400, with a mode titer (8/15 groups) of 1:1600 that represents a 4-fold increase from day 10. In general, days 10 and 17 endpoint titers among the groups for a given CHO ratio and day did not differ by more than 4-fold. The exceptions to this (e.g., compare groups 7 and 15, day 10 versus day 17, Fig. 3A) did not correlate with a particular immunogen or serum

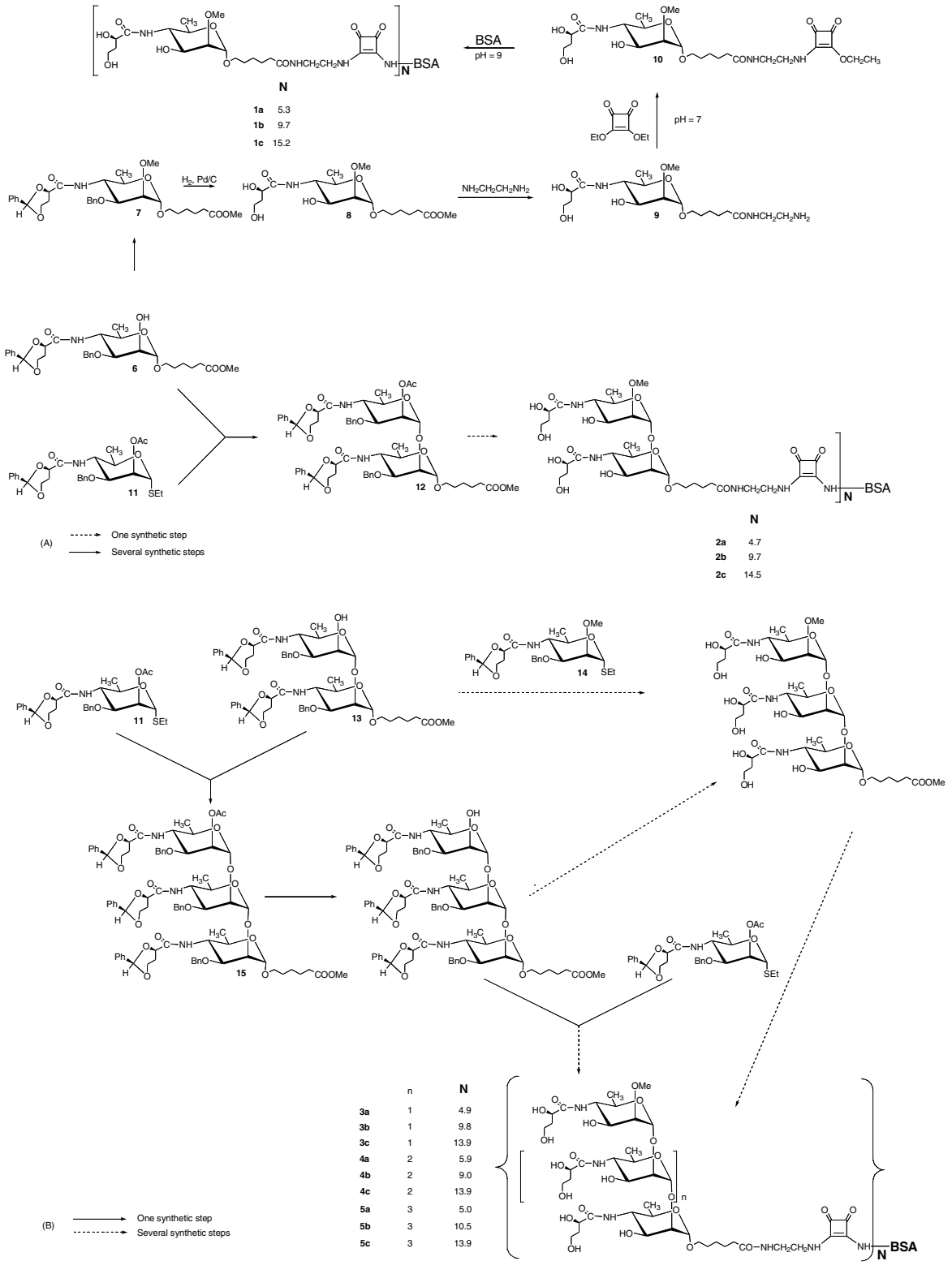


Figure 1. Routes for the generation of Ogawa neoglycoconjugate immunogens, Ogawa CHO–BSA.

sample date. Anti-Ogawa LPS IgM titers at day 35, ranged from 1:200 to 1:1600 (8/15 groups, mode 1:800). Day 85 titers also ranged from 1:200 to 1:1600 (7/15 groups, mode 1:400).

2.1.2. Equal molar (0.0065 mM) amounts (four doses of either 1.8, 3.4, 5.0, 6.6, or 8.2 μg of mono- through pentasaccharide, respectively). Mice immunized with equal moles (Fig. 2B) of Ogawa neoglycoconjugates,

Table 1. Characterization of neoglycoconjugates prepared from synthetic fragments of the O-SP of *Vibrio cholerae* O1, serotype Ogawa

Group #	mol CHO/mol BSA	O-SP length	MW neoglycoconjugate	μg CHO/0.0065 mM	μg BSA/9 μg CHO
Groups 1, 16	4.9	Mono	68,936	1.8	236.9
Groups 2, 17	10	Mono	71,588	1.8	116.2
Groups 3, 18	15.2	Mono	74,280	1.8	76.4
Groups 4, 19	5.2	Di	70,360	3.4	150.9
Groups 5, 20	9.8	Di	73,872	3.4	80
Groups 6, 21	14.5	Di	77,485	3.4	54.2
Groups 7, 22	4.9	Tri	71,426	5	121.1
Groups 8, 23	9.6	Tri	76,122	5	61.9
Groups 9, 24	13.9	Tri	80,461	5	42.7
Groups 10, 25	4.8	Tetra	72,333	6.6	99
Groups 11, 26	9	Tetra	77,693	6.6	53.1
Groups 12, 27	13.9	Tetra	84,016	6.6	34.5
Groups 13, 28	5	Penta	73,963	8.2	79.2
Groups 14, 29	8.9	Penta	79,939	8.2	44.9
Groups 15, 30	14.1	Penta	87,651	8.2	28.2

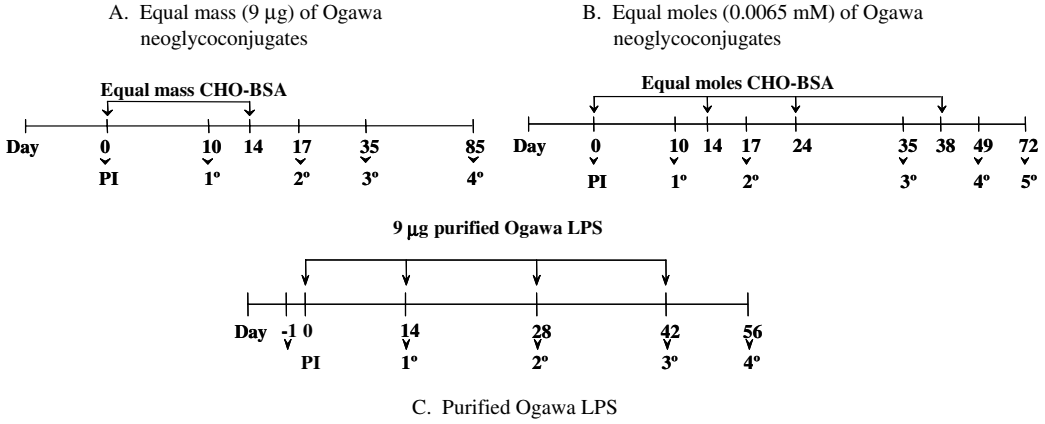


Figure 2. Timelines for mouse immunizations and serum collection for the various experimental vaccine protocols. Each treatment group consisted of five female BALB/c mice that were seven weeks old at the start of the treatment regimen. Mice received injections of Ogawa CHO–BSA neoglycoconjugate immunogen mixed 1:1 in RIBI® adjuvant in 150 mM NaCl. The immunization points are shown above the line; the serum collection days are noted below the time line by arrow heads and designation of the designation of the serum type, 1° primary, 2° secondary, 3° tertiary, 4° quaternary, and 5° quintary. (A) The immunization scheme for mice immunized with equal mass of immunogen. (B) The timeline represents mice immunized with normalized moles of Ogawa neoglycoconjugates. (C) This time line depicts the immunization protocol for mice immunized with purified Ogawa O1 LPS strain O395.

but differing masses of immunogen had IgM titers (day 10) that ranged from 1:200 to 1:400 with the mode (10/15 groups) titer being 1:400 (Fig. 3B). The anti-Ogawa LPS IgM titers at day 17, 3 days after a booster, ranged from 1:400 to 1:1600 (mode titer 1:800 9/15 groups). The titers on day 35 were similar, ranging from 1:200 to 1:1600 (bimodal titer 1:800 and 1:1600 6/10 groups). Day 35 titers were similar to those of day 17 despite an additional booster.

The data (Fig. 3B) clearly show that two additional immunizations at days 28 and 35 increased the IgM response at 49 days, especially in certain groups immunized with higher oligosaccharides. The degree of CHO substitution seemed optimal at ≈10 and ≈15 mol, as there was less variability in the IgM titers at day 49 for these groups compared to the mice immunized with ≈5 mol CHO/mol BSA. This was in contrast to what we previously[1] found for the hexasaccharide conjugate, where of the conjugates with substitution ratio ranging from ~4.5 to 15 mol CHO/BSA the one having the lowest substitution ratio showed the highest efficacy. The anti-Ogawa LPS IgM titers were declining by day 72. The relative differences between groups of mice receiving different length saccharides but the same CHO ratio to carrier proteins were maintained for samples collected at days 49 and 72. If there was a difference in responsiveness to the different mass of immunogens it would be for the ≈5 mol CHO/mol BSA immunogens on days 49 and 72. These IgM titers were generally lower for mice inoculated with trisaccharide and neoglycoconjugates prepared from shorter saccharides.

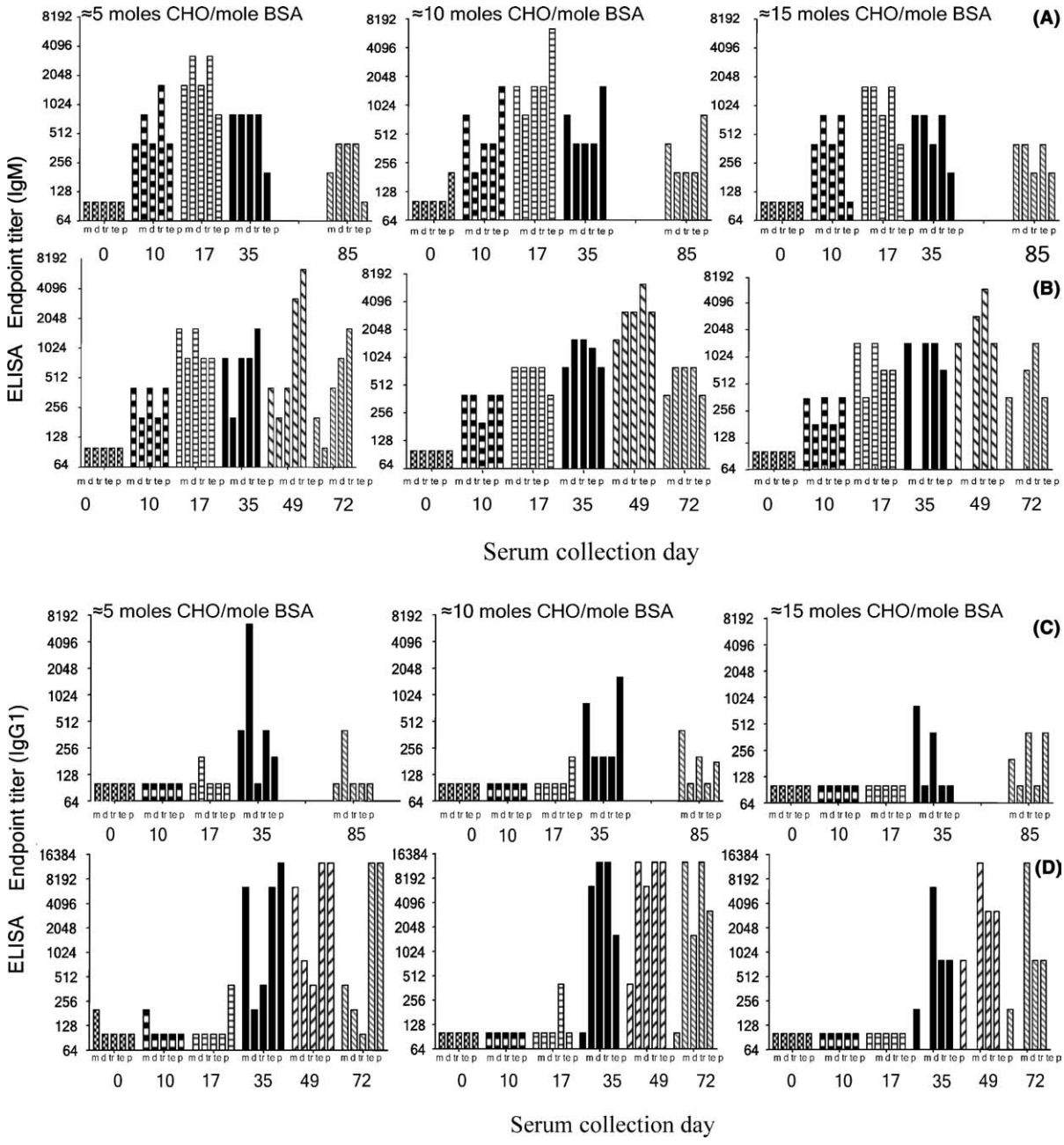


Figure 3. *Vibrio cholerae* Ogawa lipopolysaccharide (LPS) specific IgM (A and B) and IgG1 (C and D) responses following immunization with synthetic Ogawa neoglycoconjugates varying in length from mono to pentasaccharides. The conjugates differed in their mol substitutions of CHO/mol BSA carrier (≈5 mol CHO, left panels; ≈10 mol CHO, middle panels; or ≈15 mol CHO, right panels). For a description of Ogawa constructs see Table 1. Panels 3A and B represent the IgM titers of mice immunized with a constant mass of Ogawa neoglycoconjugates or normalized mol ends, respectively. Panels 3C and D represent the companion IgG1 titers for the mice immunized with the Ogawa neoglycoconjugates but with different protocols as indicated for panels 3A and B. The abbreviations below the bars represent the saccharide length of the immunogen give the mice that contributed to the pooled sera: m = monosaccharide, d = disaccharide, ti = trisaccharide, te = tetrasaccharide, and p = pentasaccharide. The individual bars represent endpoint titers from pooled sera of mice immunized with a particular CHO–BSA construct. Bars with the same fill pattern represent different responses of groups bleed on the same day. The cardinal numbers below the group designations represent the day the serum was collected for that group comparison. Group 27, the ≈15 CHO–BSA disaccharide group, does not have data for days 49, 72 in Figure 3B or D (right panels) because the mice were drowned due to an automatic watering system malfunction during the course of the experiment.

2.2. IgG1 serum responses to Ogawa neoglycoconjugates

2.2.1. Equal mass (two doses, 9 μg). The overwhelming majority of the groups of mice immunized with 9 μg of Ogawa conjugates did not have measurable serum Ogawa LPS-specific IgG1 at days 10 or 17 after immunization (Fig. 3C). At day 35, mice had Ogawa LPS-specific IgG1 titers that ranged from <1:100 to 1:6400. The

response was bimodal (4/15 groups): 1:400 and 1:200. Some groups of mice (3, 12, 14, and 15) did not make Ogawa-specific IgG1 (day 35) following two doses of Ogawa neoglycoconjugates. At day 85 post-primary immunization, approximately 46% of the groups had low IgG1 titers while the remaining groups had baseline IgG1 titers.

2.2.2. Equal molar (0.0065 mM) amounts (four doses of either 1.8, 3.4, 5.0, 6.6, or 8.2 μg of mono- through pentasaccharide, respectively). The IgG1 responses of mice receiving equal moles of immunogen were not readily apparent until the third serum sample (day 35), a time when the mice had received two boosters (Fig. 3D). At day 35, the anti-Ogawa IgG1 titers were generally >1:800 (10/15 groups). The range of the IgG1 response at day 49 was 1:400 to 1:12,800 with the mode (6/15 groups) being 1:12,800 (Fig. 3D). The additional dose of neoglycoconjugates (day 35) did not greatly enhance the day 49 titers. The trend for Ogawa-specific IgG1 titers, 72 days post-primary immunization, was that the mice immunized with varying lengths of CHO but at a mole substitution of 10 had more consistent and elevated responses. The lower IgG1 titers induced by trisaccharide or shorter carbohydrates reported for day 72 was most apparent in the ≈5 CHO/BSA groups. It is not clear if the groups that received ≈15 CHO/mol BSA immunogens fit this profile. The response to the monosaccharide was low, the response to trisaccharide length immunogen was high and the mice immunized with the disaccharide did survive till the end of the experiment. Clearly mice that received four boosters of immunogen made more consistent IgG1 responses that remained elevated longer than mice given two doses of 9 μg.

2.3. Comparisons of humoral responses induced by the two immunization methods

The ELISA anti-Ogawa LPS titers (IgM) of the pooled serum of individual mice immunized with Ogawa neoglycoconjugates made from different length saccharides did not generally induce humoral titers that were significantly different. There are two reasons for the occasionally observed 4-fold different titers within a group (for a given CHO ratio) for a particular day to be likely not biologically significant: (1) There were no trends relative to a particular immunogen that correlated with the differences in titers; and (2) The difference in titers for a particular bleed did not always maintain the relative difference for that group in the next serum sample.

To more directly compare the two immunization methods, we pooled the ELISA titer data to generate geometric means of the endpoint titers determined for the various sera (Fig. 4). The pooling of ELISA results is valid because the titers were not significantly different among the groups for a given CHO ratio. Thus pooling the endpoint titers allows the calculation of geometric means and the use of a statistical test to determine significance. The first two post-primary immunization serum samples, days 10 and 17, were from mice that all received two doses of Ogawa neoglycoconjugate, whether normalized for mass or moles of immunogen. The later bleeds, on days 35, 49, and 72, represent sera from mice that were immunized with normalized moles of the neoglycoconjugates and thus received more carbohydrate antigen. Day 85 values are from mice that received two immunizations with equal mass of CHO–BSA.

Using two different criteria for significance, the 4-fold increase in geometric mean titer or ANOVA, the IgM

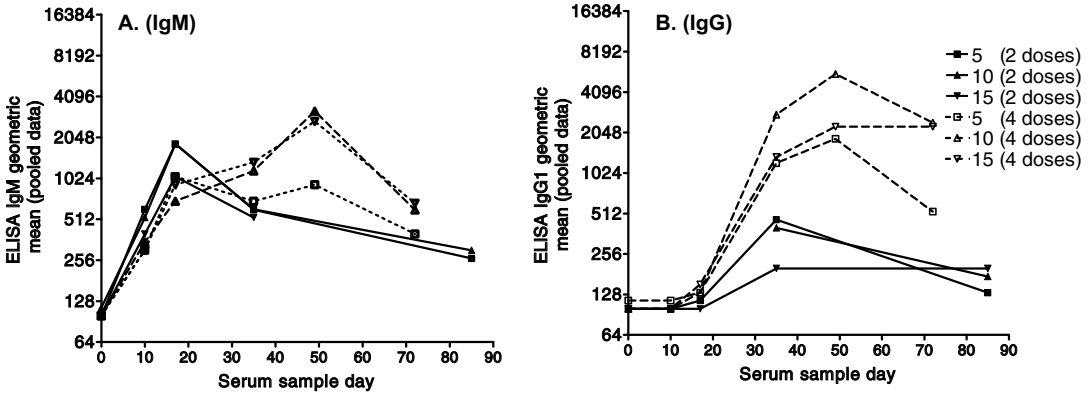


Figure 4. Anti-Ogawa LPS IgM (A) and IgG1 (B) geometric mean serum titer against Ogawa LPS. The endpoint titers of the pooled sera of individual mice immunized with Ogawa neoglycoconjugates of different saccharide lengths but the same CHO substitution ratio were pooled for a particular serum sample date. Prism® was used to calculate a geometric mean, which is less influenced by outliers. An ANOVA, using the Kruskal–Wallis test that does not require parametric data did not detect any difference between the geometric mean titers for IgM or IgG1 for any given serum sample collected on the same day.

titers do not differ (Fig. 4A). This result supports the notion that, within a certain range, the endpoint titers are independent of the mass amounts of immunogen. Thus, two doses of 9 μg of Ogawa pentasaccharide (0.007 mM) in the neoglycoconjugate are not more immunogenic than two 1.8 μg doses of Ogawa monosaccharide (0.0065 mM). This is consistent with data that reported only the terminal residue of the O-PS of *V. cholerae* O:1 (Ogawa) is fully engaged in the interaction with S-20-4, an Ogawa-specific antibody.[10] In terms of the number of B cell epitopes that an antibody like S-20-4 would bind, the molar amount of the terminal residue in 9 μg of pentasaccharide is nearly the same as that in 1.8 μg of the monosaccharide.

The geometric mean titers for the IgG1 responses were low early in the response (days 10 and 17) but increased after that (Fig. 4B). As with the IgM response, serum titers of mice immunized with additional boosters of neoglycoconjugates declined more slowly than mice receiving less immunogen. None of the geometric means differed for a particular serum sample date according to a Kruskal–Wallis ANOVA analysis. One set of comparisons differed by 4-fold or more. This was the day 72 titers for the 5 CHO/mol BSA, which was significantly lower than the other geometric means for mice immunized with more highly substituted BSA. The ANOVA results support the original analysis that showed little difference in the endpoint titers assessed as a 4-fold difference.

2.4. Cross-reactivity of antisera made to Ogawa-specific neoglycoconjugates

The two terminal sugars of the Ogawa O-SP have been shown to interact with a mAb S-20-4 that is protective against *V. cholerae* infection.[10] Thus, the configuration of the Ogawa neoglycoconjugates, especially those made with the shorter saccharides, would be expected to induce only Ogawa-specific mAb if the B cell epitope is that described by crystal structure of S-20-4 complexed to Ogawa-specific sugars. This, however, assumes that there are no other B cell epitopes in the neoglycoconjugates. It is possible that other antibodies having different geometry/conformation than that of S-20-4 can bind a slightly different B cell epitope that still involves the tetronic acid residue and the last two sugars.

To test the specificity of the antisera, we compared its reactivity to purified Ogawa or Inaba O1 LPS in the ELISA (Fig. 5). The IgM endpoint titers of pooled sera that reacted with Ogawa LPS were divided by the IgM endpoint titers of the same pooled sera that reacted with Inaba LPS. The larger the quotient, the less cross-reactive the sera are. Sixteen of the 30 groups had endpoint titers of 4-fold or greater, showing more reactivity to Ogawa LPS, suggesting that approximately 50% of the antisera was binding the terminal sugar of the Ogawa LPS. The other sera were either binding a tetronic acid-based epitope in common between the Ogawa and Inaba O-SP, or were *cross-reactive* to the Inaba terminal sugar. The cross-reactivity of the sera did not correlate with a particular immunization protocol, CHO substitution, or saccharide length. These results suggest that the B cell epitope(s) in the various Ogawa neoglycoconjugates were randomly presented, which would suggest that the affinity of the different epitopes did not effect the induction of B cells and subsequent antibody production.

2.5. Vibriocidal of anti-Ogawa CHO–BSA sera

Pooled sera from days 17, 35 (groups 1–15) and days 35, 49 (groups 16–30) were analyzed for their vibriocidal capacity against bacterial strain O395 O1 Ogawa (Fig. 6). The ODs in this assay indicate the degree of metabolic activity of treated bacteria. Non-vibriocidal sera-treated samples have high ODs while cultures

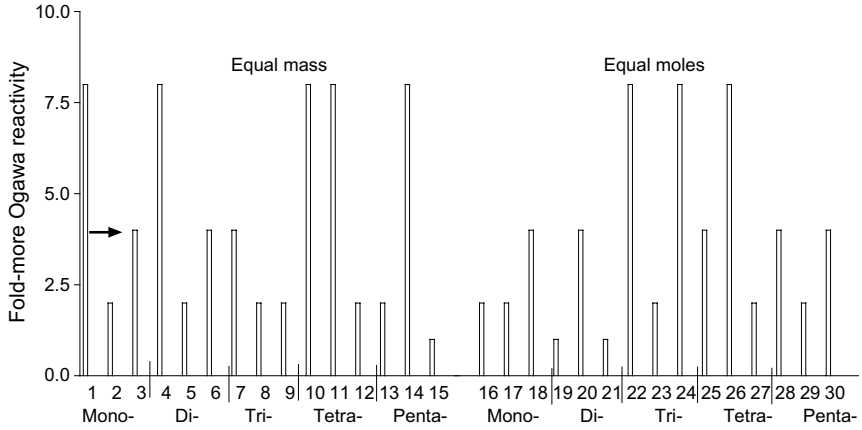


Figure 5. Cross-reactivity of anti-Ogawa CHO–BSA sera with purified Inaba LPS. ELISA (IgM) titers of pooled sera from mice immunized with Ogawa neoglycoconjugates was assessed for reactivity to purified Ogawa and purified Inaba LPS. The endpoint titers of sera that reacted with Ogawa LPS were divided by the endpoint titers of sera reacted with Inaba LPS. Values of 4-fold or greater indicate low cross-reactivity of anti-Ogawa neoglycoconjugates sera for Inaba LPS.

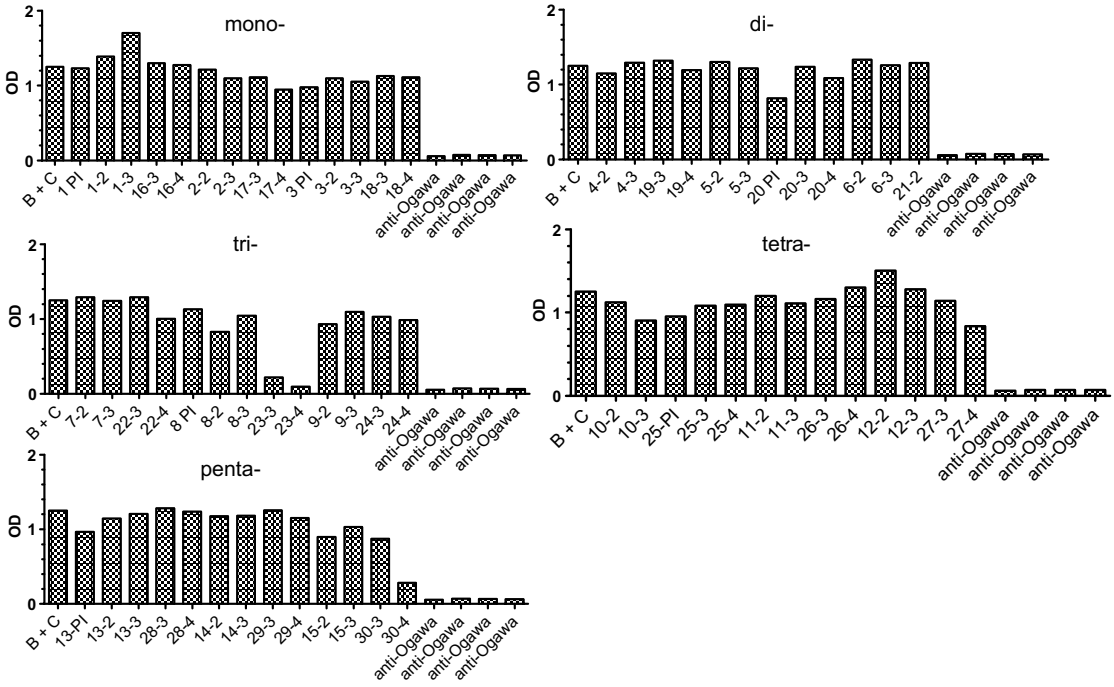


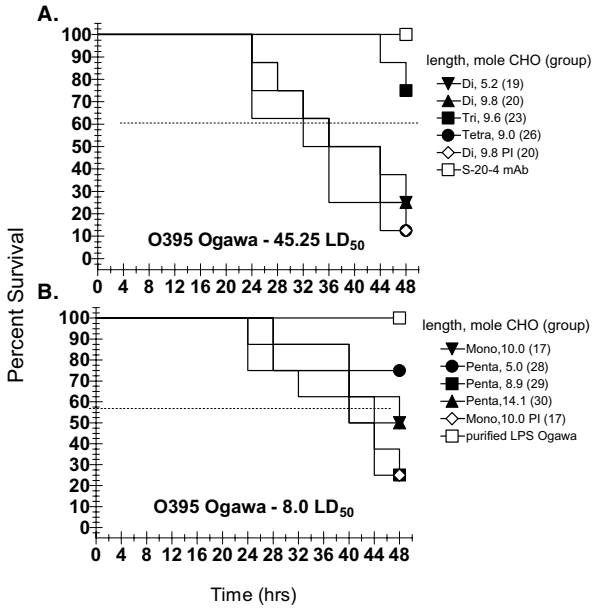
Figure 6. Vibriocidal capacity of Ogawa CHO–BSA antisera for Ogawa O395 bacteria. Pooled sera from the various groups (1–30) were tested in the microtiter vibriocidal assay. The *y*-axis represents ODs. The smaller the OD value, the more vibriocidal the sera. The pooled sera analyzed were either secondary or tertiary sera for groups 1–15 and tertiary and quaternary sera for groups 16–30. Control for non-serum killing was bacteria (B) and complement (C) only. Positive control serum (anti-Ogawa) was the tertiary sera of mice immunized with purified Ogawa LPS. The positive control serum was run in quadruplicate in random row of the 96-well plate. Preimmune (PI) were analyzed represent randomly chosen groups. The PI serum was never vibriocidal. These data are representative of two independent experiments. Only one group comparison differs by 4-fold or more.

treated with sera that contain vibriocidal antibodies have low ODs. Control anti-Ogawa LPS sera that had an ELISA IgM titer of 1:10,240 had an OD_{570} of 0.07, while the negative controls, bacteria (B) and complement (C) or preimmune sera (PI) had an OD_{570} around unity. Collectively, the sera had high anti-Ogawa LPS ELISA titers (IgM and IgG1), yet the vast majority (93.3%) of the sera were not vibriocidal. Exceptions to this were sera from groups 23 (tertiary and quaternary) and 30 (quaternary), which were vibriocidal. Group 23 had an anti-Ogawa LPS IgM ELISA titer of 1:1600, but was not particularly specific for Ogawa LPS. Thirteen other group's sera had the same IgM ELISA titer but they were not vibriocidal.

2.6. In vivo protective capacity of selected anti-Ogawa CHO–BSA sera

To further test the potential protective nature of the anti-Ogawa CHO–BSA sera, we used the infant mouse protection assay to compare sera (day 35) from selected group's that had either low or high anti-Ogawa LPS IgM and IgG1 titers. Comparison of the in vivo protective capacity of sera from groups 17, 19, 20, 23, 26, 28, 29, and 30 revealed that group 23's (trisaccharide 9.6 CHO/mol BSA) in vitro vibriocidal capacity was supported by the in vivo infant mouse protection assay results (Fig. 7A). Approximately 70% of the mice survived the challenge with forty-five 50% lethal doses of *V. cholerae* combined with pooled sera from group 23. This protection was statistically equivalent to the protective anti-Ogawa monoclonal antibody, S-20-4[1,10] (40) and clearly superior to murine preimmune sera (group 20). Group 23's anti-Ogawa LPS IgM and IgG1 ELISA titers were not different from several other groups, for example, 20, and 26 tested at this dose of virulent vibrios (Fig. 3C, table in Fig. 7). Sera collected from immunized mice had low titers of Ogawa LPS reactive IgG3, but the titers did not correlate with protection (data not shown).

Sera from groups 28, 29, and 30 (Fig. 7B) were obtained from mice immunized with pentasaccharide neoglycoconjugates but with different mol substitution ratios of CHO. A comparison of IgG1 titers between groups 28, 29, and 30 indicates that the IgG1 serologic response of group 28 is significantly higher than the other two groups. Serum pooled from group 29 was not protective, while sera from group 30 provided 50% protection. The IgM titers of these groups were not different nor were the IgG1 titers. The protective capacity of group 28 sera does not differ from that of 30, but is different from that of group 29. These data suggest that for some sera there is no strict correlation between the anti-Ogawa LPS IgM but perhaps the IgG1 titer can



C.ELISA endpoint titers

Group	IgM	IgG1
17	800	<100
19	200	<100
20	1600	6400
23	1600	12800
26	1600	12800
28	1600	12800
29	800	1600
30	800	3200
PI 17	<100	<100
PI 20	<100	<100

Figure 7. Survival of neonatal mice following oral challenge with live virulent *Vibrio cholerae*. Ten, 4–5-day-old CD-1 neonatal mice were orally gavaged with virulent *Vibrio cholerae* O1 classical, Ogawa strain O395 (panel A 45 LD_{50} panel B 8 LD_{50}) mixed 1:1 with either tertiary antisera, preimmune antisera, positive control monoclonal antibody (S-20-4) or no antibody (untreated group). The tertiary sera, unless otherwise noted were diluted with normal mouse sera for a final dilution of 1:5. 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. A *p*-value of 0.05 or less is considered significantly different for the comparisons of selected survival curves: Panel A, group 23 versus S-20-4, $p = 0.1435$; group 20 PI versus S-20-4, $p = 0.0006$; group 20 versus group 19, $p = 0.8852$; group 23 versus group 20, $p = 0.0224$; group 23 versus group 26, $p = 0.0045$. Panel B, group 29 versus purified Ogawa sera, $p = 0.002$; group 28 versus purified Ogawa sera, $p = 0.1435$; group 17 PI versus purified Ogawa sera, $p = 0.0025$; group 17 PI versus group 17, $p = 0.3521$; group 30 versus group 29, $p = 0.3182$; group 28 versus group 30, $p = 0.3681$, group 28 versus group 29, $p = 0.0656$.

predict a function. This is, however, dependent on the challenge dose as groups 20 and 26 antisera had statistically similar IgG1 titers but they were not protective whereas group 23's pooled serum was.

2.7. Purified Ogawa LPS: induction of specific IgM and vibriocidal titers

Immunization of mice, ip with 9 μg of Ogawa LPS, induced anti-Ogawa LPS IgM ELISA titers (1:3200) at day 28 after two doses of immunogen (Fig. 8A). The IgG1 titers were not measurable until day 42 and were not as high as the IgG1 titers induced by the Ogawa neoglycoconjugates (compare Fig. 8B with day 35 in Fig. 3C and D). Purified Ogawa LPS efficiently induced vibriocidal antibody titers (Fig. 8, numbers above bars). The vibriocidal titer was 1:2560 for an anti-Ogawa-specific LPS IgM titer of 1:3200. We did not consider the ELISA titer of 1:3200 significantly different from 1:1600 (typical titer of antisera induced by Ogawa neoglycoconjugates), but clearly there is a functional difference between the sera that have similar IgM ELISA titers. These data suggest that there is something unique about purified LPS immunogens that the Ogawa neoglycoconjugates made from mono- through pentasaccharides do not readily duplicate or perhaps lack.

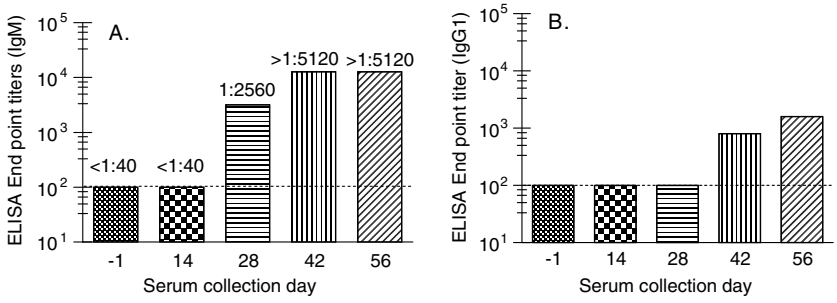


Figure 8. Immunogenicity of purified Ogawa LPS. Female BALB/c mice were immunized ip, four times with 9 μg of purified Ogawa LPS (O395) every two weeks. Serum sample dates are shown on the *x*-axis. The *y*-axis represents the ELISA endpoint titer of pooled sera. Numbers above the bars in panel A are the vibriocidal titers of the corresponding pooled sera. A. Anti-Ogawa LPS IgM. B. Anti-Ogawa LPS IgG1.

3. Discussion

V. cholerae LPS efficiently induces vibriocidal antibodies (antibodies that kill bacteria in vitro via a complement-dependent mechanism).[2,8,17–19] However, lipid A, which couples LPS to the bacterial outer membrane, is toxic and therefore, except for oral administration, LPS has limited value as a cholera vaccine component. Detoxified LPS[17,18] or a synthetic fragment of the O-SP[1,19] has been generated to eliminate the toxicity of native LPS while retaining LPS's protective B cell epitopes that are located in the O-SP and core polysaccharide structures.[1,9,10] Ogawa hexasaccharide neoglycoconjugates induced protective antibody in mice.[1] The antisera were also predominately vibriocidal (9/12 experimental groups).

We undertook the studies described herein to determine if the length of the Ogawa saccharide in the neoglycoconjugates influenced the immunogenicity and efficacy of the immunogens. This is an important question as the structural detail of the carbohydrate antigens can dictate the type of immune response.[16,20] The minimum length of Ogawa-based perosamine immunogens might be expected to be a mono- or disaccharide, based on the crystal structure of a complex of an anti-Ogawa-specific mAbs bound with saccharides of those lengths.[10] If a conjugate made with a saccharide shorter than a hexasaccharide was immunogenic and protective, the synthesis of a cholera subunit conjugate vaccine would be simpler and cheaper. The shorter length Ogawa saccharides were immunogenic and, in some cases, did induce protective antibody, but were mostly ineffective at inducing vibriocidal antibody, which has been previously correlated with protection in the infant mouse assay.[1]

A direct comparison of endpoint titers (lowest dilution of antisera) induced by the Ogawa hexasaccharide and the Ogawa mono- through pentasaccharide induced titers is difficult. The experiments were performed at different times and the serologic analyses were slightly different. However geometric means can be calculated. The range of the 95% confidence intervals of the geometric means of the IgM and IgG ELISA titers from the two studies does not demonstrate statistically different responses within a comparison group that had the same CHO loading as that of the conjugate (W. F. Wade, personal observation). The statistical similarity of the IgM and IgG titers at day 35 for both studies is at variance with the vibriocidal capacity of the various sera. Seventy-five percent of the mouse groups immunized with Ogawa hexasaccharide had vibriocidal antibody and all groups of mice had some level of protective antibody. In contrast, the vast majority of the pooled sera (tertiary and quaternary) collected from mice immunized with the Ogawa mono through pentasaccharide conjugates were non-vibriocidal. Some sera did provide for varying degrees of protection but the ELISA titers did not correlate with the protection.

We reported that anti-Inaba LPS vibriocidal antibody correlated with antibody binding LPS in situ.[19] A similar analysis of the Ogawa antisera used in the protection assay (Fig. 7) did not reveal any binding, suggesting that the affinity or concentration of anti-Ogawa antibody is limiting for this type of biochemical analyses (P. Kováč and W. F. Wade, personal observation). We hypothesize, that relatively high ELISA titers antisera that are not protective, can bind LPS in the ELISA (due to the high concentration of LPS on the plate) but not to the LPS on the bacterial cell outer membrane, due to either spatial or concentration limitations. Higher affinity antibody would be more likely to bind limiting amounts of LPS on the bacterial cell and it maybe the type of antibody required for protection in vivo.

3.1. Differences in synthetic Ogawa immunogens compared to LPS

In view of the results of the crystallography study of anti-Ogawa-specific antibody that binds the terminal sugar of Ogawa LPS,[10] it is not immediately clear why the Ogawa hexasaccharide neoglycoconjugates induce vibriocidal and protective antibodies more efficiently than neoglycoconjugates of shorter saccharide length. All Ogawa neoglycoconjugates, regardless of the saccharide length, contain the structural motif that defines the Ogawa serotype.[1,2,8,10] The only physical differences between the Ogawa neoglycoconjugates in the two studies are the lengths of the saccharide and the linker. The mono–pentasaccharide conjugates studied here had an ethylene- diamine linker while the hexasaccharide conjugates[1] had a 2-atom shorter hydrazine linker.[1] The increased saccharide length of the hexasaccharide immunogens could provide a second B cell epitope, whereby six perosamines may be large enough or form an optimum conformation to occupy the antibody combining site. Another explanation is that the efficiency of inducing antibodies to the terminal sugars of Ogawa neoglycoconjugates may be related to the length of the saccharide indirectly and not involve the size of the ligand that occupies the antibody antigen combining site. Paoletti and co-workers reported that for group B *Streptococcus* type III polysaccharide-tetanus toxoid conjugates shorter and longer saccharide lengths were less effective at inducing protective responses than an immunogen that had a length intermediate between short and long.[20] They reported similar immunogenicity (the ability to elicit antibody) of the three conjugates made from different length carbohydrates. Their hypothesis was that a certain length of sugars is required to optimally express a conformational epitope. This has not been reported for the Ogawa serologic epitope, but perhaps it also has a length-dependent conformation

that influences the ability of antibodies expressed by naïve B cells (antibody producing cells that first respond to the immunogen) to bind the immunogen, be activated, and later secrete antibodies.

A linear epitope represented by the hexasaccharide would represent an additional B cell target, a target in addition to the B cells epitope provided by the downstream perosamines.[8,10] The concept of a common B cell epitope contained within the Ogawa hexasaccharide immunogens, such as that defined by the A antigen, is supported by a retrospective analysis of the anti-Ogawa hexasaccharide sera,[1] which bound Inaba LPS in an ELISA (W. F. Wade, personal observation). In this study, we found that a high number of antisera bound Inaba LPS even though the sera were raised against Ogawa neoglycoconjugates. If the cross-reactive epitope contained within the Ogawa neoglycoconjugates examined in this study is the tetronic acid side chain, then it is difficult to understand why the shorter length immunogens are not vibriocidal like those induced by Ogawa hexasaccharide immunogens, which also are cross-reactive with Inaba LPS. The tetronic structure on the individual perosamine, more represented in the Ogawa hexasaccharide immunogens, did not differ in the fine structure of the tetronic acid in the shorter Ogawa saccharide immunogens. Characterization of the fine specificities and affinities of the antibodies made in response to the different length Ogawa neoglycoconjugates is beyond the scope of this study but it is the only means to determine the effect of the different saccharide lengths on the induction of protective antibody.

3.2. The role of structural differences between LPS-derived immunogens in immunity

One or two immunization of humans or experimental animals with whole-cell *V. cholerae* or its purified LPS results in consistent serum-based vibriocidal titers.[17–19] An experimental cholera vaccine, detoxified Inaba LPS, conjugated to cholera toxoid (CT), required 2–3 immunizations to induce vibriocidal antibodies, whereas a single dose of whole-cell *V. cholerae* vaccine was sufficient for induction of vibriocidal antibody.[17] Phase 1 efficacy studies in humans, using the detoxified Inaba-LPS-CT demonstrated equivalent IgM titers (day 42) to titers of volunteers immunized orally with the commercial, *V. cholerae* whole-cell vaccine.[18] One form of the detoxified Inaba-LPS-CT immunogen induced equivalent vibriocidal titers to that of individuals vaccinated with *V. cholerae* whole-cell vaccine while another form of the experimental vaccine was not as effective as the commercially available whole-cell vaccine.[18] In our studies, the IgM response to purified Ogawa LPS was similar to that induced by the Ogawa neoglycoconjugates, but the purified LPS induced vibriocidal antibodies.

The Inaba neoglycoconjugates (di-, tetra-, or hexasaccharide) we examined were immunogenic but failed to induce vibriocidal or protective antibody.[19] The Inaba conjugates are similar to the detoxified Inaba-LPS-CT conjugates in that both immunogens induce Ogawa LPS cross-reactive antibody. The detoxified Inaba-CT immunogens in contrast induced vibriocidal antibodies.[17,24] Are the Inaba-induced vibriocidal antibodies, induced by the detoxified full-length Inaba LPS directed at a different, or additional B cell epitope because of the additional sugar residues?[17] Perhaps the B cell epitopes that are targeted by the detoxified and the synthetic Inaba conjugates are the same but the detoxified Inaba LPS-CT conjugates, which contain a longer polymer of perosamine in the core and the transformed Lipid A, can select different affinity B cells. Of additional note is that the carrier and the site of immunization in Gupta and colleagues' and our study were different. The type of carrier is known to influence the protective response.[21]

3.3. Future studies to resolve why structural difference in immunogens elicit different sets of functional antibodies

The purified Ogawa LPS and Ogawa neoglycoconjugates were both delivered ip, therefore the immunogens likely target the same B cell populations, either in the peritoneal cavity, mesenteric lymph nodes or the spleen. Why then is the efficacy of the humoral responses so different? The answer to this question awaits a fine analysis of the Ogawa LPS-specific B cell repertoire (generation of monoclonal antibodies) to compare the selected antibody response to *V. cholerae* LPS to that of the Ogawa neoglycoconjugates. The amino acid residues of the monoclonal antibodies within the antibodies antigen binding regions can then be correlated with efficacy and avidity. These studies will provide the foundation for defining immunization regimens with Ogawa-based immunogens to optimally induce protective antibodies. It will also be informative to assess what fine differences the linker make on the selected B cell repertoire.

3.4. Conclusions

Immunogenicity of synthetic Ogawa neoglycoconjugates made from mono- through the pentasaccharide is comparable to that of the previously described conjugate made from the Ogawa hexasaccharide.[1] The use of two doses of 9 μg immunogen made from each of mono- through the pentasaccharide versus two doses of 0.0065 mM of the same immunogen (namely down to 4-fold less when going from penta- to the monosaccharide) is equally effective and does not influence the humoral response. Thus, within the range investigated, the immune response is independent of the mass of CHO–BSA administered. The shorter length CHO–BSA conjugates, however, do not readily induce

vibriocidal or protective antibody as efficiently as the hexasaccharide conjugates. The mono- through pentasaccharide CHO–BSA immunogens were variably effective at inducing Ogawa-specific responses and clearly less effective than native Ogawa LPS at inducing vibriocidal antibody. These data underscore that a protective and vibriocidal response to Ogawa synthetic epitopes is more likely induced by hexasaccharides than by conjugates of shorter saccharide lengths. The reason for the difference in the induction of a protective immune response is not known. It could be due to extra B cell epitopes in the hexasaccharide, a minimum length requirement for induction of effective antibodies that bind the Ogawa B cell epitope, and/or the adverse effect of the increased linker length present in conjugates studied here compared to the hexasaccharide conjugates.[1]

4. Experimental

4.1. Animals

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

4.2. Ogawa CHO–BSA constructs

Immunogens **1a**–**5c** were prepared by linking the chemically synthesized mono- through pentasaccharide fragments of the O-SP of *V. cholerae* O1, serotype Ogawa, to BSA using squaric acid chemistry.[22–24] The haptens (B cell epitopes) were prepared in a stepwise manner[24] (Fig. 1A and B) by coupling monosaccharide glycosyl donors **11** and **14** with the required glycosyl acceptors. Coupling of these intermediates afforded, after deprotection, haptens in the form of glycosides whose aglycon made them amenable to conjugate to proteins. The chemistry involved in the preparation of carbohydrate–BSA constructs **1a**–**1c** from the monosaccharide hapten **8** is shown in Figure 1A. Thus, treatment[25] of methyl ester **8** (prepared from alcohol **6** by sequential methylation (→**7**) and hydrogenolysis) with ethylenediamine, gave amine **9**. Reaction of the latter with squaric acid diethyl ester at pH 7, followed by treatment of the formed monoethyl ester **10** with BSA at pH 9 afforded, in one pot,[23] constructs **1a**–**c**. Compound **11** was used as a glycosyl donor to make the requisite oligosaccharides. Thus, coupling of **11** with glycosyl acceptor **6** (Fig. 1A) gave the fully protected disaccharide **12**. Alternatively, coupling (Fig. 1B) of **11** with acceptor **13**, obtained by deacetylation of **12**, gave trisaccharide **15**. Such coupling reactions were then followed by deacetylation and methylation at the terminal perosamine residue, and hydrogenolysis to remove the protecting benzyl and benzylidene groups. The final coupling step, which resulted in the targeted oligosaccharide size, was also performed using the methylated glycosyl donor **14**. For example, its reaction with the glycosyl acceptor **13** (Fig. 1B) gave a trisaccharide. The same trisaccharide was also obtained by deprotection of **15**, obtained by coupling of **11** with **13** (Fig. 1B). Deprotected, linker functionalized oligosaccharides, such as the aforementioned trisaccharide, were then converted to neoglycoconjugates applying the sequence of reactions shown in Scheme 1 for the monosaccharide (**8**→**9**→**10**→**1a**+**1b**+**1c**). The chemical structure of all intermediates in the synthesis of CHO–BSA immunogens were confirmed by NMR and mass spectrometry. Monitoring of the conjugation reactions and determination of carbohydrate–protein ratios in the assembled CHO–BSA constructs were done by surface enhanced laser-desorption ionization–time-of flight mass spectrometry.[26,27]

4.3. Immunization and sera collection

Sixteen groups of five mice each were used to test the immunogenicity of the Ogawa O-SP epitope conjugates, according to the regimen shown in Figure 2. Nine micrograms (based on carbohydrate weight) of Ogawa CHO–BSA conjugate suspended in 150 mM NaCl and mixed 1:1 in RIBI® adjuvant (Sigma; St. Louis, MO) was injected ip on days 0, and +14. Blood collection via retro-orbital sinus/plexus was done on days 0, +10, +17, +35, and 85. Anti-Ogawa monoclonal (mAb) complexed with Ogawa mono- or the disaccharide showed that only the terminal monosaccharide makes meaningful contact with the mAb. This would suggest that mono and disaccharides should be immunogenic and induce protective antibody. We tested by using an immunization protocol that normalized the 'mole ends' of the shorter immunogens to that used in our previous study, which showed protective responses to hexasaccharide. Mice were immunized four times at day 0, +14, +24, and +38 with normalized moles of immunogen. Blood was collected from this group of mice at days 0, +10, +17, +35, +49, and +72. Retro-orbital plexus bleeding routinely yielded approximately 100 μL of blood, which after processing provided 50% of that volume as serum. Resulting sera from individual mice within a group was pooled and stored at 4 or −20 °C until used. In some experiments, individual mice serum samples were not pooled and the individual samples from within the group were assessed by ELISA or the vibriocidal assay.

BALB/c female mice used to generate control sera for protection and vibriocidal assays were immunized with purified LPS from *V. cholerae* O1 Ogawa strain P1418 (a generous gift from Dr. S. Kondo, Josai University, Japan). Mice were immunized ip with 9 μg of purified Ogawa LPS on days 0, +14, +28, and +42. Blood was collected on the days of immunization, at +56 days and one day (prebleed serum) prior to the start of the immunization.

4.4. Serology

The presence of anti-O-SP Ogawa specific was measured by ELISA as described in detail.[1,19] The source of the ELISA test antigen was purified Ogawa LPS P14183 (a gift from Dr. Kondo, Japan) or Inaba LPS (Sigma, St. Louis MO). Endpoint titers for ELISA (IgM or IgG1) 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. Positive integers in wells with the highest serum dilution were considered the endpoint titer.

4.5. Vibriocidal microtiter assay

We used the microtiter test reported by Fournier's group that is described in detail elsewhere.[8,28] The OD_{600} of an overnight bacterial (*V. cholerae* O1 classical Ogawa strain O395) suspension was adjusted to 0.80 with PBS to approximate the bacteria to 1×10^9/mL. Twenty-five microliters of the complement-treated bacteria was added to each well of a 96-well plate that contained 25 μL of heat-inactivated, pooled mouse sera from the various treatment groups that had been serially diluted. The mixture was covered and incubated for 1 h at 37 °C. One hundred-fifty microliters of L-broth was then added to each well and the plate was incubated uncovered in a humidified chamber for 2 h at 37 °C. Twenty-five microliters of the indicator solution was added to each well, and incubated uncovered for 15 min at room temp before recording the optical density (OD_{570}). The plate was then placed in a humidified chamber at 4 °C overnight, and the optical density was re-recorded 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. A titer of $1 < 1{:}40$ is considered not vibriocidal.

4.6. Infant mouse challenge assay

The suckling mouse challenge model for cholera was used for assessing the protective quality of anti-O-SP Ogawa-specific antibodies in vivo.[1,19,29] Cultures of *V. cholerae* O395 Ogawa were grown for 16 h in LB pH 6.5, at 30 °C and either 8 or 45 LD50 was combined with 25 μL of either preimmune sera (negative control) or test sera immediately before administration intragastrically to 4–5-day-old CD-1 mice. Challenged mice were kept at 30 °C and monitored every 4 h starting 24 h post challenge.

4.7. Statistical analyses

The ELISA titers, anti-Ogawa LPS IgM or anti-Ogawa LPS IgG1 of pooled sera from the various groups and collection dates were compared for significant differences using the established parameter that requires a 4-fold or greater difference between endpoint titers for significance.[2,3,19] The Prism GraphPad® program was used to calculate geometric means (endpoint titers) of selected data sets to evaluate statistical significance by ANOVA. Statistical significance of the infant mouse protection assay data was calculated based on the log-rank test, which is equivalent to the Mantel–Haneszel test.[19] The null hypothesis that was tested was that selected survival curves were identical, that is, the antisera used did not change survival. A p-value of 0.05 or lower was considered significant.

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