Development of Peptide Mimics of a Protective Epitope of Vibrio cholerae Ogawa O-antigen and Investigation of the Structural Basis of Peptide Mimicry*

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As an alternative approach toward the development of a cholera vaccine, the potential of peptide mimics of Vibrio cholerae lipopolysaccharide (LPS) to elicit cross-reactive immune responses against LPS was investigated. Two closely related protective monoclonal antibodies, S-20-4 and A-20-6, which are specific for Ogawa O-antigen (O-specific polysaccharide; O-SP) of V. cholerae O1, were used as the target antibodies (Abs) to pan phage display libraries under different elution conditions. Six phage clones identified from S-20-4 panning showed significant binding to both S-20-4 and A-20-6. Thus, it is likely that these phage-displayed peptides mimic an important conformational epitope of Ogawa antigens and are not simply functionally recognized by S-20-4. Each of the six phage clones that could bind to both monoclonal antibodies also competed with LPS for binding to S-20-4, suggesting that the peptides bind close to the paratope of the Ab. In order to predict how these peptide mimics interact with S-20-4 compared with its carbohydrate counterpart, one peptide mimic, 4P-8, which is one of the highest affinity binders and shares motifs with several other peptide mimics, was selected for further studies using computer modeling methods and site-directed mutagenesis. These studies suggest that 4P-8 is recognized as a hairpin structure that mimics some O-SP interactions with S-20-4 and also makes unique ligand interactions with S-20-4. In addition, 4P-8-KLH was able to elicit anti-LPS Abs in mice, but the immune response was not vibriocidal or protective. However, boosting with 4P-8-KLH after immunizing with LPS prolonged the LPS-reactive IgG and IgM Ab responses as well as vibriocidal titers and provided a much greater degree of protection than priming with LPS alone.

Cholera is a severe diarrheal disease caused by the bacterium Vibrio cholerae, which is prevalent in many developing countries. According to the World Health Organization, an estimated 120,000 deaths occur from cholera worldwide each year. Although the establishment of clean water and food, adequate personal hygiene, and sanitation are the long term solutions for cholera control, in the short term, sufficient improvements in these areas are difficult to achieve in most cholera-endemic areas. Meanwhile, there is an urgent need for improved vaccines as an additional public health tool for cholera prevention (1). Since the affected population is very poor, development of an inexpensive vaccine is most desirable.

Currently available vaccines for cholera are based on killed, whole cell, or live attenuated formulations. The killed, whole cell (WC and WCrBS) vaccines provide only partial short term protection. The live, attenuated strains, such as CVD 103-HgR, have greatly improved efficacy in North American volunteers but have not been proven efficacious in field trials (1, 2). However, field trials of the live attenuated strain Peru-15 have proven to be safe, immunogenic, and efficacious (3). An approach that could serve as an alternative or be used to augment existing vaccines is to develop defined component formulations. One surface antigen to consider as a potential component of such a formulation is LPS2 or portions thereof.

LPS is composed of lipid A, core polysaccharide, and O-SP. Although there are over 200 serogroups of V. cholerae that have been identified on the basis of O-SP, only O1 and O139 are known to cause major epidemics. V. cholerae O1 strains are divided into two biotypes, classical and El Tor. Each of these two biotypes is further divided into serotypes Ogawa, which has an O-methyl group at the 2-position in the nonreducing terminal perosamine unit of O-SP, and Inaba, which has a free hydroxyl at this position (4).

LPS is known to induce protective Abs in animal models, and an anti-LPS response exhibited as a vibriocidal Ab response is the best correlate to protection in individuals who have been vaccinated or otherwise exposed to V. cholerae infection (5). Although native LPS is immunogenic, it is highly toxic due to the lipid A component. Systemic LPS leads to inflammation, multiple organ failure, shock, and potentially death. The O-SP by itself is poorly immunogenic (6). However, LPS can be detoxified, coupled to a protein carrier, and used as an immunogen to recruit T-cell help (7, 8). Another approach to overcome the problems associated with administration of LPS is to couple synthetic O-SP to a protein carrier. Synthetic O-SP conjugated to BSA has been shown to elicit a protective immune response in mice (9). These results, as well as the availability of mAbs S-20-4 and A-20-6, which are protective Abs specific for V.

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2 The abbreviations used are: LPS, lipopolysaccharide; Ab, antibody; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin.

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*V. cholerae* Ogawa O-SP (10), suggested that it might be possible to identify peptides that could specifically mimic the protective Ogawa epitope. Such peptide mimics have been proposed as potential surrogate antigens of carbohydrates for vaccine development against several microorganisms (11–21) and tumors (22–25). Indeed, because of their ease of production and their intrinsic immunogenic properties, peptide mimotopes may have several advantages over incorporating complex carbohydrate haptons issued from bacterial cell cultures or low yielding syntheses. Phage display libraries are commonly used to identify peptide mimics of various surface carbohydrate structures of pathogenic bacteria (26). A phage display library in which random peptides are expressed on the surface of filamentous phage provides a source of structurally diverse mimics (27). Panning and screening phage display libraries with mAbs has identified peptide mimics of surface carbohydrate structures of several pathogens and tumors that have also been used as immunogens to elicit cross-reactive carbohydrate-directed responses (11, 13–16). However, only a few peptide mimics of surface carbohydrate structures have been shown to induce a protective immune response (17–23).

Peptides identified by panning phage display libraries using an mAb directed against a carbohydrate ligand can mimic the ligand in either a structural or functional manner with respect to Ab recognition. Structural mimicry, the goal of these experiments, is achieved if the peptide and carbohydrate ligands are bound by similar groups on the Ab or any other molecule that the ligands bind. Structural mimics are generally thought to have the best chance at eliciting a robust immune response that will be directed against the mimicked antigen. Structural mimicry has been observed in the complexes of a camel heavy-chain Ab against lysozyme, where parts of some residues of the Ab mimic parts of the sugar substrate of lysozyme (28) and in the complex of porcine pancreatic α-amylase with its proteinaceous inhibitor, where several specific hydrogen-bonding and hydrophobic interactions with the carbohydrate substrate are mimicked by the inhibitor (29). However, if the peptide and carbohydrate ligands are bound by different groups on the Ab, then the nature of the mimicry is functional. The crystal structure of the Fab fragment of the Ab specific for the cell surface O-SP of the pathogen *Shigella flexneri* serotype Y in complex with O-SP pentasaccharide (30) and a peptide mimic identified from a phage display library revealed that the modes of binding of the two ligands differ considerably (31). Thus, the mode of mimicry in this case is functional. It is not yet known whether this peptide mimic can elicit anti-LPS Abs.

Although peptide mimics possess unquestionable advantages as immunogens, the success of this strategy depends on the ability of the peptides to mimic oligosaccharide epitopes. A better understanding of the molecular basis of peptide-carbohydrate mimicry could help the rational design of potent peptide mimotope-based vaccines or allow improvements to be engineered into existing peptide mimics. Such an understanding could also help to further determine whether structural mimicry is a prerequisite to elicit cross-reactive, carbohydrate-directed Abs or to elicit a protective immune response.

In this study, we have identified six peptide mimics that show significant binding to an anti-O-SP mAb in addition to the selecting anti-O-SP mAb, suggesting that these peptides are potential structural mimics. Mutagenesis of selected residues of a peptide mimic was carried out to determine what residues are important in binding to the selecting Ab. Based on the previously determined crystal structure of the selecting Ab complexed with synthetic fragments of O-SP, computer modeling studies were focused on predicting how one selected peptide mimic interacts with the Ab compared with its carbohydrate counterpart. These findings were related to the experimentally determined efficacy of the Ab.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Murine ascites fluid containing S-20-4 and A-20-6 mAbs were gifts from Dr. J. M. Fournier and Dr. Farida Nato (Pasteur Institute, France). S-20-4 mAb (IgG1) was purified using a T-gel column (Pierce) or A column (Millipore). A-20-6 mAb (IgG1) was purified using a protein A column. Purity of mAbs was determined by Coomassie stain of SDS-PAGE, and Ab was quantitated by Bradford assay (Bio-Rad). S-20-4 and A-20-6 mAbs were further standardized by their ability to bind to Ogawa LPS, which was quantitated by ELISA.

**Screening Phage Display Libraries**—Phage display libraries in which 12 (X12) or seven (X7) amino acid random peptides or seven amino acid random peptides constrained by two cysteine residues (CX,C) displayed on the minor coat protein (pIII) of M13 phage were purchased from New England Biolabs. Phage were incubated with the selection Ab in solution, followed by affinity capture of the Ab-phage complexes onto protein A/G-agarose beads as described by the manufacturer. Briefly, 50 μl of protein A/G-agarose (catalog number 53132; Pierce) were blocked for 1 h with blocking buffer (0.1 M NaHCO3, 5 mg/ml BSA, pH 8.6) and washed with TBST (50 mM Tris-HCl, 150 mM NaCl, 0.5% Tween 20). 1 μg of S-20-4 or A-20-6 and 2 × 1014 phage virions from a given phage display library were diluted with TBST to a final volume of 200 μl and incubated at room temperature for 20 min. The phage-Ab mixture was then transferred to the washed resin, incubated at room temperature for 15 min, and washed with TBST to remove the unbound phage. In some pannings, phage bound to resin were eluted with 1 ml of glycine HCl (pH 2.2) and rapidly neutralized with 150 μl of 1 M Tris-HCl (pH 9.1). Eluted phage were titered to determine the number of phage captured by the Ab-coated A/G-agarose beads and were amplified by infecting *Escherichia coli* strain ER2738 (F' proA B lacIq ΔlacZΔM15 zfd:Tn10(TetR))/θhiuA2 glnV Δ(lac-proAB) thi-1 Δ(hsdS McriB)5; New England Biolabs). In other pannings, phage bound to resin were (nonelution) directly added to a culture of *E. coli* strain ER2738 for amplification. Amplified phage were purified by precipitating the phage with polyethylene glycol/NaCl (20% polyethelene glycol, 2.5 M NaCl). Three or four rounds of panning were carried out until a large enrichment of phage was seen, indicated by a large increase in phage titer. After the large enrichment, phage plaques were randomly picked, and the gene III region was sequenced by automated dideoxyligonucleotide sequencing as described below.

**DNA Sequencing of Phage Gene III Peptide Motif Regions**—To determine the DNA sequence of the peptide displayed on the pIII protein, phage clones were expanded by propagating them
in 2-ml cultures of logarithmically growing ER2738 for 4–5 h at 37 °C. To recover the phage, the bacteria were removed from the culture suspension by a brief centrifugation (10,000 × g for 10 s). 1 ml of the culture supernatant was mixed with 400 μl of 20% polyethylene glycol, 2.5 x NaCl solution, and the mixture was centrifuged (10,000 × g) for 10 min. The pellet containing the phage was resuspended with 100 μl of iodide buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 4 mM NaI) and 250 μl of absolute ethanol and centrifuged for 10 min. The phage DNA was washed with 70% ethanol, dried, and resuspended in 30 μl of TE buffer. 100 μg of the phage DNA was subjected to dideoxyoligonucleotide termination reactions using a DNA sequencing kit (PerkinElmer Life Sciences) and −96 sequencing primer (New England Biolabs). The sequence of the gene III region was obtained by running the above reaction products through an automated DNA sequencer from Applied Biosystems (Foster City, CA).

Phage Binding Assays—ELISA was used to assess the binding of phage to mAbs. 96-well microtiter plates were coated with serial dilutions of S-20-4, A-20-6, or IgG1 isotype control Ab (Southern Biotech) in 0.1 M Na₂CO₃ (pH 8.6) overnight at 4 °C. Plates were blocked with blocking buffer containing 5 mg/ml BSA. Approximately 10⁵ purified phage particles in 100 μl of TBST were placed in each of the wells and incubated for 1 h at room temperature. The plates were washed six times and incubated with 100 μl of horseradish peroxidase-conjugated anti-M13 Ab diluted 1:3300 in TBST (Amersham Biosciences) for 1 h, followed by washing six times and the addition of 100 μl of the colorimetric substrate 3,3′,5,5′-tetramethylbenzidine (Sigma) until color developed. The reaction was stopped by the addition of 100 μl of 3 M HCl, and absorbance was measured at 450 nm with a kinetic microplate reader (Molecular Devices).

Competitive Inhibition—For the phage/LPS competition assay, microtiter plates were coated with 0.05 μg/ml mAb S-20-4, as above. A constant amount of phage was added together with different concentrations of LPS. V. cholerae O1 Ogawa LPS was a gift from Dr. S. Kondo (Josai University) or isolated using an LPS purification kit from Intron Biotechnology. E. coli LPS was from Sigma until color developed. The reaction was stopped by the addition of 100 μl of 3 M HCl, and absorbance was measured at 450 nm with a kinetic microplate reader (Molecular Devices).

Site-directed Mutagenesis of Gene III Peptide Motif Region—Each mutation was created by inserting DNA with the desired mutation between the EagI and BglII sites of the vector M13KE (New England Biolabs). Briefly, PCR was carried out using 50 pmol of oligonucleotide containing a BglII site (CTTGGTATGACTCTCTCAAAAAATAGCTACC) and 50 pmol of a 70–90-nucleotide oligonucleotide containing an EagI site and the desired mutation with PfuTurbo Cx Hotstart DNA polymerase (Stratagene) using single-stranded phage DNA as the template. The resulting ~2-kb fragment was digested with EagI and BglII (New England Biolabs) and purified with a PCR purification kit (Qiagen) to remove the digested ends. M13KE replicative form DNA was also digested with EagI and BglII, and the resulting ~5-kb vector fragment was gel-purified. 45 ng of the ~2-kb insert was ligated with 20 ng of the ~5-kb vector fragment at a 6:1 molar ratio. After electroporation into the bacterial strain ER2738, each mutation was confirmed by DNA sequencing as above.

Computational Docking of Peptide Ligand—Crystal structure of S-20-4/O-SP (32) was manually examined for binding site geometry, hydrophobicity, electrostatics, amino acid side chain chemistry, and backbone and side chain mobility using interactive molecular graphics GRASP (33) and PyMol (34) algorithms. Based on phage selection, Ab cross-reactivity, immunological data, and alanine scan binding data, peptide 4P-8 was used in all further ligand docking experiments. An initial peptide conformation for the 4P-8 sequence was obtained by using a BLAST search of the protein structure data base (available on the World Wide Web; search sequence, NHNYPPLSLTF; data base = Protein Data Bank; expectation setting of 20,000 and PAM30 as the scoring matrix). No exact matches for the 12-mer probe sequence of 4P-8 were initially found. Thus, structural analogs of 4P-8 represented in the Protein Data Bank were identified by requiring sequences to contain the P-P covalent dimer and containing amino acids that, if not identical, were at least isosteres or similar in chemical nature to 4P-8 side chains (e.g. Gln for His or Met for Leu). A sequence was identified from Protein Data Bank structure 1RC6 (A. A. Fedorov, E. V. Fedorov, R. Thirumuruhan, U. A. Ramagopal, and S. C. Almo; Protein Data Bank code 1RC6) (112–119, PPGLMTTF, referred to here as 1rc6-P) as having sequence similarity to 4P-8 for the eight carboxyl-terminal amino acids. The 1rc6-P crystallographic conformation and bond geometries were checked using PROCHECK (36), and all were found to lie within regions of normal protein geometry. The 4P-8 peptide sequence was threaded onto 1rc6-P in PyMol to make the 4P-8 sequence adopt a peptide conformation similar to a peptide reported for an x-ray-determined structure. The remaining four amino-terminal amino acids were idealized by requiring all trans-peptide bond configurations. This structural motif and alternative peptide conformations, arrived at by the threading of the full 12-mer sequence (NHNYPPLSLTF) onto similar peptides observed in similar Ab-peptide complexes (37), provided a range of similar hairpin peptide configurations. These hairpin structures were used in the manual docking of the 4P-8 sequences to the paratope of S-20-4. Peptide conformers were adjusted manually to bring all of the φ-ψ angles into the most favorable regions of the Ramachandran diagram while trying to maximize the peptide-Ab chemical complementarity (hydrophobic peptide side chains and charged side chains in close proximity to hydrophobic pockets and oppositely charged side chains of the Ab, respectively) and while trying to account for the apparent preferences identified in the alanine scan data. These manually fitted 4P-8 models were used to seed 10⁵ conformations using distance geometry constraints in the program DGEOM. The constraints used to limit the search space included support for three intrapeptide hydrogen bonds, side-chain chemical complementarity in binding His-2 local to the heavy chain (l) Asp-33 and Leu-9 of 4P-8 or mutation L9F to a shallow hydrophobic patch in the O-SP binding region made by Ab residues h-Phe-50 and light chain (l) Thr-93 and Leu-10 of 4P-8 localized in the more size- and shape-restrictive pocket made by residues l-Tyr-34, l-Trp-93, and l-Trp-98 and h-Phe-50, h-His-99, h-Tyr-101, and h-Ala-102 (Table 2). In addition to
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Leu, the larger amino acids Phe and Ile were also used at position 9 for 4P-8 modeling. It was believed that Phe was a conservative choice for size tolerance for this position, as was indicated from binding data showing that peptides with Phe, Leu, or Ile in this position bound with approximately the same affinity. All resulting DGEOM-generated models were clustered and culled to representative individual structures with root mean square deviation values of 3–4 Å using the application COMPARE (a gift from Dr. Jeffrey Blaney). These conformations were further filtered via PROCHECK and PyMol and rejected when any steric clashes occurred with the Ab or when unrecoverable bond geometry was detected (via PyMol sculpture) (34). The types of peptide-Ab interactions and potential antigen mimicry for the best bound cluster representative of the 4P-8 peptide model was tabulated using the program HBPLUS (38, 39) and listed in Table 2.

Ab Binding to 4P-8-BSA—Synthetic peptides representing 4P-8 (NHNYPLSLLTFCAGGC) were chemically synthesized and conjugated to BSA or KLH by the macromolecular facility at the University of Colorado. Binding of S-20-4, A-20-6, IgG isotype control Ab, and polyclonal anti-LPS serum obtained from LPS-immunized mice to 4P-8-BSA was determined by ELISA as below.

Immunization of Mice and Sera Collection—6-Week-old female BALB/c mice (Charles River Laboratories), four in each group, were injected intraperitoneally with 50 μg of 4P-8-KLH in RIBI adjuvant (Sigma) three times, 2 weeks apart. Mice injected with 50 μg of KLH in RIBI served as a control. Blood collection via retro-orbital sinus/plex was performed 10 days after every injection, which represent primary (1'), secondary (2'), and tertiary (3') sera or preimmune sera (P).

In an alternative immunization scheme, 6-week-old female BALB/c mice, four in each group, were injected intraperitoneally with 9 μg of V. cholerae Ogawa LPS twice, 2 weeks apart, and then injected with 50 μg of 4P-8-KLH or KLH in RIBI adjuvant (Sigma) twice, 2 weeks apart. Mice were bled periodically up to 292 days. Retro-orbital plexus bleeding yielded 80–120 μl of blood, which provided 50% of that volume as serum after processing. Resulting sera from individual mice within a group were pooled and stored at 4 or −20 °C until use.

Detection of Anti-LPS and Anti-4P-8 Immune Responses by ELISA—Microtiter plates were coated with 5 μg/ml V. cholerae LPS in 0.1 M Na2CO3/NaHCO3, pH 9.5, or 5 μg/ml 4P-8-BSA in 0.1 M NaHCO3, pH 8.6. Plates were blocked with blocking buffer containing 1% fish gelatin (BioFX Laboratories, Inc.) for 2 h. Serial dilutions of serum were added to each plate and incubated at 4 °C overnight. After washing, bound Abs were detected by horseradish peroxidase-conjugated goat antimouse IgG or IgM (Southern Biotech). End point titers were defined as the reciprocal of the Ab 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. The OD values of the preimmunization sera were averaged and then doubled. This value was subtracted from the OD of all of the wells containing titrations of the pooled serum samples. Data represent the means ± S.D. of triplicate values and are representative of three independent experiments.

Detection of Vibriocidal Antibody by the Microtiter Method—A microtiter test was performed as previously described (40). The vibriocidal assay is a well accepted in vitro assay for assessing the capability of V. cholerae anti-LPS Abs to facilitate complement-mediated killing. This assay measures the metabolic activity of the bacteria following treatment with antisera and complement. V. cholerae classical, Ogawa strain O395 was inoculated into 2 ml of APW (alkaline/peptone/water; 1.0% peptone, 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. Cold phosphate-buffered saline was applied to the plate, gently swirled to resuspend the bacteria, and then transferred to a 15-ml conical tube. The A600 of the bacterial suspension was adjusted to 0.9 with phosphate-buffered saline to adjust the bacterial concentration to ~1 × 108/ml. Seven volumes of cold phosphate-buffered saline, 2 volumes of guinea pig complement (Sigma), and 1 volume of bacterial suspension were mixed in a chilled tube and kept on ice for 20 min. 50 μl of heat-inactivated, diluted mouse sera from various treatment groups was placed in a round bottom sterile microtiter plate and serially diluted in 1:2 increments in phosphate-buffered saline. 50 μl of complement-treated bacteria were added to each well, and the plate was incubated uncovered in a humidified chamber for 2 h at 37 °C. 25 μl of an aqueous solution containing 1 volume of 1.0% neotetrazolium chloride (ICN Biochemicals) and 9 volumes of 2.7% sodium succinate (ICN Biochemicals) was added to each well and incubated uncovered at room temperature for 15 min. The plate was then placed in a humidified chamber at 4 °C overnight, and the intensity of violet color, after overnight incubation, indicating the presence of live metabolically active Vibrios, was examined. The vibriocidal titer was reported as the reciprocal of the Ab dilution with the lowest concentration that caused bacterial killing, as indicated by a clear well. Data represent the means ± S.D. of triplicate values and are representative of three independent experiments.

Infant Mouse Challenge—The infant mouse challenge model for cholera was used to assess the protective quality of anti-Ogawa O-SP Abs in vivo (41). 4–5-Day-old CD-1 infant mice were orally administered, by gavage, 25 μl of virulent V. cholerae O1 Ogawa strain O395 that contained 9 × 107 bacteria or ~300 LD50, which had been cultured overnight in LB medium at 37 °C and then mixed 1:1 with antisera from the ninth bleed or normal mouse serum. Challenged mice were kept at 30 °C and monitored after challenge until the termination of the assay.

RESULTS

Selection of S-20-4- and A-20-6-specific Phage Clones—X12, X7, and CX, C phage display libraries were biopanned against mAb S-20-4, which is specific for the protective epitope of Ogawa LPS (10), using low pH elution or nonelution. After enrichment, 12–21 plaques were randomly picked from each panning and sequenced. 19 unique sequences that could bind to S-20-4 were identified from four different pannings against S-20-4 (Table 1). Most of these sequences were identified multiple times. Although no single motif was present throughout all of the peptides that were identified, several motifs were
found to be present multiple times. Peptide sequences SH(K/R)(L/I) and HXPRXH were identified from the X₁ library under low pH elution and nonelution conditions, respectively. These two motifs were also identified from the X₁₂ library under nonelution conditions. The sequence HXXPPXXLL was found in many, but not all six, peptide mimics identified from the X₁₂ library under low pH elution. 4P-8, which also shares the HXPPXXLL sequence, was the most dominant sequence (12 of 21) identified from the X₁₂ library under low pH elution conditions. 4P-8 was also identified under the nonelution condition.

A-20-6, which is specific for the same protective epitope as S-20-4, was also used to pan X₁₂, X₇, and CX-C phage display libraries using low pH elution or nonelution. 14 unique sequences that bind to A-20-6 were identified from four different pannings with A-20-6. Almost all of the unique peptide sequences shared the common sequence motif, W(S/T)P, regardless of the phage display library or the elution conditions used (data not shown).

Binding Properties of Phage Expressing pIII Peptide Mimics of Ogawa O-SP—Each of the sequenced unique phage clones was analyzed by ELISA to determine the degree of binding to Ab used for screening (S-20-4 or A-20-6), IgG1 isotype control, and BSA. Of the unique sequences identified from different phage display libraries using different elution conditions, 14 were capable of specifically binding to S-20-4, based on negligible binding to the IgG1 isotype control (Table 1). A phage clone SP-1 that has an HPQ motif, which specifically binds to streptavidin (47), was used as a control phage and was not capable of binding to S-20-4.

Phage clones that were selected from the S-20-4 panning were also tested for their ability to bind to the related mAb A-20-6. Only the six phage clones that were identified from the X₁₂ phage display library panned by low pH elution had the ability to bind A-20-6. This binding was of similar affinity as that for S-20-4 binding at the immobilized mAb concentration used in Table 1 (0.5 μg/ml). Thus, the six amino acid residues that are not conserved between S-20-4 and A-20-6 (Fig. 1) (10) are not critical for the interaction with these six phage clones, suggesting that the peptides displayed on these phage could potentially represent structural mimics. Other phage clones from the S-20-4 panning did not show any affinity to A-20-6 and are thus likely to express peptides that are functional mimics.

14 unique sequences were identified from the A-20-6 panning, but all showed low affinity for the selecting mAb. An A-20-6 mAb concentration of greater than 8 μg/ml was needed to detect phage binding by ELISA, compared with 0.5 μg/ml mAb to detect phage selected by S-20-4. The A-20-6-selected phage were also tested for their ability to bind to the related mAb S-20-4. None of the phage identified in the A-20-6 panning showed significant binding to S-20-4 (data not shown), and they were not studied further.
Competitive Inhibition by LPS—Each of the six phage clones that was capable of binding to both S-20-4 and A-20-6 was tested for the ability to compete against binding with V. cholerae Ogawa LPS. All six phage clones were capable of competing with LPS (Fig. 2), suggesting that these phage clones encode peptide sequences that bind to, or close to, the LPS binding site of S-20-4. Unlike V. cholerae Ogawa LPS, E. coli LPS did not inhibit phage binding to S-20-4 (data not shown). An irrelevant phage clone, SP-1, which does not bind to S-20-4, was used to indicate the level of background binding.

Alanine Scanning and Site-directed Mutagenesis of Phage Expressing Peptide 4P-8—Out of the potential structural mimics identified, 4P-8 was chosen for further analysis, since it was the most dominant sequence (12 of 21) identified from the X12 library under low pH elution conditions and showed the highest degree of binding to S-20-4 and A-20-6. Each residue of 4P-8 expressed by the phage was altered to alanine. Interestingly His-2, Pro-5, Pro-6, Leu-9, and Leu-10, which are conserved among other peptide mimics, are all critical for binding to S-20-4 and A-20-6 based on the results of the alanine scanning (Fig. 3). Additional replacement of His-2 with isosteric amino acids Glu or Gln nearly abolished binding to S-20-4 or A-20-6, but substitution of positively charged Arg retained nearly full binding activity. Thus, a positive charge at position 2 seems to be an important component for binding. Replacement of Asn-3 with Ala, Ser, or Lys did not have a major effect on binding. Thus, diverse residues are tolerated at this position. Pro-5 and Pro-6 have critical roles in binding that may be due to the conformational restrictions they make rather than hydrophobicity, since replacing Pro-5 or Pro-6 with Ala at either position or Val at either position, or both together, resulted in loss of binding. Although replacing Leu-9 with Ala resulted in a dramatic drop in binding, replacing Leu-9 with the very similar residue Ile or another hydrophobic but very different residue, Phe, retained binding. In contrast, replacing Leu-10 with Phe or Ile did not retain binding, suggesting additional contributions by this residue and that Leu is very specific for this position.

Pharmacophore Features of Crystallographic Structure for Docking—In order to better understand the type of ligand-Ab interactions available for peptide mimicry of O-SP, many computer models were constructed. These models incorporated the structural information seen in the O-SP/S-20-4 complex, the S-20-4 preferred peptide sequences displayed on phage, the alanine scan results, and the site-directed mutagenesis binding studies. A single orienta-
tion for the 4P-8/S-20-4 interaction model was able to reproduce many of the O-SP interactions with no abnormal distortions to peptide geometry and conformations while bringing into register additional chemical Ab-ligand complementarity not possible with the O-SP ligand (see Table 2). This mode of binding formed a set of interactions not seen in the O-SP ligand (Table 2 and Fig. 4, A and B). We sought a model that would describe the sequence specificity indicated from the mutational analysis and the phage display results. To this end, conformations of publicly available crystallographically determined peptide-Ab structures were reviewed for examples and possible starting structural motifs for our modeling experiments (37). Several threadings of the 4P-8 sequences on these peptide conformations were used to investigate the likelihood of the 12-mer 4P-8 peptide adopting similar conformational configurations while helping to explain S-20-4 specificity for the 4P-8 sequence. Our “best” model of 4P-8 incorporates, by subjective means, 1) known peptide structural constraints represented in the conformations adopted by similar peptide sequences retrieved from the Protein Data Bank as well as common side chain rotomers, 2) amino acid preferences from mutagenesis and phage-display results, and 3) amino acids from positions 8–12 playing a critical role in binding as demonstrated that 4P-8 does not have to be in the context of MM, main chain to main chain atom interactions. 

Ab Binding to 4P-8-BSA—The 4P-8 amino acid sequence was chemically synthesized and conjugated to BSA or KLH to further characterize the properties of this sequence and to immunize mice, since the peptides are a minor component in the context of PIII displayed on the phage (<5 molecules/phage). As shown in Fig. 5, S-20-4 and A-20-6 mAb bound the synthetic 4P-8-BSA but not IgG1 isotype control Ab, demonstrating that 4P-8 does not have to be in the context of

| Ab chain, residue, atom | Ogawa O-SP contact atom (interactions: S-CH = 10, M-CH = 7) | 12-mer modeled 4P-8 peptide contact atom (interactions: SS = 6, MS = 4, SM = 3, MM = 1) |
|------------------------|---------------------------------|---------------------------------|
| h-Thr-31 O             | C12                             | His-2 Cε1                       |
| h-Tyr-32 Cε2           | O10                             | His-2 Nε1                       |
| h-Asp-33 Cβ            | O10                             | His-2 Nε1                       |
| h-Asp-33 N             | O3                             | Phe-9 Cε2                       |
| h-Phe-50 Cε1           | O3                             | Phe-9 Cε2                       |
| h-His-99 C             | O10                             | Phe-9 Cε2                       |
| h-His-99 Cβ            | O10                             | Phe-9 Cε2                       |
| h-His-99 Cε1           | C8                             | Phe-9 Cε2                       |
| h-His-99 Cγ            | C8, O3                          | Phe-9 Cε2                       |
| h-His-99 Nε1           | C3                             | Leu-10 Cε2                      |
| h-His-99 O             | C10, C9                         | Leu-10 Cε2                      |
| h-Tyr-101 C            | C6, O9                          | Leu-10 Cε2                      |
| h-Tyr-101 Cα           | C10, C6, C9, O9                 | Leu-10 Cε2                      |
| h-Tyr-101 Cβ           | C11                             | Leu-10 Cε2                      |
| h-Tyr-101 N            | C10, C9                         | Leu-10 Cε2                      |
| h-Asp-33 Cβ            | C6, C8, O3, O9                  | Leu-10 Cε2                      |
| h-Asp-33 N             | C4, C6, C8, C9, O9              | Leu-10 Cε2                      |
| h-Asp-33 Cε1           | C1                               | Leu-10 Cε2                      |
| i-Tyr-34 Cε3           | C1, C22                         | Leu-10 C ε2                     |
| i-Tyr-34 OH            | C22, C23, O1, O23               | Leu-10 C ε2                     |
| i-Val-93 Cε1           | O23                             | Leu-10 C ε2                     |
| i-Val-93 Cε2           | O23                             | Leu-10 C ε2                     |
| i-Val-93 Cε3           | O23                             | Leu-10 C ε2                     |
| i-Ser-95 Cα            | O30                             | Leu-7 O                         |
| i-Ser-95 Cβ            | O30                             | Leu-7 O                         |
| i-Ser-95 Cγ            | O30                             | Leu-7 O                         |
| i-Ser-95 O             | O30                             | Leu-7 O                         |
| i-Ser-95 O             | O30                             | Leu-7 O                         |
| i-Thr-96 Cε1           | O30                             | Leu-7 O                         |
| i-Thr-96 Cε2           | O30                             | Leu-7 O                         |
| i-Thr-96 Cε3           | O30                             | Leu-7 O                         |

| Atoms          | Interactions | Residues and atoms | Interaction |
|---------------|--------------|--------------------|-------------|
| S-CH           | M-CH         | His-2 Cε1          | MS          |
| S-CH           | M-CH         | His-2 Nε1          | SS          |
| S-CH           | M-CH         | His-2 Nε1          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |
| S-CH           | M-CH         | Phe-9 Cε2          | SS          |

Ad heavy chain and light chain are indicated by h and i, respectively. Atoms such as carbon, hydrogen, nitrogen, and oxygen are indicated by C, H, N, and O, respectively.

Ab to carbohydrate interactions. S-CH and M-CH, side chain and main chain to carbohydrate atom interactions, respectively.

Ab to 4P-8 peptide interactions. SS, side chain to side chain atom interactions; MS, main chain to side chain atom interactions; SM, side chain to main chain atom interactions; MM, main chain to main chain atom interactions.

Ligand binding site amino acid side chains that could potentially interact with the modeled 4P-8 peptide ligand and have rotational freedom that do not break or create intra-Ab contacts but could potentially form intermolecular contacts and bonds with a docked peptide ligand. The number of rotamers considered is noted in parentheses as follows: 1-3, 5 molecules/atom, respectively.

| Atom | Interaction |
|------|-------------|
| Cε1  | MS          |
| Cε2  | SS          |
| Cε3  | SS          |

* Ab heavy chain and light chain are indicated by h and i, respectively. Atoms such as carbon, hydrogen, nitrogen, and oxygen are indicated by C, H, N, and O, respectively.

* Ab to carbohydrate interactions. S-CH and M-CH, side chain and main chain to carbohydrate atom interactions, respectively.

* Ab to 4P-8 peptide interactions. SS, side chain to side chain atom interactions; MS, main chain to side chain atom interactions; SM, side chain to main chain atom interactions; MM, main chain to main chain atom interactions.

* Ligand binding site amino acid side chains that could potentially interact with the modeled 4P-8 peptide ligand and have rotational freedom that do not break or create intra-Ab contacts but could potentially form intermolecular contacts and bonds with a docked peptide ligand. The number of rotamers considered is noted in parentheses as follows: 1-3, 5 molecules/atom, respectively.

* NI, no interactions.

**Peptide Mimics of V. cholerae LPS**
Peptide Mimics of V. cholerae LPS

pIII to bind to S-20-4 or A-20-6. In addition, polyclonal anti-LPS IgG and IgM Abs in serum obtained from mice immunized with LPS bound to 4P-8-BSA, whereas preimmune sera did not. None of the Abs bound to plates coated with BSA (data not shown). These data further demonstrate that anti-LPS Abs other than the selecting Ab specifically recognize the 12-amino acid 4P-8 peptide sequence.

**Immune Responses Elicited by 4P-8**—In order to determine whether 4P-8 could elicit cross-reactive anti-LPS Abs, 4P-8-KLH plus RIBI adjuvant were injected into mice at 2-week intervals, and serum was collected 10 days after each injection (see “Experimental Procedures”). Each of the serum samples collected from 4P-8-injected mice was tested by ELISA for the presence of anti-4P-8 IgG and IgM (Fig. 6, A and B). Serum collected from 4P-8-KLH-injected mice showed anti-4P-8 IgG and IgM Ab titers significantly higher than for preimmune sera or serum collected from KLH-immunized mice.

Each of the serum samples was also tested by ELISA for the presence of anti-LPS IgM and IgG Abs (Fig. 6, C and D). Anti-LPS IgG and IgM Ab titers were significantly higher than for preimmune sera or serum collected from KLH-immunized mice. Since 4P-8 elicits an anti-LPS immune response, 4P-8 is a peptide mimotope of V. cholerae Ogawa O-SP. The anti-LPS IgM response (Fig. 6C) is comparable with the anti-4P-8 IgM response (Fig. 6A). However, there was only a marginal increase in the IgG anti-LPS immune response with every peptide injection (Fig. 6D) compared with the anti-4P-8 IgG Ab response (Fig. 6B). Unlike the anti-LPS IgG response, there were increases in the anti-4P-8 IgG with the second and third 4P-8-KLH injections.

Serum collected from mice immunized with three injections of 4P-8-KLH was tested for the presence of vibriocidal Abs and for its ability to protect infant mice from a pathogenic V. cholerae challenge. None of these pooled serum samples showed any vibriocidal activity (data not shown). Consistent with this finding, pooled tertiary serum from these mice did not provide any protection against a pathogenic V. cholerae challenge, and survival curves were comparable with the negative control preimmune serum (data not shown).

**Immune Responses Induced by a Priming and Boosting Immunization Scheme**—To determine the effectiveness of 4P-8 as a secondary immunogen, mice were primed with LPS prior to boosting with 4P-8-KLH. Serum collected from this immunization scheme was tested for anti-LPS Abs by ELISA. The first LPS injection failed to induce a robust IgG or IgM Ab response, as indicated by low end point titers. The second LPS injection increased the anti-LPS IgM and IgG end point titer to 1:12,800 in both groups of mice. The two injections of 4P-8-KLH did not further increase the titers. However, the anti-LPS IgM or IgG response in the group of mice that were boosted with KLH
diminished rapidly, whereas mice that were boosted with 4P-8-KLH retained stable, long lasting LPS-reactive IgG and IgM responses (Fig. 7, B and C).

Serum collected from the priming and boosting immunization scheme was tested for the presence of vibriocidal Abs by vibriocidal assay. In agreement with the humoral response, priming with LPS and boosting with 4P-8-KLH did not increase the vibriocidal titers but did extend the longevity of the titers when compared with priming with LPS and boosting with KLH (Fig. 7D). The vibriocidal titer was reflected in the ability to protect infant mice from V. cholerae challenge. Serum collected from mice primed with LPS and boosted with 4P-8-KLH was 100% protective in infant mice challenged with ~300 LD<sub>50</sub> V. cholerae. Serum collected from mice primed with LPS and boosted with KLH protected only 20% of the infant mice (Fig. 7E).

**DISCUSSION**

We identified peptides that can bind to mAbs S-20-4 and A-20-6, which are specific for the terminal perosamine of the Ogawa O-SP. Of all of the unique peptides identified from different phage display libraries under different elution conditions, only six sequences bound both S-20-4 and A-20-6. The specificity of these six peptides was demonstrated by their ability to inhibit binding of S-20-4 by V. cholerae Ogawa LPS. Each of these sequences has one or two aromatic residues. The presence of aromatic residues is often a characteristic of peptides that mimic carbohydrates. This may be because aromatic amino acids resemble sugar moieties in their size and cyclic shape. In addition, each of these sequences contain many amino acid side chains that are hydrophobic and can form hydrogen bonds, which are also commonly seen in carbohydrate mimics (42).

The 4P-8 peptide sequence was chosen for further analysis by mutagenesis and computer modeling to predict the nature of its interaction with S-20-4 as compared with its carbohydrate counterpart. A study by Wang et al. (10) has shown that the terminal O-SP monosaccharide is the primary antigenic determinant. Additional perosamine residues contribute only marginally to the binding affinity and specificity. The crystal structure of the murine Fab S-20-4 complexed with synthetic Ogawa O-SP monosaccharide has been determined. It shows that a complementary water-excluding hydrophobic interface and six Ab-antigen hydrogen bonds are crucial for carbohydrate recognition. Four of the six hydrogen bonds are formed between the hydroxyl groups of the terminal sugar residue and h-Asp-33 and h-His-99 (32). A-20-6 is also a protective Ab that is specific for the same terminal monosaccharide as S-20-4. These two mAbs have identical light chains, and only six amino acids differ in the heavy chains (10). In addition, amino acids important for hydrogen bonding of S-20-4 to O-SP monosaccharide epitopes in the crystal structure are conserved in both mAbs (Fig. 1). Thus, it is likely that these two mAbs make identical hydrogen bonds with O-SP monosaccharide (32).

An important feature revealed by the crystallographic study is a
Peptide Mimics of V. cholerae LPS

A, time line for immunization and bleeding of mice. Two groups of mice were primed with two injections of LPS 2 weeks apart and boosted with 4P-8-KLH or KLH 2 weeks apart. The first four bleeds were obtained 10 days after each injection. The fifth, sixth, and seventh bleeds were obtained at 30-day intervals. The eighth bleed was obtained 60 days after the seventh bleed, and the ninth bleed was obtained 90 days after the eighth bleed. Shown are IgM (B) and IgG (C) responses to V. cholerae Ogawa anti-LPS following intraperitoneal immunization with LPS followed by (f) 4P-8-KLH or KLH. End point titers are shown on the y axis. Two groups of mice that received either LPS followed by KLH or 4P-8-KLH represent the x axis. Data represent the means ± S.E. of triplicate values and are representative of three independent experiments. *p < 0.05 (significant) when vibriocidal titers of bleed 3–9 in LPS followed by 4P-8-KLH group are compared with the LPS followed by KLH group. 

D, vibriocidal titers of pooled sera from mice immunized with LPS followed by 4P-8-KLH or KLH. End point titers are shown on the y axis. Groups of mice are indicated on the x axis. *p < 0.05 (significant) when vibriocidal titers of bleed 3–9 in the LPS followed by 4P-8-KLH group are compared with the LPS followed by KLH group. 

End point titers of bleed 3 are shown on the x axis. Data represent the means ± S.E. of triplicate values and are representative of three independent experiments. *p < 0.05 (significant) when vibriocidal titers of bleed 3–9 in LPS followed by 4P-8-KLH group are compared with the LPS followed by KLH group. 

FIGURE 7. Immune responses to the priming and boosting immunization scheme. A, time line for immunization and bleeding of mice. Two groups of mice were primed with two injections of LPS 2 weeks apart and boosted with 4P-8-KLH or KLH 2 weeks apart. The first four bleeds were obtained 10 days after each injection. The fifth, sixth, and seventh bleeds were obtained at 30-day intervals. The eighth bleed was obtained 60 days after the seventh bleed, and the ninth bleed was obtained 90 days after the eighth bleed. Shown are IgM (B) and IgG (C) responses to V. cholerae Ogawa anti-LPS following intraperitoneal immunization with LPS followed by (f) 4P-8-KLH or KLH. End point titers are shown on the y axis. Two groups of mice that received either LPS followed by KLH or 4P-8-KLH represent the x axis. Data represent the means ± S.E. of triplicate values and are representative of three independent experiments. *p < 0.05 (significant) when end point titers of bleed 3–9 in LPS followed by 4P-8-KLH group are compared with the LPS followed by KLH group. 

D, vibriocidal titers of pooled sera from mice immunized with LPS followed by 4P-8-KLH or KLH. End point titers are shown on the y axis. Groups of mice are indicated on the x axis. *p < 0.05 (significant) when vibriocidal titers of bleed 3–9 in the LPS followed by 4P-8-KLH group are compared with the LPS followed by KLH group. 

E, percentage survival of infant mice following oral challenge with live V. cholerae. Five mice were used for each group that received normal mouse serum. Survival curves were drawn using GraphPad Prism 3.0.

hydrophobic pocket that accommodates the 2-O-methyl group on the terminal perosamine of O-SP monosaccharide. This pocket is made of predominantly hydrophobic side chains of both the light chain (Tyr-34, Trp-93, and Trp-98) and the heavy chain (Phe-50, His-99, Tyr-101, and Ala-102). It is ~5 Å in diameter and ~5 Å deep (seen in Fig. 4B) and is at the center of the Ab-antigen interface. The 2-O-methyl group of the terminal perosamine, which is recognized by the hydrophobic pocket, is critical for antigen recognition and accounts for 90% of the maximal binding energy (10, 32).

Preliminary electrostatic calculations for S-20-4, using Poisson-Boltzmann equations for electrostatics and Amber-derived charges, indicate that this pocket is bathed in a negative electrostatic field. This is intensified, with the two amino acid differences of Asp for Val and Asp for Arg found at heavy chain positions 28 and 98, respectively, of the A-20-6 Ab (Fig. 1). The model of 4P-8 complexed with S-20-4 suggests that a positively charged amino acid side chain at position 2 in the 4P-8 peptide would be complementary to the electrostatic nature of the Ab in this region and could form a salt bridge with h-Asp-33. The O-SP makes 10 and 7 contacts with the Ab side-chain and main-chain atoms, respectively. The 4P-8 model makes fewer Ab side-chain contacts and fewer Ab main-chain contacts than O-SP (Table 2). There are fewer hydrogen bonds between modeled 4P-8 and the Ab than O-SP Ab. The 4P-8 model places the highly specific Leu-10 in the hydrophobic pocket (Fig. 4A). With a consideration of more Ab flexibility than we show in this report, a charge relay system can be arranged between h-His-99 and h-Asp-33 and could increase the likelihood that bound peptide side chain interactions in the major pocket could include hydrogen bonding. A more thorough treatment of protein electrostatics and protein flexibility should help address these longer range effects on the pKa of the buried h-His-99, its availability for hydrogen bonding interactions, and how h-His-99 might alter the hydrophobic nature of this pocket in S-20-4 versus A-20-6 mAbs. There is a considerable amount of conformational constraint due to the P-P internal sequence in the 4P-8 peptide. The preference for this portion of the peptide sequence could be in part due to the conformational constraints needed to form the modeled hairpin structure. The hairpin conformation of modeled 4P-8 helps bring the peptide side chains with positive charge (H2 or R2) in close proximity to h-Asp-33 of S-20-4 while allowing the highly specific L10 to interact with the hydrophobic pocket. Additionally, the modeled 4P-8 energetically supports the hairpin bend through stabilizing intrapeptide hydrogen bonds and suggests that further mutations as well as more exotic chemical modifications, through covalent cross linking, could help stabilize a bound conformer. Our ongoing and increasingly more sophisticated computer-aided explorations of peptide mimicry for O-SP-Ab interactions will help in describing some of the possible underlying physical chemistry governing the differential binding of the 4P-8 peptide and its derivatives to S-20-4 and A-20-6.

The effectiveness of 4P-8 in eliciting anti-LPS Abs was investigated. We can call 4P-8 a peptide mimotope, since mice immunized with 4P-8-KLH elicited a significantly higher cross-
reactive Ab response to Ogawa LPS compared with mice immunized with just KLH or preimmune sera. Although the second and third 4P-8-KLH injections did not significantly increase anti-LPS IgG and IgM and anti-4P-8 IgM responses, they significantly increased the 4P-8 IgG response. The specific humoral response elicited following the primary encounters is predominantly composed of relatively low affinity IgM. Later during the primary encounter and during secondary encounter with the same antigen, antigen-specific Abs usually exhibit improved affinity due to affinity maturation and isotype switching with the same antigen, antigen-specific Abs usually exhibit predominantly composed of relatively low affinity IgM. Later humoral response elicited following the primary encounters is they significantly increased the 4P-8 IgG response. The specific increase anti-LPS IgG and IgM and anti-4P-8 IgM responses, second and third 4P-8-KLH injections did not significantly immunized with just KLH or preimmune sera. Although the LPS at a young age. A recent report suggests that vibriocidal living in endemic areas are likely to be exposed to The logic of this immunization regimen is that most individuals a much greater degree of protection than boosting with KLH. and IgM Ab responses as well as vibriocidal titers and provided a much greater degree of protection than boosting with KLH. The logic of this immunization regimen is that most individuals living in endemic areas are likely to be exposed to V. cholerae LPS at a young age. A recent report suggests that vibriocidal Abs are present in 40–80% of individuals 10–15 years of age in Bangladesh and are associated with protection against cholera.

As an alternative to the use of 4P-8 as a primary immunogen, we investigated whether the 4P-8 peptide mimic could be used as a secondary immunogen in selectively stimulating those B cells producing Abs to a protective LPS epitope. Mice were primed with LPS and then boosted with 4P-8-KLH or KLH alone. Boosting with 4P-8-KLH prolonged the LPS-reactive IgG and IgM AB responses as well as vibriocidal titers and provided a much greater degree of protection than boosting with KLH. The logic of this immunization regimen is that most individuals living in endemic areas are likely to be exposed to V. cholerae LPS at a young age. A recent report suggests that vibriocidal Abs are present in 40–80% of individuals 10–15 years of age in Bangladesh and are associated with protection against cholera (45). Thus, if preexisting immunity to LPS, elicited by either natural exposure or vaccination, could be extended by vaccination with a peptide mimic, such peptides might be, in and of themselves, effective as vaccines in endemic areas. Peptide mimics have been used in prime and boost immunization strategies to obtain more robust immune responses against surface carbohydrate structures (35, 46). The effectiveness of peptide mimic 4P-8 as a secondary immunogen may be due to its ability to selectively stimulate those B cells producing Abs to a protective epitope from among a broad pool of B cells primed by V. cholerae LPS. Furthermore, our results suggest that if we primed with LPS, a secondary Ab response could then be specifically directed to a protective epitope by immunization with peptide mimic 4P-8. These results have implications for the design of peptide mimotope vaccines to carbohydrate antigens.

Taken together, we present evidence that anti-LPS Ab S-20-4 can be used to identify a mimotope from a phage display library that is likely to make similar interactions as V. cholerae Ogawa LPS and unique interactions when binding to S-20-4. Future studies will focus on improving antigen structural mimicry based on crystal structures or on computer modeling with the intention of inducing a more robust immune response in animal models.

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