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Transcutaneous immunization with a synthetic hexasaccharide-protein conjugate induces anti-*Vibrio cholerae* lipopolysaccharide responses in mice

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Abstract

Antibodies specific for *Vibrio cholerae* lipopolysaccharide (LPS) are common in humans recovering from cholera, and constitute a primary component of the vibriocidal response, a serum complement-mediated bacteriocidal response correlated with protection against cholera. In order to determine whether transcutaneous immunization (TCI) with a *V. cholerae* neoglycoconjugate (CHO-BSA) comprised of a synthetic terminal hexasaccharide of the O-specific polysaccharide of *V. cholerae* O1 (Ogawa) conjugated with bovine serum albumin (BSA) could induce anti-*V. cholerae* LPS and vibriocidal responses, we applied CHO-BSA transcutaneously in the presence or absence of the immune adjuvant cholera toxin (CT) to mice. Transcutaneously applied neoglycoconjugate elicited prominent *V. cholerae* specific LPS IgG responses in the presence of CT, but not IgM or IgA responses. CT applied on the skin induced strong IgG and IgA serum responses. TCI with neoglycoconjugate did not elicit detectable vibriocidal responses, protection in a mouse challenge assay, or stool anti-*V. cholerae* IgA responses, irrespective of the presence or absence of CT. Our results suggest that transcutaneously applied synthetic *V. cholerae* neoglycoconjugate is safe and immunogenic, but predominantly induces systemic LPS responses of the IgG isotype.

1. Introduction

Vibrio cholerae is a non-invasive organism that colonizes the human intestine and produces cholera toxin (CT), an ADP-ribosylating protein that causes a severe secretory diarrhea in

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infected humans. Strains of *V. cholerae* can be differentiated serologically by the O-specific polysaccharide of the lipopolysaccharide (LPS) component of the outer membrane. The vast majority of strains that induce epidemic cholera belong to serogroups O1 or O139. *V. cholerae* O1 is divided into two biotypes, classical and El Tor, which differ clinically and biochemically. Based on O-antigen differences, each O1 biotype can be further subdivided into three serotypes: Ogawa, Inaba, and Hikojima. During outbreaks or sustained transmission, *V. cholerae* O1 may switch between Ogawa and Inaba serotypes [1]. The Hikojima serotype is rare and thought to be unstable.

V. cholerae LPS is immunogenic in humans following wild type disease, inducing significant increases in *V. cholerae* O1 LPS IgG, IgM, and IgA serum antibody responses, as well as antibody secreting cell responses [2-6]. Wild type disease also induces intestinal secretory IgA responses, and immunity against *V. cholerae* O1 LPS of the IgA and IgM (but not IgG) isotypes is associated with protection from cholera in humans [3,4,7,8]. Passive immunization with anti-*V. cholerae* LPS antibodies protects against *V. cholerae* challenge in both mice [9,10] and rabbits [11], and immunization with purified *V. cholerae* LPS confers protection against challenge in rabbits [12] and humans [5,13,14]. *V. cholerae* specific LPS antibodies of the IgM and IgG isotypes constitute a primary component of the vibriocidal response [7], a serum complement-mediated bacteriocidal response correlate with protection against cholera [15].

V. cholerae O1 LPS is comprised of a lipid component, core polysaccharide, and an O-specific polysaccharide (O-SP). The O-SP in *V. cholerae* O1 consists of (1->2)-alpha-linked 4-amino-4,6-dideoxy-D-mannose (perosamine), in which the amino group is acylated with 3-deoxy-L-glycero-tetronic acid [16]. The Inaba O-SP has a terminal sugar characterized by a 2-O-hydroxyl group; in the Ogawa O-SP, the hydroxyl group is replaced by a 2-O-methyl group [17]. The differences in the terminal sugars of the Inaba and Ogawa are thought to define their respective serotypes.

Unfortunately, a cholera subunit vaccine based on parenteral immunization with *V. cholerae* LPS has a number of real and potential shortcomings, including difficulties in manufacturing, a high reactogenicity profile following vaccine administration, and the requirement for needle-based administration. To decrease reactogenicity, detoxified versions of LPS consisting largely of the O-polysaccharide with an altered and decreased lipid component have been developed; however, polysaccharide-based vaccines often induce low level and short term immunity via T cell independent pathways [18]. Efforts to circumvent this issue have involved coupling detoxified LPS and polysaccharides to protein carriers such as CT [19,20] or tetanus toxoid [21], and administration of such vaccines is immunogenic in animals and humans [19-21]. A different approach involves the construction of synthetic neoglycoconjugates, in which different lengths of perosamine polymers of *V. cholerae* O-SP are chemically linked to a protein carrier [16,18,22-25]. Ogawa and Inaba neoglycoconjugates are immunogenic in mice, and intra-peritoneal vaccination of mice with a synthesized hexasaccharide derived from the O-SP component of *V. cholerae* O1 Ogawa LPS bound to BSA was protective in a *V. cholerae* neonatal mouse challenge model [18,22]. We were thus interested in evaluating whether needle-free transcutaneous immunization (TCI) with an Ogawa *V. cholerae* O1 neoglycoconjugate would induce immunity in mice [26,27].

2. Materials and Methods

2.1. Bacterial strains and media

V. cholerae O1 El Tor Ogawa strain X25049 was used to prepare LPS for immunogen preparation and immunological assays, and wild type classical *V. cholerae* O1 classical Ogawa strain O395 was used in vibriocidal assays and mouse challenge models, as described below

[28]. Prior to use in challenge studies, organisms were grown for 12 hours at 37°C with aeration in Luria-Bertani broth containing streptomycin (100 µg/ml).

2.2. Vaccine antigens

V. cholerae Ogawa neoglycoconjugate was comprised of a synthesized hexasaccharide derived from the O-SP component of *V. cholerae* O1 Ogawa lipopolysaccharide (CHO) bound through a linker to BSA in a molar ratio of 5 hexasaccharides: 1 protein (CHO-BSA; Figure 1) [16, 18]. To produce the conjugate, hexasaccharide squarate (3.78 mg, 0.0021 mM) was added to a solution of BSA (Sigma A-4503, purified [29]; 20 mg, 0.0003 mM) and borate buffer pH 9.00 (0.5 M, 0.53 ml). The reaction mixture was gently stirred and the reaction was periodically monitored by SELDI TOF-MS, which, after 8 h, showed the carbohydrate-protein ratio to be 5.0 [30]. Borate buffer pH 7.00 was added to terminate the reaction, and the mixture was transferred to a centrifugal filter device (10 k, Amicon Ultra, Millipore), and dialyzed against 10 mM ammonium carbonate solution (8 times) to remove low molecular mass material. The retentate was lyophilized and resuspended in TEAN (30 mM Tris, 1mM EDTA, 3mM NaN₃, 200mM NaCl), pH7.5 prior to use in immunization regimens [18,30].

We also immunized mice with purified LPS derived from *V. cholerae* O1 Ogawa strain X25049, which was first prepared, purified, and lyophilized as previously described [6,28], and resuspended in TEAN. Cholera toxin (CT; List Biological Laboratories, Campbell, CA) was used as an immunogen and immunoadjuvant.

2.3. Immunization of mice

We transcutaneously immunized cohorts of 3-12 adult BALB/c mice (Charles River Laboratories) with CT (25 µg), CHO-BSA (10 µg of perosamine weight), or both CT (25 µg) and CHO-BSA (10 µg of perosamine weight). We subcutaneously immunized a fourth group of mice with CHO-BSA (2.5 µg of perosamine weight) and CT (10 µg), and transcutaneously immunized a fifth group of mice with purified Ogawa LPS (10 µg) and CT (25 µg). Mice were immunized on days 0, 14, and 28, and prior to collection of blood for use in the neonatal challenge assay, mice received a booster immunization on day 42 after which blood was collected on day 56. Transcutaneous immunizations were performed as previously described [26,31,32]. Briefly, we shaved the dorsum of mice using a clipper with a no. 40 blade (Wahl Clipper Corp., Sterling, IL); then rested the mice for 24 hr. We then anesthetized mice with an intraperitoneal injection of 0.017 ml/g of a 2.5% solution in normal saline of 0.25 gm of 2-2-2 tribromoethanol (Sigma) mixed with 0.25 ml of tertiary amyl alcohol (Sigma) to prevent grooming following immunization. In order to enhance absorption of reagents into the skin, the shaved area was hydrated for 5 min with sterile water, gently brushed with emery paper (to remove the outer layers of stratum corneum), and then hydrated a second time for 5 min. We then blotted the region dry and immediately applied the vaccine antigens to approximately 1cm² of shaved hydrated skin surface area. The mice were then rested and observed for 1 hr. The shaved region was then covered with a patch consisting of tape and gauze soaked in sterile phosphate buffered saline (PBS). After 24 hr, the patch was removed and the region was washed thoroughly with 1 L of warm water. The use of animals complied fully with relevant governmental and institutional requirements, guidelines, and policies.

2.4. Immunological sampling

We collected blood samples via tail bleeds on days 0, 14, 28, 42, and 56, and collected stool on day 56. Samples were collected, processed, aliquoted, and stored as previously described [26,31,33-35].

2.5. Detection of specific antibody responses in serum and stool

To detect antibody responses to LPS, we coated microtiter plates overnight at room temperature with 250 ng of LPS in PBS per well, and subsequently blocked plates for 40 minutes at 37°C with PBS-1% ovalbumin (Sigma). We added 100 µl of diluted sera (using the following dilutions by measured isotype: IgM, 1:200; IgG, 1:50; IgA, 1:50) in PBS-0.1% ovalbumin-0.05% Tween 20 (Sigma), and incubated plates at 37°C for 1.5 hr. Following washing of plates with PBS-0.05% Tween 20 (PBS-T), to detect IgG, IgA, or IgM responses, we added 100 µl of sheep anti-mouse IgG conjugated to HRP (1:1000, Amersham Biosciences, Little Chalfont, Buckinghamshire, England), goat anti-mouse IgA conjugated to HRP (1:1000, Southern Biotech, Birmingham, AL), or goat anti-mouse IgM conjugated to HRP (1:1000 Southern Biotech, Birmingham, AL), respectively, to each well. We incubated plates at 37°C for 1.5 hr, washed them with PBS-T, and developed with a 0.55 mg/ml solution of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; Sigma) with 0.03% H₂O₂ (Sigma). We determined the optical density at 405 nm with a Vmax microplate kinetic reader (Molecular Devices Corp., Sunnyvale, CA). Plates were read for 5 min at 14s intervals, and the maximum slope for an optical density change of 0.2 U was reported as milli-optical density units per minute (mOD/min), as previously described [26,31]. To compare values across plates, results were divided by a pooled plate control and reported as ELISA units.

To detect antibody responses to CT, we coated microtiter plates sequentially with 1 µg of type III ganglioside (Sigma) in 50 mM carbonate buffer (pH 9.6) per well overnight at room temperature, followed by 100 ng CT per well in PBS overnight at room temperature. Following blocking of plates for 4-6 hr at room temperature with PBS-1% ovalbumin, we added dilutions of sera (IgG, 1:6000; IgA, 1:50) in PBS-0.1% ovalbumin-0.05% Tween 20, and incubated plates overnight at room temperature. Following washing of the plates, to detect IgG and IgA responses, we added sheep anti-mouse IgG conjugated to HRP or goat anti-mouse IgA conjugated to HRP, respectively, and developed as described above.

To detect LPS specific IgA antibody in feces, we first prepared fecal extracts and measured total fecal IgA in samples as previously described [26,31]. Briefly, we added duplicate serial twofold dilutions of fecal samples (1:10 to 1:320) in PBS-T to wells previously coated with rat monoclonal anti-mouse IgA antibody C10-3 (Pharmingen, San Diego, CA) at a dilution of 1:1,000 [26,31]. We then added a 1:1,000 dilution of goat anti-mouse IgA HRP-linked antibody to wells and developed the plates as described above. Comparisons were made to a mouse IgA standard (Kappa TEPC 15; Sigma). To detect specific anti-CT or anti-LPS antibodies in feces, we added 75 ng of total fecal IgA in PBS-T to wells in enzyme-linked immunosorbent assays identical to those used to measure serum anti-CT or anti-LPS responses, and we processed plates as described above.

2.6. Measurement of serum vibriocidal responses

We measured serum vibriocidal antibody titers in a micro-assay as previously described [33, 36]. We inactivated the endogenous complement activity of mouse sera by heating sera to 56°C for 1 hr. We then added 50 µl aliquots of serial twofold dilutions of heat-inactivated sera in PBS (1:25 to 1:25,600) to wells of sterile 96-well tissue culture plates containing 50 µl per well of a 10⁸-CFU/ml culture of *V. cholerae* O395 in PBS and 22% guinea pig complement (EMD Biosciences, San Diego, CA). We then incubated the plates for 1 hr at 37°C, added 150 µl of brain heart infusion broth (Becton Dickinson, Sparks, MD) to each well, incubated the plates for an additional 2 hr at 37°C, and then measured the optical density of each well at 600 nm. We calculated the vibriocidal titer as the dilution of serum causing 50% reduction in optical density compared with that of wells containing no serum [37,38].

2.7 The infant mouse challenge model

To evaluate protective efficacy, we used a *V. cholerae* infant mouse LD50 challenge model [18,26]. In brief, we removed 3 to 5 day old CD-1 suckling mice from their non-immunized mothers at least 3 hr prior to inoculation. We then administered to pups a 50 μ l inoculum comprised of 25 μ l of LB containing 10^6 or 10^7 CFU of wild type *V. cholerae* O395 and 25 μ l of day 56 pooled serum from immunized mice ($n = 15$ mice/cohort). Following oral challenge, we kept neonates separate from dams at 30°C, and monitored animals every 3 to 6 hr for 48 hr, after which, surviving animals were euthanized.

2.8. Statistics and Graphs

We compared data from different test groups using two-tailed Mann Whitney U tests. Within each group, comparisons of data from different time points to baseline data (day 0) were carried out using one-tailed Wilcoxon Signed Ranks tests. Survival curves were analyzed by log rank testing. We used Statistical Package for Social Sciences version 12.0 for Windows (SPSS, Chicago, IL), and plotted data using Microsoft Excel 2002 and Prism5.3.

3. Results

3.1. Measurement of immune responses

In order to determine whether transcutaneous immunization with CHO-BSA and CT would elicit systemic anti-LPS humoral immune responses, we compared anti-LPS antibody responses in sera collected on days 0, 14, 28 and 42 from mice given zero, one, two, or three transcutaneous immunizations, respectively, of CHO-BSA and CT, or CT alone. Following two immunizations, we found mice transcutaneously immunized with CHO-BSA and CT had significant serum anti-LPS IgG responses compared to responses of mice receiving CT alone (day 28, $p < 0.01$; Figure 2A), and that anti-LPS IgG responses increased following a third immunization (day 42, $p < 0.01$). We found no significant anti-LPS IgG response in mice transcutaneously immunized with CHO-BSA in the absence of CT. Anti-LPS IgG responses were highest in animals receiving subcutaneously administered CHO-BSA and CT, although responses were not significantly different from those observed in mice transcutaneously immunized with CHO-BSA and CT in this study. We detected no appreciable anti-LPS response following transcutaneous immunization with LPS and CT. We detected no appreciable serum IgM (Figure 2B), serum IgA (Figure 3A), or stool IgA anti-LPS responses or vibriocidal responses (data not shown) in any vaccinated cohort.

In comparison, we detected low level but significant anti-CT IgG responses in serum following a single transcutaneous immunization with CT ($p < 0.01$; Figure 2C), and anti-CT IgG responses increased with subsequent boosters. Significant serum anti-CT responses were observed in all mice immunized with CT irrespective of route or cohort, including mice transcutaneously immunized with holo-LPS and CT ($p < 0.05$). We also observed serum anti-CT IgA responses in all groups transcutaneously immunized with CT ($p < 0.05$), but not in cohorts of mice subcutaneously immunized with CT (Figure 3B). We detected no significant anti-CT IgA responses in feces (data not shown).

3.1. Neonatal mouse challenge assay

We detected no difference in survival between mice challenged with wild type *V. cholerae* O395 mixed with sera collected from mice transcutaneously immunized with CHO-BSA and CT, compared to survival following challenge with wild type *V. cholerae* O395 mixed with sera from mice transcutaneously immunized with CT alone (Figure 4).

4. Discussion

Parenterally administered vaccines that primarily elicit anti-LPS responses (such as the killed whole cell typhoid and cholera vaccines, which are no longer manufactured) can be effective, but often require repetitive dosing in immunologically naive individuals. Parenteral administration of such vaccines is also often associated with high adverse event profiles (including moderate or severe local reactions at the inoculation site), and often induces relatively short lived immunity with immune responses often decreasing substantially within 6 months of vaccination. LPS specificity is largely directed to the O-specific polysaccharide component of LPS; however, polysaccharides, which are T cell independent antigens, are themselves often poorly immunogenic in humans. The coupling of polysaccharides to protein carriers increases T cell activation, markedly improves the magnitude and duration of immune responses following immunization, and is the basis of a number of anti-bacterial vaccines now in wide use in humans, including the *Haemophilus influenzae* b, pneumococcal, and meningococcal conjugate vaccines [39]. These organisms differ from *V. cholerae* O1, the predominant cause of cholera globally, in that a component of their polysaccharides can be relatively easily produced for manufacturing purposes through the induction of a polysaccharide capsule during in vitro growth, and capsular polysaccharide does not contain the endotoxic lipid A component of LPS. To overcome this problem, polysaccharide components may be chemically synthesized prior to conjugation to a protein carrier. Such an approach has previously been applied to *V. cholerae*, and intra-peritoneal immunization of mice with a glycoconjugate comprised of a synthetic hexasaccharide of Ogawa O1 perosamines attached to BSA was found to induce serum anti-LPS and vibriocidal antibody responses, and protection in a challenge assay [18].

Transcutaneous immunization has been shown to elicit prominent IgG antibody responses, and weaker, but still significant mucosal immune responses to highly immunogenic proteins such as CT [27,40], and other antigens, when co-administered with an immunoadjuvant [26,31,32, 41]. For instance, transcutaneous immunization with TcpA, an important virulence factor in *V. cholerae*, gives rise to systemic and mucosal anti-TcpA immune responses that are protective against *V. cholerae* challenge in mice [26]. Previous evaluation of the immunogenicity of transdermally applied polysaccharides, although less well studied, has also been successful: transcutaneous application of a *Haemophilus influenzae* b conjugate vaccine has been shown to induce in animals an anti-Hib polysaccharide immune response that is protective [42]. We therefore evaluated whether a cholera synthetic glycoconjugate could similarly induce protective immunity against cholera following transcutaneous immunization. Although we were able to induce anti-LPS IgG, anti-CT IgG, and anti-CT IgA serum responses, we were not able to detect anti-LPS IgA following transcutaneous immunization. We were also unable to detect anti-LPS IgM responses following immunization, which may in part be due to the ability of protein conjugation to alter T cell independence of polysaccharide processing to T cell dependence and isotype maturation [18]. This lack of induction of IgM may in part explain our inability to detect a vibriocidal response, since anti-LPS IgM is thought to be a primary component of the vibriocidal antibody response [7].

In this study, transcutaneous immunization with CHO-BSA was not associated with protection in a challenge assay despite the induction of serum anti-LPS IgG. In humans, serum IgG with LPS specificity is not associated with protection from cholera, although serum IgA with LPS specificity and vibriocidal antibodies (comprised in part by complement-fixing anti-LPS IgM) is associated with protection. This suggests, that while LPS may be immunogenic, the type of antibody response may be critical to mediating protection, with responses that reflect mucosal immunity and those that bind complement perhaps being the most important. Indeed, since *V. cholerae* is a non-invasive organism that does not cross the intestinal epithelial surface, the

serum vibriocidal response may in fact be a surrogate marker for as yet poorly understood mucosal immune responses.

It is unclear why intra-peritoneal immunization with a similar vaccine previously resulted in protective immune responses in mice, but trafficking and antigenic handling by intra-peritoneal macrophages and lymphocytes differ from those associated with subcutaneous and dermal structures, and immunoadjuvants used in the two studies were also different (RIBI in the previous study [18], CT in this study). Although we did not perform dose ranging studies in this experiment, the amount of antigen used in our vaccine preparation (approximately 100 µg total antigen, containing 10 µg of sugar) is equivalent to doses previously found to be effective for sugar and protein antigens when administered transcutaneously, and would probably represent the maximum for viable manufacturing purposes [18,26,31,42].

We recognize that the synthetic hexasaccharide conjugate included in our vaccine preparation represents only a small antigenic fragment of the *V. cholerae* O-SP. It was for this reason that we also evaluated immune responses following transcutaneous application of the intact LPS antigen. Interestingly, we were unable to detect any anti-LPS responses (including IgG) using transcutaneously applied holo-LPS, although we were able to detect prominent IgG and IgA responses to co-administered CT. Whether the lack of anti-LPS responses in our study reflected a lack of penetration of antigen to dermal Langerhan's cells or poor immunogenicity is currently unclear.

Although we were unable to induce protective immune responses with a synthetic *V. cholerae* LPS glycoconjugate administered transcutaneously in this study, our ability to induce anti-LPS responses in immunologically naïve mice with TCI raises the possibility that transcutaneous immunization with glycoconjugate could be used to boost pre-existing anti-LPS and vibriocidal responses in previously primed subjects [34,43]. In addition, analysis of immune cell trafficking and antigenic handling could give future insights into the potential role of transcutaneous immunization using polysaccharide-conjugate vaccines against cholera.

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Figure 1.

Structure of O-specific Ogawa synthetic hexasaccharide BSA conjugate. Molar ratio, 5 hexasaccharide molecules: 1 BSA. BSA, bovine serum albumen; Me, methyl.

Figure 2.

Serum (A) anti-LPS IgG, (B) anti-LPS IgM and (C) anti-CT IgG responses in mice transcutaneously immunized (TCI) with CT, CHO-BSA, CHO-BSA and CT, or LPS and CT, or subcutaneously immunized (SQ) with CHO-BSA and CT. Geometric mean and standard error of the mean are reported for each group.

A. IgA anti-LPS

B. IgA anti-CT

Figure 3.

Serum day 42 (A) anti-LPS IgA, and (B) anti-CT IgA responses in mice transcutaneously immunized (TCI) with CT, CHO-BSA, CHO-BSA and CT, or LPS and CT, or subcutaneously immunized (SQ) with CHO-BSA and CT. Geometric mean and standard error of the mean are reported for each group.

Figure 4.

Survival likelihoods of neonatal CD-1 mice following oral challenge with wild-type O1 Ogawa *V. cholerae* O395. Three- to five-day-old pups (cohort size 15) were gavaged with 50 μ l of a preparation containing 10^6 or 10^7 CFU of *V. cholerae* O395 mixed 1:1 with pooled day 56 serum from mice transcutaneously immunized with neoglycoconjugate (CHO-BSA) and immunoadjuvantative cholera toxin (CT) or CT alone. Challenged mice were kept at 30°C and monitored for death every 3 to 6 h starting 18 h after oral challenge till 48 h after oral challenge. Survival curves were compared by log rank testing.