Vaccine 27 (2009) 4917–4922

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

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

Julianne E. Rollenhagen[a,b], Anuj Kalsy[a], Rina Saksena[c], Alaullah Sheikh[a,d], Mohammad Murshid Alam[d], Firdausi Qadri[d], Stephen B. Calderwood[a,b,e], Pavol Kovác[c], Edward T. Ryan[a,b,f,*]

[a] *Division of Infectious Diseases, Massachusetts General Hospital, Boston, MA, USA*
[b] *Department of Medicine, Harvard Medical School, Boston, MA, USA*
[c] *NIDDK, LBC, Section on Carbohydrates, National Institutes of Health, Bethesda, MD, USA*
[d] *International Centre For Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Dhaka, Bangladesh*
[e] *Department of Microbiology and Molecular Center, Harvard Medical School, Boston, MA, USA*
[f] *Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA, USA*

ARTICLE INFO

Article history:
Received 11 March 2009
Received in revised form 29 May 2009
Accepted 9 June 2009
Available online 27 June 2009

Keywords:
Cholera
Transcutaneous immunization
Conjugate

ABSTRACT

Antibodies specific for *Vibrio cholerae* lipopolysaccaride (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 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

* Corresponding author at: Division of Infectious Diseases, Massachusetts General Hospital, Jackson 504, 55 Fruit Street, Boston, MA 02114 USA. Tel.: +1 617 726 6175; fax: +1 617 726 7416.
E-mail addresses: etryan@partners.org, julianne.rollenhagen@med.navy.mil (E.T. Ryan).

0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.06.040

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 h 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; Fig. 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, 1 mM EDTA, 3 mM NaN3, 200 mM 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.

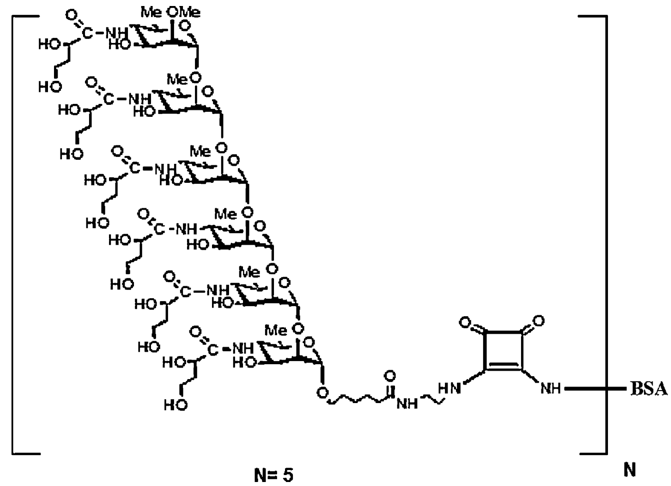

Fig. 1. Structure of O-specific Ogawa synthetic hexasaccharide BSA conjugate. Molar ratio, 5 hexasaccharide molecules: 1 BSA. BSA: bovine serum albumen; Me: methyl.

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 h. 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 1 cm^2 of shaved hydrated skin surface area. The mice were then rested and observed for 1 h. The shaved region was then covered with a patch consisting of tape and gauze soaked in sterile phosphate buffered saline (PBS). After 24 h, 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 min 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,