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Saccharide/protein conjugate vaccines for *Bordetella* species: Preparation of saccharide, development of new conjugation procedures, and physico-chemical and immunological characterization of the conjugates

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ABSTRACT

Bordetellae are Gram-negative bacilli causing respiratory tract infections of mammals and birds. Clinically important are *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. *B. pertussis* vaccines have been successful in preventing pertussis in infants and children. Veterinary vaccines against *B. bronchiseptica* are available, but their efficacy and mode of action are not established. There is no vaccine against *B. parapertussis*. Based on the concept that immunity to non-capsulated Gram-negative bacteria may be conferred by serum IgG anti-LPS we studied chemical, serological and immunological properties of the O-specific polysaccharides (O-SP) of *B. bronchiseptica* and *B. parapertussis* obtained by different degradation procedures. One type of the *B. parapertussis* and two types of *B. bronchiseptica* O-SP were recognized based on the structure of their non-reducing end saccharide; no cross-reaction between the two *B. bronchiseptica* types was observed. Competitive inhibition assays showed the immunodominance of the non-reducing end of these O-SP. Conjugates of *B. bronchiseptica* and *B. parapertussis* O-SP were prepared by two methods: using the anhydro-Kdo residue exposed by mild acid hydrolysis of the LPS or the 2,5-anhydromannose residue exposed by deamination of the core glucosamine of the LPS, for binding to an aminooxylated protein. Both coupling methods were carried out at a neutral pH, room temperature, and in a short time. All conjugates, injected as saline solutions at a fraction of an estimated human dose, induced antibodies in mice to the homologous O-SP. These methodologies can be applied to prepare O-SP-based vaccines against other Gram-negative bacteria.

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1. Introduction

Vaccination has been proven effective for preventing infection of humans and animals by *Bordetella* spp. Killed whole cell and subunit vaccines have been used to immunize infants and children against *Bordetella pertussis*, the cause of pertussis, a highly contagious, severe respiratory infection especially of young children. No vaccine is available against *B. parapertussis* which causes a milder and less frequent form of pertussis in humans and a respiratory infection in sheep [1]. *B. pertussis* is confined to humans. Infection with *B. parapertussis* does not confer immunity to pertussis [2]. *B. bronchiseptica* causes serious respiratory infections in a variety of hosts: kennel cough in dogs, atrophic rhinitis in piglets, bronchopneumonia in rabbits and guinea pigs [3]. Rarely, *B. bronchiseptica* infects humans, mostly young children, animal handlers and immuno-compromised individuals [4]. Cellular and subcellular veterinary vaccines are available for this pathogen but they are of limited efficacy [5–7]. Among all bordetellae only *B. pertussis* expresses pertussis toxin [8,9].

Serum IgG anti-LPS has been shown to confer immunity to Gram-negative bacteria [10–12]. Monoclonal antibodies to *B. pertussis* LPS were shown to have complement-dependent bactericidal activity [13]. The LPS of all three bordetellae is of low molecular weight, <10 kDa, rendering their isolated saccharides non-immunogenic. *B. pertussis* LPS is comprised of a Lipid A domain and a branched dodecasaccharide, composed of unusual sugars with free amino and carboxylic groups [14]. By SDS-PAGE, *B. pertussis* LPS shows two bands, A and B. Band B contains Lipid A and a branched nonasaccharide core, Band A contains further

Abbreviations: GM, geometric mean; SBAP, *N*-succinimidyl 3-(bromoacetamido) propionate; GalNAc3NAcA, 2,3-diacetamido-2,3-dideoxy-L-galacturonic acid; Buffer A, PBS, 0.1% glycerol, 5 mM EDTA, pH 7.4; s.c., subcutaneously.

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substituted Band B by a trisaccharide unit. An almost identical core structure to *B. pertussis* was reported for *B. bronchiseptica* LPS [15]. The core region of *B. parapertussis* has a shorter heptasaccharide structure and does not contain the Band A trisaccharide [14]. In contrast to *B. pertussis*, which produces no O-specific polysaccharide (O-SP), *B. bronchiseptica* and *B. parapertussis* synthesize short O-SP containing about 12–18 sugars. Initially, it was reported that the O-SP of both these organisms is identical and composed of a linear polymer of 1,4-linked 2,3-diacetamido-2,3-dideoxy-α-galacturonic acid (GalNAc3NAcA) [16]. Later, serological differences between *B. bronchiseptica* strains were ascribed to the structural variations of the non-reducing end-groups of the O-SP [17,18]. Similar observations were made for the serotypes Ogawa and Inaba of *Vibrio cholerae* O1 that differ only by a methyl group at the non-reducing end of Ogawa [19] and for *Salmonella* O40 and O43 serotypes [18].

The objectives of this study were to define and correlate structural and immunological data of *B. bronchiseptica* and *B. parapertussis* O-SP to provide experimental vaccines of wide coverage. Different conjugation methods were devised and the serum antibody responses elicited by these investigational vaccines in young outbred mice were assayed.

2. Materials and methods

2.1. Bacteria and cultivation

B. bronchiseptica ATCC 10580, Rb50 (ATCC BAA-588), and *B. parapertussis* ATCC 15989 and 12822 were obtained from ATCC (Manassas, VA). *B. bronchiseptica* 15374 and 3145 were obtained from Malcom Perry (NRC, Ottawa, Canada). Bacteria were grown on Bordet-Gengou (BG) agar plates and transferred to Stainer-Scholte (S-S) media [20]. After 16–24 h of cultivation at 37 °C with shaking in buffled flasks, bacteria were harvested by centrifugation, killed by boiling for 1 h and stored at −20 °C for LPS extraction. *Haemophilus ducreyi* LPS used as a control was a gift from Teresa Lagergård (Göteborg, Sweden).

2.2. Oligosaccharides

LPS was isolated by hot phenol-water extraction and purified by enzyme treatment and ultracentrifugation as described [21]. Two methods were used for LPS degradation: (1) LPS (100 mg) was heated in 10 ml 1% acetic acid for 60 min at 100 °C, ultracentrifuged at 35,000 rpm for 5 h at 4 °C and the carbohydrate-containing supernatant was passed through a 1 × 100 cm column of BioGel P-4 in pyridine/glacial acetic acid/water buffer (4/8/988 ml, pH 4.5) monitored with a Knauer differential refractometer. Twenty-eight milligrams of O-SP were recovered from the void volume; (2) LPS (100 mg) was deaminated in 18 ml of a solution containing 30% acetic acid/5% sodium nitrite/water (1/1/1) for 6 h, at room temperature, on a magnetic stirrer, followed by ultracentrifugation [22]. The supernatant was freeze-dried and purified on the BioGel P-4 column as above. Twenty-three milligrams of the saccharide fraction, designated as O-SP_{deam}, were recovered from the void volume.

For isolation of oligosaccharides used in competitive inhibition assays, LPS was dissolved in anhydrous HF (100 mg LPS, 8 ml HF, 25 °C, 24 h), then evaporated at room temperature, in a hood, on a plastic Petri dish. The residue was dissolved in pyridine/glacial acetic acid/water buffer (4/8/988 ml), passed through a 2.5 cm × 80 cm column of Sephadex G-50 and further purified by HPLC on 250 × 9.5 mm Phenomenex Aqua column in 0.1% TFA (for the first 10 min), then with a gradient of 0–50% acetonitrile in 0.1% TFA. The effluent was monitored by 220 nm absorption. The isolated oligosaccharides contained an average of 15 repeats of diacetamidouronic acid (average molecular mass of 4158 Da, as assayed by MALDI-TOF) and were chemically characterized as previously [17].

2.3. Conjugation

2.3.1. O-SP conjugates

Bovine serum albumin (BSA, Sigma, St. Louis, MO) was derivatized to aminooxylated derivatives in a two step procedure: (1) BSA was treated with succinimidyl 3-(bromoacetamido)propionate (SBAP, Pierce, Pittsburgh, PA) to introduce thiol-reactive bromoacetamido moieties (BSA-Br); (2) BSA-Br was coupled with *O*-(3-thiopropyl)hydroxylamine, a heterobifunctional linker, to form the aminooxylated protein through stable thioether linkages (BSA-ONH_2) as described [23]. For conjugation, BSA-ONH_2 (5 mg) was reacted with 10 mg of O-SP of *B. bronchiseptica* 10580 (Bb10580), *B. bronchiseptica* Rb50 (BbRb50) or *B. parapertussis* 10595 (Bpp15989) in 1.5 ml Buffer A (PBS, 0.1% glycerol, 5 mM EDTA), at pH 5.7, for 15 h with stirring, at room temperature. The reaction mixture was passed through a 1 × 100 cm Sephadex G-100 column in 0.2 M NaCl and the void volume fraction characterized by protein concentration, immunodiffusion, SDS-PAGE and MALDI-TOF spectroscopy. The conjugates were designated BSA-ONH_2/Bb10580 (#1), BSA-ONH_2/BbRb50 (#2) and BSA-ONH_2/Bpp15989 (#3).

2.3.2. O-SP_{deam} conjugates

BSA-ONH_2 (5 mg) was reacted with 10 mg of O-SP_{deam} of the listed strains using the described conditions. The products were designates BSA-ONH_2/$Bb10580_{deam}$ (#4), BSA-ONH_2/$BbRb50_{deam}$ (#5) and BSA-ONH_2/$Bpp15989_{deam}$ (#6).

2.4. Immunization

5–6 weeks-old female NIH Swiss Webster mice were injected s.c. 3 times at 2 weeks intervals with 2.5 μg saccharide as a conjugate in 0.1 ml PBS. Groups of 10 mice were exsanguinated 7 days after the second or third injections [24]. Controls received PBS. Hyperimmune sera against *B. bronchiseptica* strains 10580 and Rb50, and against *B. parapertussis* strain 15989 were prepared with heat-killed whole bacteria as described [25].

2.5. Analytic

Protein concentration was measured by the method of Lowry [26]. SDS-PAGE used 14% gels according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Double immunodiffusion was performed in 1% agarose gel in PBS. Endotoxic activity was measured by the limulus amebocyte lysate assay as described by the manufacturer (Cambrex, Walkersville, MD).

2.6. Spectroscopy

MALDI-TOF mass spectra of the derivatized carrier proteins and of the conjugates were obtained with an OmniFlex MALDI-TOF instrument (Bruker Daltonics, Billerica, MA) operated in the linear mode. Samples for analysis were desalted and 1 μl, mixed with 20 μl of sinnapinic acid matrix made in 30% CH_3CN and 0.1% trifluroacetic acid. Next, 1 μl of the mixture was dried on the sample stage and placed in the mass spectrometer. NMR spectra were recorded at 30 °C in D_2O on a Varian UNITY INOVA 600 instrument using acetone as the reference for both proton (2.225 ppm) and carbon (31.5 ppm) spectra. Varian standard programs COSY, NOESY