Oligosaccharide conjugates of *Bordetella pertussis* and *bronchiseptica* induce bactericidal antibodies, an addition to pertussis vaccine

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Pertussis is a highly contagious respiratory disease that is especially dangerous for infants and children. Despite mass vaccination, reported pertussis cases have increased in the United States and other parts of the world, probably because of increased awareness, improved diagnostic means, and waning vaccine-induced immunity among adolescents and adults. Licensed vaccines do not kill the organism directly; the addition of a component inducing bactericidal antibodies would improve vaccine efficacy. We investigated *Bordetella pertussis* and *Bordetella bronchiseptica* LPS-derived core oligosaccharide (OS) protein conjugates for their immunogenicity in mice. *B. pertussis* and *B. bronchiseptica* core OS were bound to aminooxylated BSA via their terminal Kdo residues. The two conjugates induced similar anti-*B. pertussis* LPS IgG levels in mice. *B. bronchiseptica* was investigated because it is easier to grow than *B. pertussis*. Using *B. bronchiseptica* genetically modified strains deficient in the O-specific polysaccharide, we isolated fractions of core OS with one to five repeats of the terminal trisaccharide, having at the nonreducing end a GlcNAc or GalNAc, and bound them to BSA at different densities. The highest antibody levels in mice were elicited by conjugates containing an average of 8–17 OS chains per protein and with one repeat of the terminal trisaccharide. Conjugate-induced antisera were bactericidal against *B. pertussis*, and the titers correlated with ELISA-measured antibody levels ($r = 0.74$). Such conjugates are easy to prepare and standardize; added to a recombinant pertussis toxoid, they may induce antibacterial and antitoxin immunity.

hydroxylamine | Gram-negative

The genus *Bordetella* comprises Gram-negative bacteria pathogenic for mammals and birds. The most common *Bordetella* species are the respiratory pathogens *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica*. *B. pertussis* is an exclusively human pathogen causing paroxysmal coughing in infants (whooping cough) and persistent respiratory infections in adults (1). Killed whole-cell pertussis vaccines were used to immunize infants and children worldwide for many years but are too reactogenic for adults (2). Acellular pertussis vaccines containing pertussis toxoid and other proteins, such as filamentous hemagglutinin, pertactin, and fimbriae, have recently largely replaced whole-cell vaccines in most developed countries (3).

Despite a high rate of vaccination, *B. pertussis* is responsible for an estimated 260,000 deaths annually, and a resurgence of pertussis in the United States and Europe has initiated calls for an improved vaccine (4, 5). However, there is no agreement among experts or regulatory agencies about how to achieve this goal. There is, however, agreement that pertussis toxoid is an essential component of the acellular vaccines; a postimmunization level of ≥ 100 ELISA units (EU) to pertussis toxin (PT) is the only reliable method for its serologic diagnosis, and low levels of anti-PT are related to infection with this pathogen (6). The confusion is

mainly due to the nature of pertussis vaccines' complex mode of action. The primary action of pertussis vaccines is serum IgG antitoxin immunity that blocks the inactivating action of PT on phagocytic cells, thus allowing them to opsonize the *B. pertussis*; i.e., the antibodies elicited by acellular or whole-cell vaccines do not directly kill the pathogen. There is also the "herd" immunity effect of pertussis vaccines that results in decreased transmission of *B. pertussis* in the susceptible population (2, 6, 7). Similar to the effect induced by widespread immunization with diphtheria toxoid, this indirect effect of antitoxin accounts for the incomplete immunity of both vaccines on an individual basis ($\sim 80\%$; ref. 6).

Because IgG antibodies to the lipopolysaccharide (LPS) of noncapsulated Gram-negative bacteria were protective in humans (8, 9), we studied the oligosaccharide (OS) of *B. pertussis* LPS as a potential vaccine component. Several articles indicate that serum IgG anti-OS antibodies can confer complement mediated killing effect upon *B. pertussis* (10, 11). *B. pertussis* LPS is comprised of Lipid A and a branched dodecasaccharide core, composed of unusual sugars with free amino and carboxylic acid groups but with no O-specific polysaccharide (O-SP; ref. 12). SDS/PAGE analysis of *B. pertussis* LPS reveals two bands, band A and band B. Band B is composed of Lipid A and a branched nonasaccharide core, and band A consists of band B further substituted by a trisaccharide unit. *B. bronchiseptica* LPS was reported to have an identical core structure to that of *B. pertussis* but substituted by an O-SP, composed of a linear polymer of 1,4-linked 2,3-diacetamido-2,3-dideoxy- α -L-galacturonic acid (GalNAc3NAcA), connected to the core by a five-sugar linker (13–16).

B. pertussis and *B. bronchiseptica* core OS have low molecular weights and require covalent binding to a protein to be immunogenic. The conjugate-induced antibody levels are a function of the saccharide chain length, their loading on the protein, and the structure of the nonreducing terminal monosaccharide (17, 18). To evaluate these variables, we prepared conjugates of *B. pertussis* and *B. bronchiseptica* OS isolated from native and genetically modified strains (*B. bronchiseptica* RBA2b OS differed from that of *B. pertussis* in the OS chain length by producing multiples of band A terminal trisaccharide, and *B. bronchiseptica* RBB1a OS had $\sim 50\%$ of GlcNAc replaced by GalNAc at the

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Fig. 1. Structure of *B. pertussis* LPS and *B. bronchiseptica* RB50 core-Lipid A fraction (band A). * indicates differences in methylation and phosphorylation between the strains (Results).

nonreducing end) and studied their chemical, serological, and immunological properties as an addition to pertussis vaccine.

Results

Chemical Characterization of the LPS. *B. bronchiseptica* RB50 LPS core OS structure is similar to that of *B. pertussis* Tohama I and Tax 113, with an additional component, an O-SP. The dodecasaccharide core of *B. bronchiseptica* RB50 without its O-SP (commonly referred to as “band A”) was separated on a Bio-Gel P-4 column. The first fraction, eluted in the void volume, contained band A substituted with O-SP (~80% by weight). The second fraction contained nonsubstituted band A, which was used in this study (~20% by weight). Electrospray ionization (ESI)-MS and NMR analysis confirmed the published LPS structure (13, 14). Mass spectra showed minor differences between band A of *B. pertussis* and of *B. bronchiseptica* (Fig. 1). In *B. pertussis*, phosphate (P) was not detected, and methylation (Me) of the N-4 of

FucNAc4N was complete (a peak of 2,294 Da; the Kdo was present only in the anhydro-Kdo form), whereas in *B. bronchiseptica* RB50, ~50% of N-4 of FucNAc4N were methylated and ~30% of O-4 of the Hep substituted with GlcA was phosphorylated (peaks detected were as follows: 2,374 Da, band A with P and Me; 2,360 Da, band A without Me; 2,294 Da, band A without P; and 2,280 Da, band A without both P and Me).

Three mutants deficient in O-SP, derived from *B. bronchiseptica* RB50, were used. The first was RBwbmΔ, which lacks the complete *wbm* locus and does not express O-SP, but produces core OS structurally almost identical to that of *B. pertussis*, differing from it only by the degree of phosphorylation and methylation, and identical to its parental strain RB50. RBB1a and RBA2b produced LPS containing several repeats of the band A trisaccharide. RBA2b produced oligomers of up to five repeats of the band A trisaccharide, visible by SDS/PAGE and mass spectra. OS with different numbers of repeats were separated on a Bio-Gel P-10 column from the acetic acid RBA2b LPS hydrolysate (Fig. 2). ESI-MS spectra confirmed that the OS in each fraction differed by one additional band A trisaccharide repeat (661 Da). NMR spectra of the RBA2b structures with repeats of terminal trisaccharide of various lengths were identical except for a new spin system of α-GlcNAc linking band A trisaccharide repeats together, compared with the core fraction of one band A trisaccharide only. LPS of the RBB1a strain contained mostly core with only small amounts of the band A trisaccharide repeats, but ~50% of the terminal GlcNAc was replaced by GalNAc.

Fig. 2. Characterization of *B. bronchiseptica* RBA2b LPS hydrolysate. (A) Bio-Gel P-10 gel filtration of *B. bronchiseptica* RBA2b OS with different number of band A nonreducing end trisaccharide repeats. (B) SDS/PAGE of *B. bronchiseptica* RBA2b LPS. (C and D) ESI-MS spectrum of the separated fractions (C) and their chemical structures (D). Each fraction differs by 661 Da (one repeat). Each OS is represented by several peaks due to incomplete methylation of FucNAc4N (14 Da) and/or phosphate (80 Da). F1, four and five repeats; F2, three repeats; F3, two repeats; F4, one repeat.

Fig. 4. Scheme of conjugation of *B. pertussis* and *B. bronchiseptica* core OS.

GalNAc at the nonreducing terminal (~1:1 ratio) induced antibody levels similar to those induced by conjugates containing only GlcNAc termini (conjugates 1, 3, 5, and 10). Conjugates with two to five repeats of band A trisaccharide induced significantly lower anti-*B. pertussis* LPS levels than the conjugate with only one repeat (conjugate 10 vs. 11, 12, and 13; $P < 0.005$).

Bactericidal activity and IgM antibody levels were measured in sera representing various IgG antibody levels (Table 2). The

correlation coefficients between the reciprocal bactericidal titers of these sera and their ELISA-measured antibody levels were as follows: IgG, $r = 0.66$; IgM, $r = 0.73$; and IgG plus IgM, $r = 0.74$.

Discussion

Conjugates of *B. pertussis* core OS injected s.c. in PBS, at a fraction of an estimated human dose, induced serum bactericidal antibodies in young outbred mice. A dodecasaccharide was obtained from acid-hydrolyzed *B. pertussis* and *B. bronchiseptica* LPS, and each was bound to BSA by a single-point attachment at its reducing end. Conjugation was performed between the carbonyl group of acid-released anhydro-Kdo (19–21) and an aminoxy group of a bifunctional thio-aminoxy linker bound to BSA. This efficient reaction was used for preparing conjugates of *Bordetella* and *Shigella* O-SP-core fragments (22–25). The method allows the binding of saccharide components of different lengths and at different densities per protein molecule to select the most immunogenic construct. The final product is easy to standardize, and the methodology is suitable for clinical use.

Another type of *B. pertussis* LPS-based conjugate was immunogenic in rabbits, inducing antibodies that bound to *B. pertussis* cells as assayed by immunofluorescence (26). In that study, the conjugate was composed of the nonreducing end pentasaccharide (band A trisaccharide, GlcN, Hep) with the reducing end GlcN modified to anhydro-mannose used for conjugation. The authors (26) suggested that the epitope was located predominantly at the band A distal trisaccharide.

These results, together with the fact that the immunodominant fragment of *B. bronchiseptica* LPS was its nonreducing O-SP end saccharides (22), prompted us to prepare conjugates containing several repeats of the band A nonreducing end trisaccharide produced by recombinant strains of *B. bronchiseptica* to examine whether conjugates of longer trisaccharide chains would induce higher antibody levels. We isolated OS with one to five repeats of the trisaccharide from the *B. bronchiseptica* RBA2b strain and bound them separately to BSA. The LPS with more than one repeat did not bind to the hyperimmune *B. pertussis* antiserum or to antiserum raised against conjugates prepared with OS con-

Table 2. Bactericidal activity, IgG, and IgM anti-*B. pertussis* Tohamma I LPS (ELISA) elicited by different conjugates

Conj no.	OS in a conjugate	No. of chains per BSA	Individual serum	IgG, EU	IgM, EU	Reciprocal bactericidal titers
1	B.p. Tohama I	8	1786A	3.6	3.2	20
2	B.p. Tax113	25	2058H	22.6	1.4	50
3	B.p. Tax113	17	2059A	9.4	4.2	100
3	B.p. Tax113	17	2059E	1.1	0.8	10
3	B.p. Tax113	17	2059I	13.8	9.5	750
4	B.p. Tax113	11	2060A	6.2	0.2	100
5	B.b. RB50 core	9	1787C	9.6	1.7	500
5	B.b. RB50 core	9	1787G	64.9	2.8	750
6	B.b. RBwbmΔ	19	2064A	2.4	3.1	20
7	B.b. RBwbmΔ	11	2063A	0.06	0.3	<10
7	B.b. RBwbmΔ	11	2063C	0.04	0.07	<10
9	B.b. RBB1a	12	2093H	38.4	13.9	750
9	B.b. RBB1a	12	2093J	14.0	1.6	10
10	B.b. RBA2b, F1	8	2152B	5.3	0.6	<10
11	B.b. RBA2b, F2	9	2153D	4.2	0.7	10
12	B.b. RBA2b, F3	11	2154H	4.1	0.2	10
13	B.b. RBA2b, F4	9	2155D	39.3	0.1	50
13	B.b. RBA2b, F4	9	2155F	2.1	3.8	<10
13	B.b. RBA2b, F4	9	2155H	0.8	0.5	<10
	PBS	—	2156A	<0.01	<0.01	<10

Correlation coefficients between the reciprocal bactericidal titer and IgG, $r = 0.66$; IgM, $r = 0.73$; and IgG plus IgM, $r = 0.74$. *B.p., *B. pertussis*; B.b., *B. bronchiseptica*; -, not applied.

taining only one trisaccharide. Our interpretation is that the epitope of *B. pertussis* LPS includes not only the terminal trisaccharide but also some core sugars, possibly the side chain Hep of the terminal pentasaccharide, as observed in saturation transfer difference NMR experiments (26). This suggestion could explain why conjugates containing two to five repeats induced lower antibody levels to *B. pertussis* LPS than the conjugate containing one repeat only. The ELISA-measured antibody levels were in accordance with the bactericidal data; sera induced by the conjugates of several band A trisaccharide repeats had no or very low bactericidal activity. On the bases of these experiments, we suggest a dodecasaccharide with only one trisaccharide repeat as a vaccine candidate.

Other variables reported to influence the immunogenicity of conjugates were the number of OS chains per protein molecule and the identity of the nonreducing end molecule (17, 18, 22, 27, 28). In the present study, the range of optimal chain density was wide, with an average number of chains between 8 and 17. For the evaluation of the nonreducing end molecule we used a *B. bronchiseptica* mutant RBB1a LPS, which expressed band A with a terminal GlcNAc or GalNAc in an ~1:1 ratio. Conjugates prepared with this OS at the optimal densities were as immunogenic as conjugates with terminal GlcNAc only. We were unable to obtain OS with terminal GalNAc only; therefore, the role of the end group cannot be definitively established.

To summarize, we present an easy method of preparing *B. pertussis* OS/protein conjugates using either *B. pertussis* or *B. bronchiseptica* strains. The benefits of using *B. bronchiseptica* instead of *B. pertussis* are its ability to grow on simple media, in a short time, and with higher bacterial mass yield. We plan to conjugate the band A of RBwbmΔ LPS—the same as that of *B. pertussis*—to a clinically useful protein for clinical evaluation. The “herd” immunity induced by our current acellular pertussis vaccines has been successful in reducing significantly the incidence of pertussis but leaves many individuals who are not immune. Induction of antibacterial immunity by OS conjugates could further reduce the circulation of *B. pertussis* and decrease outbreaks of this disease. We believe that our OS-based conjugate, added to a recombinant pertussis toxoid, could provide antibacterial immunity to antitoxin immunity.

Materials and Methods

Bacteria and Cultivation. *B. pertussis* Tohama I (ATCC BAA-589) was obtained from the Culture Collection of Göteborg. Construction of genetically inactivated *B. pertussis* strain Tax 113 was done as described, except that in that strain, the codon for the glutamic acid residue at position 129 of the mature S1 subunit was changed to an alanine (GCC; ref. 29). This strain produces pertussis holotoxin with R9K and E129A mutations in the S1 subunit of the toxin, which abolishes the enzymatic activity of the toxin. *B. bronchiseptica* RB50 (ATCC BAA-588) was obtained from ATCC. Genetically modified strains were as follows: RBwbmΔ is a mutant of *B. bronchiseptica* RB50 in which a large chromosomal XbaI fragment encompassing *wbmA-wbmZ* is replaced with a kanamycin-resistance cassette; RBA2b and RBB1a are *B. bronchiseptica* RB50 mutants in which *wbmA* (RBA2b) or *wbmB* (RBB1a) coding sequences are disrupted by insertion of a tetracycline resistance cassette.

B. pertussis and *B. bronchiseptica* strains were cultivated on Bordet-Gengou agar plates. *B. bronchiseptica* was then transferred to Stainer-Scholte liquid medium containing 50 mM MgSO₄ (30). After 16–24 h of cultivation at 37 °C with shaking in baffled flasks, bacteria were harvested by centrifugation, killed by boiling for 1 h, and stored at –20 °C for LPS extraction.

LPS and OS Preparation. LPS was isolated by hot phenol-water extraction and purified by enzyme treatment and ultracentrifugation as described (31). LPS was heated in 1% acetic acid for 90 min at 100 °C and ultracentrifuged at 142,000 × g for 5 h at 4 °C, and the carbohydrate-containing supernatant was passed through a 1 × 100-cm column of Bio-Gel P-4—or, in the case of *B. bronchiseptica* RBA2b, Bio-Gel P-10—in pyridine/acetic acid/water buffer (4/8/988 mL, pH 5.7) monitored with a Knauer differential refractometer.

Fractions were collected based on the refractometer profile and freeze-dried twice to remove traces of the buffer components.

Conjugation. BSA (Sigma) was converted to aminooxylated derivatives in a two-step procedure, similar to that described for *B. bronchiseptica* and *B. paraptussis* (22): (i) BSA was treated with succinimidyl 3-(bromoacetamido) propionate (Pierce) to introduce thiol-reactive bromoacetamido groups (BSA-Br); and (ii) BSA-Br was coupled to O-(3-thiopropyl)hydroxylamine, a heterobifunctional linker, to form the aminooxylated protein through stable thioether linkages (BSA-ONH₂) as described (32). For conjugation, BSA-ONH₂ (5 mg) was reacted with OS isolated from different *B. pertussis* or *B. bronchiseptica* strains in quantities of 2.5 mg (conjugates 1, 4, 5, 7, and 9–13; see Table 1), 5 mg (conjugates 3, 6, and 8), and 7.5 mg (conjugate 2) of OS in 1.5 mL of buffer A (PBS/0.1% glycerol/5 mM EDTA) at pH 5.7 for 15 h. The ratio of BSA to OS was adjusted to achieve a range of OS chains incorporated per BSA. Next, the reaction mixture was passed through a 1 × 100-cm Sephadex G-75 column in 0.2 M NaCl as eluent, and the void volume fraction was characterized by protein assay, immunodiffusion, SDS/PAGE, and MALDI-TOF spectroscopy.

Reactivity of *B. pertussis* OS with O-(3-thiopropyl)hydroxylamine. *B. pertussis* Tax 113 OS (5 mg) was reacted with 1 mg of O-(3-thiopropyl)hydroxylamine in 1.5 mL buffer A (PBS/0.1% glycerol/5 mM EDTA) at pH 5.7 for 15 h. Next, the reaction mixture was passed through a 1 × 100-cm Bio-Gel P-4 column in pyridine/acetic acid/water buffer (4/8/988 mL, pH 5.7) monitored with a Knauer differential refractometer, and then fractions were collected and freeze-dried. ESI-MS spectra of *B. pertussis* OS before and after reaction were recorded.

Immunization. For immunization, 5- to 6-week-old female NIH Swiss Webster mice were injected s.c. three times at 2-week intervals with 2.5 μg of saccharide as a conjugate in 0.1 mL of PBS. Groups of 10 mice were exsanguinated 7 d after the second or third injection (33). Controls received PBS. Hyperimmune antiserum against *B. pertussis* Tohama I was prepared with heat-killed bacteria as described (34).

Analytic. Protein concentration was measured by the method of Lowry (35). Double immunodiffusion was performed in 1% agarose gel in PBS. SDS/PAGE gel and immunoblotting used 16% Tris-glycine gels and PVDF membranes and were performed according to the manufacturer's instructions (Invitrogen). Endotoxin activity was measured by the limulus amoebocyte lysate assay as described by the manufacturer (Cambrex).

Spectroscopy. MALDI-TOF mass spectra of the derivatized carrier proteins and of the conjugates were obtained with an OmniFlex MALDI-TOF instrument (Bruker Daltonics) operated in the linear mode. Samples for analysis were desalted, and 1 μL was mixed with 20 μL of sinapinic acid matrix made in 30% CH₃CN and 0.1% trifluoroacetic acid. Next, 1 μL of mixture was dried on the sample stage and placed in the mass spectrometer. NMR spectra were recorded at 30 °C in D₂O on a Varian UNITY INOVA 600 instrument using acetone as reference for both proton (2.225 ppm) and carbon (31.5 ppm) spectra. Varian standard programs COSY, NOESY (mixing time of 400 ms), TOCSY (spinlock time 120 ms), HSQC, and gHMBC (long-range transfer delay 70 ms) were used (14).

Antibodies. Serum IgG and IgM antibodies were measured by ELISA (36). Nunc Maxisorb plates were coated with *B. pertussis* Tohama I LPS at 10 μg/mL in PBS; 1% human serum albumin in PBS was used in a blocking step. The optimal concentration of the coating antigen was determined by checkerboard titration. A PA MRX Dynatech reader was used. Antibody levels were calculated relative to the hyperimmune standard antiserum diluted 1:5,000 for IgG and 1:3,000 for IgM, and a value of 100 EU was assigned for each. Results were computed with an ELISA data processing program provided by the Biostatistics and Information Management Branch of the Centers for Disease Control and Prevention (37).

Bactericidal Assay. The bactericidal activity of sera was tested in vitro (12). Briefly, antisera were inactivated at 56 °C for 15 min and diluted 10-fold in PBS with 0.15 mM CaCl₂, 0.5 mM MgCl₂, and 0.1% BSA (buffer B, pH 7.4). *B. pertussis* Tohama I was diluted to ~300–500 bacteria per 25 μL of buffer B. A precolostral calf serum (PCS) was used as a source of complement. Then, 45 μL of antisera was mixed with 25 μL of bacteria and 15 μL of undiluted PCS. Finally, 140 μL of buffer B was added (final volume of reagents, 225 μL). Mixtures of bacteria in buffer B without PCS or without serum were used as

controls. After incubation for 60 min at 37 °C, 100 μ L of each mixture was plated onto Bordet–Gengou agar and incubated for 3–5 d, and then colonies were counted. The bactericidal titer of the antiserum was defined as the highest dilution giving killing of 50% of the inoculum. The lowest positive titer was a dilution of 1:10.

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