Epitope of the Vaccine-Type *Bordetella pertussis* Strain 186 Lipooligosaccharide and Antiendotoxin Activity of Antibodies Directed against the Terminal Pentasaccharide-Tetanus Toxoid Conjugate†

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Lipooligosaccharides (LOS) isolated from *Bordetella pertussis* strains 186 and 606 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and high-resolution magic angle spinning nuclear magnetic resonance (NMR). These analyses distinguished between the LOS of strains 186 and 606, suggesting that the structure of LOS in *B. pertussis* is heterogeneous. The pentasaccharide was selectively cleaved from LOS of *B. pertussis* strain 186, purified, and covalently linked to a monomer fraction of tetanus toxoid. Injection of rabbits with the neoglycoconjugate emulsified in complete Freund's adjuvant yielded immunoglobulin G antibodies that were reactive with the LOS. These antibodies reacted strongly with *B. pertussis* LOS possessing the complete dodecasaccharide, as determined by an enzyme-linked immunosorbent assay, immunoblotting, and flow cytometry with intact, live bacterial cells. The binding epitope within the pentasaccharide was investigated by saturation transfer difference (STD) NMR spectroscopy. Protons H-1 and H-4 of the terminal α -D-GlcNAc and proton H-6 and protons of an *N*-methyl group at H-4 of 3-substituted β -L-FucpNAc4NMe exhibited the largest saturation transfers. STD NMR experiments confirmed that the immunodominant epitope recognized by the antineoglycoconjugate antibodies is located predominantly in the distal trisaccharide of *B. pertussis* 186 LOS. The antipentasaccharide antibodies induced by the conjugate inhibited the secretion of tumor necrosis factor alpha, interleukin-6, and NO by LOS-stimulated J774A.1 cells.

Bordetella pertussis, an aerobic gram-negative rod, is the causative agent of whooping cough (pertussis) in humans. Pertussis is a highly contagious disease involving the respiratory tract and is especially dangerous for infants and young children. Increasing numbers of adolescents and adults with pertussis have also been reported (15). Widespread immunization has drastically reduced the prevalence of pertussis in many countries; however, outbreaks still occur even in well-immunized communities (13).

The main virulence factors of *B. pertussis* comprise surface adhesion molecules that are responsible for attachment of the bacteria to host cells (filamentous hemagglutinin, fimbriae, pertactin) and toxins that facilitate evasion of the host immune system by the bacterium (pertussis toxin, adenylate cyclase toxin, dermonecrotic toxin, tracheal cytotoxin) (43).

One of the virulence determinants of *B. pertussis* and the most abundant surface molecule is lipopolysaccharide (39). This molecule plays a major role in the host-pathogen interactions (46) and is responsible for endotoxic activities similar to those of enteric bacteria (1, 50).

B. pertussis produces only two types of LPS (4), which consist of a lipid A moiety linked to a core nonasaccharide or a dodecasaccharide. The LPS of *B. pertussis* is devoid of an O-specific polysaccharide chain; instead, it contains a single distal trisaccharide and thus structurally is a lipooligosaccharide.

Although whole-cell pertussis vaccines are effective in controlling whooping cough, concerns related to adverse effects following vaccination have led to the development of acellular pertussis vaccines composed of different surface proteins (filamentous hemagglutinin, pertactin, fimbriae 2 and 3, inactivated pertussis toxin) in various combinations. Two- and five-component acellular vaccines have been shown to protect against pertussis with 58% and 85% efficacy, respectively (12). Despite the high vaccination coverage in some countries (e.g., The Netherlands, Australia, and Canada) (33), a substantial increase in the incidence of pertussis has been observed in the last few decades (6, 47).

There are conflicting data regarding the role of LOS in protection against whooping cough. It was found that most of the sera obtained from children with diagnosed pertussis, as well as sera from children vaccinated with a whole-cell pertussis vaccine, contained specific anti-LOS antibodies, suggesting that LOS present on *B. pertussis* cells is immunogenic. On the other hand, in an animal model of *B. pertussis* infection, anti-LOS antibodies did not protect against a subsequent challenge with virulent bacteria. However, the lack of an immune re-

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sponse against LOS in this experiment was probably due to the inappropriate form of antigen and the route of administration (5).

The biological activities of *B. pertussis* LOS are similar to those of endotoxins isolated from other gram-negative bacteria. It has been shown that LOS of *B. pertussis* is an antigen in natural infection (44), and new findings suggest that it is an important etiological factor in whooping cough pathogenesis and is directly involved in destruction of the ciliated cells of the human airways mucosa (8). LOS, working synergistically with tracheal cytotoxin, takes part in induction of nitric oxide synthase in nonciliated epithelial cells, and this is followed by NO release and subsequent damage to the ciliated cells (8, 9).

The importance of the *B. pertussis* endotoxin as a virulence factor is further indicated by its involvement in the interaction with SP-A of the respiratory tract (41). SP-A binds to gram-negative microorganisms via lipid A, inducing destabilization of bacterial membranes, and it plays an important role in innate immunity. Surprisingly, it was found that SP-A neither bound nor permeabilized wild-type *B. pertussis* cells. The lack of a bactericidal effect of SP-A on *B. pertussis* was explained by the presence of the trisaccharide at the nonreducing end of the LOS core oligosaccharide, which hindered access of the protein to the lipid A (41). Previously, it has also been demonstrated that a mouse monoclonal antibody specific for the dodecasaccharide-containing LOS of *B. pertussis* has complement-dependent bactericidal activity and substantially reduces colonization of the respiratory tract of mice following aerosol infection (35). These data further suggest that epitopes specific for the trisaccharide in the LOS of *B. pertussis* are involved in complement-dependent bactericidal activity.

As LOS is also the most abundant cell surface component of *B. pertussis*, it was of interest to evaluate LOS epitopes as a target for antibodies that might be protective against biological activities of *B. pertussis*. For these experiments we used the LOS of *B. pertussis* strain 186, a wild-type strain and a component of the whole-cell pertussis vaccine which has been used in Poland since 1978 (53). The vaccine also contains strains 606 and 629. So far, data on the structural details of LOS isolated from a single strain, strain 1414, have been published (3).

Thus, here we describe structural details of the *B. pertussis* strain 186 LOS and the antiendotoxin activity of polyclonal antibodies against a covalent conjugate of the pentasaccharide with tetanus toxoid (17). We also performed STD NMR experiments for identification of the binding epitope in the pentasaccharide investigated. Additionally, by using HR-MAS NMR analysis, we were also able to identify the pentasaccharide directly on intact LOS and to distinguish the LOS of *B. pertussis* strain 186 from the LOS of strain 606.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this paper: LPS, lipopolysaccharide; LOS, lipooligosaccharide; SP-A, surfactant protein A; TT, tetanus toxoid; PBS, phosphate-buffered saline; MALDI-TOF, matrix-assisted laser desorption ionization–time of flight; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; FACS, fluorescence-activated cell sorter; NMR, nuclear magnetic resonance; COSY, correlated spectroscopy; TOCSY, total correlation spectroscopy; ROESY, rotating frame nuclear Overhauser effect spectroscopy; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; DEPT, distortionless enhancement by polarization transfer; HR-MAS, high-resolution magic angle spinning; STD, saturation

transfer difference; TNF- α , tumor necrosis factor alpha; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; IL-6, interleukin-6; LD-Hep, L-glycero-D-mannoheptose; D-ManpNAc3NAcA, 2,3-diacetamido-2,3-dideoxy-mannuronic acid; L-FucNAc4NMe, 2-acetamido-4-N-methyl-2,4,6-trideoxy-galactose; 2,5-anhydromannan, 2,5-anhydro-D-mannose; MTT, 1-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Bacteria. *B. pertussis* strains 186 and 606, which are used by Biomed, Krakow, Poland, for production of pertussis vaccine, and two clinical isolates, strains 3210 and 3225 (National Institute of Public Health, Warsaw, Poland), were used in this study. The bacteria were grown on Bordet-Gengou medium and solidified Cohen-Wheeler charcoal-containing medium, collected as a suspension in PBS, and killed by incubation at 60°C for 45 min.

Cell lines. Cells of mouse macrophage-like cell line J774A.1 were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), and TNF- α -sensitive WEHI 164 clone 13 mouse fibrosarcoma cells were kindly donated by M. Zimecki (Institute of Immunology and Experimental Therapy, Wrocław, Poland). J774A.1 cells were grown in Dulbecco's medium supplemented with 10% FBS (Gibco, Biocult, Glasgow, United Kingdom). WEHI 164.13 cells were grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM pyruvic acid, and 4 mM 2-mercaptoethanol. Cells were grown and maintained during the tests at 37°C in the presence of 5% CO₂.

Preparation of lipooligosaccharide of *B. pertussis* strain 186 and isolation of the pentasaccharide. LOS was extracted from bacterial cells by the hot phenol-water method (52) and was purified by ultracentrifugation as previously described (36). The pentasaccharide was selectively cleaved from the LOS of *B. pertussis* strain 186 by treatment with nitrous acid (22). Briefly, the lipooligosaccharide (50 mg) was suspended in a freshly prepared solution (180 ml) containing water, 5% sodium nitrite, and 30% acetic acid (1:1:1, vol/vol/vol) and incubated for 4 h at 25°C, and this was followed by ultracentrifugation (200,000 $\times$ g, 2 h). The supernatant was freeze-dried, and the product was purified on a column of Bio-Gel P-2 (Bio-Rad), yielding ~10 mg, and analyzed by MALDI-TOF MS and NMR spectroscopy.

Conjugation of the isolated pentasaccharide with tetanus toxoid. A conjugate was prepared by the method of Jennings and Lugowski (17). A tetanus toxoid preparation was obtained from Biomed, Krakow, Poland. The pentasaccharide of *B. pertussis* strain 186 (10 mg) was dissolved in 1 ml of 0.5 M K₂HPO₄, pH 9.0. Tetanus toxoid (2.5 mg), NaBH₃CN (10 mg), and 1 drop of chloroform were added to the solution. The reaction mixture was kept in a sealed vial for 12 days at 37°C and then applied to a Sephadex G-100 column (1.6 by 100 cm) equilibrated with PBS. Three batches of the conjugate were prepared. Fractions containing the conjugate were concentrated by ultrafiltration to obtain 2.5 mg of product. The calculated molar ratio of the pentasaccharide to tetanus toxoid obtained from a carbohydrate and protein determination was 8:1 to 10:1.

Mass spectrometry. MALDI-TOF MS of the oligosaccharide investigated, in the positive or negative mode, was performed with a Bruker Reflex III instrument. 2,5-Dihydroxybenzoic acid was used as the matrix.

NMR spectroscopy. NMR spectra of the isolated oligosaccharide were obtained for ²H₂O solutions at 40°C with a Bruker DRX 600 spectrometer, using acetone (δ_H 2.225 and δ_C 31.05) as the internal reference. The oligosaccharide was repeatedly exchanged with ²H₂O with intermediate lyophilization prior to analysis. The signals were assigned by one- and two-dimensional experiments (COSY, clean-TOCSY, ROESY, HMBC, and HSQC-DEPT with and without carbon decoupling). In the clean-TOCSY experiments the mixing times were 30, 60, and 100 ms. The delay in the HMBC experiments and the mixing time in the ROESY experiments were 60 and 200 ms, respectively. The data were acquired and processed using standard Bruker software. The processed spectra were assigned with help of the SPARKY program (10).

An analysis of pH dependence of the N-methyl resonance in the isolated pentasaccharide was carried out in 10% ²H₂O by using a pH range of 2 to 11, with the pH adjusted with 1 M HCl and 1 M NaOH. Spectra were obtained at 30°C with the WATERGATE (37) pulse program.

NMR spectra of suspensions of *B. pertussis* strains 186 and 606 LOS (~0.5 mg) in ²H₂O (~10 μ l) were obtained using the HR-MAS technique with a Bruker DRX 600 spectrometer. The experiments were carried out at a spin rate of 5 kHz at 23°C (the measured temperature of the pressurized air used for sample spinning) using a Bruker 4-mm HR-MAS probe and a ZrO₂ rotor. The one-dimensional ¹H NMR spectra of the LOS were acquired using a Carr-Purcell-Meibom-Gill pulse sequence 90-(τ -180- τ)_n (31) as a T₂ filter before acquisition to reduce the broad signals of lipids (16). The total delay time was 12 ms. The data were acquired and processed using standard Bruker software.

One-dimensional STD NMR experiments were performed at 37°C with a

2.5-mm inverse-detection probe head equipped with z-gradient. Spectra were recorded at 37°C using the pulse programs described previously (29). The WATERGATE pulse sequence was used to suppress water signals in the spectra, and the broad resonances of a protein were suppressed with a 10-ms spin-lock pulse. The protein was irradiated at δ_H -2.5 ppm (on-resonance) and δ_H 40 ppm (off-resonance) with a train of n Gaussian shaped pulses (50 ms) separated by 20- μ s delays, and the saturated spectrum was subtracted from the reference via phase cycling. The setup of the STD NMR experiments was optimized in a series of experiments with ligand-protein and ligand-only samples to ensure that the irradiation at the selected frequency did not affect the ligand. The overall saturation time (0.1 to 3 s) was controlled with different values of n . The relaxation delay used in all the experiments was 0.5 s. Samples (total volume, 35 μ l) were prepared in PBS made with $^2\text{H}_2\text{O}$, pH 7.5 (not corrected for $^2\text{H}_2\text{O}$).

Analytical procedures, immunizations, and polyclonal antibodies. The LOS were analyzed by SDS-PAGE using the method of Laemmli (21) with modifications described previously (40); a 17.5% (wt/vol) polyacrylamide-bisacrylamide gel was used as the separating gel, and a cathode buffer containing Tricine was used as described previously (23). The LOS bands were visualized by the silver staining method (45).

Four Belgian Giant rabbits were immunized with the *B. pertussis* pentasaccharide-TT conjugate suspended in complete Freund's adjuvant, and polyclonal antibodies against the conjugate were obtained by the procedures described previously (27). Additionally, polyclonal antibodies against whole bacterial cells were obtained by intravenous immunization of four Belgian Giant rabbits with a PBS suspension of *B. pertussis* strains 186 and 606 (27). Rabbits were housed at the animal facility of the Institute of Immunology and Experimental Therapy, Wrocław, Poland, and all the experiments were carried out by using procedures approved by the Local Ethical Commission for Animal Experimentation.

As antisera give complex ^1H NMR spectra, containing signals of proteins, lipoproteins, and lipids, as well as low-molecular-weight metabolites that can interfere with the signals of ligand in the spectra during the STD NMR experiments, a crude ammonium sulfate-cut IgG-enriched fraction was prepared. Briefly, antiserum (550 μ l) was centrifuged (3,000 $\times$ g, 15 min, 4°C) to remove any insoluble particles. A saturated ammonium sulfate solution (450 μ l) was added to the supernatant collected (final saturation, 45% [vol/vol]) and left overnight at 4°C. The mixture was then centrifuged, the supernatant was discarded, and the pellet was dissolved in PBS. The solution was dialyzed against PBS, using Microcon YM-50 filters with a 50-kDa cutoff (Millipore, Bedford, Mass.). The immunoglobulin fraction isolated was tested with an ELISA. The protein concentration was estimated and adjusted to 14 μ M by using UV absorbance at 280 nm. As no titration experiments were performed, the estimates were used for calculation of the approximate ligand-to-protein ratio (ratio of pentasaccharide to antibody binding sites, 140:1).

Immunofluorescence procedures and serological methods. Bacteria, cultured as described above, were washed and suspended in PBS to an optical density at 600 nm of 0.6 (4×10^8 CFU/ml). Appropriate antisera (0.5 ml, 1/50 and 1/200 dilutions) were added to the bacterial suspension and incubated at 25°C for 2 h. Preimmune sera were employed as negative controls. Bacteria were washed three times with PBS and incubated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (3 h, 25°C). After thorough washing with PBS, bacteria were resuspended in PBS containing 0.5% paraformaldehyde. An analysis of the fluorescein isothiocyanate-labeled bacteria was performed using a FACSScan (Becton Dickinson) fluorescence-activated cell sorter. In each analysis 4×10^4 bacterial cells were evaluated. Narrow-angle forward light scatter and green fluorescence emission signals were collected. Bacterial aggregates were electronically excluded on the basis of light scatter signals.

An ELISA, using LOS as the solid-phase antigen, was performed by a modification (17) of the method described by Voller et al. (49), and immunoblotting was performed as previously described (36). The detection systems consisted of a goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad) as a second antibody and *p*-nitrophenylphosphate and 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium for the ELISA and immunoblotting, respectively.

A quantitative microprecipitin test was carried out by the method of Kabat and Mayer (19). The reaction mixture contained 2% polyethylene glycol 6000 to enhance the rate of immunoprecipitate formation (38).

Stimulation of TNF- α , IL-6 release, and NO production in J774A.1 cells. J774A.1 cells were plated in 24-well tissue culture plates (Nunc, Denmark) at a concentration of 1×10^6 cells/well in 1 ml of Dulbecco's medium supplemented with 10% (vol/vol) FBS, incubated for 2 h, and washed three times with serum-free medium. The cells were then incubated with serial dilutions of stimulants in the medium (total volume, 1 ml). The supernatants were collected after 6, 24,

and 48 h for determination of TNF- α , IL-6, and NO, respectively, and stored at -80°C until measurements were obtained.

TNF- α bioassay. TNF- α activity was determined by a cytotoxic assay using WEHI 164 clone 13 cells seeded at a concentration of 2×10^4 cells/well in 96-well plates (Nunc, Denmark) in the presence of actinomycin D (1 μ g/ml), as previously described (7, 26). The viability of the cells was determined by quantitative colorimetric staining with MTT as described by Mossmann (34), using 20% SDS with 50% dimethylformamide, pH 4.7 (100 μ l), as the extraction buffer according to the modification described by Hansen et al. (14). The amount of TNF- α released upon stimulation was interpolated from the calibration curve obtained for murine recombinant TNF- α (PharMingen, San Diego, Calif.). The detection limit of the WEHI 164.13 cytotoxicity assay was 0.15 pg/ml.

IL-6 assays. The IL-6 content of the culture medium was quantified using a commercially available ELISA kit, an OptEIA mouse IL-6 set (PharMingen, San Diego, Calif.). The assay was performed according to the manufacturer's instructions. Recombinant mouse IL-6 was used as a standard. The dilutions of the samples tested were adjusted to the working range of the test (i.e., 15.6 to 1,000 pg/ml).

NO determination. The NO contents in the supernatants collected from stimulated J774A.1 cells were determined as the total nitrite (42), using the colorimetric assay with the Griess reagent described previously (11).

Inhibition of TNF- α , IL-6 release, and NO production in J774.1 cells. Appropriate antisera, including anti-*B. pertussis* pentasaccharide-TT conjugate serum, anti-whole-bacterium serum, and serum of a nonimmunized rabbit (100 μ l), were preincubated with *B. pertussis* 186 LOS (10 ng) for 1 h at 37°C, followed by transfer to a culture and incubation as described above. Serum of nonimmunized rabbits was used as a control. Samples (100 μ l) of the supernatants were collected after 6, 24, and 48 h and assayed for TNF- α , IL-6, and NO release as described above.

Statistical analysis. A one-way analysis of variance test was used to analyze the significance of differences in the inhibition tests for TNF- α , IL-6 release, and NO secretion. Assays were performed using duplicate cultures. The data are expressed as means $\pm$ standard deviations for three replicates.

RESULTS

Bacteria and lipooligosaccharide preparation. *B. pertussis* strains 186 and 606 and two clinical isolates (strains 3210 and 3225) were used in this study. The lipooligosaccharides of *B. pertussis* strains were isolated by the water-phenol method and analyzed by SDS-PAGE (Fig. 1A), which revealed the presence of incomplete LOS in strain 606 (fast-migrating band) and complete LOS in strain 186 (slowly migrating band) and strains 3210 and 3225 (not shown). Using HR-MAS NMR analysis of the intact LOS of *B. pertussis* strains 186 and 606, the *N*-methyl and *N*-acetyl groups present in dodecasaccharide-containing LOS of *B. pertussis* as described by Le Blay et al. (22) were identified and distinguished directly (Fig. 2). Therefore, the characteristic *N*-acetyl and *N*-methyl resonances in the spectra could be used as structure reporter groups (48) for identification of the complete LOS of *B. pertussis*.

B. pertussis strain 186, which is used for production of the pertussis cellular vaccine in Poland (Biomed, Krakow, Poland) and has the complete LOS structure as determined by SDS-PAGE and HR-MAS NMR, was chosen for preparation of the pentasaccharide.

Isolation of the pentasaccharide, preparation of the neoglycoconjugate, and reactivity of anticonjugate antibodies. The pentasaccharide was selectively cleaved from LOS of *B. pertussis* strain 186 by deamination with nitrous acid. The soluble oligosaccharide fraction isolated by ultracentrifugation was purified on a Bio-Gel P-2 column and analyzed by ^1H NMR spectroscopy and MALDI-TOF MS.

The ^1H (Fig. 2) and HSQC-DEPT NMR (see Fig. SA1 in the supplemental material) spectra of the *B. pertussis* strain 186 oligosaccharide contained signals for four anomeric protons

FIG. 1. Reactivities of polyclonal antibodies against *B. pertussis* pentasaccharide-TT conjugate with LOS of *B. pertussis* strains 186 and 606 as determined by ELISA and immunoblotting (inset). Each line in the groups of LOS represents a different rabbit. LOS of strains 186 (open symbols) and 606 (solid symbols) were used as solid-phase antigens (10 μ g/ml) in the ELISA and were also analyzed by SDS-PAGE (1 μ g/lane) using a 17.5% polyacrylamide-bisacrylamide separating gel, and they were visualized by silver staining (A) or transblotted onto nitrocellulose (B). For immunoblotting antipentasaccharide-TT conjugate serum was diluted 250-fold. The ELISA values are the means of three replicates. The standard deviations did not exceed 5% and are not shown.

and carbons and also a spin system of 2,5-anhydroMan as a hydrate, confirming that the compound is a pentasaccharide. All signals and spin systems containing ^1H and ^{13}C resonances were assigned by COSY, TOCSY with different mixing times, HSQC, and HMBC experiments. The chemical shifts of all proton and carbon resonances (see Table SA1 in the supplemental material) except those for a 4,6-disubstituted 2,5-anhydroMan residue were the same as those described for the LOS of *B. pertussis* 1414 (3). However, the MALDI-TOF MS spectrum (Fig. 3) showed that the molecular mass of the isolated pentasaccharide did not match the expected mass (mass difference, 29).

The combined data from the MALDI-TOF spectra of the

pentasaccharide and HR-MAS NMR experiments performed for the LOS suggested that the amino group at C-4 of the $\rightarrow 3$)- β -L-FucpNAc4NMe-(1 $\rightarrow$ methylated in the native LOS was also nitrosylated, forming an *N*-nitroso compound during the deamination procedure.

This observation was confirmed by the lack of pH dependence of the *N*-methyl resonance chemical shift (δ_{H} 3.26 ppm) in the pentasaccharide.

The MALDI-TOF mass spectrum of the oligosaccharide (Fig. 3) had a main ion at m/z 1068.0, corresponding to the pentasaccharide as $[\text{M}+\text{Na}]^+$ consisting of one GlcNAc, one Hep, one Man2NAc3NAcA, one 2,5-anhydroMan, and one FucNAc4NRMe with an *N*-methyl group nitrosylated (R) during the deamination procedure.

A single aldehyde group in the pentasaccharide (Fig. 2), which was formed during the deamination procedure, was used to form the covalent linkage with a monomeric fraction of tetanus toxoid by reductive amination (17). The neoglycoconjugate obtained was used for immunization of rabbits (four animals). The reactivities of antipentasaccharide-TT conjugate antibodies with the dodecasaccharide-containing LOS of homologous *B. pertussis* strain 186 and the nonasaccharide-containing LOS of strain 606 were determined by immunoblotting and by an ELISA. The antibodies reacted strongly, at both high and low concentrations, with LOS of strain 186 in the ELISA (Fig. 1). However, they showed only weak reactions at a high concentration with LOS of strain 606, suggesting that the binding epitope is located in the distal trisaccharide, which occurs only in the LOS with a complete dodecasaccharide. The antisera obtained from different animals showed similar antibody reaction patterns with the LOS of strains 186 and 606 (Fig. 1), and one of them was used for a detailed analysis by STD NMR and the immunochemical and biological methods. The level of antipentasaccharide antibodies of the IgG class was determined by the quantitative microprecipitin test. At the equivalence point the LOS of *B. pertussis* strain 186 precipitated 1.5 mg of antibody per ml of serum.

The reactivities of antipentasaccharide-TT conjugate antibodies with LOS of different *B. pertussis* strains were also tested by immunoblotting of antigens separated by SDS-PAGE. The antibodies reacted strongly with the slowly migrating LOS fraction of strain 186, but no reaction was observed with the fast-migrating fraction (devoid of the distal trisaccharide) of strain 606 (Fig. 1B). The reactions observed were in agreement with the data obtained by the ELISA.

The binding of anticonjugate antibodies and antibodies

FIG. 2. 600-MHz HR-MAS ^1H NMR spectra of the *B. pertussis* LOS containing dodecasaccharide (LOS 186) and nonasaccharide (LOS 606) and epitope mapping of the LOS-derived pentasaccharide in the presence of polyclonal anticonjugate antibodies. The HR-MAS NMR spectra (upper panel) were obtained for $^2\text{H}_2\text{O}$ suspensions of LOS at a spin rate of 5 kHz and 23°C. Structure reporter groups of the dodecasaccharide are labeled as follows: NMe E4, resonance of the *N*-methyl group of $\rightarrow 3$)- β -L-FucpNAc4NRMe-(1 $\rightarrow$; and NAc A2, NAc D2, and NAc D3 and NAc E2, resonances of the *N*-acetyl groups of α -D-GlcpNAc-(1 $\rightarrow$, $\rightarrow 4$)- β -D-Manp2NAc3NAcA-(1 $\rightarrow$, and $\rightarrow 3$)- β -L-FucpNAc4NMe-(1 $\rightarrow$, respectively. The STD NMR and reference ^1H NMR spectra (bottom panel) of the pentasaccharide (diagram) in the presence of anticonjugate antibodies were acquired at 600 MHz and 37°C for samples (35 μ l, in a capillary tube) prepared in PBS made with $^2\text{H}_2\text{O}$, pH 7.5. The protein was irradiated at δ_{H} -2.5 ppm (on-resonance) and δ_{H} 40 ppm (off-resonance) with a saturation time of 2 s. The WATERGATE pulse sequence was used to suppress water signals. The capital letters and numbers indicate the proton resonances in the carbohydrate residues that were enhanced in the STD experiments. The protons in close contact with antibodies are indicated by asterisks in the diagram. The unresolved resonances are in parentheses. The remaining signals in the region of *N*-acetyl groups might be subtraction artifacts of the originally most intense proton resonances. The aldehyde group in the pentasaccharide used for the formation of the covalent linkage to tetanus toxoid is circled in the diagram.

HR-MAS NMR (LOS 186)

HR-MAS NMR (LOS 606)

¹H-NMR (reference)

STD-NMR

against LOS of *B. pertussis* strain 186 was also investigated, using flow cytometry with whole bacterial cells. Both live bacteria (strain 186) and heat-killed bacteria (clinical isolates 3210 and 3225) were incubated with anticonjugate antibodies and antibodies against the whole bacterial cells. FACS analysis showed that anticonjugate antibodies reacted strongly and similar to the antibodies against the whole bacterial cells with live cells of *B. pertussis* strain 186 (Fig. 4A). A similar pattern was observed for the reactions of the anticonjugate antibodies with heat-killed cells of clinical isolates 3210 (Fig. 4B) and 3225 (Fig. 4C). FACS analysis carried out with live and heat-killed bacteria showed that the anticonjugate antibodies labeled with high intensity more than 95% of the bacterial cells counted.

Mapping of the binding epitope within the pentasaccharide by saturation transfer difference NMR experiments. The reactions of anticonjugate antibodies with dodecasaccharide-containing LOS of *B. pertussis* observed in the ELISA and immunoblotting assays suggested that the binding epitope is localized within the pentasaccharide which was investigated. Therefore, the structural elements within the pentasaccharide that could contribute to the binding epitope were investigated by STD NMR (30, 32), using the IgG-enriched fraction of the antipentasaccharide-TT conjugate antibodies. The STD NMR technique allows identification of the proton resonances of a ligand that are in close contact with an antibody, and it was used to identify interactions between the pentasaccharide and antipentasaccharide-TT conjugate antibodies.

It was found that the enhanced resonances in the STD NMR spectrum belonged to protons H-1 and H-4 of the terminal α -D-GlcpNAc (residue A) and proton H-6 and protons of an *N*-methyl of the 3-substituted β -L-FucpNAc4NMe (residue E), defining the immunodominant region of the antigen (the enhanced resonances are indicated in the diagram in Fig. 2) recognized by the antipentasaccharide-TT conjugate antibodies. Minor enhancements of the resonances of protons H-1 and H-5 belonging to the terminal L-glycero- α -D-manno-Hepp residue were also observed (residue B). The enhanced resonance at ~ 3.8 ppm could not be assigned unequivocally as H-6 of α -D-GlcpNAc (residue A), H-3 of the terminal L- α -D-Hepp (residue B), and H-2 and H-6 of 2,5-anhydroMan (residue C) were not resolved.

STD NMR experiments confirmed that the immunodominant epitope recognized by the anticonjugate antibodies is located predominantly in the distal trisaccharide of *B. pertussis* 186 LOS.

Inhibition of LOS induced TNF- α and IL-6 secretion. The J774A.1 cells stimulated with LOS of *B. pertussis* strain 186 released TNF- α and IL-6 in a dose-response mode. The concentrations of TNF- α and IL-6 in the supernatants were calculated from the calibration curves for mouse recombinant TNF- α and recombinant IL-6, respectively. The anticonjugate antibodies at a concentration of 150 μ g/ml inhibited the secretion of TNF- α by LOS-induced J774A.1 cells so that the level was almost the same as the level detected in the supernatant of nonstimulated cells. There was a 10-fold decrease in both the TNF- α and IL-6 levels (Fig. 5) compared to the levels determined for the supernatants of the J774A.1 cells stimulated with the preimmune antibodies used for inhibition.

Inhibition of LOS induced NO release. The NO secretion by J774A.1 cells stimulated with LOS of *B. pertussis* strain 186 (10

FIG. 3. MALDI-TOF spectrum of the isolated pentasaccharide of *B. pertussis* strain 186. The MALDI-TOF mass spectrum was obtained in the positive reflectron mode with 2,5-dihydroxybenzoic acid as the matrix. The m/z values represent monoisotopic masses.

ng/ml) was determined by determining the total nitrite by the colorimetric assay using the Griess reagent. The preimmune antibodies were used as the control for inhibition of NO production. A threefold reduction in the nitrite concentration in the supernatants of J774A.1 cells was observed when the antipentasaccharide-TT conjugate antibodies (150 μ g/ml) were used for inhibition of NO production by LOS-induced J774A.1 cells (Fig. 5).

DISCUSSION

LPS is a major component of the cell membrane of gram-negative bacteria, is the most exposed antigen in nonencapsulated bacteria, is an important virulence factor, and is the target for specific antibodies (39). It is well known that protective human immunity to many gram-negative diseases correlates with the presence of serum bactericidal antibodies that recognize surface carbohydrate antigens (20).

Therefore, the conclusion reached in the study of Chaby and Caroff (5) which suggested that endotoxin is not very important in the pertussis syndrome in humans seemed to be unlikely and not fully supported by experimental data. Contrary evidence, presented by Flak and Goldman (8), indicated that *B. pertussis* endotoxin plays a crucial role in the selective destruction of the ciliated cells of human airway mucosa. In this process the endotoxin works synergistically with tracheal cytotoxin by inducing a toxic level of NO released from secretory epithelial cells. In addition, the exposure of humans to *B. pertussis* during infection can induce an immune response against LOS, the main surface antigen of this bacterium (44). However, these molecules in a free form or present on bacterial cells are toxic, reactogenic, T-independent antigens. It was found that in some cases LOS can stimulate the production of bactericidal antibodies even if it is present on bacteria in the form of T-independent antigen (51). The carbohydrate component of LPS, when linked to a protein carrier, becomes a nontoxic, highly immunogenic, T-dependent antigen (18, 24).

We demonstrated previously that covalent conjugates of oli-

FIG. 4. Binding of the antipentaccharide-TT antibodies and anti-whole-bacterium antibodies to live cells of *B. pertussis* strain 186 (A) and heat-killed clinical isolates *B. pertussis* 3210 (B) and 3225 (C). The binding was evaluated by flow cytometry, and the histograms show the results for control serum (CS), antipentaccharide-TT conjugate serum (OS-TT), and serum against whole cells of strain 186 (WB). The antisera were diluted 200-fold. A total of 4×10^4 bacteria were counted in each analysis. The laser power used was 14 mW. Narrow-angle forward and side scatter and green fluorescence (FL1-H) emission signals were collected. Bacterial aggregates were excluded electronically.

gosaccharides with tetanus toxoid were immunogenic and induced specific antibodies belonging to the IgG class in animals. The antibodies produced against complete core structures of *Escherichia coli* reacted strongly with LPS in the presence of serum proteins and were able to recognize LPS present on the surface of live bacteria (25, 26, 28).

Here we determined the structural details of the pentasac-

FIG. 5. Inhibitory effects of antibodies against the pentasaccharide-TT conjugate and antibodies against whole cells of *B. pertussis* on the production of TNF- α , IL-6, and NO by J774A.1 cells stimulated with LOS of strain 186. J774A.1 cells (1×10^6 cells) were incubated with LOS of *B. pertussis* strain 186 (10 ng) in the presence of antipentaccharide-TT conjugate serum (OS-TT), serum against whole cells of strain 186 (WB), control serum (CS), and a nonstimulated control serum (NS). The levels of TNF- α , IL-6, and NO in the media were determined after incubation for 6, 24, and 48 h, respectively. The values are means $\pm$ standard deviations for three parallel cultures with duplicate samples. Differences between groups receiving antibodies and the control were significant at P values of <0.001 (one asterisk) and <0.05 (two asterisks). (r), error bar is smaller than the resolution of the graph.

charide isolated from the LOS of *B. pertussis* strain 186 and examined the specificity and the endotoxin-neutralizing activity of antibodies against a neoglycoconjugate of the pentasaccharide with tetanus toxoid. The anticonjugate antibodies reacted specifically with the wild-type LOS of *B. pertussis* and substantially reduced its ability to activate J774A.1 cells for production of NO, TNF- α , and IL-6. These antibodies were able to react with the isolated LOS in the presence of serum proteins, as well as with LOS present on the surface of live bacteria. The reactivities of the monospecific antibodies against the neoglycoconjugate of a single antigen (i.e., the LOS pentasaccharide) and the antibodies raised against the whole bacteria (i.e., antibodies against a variety of bacterial surface antigens exposed on the surface of *B. pertussis* cells) were similar. These data confirm the importance of a proper form of antigen used for immunization.

The STD NMR experiments used for the interaction studies demonstrated that the immunodominant epitope recognized by the anticonjugate antibodies within the pentasaccharide is located predominantly in the distal trisaccharide. The data are in agreement with previous observations of Le Blay et al. (22) concerning the specificities of three different monoclonal antibodies against *B. pertussis* LOS. These antibodies recognized three distinct epitopes in the distal pentasaccharide of the *B. pertussis* LOS, (i) the terminal α -D-GlcpNAc-(1 $\rightarrow$), linked to and modulated by the presence of $\rightarrow$ 4)- β -D-ManpNAc3NAcA-(1 $\rightarrow$), (ii) the $\rightarrow$ 3)- β -L-FucpNAc4NMe-(1 $\rightarrow$ 6)- α -D-GlcpN-(1 $\rightarrow$ disaccharide, and (iii) the L- α -D-Hepp-(1 $\rightarrow$ 4)- α -D-GlcpN-(1 $\rightarrow$ segment of the pentasaccharide.

We believe that such a specific antibody directed against the terminal trisaccharide could compensate for the lack of SP-A interaction with *B. pertussis* LOS in vivo and promote agglutination, phagocytosis, and bacterial killing, as well as inhibition of the endotoxin activity of the LOS.

Weiss and coworkers screened a set of human immune sera to identify the antibodies which can promote killing of *B. pertussis* cells (51). They found that antibodies to the *B. pertussis* LOS, in the presence of complement, could be bactericidal against wild bacterial strains producing BrkA protein, a virulence factor which inhibits the classical pathway of complement activation (2). However, not all anti-LOS antibodies were bactericidal. Antibodies produced against the components of acellular pertussis vaccine, which is devoid of LOS, did not promote the complement-dependent killing of bacteria.

Thus, finding a simple method for distinguishing between protective and nonprotective immune responses to *B. pertussis* infection and defining the additional components of acellular pertussis vaccine seem to be very important. We anticipate that doing these things could improve the efficiency of the vaccine by promoting the immune defense that can kill bacteria and neutralize the harmful effects of endotoxin.

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