The O Antigen Is a Critical Antigen for the Development of a Protective Immune Response to *Bordetella parapertussis*[▽]

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Despite excellent vaccine coverage in developed countries, whooping cough is a reemerging disease that can be caused by two closely related pathogens, *Bordetella pertussis* and *B. parapertussis*. The two are antigenically distinct, and current vaccines, containing only *B. pertussis*-derived antigens, confer efficient protection against *B. pertussis* but not against *B. parapertussis*. *B. pertussis* does not express the O antigen, while *B. parapertussis* retains it as a dominant surface antigen. Since the O antigen is a protective antigen for many pathogenic bacteria, we examined whether this factor is a potential protective antigen for *B. parapertussis*. In a mouse model of infection, immunization with wild-type *B. parapertussis* elicited a strong antibody response to the O antigen and conferred efficient protection against a subsequent *B. parapertussis* challenge. However, immunization with an isogenic mutant lacking the O antigen, *B. parapertussis* Δ*wbm*, induced antibodies that recognized other antigens but did not efficiently mediate opsonophagocytosis of *B. parapertussis*. The passive transfer of sera raised against *B. parapertussis*, but not *B. parapertussis* Δ*wbm*, reduced *B. parapertussis* loads in the lower respiratory tracts of mice. The addition of 10 μg of purified *B. parapertussis* lipopolysaccharide (LPS), which contains the O antigen, but not *B. parapertussis* Δ*wbm* LPS drastically improved the efficacy of the acellular vaccine Adacel against *B. parapertussis*. These data suggest that the O antigen is a critical protective antigen of *B. parapertussis* and its inclusion can substantially improve whooping cough vaccine efficacy against this pathogen.

Bordetella pertussis and *B. parapertussis* are the causative agents of whooping cough, resulting in approximately 50 million cases and 300,000 deaths annually worldwide (28). While whooping cough is considered by the CDC to be a reemerging disease (5), the relative incidences of *B. pertussis* and *B. parapertussis* are not clear (50). It is known, however, that the resurgence of whooping cough roughly correlates with the introduction of acellular pertussis vaccines (5). These vaccines contain only *B. pertussis*-derived antigens and confer little to no protection against *B. parapertussis* (9, 14, 15, 23, 27, 28). Current acellular pertussis vaccines contain some combination of filamentous hemagglutinin, pertactin, and fimbriae 2 and 3, all of which are expressed by both *B. pertussis* and *B. parapertussis*, and pertussis toxin, which is *B. pertussis* specific (33, 34). Based on genome sequences, the levels of amino acid sequence identity between *B. pertussis* and *B. parapertussis* filamentous hemagglutinin, pertactin, and fimbria 2 and 3 proteins are about 98, 91, 71, and 92% (35), and antibodies raised against these antigens from *B. pertussis* cross-react with *B. parapertussis* (17, 31). However, immunization with purified *B. pertussis* filamentous hemagglutinin or pertactin does not con-

fer protection against *B. parapertussis* (17). *B. pertussis* fimbriae confer some protection against *B. parapertussis*, but at much lower levels than they protect against *B. pertussis* (52). Based on these observations and the fact that *B. parapertussis* infection induces protective immunity to itself (56, 58), we hypothesized that the lack of protective antigens from *B. parapertussis* may be part of the reason why current whooping cough vaccines are ineffective against this bacterium.

Although *B. pertussis* and *B. parapertussis* are very closely related (8, 35, 48), they differ in the structure of their lipopolysaccharides (LPS) (1, 2, 39, 40, 47). *B. pertussis* produces a lipooligosaccharide containing lipid A and a branched-chain core oligosaccharide with a complex trisaccharide modification but lacks the O antigen due to a natural deletion of the *wbm* locus responsible for its synthesis (39, 47). *B. parapertussis* LPS is similar to *B. pertussis* LPS but lacks the trisaccharide modification and includes an O antigen (39, 40). In addition to conferring serum resistance by inhibiting C3 deposition onto the surfaces of bacteria (11), the O antigen enables *B. parapertussis* to avoid *B. pertussis*-induced immunity by preventing antibody binding to cross-reactive antigens on the surfaces of *B. parapertussis* cells (56, 59). Since the O antigen is one dominant surface antigen recognized by *B. parapertussis* immune sera (56) and has been shown previously to be a protective antigen of various pathogenic bacteria (22, 36), we hypothesized that the O antigen is a protective antigen of *B. parapertussis*.

To assess the role of the O antigen in the generation of an

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adaptive immune response to *B. parapertussis*, the immunity and protection generated by *B. parapertussis* infection or vaccination were compared to those generated by an isogenic mutant of *B. parapertussis* lacking the O antigen (Δ*wbm*) (39). Animals immunized with *B. parapertussis*, but not *B. parapertussis* Δ*wbm*, were protected against subsequent challenge with *B. parapertussis*. Mice immunized with *B. parapertussis* Δ*wbm* were also deficient in the production of *B. parapertussis*-specific antibodies, and sera collected from these mice were less effective at reducing *B. parapertussis* colonization upon passive transfer than sera raised against *B. parapertussis*. The inclusion of LPS from *B. parapertussis*, but not from *B. parapertussis* Δ*wbm*, rendered the acellular *B. pertussis* vaccine Adacel efficacious against *B. parapertussis* challenge. Together, these data indicate that the O antigen is an important protective antigen of *B. parapertussis*.

MATERIALS AND METHODS

Bacterial strains and growth. *B. pertussis* strain 536, *B. parapertussis* strain CN2591, and the isogenic *B. parapertussis* mutant strain lacking the O antigen, CN2591 Δ*wbm*, have been described previously (39, 46). For opsonization, attachment, and phagocytosis experiments, these strains were transformed with plasmid pCW505 (kindly supplied by Alison Weiss, Cincinnati, OH), which induces cytoplasmic expression of green fluorescent protein (GFP) without affecting growth or antigen expression (51). Bacteria were maintained on Bordet-Gengou agar (Difco) supplemented with 10% sheep blood (Hema Resources) and 20 μg/ml streptomycin (Sigma-Aldrich). Liquid cultures were grown overnight on a roller drum at 37°C to mid-log phase in Stainer-Scholte broth (44, 49).

Cells. Peripheral blood polymorphonuclear leukocytes (PMNs) were isolated from heparinized venous blood by using Ficoll-Histopaque (Sigma, St. Louis, MO) gradient centrifugation. PMNs were harvested, and the remaining erythrocytes were removed by hypotonic lysis. Cell viability was >99% as determined by trypan blue exclusion. Prior to functional assays, PMNs were washed twice with Dulbecco's modified Eagle medium (HyClone) supplemented with 10% fetal calf serum (HyClone) and resuspended, and the cells were used immediately. All experiments were carried out with freshly isolated PMNs lacking FcγRI (CD64) expression, as monitored by fluorescence-activated cell sorter analysis using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) with anti-FcγRI monoclonal antibody 22 (41).

Opsonization. GFP-expressing strains were opsonized by incubation at 37°C for 30 min in a final volume of 50 μl containing 5% heat-inactivated serum samples from naïve $C3^{-/-}$ mice or convalescent $C3^{-/-}$ mice challenged with CN2591 or CN2591 Δ*wbm*. Serum-opsonized bacteria were incubated with R-phycoerythrin (RPE)-labeled goat F(ab′)₂ fragments of anti-mouse immunoglobulin G (IgG; Southern Biotechnology, Birmingham, AL) for 30 min at 4°C. The opsonization of each strain was assessed by fluorescence-activated cell sorter analysis (43).

Attachment and phagocytosis. Attachment and phagocytosis of the *B. parapertussis* strains were evaluated as described previously, with a few modifications (42). Briefly, serum-opsonized, GFP-expressing bacteria were subsequently incubated with PMNs at a multiplicity of infection of 30 for 20 min at 37°C to allow binding. In selected experiments, 200 ng/ml cytochalasin D (Sigma-Aldrich) was added to inhibit phagocytosis. After extensive washing to remove unattached bacteria, an aliquot was maintained on ice to be used as a bacterial attachment control. Another aliquot was further incubated for 1 h at 37°C to allow internalization. Phagocytosis was stopped by placing PMNs on ice. Cell surface-bound bacteria in both aliquots (obtained before and after 1 h of incubation at 37°C) were detected by incubation with RPE-labeled goat F(ab′)₂ fragments of anti-mouse IgG at 4°C for 30 min. To avoid eventual cytophilic binding of antibodies, all incubations were done in the presence of 25% heat-inactivated human serum. After being washed, samples were analyzed by flow cytometry. Ten thousand cells per sample were analyzed. Green fluorescence intensity associated with PMNs maintained at 37°C for 20 min was determined to indicate the level of bacterial attachment. The decrease in red fluorescence after incubation for 1 h at 37°C reflects bacterial phagocytosis. Phagocytosis was calculated from the drop in the mean red fluorescence intensity of green fluorescence-positive cells as described previously (42).

Animal experiments. C57BL/6 mice were obtained from Jackson Laboratories. $C3^{-/-}$ mice were kind gifts from Rick Wetsel and have been described elsewhere (7). All mice were bred in our *Bordetella*-free, specific-pathogen-free breeding rooms at The Pennsylvania State University. Four- to six-week-old mice were sedated with 5% isoflurane (Abbott Laboratory) in oxygen and inoculated by pipetting of 50 μl of phosphate-buffered saline (PBS; Omnipur) containing 5 × 10^5 CFU of bacteria onto the external nares (18). This method reliably distributes the bacteria throughout the respiratory tract (13). For rechallenge experiments, mice were treated with gentamicin in drinking water (10 mg/ml) for 7 days starting on day 21 postinoculation (57). Mice were challenged with 5 × 10^5 CFU of bacteria on day 30 postinoculation and dissected 3 days postchallenge (57). For passive transfer of sera, 200-μl serum samples (collected on day 28 postinoculation) from naïve or convalescent $C3^{-/-}$ mice were intraperitoneally (i.p.) injected at the time of inoculation (19, 38). For vaccination, mice were i.p. injected with 10^8 CFU of heat-killed CN2591 or CN2591 Δ*wbm* in 200 μl of PBS with Imject Alum adjuvant (Pierce) on days 0 and 14. For acellular *B. pertussis* vaccinations, mice were i.p. injected with one-fifth of a human dose of Adacel (Sanofi Pasteur; 0.5 μg of pertussis toxin, 1 μg of filamentous hemagglutinin, 0.6 μg of pertactin, and a 5-μg mixture of fimbriae 2 and 3 per mouse) in a 200-μl volume containing PBS and Imject Alum adjuvant with or without 10 μg of purified CN2591 LPS or CN2591 Δ*wbm* LPS (45) on days 0 and 14. Vaccinated mice were challenged with bacteria on day 28. Mice were sacrificed via CO_2 inhalation, and the lungs, tracheae, and nasal cavities were excised. Tissues were homogenized in PBS, serially diluted, and plated onto Bordet-Gengou agar, and colonies were counted after incubation at 37°C for 3 to 4 days (25). All protocols were reviewed and approved by The Pennsylvania State University Institutional Animal Care and Use Committee, and all animals were handled in accordance with institutional guidelines.

Splenocyte restimulations. Spleens were taken from C57BL/6 mice immunized with CN2591 or CN2591 Δ*wbm* on day 28 postinoculation. Splenocytes were isolated as described previously (25, 37). In brief, spleens were homogenized and red blood cells were lysed by 0.84% ammonium chloride treatment. Aliquots of cells (2 × 10^6) were resuspended in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate (HyClone), and 100 μg/ml penicillin-streptomycin (HyClone) and placed into each well of a 96-well tissue culture plate. Splenocytes were stimulated with either medium alone or medium containing 10^7 CFU of heat-killed CN2591 or CN2591 Δ*wbm* (multiplicity of infection of 5) (37). After 3 days, the supernatants were collected and analyzed for gamma interferon (IFN-γ) and interleukin-10 (IL-10) production via enzyme-linked immunosorbent assays (ELISAs) per the instructions of the manufacturer (R&D Systems).

Titer ELISAs. Antibody titers were determined as described previously (25, 56). In brief, exponential-phase live CN2591 or CN2591 Δ*wbm* bacteria were diluted to 5 × 10^7 CFU/ml in a 1:1 mix of 0.2 M sodium carbonate and 0.2 M sodium bicarbonate buffers. The wells of 96-well plates were coated with these antigens, and the plates were incubated for 2 h at 37°C in a humidified chamber and then washed and blocked. Serum samples from individual mice were diluted 1:50, added to the first wells of the plates, and serially diluted 1:2 across the plates, and the plates were incubated for 2 h at 37°C. Plates were washed, probed with a 1:4,000 dilution of goat anti-mouse Ig horseradish peroxidase-conjugated antibodies (Southern Biotech) for 1 h, and washed again prior to visualization with 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt in phosphate-citrate buffer with hydrogen peroxide at an absorbance of 405 nm. Titers were determined via the end point method based on optical densities in identically treated wells probed with naïve sera.

Western blot analysis. Lysates containing 10^7 CFU of heat-killed CN2591 or CN2591 Δ*wbm* were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels under denaturing conditions. Polyvinylidene difluoride membranes (Millipore) were probed overnight with either naïve sera or sera from CN2591- or CN2591 Δ*wbm*-inoculated mice at a 1:500 dilution. Goat anti-mouse Ig horseradish peroxidase-conjugated antibodies (Southern Biotech) were used at a dilution of 1:10,000 as the detector antibody (56, 57). The membrane was visualized with enhanced chemiluminescence Western blotting detection reagent (Pierce Biotechnology).

Statistical analysis. The mean ± standard error was determined for all appropriate data. Two-tailed, unpaired Student's *t* tests were used to determine the statistical significance of differences between groups. All experiments were performed at least twice with similar results.

RESULTS

The O antigen is required for efficient generation of protective immunity against *B. parapertussis* infection. To determine
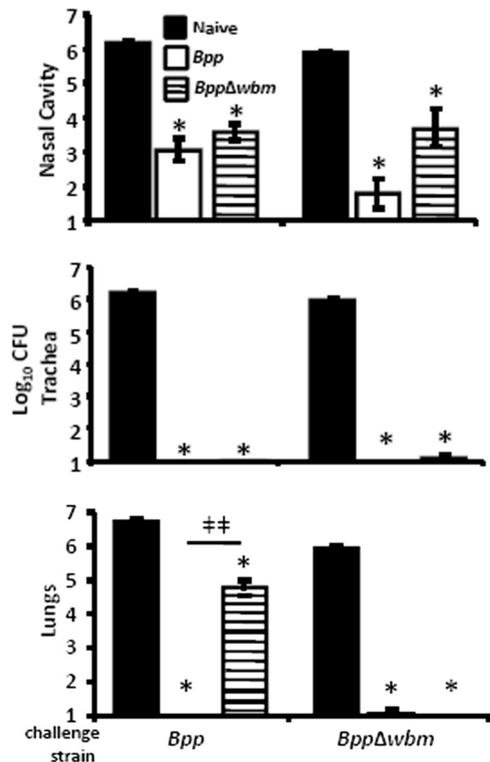
FIG. 1. The O antigen contributes to the generation of protective immunity to *B. parapertussis*. Groups of four C57BL/6 mice were inoculated with *B. parapertussis* (*Bpp*) or *B. parapertussis* Δ*wbm* (*Bpp*Δ*wbm*) and allowed to convalesce. Naïve and immunized mice were challenged with the indicated bacteria. The numbers of CFU recovered from the nasal cavities, tracheae, and lungs at day 3 postchallenge are expressed as the $\log_{10}$ means ± the standard errors. * indicates a P value of ≤0.05 for comparison to results for naïve mice; ‡‡ indicates a P value of ≤0.01. The limit of detection is indicated by the y axis.
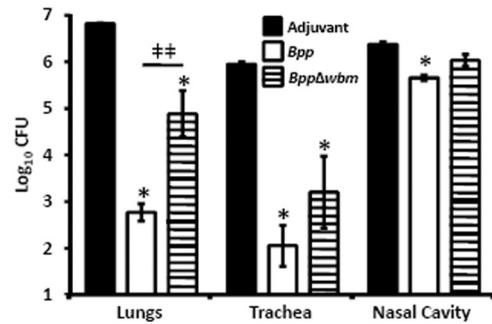

FIG. 2. A response against the O antigen contributes to effective vaccine-induced immunity. Groups of four C57BL/6 mice were vaccinated with adjuvant only, *B. parapertussis* with adjuvant (*Bpp*), or *B. parapertussis* Δ*wbm* with adjuvant (*Bpp*Δ*wbm*) and challenged with *B. parapertussis*. The numbers of CFU recovered from the nasal cavities, tracheae, and lungs at day 3 postchallenge are expressed as the $\log_{10}$ means ± the standard errors. * indicates a P value of ≤0.05 for comparison to results for mice vaccinated with adjuvant only; ‡‡ indicates a P value of ≤0.01. The limit of detection is indicated by the y axis.

whether the O antigen contributes to the generation of *B. parapertussis* infection-induced protective immunity against secondary challenge, mice were intranasally inoculated with either *B. parapertussis* or *B. parapertussis* Δ*wbm* and challenged with either *B. parapertussis* or *B. parapertussis* Δ*wbm*. Naïve animals challenged with wild-type *B. parapertussis* had mean loads of $10^{6.2}$, $10^{6.1}$, and $10^{6.7}$ CFU in the nasal cavity, trachea, and lungs on day 3 postchallenge (Fig. 1, black bars). Mice previously inoculated with *B. parapertussis* were substantially immune to subsequent challenge, harboring approximately 10^3 CFU in the nasal cavity, and had cleared the bacteria from both the trachea and lungs by 3 days postchallenge (Fig. 1, white bars). Prior infection with *B. parapertussis* and *B. parapertussis* Δ*wbm* conferred similar levels of protection in the lower respiratory tract (LRT) against subsequent *B. parapertussis* Δ*wbm* challenge (Fig. 1, right). Interestingly, *B. parapertussis* infection induced more protection against the O-antigen-deficient strain in the nasal cavity than *B. parapertussis* Δ*wbm* infection did (Fig. 1, top right). However, mice immunized with *B. parapertussis* Δ*wbm* harbored at least 8,000-fold more *B. parapertussis* bacteria in the lungs, $10^{4.9}$ CFU, than *B. parapertussis*-immunized mice (P < 0.01) (Fig. 1, bottom), indicat-

ing that the mutant lacking the O antigen did not induce effective protective immunity.

Effective vaccine-induced immunity requires a response against the O antigen. *B. parapertussis* Δ*wbm* is known to colonize at a lower level than *B. parapertussis* in the presence of complement (11), raising the possibility that its defect in colonization contributes to the decreased protection against subsequent challenge (Fig. 1). To deliver equivalent amounts of antigens, mice were vaccinated with heat-killed *B. parapertussis* or *B. parapertussis* Δ*wbm*. Sham-vaccinated control mice challenged with *B. parapertussis* harbored $10^{6.4}$, $10^{5.8}$, and $10^{6.8}$ CFU in the nasal cavity, trachea, and lungs 3 days later (Fig. 2, black bars). Vaccination with *B. parapertussis* effectively decreased *B. parapertussis* numbers by 99.99% in the LRT and by 80% in the nasal cavity (Fig. 2, white bars). Although vaccination with *B. parapertussis* Δ*wbm* reduced *B. parapertussis* numbers in the LRT (Fig. 2, striped bars), animals vaccinated with *B. parapertussis* Δ*wbm* had 160-, 16-, and 3-fold more bacteria in the lungs, trachea, and nasal cavity than *B. parapertussis*-vaccinated animals (Fig. 2). This decreased protection conferred by *B. parapertussis* Δ*wbm* vaccination further strengthens the conclusion that the O antigen is required for the efficient generation of an adaptive immune response against *B. parapertussis*.

The O antigen is not required for the development of splenic IFN-γ and IL-10 responses to *B. parapertussis*. Since the O antigen contributes to the generation of efficient protective immunity against *B. parapertussis*, we investigated whether the O antigen is involved in the generation of a T-cell response. Splenocytes from naïve or *B. parapertussis*- or *B. parapertussis* Δ*wbm*-vaccinated mice were stimulated with medium alone or with heat-killed *B. parapertussis* or *B. parapertussis* Δ*wbm*, and 3 days later, IFN-γ and IL-10 concentrations in the culture supernatants were measured. Vaccination with either strain resulted in increased IFN-γ and IL-10 production. There was no significant difference in IFN-γ or IL-10 production in response to *B. parapertussis* or *B. parapertussis* Δ*wbm* between mice vaccinated with *B. parapertussis* and those vaccinated with
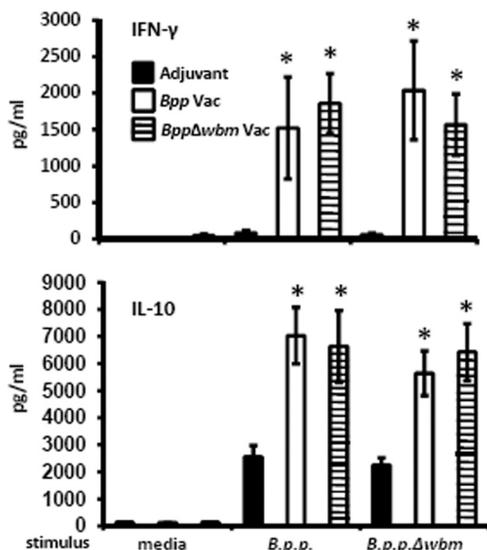
FIG. 3. The O antigen is not required for the development of splenic IFN-γ or IL-10 responses to *B. parapertussis*. Splenocytes from groups of four C57BL/6 mice vaccinated with adjuvant only, *B. parapertussis* with adjuvant (*Bpp* Vac), or *B. parapertussis* Δ*wbm* with adjuvant (*BppΔwbm* Vac) were stimulated with the indicated bacteria, and the resulting IFN-γ and IL-10 production levels are expressed as mean concentrations ± standard errors. ∗ indicates a *P* value of ≤0.05 for comparison to results for medium-stimulated groups. *B.p.p.*, wild-type *B. parapertussis*; *B.p.p.*Δ*wbm*, *B. parapertussis* Δ*wbm*.

B. parapertussis Δ*wbm* (Fig. 3). Since the splenic IFN-γ and IL-10 responses are T-cell dependent (D. N. Wolfe, M. J. Kennett, S. E. Hester, and E. T. Harvill, unpublished data), these data suggest that the O antigen is not required for the generation of a T-cell response to *B. parapertussis*.

The O antigen is required for the generation of an efficient antibody response against *B. parapertussis*. As the O antigen is required for the generation of anamnestic immunity to *B.*

parapertussis but not an efficient T-cell response, we assessed whether the O antigen contributes to efficient antibody generation. In ELISAs using either strain as the antigen, *B. parapertussis* immune serum had significantly less recognition of the O antigen mutant than of wild-type bacteria (Fig. 4A, left). *B. parapertussis* Δ*wbm* immunization sera had similar Ig titers when probed with the wild-type and O antigen mutant *B. parapertussis* strains (Fig. 4A, right). Sera raised against *B. parapertussis* Δ*wbm* showed a 44% reduction in *B. parapertussis*-specific antibody titers compared to those in sera raised against *B. parapertussis* (Fig. 4A, first and third bars). These data suggest that vaccination with *B. parapertussis* induces a robust antibody response against the O antigen and that vaccination with *B. parapertussis* Δ*wbm* induces an antibody response to other antigens that are shared.

To compare the antigens recognized by sera from different groups, Western blotting analyses were performed with lysates of *B. parapertussis* and *B. parapertussis* Δ*wbm* probed with naïve sera or *B. parapertussis* or *B. parapertussis* Δ*wbm* immune sera (Fig. 4B). Naïve sera appeared to minimally bind antigens from either lysate (Fig. 4B, lanes 1 and 2). *B. parapertussis*-induced serum antibodies recognized a broad band or smear, band i, present in *B. parapertussis* lysate but not in *B. parapertussis* Δ*wbm* lysate (Fig. 4B, lanes 3 and 4), suggesting that it represents LPS containing the O antigen and that the O antigen is one of the dominant antigens of *B. parapertussis*. Several higher-molecular-mass antigens shared by the two strains, for example, those represented by bands iii and iv, were also recognized by *B. parapertussis* immune serum antibodies. Interestingly, although *B. parapertussis* Δ*wbm*-induced serum antibodies showed recognition of antigen(s) in band iii, these antibodies lacked recognition of antigen(s) in band iv and had strong recognition of additional antigen(s) in bands ii and v, not recognized by *B. parapertussis*-induced serum antibodies. As expected, the O antigen (band i) was not recognized by *B. parapertussis* Δ*wbm*-induced serum antibodies. Together, these
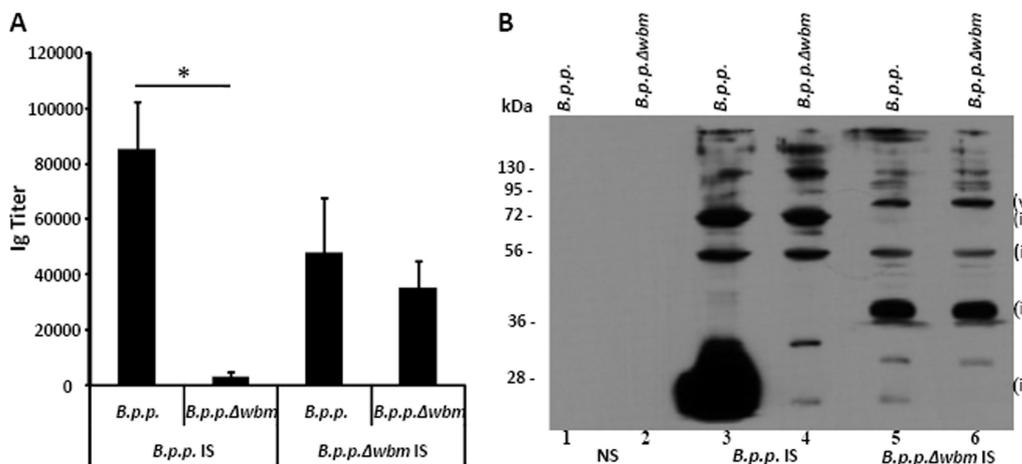

FIG. 4. The O antigen contributes to the production of a robust anti-*B. parapertussis* antibody response. (A) Ig titers in sera from groups of four C57BL/6 mice immunized with *B. parapertussis* (*B. parapertussis* immune sera [*B.p.p.* IS]) or *B. parapertussis* Δ*wbm* (*B.p.p.*Δ*wbm* IS) supplemented with adjuvant were determined via *B. parapertussis* (*B.p.p.*)- or *B. parapertussis* Δ*wbm* (*B.p.p.* Δ*wbm*)-specific ELISAs. Titers are expressed as means ± standard errors. ∗ indicates a *P* value of ≤0.05. (B) Lysates (10⁷ CFU) from *B. parapertussis* (*B.p.p.*) or *B. parapertussis* Δ*wbm* (*B.p.p.*Δ*wbm*) were probed with naïve sera (NS), sera from *B. parapertussis*-immunized mice (*B.p.p.* IS), or sera from *B. parapertussis* Δ*wbm*-immunized mice (*B.p.p.*Δ*wbm* IS), as indicated. Roman numerals to the right of the gel identify bands.

data indicate that immunization with *B. parapertussis* induces a measurably stronger antibody response, dominated by the O antigen, than that induced by *B. parapertussis* Δ*wbm* and that immunization with *B. parapertussis* Δ*wbm* induces a different antigen recognition profile from that induced by immunization with the wild-type counterpart.

The O antigen contributes to the generation of antibodies that mediate opsonophagocytosis of *B. parapertussis* by PMNs. To determine whether antibodies against the O antigen are important for some key antibody functions, we assessed the opsonization of bacteria and subsequent attachment to, and phagocytosis by, PMNs mediated by antibodies raised against wild-type or O-antigen-deficient *B. parapertussis*. Because *B. parapertussis* Δ*wbm* is not defective in colonization of mice lacking complement (11), sera were generated in complement-deficient mice, thereby removing the difference in bacterial load as a factor affecting antibody production. Compared to the naïve sera, *B. parapertussis* immune sera mediated efficient opsonization of wild-type *B. parapertussis* and subsequent attachment to and phagocytosis by PMNs (Fig. 5, middle black bars). *B. parapertussis* immune sera were less effective against O-antigen-deficient *B. parapertussis* in all three assays (Fig. 5, middle white bars), suggesting that antibodies recognizing the O antigen, rather than the shared antigens, are involved. Sera from mice immunized with O-antigen-deficient *B. parapertussis* were similarly effective against the wild-type and O-antigen-deficient strains (Fig. 5, right). Control PMNs treated with cytochalasin, a phagocytosis inhibitor, showed no phagocytosis (data not shown), indicating that although indirect, the assay measured phagocytosis. The observed high levels of activity of *B. parapertussis* immune sera against wild-type but not O-antigen-deficient *B. parapertussis* suggest that much of this activity is mediated by antibodies to the O antigen.

The O antigen is required for the generation of antibodies that efficiently clear *B. parapertussis*. To determine if the decreased *B. parapertussis*-specific antibody titers of, and opsonophagocytosis mediated by, sera raised against *B. parapertussis* Δ*wbm* result in decreased antibody-mediated clearance in vivo, mice received passively transferred naïve sera or sera raised against wild-type or O-antigen-deficient *B. parapertussis* in C3$^{-/-}$ mice. Mice were then challenged with *B. parapertussis* and sacrificed on day 14 postchallenge for bacterial enumeration, since *B. parapertussis* poorly stimulates Toll-like receptor 4 (TLR4) and antibodies therefore have no effect until around day 14 after T cells have been generated (19, 55; D. N. Wolfe, unpublished data). Naïve sera had no effect on bacterial loads throughout the respiratory tract on day 14 postchallenge (Fig. 6). As seen in previous studies (19, 56), *B. parapertussis* immune sera decreased the bacterial loads in the trachea and lungs by 96 and 99.6% at this time point. However, *B. parapertussis* Δ*wbm* immune sera failed to significantly reduce *B. parapertussis* colonization, indicating that the O antigen is required for the generation of antibodies that clear *B. parapertussis* from the LRT in vivo upon adoptive transfer. Neither serum treatment affected bacterial numbers in the nasal cavity.

Supplementing Adacel with *B. parapertussis* LPS containing the O antigen confers protection against *B. parapertussis* challenge. Since the O antigen is necessary for the generation of
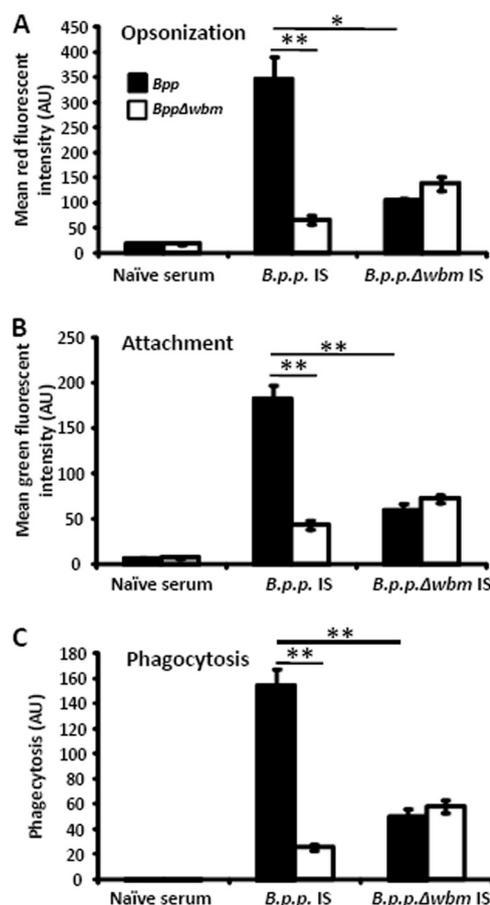

FIG. 5. Generation of antibodies that mediate efficient opsonophagocytosis of *B. parapertussis* by PMNs requires the O antigen. GFP-expressing wild-type *B. parapertussis* (*Bpp*) or O-antigen-deficient *B. parapertussis* (*Bpp*Δ*wbm*) was opsonized with naïve sera or sera from C3$^{-/-}$ mice challenged with *B. parapertussis* (*B. parapertussis* immune sera [*B.p.p.* IS]) or *B. parapertussis* Δ*wbm* (*B.p.p.*Δ*wbm* IS) and stained with RPE-labeled goat F(ab')$_2$ fragments of anti-mouse IgG. (A) Opsonization levels were measured as mean intensities ± standard errors of red fluorescence from bacteria opsonized with the indicated sera from four individual mice. (B) Opsonized bacteria were incubated with freshly isolated human peripheral blood PMNs for 20 min or 1 h and 20 min. Attachment levels were measured as mean intensities ± standard errors of green fluorescence associated with PMNs incubated for 20 min with bacteria opsonized by the indicated sera from four individual mice. (C) The cell surface-bound bacteria on PMNs were detected by incubation with RPE-labeled goat F(ab')$_2$ fragments of anti-mouse IgG. Mean phagocytosis levels ± standard errors were calculated from the drop in red fluorescence of green fluorescence-positive cells incubated for 1 h and 20 min compared to that of cells incubated for 20 min. Results were obtained from experiments done with four independent serum samples. AU indicates arbitrary units; * indicates a *P* value of ≤0.05; ** indicates a *P* value of ≤0.01.

efficient protective immunity to *B. parapertussis* (Fig. 1, 2, and 4), we examined whether *B. parapertussis* LPS alone, containing the O antigen, is sufficient to induce protective immunity against this pathogen and whether supplementing Adacel with *B. parapertussis* LPS renders this vaccine effective against *B. parapertussis*. Mice were vaccinated with an adjuvant alone, the acellular pertussis vaccine Adacel with an adjuvant, or Adacel with an adjuvant supplemented with purified LPS from *B. parapertussis* or *B. parapertussis* Δ*wbm*. Vaccination with adju-
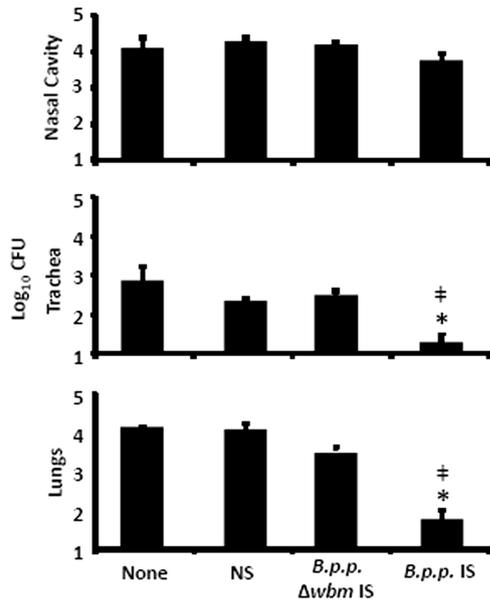
FIG. 6. Antibodies to the O antigen are required for efficient antibody-mediated clearance of *B. parapertussis*. Groups of four C57BL/6 mice were inoculated with *B. parapertussis* and i.p. injected with the indicated serum. Bacterial loads in the nasal cavities, tracheae, and lungs at 14 days postinoculation are expressed as the $\log_{10}$ means $\pm$ standard errors. * indicates a P value of ≤ 0.05 for comparison between results for groups receiving naïve serum (NS) and *B. parapertussis* immune serum. ‡ indicates a P value of ≤ 0.05 for comparison between results for groups receiving *B. parapertussis* Δwbm immune serum (*B.p.p.* Δwbm IS) and wild-type *B. parapertussis* immune serum (*B.p.p.* IS). The limit of detection is indicated by the y axis.

vant alone or Adacel had no effect on *B. parapertussis* loads throughout the respiratory tract 3 days postchallenge (Fig. 7A). In contrast, vaccination with *B. parapertussis* LPS, but not *B. parapertussis* Δwbm LPS, significantly reduced *B. parapertussis* loads in the lungs by 93.8% compared to those in the group vaccinated with adjuvant alone. Moreover, the addition of *B. parapertussis* LPS, but not *B. parapertussis* Δwbm LPS, to Adacel caused significant decreases in bacterial loads, by 70.7, 99.6, and 96.2% in the nasal cavity, trachea, and lungs, respectively, suggesting that the efficacy of an acellular pertussis vaccine against *B. parapertussis* may be increased if *B. parapertussis* LPS containing the O antigen is included. To ensure that the addition of *B. parapertussis* LPS did not have an impact on the efficacy of Adacel against *B. pertussis*, mice were immunized with this vaccine with or without *B. parapertussis* LPS and challenged with *B. pertussis*. As expected, vaccination with the adjuvant alone did not affect the colonization by *B. pertussis* compared to that of naïve animals (Fig. 7B). Vaccination with Adacel reduced the *B. pertussis* load in the lungs by >99.5% (Fig. 7B, bottom). This vaccine supplemented with *B. parapertussis* LPS caused a similar reduction of *B. pertussis* numbers (Fig. 7B, bottom), suggesting that the inclusion of *B. parapertussis* LPS does not affect the efficacy of the vaccine against *B. pertussis*. All together, our data suggest that the addition of *B. parapertussis* LPS containing the O antigen to a current acellular vaccine extended its utility to include protective immunity to *B. parapertussis*.
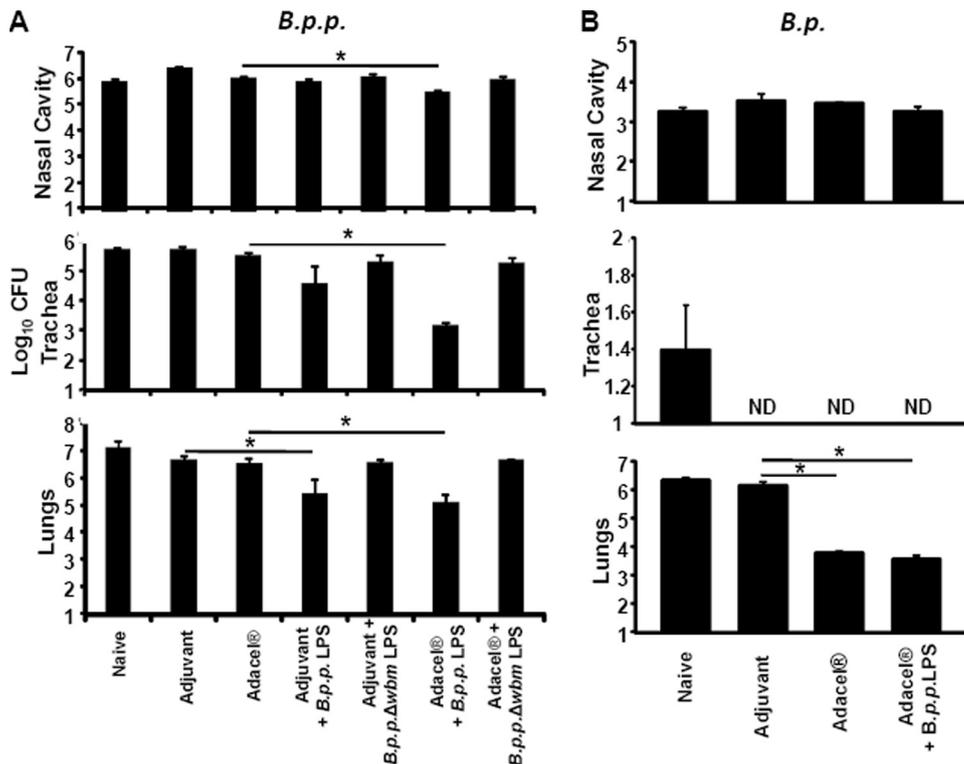
FIG. 7. Addition of purified *B. parapertussis* LPS to an acellular *B. pertussis* vaccine confers protection against *B. parapertussis* challenge. Groups of four C57BL/6 mice were vaccinated as indicated and then challenged with *B. parapertussis* (*B.p.p.*) (A) or *B. pertussis* (*B.p.*) (B) and dissected at day 3 postchallenge. The numbers of CFU recovered from the nasal cavities, tracheae, and lungs are expressed as the $\log_{10}$ means $\pm$ the standard errors. ND indicates that CFU were not detectable. * indicates a P value of ≤ 0.05. The limit of detection is indicated by the y axis.

DISCUSSION

A clear picture of *B. parapertussis* epidemiology is not available because differential diagnostic methods to distinguish the two causative agents of whooping cough are rarely performed at the clinical level and diseases caused by *B. parapertussis* are not reportable to the CDC. However, when carefully monitored, *B. parapertussis* has been found to cause a substantial proportion of whooping cough cases and even larger proportions among vaccinated groups (4, 23, 24, 50). Although the mouse model does not replicate coughing symptoms of the disease, mechanisms of immune control and clearance of the bordetellae are consistent with what is known of these mechanisms in humans (19, 29, 30). The data presented here are consistent with the findings of experimental studies using a mouse infection model, as well as those of clinical studies, in which *B. pertussis* immunity failed to induce protective immunity to *B. parapertussis* (Fig. 7A) (9, 10, 14, 23, 27, 52, 56, 59). This work extends the findings of those previous studies to examine the role of the O antigen in the generation of *B. parapertussis*-specific immunity.

We found that although immunization with wild-type *B. parapertussis* induced protective immunity to both the wild-type and the O-antigen-deficient *B. parapertussis* strains, prior infection or vaccination with the O-antigen-deficient strain conferred significantly less protection against the wild type in the lungs (Fig. 1 and 2). Immunization with *B. parapertussis* Δwbm induced splenic cytokine production similar to that induced by wild-type vaccination (Fig. 3), indicating that the decrease in protection conferred by the O-antigen-deficient strain was not due to inefficient T-cell cytokine production. Interestingly, *B. parapertussis*-induced antibodies recognized the O antigen as a dominant antigen (Fig. 4A and B, lanes 3 and 4). Serum antibodies raised against the wild type, but not the O-antigen-deficient strain, mediated efficient opsonophagocytosis and reduced *B. parapertussis* colonization upon passive transfer (Fig. 5). Together, these data suggest that the O antigen is required for the generation of an effective antibody response against *B. parapertussis*.

Antibodies raised against *B. parapertussis* Δwbm lacked recognition of the O antigen but recognized different antigens from those recognized by antibodies raised against wild-type *B. parapertussis* (Fig. 4B) and efficiently cleared *B. parapertussis* Δwbm (Fig. 1). These antigens are present in the *B. parapertussis* lysate (Fig. 4B), but *B. parapertussis* Δwbm immune serum is much less effective at binding live bacteria, mediating opsonophagocytosis in vitro, or mediating bacterial clearance in vivo than *B. parapertussis* immune serum (Fig. 4 to 6), suggesting that these antigens may not be recognized on the surfaces of live *B. parapertussis* cells expressing the O antigen. These data further indicate that the O antigen is a dominant surface antigen of *B. parapertussis* and that antibodies against it are required for efficient clearance of this bacterium.

The O antigen seems to contribute to the generation of effective protective immunity against *B. parapertussis* in the lungs but not in the trachea or nasal cavity (Fig. 1 and 2). Wolfe et al. observed that B cells and T cells are required for clearance of *B. parapertussis* from the lungs and that CD4$^+$ T cells, complement, and neutrophils are required for antibody-mediated clearance in this organ (58). What immune compo-

nents are required for *B. parapertussis* clearance in the trachea and nasal cavity is less understood. Infection-induced immunity appeared to be more effective than vaccination-induced immunity in the nasal cavity and trachea (Fig. 1 and 2). This pattern may be due to different clearance mechanisms in infection- and vaccination-induced immunity to bordetellae (12). Vaccination is efficient in controlling disease but may be less effective in preventing subclinical colonization, as observed with *B. pertussis* (28). While the nasal cavity may be a reservoir of asymptomatic carriage of *B. parapertussis*, the protection in the lungs correlates with vaccine efficacy against severe disease and is thus the focus of this study (9).

The incidence of whooping cough has increased over the past 20 years, despite the maintenance of excellent vaccine coverage in developed countries (5). This trend may be due, at least in part, to vaccines' being ineffective against *B. parapertussis*-induced disease (9, 16, 23). Of note, the switch from whole-cell to acellular vaccines correlates with increased prevalence of *B. parapertussis* (23). Moreover, whooping cough vaccinations have been proposed to shape the age-incidence patterns of the two causative agents. *B. pertussis* is more common in infants prior to vaccination and adolescents in whom vaccine-induced immunity has waned (6, 53), whereas *B. parapertussis* is most common in young children who have been recently vaccinated (3, 21, 54; J. Lavine, L. Han, E. T. Harvill, and O. Bjornstad, unpublished data). All these observations suggest that current whooping cough vaccines confer a selective advantage on *B. parapertussis* in its ongoing competition with *B. pertussis*.

We have shown that supplementing the acellular pertussis vaccine Adacel with 10 μg of purified *B. parapertussis* LPS containing the O antigen reduced *B. parapertussis* numbers in the LRT by more than 90% within 3 days compared to the numbers in the group receiving Adacel alone (Fig. 7). Thus, the addition of this single antigen increased the efficacy of this vaccine against *B. parapertussis* in the mouse model. These results are not necessarily easily translated to improved human vaccines, since vaccine reactogenicity has been associated with LPS of *B. pertussis*. However, *B. parapertussis* LPS is less stimulatory toward TLR4 than *B. pertussis* LPS, and it is possible to purify the O antigen portion of the LPS (20, 26, 55), thereby removing the TLR4 agonist, lipid A, to which is attributed most of the proinflammatory stimulation (32). Alternatively, other, as-yet-unidentified antigens of *B. parapertussis* may prove to be protective and could be added to acellular whooping cough vaccines. However, the poor protection conferred by the O-antigen-deficient strain and the ability of the O antigen to block the effects of antibodies recognizing other antigens (56, 59) suggest that the inclusion of the O antigen in the whooping cough vaccines should be favored over other, as-yet-unidentified protein antigens.

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