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Nasal Immunization with *Burkholderia multivorans* Outer Membrane Proteins and the Mucosal Adjuvant Adamantylamide Dipeptide Confers Efficient Protection against Experimental Lung Infections with *B. multivorans* and *B. cenocepacia*[▽]

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Chronic lung infection by opportunistic pathogens, such as *Pseudomonas aeruginosa* and members of the *Burkholderia cepacia* complex, is a major cause of morbidity and mortality in patients with cystic fibrosis. Outer membrane proteins (OMPs) of gram-negative bacteria are promising vaccine antigen candidates. In this study, we evaluated the immunogenicity, protection, and cross-protection conferred by intranasal vaccination of mice with OMPs from *B. multivorans* plus the mucosal adjuvant adamantylamide dipeptide (AdDP). Robust mucosal and systemic immune responses were stimulated by vaccination of naive animals with OMPs from *B. multivorans* and *B. cenocepacia* plus AdDP. Using a mouse model of chronic pulmonary infection, we observed enhanced clearance of *B. multivorans* from the lungs of vaccinated animals, which correlated with OMP-specific secretory immunoglobulin A responses. Furthermore, OMP-immunized mice showed rapid resolution of the pulmonary infection with virtually no lung pathology after bacterial challenge with *B. multivorans*. In addition, we demonstrated that administration of *B. multivorans* OMP vaccine conferred protection against *B. cenocepacia* challenge in this mouse infection model, suggesting that OMPs provide cross-protection against the *B. cepacia* complex. Therefore, we concluded that mucosal immunity to *B. multivorans* elicited by intranasal vaccination with OMPs plus AdDP could prevent early steps of colonization and infection with *B. multivorans* and also ameliorate lung tissue damage, while eliciting cross-protection against *B. cenocepacia*. These results support the notion that therapies leading to increased mucosal immunity in the airways may help patients with cystic fibrosis.

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the CF transmembrane regulator gene, which encodes the CFTR chloride channel (35, 45). Chronic lung infection by opportunistic pathogens, such as *Pseudomonas aeruginosa* and the *Burkholderia cepacia* complex (Bcc) (12), causes significant morbidity and mortality in CF patients. The Bcc currently consists of at least nine species: *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina*, and *B. pyrrocinia* (13). Isolates of all Bcc species have been recovered from the sputum of patients with CF (12). *B. cenocepacia* and *B. multivorans* comprise about 83 and 10% of all Bcc isolates from CF patients in Canada, respectively (43). In the United States, *B. cenocepacia* and *B. multivorans* account for about 45 and 39% of all isolates recovered from CF patients, respectively (41). *B. cenocepacia* isolates are also prevalent in pediatric CF patients admitted to the Children's Hospital of Buenos Aires (L. Galanternik and M. A. Valvano, unpublished).

Bcc bacteria are not usually part of the normal flora of humans, and they do not commonly pose a risk to healthy individuals. However, a proportion of CF patients infected with Bcc can develop "cepacia syndrome," a devastating illness characterized by a fatal acute necrotizing pneumonia that causes rapid and progressive respiratory failure, often leading to the patient's death (22). Intrinsic resistance of Bcc bacteria to many commonly used antibiotics (1) and induction of cross-resistance to unrelated antimicrobial agents (40) make it difficult to eradicate these bacteria from CF patients.

The specific mechanisms by which Bcc bacteria can subvert host defenses, invade deeper tissues of the lung, and ultimately become blood borne are poorly understood (26, 33). Chronic airway infection and exacerbated inflammation are significant clinical problems for CF patients, since ultimately these processes lead to destruction of the lung tissue. Given the morbidity, mortality, and health care costs associated with Bcc infection in CF patients and the growing concerns about increased antimicrobial resistance, it would be desirable to have therapeutic alternatives for protecting patients against early infection of the lungs. Strategies that prevent colonization or reduce bacterial transmission among CF patients while minimizing lung inflammation would help control the progression of CF lung disease.

Little is known about the humoral immune response to Bcc

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infection in CF patients. Immunoglobulin G (IgG) antibodies to *B. cepacia* outer membrane proteins (OMPs) have been detected in sera of CF patients colonized with both *B. cepacia* and *P. aeruginosa* (3, 4), suggesting cross-reactivity between the OMPs of these organisms. Another study showed that the antibody response was specific to *B. cepacia* antigens (29). Furthermore, serum IgG and sputum IgA titers against *B. cepacia* lipopolysaccharide (LPS) were significantly greater in CF patients colonized with *B. cepacia* than in age- and sex-matched CF patients colonized with *P. aeruginosa* or in healthy individuals without CF harboring neither organism (36).

To our knowledge, the protective value of anti-Bcc immune responses has not been explored. Since Bcc bacteria cause mucosal infections, a vaccine generating a mucosal immune response would be an effective approach for preventing bacterial colonization. The mucosal immune system is the first line of defense against invading pathogens. Nasopharynx-associated lymphoid tissue (NALT) and Peyer's patches are important inductive sites for the initiation of antigen-specific mucosal IgA and serum IgG responses, as well as cytotoxic T-lymphocyte immune responses, at both mucosal and systemic sites. Thus, both NALT and Peyer's patches maximize the two-tiered immunological barrier of the host. Intranasal (i.n.) delivery of vaccines is an attractive mode of immunization. The nose, like the mouth, is a practical site for vaccine administration, and NALT stimulation efficiently induces antigen-specific immune responses in both mucosal and systemic compartments (15, 28). In the past decade, several clinical studies have confirmed that local immunity and systemic immunity are generated after nasal immunization of humans against diphtheria and tetanus (2), influenza (21), and infection with *Streptococcus mutans* (32). A large number of studies performed with mice, pigs, and monkeys have also confirmed the effectiveness of nasal immunization with a variety of vaccines (15). We have previously reported that the adjuvant adamantylamide dipeptide (AdDP) can enhance protective immune responses against antigens administered by a mucosal route (5, 6).

We hypothesize that generating a mucosal specific immune response in the respiratory tract could prevent early steps of colonization and infection by Bcc bacteria and thus could prevent or ameliorate lung damage due to inflammation during subsequent infection. Using a murine model of chronic pulmonary infection with *B. multivorans*, we show here that i.n. immunization with a *B. multivorans* OMP preparation can induce specific mucosal immune responses in the respiratory tract, which in turn enhance the clearance of *B. multivorans* and minimize lung inflammatory damage after bacterial challenge. In addition, we demonstrated that administration of the *B. multivorans* OMP vaccine conferred protection against *B. cenocepacia* challenge in this mouse infection model, suggesting that OMPs may provide cross-protection against other Bcc members.

MATERIALS AND METHODS

Bacterial strains and media. The Bcc strains used in this study included *B. multivorans* ATCC 17616, *B. vietnamiensis* LMG16232, and clinical isolates of *B. cenocepacia*, *B. stabilis*, and *B. ambifaria* obtained from sputum of CF patients at the Ricardo Gutiérrez Children's Hospital, Buenos Aires, Argentina. Bacteria were maintained in Luria-Bertani (LB) broth containing 20% (vol/vol) glycerol at −80°C until they were used, and they were streaked onto LB agar or grown in LB broth and incubated at 37°C overnight, as required.

Adjuvant. AdDP was synthesized by Bachem, Switzerland, using a previously described procedure (18).

Animals. Female BALB/c mice that were 8 to 12 weeks old were obtained from Gador S.A. Laboratory (Buenos Aires, Argentina). Mice were housed in groups of five or six, and food and water were provided ad libitum. All procedures were in compliance with U.S. National Institutes of Health guidelines for handling laboratory animals.

Preparation of OMPs. OMPs were prepared by using a previously described method (7). In brief, bacteria were grown overnight in 10 ml of LB broth, harvested by centrifugation at 5,000 × *g* for 20 min at room temperature, and washed twice with saline. The bacterial pellet was suspended in 3 ml of 10 mM Tris-HCl (pH 8.0) and sonicated six times for 20 s at 40 W. The suspension was centrifuged at 10,000 × *g* for 1 min to remove debris and unbroken bacteria, and the supernatant was centrifuged at 40,000 × *g* for 30 min at 4°C. The pellet containing total membranes was resuspended in distilled H_2O, and an equal volume of a 20 mM Tris solution containing 1.5% Sarkosyl was added. The suspension was incubated for 20 min at room temperature to solubilize the inner membranes and then centrifuged at 40,000 × *g* for 30 min at 4°C. The resulting pellet was highly enriched for OMPs. The protein concentration was determined by using a protein assay kit (Bio-Rad Laboratories, Richmond, CA). OMP preparations were tested for the presence of endotoxin by chromogenic *Limulus* amebocyte lysate (LAL Endochrome; Charles River Endosafe, Charleston, SC) according to the manufacturer's instructions. Endotoxin levels were less than 30,000 endotoxin units/mg in OMP preparations, which corresponded to approximately 70 μg of LPS per mg of protein.

LPS extraction. LPS was extracted from *B. multivorans* by the method described by Darveau and Hancock (14). LPS samples were resuspended in pyrogen-free water, and the activity was determined by the *Limulus* amebocyte lysate method. The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories). Protein in LPS samples accounted for less than 0.1% of the total weight of the LPS. LPS was characterized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described below, and silver staining was performed as described by Tsai and Frasch (44).

SDS-PAGE and Western blot analysis. SDS-PAGE was performed as described by Laemmli (30). Proteins present on the gels were detected by using Coomassie blue stain. For immunoblotting, *B. multivorans* OMPs, *B. cenocepacia* OMPs, or *B. multivorans* LPS samples were separated by SDS-PAGE, electroblotted onto nitrocellulose membranes, and then reacted with mouse antiserum raised against *B. multivorans* OMPs by use of standard protocols (9).

Immunization and sample collection. Groups of five or six mice were immunized by i.n. inoculation (10 μl/nostril) of OMPs purified from *B. multivorans*, *B. cenocepacia*, *B. vietnamiensis*, *B. stabilis*, or *B. ambifaria* (30 μg/dose) together with AdDP (200 μg/dose) as a mucosal adjuvant diluted in sterile phosphate-buffered saline (PBS) on days 0, 7, and 14. A control group received only AdDP (200 μg/dose). On day 21, serum, saliva, bronchoalveolar lavage (BAL), and nasal wash (NAL) samples were obtained as previously described (6) and examined for the presence of OMP-specific antibodies. Briefly, saliva samples were obtained following intraperitoneal injection of 100 μl of pilocarpine (1 mg/ml; Sigma) diluted in sterile PBS to induce salivary secretion. Blood samples were collected by cardiac puncture immediately after sacrifice. NAL specimens were obtained by gently flushing the nasal cavities from the posterior opening of the nose with 200 μl of PBS after the mandible was removed. BAL samples were obtained by irrigation with 400 μl of PBS, using a blunted needle inserted into the trachea after a tracheotomy. The wash samples recovered were centrifuged at 3,000 × *g* for 5 min to remove cellular debris, and the supernatants were examined by using an enzyme-linked immunosorbent assay (ELISA) (see below).

Assessment of the effects of LPS. Groups of eight mice were immunized i.n. on days 0, 7, and 14 with either LPS (2 or 20 μg/dose), LPS (2 μg/dose) plus AdDP (200 μg/dose), or *B. multivorans* OMPs plus AdDP. A control group received only saline. The effects of LPS were assessed by determining the body temperature, the total cell count and percentage of polymorphonuclear leukocytes in BAL samples, and lung histopathology and by detecting LPS-specific antibodies in serum samples. The body temperature was measured by determining the rectal temperature using a digital thermometer. For each measurement, after 1 h of adaptation two values were averaged to determine the baseline. The rectal temperature was determined 1 day before immunization and 1, 2, 4, 6, 8, 24, and 48 h after immunization. Twenty-four hours after immunization, three mice in each group were sacrificed to obtain BAL samples and lungs for histopathological examination (see below). The total cell counts in BAL samples were determined with an autoanalyzer hemacytometer (Cell-Dyn 1600; ABBOT). A cyto-

spin analysis of BAL cells was performed on standard microscope slides using cytobucket carriers (Fisher Scientific). A 200-μl BAL sample was centrifuged at 700 rpm for 10 min. Cells were air dried, fixed directly with methanol, and stained with Giemsa stain. Differential polymorphonuclear leukocyte counts were obtained using stained cells, and averages were determined for at least 200 cells. On day 21, serum samples were obtained from the remainder of the mice, and the presence of LPS-specific antibodies was determined by ELISA as described below.

Detection of OMP- and LPS-specific antibodies by ELISA. OMP-specific antibody titers in mucosal secretions and sera and LPS-specific antibody titers in sera were determined by ELISA. Briefly, 96-well Nunc-Immuno MaxiSorp assay plates (Nunc, Roskilde, Denmark) were coated with 0.5 μg/well of the OMPs purified from each Bcc strain or LPS in coating buffer (sodium bicarbonate, pH 9.4), as indicated in the experiment design. After overnight incubation at 4°C, the plates were blocked with 0.2% Tween 20 in PBS for 2 h at 37°C. Serial twofold dilutions of samples in PBS–0.05% Tween 20 were added (100 μl/well), and the plates were incubated for 2 h at 37°C. After four washes with PBS–0.05% Tween 20, horseradish peroxidase-conjugated γ-chain-specific goat anti-mouse IgG (Chemicon) or phosphatase alkaline-conjugated α-chain-specific rabbit anti-mouse IgA (ICN) was added as a secondary antibody. The plates were incubated for 2 h at 37°C, and after four washes, the reactions were developed with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 0.1 M citrate-phosphate buffer (pH 4.3) containing 0.01% H_2O_2 or with *p*-nitrophenyl phosphate in 10 mM diethanolamine (pH 9.5) containing 0.5 mM $MgCl_2$. The absorbance was determined at a wavelength of 405 nm. Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an optical density of ≥0.1 U for negative control samples obtained from nonimmunized animals.

Bacterial challenge. The bacterial challenge studies were performed using a chronic pulmonary model of *B. cepacia* infection described previously (10, 11), with some modifications. Briefly, cyclophosphamide (150 mg/kg of body weight; Filaxis Laboratories) was administered intraperitoneally on days −1, 4, 9, and 13 of challenge. On days −2, 0, 5, and 13 a sample of blood was obtained from the tail vein. Total peripheral leukocyte counts were determined with an autoanalyzer hemacytometer (Cell-Dyn 1600), and differential counts were determined microscopically using Giemsa-stained blood smears. A pulmonary challenge with live bacteria was performed on day 21 after the first i.n. immunization. *B. multivorans* or *B. cenocepacia* was prepared from overnight cultures as described above and resuspended in PBS. The concentration of the inoculum was estimated by determining the optical density at 630 nm and was confirmed by counting the CFU in serial dilutions of the inoculum. Mice were challenged i.n. with 2.8×10^7 CFU (11) in a 20-μl dose. For *B. multivorans* challenge, five animals were sacrificed at 4 h (day 0) and on days 5 and 15 after pulmonary infection, whereas for *B. cenocepacia* challenge mice were sacrificed at 4 h (day 0) and on day 5 after pulmonary infection. Lungs were excised, weighed, and homogenized with a pestle, and serial dilutions in PBS of the homogenate were plated on LB agar. Viable counts were determined after 24 to 48 h of incubation at 37°C and were expressed as the $\log_{10}$ CFU/g of lungs (mean ± standard error of the mean [SEM]). Blood samples and NAL samples were collected to monitor the presence of OMP-specific antibodies.

Changes in weight and clinical illness scores following challenge were determined by weighing the mice with a digital scale and by determining the appearance of the mice, respectively. Clinical illness scores were assigned by a blinded examiner using an index derived by assigning numbers to a set of clinical features seen in mice with different degrees of illness, as follows: 0, healthy; 1, barely ruffled fur; 2, ruffled fur and active; 3, ruffled fur and inactive; 4, ruffled fur, inactive, hunched posture, and gaunt; 5, dead.

Histopathology. Lungs were infused with 10% (vol/vol) neutral buffered formalin, carefully removed from the chest cavity, placed for 48 h in 10% neutral buffered formalin, and then processed for routine histological examination using paraffin-embedded sections stained with hematoxylin and eosin.

Statistical analyses. In the immunogenicity and protection studies the significance of the differences between two groups was determined by Student's unpaired two-tailed *t* test with transformed data ($\log_{10}$ or $\log_2$), and the significance of the differences between three or more groups was determined by one-way analysis of variance (ANOVA) with the Tukey-Kramer multiple-comparison test. The linear correlation between two variables was determined using the Pearson correlation coefficient. Total peripheral leukocyte and polymorphonuclear leukocyte counts were compared by the nonparametric Mann-Whitney U test for two groups and by the Krustal-Wallis test with Dunn's multiple-comparison test for three or more groups. For parametric or log-transformed data the results were expressed as means ± SEM, whereas for nonparametric data the results were expressed as medians and ranges. Differences were considered significant at a P value of <0.05.

RESULTS

Specific antibody responses in serum and mucosal secretions are elicited by i.n. immunization with OMPs from Bcc species coadministered with AdDP. We examined the ability of OMPs to elicit serum and mucosal immune responses after i.n. vaccination, using a vaccination protocol that was successful in previous studies (6). Although Bcc OMPs themselves could have immunogenic properties, we included AdDP as an adjuvant in the immunization protocol. AdDP is a mucosal adjuvant that promotes a robust T helper 2 response when it is coadministered with native and recombinant antigens by intraperitoneal, oral, or i.n. routes (5, 6). High titers of OMP-specific IgG and IgA serum antibodies were elicited by i.n. immunization with OMPs from isolates of various Bcc species plus AdDP (Fig. 1A). The titers were significantly higher than those in AdDP-immunized controls ($P < 0.001$) in all cases except the serum IgA titers induced by OMPs from *B. stabilis* ($P < 0.03$). OMPs from *B. multivorans*, *B. vietnamiensis*, and *B. ambifaria* elicited higher endpoint titers of serum IgG antibodies than OMPs from *B. cenocepacia* and *B. stabilis* ($P < 0.002$). Since secretory antibodies are the main effectors in protection against pathogens at mucosal sites, we evaluated the efficacy of OMPs plus AdDP to elicit specific antibody production in the respiratory mucosa. Significant increases in OMP-specific IgA titers were observed in BAL, NAL, and saliva samples from mice immunized with OMPs plus AdDP (Fig. 1B), and these titers were significantly higher than all the titers obtained for mice immunized with AdDP ($P < 0.0001$) except the titers in BAL samples for OMPs derived from *B. cenocepacia* ($P < 0.05$), *B. stabilis* ($P < 0.03$), and *B. vietnamiensis* ($P < 0.005$). No statistically significant differences among the endpoint titers were observed for BAL samples, but, as we observed for serum IgA, OMPs from *B. multivorans*, *B. ambifaria*, *B. cenocepacia*, and *B. vietnamiensis* elicited greater specific secretory IgA responses than OMPs from *B. stabilis* elicited ($P < 0.0001$) in NAL and saliva samples. Furthermore, we detected OMP-specific IgG antibody responses in BAL samples from OMPs-vaccinated mice (data not shown). Together, these results demonstrated that the Bcc OMPs plus AdDP can elicit serum and mucosal antibody responses under our experimental conditions.

OMP-mediated immune response is independent of endotoxin. To investigate the possible role of endotoxin in OMP preparations, we examined the potential proinflammatory effect of *B. multivorans* LPS after i.n. immunization by evaluating the body temperature response, the polymorphonuclear leukocyte counts in BAL samples, and the lung histopathology of mice immunized with *B. multivorans* LPS. There were no significant differences in the baseline body temperature among the groups. For animals inoculated i.n. with *B. multivorans* LPS (at the same concentration that was present as a contaminant in *B. multivorans* OPM preparations) or with *B. multivorans* OMPs plus AdDP we did not observe any change in the baseline body temperature compared to the baseline body temperature of the control group (Fig. 2). We observed an approximately 2°C increase in the baseline body temperature at 6 h postinoculation, which remained elevated throughout the 24-h recording period, when mice were inoculated with a 10-fold-higher dose of *B. multivorans* LPS (Fig. 2). No histological

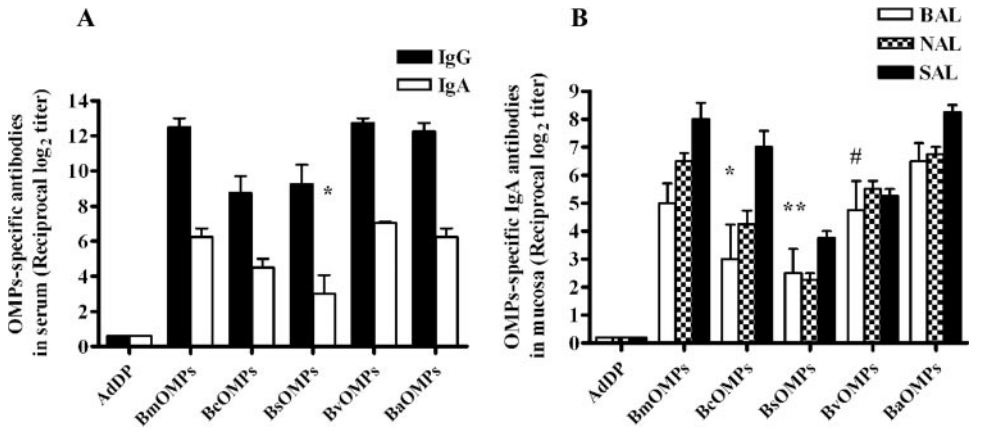

FIG. 1. Systemic and mucosal humoral immune responses directed against OMPs in mice vaccinated i.n. with OMPs purified from *B. multivorans* (BmOMPs), *B. cenocepacia* (BcOMPs), *B. stabilis* (BsOMPs), *B. ambifaria* (BaOMPs), and *B. vietnamiensis* (BvOMPs) coadministered with AdDP. The results are expressed as the reciprocal $\log_2$ of the mean endpoint titer; the error bars indicate SEM. The AdDP data show the AdDP-immunized mouse IgG and IgA serum or mucosal IgA antibody response against OMPs, determined separately for each strain. (A) Specific IgG and IgA titers in the sera of control mice (AdDP) and mice vaccinated with each OMP preparation from a Bcc species plus AdDP. Most differences were statistically significant at a P value of < 0.0001 when values were compared with the value for the AdDP-vaccinated mice, as determined by Student's unpaired two-tailed t test); the only exception is indicated by an asterisk ($P < 0.03$). For differences in the endpoint titers for IgG between *B. multivorans* OMPs, *B. vietnamiensis* OMPs, and *B. ambifaria* OMPs and between *B. cenocepacia* OMPs and *B. stabilis* OMPs, the P value was <0.002, as determined by one-way ANOVA with the Tukey posttest. For differences in the endpoint titers for IgA between *B. stabilis* OMPs and all other Bcc OMPs, the P value was <0.002, as determined by one-way ANOVA with the Tukey posttest. (B) Specific IgA antibodies in BAL, NAL, and saliva (SAL) samples from control mice (AdDP) and mice vaccinated with Bcc OMPs plus AdDP. Most differences were statistically significant at a P value of <0.0001 when values were compared with the values for the AdDP-vaccinated mice; the exceptions are indicated by one asterisk ($P < 0.05$), two asterisks ($P < 0.03$), and a number sign ($P < 0.005$). For differences in the endpoint titers for secretory IgA in NAL and saliva samples between *B. stabilis* OMPs and all other Bcc OMPs, the P value was <0.0001, as determined by a one-way ANOVA with the Tukey posttest.

changes indicating inflammation or membrane barrier disruption were observed in any of the mice immunized with *B. multivorans* LPS (data not shown). Also, a 10-fold increase in the i.n. LPS dose did not have adverse effects on the mice. Consistent with these results, we observed no differences in the percentage of polymorphonuclear leukocytes present in the BAL samples for any of the immunized mouse groups (Table 1). Therefore, when administrated by the i.n. route, the OMP preparation appeared to be safe and nontoxic and did not trigger any significant inflammatory response.

We also analyzed whether endotoxin contamination could have been responsible for the specificity of the immune response against OMPs. *B. multivorans* LPS with endotoxin activity comparable to the activity of the contaminating endotoxin in the *B. multivorans* OMP preparation did not induce serum anti-LPS immune responses (Fig. 3A). LPS doses that were at least 10-fold higher were required to detect a weak effect on serum anti-LPS responses (Fig. 3A). The presence of LPS as a contaminant in *B. multivorans* OMP preparations raised the question of whether the antibodies induced by i.n. immunization with *B. multivorans* OMPs plus AdDP were directed at or cross-reacted with the LPS core oligosaccharide

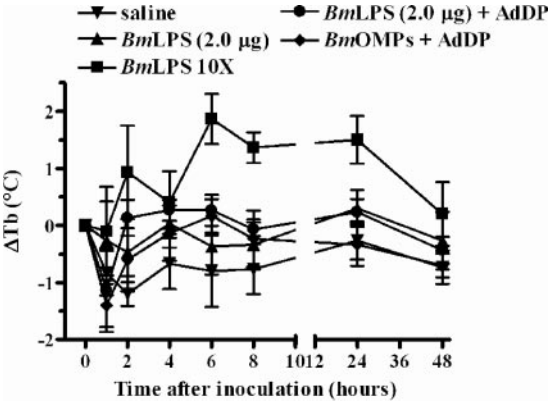

FIG. 2. Effects of i.n. immunization with *B. multivorans* LPS (*Bm*LPS) on the body temperature response in mice. Mice were immunized i.n. with either *B. multivorans* LPS (2 or 20 μg/dose), *B. multivorans* LPS (2 μg/dose) plus AdDP (200 μg/dose), or *B. multivorans* OMPs (*Bm*OMPs) plus AdDP. A control group received only saline. The data are the means ± SEM. ΔTb, change in body temperature from the baseline (time zero).

TABLE 1. Total leukocytes and polymorphonuclear cells in BAL samples from LPS-immunized mice

Group	Median total leukocyte concn (range) (10^9/liter)	Polymorphonuclear leukocytes	
		Median concn (range) (10^9/liter)	%
Saline	0.9 (0.8–1.0)	0.102 (0.02–0.135)	11.3
B. multivorans LPS (2 μg)	0.9 (0.8–1.0)	0.092 (0.04–0.145)	10.2
B. multivorans LPS (20 μg)	0.65 (0.4–0.9)	0.067 (0.02–0.113)	10.3
B. multivorans OMPs + AdDP	1.2 (1.1–1.3)	0.051 (0.011–0.09)	4.3
B. multivorans LPS (2 μg) + AdDP	1.15 (1.1–1.2)	0.042 (0.011–0.012)	4.5

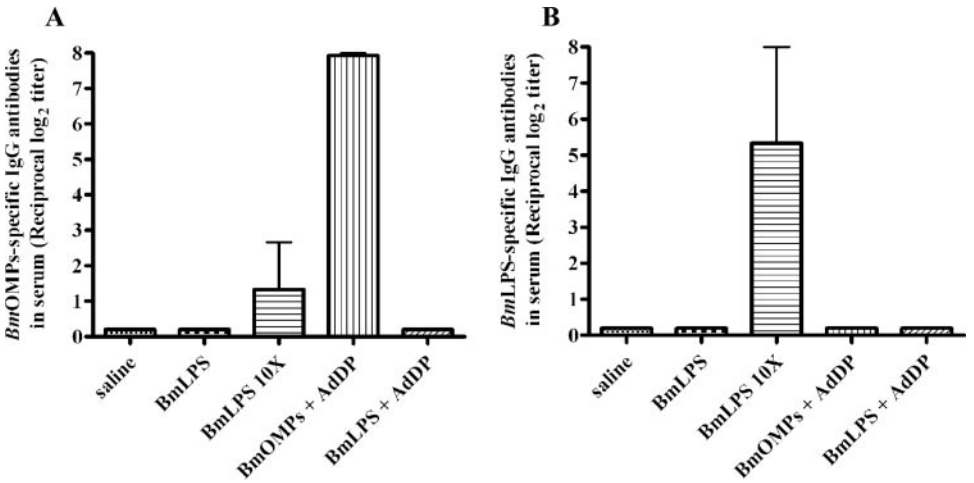

FIG. 3. Effects of i.n. immunization with *B. multivorans* LPS (BmLPS) on the immunogenicity of OMPs. Mice were immunized i.n. on days 0, 7, and 14 with either *B. multivorans* LPS (2 or 20 μg/dose), *B. multivorans* LPS (2 μg/dose) plus AdDP (200 μg/dose), or *B. multivorans* OMPs (BmOMPs) plus AdDP. A control group received only saline. The results are expressed as the reciprocal $\log_2$ of the mean endpoint titer; the error bars indicate SEM. (A) *B. multivorans* LPS-specific IgG antibodies present in the serum of control (saline) and vaccinated mice. The differences were not statistically significant, as determined by a one-way ANOVA with the Tukey-Kramer posttest. (B) OMP-specific IgG antibodies in serum of control (saline) and vaccinated mice. The differences were statistically significant at a P value of <0.0005, as determined by a one-way ANOVA with the Tukey-Kramer posttest.

region. We demonstrated that this immune response was specific against *B. multivorans* OMPs and not against contaminating endotoxin (Fig. 3B). No immunoreactive bands were detected on immunoblots when purified *B. multivorans* LPS was used as the antigen and sera from *B. multivorans* OMP-immunized mice were used as the staining antibody (data not shown). Collectively, these results confirmed that the presence of LPS has no effect on the immunogenicity of OMPs.

i.n. immunization with OMPs plus AdDP enhances the clearance of *B. multivorans* from the lungs. We used the immunocompromised mice model of lung infection (10, 11) to investigate the kinetics of infection by *B. multivorans* in mice that were immunized with AdDP and mice that were immunized with *B. multivorans* OMPs plus AdDP. The two groups exhibited different rates of bacterial clearance, as shown by the number of *B. multivorans* CFU cultured from the lungs (Fig. 4A). The difference was significant throughout the experiment ($P < 0.001$ at days 5 and 15 after challenge with *B. multivorans*). The initial pulmonary bacterial load was 4.10 ± 0.12 $\log_{10}$ CFU/g of lungs (mean ± SEM), and the AdDP-vaccinated mice maintained a pulmonary bacterial load of 3.43 ± 0.19 $\log_{10}$ CFU/g of lungs for up to 15 days. In contrast, mice vaccinated with OMPs plus AdDP and infected with *B. multivorans* exhibited rapid and almost complete clearance of the infection after 2 weeks, and the bacterial load was 1.39 ± 0.461 $\log_{10}$ CFU/g of lungs. On day 15 postinfection, there was a nearly 3-log reduction in the bacterial load in OMP-vaccinated animals, compared with a 1-log reduction in the bacterial load in the AdDP-immunized mice.

Protection was directly correlated with the magnitude of the immune response after vaccination (Fig. 4B and C). Prior to infection (day 0), NAL samples from mice immunized with *B. multivorans* OMPs plus AdDP had a statistically significant high titer of IgA OMP-specific antibodies ($P < 0.004$), and the level of this response was maintained throughout the experiment ($P < 0.0002$ at day 5 and $P < 0.002$ at day 15). Moreover, the titers of OMP-specific IgA antibodies in NAL samples from mice immunized with OMPs plus AdDP were higher on day 5 postinfection than on day 0 ($P < 0.05$) (Fig. 4B). There was also a statistically significant negative correlation between the titers of OMP-specific IgA in mucosa and the number of *B. multivorans* CFU in lungs ($r = -0.9428$; $P < 0.04$).

As observed for i.n. immunization with *B. multivorans* OMPs and AdDP, infection of AdDP-immunized mice with *B. multivorans* induced increased OMP-specific IgG serum antibodies on day 15, but this immune response did not correlate with prevention of bacterial infection (Fig. 4C). These results indicate that i.n. immunization with *B. multivorans* OMPs plus AdDP enhanced the clearance of *B. multivorans* from the lungs, and the protective effect was associated with OMP-specific IgA antibody titers in mucosal secretions.

i.n. immunization with OMPs plus AdDP is associated with reduced disease signs after *B. multivorans* infection. *B. multivorans* infection caused more weight loss in AdDP-immunized mice than in mice immunized with *B. multivorans* OMPs plus AdDP (Fig. 5A). The mean body weight of mice infected with *B. multivorans* was significantly lower for the former group than for the latter group on days 13 ($P < 0.03$) and 15 ($P < 0.005$). The median (minimum, maximum) percentages of weight loss for AdDP-vaccinated mice were 17.7% (10.6%, 23.3%) and 20.4% (10%, 31%) for days 13 and 15, respectively.

The illness score was also significantly higher for AdDP-immunized mice than for mice immunized with *B. multivorans* OMPs plus AdDP (Fig. 5B). The difference was significant throughout the experiment ($P < 0.005$ at day 9 and $P < 0.0001$ at day 15 after challenge with *B. multivorans*). On day 4 postinfection, 40% of AdDP-immunized mice and 10% of mice immunized with OMPs plus AdDP ($n = 10$ for each group) exhibited signs of illness. This was more evident on day 15

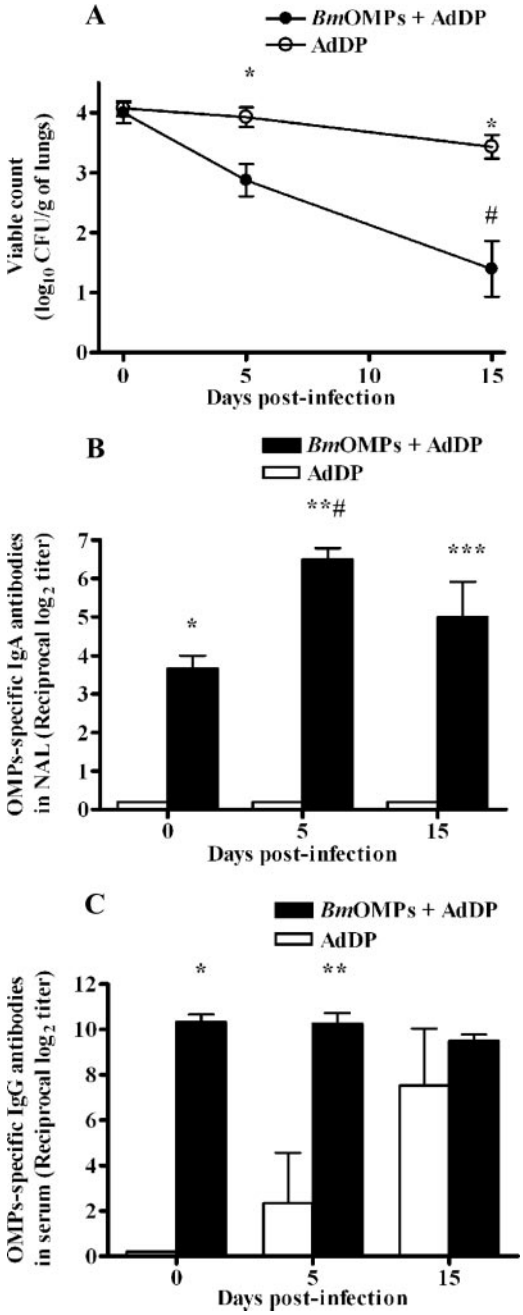

FIG. 4. Kinetics of *B. multivorans* clearance from lungs of infected mice. (A) Mice immunized with AdDP and *B. multivorans* OMPs (*Bm*OMPs) were challenged i.n. with 2.8×10^7 CFU of *B. multivorans*. Pulmonary bacterial clearance was assessed after 0 (4 h), 5, and 15 days by plating dilutions of lung homogenates on LB agar. The results are expressed as the mean $\log_{10}$-transformed CFU/g of lungs; the error bars indicate SEM. The differences were statistically significant when values were compared with the values for the AdDP-immunized mice ($P < 0.001$, as determined by Student's unpaired two-tailed *t* test) (asterisks), and with the values for *B. multivorans* OMP-immunized mice on day 0 ($P < 0.003$, as determined by a one-way ANOVA with the Tukey-Kramer posttest) (number sign). (B) OMP-specific NAL IgA antibodies of infected mice. The results are expressed as the mean reciprocal $\log_2$ of the endpoint titer; the error bars indicate SEM. The differences were statistically significant when values were compared with the values for the AdDP-immunized mice, as determined by Student's unpaired two-tailed *t* test (one asterisk, $P < 0.004$; two asterisks, $P < 0.0002$; three asterisks, $P < 0.002$), and with the values for mice immunized with OMPs and AdDP on day 0 ($P < 0.05$, as determined by one-way ANOVA with the Tukey-Kramer posttest) (number sign). (C) OMP-specific serum IgG antibodies of infected mice. The results are expressed as the mean reciprocal $\log_2$ endpoint titer; the error bars indicate SEM. The differences were statistically significant when values were compared with the values for the control group, as determined by Student's unpaired two-tailed *t* test (one asterisk, $P < 0.0002$; two asterisks, $P < 0.01$).

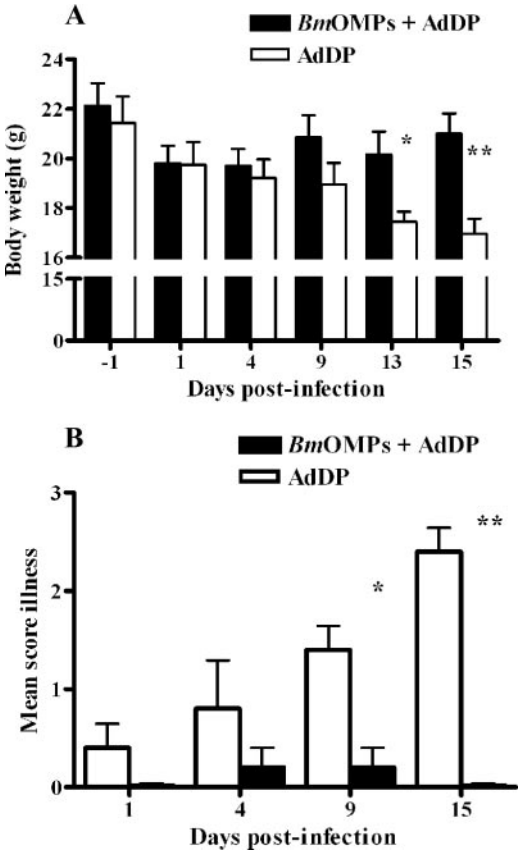

FIG. 5. Effects of i.n. immunization with *B. multivorans* OMPs (*Bm*OMPs) plus AdDP and *B. multivorans* infection on animal health over time. Mice immunized with AdDP and *B. multivorans* OMPs were challenged with 2.8×10^7 CFU of *B. multivorans*. Animals were monitored for the duration of the experiment. (A) Body weight. The results are expressed as the mean body weight; the error bars indicate SEM. The differences were statistically significant when values were compared with the values for the AdDP-immunized mice, as determined by Student's unpaired two-tailed *t* test (one asterisk, $P < 0.03$; two asterisks, $P < 0.005$). (B) Clinical illness score (0, healthy; 1, barely ruffled fur; 2, ruffled fur and active; 3, ruffled fur and inactive; 4, ruffled fur, inactive, hunched posture, and gaunt; 5, dead). The results are expressed as the means of the illness score; the error bars indicate SEM. The differences were statistically significant when values were compared with the values for the AdDP-immunized mice, as determined by Student's unpaired two-tailed *t* test (one asterisk, $P < 0.005$; two asterisks, $P < 0.0001$).

postinfection, when all five mice that received AdDP were ill, while no disease signs were observed in the animals that received *B. multivorans* OMPs plus AdDP (illness scores, 2.4 ± 0.24 and 0, respectively; $P < 0.0001$).

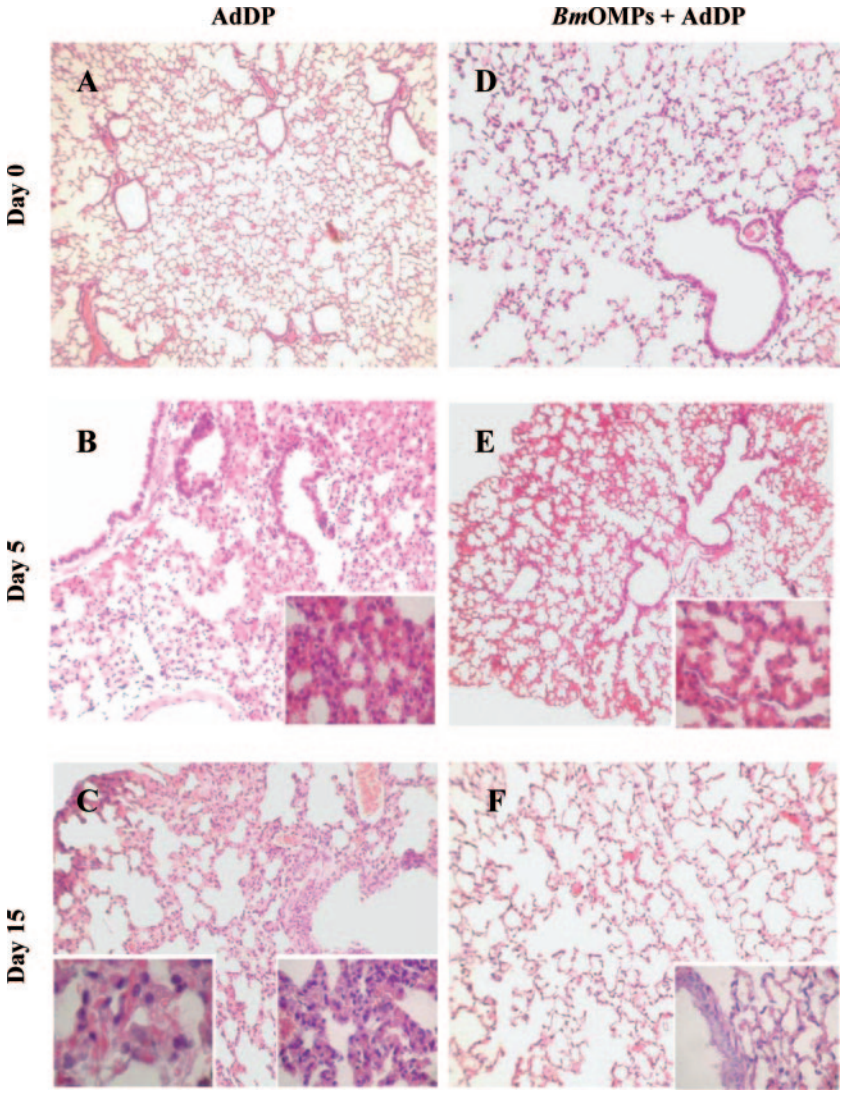

FIG. 6. Lung histopathology in mice immunized with *B. multivorans* OMPs (*Bm*OMPs) following infection with *B. multivorans*. (A, B, and C) Representative hematoxylin- and eosin-stained sections of mouse lung samples from AdDP-immunized mice at 0, 5, and 15 days postinfection, respectively. (D, E, and F) Representative hematoxylin- and eosin-stained sections of mouse lung samples from *B. multivorans* OMP-immunized mice at 0, 5, and 15 days postinfection, respectively. Magnifications: ×5 (A to F), ×40 (insets in panels B, E, and F and right inset in panel C), and ×100 (left inset in panel C).

OMP-vaccinated mice infected with *B. multivorans* exhibit minimal evidence of lung pathology. We hypothesized that the rapid clearance of bacteria from the lungs and the absence of disease signs in OMP-immunized mice were associated with mild changes in lung histology. The lungs of AdDP- and *B. multivorans* OMP-immunized mice were examined 0, 5, and 15 days after challenge with *B. multivorans*. On day 5, in lung sections from control mice infected with *B. multivorans* there were focal cellular infiltrates of inflammatory cells (mainly macrophages with limited lymphocytes and polymorphonuclear leukocytes) in alveolar, peribronchial, and perivascular areas (Fig. 6B). On day 15, these areas showed a more intense compromise characterized by diffuse and extended cellular infiltrates (mainly macrophages, lymphocytes, and polymorphonuclear leukocytes), along with disruption of the normal architecture of the parenchyma (Fig. 6C). In contrast, the histological changes in lung sections from *B. multivorans* OMP-immunized mice infected with *B. multivorans* were less pronounced. These animals exhibited reduced and limited parenchymal involvement and inflammatory cell infiltrates, and the overall architecture of the respiratory areas was conserved (Fig. 6E). Furthermore, on day 15 the lungs of *B. multivorans* OMP-immunized mice showed minimal evidence of pathology (Fig. 6F).

Supporting these results, the total peripheral leukocyte counts determined on days 0, 5, and 13 after challenge for *B. multivorans* OMP- and AdDP-immunized mice were not significantly different from the values observed for preinfection animals (day −2) except for day 13, when the number of total leukocytes in control mice was higher than the number in *B. multivorans* OMP-immunized mice (Table 2). Differential counts for blood smears showed that on day 5 postinfection,

TABLE 2. Total peripheral leukocytes and polymorphonuclear blood cells in *B. multivorans* OMP-immunized mice following infection with *B. multivorans*

Day postinfection	Median total peripheral leukocyte concn (range) (10^9/liter)		Median polymorphonuclear leukocyte concn (range) (10^9/liter)	
	AdDP	*B. multivorans* OMPs + AdDP	AdDP	*B. multivorans* OMPs + AdDP
−2	8.9 (6.8–17.0)	9.4 (6.2–12.8)	3.39 (1.77–7.34)	2.98 (2.15–3.34)
0	9.4 (6.5–15.9)	7.9 (6.1–11.4)	3.40 (1.76–7.08)	2.35 (1.77–2.99)
5	7.0 (3.9–8.0)	7.3 (5.4–8.0)	1.90 (1.33–2.32)	1.46 (1.40–2.30)
13	19.1 (14.5–30.1)[a]	8.1 (6.5–11.8)	11.50 (10.36–15.0)[b]	2.43 (1.18–4.03)

[a] $P < 0.01$ for a comparison with the mice immunized with *B. multivorans* OMPs plus AdDP, as determined by the Mann-Whitney U test.
[b] $P < 0.0001$ for a comparison with the mice immunized with *B. multivorans* OMPs plus AdDP, as determined by the Mann-Whitney U test.

the numbers of polymorphonuclear leukocytes were reduced to the same extent in AdDP-immunized mice and mice immunized with *B. multivorans* OMPs plus AdDP, as expected from the cyclophosphamide treatment. On day 13, the polymorphonuclear leukocytes counts for the AdDP-immunized mice were significantly higher than the polymorphonuclear leukocyte counts for the *B. multivorans* OMP-immunized mice (Table 2). The observation of an extended cellular infiltrate on day 15 in the lungs of infected control mice is consistent with the increased number of polymorphonuclear leukocytes in these mice.

i.n. immunization with *B. multivorans* OMPs plus AdDP induces cross-reactivity and confers protection against *B. cenocepacia* lung infection. As immunization with *B. multivorans* OMPs resulted in a strong immune response and protection of vaccinated mice against pulmonary infection with *B. multivorans*, we assessed whether immunization with this preparation could cross-protect mice against challenge with *B. cenocepacia*. In these experiments, mice in the AdDP-vaccinated control group exhibited more severe illness than the animals immunized with *B. multivorans* OMPs plus AdDP exhibited after *B. cenocepacia* challenge. Therefore, animals were sacrificed on day 5 postinfection, and samples were collected to evaluate cross-protection. Based on clinical illness scores, lung histopathology, and clearance of the bacterial load, mice immunized with *B. multivorans* OMPs were protected against pulmonary challenge with *B. cenocepacia*. As we observed in the experiment described above (Fig. 4A) and confirmed in this experiment (Fig. 7A, right panel) for *B. multivorans* pulmonary infection, the control group and the group treated with *B. multivorans* OMPs plus AdDP exhibited different rates of bacterial clearance after challenge with *B. cenocepacia*, as shown by the number of bacteria recovered from the lungs (Fig. 7A, left panel). The initial pulmonary bacterial load was 4.2 ± 0.2 $\log_{10}$ CFU/g of lungs (mean ± SEM), and by day 5 the load in the AdDP-vaccinated mice was 3.83 ± 0.13 $\log_{10}$ CFU/g of lungs ($P < 0.03$). In contrast, mice vaccinated with *B. multivorans* OMPs plus AdDP and infected with *B. cenocepacia* exhibited more rapid clearance of the infection, and the load was 2.28 ± 0.3 $\log_{10}$ CFU/g of lungs, which corresponded to a nearly 2-log reduction in the bacterial load for this group ($P < 0.001$ for a comparison with day 0 data and $P < 0.006$ for a comparison with the AdDP-immunized group).

On day 5 postinfection, examination of lung sections from control mice infected with *B. cenocepacia* revealed a focal cellular infiltrate of inflammatory cells that were mainly mononuclear cells, which also included a limited number of polymorphonuclear leukocytes in alveolar, peribronchial, and perivascular areas. In contrast, the histological changes in lung sections from *B. multivorans* OMPs-immunized mice infected with *B. cenocepacia* were less pronounced. These animals exhibited reduced and limited parenchymal involvement and inflammatory cell infiltrates, and the overall architecture of the respiratory areas was conserved. The histological changes were similar to those shown in Fig. 6B and E.

The mean body weight of AdDP-immunized mice infected with *B. cenocepacia* was significantly lower than the mean body weight of the *B. multivorans* OMP-immunized group on day 5 ($P < 0.005$) (Fig. 7B, left panel). On day 5 the median (minimum, maximum) percentage of weight loss for AdDP-vaccinated mice was 12% (9%, 13%). The illness score was also significantly higher for the AdDP-immunized mice than for the mice immunized with *B. multivorans* OMPs plus AdDP (Fig. 7B, right panel). The difference was significant throughout the experiment for AdDP-immunized mice ($P < 0.0001$, as determined by ANOVA). This was more evident on day 5 postinfection, when all five mice that received AdDP had signs of severe disease, while no signs of disease were observed in the mice immunized with *B. multivorans* OMPs plus AdDP (illness scores, 3.6 ± 0.24 and 0.8 ± 0.2, respectively; $P < 0.0001$).

Cross-protection directly correlated with the immune response elicited by vaccination cross-reactivity (Fig. 7C), as indicated by a comparison of the reactivities of OMP-specific antibodies of *B. multivorans* OMP-vaccinated mice challenged with *B. multivorans* or *B. cenocepacia* against homologous antigens (Fig. 7C, right panel) and nonhomologous antigens (Fig. 7C, left panel). The *B. multivorans* OMP serum IgG (Fig. 7C, upper right panel) and NAL *B. multivorans* OMP IgA (Fig. 7C, lower right panel) antibodies induced by vaccination with *B. multivorans* OMPs plus AdDP in both *B. multivorans*- and *B. cenocepacia*-challenged mice also cross-reacted with the nonhomologous antigens of *B. cenocepacia* OMPs (Fig. 7C, upper left and lower left panels). There were no statistically significant differences between the endpoint titers obtained for mice challenged with *B. multivorans* and the endpoint titers obtained for mice challenged with *B. cenocepacia*. Moreover, this level of response was maintained in both conditions throughout the experiment.

We observed similar patterns of *B. multivorans* OMPs and *B. cenocepacia* OMPs using SDS-PAGE with Coomassie blue staining (Fig. 7D). There were polypeptide bands at apparent molecular masses of 97, 91, 72, 45, 42, 37, 26, 22, and 20 kDa and some faint bands at 60 to 66 kDa. IgG serum antibodies

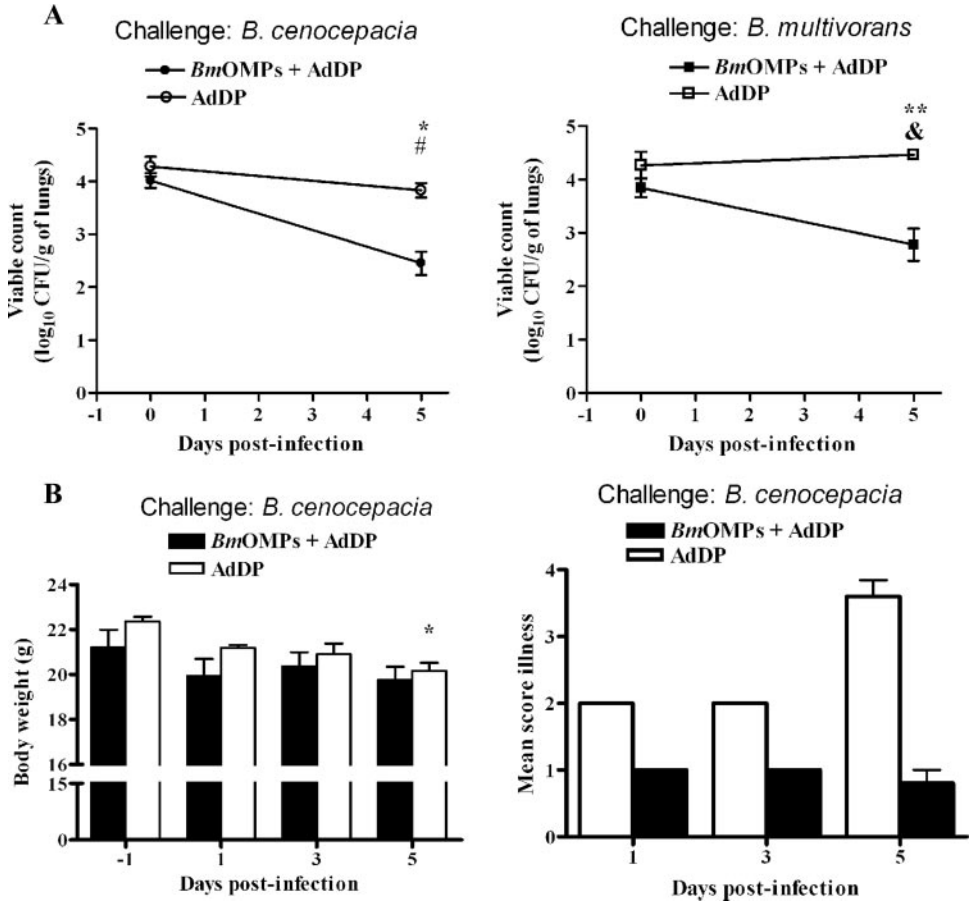

FIG. 7. i.n. immunization with *B. multivorans* OMPs (*Bm*OMPs) plus AdDP induces cross-reactivity and confers protection against *B. cenocepacia* lung infection. Mice immunized with AdDP and *B. multivorans* OMPs were challenged i.n. with 2.8×10^7 CFU of *B. multivorans* or *B. cenocepacia*. (A) Kinetics of *B. cenocepacia* (left panel) or *B. multivorans* (right panel) clearance from lungs of infected mice. Pulmonary bacterial clearance was assessed after 0 (4 h) and 5 days by plating dilutions of lung homogenate on LB agar. The results are expressed as the mean $\log_{10}$-transformed CFU/g of lungs; the error bars indicate SEM. One asterisk indicates that the *P* value is <0.0066 and two asterisks indicate that the *P* value is <0.009 for a comparison with the AdDP-immunized group, as determined by Student's unpaired two-tailed *t* test; a number sign indicates that the *P* value is <0.001 and an ampersand indicates that the *P* value is <0.04 for comparison with the *B. multivorans* OMP-immunized group on day 0, as determined by Student's unpaired two-tailed *t* test. (B) Changes in body weight (left panel) and the clinical illness score (right panel) of *B. cenocepacia*-infected mice. The body weight results are expressed as the mean body weight; the error bars indicate SEM. The asterisk indicates that the *P* value is <0.005 for a comparison with the AdDP-immunized group, as determined by a Student's unpaired two-tailed *t* test. The following clinical illness scores were used: 0, healthy; 1, barely ruffled fur; 2, ruffled fur and active; 3, ruffled fur and inactive; 4, ruffled, inactive, hunched posture, and gaunt; 5, dead. The results are expressed as the means of the illness score; the error bars indicate SEM. The differences were statistically significant when values were compared with the values for the AdDP-immunized group at $P < 0.0001$, as determined by Student's unpaired two-tailed *t* test. (C) Comparison of the reactivities of OMP-specific antibodies of infected mice as determined by ELISA using *B. multivorans* OMPs (right panels) or *B. cenocepacia* OMPs (*Bc*OMP) (left panels) as the coating antigens. The titers of OMP-specific serum IgG antibodies (upper panels) and OMP-specific NAL IgA antibodies (lower panels) were determined. The results are expressed as the mean reciprocal $\log_2$ of the endpoint titer; the error bars indicate SEM. Mice vaccinated with AdDP and with *B. multivorans* OMPs plus AdDP were challenged with *B. cenocepacia* and with *B. multivorans*. Most differences were statistically significant at a *P* value of <0.0001 when values were compared with the values for the AdDP-immunized group, as determined by Student's unpaired two-tailed *t* test; the exceptions are indicated by one asterisk ($P < 0.02$), two asterisks ($P < 0.002$), three asterisks ($P < 0.001$), one number sign ($P < 0.05$), two number signs ($P < 0.03$), and an ampersand ($P < 0.01$). (D) Coomassie blue staining of *B. multivorans* OMPs and *B. cenocepacia* OMPs. OMPs were separated by 12% SDS–PAGE and analyzed by Coomassie blue staining. Lane 1, molecular weight markers; lane 2, *B. multivorans* OMPs; lane 3, *B. cenocepacia* OMPs. (E) Comparison of the reactivities of *B. multivorans* OMP-specific serum IgG antibodies (left panel) and *B. multivorans* OMP-specific NAL IgA antibodies (right panel) of infected mice by Western blotting using *B. multivorans* OMPs as the immobilized antigens. Lanes 1, 2, 5, and 6, mice infected with *B. cenocepacia*; lanes 1 and 5, *B. multivorans* OMP-immunized mice; lanes 2 and 6, AdDP immunized mice; lanes 3, 4, 7, and 8, mice infected with *B. multivorans*; lanes 3 and 7, *B. multivorans* OMP-immunized mice; lanes 4 and 8, AdDP-immunized mice; lane 9, conjugate control. (F) Comparison of the cross-reactivities of *B. multivorans* OMP NAL IgA antibodies of infected mice by Western blotting using *B. cenocepacia* OMPs as the immobilized antigens. Lanes 1, 2, 5, and 6, mice infected with *B. cenocepacia*; lanes 1 and 5, *B. multivorans* OMP-immunized mice; lanes 2 and 6, AdDP-immunized mice; lanes 3, 4, 7, and 8, mice infected with *B. multivorans*; lanes 3 and 7, *B. multivorans* OMP-immunized mice; lanes 4 and 8, AdDP-immunized mice; lane 9, conjugate control. The positions of the molecular mass standards are indicated on the right (in kDa). DPI, days postinfection.

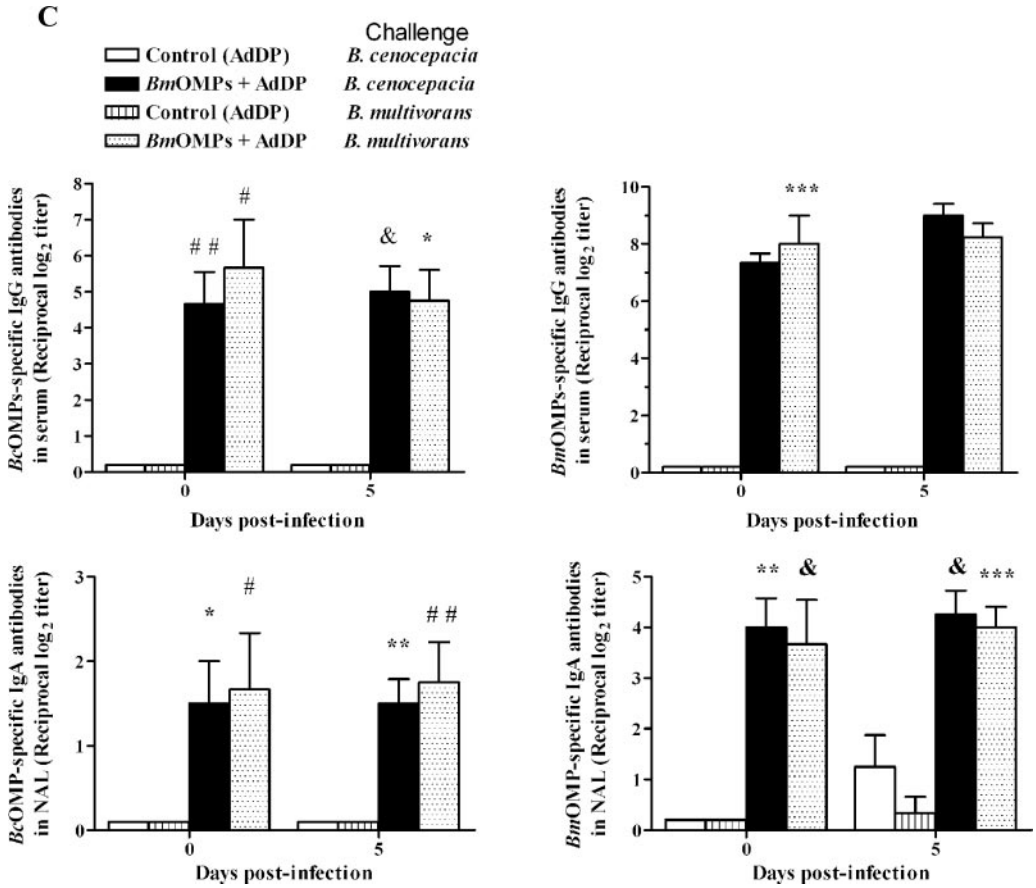

FIG. 7—*Continued*.

from *B. multivorans* OMP-vaccinated mice after challenge with the homologous and nonhomologous species against *B. multivorans* OMPs showed similar patterns of specific reactivity to the 97-, 91-, 72-, and 45-kDa polypeptides throughout the experiment and bands between 60 and 66 kDa on day 5 after the challenge (Fig. 7E, left panel). On the other hand, the reactivity patterns of antibodies present in NAL samples indicated that secretory IgA antibodies recognized the 90-, 72-, 66- to 60-, and 45-kDa polypeptides and some other faint bands with molecular masses ranging from 26 to 18 kDa on day 5 postchallenge in both *B. multivorans*- and *B. cenocepacia*-infected mice (Fig. 7E, right panel). A comparison of the results of the immunoblotting analysis of the reactivity patterns for cross-reactive NAL antibodies of *B. multivorans* OMP-vaccinated mice after challenge with nonhomologous OMPs showed that the antibodies directed against *B. multivorans* OMP antigens also recognized several OMP antigens from *B. cenocepacia*. The cross-reactivity was observed mainly with the 90-, 72-, and 60- to 66-kDa antigens for NAL secretory IgA antibodies (Fig. 7F).

These results indicate that i.n. immunization with *B. multivorans* OMPs plus AdDP enhanced the clearance of the nonhomologous species *B. cenocepacia* from the lungs, and the protective effect was associated with cross-reactivity of OMP-specific IgA antibody titers in NAL samples that recognized 90-, 72-, and 60- to 66-kDa antigens.

DISCUSSION

We demonstrate here that i.n. immunization with OMPs from Bcc species plus AdDP, a mucosal adjuvant with an adequate safety profile (8, 25), elicited significant Bcc-specific serum and mucosal immune responses. All titers were significantly higher in OMP-immunized animals than in AdDP-immunized controls. Given its pharmacokinetics and safety profiles, AdDP is a promising candidate for incorporation in mucosal vaccine formulations. The inclusion of AdDP in this immunization protocol should be relevant for future studies on the use of potential candidate antigens for developing recombinant subunit protein-based vaccines, since soluble antigens generally must be administered in combination with suitable adjuvants to evoke effective immune responses (42).

Although the level of endotoxin contamination was low in OMP preparations, we wanted to be sure that the amount of endotoxin present did not have a toxic effect or influence the specificity of the immune response. Our results demonstrated that the level of LPS present in the *B. multivorans* OMP preparation did not have a pyrogenic effect and that the OMP preparation not only was safe and nontoxic but also did not trigger any inflammatory responses when it was administrated by the i.n. route. Furthermore, our results confirmed that LPS has no effect on the immunogenicity of OMPs. These results are consistent with the results of previous i.n. immunization

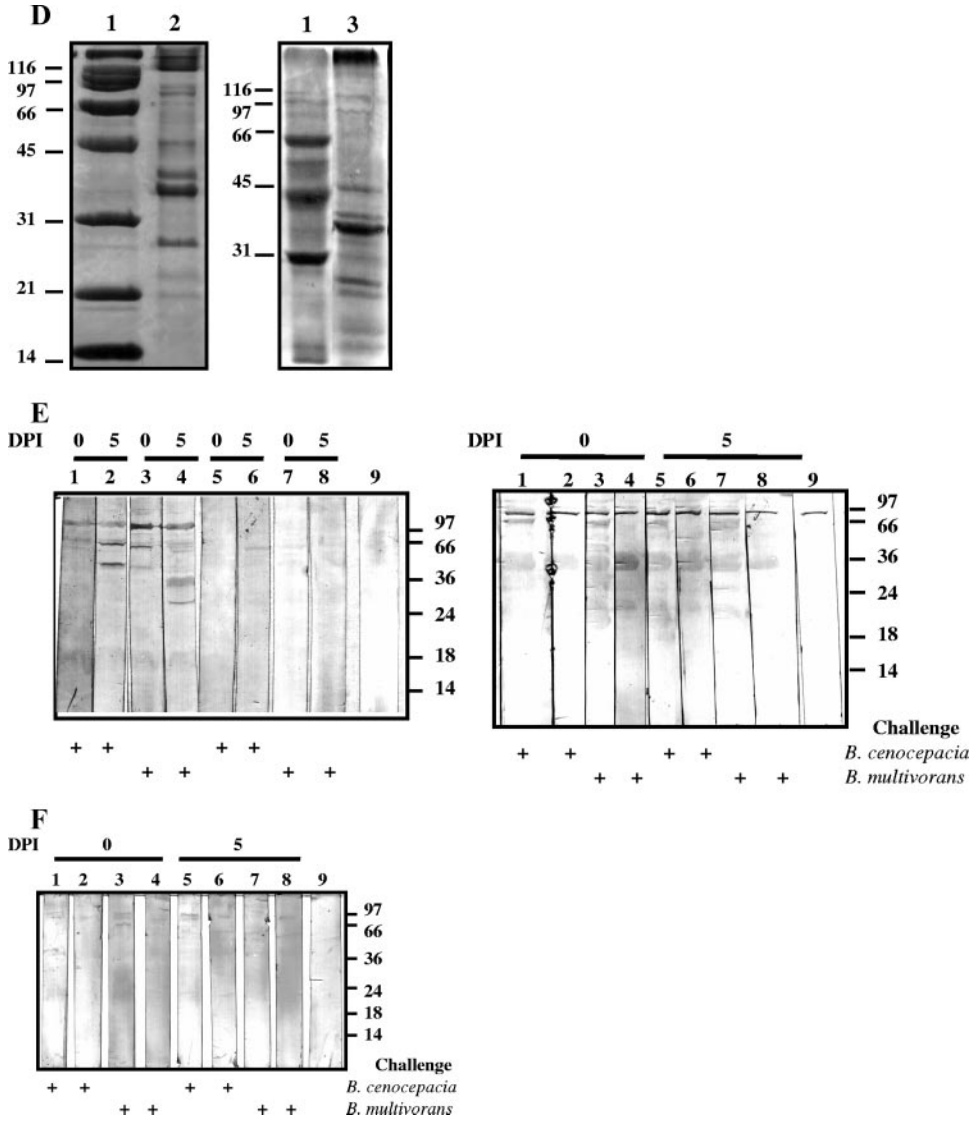

FIG. 7—*Continued*.

studies of humans with OMP vesicle preparations containing LPS, which revealed no problems with LPS in the nasal vaccine formulation (16, 27). Moreover, LPS is used as the protective antigen component of anti-*Shigella* vaccines that have advanced to clinical trials, and when given i.n. to laboratory rodents or nonhuman primates, the vaccine proved to be safe, nontoxic, well tolerated, and highly immunogenic and protected against *Shigella* challenge in various models of *Shigella* infection (34, 38). Similarly, the vaccine was safe, nontoxic, and well tolerated in phase I and II clinical trials in which more than 100 volunteers received doses of up to 1.5 mg of LPS (20).

The mouse model of chronic lung infection allowed us to evaluate the protective effect of i.n. immunization after challenge with *B. multivorans*. This species was chosen since previous studies have shown that, unlike *B. cenocepacia*, *B. multivorans* can establish a chronic infection in the mildly neutropenic mouse model (10, 11). Also, *B. multivorans* is regularly isolated from CF patients (41). Although this model does not exactly mimic CF disease, it provides a cost-effective screening tool for selecting the most promising vaccine formulation for further development and determining a correlation with protection. Our results clearly demonstrated that immunization with *B. multivorans* OMPs plus AdDP, followed by challenge with *B. multivorans*, dramatically decreased the lung pathology compared to that in AdDP-immunized mice. The reduction in the severity of lung disease correlated with significantly enhanced and almost complete bacterial clearance in the lungs of *B. multivorans* OMP-vaccinated mice compared to the control groups. Also, all control animals exhibited signs of illness and weight loss at the end of the experiment, whereas no evidence of clinical disease was observed in *B. multivorans* OMP-immunized mice.

Specific IgA antibodies elicited by mucosal immunization are likely to play an important role in the adaptive immune system, inhibiting adhesion and colonization of bacterial pathogens (48). This indicates that IgA is the first line of

defense in the mucosal compartment (31). Although serum IgA exhibits both pro- and anti-inflammatory activities (17, 19, 37, 39, 49, 50), secretory IgA is generally considered a noninflammatory antibody because it does not elicit inflammatory processes after binding to antigens (23, 24, 47). Our data suggest that local immunity in the respiratory tract before exposure to Bcc bacteria would be beneficial for the host. Indeed, the enhanced bacterial clearance in the lungs of OMP-vaccinated animals correlated with the anti-OMP secretory IgA response observed in NAL samples. Although we demonstrated that the 97-, 91-, 72-, 66- to 60-, and 45-kDa OMPs are recognized by serum of *B. multivorans* OMP-vaccinated mice, we found that the principal targets of secretory IgA were the 90-, 72-, 66- to 60-, and 45-kDa antigens.

We cannot rule out the possibility that specific IgG detected in respiratory secretions also contributes to bacterial clearance. Like IgA, IgG may limit the entry of mucosal pathogens into the host and their multiplication in the host, thereby preventing systemic infection (46). Although in the present study we did not examine these mechanisms directly, we propose that the more rapid resolution of pulmonary infection and the absence of disease signs in OMP-vaccinated animals were probably due to the inhibition of adhesion or colonization by bacterial pathogens and to the anti-inflammatory role of secretory IgA that may have limited lung damage caused by inflammation during infection. This is supported by the mild disease process observed to occur in OMP-vaccinated mice during the first few days after infection, which finally progressed to complete resolution of lung inflammation and no signs of lung pathology.

The range of cross-species protection that may be achieved has particular relevance since Bcc species show a great deal of diversity at the subspecies level. We demonstrated that administration of the *B. multivorans* OMP vaccine enhanced the clearance of *B. cenocepacia* from the lungs, and the protective effect was associated with cross-reactivity of OMP-specific IgA antibody titers in NAL samples that recognized the 90-, 72-, and 66- to 60-kDa antigens. Therefore, our results suggest that *B. multivorans* OMPs have determinants that are exposed on the surface of bacterium and are antigenically conserved in *B. multivorans* and *B. cenocepacia*, two species belonging to the Bcc.

In conclusion, our data demonstrate the important role of mucosal antibodies as a defense mechanism against infection with *B. multivorans or B. cenocepacia*, suggesting that increased mucosal immunity in the airways may help patients with CF. Moreover, the 90-, 72-, and 66- to 60-kDa OMPs targeted by secretory IgA and conserved in *B. multivorans* and *B. cenocepacia* may be promising candidates for formulations of recombinant subunit protein-based vaccines, providing a basis for rational vaccine design. Studies to identify these proteins and individually investigate their antigenicities and protective effects are under way in our laboratories.

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