# Identification of a 68-Kilodalton Protective Protein Antigen from *Bordetella bronchiseptica*

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A 68-kilodalton (kd) outer membrane protein antigen of *Bordetella bronchiseptica* has been identified by using monoclonal antibodies that recognized two nonoverlapping determinants. Antibody BB05 also reacted with homologous proteins from *Bordetella pertussis* and *Bordetella parapertussis* but not with another 12 organisms from various bacterial genera. Passive injection of BB05 antibody protected mice from aerosol infection with *B. bronchiseptica* as shown by reduced mortality and reduced pathology of turbinate bones. The 68-kd *B. bronchiseptica* antigen was purified by BB05-based affinity chromatography and evaluated for its potency to immunize mice actively against either intraperitoneal or aerosol challenge with *B. bronchiseptica*. Immunization with the 68-kd antigen in incomplete Freund adjuvant significantly reduced the levels of mortality in intraperitoneally challenged mice. In the aerosol infection model, injection of the 68-kd antigen with complete or incomplete Freund adjuvant or saponin reduced the bacterial counts in the lungs of infected mice. These results suggest that the 68-kd protein may represent a potential "protective" antigen of *B. bronchiseptica*.

*Bordetella bronchiseptica* is a microorganism associated with respiratory infections in animals, particularly atrophic rhinitis (AR) of pigs (8, 23). This disease is characterized by atrophy of the nasal turbinate bones, pneumonia, and growth retardation (1, 12). Vaccination of sows and piglets with *Bordetella bronchiseptica* vaccines has produced some positive results, although no detailed information is available as to which bacterial components are responsible for inducing protective immunity (reviewed in reference 7). A correlation has been found previously between antibody titers to proteins present in a cell-surface antigen (CSA) preparation from *Bordetella bronchiseptica* and resistance to AR in piglets born from immunized sows (P. Novotny, A. P. Chubb, K. Cownley, and J. A. Montaraz, manuscript in preparation). This extract contains lipopolysaccharide, outer membrane proteins, and fibrilar material. For further investigation of the molecular nature of protective antigens contained among the components of CSA, monoclonal antibodies (MABs) were raised against components of the extract and tested for passive protection of mice against *Bordetella bronchiseptica* infection. The corresponding antigen was purified by MAB-based affinity chromatography and appraised for protective potency by active immunization. Two murine models of infection were adopted: (i) intraperitoneal (i.p.) injection of the microorganism as used previously to test whole-cell *Bordetella bronchiseptica* vaccines (9, 22), and (ii) a newly developed aerosol infection model resulting in pneumonia, AR, and death (Novotny et al., in preparation). Significant protection was conferred by one protein

antigen (68 kilodaltons [kd]), although this was less than that produced by the whole-cell vaccine.

## MATERIALS AND METHODS

**Bacteria.** *Bordetella bronchiseptica* LBF, *Bordetella pertussis* (CN2992) or *Bordetella parapertussis* (2591) was used for the preparation of antigenic extracts. *Bordetella bronchiseptica* 134 was used as a challenge strain in animal experiments. The *Bordetella bronchiseptica* LBF whole-cell vaccine contained $2 \times 10^9$ microorganisms per ml, killed with Formalin and adsorbed to $Al(OH)_3$ (Alhydrogel).

The following microorganisms, obtained from the Wellcome Bacterial Collection, were used to test MAB specificity: *Streptococcus suis* type 2 (CN 7535); *Staphylococcus aureus* Cowan, serotype 1 (CN 6626); *Haemophilus parainfluenzae* (CN 3572); *Haemophilus aegyptius* (CN 7820); *Haemophilus haemoglobinophilus* (CN 3568); *Haemophilus influenzaemurium* (CN 6935); *Escherichia coli* K-88 (CN 6845), K-99 (CN 7958), and 987P (CN 7872); *Pasteurella multocida* serotypes A and D (CN 4022, CN 8151); *Pasteurella haemolytica* serotype $A_1$ (CN 7827). *Brucella abortus* was a standardized agglutination concentrate (Central Veterinary Laboratory, Weybridge, U.K.). Cultures of *Mycoplasma hyopneumoniae* (10110) and *Mycoplasma hyorhinis* (BBX/BSA) were kindly donated by G. D. Windsor (Wellcome Research Laboratories). Bacteria were tested as freshly harvested whole-cell suspensions, with the exception of *P. multocida* and *P. haemolytica*, which were mechanically disintegrated on a rotary disintegrator (15) at 4,000 rpm for 2 min in the presence of Ballotini glass beads; the slurry freed of glass beads was centrifuged in the following way: (i) at $2,000 \times g$ for 5 min, after which the sediment was discarded; (ii) at $12,000 \times g$ for 30 min, after which the sediment was discarded; and (iii) at $50,000 \times g$ for 2 h, after which the supernatant was discarded.

**Bacterial extracts.** The techniques used for the preparation of antigenic extracts from *Bordetella bronchiseptica* will be

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described in detail elsewhere (P. Novotny, A. P. Chubb, K. Cownley, and J. A. Montaraz, submitted for publication). Briefly, the extracts were prepared by the following methods.

**CSAs.** CSA was prepared by a modified technique used for the preparation of K-antigen from *E. coli* (17). Essentially, *Bordetella bronchiseptica* cells at the exponential growth phase were incubated for 30 min at 60°C and sedimented by centrifugation for 30 min at 10,000 × $g$. The supernatant was centrifuged for 2 h at 50,000 × $g$, cooled, and precipitated by 67% (vol/vol) ethanol. The precipitate was dissolved in distilled water and cleared of insoluble residues by centrifugation for 2 h at 100,000 × $g$. The supernatant fraction (CSA) was stored freeze-dried.

**Acid glycine hydrolysate.** Cells were suspended in 250 mM glycine–5 mM EDTA buffer (pH 3), incubated for 18 h at 37°C, neutralized, and centrifuged. One volume of supernatant, mixed with 2 volumes of acetone, was incubated for 3 h at −20°C, and the resulting precipitate was centrifuged at 4,000 × $g$ and dissolved in water. The solution, freed of insoluble material by centrifugation, was stored freeze-dried.

**Outer membrane vesicles (OMVs).** OMVs were prepared by differential centrifugation methods described previously for the fractionation of *Bordetella pertussis* (16). Cells were broken with a rotary disintegrator (15), and the OMV fraction was sedimented by centrifugation at 50,000 × $g$ for 1 h. This material, when centrifuged through a 55% Percoll solution, showed a peak in the 1.032 density region (Novotny et al., submitted for publication).

**Production of MABs.** BALB/c mice were immunized i.p. with 0.5-ml doses of a *Bordetella bronchiseptica* vaccine diluted 1:10 in phosphate-buffered saline at 26-day intervals. Mice were challenged with the same antigen 2 to 3 days before fusion.

Fusions were performed by using spleen cells from immunized mice and P3-NSI/1-Ag4-1 myeloma cells in the presence of polyethylene glycol (6). Cells were plated in RPMI medium (GIBCO Laboratories) supplemented with 10% fetal calf serum, 0.1 mM hypoxanthine, 15 mM thymidine, and 0.43 M aminopterin (HAT medium). Tissue culture supernatants were screened for antibody activity by a solid-phase radioimmunoassay (RIA), using CSA as antigen. Positive cultures were cloned by limiting dilution and expanded either in culture to produce maximum growth medium or as ascitic tumours in the peritoneal cavities of mice. The globulin fraction was prepared from ascitic fluid by precipitation in the presence of 18% (wt/vol) sodium sulfate and adjusted to a 5-mg/ml concentration on the basis of protein content (10).

Two IgG1 MABs, designated BB05 and BB07, were chosen for detailed characterization.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/immunoblot technique.** The SDS-PAGE system used consisted of slab gels with a discontinuous buffer system (14). A 12% polyacrylamide separating gel in combination with a 4.5% stacking gel was used in all analyses. Samples, usually containing 5 to 50 μg of protein, were run under reducing conditions (0.2 M dithiothreitol).

Proteins separated on polyacrylamide gels were electrophoretically transferred onto sheets of nitrocellulose by the method of Towbin et al (24). After an overnight run at 250 mA, the nitrocellulose strips were incubated with MAG-maximum growth medium 1:10 or mouse polyclonal antiserum 1:100 for 2 h at 20°C and thoroughly washed in PBS. The strips were developed with a ^{125}I-labeled rabbit anti-

mouse immunoglobulin conjugate followed by autoradiography.

**Affinity chromatography.** The BB05 globulin (500 mg) was coupled to 70 mg of CNBr-Sepharose CL4B (Pharmacia Fine Chemicals, Inc.) by the manufacturer's instructions. Briefly, BB05 globulin at 10 mg/ml in 0.1 M borate buffer (pH 8.3) and CNBr-Sepharose were incubated with gentle rotation for 20 h at 4°C. Then the Sepharose was washed with PBS, incubated with 1 M ethanolamine (pH 8) for 2 h at 20°C, washed again with PBS, and stored in the presence of 0.1% sodium azide at 4°C.

The acid glycine hydrolysate extract used as starting material was initially applied to a column of DEAE-Trisacryl (Pharmacia) equilibrated with 0.025 M Tris–0.035 M NaCl (pH 8.8) buffer. The unretained filtrate fraction (200 mg) was dialyzed against PBS and subsequently applied onto the BB05-Sepharose column (50 by 2.6 cm). After extensive washing with 0.2 M ammonium bicarbonate (pH 7.0), the bound antigen was eluted with 3 M NaSCN.

**Murine models of *Bordetella bronchiseptica* infection.** MF-1 specific-pathogen-free (OLAC), 3-week-old mice were used throughout the study.

**(i) Intraperitoneal infection.** The virulent strain of *Bordetella bronchiseptica* 134, originally isolated from a pig suffering from AR, was used for the challenge of mice. Bacteria were grown on Cohen-Wheeler agar containing 5% horse blood and were harvested after 18 h of incubation at 37°C. The cells were suspended in PBS, and the suspension was adjusted to an optical density of 0.5 at 650 nm, equivalent to a bacterial concentration of 2 × $10^9$ CFU/ml. Mice were injected with 0.5 ml of this suspension with 90 min of its reading, and mortality was recorded daily for a period of 10 days.

**(ii) Aerosol infection.** The aerosol infection model has been described comprehensively elsewhere (Novotny et al., submitted for publication). Briefly, mice were exposed to an aerosol generated from a *Bordetella bronchiseptica* suspension containing 2 × $10^9$ CFU/ml. After 10 min of exposure, a very consistent count of $10^3$ to $10^4$ CFU of *B. bronchiseptica* per lung was obtained 30 to 60 min after infection. Mortality was recorded daily for 21 days, and periodically mice were killed for the assay of CFU in the lungs. For this purpose, the right lung was aseptically removed and homogenized. Dilutions of the homogenate were dropped onto Cohen-Wheeler agar and counted after 48 h of incubation at 37°C.

Determination of AR was carried out 5 weeks postinfection. Mice killed by cervical dislocation were decapitated, the hard palate was exposed, and the skull was frozen. The skull was cut twice in front of and behind the most prominent ridge of the rugae palatinae with a tungsten carbide circular saw driven by a dental drill. Three adjacent sections were obtained: the nose end (snout), a 1-mm-thick intermediary slice, and the skull end. The first two sections only were examined under a dissecting microscope immediately after cutting (i.e., while still frozen) and after fixation in 10% Formalin. In normal mice, the turbinate bones give a solid appearance, with very narrow gaps between the turbinates and the nasal septum. As a result of AR, the gaps are enlarged and cavities, very often asymmetrical, have developed.

**Determination of serum antibody levels.** Sera were titrated in a solid-phase RIA. Polyvinyl microtiter plates (Dynatech Laboratories), coated with either CSA (1 μg in 100 μl of PBS per well) or 68-kd antigen (0.5 μg in 50 μl of PBS per well), were reacted with dilutions of mice sera for 2 to 4 h at 4°C,

and bound antibody was detected by iodinated rabbit anti-mouse immunoglobulin (50,000 cpm per well) incubated overnight at 4°C. Antibody titers were expressed as serum dilutions giving 50% of specific binding ($ABT_{50}$). Specific binding was calculated with the formula percent specific binding = [(Test serum cpm − normal serum cpm)/(positive standard cpm − normal serum cpm)] × 100.

**Polyclonal antiserum.** BALB/c 10-week-old female mice were injected i.p. with 30 μg of CSA in incomlete Freund adjuvant followed by four injections of the same dose in saline over a period of 2 months. Mice were bled out 4 days after the last injection. The pool of antisera from 20 mice (PII) was titrated by the solid-phase RIA and showed a 630 $ABT_{50}$ antibody titer.

## RESULTS

**Comparative analysis of MABs BB05 and BB07.** As measured by the SDS-PAGE/immunoblot technique, the *Bordetella bronchiseptica* antigen bound by the BB05 antibody had an apparent molecular weight of 68,000 (Fig. 1). Antibody BB05 cross-reacted with *Bordetella pertussis*, showing two bands corresponding to molecular weights of 68,000 and 40,000, and with *Bordetella parapertussis* by a band of 70.7 kd. By the same technique, BB05 failed to bind with whole cells or membranes from *E. coli* K-88, K-99, and 987P; *M. hyopneumoniae*, *M. hyorhinis*, *Brucella abortus*, *H. parainfluenzae*, *H. haemoglobinophilus*, *H. aegyptius*, *H. influenzaemurium*, *P. multocida* (serotypes A and D), *P. haemolytica* (serotype $A_1$), *Streptococcus suis*, and *Staphylococcus aureus* (data not shown).

Subsequently, the binding profile of antibody BB07 was evaluated. The immunoblot analysis showed that BB07, like BB05, reacted with a 68-kd antigen present in both CSA and *Bordetella pertussis* extracts. The possibility that two different molecules of the same molecular weight are involved was ruled out by the immunoassay of eluates and filtrate fractions obtained from BB05-MAB-based affinity fractionation of *Bordetella bronchiseptica* and *Bordetella pertussis* extracts (Table 1). MAB-BB07 reacted with a 68-kd antigen present in both extracts, whereas the filtrates from the immunosorbent column showed no reaction with either BB05 or BB07 antibodies. This result suggested that the
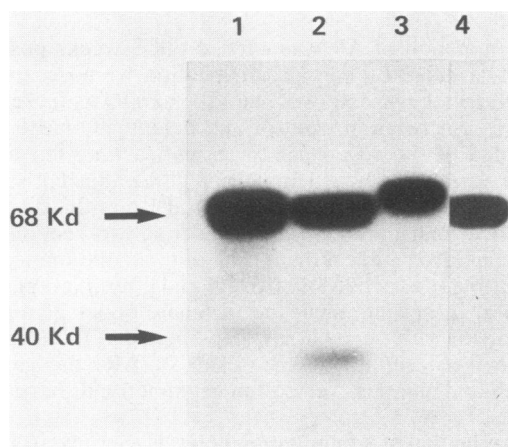
TABLE 1. Analysis of fractions from a BB05 affinity column[a]

| Extract from: | Fraction from BB05 column | Reaction with antibody: | |
|---|---|---|---|
| | | BB05 | BB07 |
| *Bordetella bronchiseptica* | Eluate | + | + |
| | Filtrate | − | − |
| *Bordetella pertussis* | Eluate | + | + |
| | Filtrate | − | − |

[a] Bacterial extracts were passed through the BB05 column and eluted with 3 M NaSCN. The fractions obtained were analyzed by SDS-PAGE immunoblot, using either BB05 or BB07 antibody.

determinants recognized by either of the two MABs are expressed by the same 68-kd molecule.

A competition RIA was set up to investigate whether BB05 and BB07 reacted with the same or distinct epitopes of the 68-kd molecule. In principle, if the two antibodies reacted with the same epitope, binding of the unlabeled antibody would prevent the binding of the second radiolabeled antibody. However, if the two antibodies reacted with different, nonoverlapping epitopes, the binding of the radiolabeled antibody would be detected regardless of the presence of the unlabeled antibody. The results of reciprocal competition assays of radiolabeled BB05 or BB07 showed that the homologous antibody competed with the binding of the corresponding iodinated globulin fraction in a dose-response fashion (Fig. 2). However, cross-competition was not observed, indicating that each MAB reacted with a different nonoverlapping epitope on the 68-kd molecule.

**Location of the 68-kd antigen within bacteria.** The CSA preparation of *Bordetella bronchiseptica* contains lipopolysaccharides, fibers, and outer membrane proteins, of which the latter component seemed the most likely to contain the 68-kd molecule. To test this assumption, OMVs were run in SDS-PAGE and the electroblot was reacted with MAB BB05. Binding to the 68-kd component of the OMV preparation was observed (Fig. 1, lane 4), suggesting that this antigen is a component of the outer membrane of *Bordetella bronchiseptica*. An analysis of the enzymatic susceptibility of the 68-kd antigen showed that the BB05 epitope was destroyed by *Bacillus subtilis* protease or trypsin treatment. However, the epitope was resistant to neuraminidase or sodium periodate oxidation (data not shown). These results further support the view that the 68-kd antigen is probably of a protein nature.

**Purification of the 68-kd antigen of *Bordetella bronchiseptica* by MAB BB05-based affinity chromatography.** The 68-kd antigen was purified from an acid glycine hydrolysate extract of *Bordetella bronchiseptica*. Preliminary experiments suggested that passage of the extract through an anionic exchange column eliminates certain bacterial components that may stick to Sepharose affinity chromatography columns. Therefore, the *Bordetella bronchiseptica* extract was first passed through a DEAE-Trisacryl column (LKB Instruments Inc.), and the filtrate was subsequently applied to the BB05-based affinity chromatography column. By the use of 327 ml of starting material (0.64 mg of protein per ml) for consecutive affinity purification, three fractions eluted with 3 M NaSCN were obtained and evaluated by SDS-PAGE and immunoblot. A considerable degree of purity was achieved, particularly in the eluates of the first and second chromatographic runs (Fig. 3A). The corresponding immunoblot of fractions is also shown (Fig. 3B). For subsequent in vivo experiments, the 68-kd antigens from the first and second eluates were pooled.



FIG. 1. Immunoblot of *Bordetella* preparations. Lanes: 1, 30 μg of *Bordetella bronchiseptica* extract; 2, 30 μg of *Bordetella pertussis* extract; 3, 30 μg of *Bordetella parapertussis* extract; 4, 20 μg of *Bordetella bronchiseptica* OMVs. Binding was with antibody BB05.
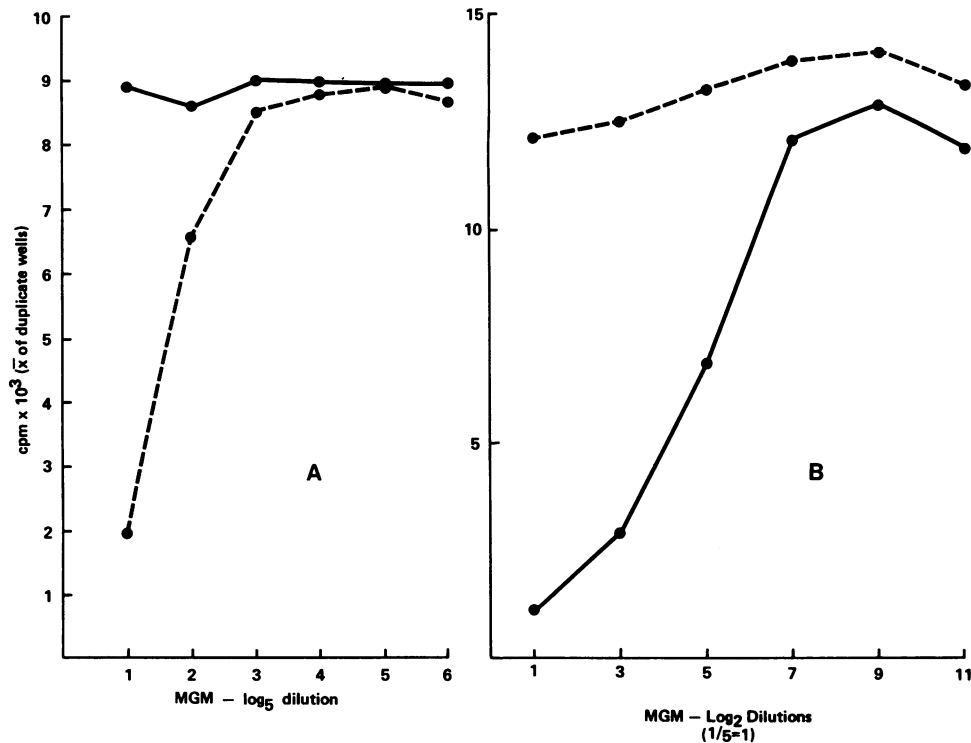
FIG. 2. Competition RIA of BB05 (- - -) and BB07 (———) MABs. *Bordetella bronchiseptica* antigen-coated plates were incubated with dilutions of MAB-MGM for 20 h at 4°C. Subsequently, either $^{125}$I-labeled BB05 (A) or BB07 (B) globulins were added to the wells, and after a further 20-h incubation, plates were washed and counted. MAB globulins were iodinoted by the iodogen technique (5).

**Passive immunization of mice against *Bordetella bronchiseptica* infection.** Mice were injected i.p. with either MAB globulin or with a polyclonal pooled murine antiserum (PII) 4 h before aerosol infection with *Bordetella bronchiseptica*. The protective effect was evaluated in terms of bacterial CFU in the lungs at different time intervals after infection, by mortality, and also on the basis of development of AR 5 weeks after challenge. The effect of MAB BB05 was compared with that of the polyclonal PII antiserum (see above). The bacterial CFU values in the lungs represent means of two mice per group harvested on different days after infection (Fig. 4). The initial 2.3 × 10⁴ CFU count immediately after challenge in the control group rose to 4.5 × 10⁹ at day 7 postinfection. Though no difference was apparent at day 2 or 5, the CFU counts in the lungs of immunized mice were lower by at least two orders of magnitude when compared with controls at the peak (day 7) of infection. The mortality of control mice ensued after the peak of CFU values was reached, i.e., during week 2 after infection. However, by day 13 postinfection, the bacterial load was similar in all groups. Although the bacterial counts in immunized and control mice were similar at day 27, the survival in the passively immunized groups (80 to 90%) was significantly better than in controls (45%). Five weeks after challenge, all surviving mice were sacrificed and their turbinate bones were examined for the presence of AR. All control mice showed signs of AR, but only one of eight mice ($P = 0.015$) injected with undiluted BB05 showed signs of the disease (Table 2, experiment 1).

In subsequent experiments, the contact infectivity of passively immunized and control aerosol-infected mice was investigated. Clean, nonimmunized mice were mixed with the experimental and control groups and monitored for mortality and incidence of AR (Table 2, experiment 2). In groups that served as the source of infection, three of four surviving mice in the control group presented AR, whereas all survivors in the MAB-injected group ($P = 0.02$) were free of the disease. Three of five mice in contact with the nonimmunized group developed AR, whereas none of the mice mixed with the BB05-injected group showed indications of AR. Hence, passive immunization with MAB BB05 appeared to prevent the spreading of the infection to nonimmunized mice.

The protective capacity of MAB BB07 was subsequently compared with that of BB05 (Table 2, experiment 3). All 15 BB05-injected mice ($P < 0.001$) survived the challenge infection, whereas only 1 of the 16 BB07-injected mice survived. A mere difference in antibody titer (7 × 10⁴ for BB05 and 7 × 10³ for BB07) seems insufficient to explain the observed differences in potency. Hence, the group that received the nonprotective BB07 antibody also served as a negative control for the BB05-injected mice. However, these results were to some extent diminished in value by the failure of the survivors in the control group to develop signs of AR.

The effects of passive injection of MABs BB05 and BB07 were also evaluated against i.p. infection with *Bordetella bronchiseptica*. Mice were injected i.p. with 2.5 mg MAB globulin, followed 4 h later by i.p. injection with 10⁹ CFU of *Bordetella bronchiseptica* per mouse. However, none of the MABs protected, and all mice died within 48 h of infection (data not shown).

**Active immunization of mice with the 68-kd antigen against *Bordetella bronchiseptica* infection.** The efficacy of active

immunization with the 68-kd antigen was evaluated in mice which were subsequently challenged by either i.p. or aerosol infection. In the case of i.p. infection, immunization with the 68-kd antigen in incomplete Freund adjuvant (IFA) or Alhydrogel was compared with immunization with a whole-cell *Bordetella bronchiseptica* vaccine. The neat ($10^9$ CFU per mouse) or 1:50 diluted ($2 \times 10^7$ CFU per mouse) challenge dose produced 100% mortality in the control groups (Table 3). Mortality was reduced to 0 or 7% ($P <$ 0.001) in mice immunized with the neat and 1:25 diluted *Bordetella bronchiseptica* vaccines, respectively. The 68-kd antigen in IFA protected 37% of mice ($P = 0.03$) irrespective



FIG. 4. Level of *Bordetella bronchiseptica* CFU in the lungs of aerosol-infected mice after passive immunization. Mice were injected i.p. with 0.5 ml of the polyclonal mouse antiserum, pool PII diluted 1:10 (△); or MAB BB05, 2.5 mg of globulin (○); or MAB BB05, 0.25 mg of globulin (□); or nonimmunized (●). Four hours later, all groups were infected with an aerosol of *Bordetella bronchiseptica*. Bacterial CFU counts in lungs were determined at different time intervals postinfection. Each point represents the mean value from two mice.

of the dose, whereas the adjuvant Alhydrogel supported the survival of 1 of 16 mice ($P > 0.5$) in each group. Antibody titrations of sera obtained 7 days before challenge against CSA did not reveal any obvious differences among the various groups (Table 3). Antibody titers against the 68-kd antigen were significantly lower in the groups immunized with the whole-cell vaccine than in mice immunized with the 68-kd antigen, in which titers up to 1:1,000,000 were reached. The specificity of antibodies towards the 68-kd antigen was also confirmed by SDS-PAGE/immunoblot analysis (data not shown).
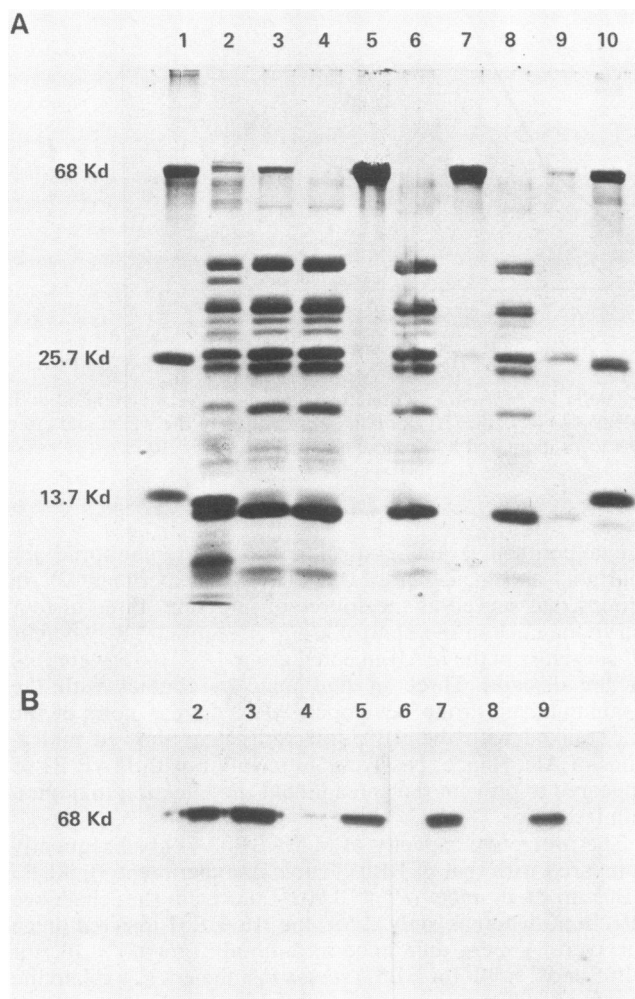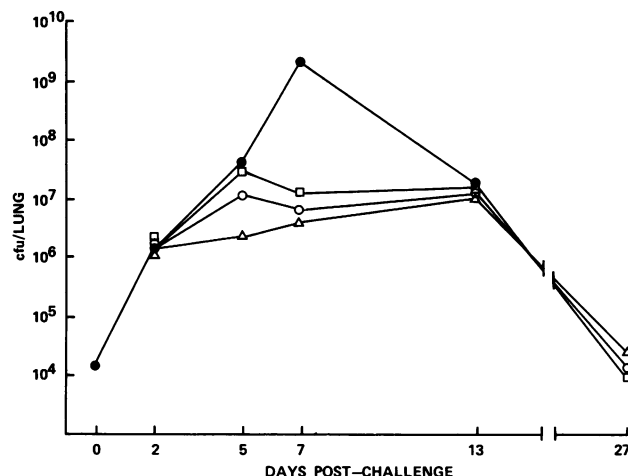


FIG. 3. Analysis of fractions obtained by BB05-based affinity chromatography purification of the 68-kd antigen. *Bordetella bronchiseptica* extract (209 mg of protein) filtered through a DEAE-Trisacryl column was applied to the BB05 immunosorbent column and eluted with 5 ml of 3 M NaSCN. From the first chromatographic run 4.73 mg of protein was eluted. The filtrate was twice recycled through the affinity column, resulting in a further 5.4 and 0.97 mg, respectively, of protein. (A) SDS-PAGE (Coomassie blue stained). (B) Immunoblot reacted with MAB BB05. Lanes: 1, Molecular weight markers; 2, *Bordetella bronchiseptica* extract (25 μg); 3, DEAE-Trisacryl filtrate (19 μg); 4, filtrate first run (17 μg); 5, eluate first run (10 μg); 6, filtrate second run (17 μg); 7, eluate second run (10 μg); 8, filtrate third run (11 μg); 9, eluate third run (10 μg); 10, molecular weight markers (bovine serum albumin, chymotrypsinogen A, RNase [as in lane 1]).

TABLE 2. Cumulative mortality and morbidity in passively immunized and *Bordetella* bronchiseptica aerosol-infected mice

| Expt no. | Antibody injected (0.5 ml) | No. of mice | | | |
|---|---|---|---|---|---|
| | | Dead/total[a] | P[b] | AR/survivors[c] | P |
| 1 | None | 6/11 | | 5/5 | |
| | BB05 (2.5 mg of globulin) | 2/10 | 0.12 | 1/8[d] | 0.015 |
| | BB05 (0.25 mg of globulin) | 1/10 | 0.03 | 5/9[d] | 0.39 |
| | PII antiserum diluted 1:10 | 1/9 | 0.06 | 5/8 | 0.39 |
| 2 | None | 7/11 (0/5)[e] | | 3/4 (3/5) | |
| | BB05 (2.5 mg of globulin) | 1/9 (0/5) | 0.03 | 0/8 (0/5) | 0.02 |
| 3 | None | 8/13 | | 0/5 | |
| | BB05 (2.5 mg of globulin) | 0/15 | <0.001 | 3/15 | >0.50 |
| | BB07 (2.5 mg of globulin) | 15/16 | 0.05 | 1/1 | 0.16 |

[a] Three weeks after infection.
[b] P, Significance of difference between experimental and control-infected group, using the one-sided Fisher exact test.
[c] Five weeks after infection.
[d] One turbinate bone could not be scored because of accidental damage.
[e] Numbers in parentheses relate to "clean" mice which had been put in contact (in the same cage) with the experimental mice 5 days after aerosol infection.

TABLE 3. Efficacy of active immunization with the 68-D antigen in protection against i.p. infection with *Bordetella bronchiseptica*

| Immunogen | Adjuvant and route (day[s] of injection)[a] | Antibody level at 21 days[b] | Challenge dose (CFU per mouse)[c] | No. of survivors/total | $P^d$ | % Survival |
|---|---|---|---|---|---|---|
| Whole-cell vaccine (neat) | Alhydrogel i.p. (1 + 14) | 2.6/2.9 | $10^9$ | 13/13 | <0.001 | 100 |
| Whole-cell vaccine (1:25) | Alhydrogel i.p. (1 + 14) | NT[e] | $10^9$ | 14/15 | <0.001 | 92 |
| 68-kd antigen (25 μg) | IFA/i.m. (1) None/i.p. (14) | 2.9/5.5 | $10^9$ | 6/16 | 0.03 | 37 |
| 68-kd antigen (5 μg) | IFA/i.m. (1) None/i.p. (14) | NT | $10^9$ | 6/16 | 0.03 | 37 |
| 68-kd antigen (25 μg) | Alhydrogel/i.m. (1) None/i.p. (14) | 2.1/6.0 | $10^9$ | 1/16 | >0.50 | 6 |
| 68-kd antigen (5 μg) | Alhydrogel/i.m. (1) None/i.p. (14) | | $10^9$ | 1/16 | >0.50 | 6 |
| Challenge control | | | $10^9$ | 0/10 | | 0 |
| | | | $2 \times 10^7$ | 0/10 | | 0 |
| | | | $10^7$ | 5/10 | | 5 |
| | | | $5 \times 10^6$ | 9/10 | | 90 |

[a] i.m., Intramuscularly.
[b] $Log_{10}$ $ABT_{50}$ serum dilution (see the text) to CSA/68-kd antigen.
[c] Given 28 days after the first immunization.
[d] Significance of difference between experimental and control-infected groups, using the one-sided Fisher exact test.
[e] NT, Not tested.

The protective potency of the 68-kd antigen towards aerosol infection was examined in groups of mice immunized with 10 μg of 68-kd antigen with various adjuvants. Mice were challenged 8 days later by an aerosol infection with *Bordetella bronchiseptica*, and bacterial CFU were determined at different days postinfection in the lungs of two mice from each group (Table 4). The decrease in lung CFU counts in immunized mice was apparent at 5 and 7 days postinfection by a 100-fold difference in comparison with controls. However, 14 days after infection, the CFU counts in all groups appeared similar. The data from Table 4 were analyzed by analysis of variance. A comparison of immunized versus nonimmunized animals on each day showed differences at day 5 ($P < 0.02$ by the Mann-Whitney test) and day 7 ($P < 0.001$ by the two-sample $t$ test, unequal variances) but not on day 14, 22, or 35. The age of mice at challenge appears to be critical with respect to mortality and development of AR. Consequently, mortality and AR rates in this experiment, in which the mice were 7 days older than in the passive immunization experiment (Table 2), were substantially lower.

## DISCUSSION

The antigen detected by the two MABs (BB05 and BB07) with specificity to a component of the outer membrane of *Bordetella bronchiseptica* was characterized. Both MABs reacted with distinct, nonoverlapping epitopes of the same 68-kd antigen. MAB BB05 cross-reacts with a 68-kd antigen of *Bordetella pertussis* and a 70.7-kd antigen of *Bordetella parapertussis*. Serological cross-reactivity among the species of the genus *Bordetella* has been known since the early studies of Eldering and Kendrick (3) showed that the three species possessed common O antigens but that each of them had its own specific O antigen in addition to the overlapping K antigens. More recently, Ezzell et al. (4) have studied the envelope proteins of bordetellas. They found that all three species possessed a major protein of molecular weight in the range of 37,000 to 41,000 and at least six other proteins of lower molecular weight. These proteins could not be assigned to either inner or outer membranes. Robinson and Hawkins (19) found that the major envelope proteins of *Bordetella bronchiseptica* and *Bordetella parepertussis* had

TABLE 4. CFU counts in the lungs of mice immunized with the 68-kd antigen (Ag) followed by *Bordetella bronchiseptica* aerosol challenge

| Immunogen[a] | CFU at days post-aerosol challenge[b] | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 5 | 7 | 14 | 22 | 35 |
| 68-kd Ag + CFA | NT[c] | <$10^4$ | $4.5 \times 10^4$ | $5.5 \times 10^5$ | <$10^4$ | $8.5 \times 10^4$ |
| | NT | <$10^4$ | $7 \times 10^3$ | $3.5 \times 10^3$ | $10^4$ | $3.5 \times 10^3$ |
| 68-kd Ag + IFA | NT | —[d] | $8 \times 10^4$ | $1.1 \times 10^5$ | $4 \times 10^4$ | $10^3$ |
| | NT | <$10^4$ | $1.1 \times 10^4$ | $7 \times 10^2$ | $7 \times 10^5$ | $5 \times 10^3$ |
| 68-kd Ag + saponin | NT | $3.5 \times 10^4$ | $1.6 \times 10^5$ | $9 \times 10^4$ | $7.5 \times 10^2$ | $2 \times 10^2$ |
| | NT | <$10^4$ | $9.5 \times 10^2$ | $1.3 \times 10^5$ | $8 \times 10^4$ | $1.3 \times 10^3$ |
| Controls | NT | $7 \times 10^6$ | $3 \times 10^6$ | $1.5 \times 10^4$ | $5.5 \times 10^4$ | NT |
| | NT | $1.6 \times 10^7$ | $4 \times 10^6$ | $9.5 \times 10^4$ | $1.1 \times 10^5$ | NT |
| CFA alone | $10^4$ | $5 \times 10^6$ | $1.8 \times 10^6$ | $1.4 \times 10^5$ | $10^4$ | $10^4$ |
| | NT | $9.5 \times 10^6$ | $6 \times 10^6$ | $10^4$ | $1.4 \times 10^4$ | $4 \times 10^4$ |

[a] Mice were immunized once with 10 μg of 68-kd antigen intramuscularly, with the exception of i.p.-injected 68-kd antigen in saponin. CFA, Complete Freund adjuvant.
[b] Values indicate CFU individual mice. Aerosol challenge was 8 days after immunization.
[c] NT, Not tested.
[d] This mouse showed a peritoneal exudate of unknown origin, and the bacterial counts were not considered.

a different profile than those of *Bordetella pertussis*. However, certain proteins with molecular weights of 77,000, 63,000, and 49,000 were common to the three species. Our results provide evidence of common determinants on homologous 68-kd molecules from *Bordetella bronchiseptica* and *Bordetella pertussis* and a 70.7-kd molecule from *Bordetella parapertussis*.

Passive immunization with MABs BB05 and BB07 was tested in *Bordetella bronchiseptica* aerosol-infected mice. Antibody BB05 showed a protective effect in terms of reduced mortality, incidence of AR, and number of lung CFU counts. The latter is of particular interest since the effect of the passively infected antibody was not patent until day 5 postinfection. Three possibilities may be put forward to explain this time course.

(i) During the first days of infection, the MAB could not reach the tissue in which the microorganisms were multiplying. However, once an inflammatory response developed, changes in permeability allowed the plasma exudation and the penetration of antibody into the tissues.

(ii) The lag period may represent the time needed for the recruitment of leukocytes and macrophages for the antibody-mediated opsonization.

(iii) Although *Bordetella bronchiseptica* is considered an extracellular pathogen with marked predilection for the ciliated epithelium of the respiratory tract (2, 25), it is possible that intracellular infection may also take place and hence that antibodies could not reach the bacteria in tissues until sufficient damage exposing the microorganism had occurred.

Irrespective of the lag effect of the MAB, it is pertinent that *Bordetella bronchiseptica* persist in the lungs of infected mice for at least 35 days postinfection. It has been reported that immunization of mice with whole *Bordetella bronchiseptica* cells followed by intranasal infection effectively clears the organisms from the lungs and protects against mortality, whereas organisms still persist in the nasal cavity for up to 60 to 150 days after challenge (21). Although passive injection of MAB BB05 did not eradicate the infection, it prevented mortality, the development of AR, and the spread from infected to noninfected mice. It is conceivable that the passive protection obtained in this study with MAB BB05 may be mediated by mechanisms similar to those that are effective in piglets receiving immune colostrum from sows (11, 13). Presumably, colostral antibodies (79% immunoglobulin G and 14% immunoglobulin A from total immunoglobulin (18) are traversing the intestinal mucosa and the general circulation from which they have to reach the respiratory tract to exercise protective immunity.

Antibody BB07, in contrast to MAB BB05, failed to protect mice against aerosol infection. This result was particularly interesting considering that both antibodies react with the same antigen. Although the explanation is not known, it is possible to speculate that the two epitopes may differ in their orientation on the bacterial cell surface.

Immunization with the purified 68-kd antigen against i.p. infection resulted in 37% protection when the antigen was injected in IFA. Immunization resulted in a significant reduction in the number of CFU of *Bordetella bronchiseptica* in the lungs of aerosol-infected mice. However, the evaluation of results in preventing AR was hampered by the apparent resistance of 4-week-old mice to develop the disease. Others have observed that 5-day-old intranasally inoculated mice developed AR, whereas 7- to 14-day-old mice were resistant (20). A possible way to assess the effect of 68-kd immunization while avoiding the age-related resist-

ance would be to immunize pregnant female mice and then aerosol challenge their progeny.

Monitoring of the content of the 68-kd antigen as a representative protective antigen of *Bordetella bronchiseptica* may be useful in the quality control testing of *Bordetella bronchiseptica* vaccines. The possibility of using the 68-kd antigen as a subcellular vaccine for pigs requires extensive investigations; at present, only a few general considerations can be raised. It is conceivable that other bacterial components also play a protective role and that therefore a heterogeneous immune response may confer more effective immune protection to the host. A suitable adjuvant to support the immunization in pigs would also need to be defined. It remains a possibility that other bacterial components may potentiate the immune response to the 68-kd antigen when whole cells are injected.

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