

Identification of a 69-Kilodalton Nonfimbrial Protein As an Agglutinin of *Bordetella pertussis*

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Cells of *Bordetella pertussis* BP353, a nonfimbriated Eldering serotype 1.3 strain, were used as an immunogen to produce three monoclonal antibodies, BPE3, BPD8, and BPE8, that agglutinated the immunizing cells, as well as certain other nonfimbriated and fimbriated serotype 3-containing *B. pertussis* strains. The antibodies did not agglutinate serotype 1 or nontypable *B. pertussis* cells. These monoclonal antibodies specifically detected a 69-kilodalton (kDa) band on Western blots (immunoblots) containing whole *B. pertussis* cell lysates of Eldering agglutinin serotypes 1.3, 1.3.6, 1.2.3.4, and 1.2.3.4.6. This 69-kDa antigen was released from the bacteria by cell incubation for 60 min at 60°C, and it was purified by affinity chromatography with a BPE3-agarose affinity matrix. Purified material was used to produce a polyclonal antiserum that agglutinated all nonfimbriated and fimbriated *B. pertussis* cells containing serotype 3 agglutinin. Immunogold electron microscopy and indirect immunofluorescence studies demonstrated that it is an outer membrane constituent but nonfimbrial in appearance. BPE3 did not detect purified fimbriae on Western blots, and antibodies to these fimbriae did not bind to the 69-kDa component. Although *B. bronchiseptica* and *B. parapertussis* cells were not agglutinated by the monoclonal antibodies, antigenically similar proteins were detected in extracts of the bacteria. These results identify the 69-kDa protein as a nonfimbrial agglutinin present on all virulent strains of *B. pertussis*. The monoclonal antibodies described here should be useful for further studies on the structure and function of this protein.

Serotype markers for *Bordetella pertussis* have historically been defined by the ability of strain-specific polyclonal antisera to agglutinate the bacteria. Andersen first identified five distinct agglutinin factors (1), and subsequently Eldering et al. added agglutinin factor 6 (6). The U.S. Reference Factor 1 to 6 antisera prepared by Eldering et al. (6) and three monospecific polyclonal antisera (Factors 1 to 3) produced by Preston (23) have been used to identify the serotypes of human isolates and to distinguish *B. pertussis* from the closely related species *B. bronchiseptica* and *B. parapertussis* (5, 14, 25). The prevalence of certain serotypes following outbreaks of whooping cough has been noted (8, 25), and there is some evidence that serum agglutinin titers of human vaccinees correlate with clinical protection from pertussis (16). Therefore, the agglutinin factors present on *B. pertussis* may contribute to the formulation of better-defined protective vaccines.

Some of these agglutinin factors have been defined, at least in part, at the molecular level. The expression of lipooligosaccharide A by *B. pertussis* cells is correlated with the presence of the serotype 1 agglutinin factor (12), and a fimbrial agglutinin present on serotype 2-containing cells has been purified and well characterized (3, 9, 26, 31). Fimbriae isolated from strains agglutinated by U.S. Reference Factor 6 antiserum (type 6 fimbriae) and Preston agglutinin 3 antiserum (type 3 fimbriae) appear to be antigenically identical (4, 9, 19). The agglutinins recognized by U.S. Reference Factor 3 antiserum have not been well characterized.

To identify agglutinins of *B. pertussis*, we have produced a number of monoclonal antibodies that agglutinate serotype-specific strains. Monoclonal antibodies that agglutinate *B. pertussis* cells containing serotype 1 agglutinin were found to be uniquely reactive with lipooligosaccharide A (12). Other monoclonal antibodies have been identified which recognize the serotype 2 and 6 fimbriae of *B. pertussis* (11). In this study, monoclonal antibodies were used to characterize a 69-kilodalton (kDa) nonfimbrial outer membrane protein found on *B. pertussis* cells agglutinated by U.S. Reference Factor 3 antiserum. This protein is expressed by virulent strains of *B. pertussis* and is antigenically identical to a protein previously correlated with the adenylate cyclase activity of *Bordetella* species (20).

MATERIALS AND METHODS

Microorganisms. Cells of *Bordetella* species were cultured on plates of Bordet-Gengou medium (Difco Laboratories, Detroit, Mich.) containing 15% defibrinated sheep blood, and growth was continued in liquid Cohen-Wheeler medium with constant agitation. Modified Stainer-Scholte medium was used for the liquid culture of *B. pertussis* 114 and 432. A detailed description of the bacterial growth conditions is provided elsewhere (4, 32). The bacteria were used for protein purification as described below or fixed in 0.2% formaldehyde and serotyped by the microagglutination assay (15) with U.S. Reference Factor 1 to 6 antisera (Eldering agglutinin 1 to 6 polyclonal antisera) (6). The transposon Tn5 insertion mutants of *B. pertussis* used in this study (28, 29) were provided by Alison Weiss, Medical College of Virginia, Richmond, and the source of the *B. bronchiseptica*

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Monoclonal antibodies. Hybridomas BPE3, BPD8, and BPE8 were obtained by fusion of spleen cells from BALB/c mice immunized with *B. pertussis* BP353 (serotype 1.3) to the SP2/0 cell line (7). Mice were injected with 5×10^8 formaldehyde-fixed bacteria intraperitoneally three times at 2-week intervals, and an intravenous injection was given 3 days before fusion. Hybridoma supernatants were initially screened by bacterial agglutination (15) and by indirect enzyme-linked immunosorbent assay with microtiter plates coated with fixed *B. pertussis* BP353 cells. Immunoglobulin isotypes were determined by enzyme-linked immunosorbent assay with specific anti-mouse immunoglobulin reagents (Southern Biotechnology Associated, Inc., Birmingham, Ala.). Antibodies were purified from ascitic fluid by 50% ammonium sulfate precipitation followed by chromatography on DEAE-cellulose for antibodies of the immunoglobulin G isotype or by gel filtration on Sepharose 4B for those of the immunoglobulin M isotype. The purity of the immunoglobulin fractions was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The monoclonal antibodies to fimbriae, BPF2 and BPC10, were produced by immunizing mice with crude preparations of fimbriae partially purified from *B. pertussis* 325 (serotype 1.2.3.4) or 114 (serotype 1.3.6) (11). The production and characterization of anti-*B. bronchiseptica* monoclonal antibody BB05 have been described previously (18).

SDS-PAGE and Western blot (immunoblot) analysis. Whole-cell lysates, bacterial extracts, or purified proteins were analyzed by SDS-PAGE on a 10 or 15% SDS resolving gel with a 3% stacking gel (10). Most samples in electrophoresis buffer containing SDS were boiled for 5 min in the presence of 0.1 M dithiothreitol before analysis with a mini-Protein II slab gel (Bio-Rad Laboratories, Richmond, Calif.). For detection of fimbrial antigen, samples were applied to the gel without boiling. Protein bands in the gels were visualized by Coomassie blue or silver staining (30). The molecular mass protein standards (Bio-Rad) included myosin (200 kDa), β -galactosidase (116.25 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa). Prestained protein standards (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used to estimate the molecular masses of proteins transferred to nitrocellulose filters.

For Western blot analysis, proteins were electroblotted from the gel onto nitrocellulose paper (BA-85; Schleicher & Schuell, Inc., Keene, N.H.) for 1 h at 100 V in 0.025 M Tris-0.192 M glycine (pH 8.3) buffer containing 20% methanol (27). Nitrocellulose filters were routinely blocked in Tris-buffered saline (0.02 M Tris-0.5 M NaCl, pH 7.5) containing 0.5% bovine serum albumin with shaking overnight at 25°C. The filters were incubated with hybridoma supernatants concentrated 10-fold with 50% ammonium sulfate or ascitic fluid diluted 1:1,000 in Tris-buffered saline containing 0.05% Tween 20 and 0.2% sodium azide for 2 h at 25°C. After extensive washing, the filters were incubated for 2 h in a 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Sigma Chemical Co., St. Louis, Mo.) or, in some cases, peroxidase-conjugated goat anti-mouse immunoglobulin (Bio-Rad). The filters were developed with the Protoblot substrate (Promega Biotec, Madison, Wis.) to detect alkaline phosphatase-conjugated antibodies or with 4-chloro-1-naphthol as the peroxidase substrate. A similar procedure was used to detect bands

reactive with U.S. Reference Factor 3 antiserum with this rabbit polyclonal antiserum diluted 1:300, followed by a 1:1,000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin (Bio-Rad).

Protein purification. The 69-kDa protein was initially extracted from *B. pertussis* cells by incubating 6×10^{12} washed bacteria in 30 ml of phosphate-buffered saline (PBS; 0.01 M PO_4 -0.15 M NaCl, pH 7.2) for 1 h at 60°C. The cell suspension was centrifuged for 20 min at $10,000 \times g$, and the supernatant was dialyzed against Tris-buffered saline (0.01 M Tris-0.15 M NaCl, pH 8) containing 0.001 M EDTA, 0.001 M phenylmethylsulfonyl fluoride, and 0.1% Brij 35. With this buffer, the extract was applied to a column of fetuin-Sepharose 4B (10-ml column volume) to remove pertussis toxin. The flow-through material was chromatographed on a 10-ml column of BPE3 immunoglobulin linked to agarose (Reacti-gel 6X; Pierce Chemical Co., Rockford, Ill.) in the same buffer. The column was washed with 0.5 M NaCl containing 0.5% Zwittergent 3-14 (Calbiochem-Behring, La Jolla, Calif.), and the bound protein was eluted with 6 M urea. The purity of the eluted fractions was assessed by SDS-PAGE, followed by silver staining and Western blot analysis.

Purified material was used to produce polyclonal antisera by immunizing five mice subcutaneously with 20 μg of protein per mouse in Freund complete adjuvant, followed in 4 weeks by a secondary injection with incomplete adjuvant. The mice were bled 7 days after the second injection, the sera were pooled, and agglutination tests were performed after nonspecific agglutination was reduced by adsorption of the sera with 25% kaolin (31). Mouse sera obtained prior to immunization were negative in the agglutination assays.

Immunogold electron microscopy and indirect immunofluorescence. Formaldehyde-fixed *B. pertussis* cells (2×10^{10}) were washed with PBS containing 0.5% bovine serum albumin and incubated with 100 μg of immunoglobulin per ml for 1 h at 25°C. Bacteria were washed twice and incubated with a 1:2 dilution of gold-conjugated goat anti-mouse immunoglobulin (10 nm; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for an additional hour and then washed three times with PBS. Samples were fixed with 1% glutaraldehyde in PBS for 30 min at room temperature, washed three times with PBS, postfixed with 2% osmium tetroxide for 30 min, serially dehydrated in alcohol, and embedded in an Epon-Araldite resin mixture. Thin sections were cut on a Reichert Ultracut E with a diamond knife and stained with Reynolds lead citrate and 5% uranyl acetate. Samples were observed with a Philips EM400HM electron microscope at 80 kV at a magnification of $\times 30,000$ with a liquid N_2 anticontamination device routinely in use.

For indirect immunofluorescence assays, 20 μl of a formaldehyde-fixed suspension of bacteria was heat dried on a glass cover slip and washed with PBS. The cells were incubated with 25 μg of purified immunoglobulin per ml for 1 h, washed with PBS three times, and then incubated with a 1:20 dilution of rhodamine-conjugated goat anti-mouse immunoglobulin (Southern Biotechnology Associated). After extensive washing, labeled cells were observed with a Nikon optiphot fluorescence microscope.

RESULTS

Identification of monoclonal antibodies that agglutinate *B. pertussis* cells. To identify the agglutinin(s) on nonfimbriated serotype 1.3 BP353 cells, mice were immunized with fixed bacteria and the abilities of the resulting hybridoma

TABLE 1. Agglutination of serotype-specific *B. pertussis* strains by monoclonal antibodies and polyclonal antiserum

<i>B. pertussis</i> strain	Agglutinin serotype ^a	Agglutination titer with the following monoclonal antibody: ^b					Agglutination titer with mouse anti-69-kDa protein antiserum
		BPE3	BPE8	BPD8	BPF2	BPC10	
10901	Nontypable	— ^c	—	—	—	—	—
11615	Nontypable	—	—	—	—	—	—
Tohama III	1	—	—	—	—	—	—
BP326	1	—	—	—	—	—	—
BP353	1.3	32,768	4,096	512	—	—	512
BP354	1.3	32,768	4,096	512	—	—	512
432	1.3.6	2,048	—	—	—	8,192	256
114	1.3.6	—	—	—	—	4,096	64
BP338	1.2.3.4	2,048	1,024	128	2,560	—	128
150	1.2.3.4	—	—	—	2,560	—	128
460	1.2.3.4.6	4,096	—	—	4,096	2,048	128
165	1.2.3.4.6	—	—	—	8,192	4,096	64

^a *B. pertussis* strains were serotyped by agglutination, as described in Materials and Methods, with U.S. Reference Factor 1 to 6 antisera.

^b Agglutination assays were performed with concentrated hybridoma supernatants or mouse sera pretreated with kaolin, as described in Materials and Methods. Titers are reported as the reciprocal of the maximum antibody dilution which agglutinated the bacteria. Monoclonal antibodies were produced by immunizing mice with *B. pertussis* BP353 cells (BPE3, BPE8, and BPD8) or partially purified type 2 (BPF2) or 6 (BPC10) fimbriae, as described in Materials and Methods.

^c —, Not agglutinated by antibodies or antiserum at a 1:2 dilution.

supernatants to agglutinate various *B. pertussis* strains were assessed (Table 1). Three hybridomas, BPE3, BPE8, and BPD8, produced monoclonal antibodies that strongly agglutinated the immunizing BP353 cells and an additional serotype 1.3 strain, BP354. These monoclonal antibodies also agglutinated some (but not all) *B. pertussis* cells of serotypes 1.3.6, 1.2.3.4, and 1.2.3.4.6. In all cases, the agglutination titer was highest with BPE3 (immunoglobulin M isotype). The monoclonal antibodies did not agglutinate serotype 1 or nontypable strains of *B. pertussis* (Table 1), four strains of *B. bronchiseptica*, three strains of *B. parapertussis*, and other gram-negative bacteria, including *Escherichia coli*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Salmonella typhimurium* (data not shown). For comparison, monoclonal antibodies to type 2 (BPF2) or 6 (BPC10) fimbriae (11) agglutinated *B. pertussis* cells reactive with U.S. Reference Factor 2 (serotypes 1.2.3.4 and 1.2.3.4.6) or 6 antiserum (serotypes 1.3.6 and 1.2.3.4.6), respectively (Table 1).

Western blot analysis with agglutinating monoclonal antibodies and U.S. Reference Factor 3 antiserum. Monoclonal antibody BPE3 specifically detected a single band on Western blots containing cell lysates of serotypes 1.3, 1.3.6, 1.2.3.4, and 1.2.3.4.6 *B. pertussis* cells (Fig. 1A, lanes 1 to 3, and B, lane 1). This band, at about 69 kDa, was present in all strains containing serotype 3 agglutinin, including those not agglutinated by the monoclonal antibody, such as strains 150 (Fig. 1A, lane 2) and 114 (Fig. 1A, lane 3). No reactivity was observed on immunoblots containing cell lysates of serotype 1 or avirulent strains of *B. pertussis* (Fig. 1B, lanes 2 and 3) or other gram-negative organisms (data not shown). The two immunoglobulin G1 monoclonal antibodies, BPD8 and BPE8, also reacted with the 69-kDa band by Western blot analysis, but much greater amounts of antibody were required for detection. U.S. Reference Factor 3 antiserum (Eldering agglutinin 3 polyclonal antiserum) detected a 69-kDa band on identical immunoblots containing *B. pertussis* cell lysates in a pattern consistent with that of monoclonal antibody BPE3 (Fig. 1A and B, lanes 4 to 6). This polyclonal antiserum was also reactive with a number of other heterogeneous bands on these Western blots.

Purification of the 69-kDa antigen and production of an agglutinating polyclonal antiserum. *B. pertussis* BP353 cells

were incubated for 60 min at 60°C in PBS, and the 69-kDa component that was detached from the bacteria was assessed by SDS-PAGE (Fig. 2A, lane 1) and immunoblotting (Fig. 2B, lane 4). This material was chromatographed on a BPE3-agarose affinity matrix, and the 69-kDa antigen was specifically eluted from the column with 6 M urea (Fig. 2A, lane 3, and B, lane 6). The 69-kDa antigen occasionally appeared as a doublet on SDS-PAGE (Fig. 2). Reactivity of BPE3 with the purified 69-kDa band was substantially diminished after treatment of the blotted antigen with 5 µg of trypsin per ml for 10 min at 35°C but not after treatment with periodate (data not shown).

Mice were immunized with the affinity-purified 69-kDa material, and the polyclonal antiserum obtained was tested

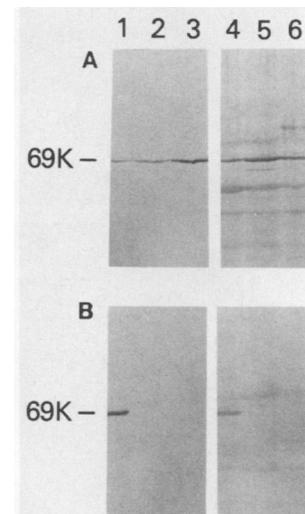


FIG. 1. Detection of a 69-kDa band on Western blots of *B. pertussis* cell lysates. Nitrocellulose filters that contained whole-cell lysates of *B. pertussis* (panel A) 460 (lanes 1 and 4), 150 (lanes 2 and 5), 114 (lanes 3 and 6), (panel B) BP353 (lanes 1 and 4), Tohama III (lanes 2 and 5), and 10901 (lanes 3 and 6) were reacted with monoclonal antibody BPE3 (lanes 1 to 3) or U.S. Reference Factor 3 antiserum (lanes 4 to 6), and binding was detected with peroxidase-conjugated species-specific antibodies, as described in Materials and Methods. The position of the 69-kDa band is shown.

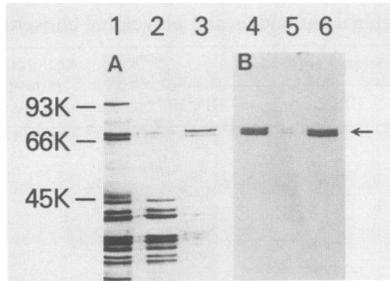


FIG. 2. Purification of the 69-kDa antigen by immunoaffinity chromatography. (A) SDS-polyacrylamide gel stained with silver. (B) Immunoblot detected with monoclonal antibody BPE3. Lanes: 1 and 4, pre-column 60°C, 60-min extract; 2 and 5, unbound material; 3 and 6, 6 M urea eluate. Further analysis of the purified material indicated that the faint lower-molecular-weight bands in lane 3 were a result of sample spillover from lane 2. Molecular mass markers (in kilodaltons) are listed to the left. The position of the 69-kDa band is indicated by the arrow.

for its ability to agglutinate serotype-specific strains of *B. pertussis* (Table 1). The pooled mouse serum agglutinated all of the strains tested except serotype 1 and nontypable strains. As with monoclonal antibodies BPE3, BPD8, and BPE8, the agglutination titers were greatest with serotype 1.3 *B. pertussis* cells. Also, like the monoclonal antibodies, this polyclonal antiserum specifically detected a 69-kDa

band on Western blots containing BP353 cell lysates (data not shown).

Identification of the 69-kDa antigen as a nonfimbrial component of the outer membrane. Immunogold electron microscopy was used to compare the distribution of the 69-kDa antigen and type 6 fimbriae on *B. pertussis* cells, with gold-labeled secondary antibodies used to visualize the binding of primary monoclonal antibodies. Following incubation of BP353 (Fig. 3A) or 432 (Fig. 3B) cells with the anti-69-kDa antibody BPD8, numerous gold particles were observed in close apposition to their outer membranes. Frequently, small clusters of two to five gold particles were observed, suggesting an oligomeric arrangement or local concentrations of the antigen. Different results were obtained with anti-fimbrial antibody BPC10. This antibody did not label BP353 cells (Fig. 3C), and with fimbriated 432 cells its labeling pattern included characteristic constellations of gold particles extending to considerable distances from the bacterial surface (Fig. 3D, arrow). These constellations are consistent with multiple labeling of fimbriae that extend from the bacterial surface while remaining within the plane of the section.

These results and the fact that fimbriae were not observed on cells of the immunizing strain, BP353, by electron microscopy of either negatively stained or rotary shadowed specimens (data not shown) indicate that the 69-kDa antigen is not a fimbrial protein. To substantiate further the conclu-

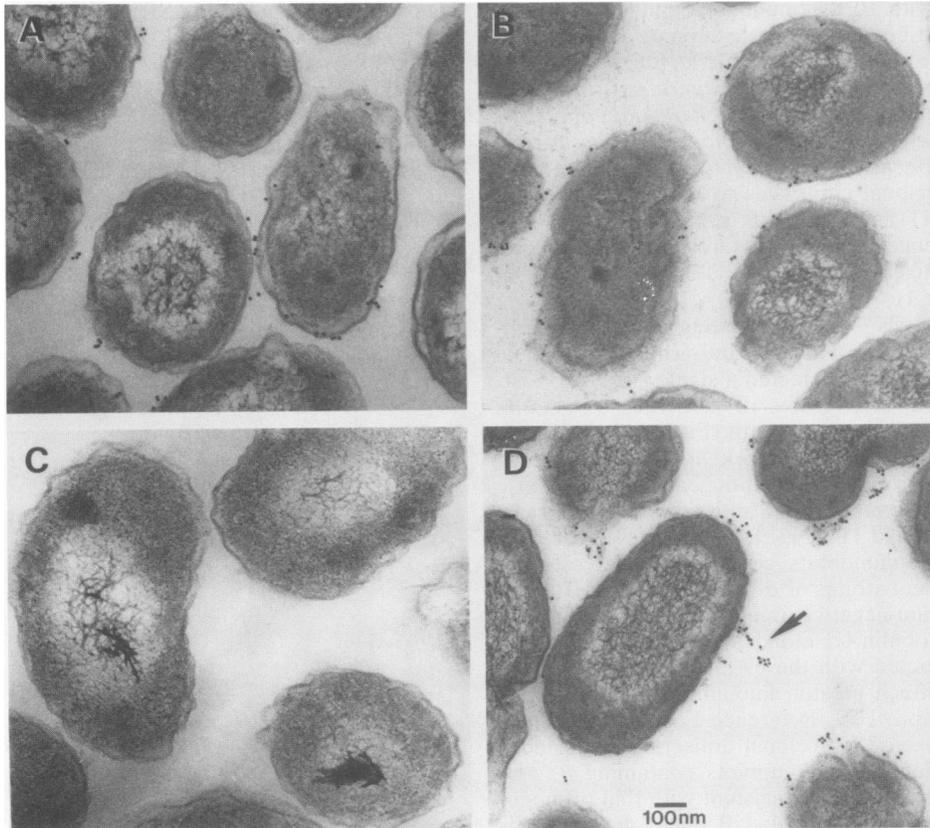


FIG. 3. Immunogold electron microscopy. *B. pertussis* BP353 (A and C) and 432 (B and D) cells were incubated with monoclonal antibody BPD8 to the 69-kDa antigen (A and B) or BPC10 to type 6 fimbriae (C and D), reacted with gold-conjugated anti-mouse immunoglobulin, and subsequently processed as described in Materials and Methods. The arrow in panel D designates multiple labeling of a single fimbria (or bundle of fimbriae) standing out from the bacterial surface.

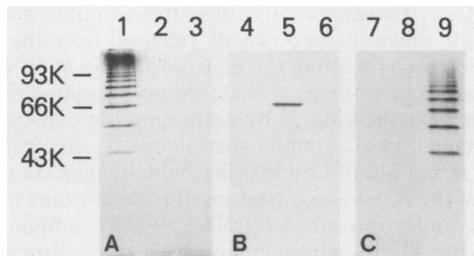


FIG. 4. Comparison of the antigenic reactivities of purified proteins by Western blot analysis. Immunoblots containing purified type 2 fimbriae (lanes 1, 4, and 7), 69-kDa protein (lanes 2, 5, and 8), and type 6 fimbriae (lanes 3, 6, and 9) were detected with monoclonal antibody BPF2 (A), BPE3 (B), or BPC10 (C). Molecular mass markers (in kilodaltons) are listed to the left.

sion that the 69-kDa antigen is not a fimbrial agglutinin, the reactivity of BPE3 and the monoclonal antibodies to type 2 (BPF2) and 6 (BPC10) fimbriae were compared by using Western blots containing purified proteins. On immunoblots containing SDS-treated, nonboiled, purified type 2 fimbriae, 69-kDa protein, and type 6 fimbriae, anti-type 2 fimbrial monoclonal antibody BPF2 detected bands only in the lane containing type 2 fimbriae (Fig. 4A, lane 1). This appearance of uniformly repeating oligomeric bands or laddering is characteristic of fimbriae detected by this method (11). BPE3 detected only the 69-kDa band and did not show the laddering effect (Fig. 4B, lane 5). In contrast, the laddering pattern was apparent in the lane containing purified type 6 fimbriae on the filter reacted with anti-type 6 fimbrial monoclonal antibody BPC10 (Fig. 4C, lane 9). With these monoclonal antibodies no antigenic cross-reactivity was observed.

Recognition of an antigenically similar protein on *B. bronchiseptica* and *B. paraptussis*. Although *B. bronchiseptica* and *B. paraptussis* strains were not agglutinated by BPE3, the monoclonal antibody did detect a band(s) that migrated slightly slower than the *B. pertussis* 69-kDa band (Fig. 5A, lane 1) on Western blots containing cell extracts of *B. bronchiseptica* 058 (Fig. 5A, lane 2) and *B. paraptussis* 497 (Fig. 5A, lane 3). These results suggest that the 69-kDa antigen is similar to an outer membrane protein first identified on *B. bronchiseptica* with monoclonal antibody BB05 (18). A duplicate filter containing *B. pertussis*, *B. bronchiseptica*, and *B. paraptussis* cell extracts was reacted with BB05 and gave results similar to those obtained with BPE3 (Fig. 5B). However, some additional bands were detected by BB05. Indirect immunofluorescence assays demonstrated

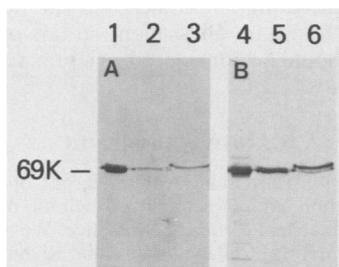


FIG. 5. Identification of antigenically similar proteins in *B. bronchiseptica* and *B. paraptussis*. Immunoblots containing 60°C, 60-min extracts from *B. pertussis* BP353 (lanes 1 and 4), *B. bronchiseptica* 058 (lanes 2 and 5), and *B. paraptussis* 497 (lanes 3 and 6) were reacted with monoclonal antibody BPE3 (A) or BB05 (B). The position of the 69-kDa *B. pertussis* band is shown.

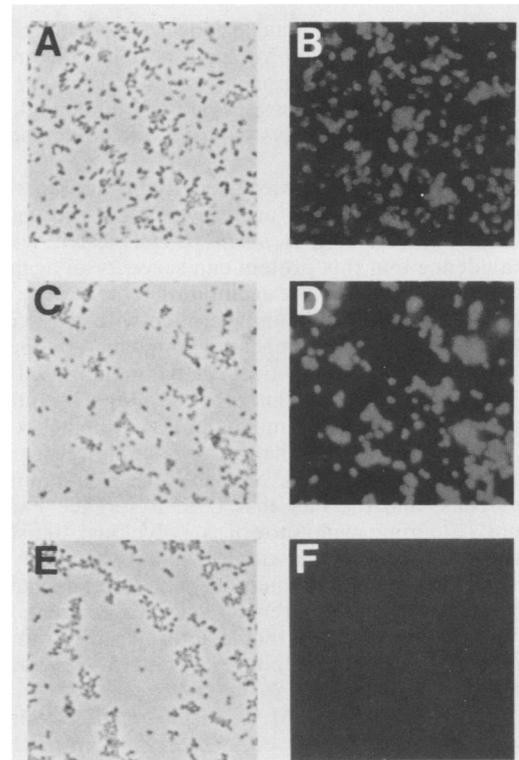


FIG. 6. Cell surface antigen detected by indirect immunofluorescence assay. *B. pertussis* BP353 (A and B), *B. bronchiseptica* 058 (C and D), or *B. paraptussis* 497 (E and F) cells were heat fixed on glass cover slips, as shown by phase microscopy (A, C, and E), and incubated with monoclonal antibody BPE3. Antibody binding was detected by indirect immunofluorescence (B, D, and F), as described in Materials and Methods. (Magnification, $\times 540$).

that BPE3 recognizes a cell surface antigen on *B. bronchiseptica* (Fig. 6C and D) as well as on *B. pertussis* (Fig. 6A and B). However, no binding to *B. paraptussis* cells was observed (Fig. 6E and F). Five different *B. bronchiseptica* strains and three strains of *B. paraptussis* gave results similar to those shown in Fig. 5 and 6.

DISCUSSION

The monoclonal antibodies described in this report recognize a 69-kDa antigen found on the surface of all virulent strains of *B. pertussis* that have been analyzed by agglutination, Western blot analysis, and indirect immunofluorescence assays. This 69-kDa antigen is apparently not produced by serotype 1 (phase III) or nontypable, avirulent (phase IV) strains of *B. pertussis* or by a number of other gram-negative organisms. It can serve as an agglutinin, as demonstrated by the ability of the monoclonal antibodies to strongly agglutinate *B. pertussis* serotype 1.3 strains and to agglutinate certain other serotype 1.3.6, 1.2.3.4, or 1.2.3.4.6 strains. However, a number of *B. pertussis* cells, as well as *B. bronchiseptica* and *B. paraptussis* strains, were not agglutinated by the monoclonal antibodies even though they express a similar antigen, as demonstrated by Western blot analysis. This suggests that for these monoclonal antibodies the antigen is not available in sufficient density to serve as an agglutinin on these strains. Alternatively, the presence of other surface components, such as fimbrial agglutinogens or flagellar or capsular components, may mask the ability of the

69-kDa antigen to act as an agglutinin. Monoclonal antibody BPE3 (immunoglobulin M) apparently has the greatest avidity for the 69-kDa component, since it is much more reactive than either BPD8 or BPE8 (both immunoglobulin G1) in all of the immunoassays we used, including bacterial agglutination.

Purified 69-kDa protein was used to produce a mouse polyclonal antiserum which agglutinated all serotype 1.3, 1.3.6, 1.2.3.4, and 1.2.3.4.6 *B. pertussis* cells tested, which is further evidence that this protein can serve as an agglutinin. However, in general, the agglutination titers were lower than those obtained by immunizing mice with purified fimbriae (4, 31) which contain repeating antigenic epitopes.

The results which demonstrate that nonfimbriated serotype 1.3 cells, as well as certain other serotype 3-containing cells, are agglutinated by monoclonal antibodies to the 69-kDa antigen, the finding that polyclonal antiserum to the purified protein agglutinates all serotype 3-containing *B. pertussis* cells, and the fact that U.S. Reference Factor 3 antiserum (Eldering agglutinin 3 polyclonal antiserum) contains antibodies reactive with the 69-kDa band on Western blots suggest that this protein is a nonfimbrial agglutinin recognized by U.S. Reference Factor 3 antiserum. Other results indicate, however, that Factor 3 antiserum also contains agglutinating antibodies to fimbriae present on serotype 1.3.6 and 1.2.3.4.6 *B. pertussis* cells (M. J. Brennan, Z. M. Li, J. L. Cowell, M. E. Bisher, A. C. Steven, and C. R. Manclark, in S. Mebel, ed., *Proceedings of the FEMS Symposium—Pertussis*, in press) which indicates that the U.S. Reference Factor 3 agglutinin is not a single component. Identification of the serotype 3 agglutinin is also complicated by the fact that U.S. Reference Factor 3 antiserum cannot be equated with Preston serotype 3 antiserum, which is also used to establish serogroups for *B. pertussis*. Preston serotype 3 antiserum appears to be specific for the fimbriae also recognized by U.S. Reference Factor 6 antiserum (11, 24; Brennan et al., in press).

Several lines of evidence indicate that the 69-kDa protein is not a fimbrial antigen. The immunizing *B. pertussis* strain, BP353, is nonfimbriated, as determined by electron microscopy and lack of interaction with fimbria-specific antiserum. The 69-kDa antigen was localized to the outer membranes of both this strain and the fimbriated strain 432 (serotype 1.3.6) by immunogold electron microscopy, and the distribution of bound gold particles on the 432 cells contrasted with the pattern observed when an anti-fimbrial monoclonal antibody was used. Also, monoclonal antibody BPE3 did not bind to purified type 2 or type 6 fimbriae and, conversely, monoclonal antibodies specific for these fimbriae did not recognize the 69-kDa protein. In addition, the estimated molecular mass of 69 kDa is not typical of the subunit molecular weight of fimbrial proteins present on gram-negative organisms (4, 9, 13, 31). These findings indicate that the 69-kDa antigen is not related to the serotype 2 or 6 fimbrial agglutinin of *B. pertussis*. Our electron microscopy studies do not rule out the possibility that the 69-kDa antigen is associated with outer membrane sites from which fimbriae, if present, may protrude. Further studies are required to determine whether it is a fimbria-associated antigen such as those recently observed on other gram-negative organisms (13, 17).

The electrophoretic and staining properties of the 69-kDa band, as well as its sensitivity to trypsin, indicates that it is a protein. Minor, faster migrating bands reactive with the monoclonal antibodies were often observed, suggesting that it is sensitive to proteolytic degradation. This may account for the appearance of a doublet band sometimes observed on

SDS-PAGE. It is apparently an extrinsic outer membrane constituent, since it can be easily released from the surface of the bacteria by heating the cells for 60 min at 60°C. Also, it is a major constituent of bacterial preparations produced by mechanical shearing or by extracting the cells with 4 M urea (Brennan et al., unpublished data). Its electrophoretic mobility is not altered by heating, which suggests that it is not one of the *B. pertussis* heat-modifiable proteins that have been previously described (2). The 69-kDa component was purified by affinity chromatography from extracts of *B. pertussis* BP353 and the nonagglutinating strains 114 and 165, and no apparent differences in electrophoretic mobility or immunologic reactivity were observed. Differences in migration on SDS-polyacrylamide gels were observed for the antigenically reactive bands found in extracts of *B. bronchiseptica* and *B. parapertussis* cells, which suggests that this protein is slightly different in these closely related *Bordetella* species.

The antigen defined here by the three anti-*B. pertussis* monoclonal antibodies was found to be antigenically similar to a protein first identified on *B. bronchiseptica* cells by a monoclonal antibody, BB05, raised against a *B. bronchiseptica* vaccine (18). Monoclonal antibody BB05 recognizes an antigen on *B. pertussis* and *B. parapertussis*, as does BPE3, although slight differences in the number and sizes of the bands recognized by these antibodies were observed on Western blots. Also, BB05 binds to the surface of *B. parapertussis* cells (18), whereas there is no evidence that BPE3 binds to these cells. BPE3 and BB05 recognize different epitopes, as determined by competitive inhibition studies (P. Novotny, unpublished data), which may account for these findings.

Previous evidence suggests that the 69-kDa protein is associated with the adenylate cyclase activity of *Bordetella* species (20), and we have observed that all strains which produce adenylate cyclase also express the 69-kDa protein. However, they apparently are separate gene products, since transposon Tn5 insertion mutant BP348, which is defective in adenylate cyclase activity (29), does contain the 69-kDa protein (Brennan et al., unpublished data). The monoclonal antibodies described here should be useful for investigating the relationship of the 69-kDa protein with the *Bordetella* adenylate cyclase toxin. There is evidence that this protein is also a protective antigen in animal models (21, 22); therefore, the monoclonal antibodies can be used in passive immunization studies to further assess its role as a protective antigen. The antibodies should also be useful for further structural and functional analysis of this protein and for establishing a better-defined serotyping system for *B. pertussis*. The finding that this protein can stimulate the production of agglutinating antibodies and the fact that it is a surface protein found on all virulent strains of *B. pertussis* suggest that it should be considered as a prospective acellular vaccine candidate.

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