INFECTION AND IMMUNITY, Apr. 1989, p. 1240–1247
0019-9567/89/041240-08$02.00/0

Vol. 57, No. 4

Bordetella parapertussis Invasion of HeLa 229 Cells and Human Respiratory Epithelial Cells in Primary Culture

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Received 3 November 1988/Accepted 9 January 1989

***Bordetella parapertussis*, a respiratory tract pathogen commonly regarded as noninvasive, was found to invade HeLa 229 cell monolayers. Following treatment of the monolayers with gentamicin, numbers of viable *B. parapertussis* recovered were comparable to those of invasive *Salmonella* and *Shigella* isolates. Invasion occurs through a cytochalasin-sensitive process which appears to be distinct from receptor-mediated endocytosis. Hyperimmune antisera raised against filamentous hemagglutinin, a major adhesin of *B. pertussis*, did not inhibit invasion by *B. parapertussis*, suggesting that alternate adhesin(s) are required for invasion. In addition, *B. parapertussis* was found to invade human respiratory epithelial cells in primary culture, as demonstrated in ultrathin sections viewed by transmission electron microscopy. Although viable intracellular *B. parapertussis* persist within HeLa cells, they do not multiply there and the monolayers remain intact, suggesting a possible mechanism of carriage for these organisms.**

Within the genus *Bordetella*, the species *Bordetella pertussis* is most frequently associated with cases of whooping cough. This distinction is afforded by the ability of virulent (phase 1) organisms to synthesize a number of unique virulence determinants associated with pathogenesis. Pertussis toxin, which is cytotoxic and probably responsible for numerous systemic manifestations of whooping cough, is a major virulence determinant of *B. pertussis* that is not shared by *B. parapertussis* (15, 20). Despite the lack of pertussis toxin, *B. parapertussis* is also capable of causing disease in humans. Although most infections caused by *B. parapertussis* are mild, cases of severe infections involving fatal bronchopneumonia have been reported (14, 29). As noted by Linneman (14), the actual frequency of infection with *B. parapertussis* is difficult to assess owing to discrepancies that exist among the limited number of available studies. Linneman favors serologic surveys over culture surveys for purposes of quantitating frequency of *B. parapertussis* infection because serologic surveys can detect asymptomatic as well as symptomatic infections. Serology reveals a very high frequency of *B. parapertussis* infection, with specific antibodies ranging from 40% in a population of children (17) to 91% in one study of adults (8).

Humans appear to be the sole reservoir for *B. pertussis* and *B. parapertussis*; however, bacteriologic surveys have failed to identify a carrier state in pertussis (10, 13). This poses a problem in attempting to determine the source of infection in isolated cases in which there is no obvious contact with symptomatic individuals. We wondered whether the absence of evidence of carriage was linked to results indicating that *B. pertussis* survives within alveolar macrophages (3) and HeLa 229 cells (4). Thus, we decided to examine *B. parapertussis* for invasive behavior. Using HeLa 229 cell monolayers and primary cultures of human respiratory epithelium, we demonstrated the ability of limited numbers of *B. parapertussis* to invade such cells through a cytochalasin-sensitive endocytic process. Viable counts and transmission electron microscopic (TEM) examination of infected monolayers were used to obtain these results. Polyclonal anti-*B. parapertussis* and anti-*B. pertussis* antisera reduced levels of invasion to 16.4% and 27.6% those of controls, respectively, whereas antisera raised against filamentous hemagglutinin (FHA) was not found to neutralize *B. parapertussis* invasion. This suggested the presence of alternate cell surface components required for entry of *B. parapertussis* into eucaryotic cells.

MATERIALS AND METHODS

Bacterial strains. *B. parapertussis* 17903 was originally obtained from the Michigan Department of Public Health, Grand Rapids. Organisms were routinely passaged on Bordet-Gengou agar containing 15% sheep blood (BGA). Positive control strains for invasion included fresh clinical isolates of *Shigella flexneri* and *Salmonella hadar*, which were obtained from the University of Alberta Hospitals microbiology laboratory. *Yersinia pseudotuberculosis* type 1A and *Yersinia enterocolitica* serotype O:3 strain E2549 were provided by the Enteric Division of the Provincial Laboratory of Public Health, Edmonton, Alberta. Virulence of *Shigella flexneri* mediated by chromosomal and plasmid-encoded determinants was ascertained on the basis of the presence of an O side chain on lipopolysaccharide, as seen in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels visualized by the silver staining technique of Tsai and Frasch (23), and the dye-binding ability on Congo red agar (5), respectively. Frozen stock cultures were passaged once only on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) prior to use in invasion assays in order to minimize reduction in virulence due to plasmid loss. Negative invasion controls included *Escherichia coli* 10418 (type 1 pili), 10407 P^- (nonpiliated variant obtained from the laboratory of Glen Armstrong, University of Alberta), and SA 1377 (rough lipopolysaccharide mutant obtained from K. Sanderson, University of Calgary), passaged routinely on trypticase soy agar.

Human respiratory epithelial tissue. Following surgical removal, nasal turbinates were washed in physiological saline to remove excess blood. Samples were placed in Eagle minimal essential medium (MEM) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with penicillin (50 μg/

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ml), streptomycin (50 μg/ml), gentamicin (50 μg/ml), and 0.1% protease (type XIV; Sigma Chemical Co., St. Louis, Mo.). After 20 to 24 h of treatment with cold protease at 4°C, mechanical agitation was used to free epithelial cells from the specimen. Ten percent (vol/vol) fetal bovine serum (FBS) was added to neutralize the protease. The detached cells were filtered through a 60-μm-pore-size Vitex mesh, centrifuged at 150 to 200 × *g* for 10 min, and washed once with MEM plus 10% FBS and once in MEM plus 2% FBS prior to being placed in plastic culture dishes on glass cover slips that has been coated with a collagen gel; collagen gels were prepared from rat tail collagen (type VII, Sigma) by the method of Yang et al. (28). A cell density of 10,000 cells per cm^2 was used. Confluence was achieved in 3 to 4 days.

HeLa cell culture methods and invasion assays. The established HeLa 229 human epithelium-like cell line (ATCC CCL 2.1) was maintained in MEM containing 5% FBS (GIBCO) without antibiotics in an atmosphere of 5% CO_2. Confluent stock monolayers were used to seed 25-cm^2 tissue culture flasks (Corning Glass Works, Corning, N.Y.) at a concentration of 9×10^5 cells per flask. Following overnight incubation, the semiconfluent monolayers were infected with organisms, which had been resuspended in MEM-FBS to an optical density of 0.15 at 540 nm and diluted to obtain an appropriate multiplicity of infection (MOI). MOIs were confirmed retrospectively for each experiment by determining the number of viable bacteria in the inocula. Organisms and HeLa cells were coincubated for 5 h, washed twice to remove unbound or loosely bound bacteria, and then reincubated for an additional 2 h in the presence of 100 μg of gentamicin per ml (GIBCO) to inactivate the remaining extracellular organisms. Viable intracellular organisms were recovered from trypsinized monolayers, followed by lysis in distilled water and sedimentation at 7,700 × *g*, and enumerated by plating appropriate dilutions in triplicate onto freshly prepared Trypticase soy agar.

Alteration of HeLa cells. Effects of cytochalasins on internalization were determined by preincubation of HeLa cell monolayers with cytochalasins B and D (Sigma) at concentrations of 1.0 and 2.5 μg/ml, respectively, containing final concentrations of dimethyl sulfoxide of 0.2 and 0.5%, respectively. Control monolayers were preincubated with 0.5% dimethyl sulfoxide. Viability of cytochalasin-treated monolayers compared with that of controls was determined by trypan blue exclusion following a total incubation time of 8 h. Monolayers were similarly treated with monodansylcadaverine (MDC, Sigma), solubilized in MEM by the addition of 1 N HCl, and adjusted to concentrations of 100, 200, and 300 μM in MEM-FBS prior to infection. Invasion assays in the presence of inhibitors were performed as described above.

Effect of antibody on internalization. The anti-FHA-purified immunoglobulin (IgG) fraction of goat anti-FHA used was kindly provided by Jim Cowell and Michael Brennan, Division of Bacterial Products, Office of Biologics Research and Review, Center for Drugs and Biologics, Food and Drug Administration, Bethesda, Md. This antiserum was used under the same conditions in which it has been previously shown to inhibit adherence of phase 1 *B. pertussis* Tohama to WiDr cells, an epithelium-like cell line derived from a human intestinal carcinoma (25). The IgG fraction was diluted with Hanks balanced salt solution (HBSS) containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2) (HBSS-HEPES) and added to suspensions of *B. parapertussis* 17903 (approximately 10^9 bacteria per ml) to obtain final protein concentrations of 0.25 and 0.50 mg/ml. Control organisms were incubated in HBSS-HEPES with normal rabbit serum added to a final serum protein concentration of 0.50 mg/ml. After incubation at 37°C for 60 min, bacteria were diluted in MEM-FBS and used in invasion assays as described above. Rabbit anti-*B. parapertussis* and anti-*B. pertussis* antisera, obtained by repeated intramuscular injections of *B. parapertussis* 17903 and *B. pertussis* 2231, were provided by Jack Munoz. Monovalent Fab fragments were isolated from purified IgG fractions following overnight incubation with papain (Boehringer Mannheim, Dorval, Quebec, Canada) by passage through protein A-Sepharose CL-4B (Sigma) to remove Fc fragments. Completion of cleavage by papain was confirmed by SDS-PAGE. Fab fragments were added to suspensions of *B. parapertussis* 17903 diluted in HBSS-HEPES to obtain final antiserum protein concentrations of 0.25 mg/ml and were used as outlined above.

SDS-PAGE and Western blotting (immunoblotting). SDS-PAGE with 10% acrylamide slab gels was performed by the method of Laemmli (12). Bacteria from 3-day-old BGA cultures were suspended in 50 mM Tris–63 mM glutamate–43 mM saline buffer (pH 7.4) to an optical density of 0.12 at 540 nm. A 1.5-ml portion of the bacterial suspension was centrifuged at 8,000 × *g* and suspended in Laemmli digestion buffer with dithiothreitol to a final volume of 50 μl. Samples were boiled, and a 10-μl portion was loaded on each lane. Proteins were electrophoretically transferred to nitrocellulose (1) overnight at 27 V in a 25 mM sodium phosphate buffer, pH 7.4. Membranes were blocked with phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween 20 (PBS-T) for 2 h at 37°C, incubated with goat anti-FHA diluted in PBS-T for 2 h at 37°C, and then washed extensively with PBS. Alkaline phosphatase-conjugated rabbit anti-goat antiserum (Sigma) was diluted in PBS-T and incubated with blots at 37°C for 1 h. After extensive washing with PBS, bands were visualized with the color development reagents 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (Bio-Rad Laboratories, Mississauga, Ontario, Canada), 15 mg/ml in *N',N'*-dimethylformamide, and *p*-Nitro Blue Tetrazolium chloride (Bio-Rad), 30 mg/ml in 70% dimethylformamide.

TEM. HeLa cell monolayers (non-contact-inhibited HeLa 229 cell monolayers) were infected with *B. parapertussis* 17903 at an MOI of approximately 1, confirmed retrospectively by viable counts. At intervals of 24, 48, and 72 h, infected and uninfected control monolayers were washed twice with a buffered EDTA solution, fixed in situ with 3.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, at 4°C for 1 h, washed twice in cold cacodylate buffer for 15 min each time, and then fixed with 1% OsO_4 in cacodylate buffer for 1 h and recovered by mechanical disruption. The cells were then washed twice for 15 min each time with buffer, dehydrated with a graded series of alcohols, and transferred to propylene oxide for 30 min and finally to a 1:1 mixture of propylene oxide and LX112 embedding monomer for 24 h in an unstoppered container. This mixture was then replaced with undiluted LX112 embedding monomer and cured at 60°C for 24 h. Sections of embedded cells (<50 nm thick) were examined with a model 300 Philips transmission electron microscope or a model 410 microscope equipped with a goniometer stage.

Confluent human respiratory epithelium monolayers cultured on collagen gel-coated glass cover slips were initially infected with *B. parapertussis* 17903 at MOIs of 1 and 10. Following an 18-h coincubation period, monolayers were washed twice, fixed in situ with 3.5% glutaraldehyde in PBS

TABLE 1. Comparison of uptake of invasive and noninvasive bacterial strains by HeLa 229 cell monolayers[a]

Strain	CFU/monolayer (10^5)
Y. enterocolitica O:3 E2549	490.7 ± 15.3
Y. pseudotuberculosis type 1A	89.8 ± 9.7
Salmonella hadar	25.9 ± 6.1
Shigella flexneri[b]	14.8 ± 0.3
B. parapertussis 17903	24.3 ± 2.3
E. coli 10418[c]	1.1 ± 0.8
E. coli 10407[d]	0.3 ± 0.1
E. coli SA 1377[e]	1.2 ± 0.7

[a] HeLa 229 cell monolayers (ca. 1.5×10^6 cells per 25-cm^2 flask) were infected with 1.5×10^8 (*B. parapertussis*) or 1.5×10^7 (all other strains) organisms. Data represent the mean number of CFU per monolayer ± the SD of triplicate independent determinations.

[b] Strain contains the 140-megadalton plasmid and the lipopolysaccharide O side chain required for virulence.

[c] Highly piliated strain.

[d] Nonpiliated variant.

[e] Rough lipopolysaccharide mutant, chemotype Re.

(pH 7.3) overnight at 4°C, washed twice in PBS, and fixed with 1% OsO_4 in PBS for 1 h. Intact monolayers were washed twice in PBS, dehydrated, embedded, sectioned, and examined as described above.

Indirect immunofluorescence detection of FHA. Surface-exposed FHA on methanol-fixed organisms was labeled for fluoresence visualization by sequential incubation with goat anti-FHA, rabbit anti-goat IgG (Sigma), and goat anti-rabbit IgG fluorescein isothiocyanate conjugate (Sigma).

RESULTS

Uptake comparisons. The relative numbers of *B. parapertussis* organisms internalized by HeLa 229 cell monolayers were compared with numbers control invasive and noninvasive genera internalized (Table 1). Significantly fewer *B. parapertussis* invaded monolayers compared with *Y. enterocolitica* serotype O:3 and *Y. pseudotuberculosis* type 1A; however, values for *B. parapertussis* were similar to those for the other tested invasive organisms, *Salmonella hadar* and *Shigella flexneri*. No obvious effects on monolayer integrity caused by invasive strains were apparent following the 5-h coincubation period. Despite efficient binding of the type 1 piliated *E. coli* control strains, 10418 and SA 1377, to HeLa cell monolayers on the basis of light microscopy, the recovery of viable organisms was markedly lower than the recovery of *B. parapertussis*. Lower still was the recovery of the nonpiliated negative *E. coli* control strain, 10407.

In separate experiments, exposure to gentamicin at a concentration of 100 μg/ml for 2 h was found to inactivate 99.99% of an initial concentration of *B. parapertussis* of 10^{10}/ml (data not shown). Furthermore, MIC determinations on inoculum *B. parapertussis* organisms and on organisms recovered from gentamicin-treated monolayers demonstrated identical sensitivities, indicating that viable organisms recovered after antibiotic treatment did not represent a resistant population selected by gentamicin. Viable intracellular *B. parapertussis* organisms could be recovered from monolayers incubated in the continued presence of gentamicin for at least 10 days postinfection.

TEM examination of infected HeLa cell monolayers. The data presented above, suggesting the intracellular presence of viable *B. parapertussis*, were confirmed by TEM examination of infected HeLa cell monolayers. Sparsely seeded HeLa cell monolayers were initially infected with *B. parapertussis* at an MOI of 0.1 and coincubated for intervals of 24, 48, and 72 h postinfection. At each interval, duplicate monolayers were either fixed and processed for microscopic examination or treated with gentamicin for quantitation of viable intracellular bacteria. At 24 h postinfection, random examination of ultrathin sections prepared from these monolayers demonstrated very few intracellular or extracellular adherent organisms. By 48 h postinfection, numerous adherent extracellular organisms were present, although few were observed to be free within the surrounding media. TEM examination revealed a moderate number of intracellular organisms within tight endocytic vacuoles (Fig. 1A and B), without obvious evidence of phagosome-lysosome fusion. In some instances, organisms appeared to be free within the cytoplasm; however, when examined later by using a tilting goniometer stage apparatus, endocytic vacuoles in contact with the outer membranes of most of these organisms were observed. Numbers of viable intracellular bacteria per cell remained relatively constant. Heavy infection of monolayers occurred by 72 h postinfection. TEM examination demonstrated the presence of large numbers of adherent bacteria per cell, with a similar increase in the numbers of intracellular organisms (Fig. 1C). Most bacteria occurred singly within tight endocytic vacuoles; vacuoles containing two or more bacteria (some undergoing fission) were occasionally seen.

In order to facilitate visualization of the entry process, we coincubated HeLa cell monolayers and *B. parapertussis* at a higher initial MOI (10) for a 24-h period. TEM examination demonstrated organisms in various stages of internalization (Fig. 2). In Fig. 2, organisms are entering HeLa cells through an endocytic process, embraced by outstretched microvilli of the HeLa cell which ultimately met and fused, reforming the continuous plasma membrane of the cell while directing the bacterium into the resultant phagosome. Although numerous structures resembling clathrin-coated pits were observed on the cytoplasmic surface of plasma membranes, none were observed to be in association with membrane regions involved with bacteria in the process of being internalized.

Alteration of HeLa cells. The TEM results suggested that receptor-mediated endocytosis did not contribute to entry of *B. parapertussis*. This interpretation was supported by the use of monodansylcadaverine (MDC), a known inhibitor of this process (22). Monolayers of HeLa cells were preincubated for 1 h with MDC at concentrations of 100 to 400 μM. The monolayers were subsequently challenged with *B. parapertussis* in the continued presence of MDC by using the standard invasion assay. No significant reduction in numbers of intracellular viable organisms was observed, further suggesting that receptor-mediated endocytosis is probably not involved in uptake of *B. parapertussis* into HeLa cells (data not shown).

The role of the host cytoskeleton in internalization of *B. parapertussis* was evaluated by using cytochalasins B and D, inhibitors that disrupt microfilament function and prevent particle phagocytosis. The protocol used in the standard invasion assay was followed after monolayers were preincubated for 1 h in the presence of cytochalasin B or D at a concentration of 2.5 or 1.0 μg/ml. Owing to the reversibility of their actions, the cytochalasins were present in the medium throughout all assays. HeLa cells treated for 8 h with cytochalasin showed profound morphological changes. The viability of these cells was unchanged, however, when compared with that of untreated cells by trypan blue exclusion. Exposure of monolayers to cytochalasin D resulted in

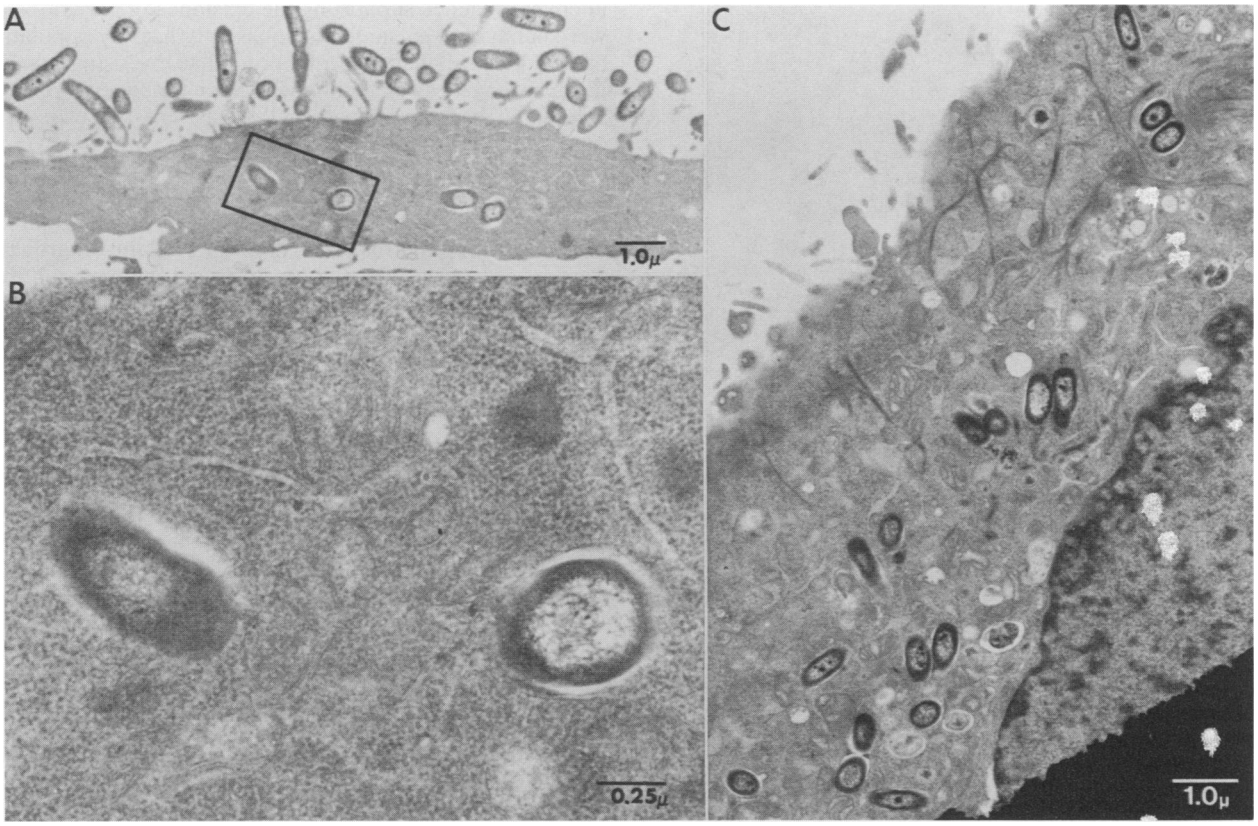

FIG. 1. Transmission electron micrographs of intracellular *B. parapertussis* 17903 within HeLa 229 cell monolayers. Monolayers were sectioned 48 h (A and B) or 72 h postinfection (C). Intracellular bacteria are bound by tight endocytic vacuoles.

a dramatic reduction in the numbers of organisms internalized. Expressed as a percentage of untreated controls, invasion was reduced to 0.7 ± 0.4% for cytochalasin D at a concentration of 2.5 μg/ml and to 1.0 ± 0.4% at 1.0 μg/ml (mean ± standard deviation [SD] of 4 independent determinations). A similar reduction, although less pronounced, occurs following treatment of monolayers with cytochalasin B. Here invasion was reduced to 16.2 ± 7.7% in the presence of 2.5 μg/ml cytochalasin B and 17.6 ± 5.2% in 1.0 μg/ml (mean ± SD of 4 independent determinations). In separate experiments, exposure to either cytochalasin (2.5 μg/ml) for a 5-h period was shown to have no effect on viability of *B. parapertussis* (data not shown).

Effect of antisera to surface structures on invasion. We used hyperimmune serum raised against FHA, a putative ligand of *B. pertussis* that mediates adhesion of these bacteria to a variety of cell types (including HeLa), to determine whether these antibodies could inhibit or reduce invasion of *B. parapertussis* by preventing initial attachment. This preparation has been previously shown to inhibit adhesion of phase 1 *B. pertussis* Tohama to WiDr cells (25). We used this antiserum under conditions identical to those described by Urisu et al. (25) in conjunction with WiDr cells. Goat anti-FHA diluted to 1/1,000 (the highest dilution tested) was first shown to recognize FHA in both phase 1 *B. pertussis* 3779^+ and *B. parapertussis* 17903 in Western blots probed with alkaline phosphatase-conjugated rabbit anti-goat immunoglobulins. It was interesting that initial attempts to demonstrate antibody specificity by using ^{125}I-protein A as a probe failed owing to the relatively inefficient binding of protein A to caprine IgG (9). Accessibility of anti-FHA to native FHA exposed on the surface of *B. parapertussis* was also confirmed by indirect immunofluorescence. Having confirmed the ability of this antiserum to bind FHA in a *Bordetella* sp. other than that against which it was raised, we sought to determine whether its presence could protect HeLa cells from invasion by *B. parapertussis* under standard assay conditions. No significant difference in invasive capacity of organisms preincubated with anti-FHA at final concentrations of 0.25 and 0.50 mg of IgG per ml was demonstrated compared with untreated controls. *B. parapertussis* pretreated with anti-FHA at a final protein concentration of 0.25 mg/ml invaded 101.1 ± 7.1% of untreated controls and invaded 104.4 ± 5.2% of cells after it was pretreated with immunoglobulin at a concentration of 0.50 mg/ml (mean ± SD of 4 independent determinations). Preincubation of *B. parapertussis* 17903 with monovalent Fab fragments isolated from polyclonal antiserum raised against this strain at a protein concentration of 0.25 mg/ml resulted in a marked decrease in invasion to 16.4 ± 3.6% of untreated control organisms (mean ± SD of 6 independent determinations). A similar effect was observed with monovalent Fab fragments isolated from antiserum raised against *B. pertussis* 2231; invasion was reduced to 27.6 ± 8.4% of that of control values (mean ± SD of 7 independent determinations).

Demonstration of *B. parapertussis* invasion of human respiratory epithelium in primary culture. Human respiratory epithelial cells were used as a more relevant cell line for testing the ability of *B. parapertussis* to invade human cells. Human respiratory epithelial cells were obtained from proteolytically disrupted nasopharyngeal turbinate tissue. Organisms were added to confluent monolayers at initial MOIs

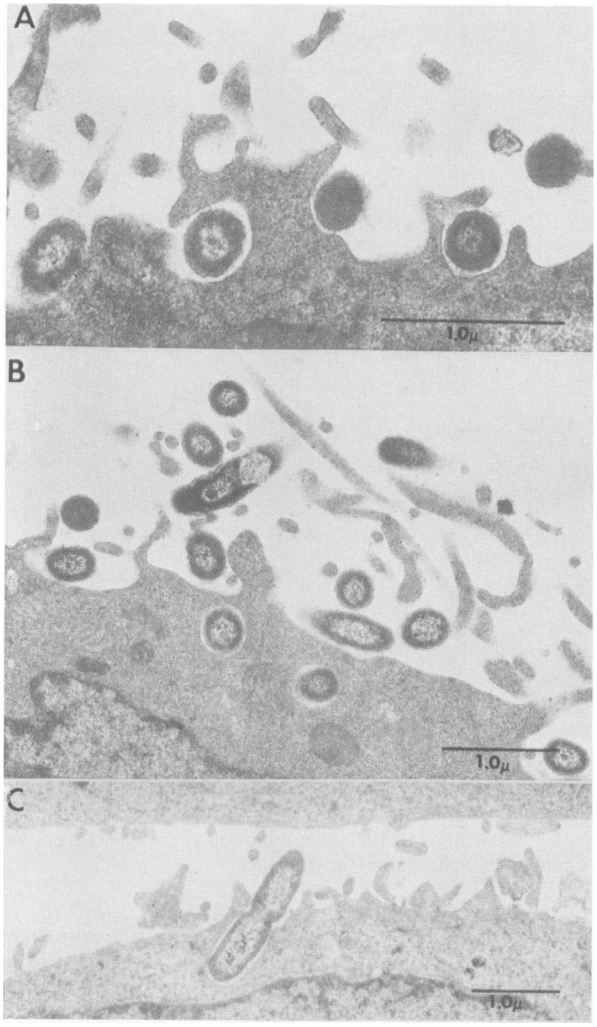

FIG. 2. Transmission electron micrographs of HeLa 229 cell monolayers infected with *B. parapertussis* 17903. The three panels depict bacteria in various stages of internalization.

of 0.1 and 1 and incubated for 24 h. Viable count determinations following the 24-h incubation period indicated an increase in MOIs to 10 and 100, respectively. Following an extensive washing, intact monolayers were fixed in situ with 3.5% glutaraldehyde, processed, and sectioned laterally from the apical face of the monolayer. TEM examination of ultrathin sections revealed moderate numbers of organisms within the cytoplasm of infected cells (Fig. 3). As were those visible in sections of infected HeLa cells, intracellular organisms appeared to be bound by tight endocytic vacuoles.

DISCUSSION

Humans are the sole reservoir for transmission of *B. pertussis* and *B. parapertussis*. Implementation of extensive pertussis immunization programs has dramatically reduced the worldwide morbidity and mortality (2, 19, 26). Apart from rare cases involving inadequately immunized or unimmunized individuals, eradication of pertussis seemed a realistic objective. The apparent lack of a carrier state (10, 13) further supported the possibility of complete eradication of this disease. Results of bacteriologic surveys, which failed to uncover evidence supporting the existence of a carrier state, were conducted by sampling apparently healthy populations for evidence of carriage of bordetellae by using standard nasopharyngeal swab techniques. Such specimens were considered to be appropriate on the basis of the widespread belief that these organisms are strictly noninvasive and possess a completely extracellular life cycle. However, evidence for the intracellular existence of the pertussis agent was previously reported by two separate investigators. In 1959, Crawford and Fishel (4) first reported the outgrowth of *B. pertussis* from several transformed human cell lines following antibiotic exposure, suggesting intracellular localization in at least one stage of their life cycle. This observation was followed in 1969 by the reported survival of *B. pertussis* within murine alveolar macrophages during the complaisant (steady-state) phase of pertussis, during which viable organisms persist within the host amidst an active immune response (3). Intracellular localization of these organisms may offer an explanation for the inability of investigators to identify a carrier state by using conventional sampling techniques and for disease persistence despite a seemingly effective vaccination program.

The work reported here used HeLa 229 cell monolayers because their ease of manipulation and relatively simple growth requirements facilitated the study of invasiveness of bordetellae. We first examined *B. parapertussis* for invasive behavior because it was much less cytotoxic than *B. pertussis* during prolonged coincubation periods.

In order to define the extent of *B. parapertussis* invasiveness, we compared it with several known invasive and noninvasive control bacterium species. *B. parapertussis* has a 3-h lag phase and a lengthy generation time in MEM (3 h). Thus, an initial 10-fold-higher MOI was used for *B. parapertussis* than for control organisms. Although this appears to be an unfair advantage, the significantly shorter generation time of the control strains resulted in a dramatic increase in their numbers during the 5-h incubation period, resulting in final MOIs at least fivefold greater than that of *B. parapertussis*. Final numbers of viable organisms remaining after gentamicin treatment reflect a combination of attachment, invasion, and intracellular multiplication potential of each strain; therefore, actual rates of invasion cannot be determined. *Bordetella* spp. attach efficiently to HeLa cell monolayers but have a limited capacity for intracellular growth during the assay period. In contrast, both *Salmonella hadar* and *Shigella flexneri* are capable of rapid intracellular multiplication once internalized, although binding efficiencies differ. When these differences are considered, the data indicate that, following a 7-h exposure period, approximately equal numbers of viable *B. parapertussis*, *Salmonella hadar*, and *Shigella flexneri* exist within HeLa cells.

The intracellular existence of *B. parapertussis* was confirmed by TEM examination of infected monolayers. At intervals of 24, 48, and 72 h following infection with *B. parapertussis* at an initial MOI of 1, monolayers were examined for intracellular organisms and relative changes in their numbers. Although intracellular bacterium numbers progressively increased, the process appeared limited, as cells did not contain large numbers of intracellular organisms even at 72 h postinfection. This is supported by viable count data, which indicated that intracellular organisms did not replicate to any appreciable extent. Consistent with these observations, most intracellular bacteria occurred singly within endocytic vacuoles. Although organisms undergoing fission were occasionally observed, these may have been in the process of division during internalization (Fig. 2C).

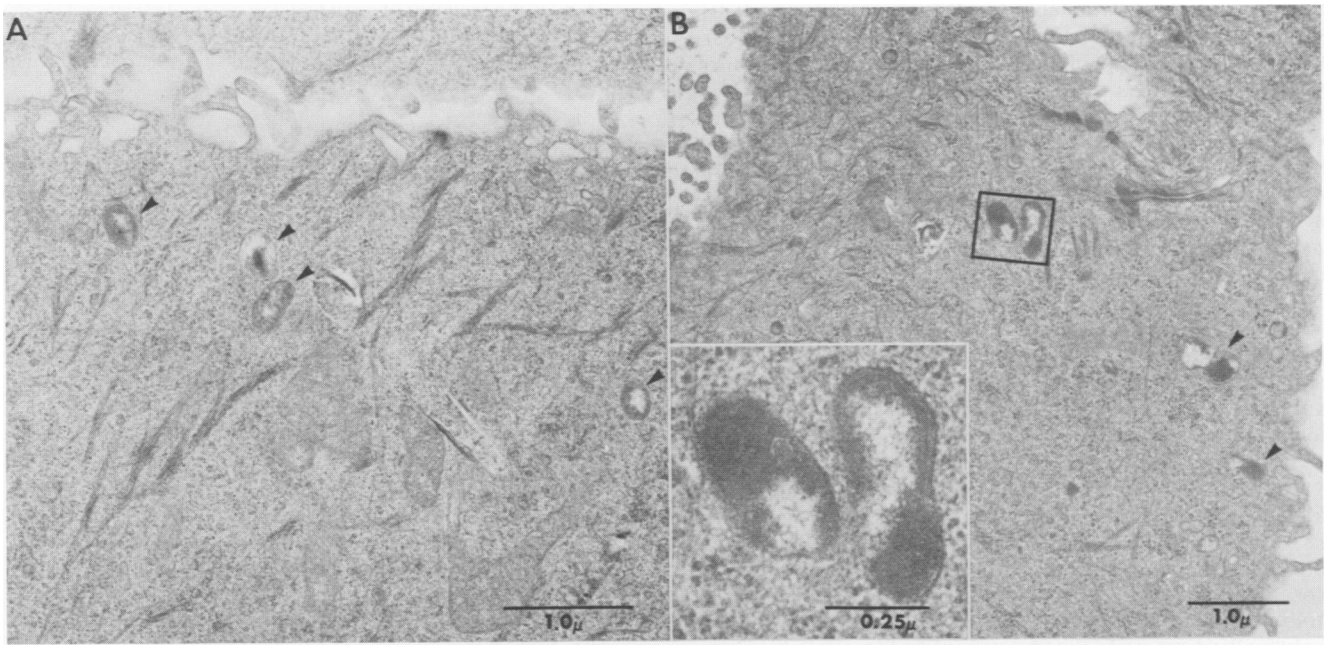

FIG. 3. Transmission electron micrograph of human respiratory epithelial cells in primary culture infected with *B. parapertussis* 17903. Arrowheads indicate intracellular bacteria bound by tight endocytic vacuoles.

The ability of invasive bacteria to self-regulate their intracellular numbers would provide the organisms with a definite survival advantage. This is especially true for *B. parapertussis*, which produces a number of potentially cytotoxic substances. Fewer intracellular organisms would minimize disruption of the host cell, thereby providing for these organisms shelter from the active immune response occurring in the extracellular environment. Alternatively, phase transition or phenotypic modulation to an avirulent phenotype, as described for *B. pertussis* (11, 26), may follow internalization of *B. parapertussis* into an intracellular environment. Once established within the host cell, a complete or partial loss of virulence-associated determinants would reduce the disruption of host cell metabolism caused by an intracellular organism, thereby potentiating its quiescent masquerade.

Bacterial "invasion" of nonprofessional phagocytes is probably accomplished by an endocytic process (7). In this study, we examined the mechanism of *B. parapertussis* invasion with the assumption that a similar process would be involved. Receptor-mediated endocytosis (RME) is a common mechanism of internalization of several types of viruses which enter eucaryotic cells during normal RME-mediated uptake of physiologically important external proteins (16, 18). We did not anticipate a role for RME in uptake of *B. parapertussis* owing to the absence of characteristic coated pits observed in association with *B. parapertussis* endocytic vesicles and to the large size of bacteria in comparison to clathrin-coated pits. Since the width of the bacteria is similar to that of chlamydia (0.3 μm), it was possible that RME could have been involved in an "end-on" internalization mechanism similar to that evident in Fig. 2C. To investigate this possibility, we examined the effects of an RME inhibitor on uptake of *B. parapertussis*. Primary amines, such as MDC, inhibit RME-mediated uptake of virus particles (22), presumably by inactivation of Ca^{2+}-dependent transglutaminases, which normally stabilize protein ligand-receptor clustering in coated pits through covalent cross-bridge formation (6). As expected, pretreatment of HeLa cells with MDC had no effect on uptake of *B. parapertussis*, thereby suggesting that RME is probably not a significant route of entry. However, further experimentation with other inhibitors of RME, such as amantidine (22), could be performed to further discount this possibility.

A microfilament-dependent endocytic process did appear to be involved, however, because adherent bacteria in the process of entry were usually observed to be circumscribed by outstretched microvilli. Pretreatment of HeLa cell monolayers with the microfilament inhibitors cytochalasins B and D markedly inhibited *B. parapertussis* uptake. This reduction was especially pronounced in the presence of cytochalasin D, the more potent microfilament inhibitor of the two cytochalasins tested. These data indicate that uptake of *B. parapertussis* proceeds via an endocytic process, possibly as a result of a bacterium-host cell interaction that stimulates uptake of the bacterium (i.e., parasite-specified endocytosis).

Recognition and adhesion are logical prerequisites to endocytosis by nonphagocytic eucaryotic cells. For example, the act of attachment of a bacterium to its corresponding cell surface receptor could be an important stimulus which initiates endocytosis by the host cell. We attempted to inhibit endocytosis of *B. parapertussis* by pretreatment with hyperimmune serum raised against FHA, the putative major adhesin of *B. pertussis* (21, 24). *B. pertussis* and *B. parapertussis* possess forms of FHA which are similar in morphology, hemagglutinating ability, and antigenic specificity; thus, FHA is the presumed ligand of *B. parapertussis*, even though the actual adhesin of this species has never been formally identified. Our data demonstrated that invasion is unaffected by high concentrations of anti-FHA. This suggests that adherence of these organisms is mediated by a ligand(s) in addition to FHA. This was supported by the observation that numerous adherent bacteria were observed in Giemsa-stained HeLa cell monolayers infected with anti-FHA-treated organisms. These data may be especially sig-

nificant with respect to Japanese B-type acellular pertussis vaccines consisting of FHA and pertussis toxin that are presently undergoing clinical trials. If the currently used whole-cell vaccines containing antigens common to both pertussis and parapertussis fortuitously provide cross-protection against both species, as indicated by the ability of polyclonal anti-pertussis sera to reduce invasion of *B. parapertussis* shown here, exclusion of all antigens except FHA and pertussis toxin that do not protect against *B. parapertussis* invasion may result in a higher incidence of infection and/or disease caused by this organism.

HeLa and other tissue culture cell lines provide simple and convenient in vitro systems in which to investigate the processes of adherence and invasion. However, the pertinence of data obtained from continuous cell culture methods is limited because of important differences between continuous tissue culture cells and the environment of host tissues, such as structural complexity, representative cell receptor types and numbers, and immune modulation. Although it is difficult to closely simulate the milieu in which *B. parapertussis* becomes established in the human lung, it is possible to provide the bacteria with a stratum for invasion that more closely parallels that which is naturally encountered. In this study, human respiratory epithelial cells obtained from proteolytically disrupted nasopharyngeal turbinate tissues were used to represent cell types normally colonized by *B. parapertussis* in the infected host. Monolayers initially infected with *B. parapertussis* at MOIs of 0.1 and 1 were fixed in situ following a 24-h coincubation period and examined by TEM. Ultrathin sections revealed clear evidence of bacterial invasion. Organisms were observed within the cytoplasm of infected cells, again bound by endocytic vacuoles whose limiting membranes appeared to be in close contact with the bacterial cell wall. Host cells and intracellular bacteria both appeared healthy; there was no evidence of death or disruption after the 24-h coincubation period used here. These data provide additional evidence for the invasion potential of *B. parapertussis* indicated in the HeLa cell system and extend it to a more relevant model which more closely resembles the situation naturally encountered by respiratory pathogens.

In summary, our data describe the unusual phenomenon of invasive behavior in a bacterial species that until now was strictly regarded as an extracellular pathogen. *B. parapertussis* appears to be capable of provoking its own uptake through endocytosis by nonphagocytic human cells. As entry without a means of intracellular survival would be suicidal, *B. parapertussis* must also possess a mechanism(s) that thwarts rapid lysis by lysosomal contents following phagosome-lysosome fusion. Clearly, adaptation to an intracellular existence is no simple task and must therefore offer a significant survival and/or pathogenic advantage to the species involved. *B. parapertussis* is no exception. Although perhaps not a pathogenic mechanism, limited invasive behavior would lend a definite survival advantage to this species, whose only host appears to possess a significant incidence of specific humoral immunity. These insights suggest an alternate mechanism of carriage which might also be applicable to other members of the genus *Bordetella*.

ACKNOWLEDGMENTS

We thank James Cowell and Michael Brennan for kindly donating anti-FHA antisera and Stanley Falkow, Brett Finlay, and Glen Armstrong for valuable and constructive criticism of the manuscript.

C.A.E. is the recipient of a studentship grant administered by the Alberta Heritage Foundation for Medical Research. M.S.P. is supported by grant MA 10415 from the Medical Research Council of Canada.

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