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Invasion of HeLa 229 Cells by Virulent *Bordetella pertussis*

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Phase-dependent invasive behavior of *Bordetella pertussis* was demonstrated by recovery of viable organisms from gentamicin-treated HeLa cell monolayers and by transmission electron microscopy. Several mutants of *B. pertussis* with Tn*5* or Tn*5 lac* inserted into various *vir*-regulated genes were evaluated for differences in their invasive abilities. Mutants lacking filamentous hemagglutinin, pertussis toxin, and two as yet uncharacterized *vir*-regulated products had levels of invasion significantly lower than that of the parent strain BP338. In contrast, invasion by mutants lacking adenylate cyclase toxin was significantly increased compared with that of wild-type *B. pertussis*. This increase in invasion was eliminated when concentrations of intracellular cyclic 3′-5′ AMP were stimulated by treating HeLa cells with cholera toxin or forskolin. Entry of *B. pertussis* occurred through a microfilament-dependent phagocytic process, as evidenced by the marked reduction in uptake following treatment of HeLa cells with cytochalasin D. Invasion was inhibited with polyclonal anti-*B. pertussis* and anti-filamentous hemagglutinin antisera. In addition, a monoclonal antibody against lipooligosaccharide A reduced uptake by 65.5%. The preservation of HeLa cell integrity and the limited replication of intracellular bacteria suggest that invasion may represent a means by which *B. pertussis* evades an active host immune response.

Bordetella pertussis is generally thought to be a noninvasive organism which causes disease in its human host through the action of several potent toxins. Classical whooping cough is believed to occur in two main stages (19). Initially, inspired bacilli colonize epithelial surfaces in the upper respiratory tract. Candidate adhesins include filamentous hemagglutinin (FHA) (23, 24), pertussis toxin (23), and fimbriae (10, 20). Once established, pathogenesis is caused by a number of toxins produced by *B. pertussis*. Local cytotoxic effects (9) and systemic metabolic disturbances (8, 17, 18) combine to produce the characteristic symptoms of a disease which is primarily regarded as a toxicosis (19, 21).

Several earlier reports have suggested the possibility that *B. pertussis* may shift to an intracellular location in vitro and in vivo. A report published in 1959 by Crawford and Fishel (5), in which the use of tissue culture was described as a means of propagation of *B. pertussis*, described the outgrowth of viable organisms from antibiotic-treated cell lines which were previously infected with *B. pertussis*. Cheers and Gray (2, 11) later described a complaisant phase of murine pertussis in which an active immune response kept an equilibrium between viable extracellular and intracellular (macrophage) populations. Finally, Hopewell et al. (14) used transmission electron microscopy (TEM) to examine the site of infection of *B. pertussis* in the mouse brain following intracerebral challenge. They observed bacteria between the microvilli and within the cytoplasm of ependymal cells, which line the ventricles of the brain. They also noted that phagocytic activity of ependymal cells (which they likened to ciliated epithelial cells of the respiratory tract) was not previously reported. On the basis of these reports, we decided to critically examine bordetellae for evidence of invasive ability. Such invasive ability was first demonstrated in *Bordetella parapertussis*, a respiratory pathogen which is similar to *B. pertussis* in most aspects except for its lack of pertussis toxin and its lower incidence of infection (6). In this report we demonstrate that invasive behavior in *B. pertussis* is similar and examine the relative contributions of various virulence-associated gene products to *B. pertussis* invasion.

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MATERIALS AND METHODS

Bacterial strains. The transposon insertion derivatives of a nalidixic acid-resistant strain of *B. pertussis* Tohama 1 (BP338) used in this study are listed in Table 1. Tn*5* or Tn*5 lac* insertions in various virulence-regulated genes (denoted by prefixes BP or BPM, respectively) were constructed as described previously (25, 25a). The *Shigella*, *Salmonella*, and *Yersinia* strains have been described previously (6).

Invasion assays. HeLa 229 cells (human epithelium-like; ATCC CCL 2.1) were used in invasion assays as described previously (6), with the following modifications. Tissue culture trays (24-well) were seeded with approximately 7×10^4 cells per well 18 h prior to the assay. Cultures of *B. pertussis* grown for 48 h on Bordet-Gengou agar (BGA) were suspended in Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 3% fetal bovine serum (Flow Laboratories, McLean, Va.) to an optical density at 540 nm of 0.12 in test tubes (13 by 100 mm; path width, 1.0 cm). Approximately 9×10^6 CFU in 0.4 ml was added to each well and incubated at 37°C in 5% CO_2 under static conditions. Monolayers were then washed twice to remove nonadherent bacteria, and the medium was replaced with 0.4 ml of Eagle minimal essential medium-fetal bovine serum containing 100 μg of gentamicin (GIBCO) per ml and incubated at 37°C for 2 h to destroy the remaining extracellular bacteria. After 2 h the residual gentamicin was removed by extensive washing, and monolayers were harvested for quantitation of intracellular CFUs. In separate experiments, treatment of *B. pertussis* strains suspended to a density equivalent to that used in invasion assays with 100

TABLE 1. Strains used in this study[a]

Strain (genotype)	ACT	Hly	FHA	DNT	69-kDa protein	PTX	Source or reference
BP338 (parental)	+	+	+	+	+	+	25
BPM1579 (unknown)[b]	+	+	+	+	+	+	25a
BPM2123 (unknown)	+	+	+	+	+	+	25a
BPM2859 (unknown)	+	+	+	+	+	+	25a
BPM177 (unknown)	+	+	+	+	+	+	25a
BPM245 (unknown)	+	+	+	+	+	+	25a
BPM2055 (unknown)	+	+	+	+	+	+	25a
BPM2119 (unknown)	+	+	+	+	+	+	25a
BPM2041 (unknown)	+	+	+	+	+	+	25a
BPM3171 (unknown)	+	+	+	+	+	+	25a
BPM2375 (pleiotropic)	±	±	±	±	±	±	25a
BPM433 (*adc*-1::Tn*5 lac*)	−	−	+	+	+	+	25a
BP349 (*hly-2*::Tn*5*)	−	−	+	+	+	+	25
BP348 (*hly-1*::Tn*5*)	−	−	+	+	+	+	25
BPM3183 (*adc-2*::Tn*5 lac*)	−	−	+	+	+	+	25a
BPM1809 (*dnt-1*::Tn*5 lac*)	+	+	+	−	+	+	25a
BP357 (*ptx-2*::Tn*5*)	+	+	+	+	+	−	25
BPM1821 (*fhaB-1*::Tn*5 lac*)	+	+	−	+	+	+	25a
BPM409 (*fhaB-1*::Tn*5 lac*)	+	+	−	+	+	+	25a
BP353 (*fha-1*::Tn*5*)	+	+	−	+	+	+	25
BP347 (*vir-1*::Tn*5*)	−	−	−	−	−	−	25
Salmonella hadar[d]							6
Shigella flexneri serotype 2[e]							6
Escherichia coli 10418 (P^+)							6

[a] Abbreviations: ACT, adenylate cyclase toxin activity; Hly, hemolysin activity; FHA, filamentous hemagglutinin determined by hemagglutination and immunoblotting; DNT, dermonecrotic toxin activity assayed in infant mice; 69-kDa protein, *vir*-specific outer membrane, nonfimbrial 69-kDa protein corresponding to agglutinogen 3 defined by monoclonal antibody BB05; PTX, pertussis toxin activity.

[b] Tn*5 lac* mutants with an unknown genotype lacked production of uncharacterized proteins whose expression was virulence regulated.

[c] The strain contained a Tn*5 lac* insertion in a pertussis toxin-linked gene; it expressed functional pertussis toxin.

[d] Clinical isolate, smooth lipopolysaccharide.

[e] Clinical isolate with 140-MDa plasmid and lipopolysaccharide O side chain required for virulence.

μg of gentamicin per ml for 2 h resulted in a 99.998% decrease in CFUs.

Alteration of HeLa cells. In several experiments, HeLa cell monolayers (approx. 7×10^4 cells) were treated with individual reagents for 1 h at 37°C prior to the addition of bacteria as indicated above. Cholera toxin (List Biological Laboratories, Campbell, Calif.) was used at a final concentration of 30 ng/ml. Forskolin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 95% ethanol and used in assays at a concentration of 100 μM. Cytochalasin D (Sigma) was used as described previously (6). Because of the reversibility of the actions of cytochalasin D and forskolin, the concentrations of all reagents were maintained throughout the duration of each assay.

Pretreatment of *B. pertussis* with FHA. Purified FHA was kindly provided by John Vose and Larry Tan (Connaught Laboratories, Willowdale, Ontario, Canada). *B. pertussis* strains grown for 48 h on BGA were suspended in Hanks balanced salt solution containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.2) to an optical density at 540 nm of 0.12. FHA in 54 mM NaH_2PO_4–13 mM K_2PO_4–1 M NaCl (pH 8.0) was added to final concentrations of 25, 50, and 75 μg/ml. Control organisms received no additions. Suspensions were incubated under static conditions for 1 h at 37°C, washed once, suspended in Eagle minimal essential medium to the original volume, and used in invasion assays as described above.

Effect of antibody on internalization. The purified immunoglobulin G (IgG) fraction of goat anti-FHA was kindly provided by Jim Cowell and Michael Brennan (Division of Bacterial Products, Office of Biologics and Research and Review, Center for Drugs and Biologics, Food and Drug Administration, Bethesda, Md.). Polyclonal antisera were used as described previously (6). Monoclonal antibodies purified from ascites fluid directed against *B. pertussis* agglutinogens, lipooligosaccharide A, and a nonfimbrial 69-kilodalton (kDa) surface antigen of BP338 (serotype 1.2.3.4) (1) were kindly provided by Michael Brennan. Bacteria suspended in Hanks balanced salt solution-HEPES as described above were preincubated with antiserum at a final protein concentration of 50 μg/ml for 1 h at 37°C under static conditions, diluted, and used in standard invasion assays.

Cyclic AMP quantitation. Cyclic AMP levels in control and treated HeLa cells were determined 6 h after the addition of cholera toxin (30 ng) or forskolin (100 μM). This interval corresponded to the total time which monolayers were exposed to these reagents in invasion assays. 3-Isobutyl-1-methylxanthine (IBMX; Sigma) was present at a final concentration of 0.5 mM throughout the assays in order to minimize the destruction of cyclic AMP by cellular phosphodiesterases. HeLa cells were scraped from flasks with a rubber policeman, homogenized, and boiled for 3 min to deproteinize the samples and release the cyclic AMP. Available cyclic AMP was quantitated by an assay based on competition for a binding protein with added [^{3}H]cyclic AMP (Amersham Canada Ltd., Oakville, Ontario, Canada).

TEM. HeLa cell monolayers (approx. 7×10^4 cells) were grown for 24 h in 24-well tissue culture trays lined with glass cover slips. Approximately 9×10^6 *B. pertussis* cells from 48-h BGA cultures were added to each monolayer and coincubated for 7 h at 37°C under static conditions. At 7 h postinfection, monolayers were washed and processed for TEM examination as described previously (6).

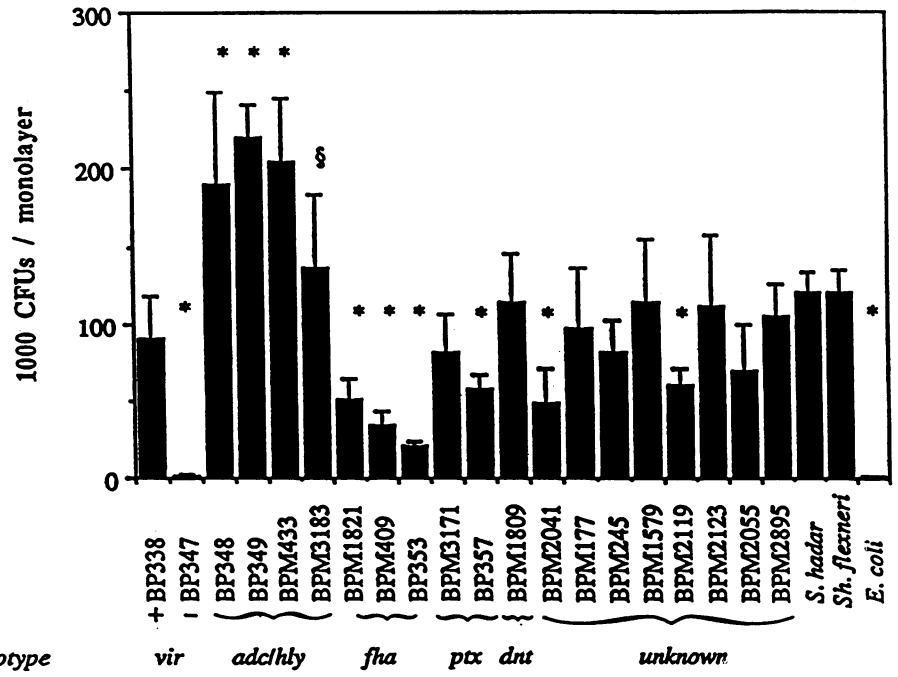

FIG. 1. Invasion of HeLa 229 cell monolayers by *B. pertussis* mutants. Values represent the mean ± standard deviation CFU (in thousands) recovered from gentamicin-treated monolayers for six replicate samples. Symbols: *, statistically significant at $P < 0.05$; §, statistically significant at $P < 0.1$ when compared with the wild type.

RESULTS

Virulence-dependent uptake of *B. pertussis* by HeLa cells. We first compared the numbers of viable intracellular phase 1 *B. pertussis* cells recovered from infected monolayers treated with gentamicin with those of known invasive and noninvasive control strains after a 7-h coincubation period (Fig. 1). The invasion process was time dependent, and theoretically, a shorter interval could have been used. However, our adherence assays required 3 h to reach binding equilibrium. Thus, we empirically decided on a 5-h invasion period to allow for maximal adherence and an additional 2 h for invasion. This interval could have been shortened by prior centrifugation of the bacteria onto the monolayers, but we did not wish to introduce potential artifacts to confuse the interpretation of the data further. After the 7-h invasion interval, the numbers of viable intracellular BP338 cells were comparable to those of invasive clinical isolates of *Shigella flexneri* and *Salmonella hadar*. Expression of virulence determinants in *B. pertussis* is coordinately regulated by an activator encoded by a gene known as *vir*. Mutations within this gene abolish expression of all virulence factors encoded by *vir*-regulated genes. Recovery of avirulent Tn*5* insertion mutant BP347 was comparable to that of noninvasive *Escherichia coli* strains, indicating that invasion by *B. pertussis* is dependent on the expression of one or more *vir*-regulated genes. In order to determine which of the numerous *vir*-regulated genes of *B. pertussis* was required for invasion, we compared the relative invasiveness of a number of Tn*5* and Tn*5* *lac* insertion mutants derived from parental strain BP338 (Fig. 1). Each of these mutants lacked one or more virulence-associated proteins, some of which have not yet been characterized.

Two virulence determinants appeared to be required for maximal internalization. The invasion levels of *B. pertussis* strains which lacked only FHA (BP353, BPM409, and BPM1821) were less than that of BP338 (FHA^+) but were significantly higher than that of avirulent BP347, which was also devoid of FHA. The Tn*5* mutant BP357 was deficient in functional pertussis toxin production and also demonstrated a significant reduction in invasion compared with BP338. Invasion of BPM3171, which produced functional pertussis toxin despite a transposon insertion into a pertussis toxin-linked gene, was not significantly reduced compared with that of the wild-type strain.

Another factor was found to influence invasion in an unexpected way. Surprisingly, invasion of *B. pertussis* strains lacking both secreted adenylate cyclase and hemolysin (BP348, BP349, BPM433, and BPM3183) exceeded that of parental strain BP338 by up to twofold.

Two mutants with Tn*5* *lac* insertions in undefined *vir* genes (BPM2119 and BPM2041) also demonstrated significant reductions in invasion compared with the wild type ($P < 0.05$). Approximate *t*-test *P* values indicating significance between values for parent strain BP338 and several strains whose variances were heterogeneous were determined (22) (see below). Adherence of strains BPM2119 and BPM2041 to HeLa cells was not significantly reduced compared with that of the wild type. Similarly, ^{125}I-labeled surface-exposed protein profiles of these strains were identical to that of BP338 (data not shown). This indicates that these strains may lack expression of a protein that is required for invasion itself or subsequent intracellular survival.

Effect of cyclic AMP on invasion. The enhanced invasiveness of *B. pertussis* mutants lacking adenylate cyclase toxin led us to question the influence of cyclic AMP on invasion by *B. pertussis*. Intracellular levels of cyclic AMP in HeLa cells were increased by prior incubation of HeLa cells with either cholera toxin or forskolin. The treated cells were then infected with *B. pertussis* mutants lacking adenylate cyclase toxin and hemolysin to determine the effect of elevated

TABLE 2. Effect of cholera toxin and forskolin on invasion of HeLa 229 cells by *B. pertussis* adenylate cyclase toxin mutants

Strain (phenotype)	Effect of[a]:		
	Control	Cholera toxin (30 ng/ml)[b]	Forskolin (100 μM)[b]
BP338 (parental)	109.7 ± 25.5	98.2 ± 12.4	117.4 ± 34.9
BPM433 (ACT⁻ Hly⁻)	212.5 ± 32.5	94.5 ± 10.5	133.2 ± 17.8
BPM3183 (ACT⁻ Hly⁻)	173.1 ± 28.8	102.8 ± 5.2	101.9 ± 14.6
BPM348 (ACT⁻ Hly⁻)	213.2 ± 12.0	97.3 ± 17.1	113.1 ± 4.1
BPM349 (ACT⁻ Hly⁻)	194.1 ± 11.3	108.5 ± 10.4	118.4 ± 7.5
BP347 (Vir⁻)	2.2 ± 1.0	1.4 ± 0.6	1.6 ± 1.1

[a] Data represent the mean ± standard deviation CFU per monolayer (in thousands) for three independent determinations. Invasion of each of the ACT mutants was significantly reduced ($P < 0.005$) in the presence of either cholera toxin or forskolin.

[b] Cholera toxin and forskolin were preincubated with monolayers for 1 h prior to the addition of *B. pertussis* in order to stimulate an increase in cyclic AMP. Because of the reversible effect of forskolin, the indicated concentrations were maintained throughout the experiment.

intracellular cyclic AMP on invasion of HeLa cells by these strains. Initial results indicated that the levels of cyclic AMP in HeLa cells exposed to cholera toxin or forskolin were below the sensitivity of the assay that was used (<2.0 pmol/10^7 cells). To confirm that these agents caused a relative increase in cyclic AMP in HeLa cells exposed to these agents compared with the cyclic AMP level in controls, we included 0.5 mM IBMX in later assays in order to prevent the destruction of newly generated cyclic AMP by phosphodiesterases. IBMX was not included with cells that were subsequently infected with *B. pertussis*. Cyclic AMP levels of HeLa monolayers (ca. 10^7 cells) were quantitated following an 8-h coincubation period with 0.5 mM IBMX with or without cholera toxin (30 ng/ml) or forskolin (100 μM) at 37°C. Concentrations of each were maintained throughout the duration of each experiment. Stimulation of cyclic AMP levels in IBMX-treated HeLa 229 cell monolayers by cholera toxin and forskolin were as follows: for control HeLa cells, 78.0 ± 2.0 pmol of cyclic AMP per 10^7 HeLa 229 cells ($n = 3$); for HeLa cells and cholera toxin (30 ng/ml), 152.8 ± 51.3 pmol of cyclic AMP per 10^7 HeLa cells; ($n = 5$); and for HeLa cells and forskolin (100 μM), 214.0 ± 72.7 pmol of cyclic AMP per 10^7 HeLa cells ($n = 5$). Forskolin and cholera toxin caused a detectable increase in intracellular cyclic AMP in HeLa cells. Furthermore, the level of invasion of each mutant lacking adenylate cyclase toxin and hemolysin was reduced to a level similar to that of the parental strain in cells exposed to either cholera toxin or forskolin (Table 2). These data indicate that elevated levels of cyclic AMP produce an inhibition of invasion of *B. pertussis*. Invasion of both BP338 and BP347 was unaffected by increases in cyclic AMP.

As a correlative experiment, we subjected treated monolayers to invasion by an unrelated species, *Yersinia pseudotuberculosis* type 1A. Invasion by *Y. pseudotuberculosis* was reduced to levels of 38.0 ± 9.1 and 34.9 ± 2.0% ($n = 2$) of the levels of untreated controls in the presence of cholera toxin and forskolin, respectively. These data suggest that increases in cyclic AMP cause a generalized reduction in the phagocytic activity of HeLa cells.

Effect of exogenous FHA on invasion of *B. pertussis* FHA⁻ mutants. The invasion data indicated that the presence of FHA was necessary for optimal invasion of *B. pertussis*. We preincubated BP353 (FHA⁻) with purified FHA prior to the invasion assays to determine whether exogenously supplied FHA would enhance invasion of these strains. The data in Fig. 2 indicate that there was a dose-dependent increase in invasion of BR353 when it was preincubated with concentrations of FHA ranging from 0 to 75 μg/ml.

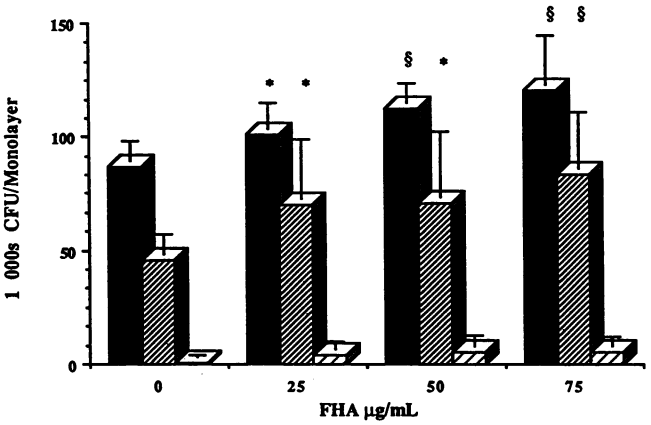

FIG. 2. Effect of exogenous FHA on invasion of parental BP338 (solid boxes), BP353 (FHA⁻) (heavily shaded boxes), and BP347 (*vir* mutant) (lightly shaded boxes). *B. pertussis* was preincubated with FHA at the indicated concentrations, washed, and then used in standard invasion assays. Each datum point represents an average of four independent determinations ± the standard deviation. Symbols: *, statistically significant compared with untreated control at $P < 0.05$; §, statistically significant at $P < 0.025$.

TEM examination of HeLa cell monolayers infected with *B. pertussis*. Sparsely seeded HeLa monolayers on glass cover slips that were initially infected with approximately 9×10^6 BP338 or BP348 for 7 h were examined by TEM in order to confirm the intracellular location of these organisms. Strain BP348 lacked both adenylate cyclase toxin and hemolysin and was included to determine whether this more invasive mutant would produce a morphological change in the intracellular appearance of these organisms. Figure 3A to D depict parental strain BP338 within the cytoplasm of infected HeLa cells. In the majority of cases, bacteria occurred singly within phagosomes, suggesting that intracellular multiplication does not occur within the 7-h interval. Phagosomal membranes surrounding the bacteria appeared to be in close contact with the circumference of the bacterial cell wall, often hindering their recognition. The intracellular appearance of mutant BP348 in Fig. 3F was not appreciably different from that of BP338. In some instances, bacteria were observed in the process of internalization and were partially or completely enveloped by microvilli. An example of this process is seen in Fig. 3E.

Entry of *B. pertussis* via a microfilament-dependent process. TEM observations suggested that *B. pertussis* enters HeLa cells by a phagocytic process. Since phagocytosis requires the action of microfilaments proximal to the site of entry, HeLa cells were pretreated with cytochalasin D, a potent microfilament inhibitor, to determine whether it had an effect on *B. pertussis* uptake. In the presence of 1.0 and 2.5 μg of cytochalasin D per ml, invasion was reduced to 1.7 ± 0.4 and 1.4 ± 0.5% that of untreated controls, respectively (mean ± standard deviation; $n = 9$). Thus, cytochalasin D lowered the levels of invasion to that of avirulent strain 347, which suggests that invasion of *B. pertussis* proceeds via a microfilament-dependent, phagocytic process.

Effect of monoclonal and polyclonal antisera on invasion of *B. pertussis*. We pretreated BP338 (serotype 1.2.3.4) with various monoclonal and polyclonal antisera in order to more clearly define the role of specific antigens in the invasion of

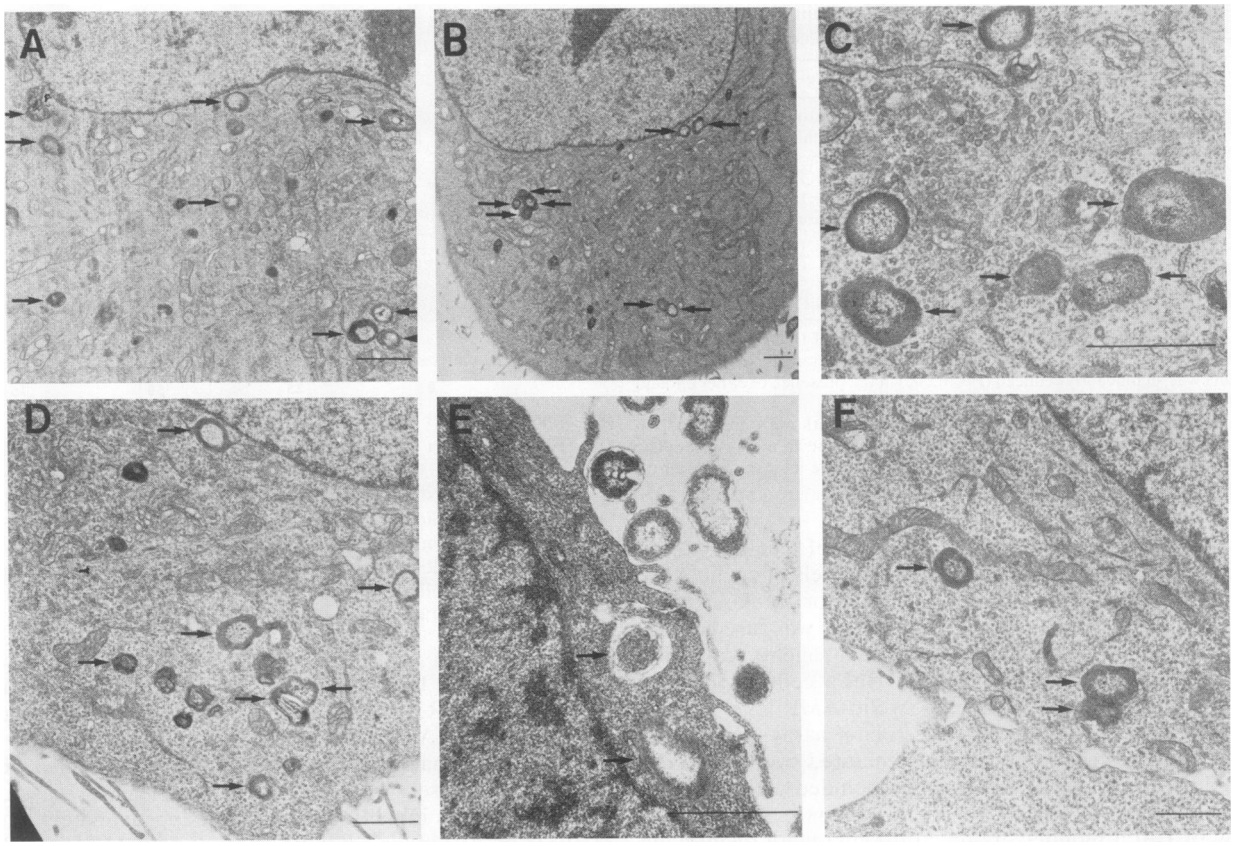

FIG. 3. Transmission electron micrographs demonstrating intracellular *B. pertussis* (arrows) within HeLa cells following a 7-h coincubation period. (A to D) Parental BP338 within endocytic vacuoles. Note the close proximity of bacterial outer membranes with membranes of vacuoles. (E) ACT Hly$^-$ mutant BP348 during the process of entry, whereupon outstretched microvilli of a HeLa cell appear to engulf bacterium. (F) Intracellular BP348. Morphologically, the lack of adenylate cyclase did not appear to render these bacteria more susceptible to damage by lysosomal enzymes.

B. pertussis. The results are given in Table 3. Preincubation of BP338 with antisera against *B. parapertussis* 17903 raised in rabbits did not reduce the invasive ability of BP338. Pretreatment of BP338 with rabbit anti-*B. pertussis* 2231, however, resulted in a dramatic reduction in invasion to a level of 4.5 ± 2.1% that of the control. Based on our results with FHA$^-$ Tn*5* *lac* mutants, we expected that pretreatment of wild-type *B. pertussis* (BP338) with anti-FHA would inhibit invasion. We used an extant anti-FHA preparation under identical conditions in which it has been proven to inhibit the binding of *B. pertussis* Tohama phase 1 to WiDr cells (24). Indeed, a reduction in invasion similar in magnitude to the reduction in binding to WiDr cells reported by Urisu et al. (24) was observed following pretreatment of BP338 with 0.25 and 0.50 mg of anti-FHA per ml. FHA appeared to play a pivotal role in the invasion of HeLa cells by *B. pertussis*, likely through adhesion to the eucaryotic cell membrane.

Invasion of BP338 was also tested after pretreatment with a number of monoclonal antisera raised to various surface antigens (Table 3). Monoclonal antisera to fimbrial antigens 2 or 3/6 had no effect on invasion. Three monoclonal antisera which recognized a newly described 69-kDa surface protein (1) did not reduce the invasive ability of BP338. One of the monoclonal antibodies (BPE3) recognized a potentially adhesive epitope, since it has been reported to reduce the adherence of CHO cells to the 69-kDa protein (E. Lieninger-Zapata, M. J. Brennan, J. G. Kenimer, I. Charles, N.

TABLE 3. Effect of monoclonal and polyclonal antisera on internalization of *B. pertussis* 338 by HeLa 229 cells

Antisera and specificity of antiserum	Protein concn (mg/ml)	Organisms/ monolayer (% of control)[a]
Polyclonal antisera		
Rabbit anti-*B. parapertussis* 17903[b]	0.25	96.3 ± 26.5
Rabbit anti-*B. pertussis* 2231[b]	0.25	4.5 ± 2.1
Goat anti-*B. pertussis* filamentous hemagglutinin[c]	0.25	40.3 ± 6.0
	0.50	27.6 ± 4.5
Monoclonal antisera[d]		
BPG10 anti-LOS[e] type A	0.05	41.5 ± 13.6
BPE8 anti-69-kDa	0.05	86.1 ± 8.2
BPE3 anti-69-kDa	0.05	91.4 ± 15.8
BPD8 anti-69-kDa	0.05	99.8 ± 1.4
BPF2 anti-fimbriae type 2	0.05	110.3 ± 13.0
BPC10 anti-fimbriae type 3/6	0.05	101.7 ± 12.7

[a] Results represent the average number of CFUs per monolayer expressed as a percentage of control ± standard deviation; $n = 6$ for anti-*B. pertussis* and anti-*B. parapertussis* data; $n = 9$ for anti-FHA data.

[b] Monovalent Fab fragments were isolated following papain digestion of IgG eluate from a protein A-Sepharose CL-4B column.

[c] Purified IgG fraction of goat anti-FHA.

[d] Monoclonal antisera were raised against *B. pertussis* 338 (serotype 1.2.3.4). Data represent the means ± standard deviations of three independent determinations for each monoclonal antiserum, except BPG10, for which $n = 9$.

[e] LOS, Lipooligosaccharide.

Fairweather, and P. Novotny. Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, B123, p. 51). In contrast, a significant reduction in invasion resulted from treatment of BP338 with a monoclonal antibody directed against lipooligosaccharide A.

DISCUSSION

In this study, we demonstrated the invasive behavior of *B. pertussis*, which is commonly regarded as a noninvasive respiratory pathogen. Virulent-phase *B. pertussis* cells invaded HeLa cells, whereas avirulent organisms did not, indicating the dependence of this behavior on *vir* expression. Mutants lacking FHA showed a significantly decreased invasive ability, presumably because of a reduction in adherence. Accordingly, preincubation of FHA-deficient strains with exogenous FHA resulted in a dose-dependent increase in uptake. While there exists strong evidence to suggest that FHA mediates the adherence of *B. pertussis* to several cell types in vitro (20, 23, 24), its role in vivo is still unclear. It is believed that it mediates the adherence of *B. pertussis* to ciliated cells of the upper respiratory tract, but its role in the interaction of *B. pertussis* with nonciliated cell types of the respiratory tract is unknown.

Expression of pertussis toxin also appears to contribute to invasion. Previous data have indicated that pertussis toxin, in combination with FHA, mediates binding of *B. pertussis* to human ciliated respiratory cells (24) and Vero cells (10). This suggests that the reduced invasion of the pertussis toxin mutant may be due to a reduction in adherence. However, the possibility that pertussis toxin may mediate the intracellular survival of *B. pertussis* cannot be excluded.

Of interest is the unusual level of invasion demonstrated by the adenylate cyclase toxin and hemolysin mutants BP348, BP349, BPM433, and BPM3183. Intoxication of professional phagocytes by adenylate cyclase toxin inhibits phagocytic and oxidative responses via increases in cyclic AMP (3, 7, 13; P. Symes, E. L. Hewlett, D. Roberts, A. Q. de Sousa, and R. D. Pearson, Clin. Res. **31**:377A, 1983). In addition, increases in cyclic AMP have also been shown to inhibit the phagocytic activities of polymorphonuclear leukocytes (4). However, the specific effect of cyclic AMP increases on parasite-directed phagocytosis by nonprofessional phagocytes is unknown. Our data suggest that increases in cyclic AMP, which were generated either by strains of *B. pertussis* which produced adenylate cyclase toxin or by the addition of exogenous agents, attenuate the phagocytic activity of HeLa cells. Conversely, the uptake of strains lacking adenylate cyclase toxin is increased, and accordingly, reconstitution of increased levels of cyclic AMP restores the attenuation and results in a reduction in phagocytosis. A similar effect on *Shigella* invasion was previously reported by Hale et al. (12). Increased cyclic AMP levels in Henle 407 cell monolayers following treatment with either dibutyryl cyclic AMP or cholera toxin caused a significant reduction in the invasion of these organisms (12). Inhibition of phagocytosis by adenylate cyclase toxin may represent a mechanism by which the bacteria limit their own uptake to minimize disruption of the host cell caused by the numerous cytotoxic substances they produce. Further experimentation is needed to determine the mechanism by which *B. pertussis* increases cyclic AMP in HeLa cells. Such studies may also reveal other effects of increased cyclic AMP levels on the recovery of viable counts of invasive bacteria.

Invasion could be markedly reduced by prior incubation of the bacteria with Fab fragments of polyclonal whole-cell anti-*B. pertussis* antiserum. In contrast, Fab fragments of polyclonal anti-*B. parapertussis* antiserum had little effect. This is important because both anti-*B. pertussis* and anti-*B. parapertussis* significantly reduced the uptake of *B. parapertussis* (6). These data suggest that *B. pertussis* possesses unique antigenic determinants that are absent from *B. parapertussis*. These determinants are important for adherence, invasion, or both. Consistent with our results is a report from Kendrick et al. (15) which demonstrated that intraperitoneal immunization with *B. parapertussis* does not protect mice against intracerebral challenge with *B. pertussis* (15).

Preincubation of the organisms with polyclonal anti-FHA showed a reduction in invasion similar in magnitude to the reduction in adherence previously described for *B. pertussis* Tohama 1 under identical conditions (24). Interestingly, the same antiserum had no effect on invasion of HeLa cells by *B. parapertussis* (6), although the FHAs of both species are presumed to be similar. These data indicate that important antigenic differences exist between the FHAs of *B. pertussis* and *B. parapertussis*.

Anti-lipooligosaccharide A caused a significant reduction in uptake. Recent data suggest that anti-lipooligosaccharide A may constitute part of the agglutinogen 1 of *B. pertussis* (16). This is relevant because a monoclonal antiserum raised against agglutinogen 1 has previously been shown to reduce the adherence of *B. pertussis* to Vero cells (10). In view of these data, the reduction in invasion caused by anti-lipooligosaccharide A may simply reflect a reduction in adherence. Alternatively, the presence of a large number of antibodies on the surface of *B. pertussis* may impair an interaction between relevant surface adhesins and the HeLa cell surface. In this case, the reduction in invasion would be a secondary effect caused by steric hindrance.

The intracellular presence of *B. pertussis* was confirmed by TEM examination of infected monolayers. The lack of an apparent vacuole enclosing each bacterium frequently complicated the recognition of intracellular bacteria. The difficulty in recognizing these bacteria suggests an explanation for why intracellular *B. pertussis* has not been observed previously.

Induction of uptake by a nonphagocytic cell type and adaptation to a hostile intracellular environment are difficult tasks that are undertaken by only a few bacterial species. This suggests that an intracellular location confers a significant pathogenic advantage, survival advantage, or both to the species which develop these abilities. In the case of *B. pertussis*, intracellular survival would provide effective protection against both nonspecific and specific host defenses, including humoral immunity. Humoral immunity is especially important, considering the high incidence of immunity to pertussis produced by immunization. As such, prolonged intracellular carriage of *B. pertussis* in asymptomatic individuals may represent a sophisticated reservoir for transmission of pertussis. Further experimentation with respect to the mechanism of survival of intracellular *B. pertussis*, the possible occurrence of antigenic modulation within an intracellular environment containing appropriate signals, and demonstration of the occurrence of this phenomenon in vivo in relevant animal models is clearly indicated.

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