

# Invasion of human respiratory epithelial cells by *Bordetella pertussis*: Possible role for a filamentous hemagglutinin Arg-Gly-Asp sequence and $\alpha 5\beta 1$ integrin

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*Bordetella pertussis*, the agent of whooping cough, is capable of invading human respiratory epithelial cells. In this study, we investigated the mechanisms by which *B. pertussis* invades the human lung epithelial cell line A549 and normal human bronchial epithelial (NHBE) cells. *In vitro* adhesion and invasion assays using both cell types with a virulent *B. pertussis* strain and its isogenic mutants revealed profound defects in a mutant deficient in filamentous hemagglutinin (FHA) expression. In addition, a mutant in which an FHA Arg-Gly-Asp (RGD) site had been changed to Arg-Ala-Asp had significantly diminished invasiveness, although its adhesiveness was comparable to that of the parental strain. Furthermore, a synthetic RGD-containing hexapeptide inhibited invasion of both cell types by the virulent strain. These results demonstrate that an RGD sequence of FHA is involved in *B. pertussis* invasion of epithelial cells *in vitro*. Monoclonal antibodies directed against human  $\alpha 5\beta 1$  integrin, but not other integrins, blocked invasion, indicating that this integrin is involved in *B. pertussis* invasion. Taken together, these findings suggest that *B. pertussis* FHA may promote invasion of human respiratory epithelial cells through the interaction of its RGD sequence with host cell  $\alpha 5\beta 1$  integrin.

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**Key words:** *Bordetella pertussis*, integrin, human epithelial cell, invasion.

## Introduction

The causative agent of whooping cough, *Bordetella pertussis*, is a Gram-negative coccobacillus

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which attaches to ciliated epithelial cells in the respiratory tract and causes disease through the action of several bacterial toxins [1]. Various *B. pertussis* virulence factors have been identified and characterized; some virulence factors including filamentous hemagglutinin (FHA), fimbriae (Fim), pertactin (Prn) and pertussis toxin

(PT) mediate adherence to mammalian cells; others such as adenylate cyclase toxin (ACT), tracheal cytotoxin (TCT), dermonecrotic toxin (DNT) and PT exert toxic effects on a variety of host cells [2, 3]. The expression of most of these virulence factors is coordinately regulated by the two-component response regulatory system BvgAS that is encoded by the *bvg* locus, that responds to environmental factors such as  $MgSO_4$ , nicotinic acid and temperature [4–6]. Although *B. pertussis* has long been considered to be an exclusively extracellular pathogen, recent findings have shown that *B. pertussis* can invade and survive in monocytes/macrophages and several types of epithelial cells [7–11]. The ability of *B. pertussis* to survive within host cells could provide mechanisms for escape from host immune defenses, and/or the establishment of a quiescent carrier state in an intracellular environment. However, the precise mechanisms by which *B. pertussis* invade human respiratory epithelial cells remains to be elucidated. Previous studies of intracellular invasion by some bacterial pathogens have revealed that entry of bacteria into non-phagocytic cells is a multifactorial process with participation of both bacterial factors and host cell surface components [12–15]. In the case of *Yersinia*, the outer membrane protein invasin binds to  $\beta 1$  integrin receptors, leading to bacterial entry [12]. *Listeria monocytogenes* also relies on ligand–receptor, protein–protein interactions to enter the human enterocyte-like epithelial cell line Caco-2 and some other epithelial cells; i.e. host E-cadherin serves as a receptor for the bacterial protein internalin (InlA) [13, 14]. For *Shigella flexneri*, the interaction of Ipa proteins with  $\alpha 5\beta 1$  integrin promotes entry of the bacteria into epithelial cells [15]. Therefore, it can be postulated that a bacterial factor and its complementary receptor on the host cell surface probably have considerable bearing on the success of bacterial invasion.

Invasion of epithelial cells by *B. pertussis* appears to be a *bvg*-dependent process, as *bvg* mutants were much less invasive [11, 16]. Recently, Bassinet *et al.* [7] have shown that *B. pertussis* FHA promotes invasion of human tracheal epithelial cells, whereas ACT and Prn impair this process. FHA is a 220 kDa protein that is both secreted and cell-associated [17]. FHA contains at least four distinct binding domains: (i) the Arg-Gly-Asp (RGD) sequence for leukocyte response integrin/integrin-

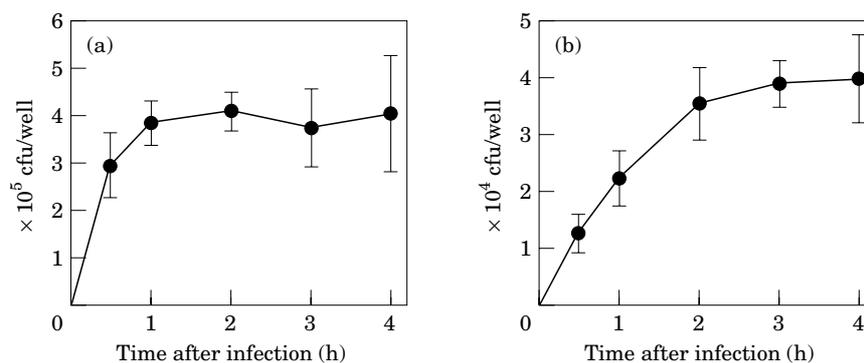
associated protein complex which mediates binding to monocytes through the up-regulation of the complement receptor type 3 (CR3) [18, 19]; (ii) a CR3 recognition domain [18]; (iii) a carbohydrate recognition domain (CRD) which mediates attachment to ciliated respiratory epithelial cells as well as to macrophages [20]; and (iv) a binding domain for heparin and other sulfated carbohydrates which can mediate adherence to nonciliated epithelial cell lines *in vitro* [21]. None of these FHA domains, or host receptors, have been implicated in invasion of respiratory epithelial cells to date. In this study, using the cultured human lung epithelial cell line A549 and primary normal human bronchial epithelial cells, we investigated the mechanism by which *B. pertussis* invades respiratory epithelial cells. Our results suggest that *B. pertussis* FHA may promote the invasion of human respiratory epithelial cells through the interaction of its RGD sequence with host cell  $\alpha 5\beta 1$  integrin.

## Results

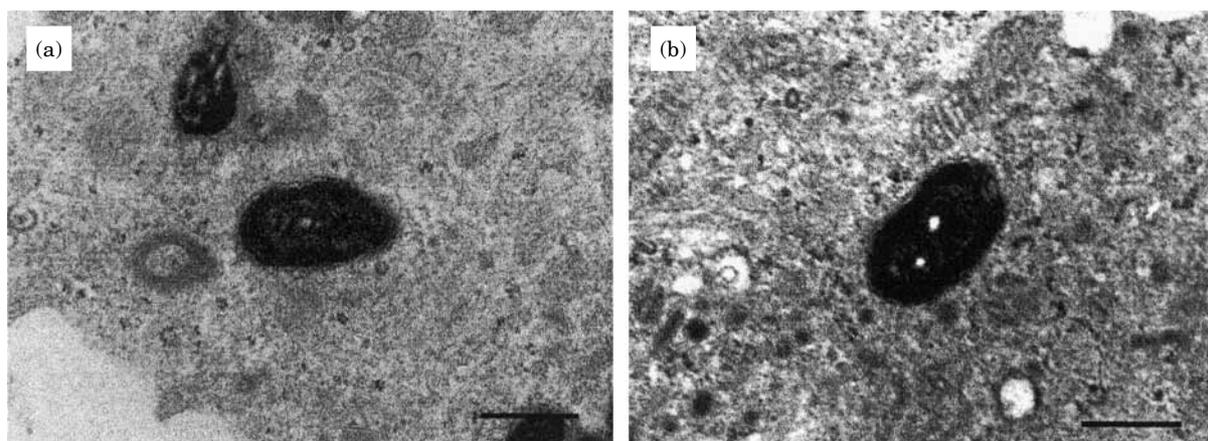
### Kinetics of the adherence to, and invasion of, A549 epithelial cells by *B. pertussis* BP536

In order to define the characteristics of our assays, we examined a time course of adherence to, and invasion of, A549 cells by *B. pertussis*. A549 cells were infected with *B. pertussis* BP536 at an moi of 100, and the numbers of viable cell-associated and intracellular bacteria were determined after various time intervals (Fig. 1). The adherence to and invasion of A549 cells by BP536 were complete after 1 and 2 h incubation, respectively. An incubation as long as 4 h showed no significant increase in the numbers of cell-associated and intracellular bacteria. In this assay, after 2 h  $9.7 \pm 2.3\%$  of the total number of A549-associated bacteria were resistant to gentamicin, and hence, were defined as intracellular.

To confirm the intracellular presence of bacteria, A549 cell monolayers were infected with *B. pertussis* BP536 at an moi of 100 at 37°C for 2 h and were examined by TEM (Fig. 2). TEM showed that most intracellular bacteria were entrapped in tight endocytic vacuoles [Fig. 2(a) and (b)]. Similar results were obtained with NHBE cells (data not shown).



**Figure 1.** Time course of adherence to and invasion of A549 cells by *B. pertussis* BP536. A549 cell monolayers ( $1 \times 10^5$  cells/well) were infected with  $10^7$  cfu *B. pertussis* BP536 for 30 min, 1, 2, 3 and 4 h at 37°C. The infected cells were treated or not treated with gentamicin for 2 h at 37°C. The cells were washed and lysed. Appropriate dilutions of the cell lysates were plated onto BG agar plates. The numbers of viable bacteria from the cells untreated with gentamicin were counted as the total number of cell-associated bacteria (a). The gentamicin-resistant bacteria were defined as intracellular bacteria (b). The values represent the means  $\pm$  SE of four experiments.

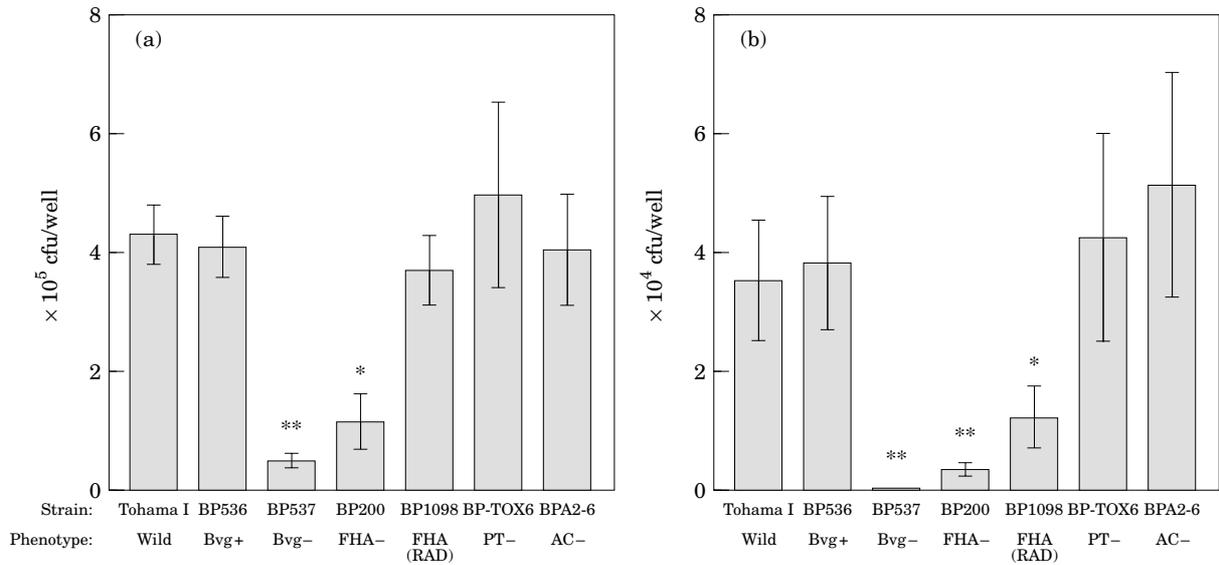


**Figure 2.** Transmission electron micrographs demonstrating *B. pertussis* invasion of A549 cells. A549 cell monolayers ( $1 \times 10^5$  cells/well) were infected with *B. pertussis* BP536 at an moi of 100 at 37°C for 2 h. In (a) and (b), intracellular bacteria are entrapped in tight endocytic vacuoles. Bar = 0.5 μm.

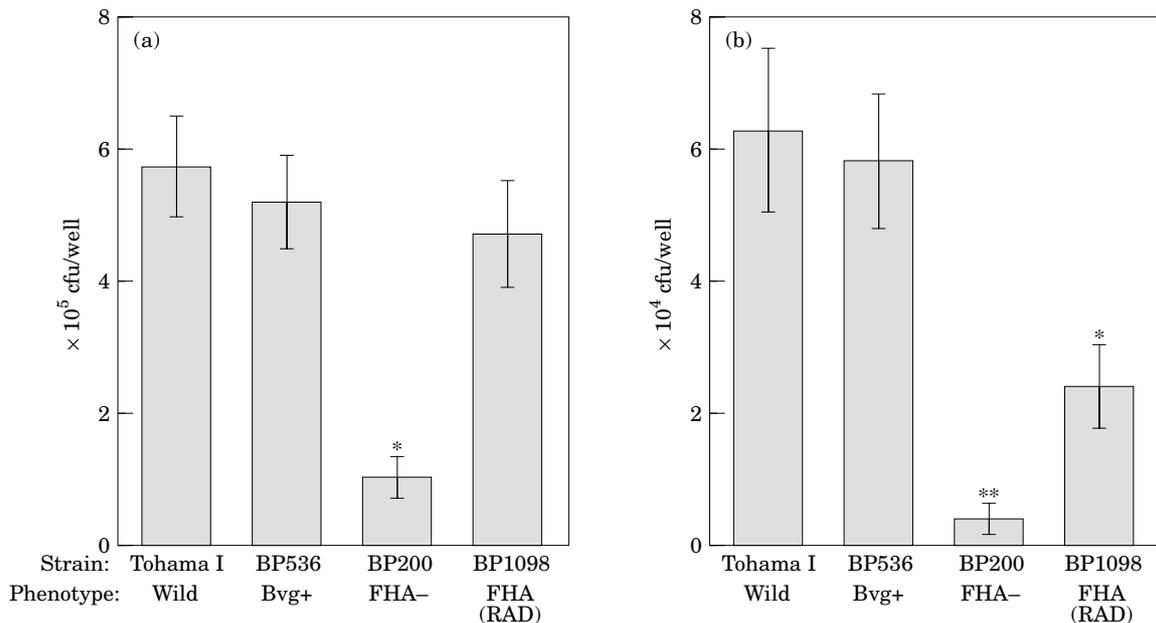
### RGD sequence of FHA is required for maximal invasion of human respiratory epithelial cells by *B. pertussis*

In order to assess the role of bacterial factors in the invasion of epithelial cells, A549 cells were infected with various isogenic mutant strains of *B. pertussis*. After 2 h incubation at 37°C, bacterial adherence and invasion were assessed (Fig. 3). The ability of *B. pertussis* BP536 to adhere and invade A549 cells was similar to that of wild type strain Tohama I. *B. pertussis* BP537, the avirulent (Bvg<sup>-</sup>) derivative of strain BP536, did not adhere to, or invade A549 cells, indicating a role for *bvg*-regulated virulence factors in the

interaction between *B. pertussis* and respiratory epithelial cells. Mutant strain BP200, deficient in FHA, was reduced in its ability to adhere to (27.6% of the parental strain BP536;  $P < 0.05$ ) and invade (9.1% of BP536;  $P < 0.01$ ) A549 cells. Strain BP1098, an isogenic derivative of BP536 that contains a site-directed chromosomal mutation resulting in a Gly  $\rightarrow$  Ala substitution at the RGD site of FHA, showed a significantly reduced invasiveness (43.2% of BP536;  $P < 0.05$ ), although its adhesiveness was similar to that of the parental strain BP536. In contrast, the adhesive and invasive properties of strain BP-TOX6, deficient in PT, or strain BPA2-6, deficient in AC activity, were indistinguishable from those of



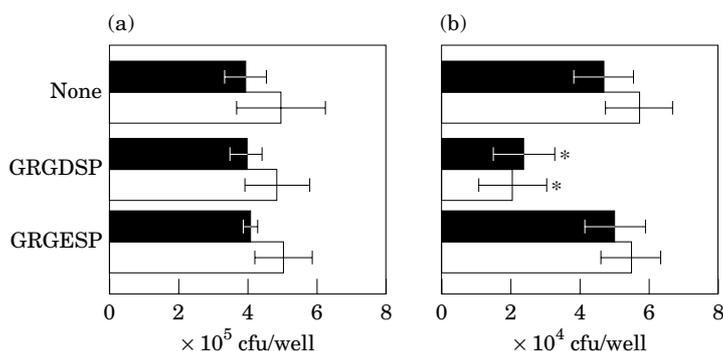
**Figure 3.** Adhesion and invasion profiles of strain BP536 and its isogenic derivatives for A549 cells. A549 cells ( $1 \times 10^5$  cells/well) were infected with  $10^7$  cfu *B. pertussis* mutant strains at  $37^\circ\text{C}$  for 2 h. The numbers of viable cell-associated bacteria (a) and intracellular bacteria (b) were determined. The values represent the means  $\pm$  SE of four experiments. \*  $P < 0.05$ ; \*\*  $P < 0.01$  vs parental strain.



**Figure 4.** Adhesion and invasion profiles of strain BP536 and its isogenic derivatives for NHBE cells. NHBE cells ( $1 \times 10^5$  cells/well) were infected with  $10^7$  cfu *B. pertussis* at  $37^\circ\text{C}$  for 2 h. The numbers of viable cell-associated bacteria (a) and intracellular bacteria (b) were determined. The values represent the means  $\pm$  SE of four experiments. \*  $P < 0.05$ ; \*\*  $P < 0.01$  vs parental strain.

the parental strain BP536. These results suggest that *B. pertussis* FHA plays a major role in the adherence to, and invasion of A549 cells, and that the 1097RGD1099 sequence of FHA may contribute to the invasion process.

Next, we used the normal human bronchial epithelial (NHBE) cells as a more relevant cell for testing the role of FHA in *B. pertussis* invasion. NHBE cells were infected with *B. pertussis* mutant strains at an moi of 100, and bacterial ad-



**Figure 5.** Effect of synthetic peptide on adherence and invasion of *B. pertussis* BP536 to A549 and NHBE cells. A549 (■) and NHBE (□) cells ( $1 \times 10^5$  cells/well) were infected with  $10^7$  cfu *B. pertussis* in the presence of 1.5 mM synthetic peptides, and the numbers of viable cell-associated bacteria (a) and intracellular bacteria (b) were determined. The values represent the means  $\pm$ SE of five experiments. \*  $P < 0.05$  vs control.

herence and invasion were determined after 2 h incubation (Fig. 4). The adhesive and invasive properties of strain BP536 were similar to those of wild type strain Tohama I. Strain BP200 (FHA-) adhered to NHBE cells at considerably lower levels than did strain BP536 (20.6% of BP536;  $P < 0.05$ ). Strain BP1098 (FHA RAD) adhered equally well to NHBE cells as did BP536, but invaded less well (42.1% of BP536;  $P < 0.05$ ). There was no difference in adhesive and invasive properties of strain BP-TOX6 and strain A2-6 relative to strain BP536 (data not shown). These results were essentially identical to those obtained with A549 cells.

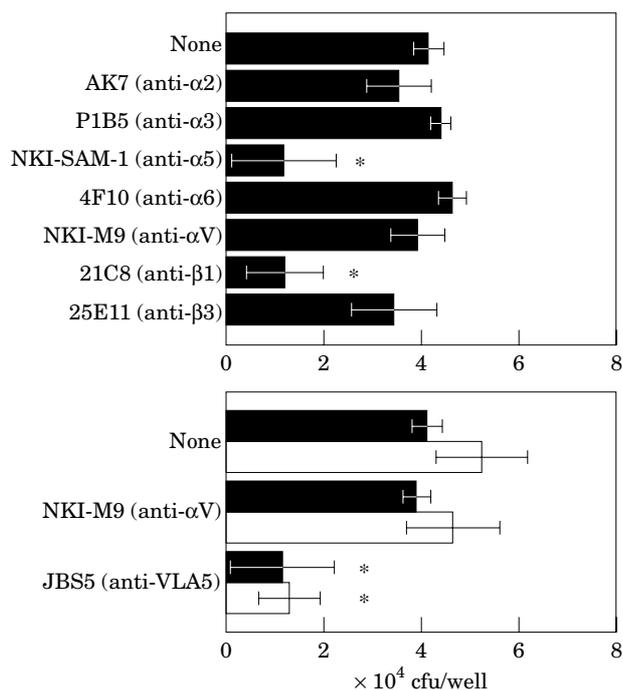
#### RGD peptide inhibits invasion of human respiratory epithelial cells by *B. pertussis*

To corroborate the involvement of the FHA RGD sequence in bacterial invasion, we examined the ability of an RGD-containing synthetic peptide to inhibit invasion by *B. pertussis*. A549 and NHBE cells were infected with strain BP536 in the presence or absence of synthetic GRGDSP peptide (1.5 mM) for 2 h at  $37^\circ\text{C}$ , and the numbers of cell-associated and intracellular bacteria were determined (Fig. 5). The invasion of A549 and NHBE cells by *B. pertussis* BP536 was significantly reduced to 50.0% ( $P < 0.05$ ) and 39.2% ( $P < 0.05$ ) of control, respectively, in the presence of the GRGDSP peptide [Fig. 5(b)]. In contrast, no inhibition was observed when the non-active GRGESP peptide was present. Neither the GRGDSP or the GRGESP peptide influenced the adhesion process [Fig. 5(a)]. These results

confirmed a role for the FHA RGD sequence in the invasion process.

#### Human $\alpha 5\beta 1$ integrin promote bacterial invasion of respiratory epithelial cells

The RGD sequence motif represents one of the major cell-attachment domains of the extracellular matrix (ECM) proteins and is recognized by integrin receptors [22, 23]. Integrins have also been found to mediate intracellular invasion by some microbial pathogens [12, 15]. To examine if RGD-binding integrins are involved in *B. pertussis* invasion of respiratory epithelial cells, we assessed the ability of several anti-integrin monoclonal antibodies to inhibit invasion of A549 cells by *B. pertussis* BP536. A549 cells were preincubated with one of several monoclonal antibodies (25  $\mu\text{g}/\text{ml}$ ) for 30 min at  $37^\circ\text{C}$ , and then infected with strain BP536 for 2 h (Fig. 6). Monoclonal antibodies directed against the integrin  $\alpha 5$  and  $\beta 1$  subunits were found to inhibit invasion of A549 cells by *B. pertussis* (29.1 and 29.7% of control;  $P < 0.05$ , respectively). In contrast, monoclonal antibodies directed against integrin chains  $\alpha V$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 6$  and  $\beta 3$  had no inhibitory effect. None of anti-integrin monoclonal antibodies influenced adhesion process (data not shown). We further tested the ability of a monoclonal antibody directed against an epitope found only in the  $\alpha 5\beta 1$  heterodimer (very late antigen-5; VLA-5) to inhibit invasion of A549 and NHBE cells (Fig. 6). This monoclonal antibody also inhibited invasion of both cell types by *B. pertussis*, suggesting that the  $\alpha 5\beta 1$



**Figure 6.** Effects of anti-integrin monoclonal antibodies on *B. pertussis* invasion. A549 monolayers were incubated with monoclonal antibodies recognizing the indicated integrin subunits for 30 min prior to addition of bacteria (upper panel). A549 (■) and NHBE (□) monolayers were incubated with monoclonal antibodies directed against  $\alpha$ V integrin subunit or VLA-5 for 30 min prior to addition of bacteria (lower panel). Thereafter, the standard assay procedure was followed. The values represent the means  $\pm$  SE of four experiments. \*  $P < 0.05$  vs control.

integrin may be required for bacterial invasion of human respiratory epithelial cells. Control experiments were performed to verify that the inhibitory monoclonal antibodies did not adversely affect bacterial and host cell viabilities (data not shown).

## Discussion

There is increasing evidence that adults serve as the primary reservoir for *B. pertussis* and facilitate its spread to susceptible children [24, 25]. *B. pertussis* is capable of invading monocytes/macrophages and respiratory epithelial cells [7–9], although it does not appear to replicate to a significant degree inside these cells. The ability to enter host cells may lead to the establishment of an intracellular reservoir of

viable *B. pertussis*. In the present study we investigated the mechanisms by which *B. pertussis* invade human respiratory epithelial cells. *B. pertussis* FHA appears to play a major role in adherence to, and invasion of, A549 and NHBE cells based upon the impaired activities of a FHA-deficient isogenic strain. This observation is consistent with a previous report describing a role for FHA in invasion of human tracheal epithelial cells [7]. Our study also demonstrated that the FHA RGD sequence at residues 1097–1099 is involved in invasion of human respiratory epithelial cells by *B. pertussis*, and that human  $\alpha$ 5 $\beta$ 1 integrin may serve as a receptor for *B. pertussis* invasion. Several types of evidence favour the roles of the FHA RGD sequence and human  $\alpha$ 5 $\beta$ 1 integrin in the invasion process: (1) a mutant strain, in which the FHA RGD motif had been changed to RAD, was much less invasive than the parental strain; and (2) both, a synthetic GRGDSP peptide and a monoclonal antibody directed against the  $\alpha$ 5 $\beta$ 1 integrin were able to inhibit invasion of A549 and NHBE cells by *B. pertussis*.

Exploitation of  $\alpha$ 5 $\beta$ 1 integrin by *B. pertussis* for the purposes of invasion is not a unique strategy among microbial pathogens. *Yersinia pseudotuberculosis* and *Y. enterocolitica* can bind to various  $\beta$ 1 integrins on several mammalian cells via the Inv protein, thereby promoting their ingestion [12]. *Shigella flexneri* can penetrate into mammalian cells through the interaction of IpaB, IpaC and IpaD proteins with  $\alpha$ 5 $\beta$ 1 integrin [15]. Furthermore, *Trypanosoma cruzi*, *Leishmania* sp., *Streptococcus pyogenes* and various *Mycobacteria* species can bind fibronectin and interact with VLA-5 via this molecular bridge [26–31]. It is worth noting that some properties of the interaction of FHA with  $\alpha$ 5 $\beta$ 1 integrin are apparently different from the characteristics reported for *Yersinia* Inv protein and *Shigella flexneri* Ipa protein. Inv protein interacts with  $\beta$ 1 integrins in a manner independent of RGD, and the Ipa protein- $\alpha$ 5 $\beta$ 1 integrin interaction is not inhibited by RGDS peptide [12, 15, 32]. In contrast, FHA/ $\alpha$ 5 $\beta$ 1 integrin-mediated *B. pertussis* invasion appears to be an RGD-dependent. It would be of great interest to distinguish further the functional consequences of the interaction of the FHA RGD with  $\alpha$ 5 $\beta$ 1 integrin from those of Inv or Ipa protein at the molecular level. Interestingly, the FHA RAD mutant strain retained the ability to adhere to A549 and NHBE cells, although its invasiveness was significantly impaired. This observation was corroborated by

the finding that the RGD-containing peptide had no effect on adherence of *B. pertussis*. These data suggest that the RGD sequence of FHA contributes to the invasion process rather than to the adhesion process; other portions of the FHA protein such as the carbohydrate recognition domain or heparin-binding domain may contribute to the adhesion process.

Menozzi *et al.* [21] have shown that the heparin-inhibitable binding activity of FHA is largely responsible for attachment of *B. pertussis* to epithelial cells. They have postulated that low-affinity ligands, like heparan sulfate, may help to attach the pathogen to the target cell, and a specific interaction with target cell proteins may then be required for internalization [21]. It is therefore possible that the heparin-binding domain of FHA provides the molecular basis of initial attachment of *B. pertussis* to the surface of human respiratory epithelial cells. This initial binding might then be followed by interaction of the RGD sequence of FHA with  $\alpha 5\beta 1$  integrin to mediate bacterial entry. This idea was supported by evidence that heparin (100  $\mu\text{g}/\text{ml}$ ) was able to inhibit adherence of the BP536 strain to A549 and NHBE cells by 18.2 and 20.4% of control, respectively (data not shown). The PT-deficient strain showed similar adhesive and invasive properties to those of the parental strain, consistent with a previous report that PT does not play a role in the invasion of epithelial cells by *B. pertussis* [7]. However, in contrast to an earlier report describing an inhibitory effect of AC on entry of *B. pertussis* into HTE and HeLa cells [7, 8], we found no evidence for a significant inhibitory effect of AC in the entry of *B. pertussis* into A549 cells. In our assay system, with the AC-deficient mutant strain as well as with the parental strain, the standard errors of the measurements could have masked an inhibitory effect of AC (Fig. 3). We did not address the roles of Prn and Fim in the invasion process. It is intriguing that Fim can interact with VLA-5 on monocytes, resulting in enhanced CR3 activity [33]. However, Bassinet *et al.* [7] have recently demonstrated that Fim is not involved in invasion of HTE cells by *B. pertussis*, and that Prn inhibits the invasion process.

It is unclear whether FHA-mediated *B. pertussis* invasion takes place *in vivo*.  $\beta 1$  integrin expression is polarized to the basolateral membrane of epithelium in the respiratory airway [34, 35]. Injury of the airway epithelium can occur during exposure to a variety of infectious

agents such as *Haemophilus influenzae* and *Pseudomonas aeruginosa*, in addition to *B. pertussis*, leading to epithelial wound repair following the shedding of the surface epithelial cells [36–38]. Regenerating airway epithelial cells display enhanced expression and apical distribution of  $\alpha 5\beta 1$  integrin [34, 35]. Roger *et al.* [35] have demonstrated that *P. aeruginosa* can adhere to the repairing airway epithelium through the interaction of a 50 kDa outer membrane protein with apically distributed  $\alpha 5\beta 1$  integrin. *B. pertussis* is known to destroy the ciliated cells that line the respiratory tract [38]. Thus, the over-expression and apical distribution of  $\alpha 5\beta 1$  integrin on the respiratory epithelium during the repair process after injury could contribute to FHA RGD-mediated *B. pertussis* invasion. Injured or activated epithelial cells (during infection) may show various inflammatory responses, including secretion of chemokines, and expression of adhesion molecules [39, 40]. Studies are currently under way in our laboratory to examine the regulation of adhesion molecule expression as well as the cytokine production in response to *B. pertussis* invasion of respiratory epithelial cells.

In conclusion, the data from our study suggest that *B. pertussis* FHA may promote invasion of human respiratory epithelial cells through the interaction of its RGD sequence with host cell  $\alpha 5\beta 1$  integrin. The identification of a bacterial ligand and its host receptor involved in *B. pertussis* invasion provides an important insight into the understanding of *B. pertussis* pathogenesis.

## Materials and Methods

### Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. Bacteria were cultured for 2 days on Bordet-Gengou (BG) agar plates (Difco Laboratories, Detroit, MI, U.S.A.) supplemented with 15% sheep blood. Before use, bacteria were harvested from the plates and suspended in Dulbecco's phosphate-buffered saline (PBS; pH 7.4) to a concentration of  $1 \times 10^8$  colony forming unit (cfu)/ml as estimated by optical density at 600 nm. Actual concentrations of viable bacteria were determined by colony counts after plating on BG agar.

**Table 1.** Strains of *B. pertussis* used in this study

Strain	Relevant phenotype or genotype	Reference
Tohama I	Virulent phase (wild type)	[41]
BP536	Str <sup>R</sup> derivative of Tohama I (Bvg +)	[41]
BP537	Contains frameshift mutation in <i>bvgS</i> resulting in avirulent-phase phenotype (Bvg-) and deficient expression of multiple proteins including FHA, AC and PT	[6], [41]
BP200	Contains a complete deletion of the FHA structural gene, <i>fhaB</i> , resulting in deficient expression of FHA protein	[18]
BP1098	Contains single amino acid substitution in FHA sequence RGD (1097-9): G1098A ( <i>fhaB1098</i> )	[19]
BP-TOX6	Contains deletion of PT operon ( <i>ptxA6</i> )	[19]
BPA2-6	Contains amino acid substitution in CyaA AC catalytic site (K58M) resulting in loss of enzymatic activity	[42]

### Monoclonal antibodies and peptides

The following monoclonal antibodies against human cell surface proteins were used: AK7 (anti-integrin  $\alpha 2$ , IgG1; CD49b), P1B5 (anti-integrin  $\alpha 3$ , IgG1; CD49c), NKI-SAM-1 (anti-integrin  $\alpha 5$ , IgG2b; CD49e), 4F10 (anti-integrin  $\alpha 6$ , IgG2b; CD49f), NKI-M9 (anti-integrin  $\alpha V$ , IgG1; CD51), 21C8 (anti-integrin  $\beta 1$ , IgG1; CD29), 25E11 (anti-integrin  $\beta 3$ , IgG2a; CD61) and JBS5 (anti-integrin  $\alpha 5\beta 1$ , IgG; VLA-5) (Chemicon International Inc., Temecula, CA, U.S.A.). The six-amino-acid peptides GRGDSP and GRGESP were purchased from Takara Biochemicals (Otsu, Japan). Lyophilized peptides were dissolved in RPMI1640 medium and filter sterilized; aliquots were stored at  $-80^{\circ}\text{C}$ .

### Eukaryotic cells and their cultivation

The human lung epithelial cell line A549 was obtained from Riken Cell Bank, Japan

(RCB0098), and normal human bronchial epithelial (NHBE) cells from BioWhittaker, Inc. (Walkersville, MD, U.S.A.). A549 cells were cultured in RPMI1640 medium (Nissui Co., Japan) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS), 100 U of sodium penicillin G/ml, and 100  $\mu\text{g}$  of streptomycin/ml. NHBE cells were grown in bronchial epithelial growth medium (BEGM; BioWhittaker, Inc.). BEGM consists of bronchial epithelial basal medium (BEBM) supplemented with recombinant human epidermal growth factor (rhEGF), insulin, hydrocortisone, transferrin, tri-iodothyronine, epinephrine, pituitary extract (PE) and retinoic acid. Cells were maintained at  $37^{\circ}\text{C}$  in a humidified atmosphere containing 5%  $\text{CO}_2$ .

### Bacterial adherence and invasion assays

For adherence and invasion assays,  $1 \times 10^5$  cells/well were seeded into 24 well tissue culture plates 18 h before the assays. The epithelial cell monolayers in 24 well tissue culture plates were washed three times and incubated with un-supplemented medium for 2 h prior to bacterial challenge. The epithelial cell monolayers were infected with  $1 \times 10^7$  bacterial cfu and incubated at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  for 2 h, unless otherwise noted. To determine the number of cell-associated bacteria, infected monolayers were washed three times with PBS to remove non-associated bacteria, lysed by adding 200  $\mu\text{l}$  of a saponin (Sigma) solution (1  $\mu\text{g}/\text{ml}$  in PBS) for 15 min at  $37^{\circ}\text{C}$ . After repeated pipetting and vortexing, the suspension was plated out in serial dilutions on BG agar to determine the number of cfu. To quantitate bacterial invasion, a gentamicin-based protection method was performed [3, 8]. The epithelial cell monolayers infected with bacteria at a multiplicity of infection (moi) of 100 for 2 h were washed three times and further incubated with the appropriate medium containing 100  $\mu\text{g}$  gentamicin/ml for 2 h to kill extracellular bacteria. In separate experiments, *B. pertussis* strains suspended at a density equivalent to that used in invasion assays were treated by incubation with 100  $\mu\text{g}$  gentamicin/ml for 2 h in the absence of epithelial cells. This procedure reduced the number of viable bacteria from  $1 \times 10^7$ – $20$  cfu, indicating that gentamicin efficiently killed extracellular bacteria. After washing three times with PBS, intracellular bacteria were released by saponin

treatment and the number of cfu was determined as described above. All samples were assayed in triplicate, and each assay was repeated at least four times. The statistical significance of data was determined by the Students *t*-test. *P* values of <0.05 were considered significant.

### Transmission electron microscopy (TEM)

The epithelial cell monolayers were infected with bacteria at an moi of 100 at 37°C for 2 h. The infected cell monolayers were fixed with 2% glutaraldehyde, post fixed with 1% osmium tetroxide, dehydrated, infiltrated and embedded in epoxy resin for sections. Thin sections of the cells were contrasted with uranyl acetate and lead citrate, and observed under a Hitachi HU-12A electron microscope.

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