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Comparative Roles of the Arg-Gly-Asp Sequence Present in the *Bordetella pertussis* Adhesins Pertactin and Filamentous Hemagglutinin

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Pertactin and filamentous hemagglutinin (FHA), proteins present on the surface of the gram-negative organism *Bordetella pertussis*, have been shown to contain the putative cell-binding sequence arginine-glycine-aspartic acid (RGD) and to promote eukaryotic cell attachment. The attachment of epithelial cells to purified pertactin and the entry of *B. pertussis* into human HeLa cells are both inhibited by an RGD-containing peptide derived from the pertactin sequence. In contrast, an RGD-containing peptide derived from the FHA sequence has no effect on either the attachment of epithelial cells to purified FHA or the entry of *B. pertussis* into HeLa cells. *Staphylococcus aureus* organisms coated with pertactin or FHA, purified from *B. pertussis*, enter HeLa cells more efficiently than *S. aureus* cells coated with bovine serum albumin. The pertactin-enhanced entry of *S. aureus* is inhibited by 75% in the presence of the RGD peptide from pertactin, whereas the RGD peptide derived from FHA has no effect on the increased entry promoted by the pertactin-coated or by the FHA-coated *S. aureus*. These results indicate that the active uptake of *B. pertussis* by certain mammalian cells may be mediated by the interaction of the RGD site found in pertactin with eukaryotic cell surface receptors.

Bordetella pertussis expresses virulence-associated attachment factors including filamentous hemagglutinin (FHA) (2, 23, 24) and pertactin (2, 15) as well as a number of toxins such as pertussis toxin and adenylate cyclase toxin which contribute to an infectious process resulting in the human respiratory illness whooping cough (25). Although the mechanism of *B. pertussis* pathogenesis is not well understood, adherence to the host cell is most likely one of the first steps in infection. It is not known whether *B. pertussis* can invade and survive within epithelial cells during the natural course of infection. Recently, it has been shown that virulent *B. pertussis* as well as *Bordetella parapertussis* can enter human epithelial cells in vitro through a phagocytic, microfilament-dependent process (8, 9, 13), an event that could further complicate our understanding of the mechanism(s) of *Bordetella* pathogenesis. Avirulent strains cannot attach to or enter mammalian cells (8, 13, 24), suggesting that bacterial adherence and penetration are mediated by proteins which are regulated by the virulence gene operon (*vir* or *bvg*) of *B. pertussis* (1, 21). It has not yet been established which of these *vir*-regulated proteins are involved in each of these processes. Some *B. pertussis* Tn*5* or Tn*5* *lac* insertion mutants, which do not express either FHA or pertussis toxin, are not effectively internalized by HeLa cells (8); however, certain other pertussis toxin-negative mutants or FHA-negative mutants (8, 13) have been shown to effectively invade HeLa cells.

Molecular analyses of FHA (7, 17) and pertactin (5) have shown that both contain the amino acid triplet arginine-glycine-aspartic acid (RGD), an amino acid sequence which functions as a cell-binding site in a number of mammalian proteins (20). We have previously shown that the RGD sequence of pertactin is involved in the adherence of mammalian cells to the purified pertactin protein (15). In this study, we used synthetic RGD-containing peptides, corresponding to pertactin or FHA sequences, to investigate whether interactions of *B. pertussis* with mammalian cells may be mediated through this potential cell-binding site.

MATERIALS AND METHODS

Materials. Preparations of purified pertactin were obtained from Pavel Novotny, Wellcome Biotech, Kent, United Kingdom, and Pasteur Merieux Sérums et Vaccines, Marcy l'Etiole, France. FHA was provided by Pasteur Merieux Sérums et Vaccines. The antipertactin monoclonal antibody (MAb) BPE3 has been described previously (4), and the MAbs E4D7 and F6E5, directed against pertactin, were gifts from Iver Heron, Statens Seruminstitut, Copenhagen, Denmark. MAbs X3C and X4B are specific for FHA and were established at the Laboratory of Cellular Physiology, Center for Biologics Evaluation and Research, Food and Drug Administration.

Cell attachment to bacterial proteins. Epithelial cells were attached to pertactin-coated or FHA-coated 96-well plastic plates (Costar no. 3590) as described previously for pertactin (15). Chinese hamster ovary (CHO; ATCC CCL L61) cells were grown in Ham's F12 medium (S&S Media, Rockville, Md.) supplemented with 2 mM glutamine, gentamicin (Flow Laboratories) at 20 μg/ml, and 10% fetal calf serum (Hyclone). HeLa 229 cells (ATCC CCL 2.1) were cultured in Dulbecco's modified Eagle's medium (Flow Laboratories) containing similar supplements. The cells were labeled overnight with [^{3}H]thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq; Dupont, NEN Research Products) at 25 μl/75-cm^2 flask to a specific activity of 1 to 2 cpm per cell. Plastic wells were coated with purified pertactin or FHA at 30 μg/ml (60 μl per

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well). For inhibition experiments, ^{3}H-labeled mammalian cells (5×10^5 cpm/ml) were mixed with the RGD-containing peptides to a final peptide concentration of 1 mg/ml. The cell-peptide mixture (200 μl) was added to each protein-coated well and incubated at 37°C for 1 h. After unattached cells were gently washed out three times with 100 μl of Dulbecco's phosphate-buffered saline (DPBS) per well, attached cells were solubilized in 10% sodium dodecyl sulfate (SDS) and quantitated by liquid scintillation counting.

***B. pertussis* entry into HeLa cells.** The entry of *B. pertussis* BP338 into HeLa cells was performed as described previously (9). *B. pertussis* BP338, a naladixic acid-resistant derivative of Tohama I, was obtained from Alison Weiss, Virginia Commonwealth University, Richmond. Human epithelial cells, HeLa 229, were seeded 18 h before the assay in 24-well tissue culture wells at approximately 7×10^4 cells per well in Eagle's minimal essential medium (MEM) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 3% fetal calf serum (Flow Laboratories, Inc., McLean, Va.) (MEM–3% FCS). Approximately 9×10^6 CFU of *B. pertussis* in 400-μl aliquots was added to wells containing monolayers of HeLa cells which had been washed three times in MEM–3% FCS, and incubation continued at 37°C in 5% CO_2 for 5 h. The monolayers were then washed twice and incubated with 1 ml of MEM–3% FCS plus gentamicin (100 μg/ml) (GIBCO) for 2 h to kill all remaining extracellular bacteria. After additional washing, the epithelial cells were lysed, and intracellular bacteria were harvested and plated onto Bordet-Gengou agar (Difco Laboratories, Detroit, Mich.) for quantitation of viable intracellular CFU. In some experiments, gentamicin was not added, and the total number of CFU associated with the epithelial cells was determined. For peptide inhibition studies, RGD-containing peptides were diluted to 10 μg/ml in MEM–3% FCS, and 400 μl of each sample was added to washed HeLa cell monolayers and incubated for 1 h at 37°C before the bacteria were added. At the concentrations of peptides used in these assays, no alteration of HeLa cell adherence to the tissue culture plate was observed. For antibody inhibition experiments, the MAbs F6E5 and X3C were preincubated with the *B. pertussis* suspension at a final concentration of 50 μg of immunoglobulin G (IgG) per ml of bacteria for 1 h at 37°C and then added to the epithelial cell monolayer as described above.

***Staphylococcus* internalization assay.** *Staphylococcus aureus* 12598 (NCTC 8530) was purchased from American Type Culture Collection (Rockville, Md.) and cultured on plates of Luria broth (LB) agar at 37°C for 18 to 21 h. Cells were harvested in 10 ml of DPBS and adjusted to 2×10^9 bacteria per ml. They were then incubated in a rotating mixer for 90 min at room temperature with 50 μg of pertactin, FHA, or bovine serum albumin (BSA) per ml in DPBS. This concentration of protein resulted in optimal adsorption of protein to the surface of the *S. aureus* cells as determined by preliminary experiments with various concentrations of protein. To estimate the amount of pertactin or FHA bound, aliquots of bacterial lysates in 0.1% SDS sample buffer were separated on an SDS–4 to 20% polyacrylamide gradient gel (Integrated Separation Systems, Hyde Park, Md.) and then transferred to Immobilon (Millipore Corp., Bedford, Mass.) by electroblotting. Western blots (immunoblots) were performed as previously described (4), using purified antipertactin MAb BPE3 at a 1:1,000 dilution and ascites of the anti-FHA MAb X3C at a 1:500 dilution. Alkaline phosphatase-labeled goat anti-mouse Ig (Sigma) and nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (NBT/BCIP) substrates (Promega, Madison, Wis.) were used to visualize the specific protein bands. The protein bands were quantified by using the calculated peak area obtained with the NIH Image 1.43 gel analysis system.

After three washes in DPBS, the protein-coated *S. aureus* cells were resuspended in Dulbecco's modified Eagle's medium containing 2% FCS, and 100-μl aliquots (2×10^7 bacteria) were added to HeLa cell monolayers in 24-well plates and incubated for 90 min at 37°C. Intracellular bacteria were determined as described above for *B. pertussis*. For peptide inhibition studies, the HeLa cell monolayer was incubated at room temperature with 100 μl of a 100-μg/ml peptide solution per well for 30 min before the addition of the protein-coated *S. aureus* cells. Wells were visually examined at the end of the incubation period to verify the integrity of the epithelial cell monolayer.

Electron microscopy. *B. pertussis* 18323 (Laboratory of Pertussis culture collection, Center for Biologics Evaluation and Research, Food and Drug Administration) was incubated with HeLa cell monolayers for 4 h as described above for the entry assays except that the incubation buffer was Hanks balanced salt solution containing 0.2% BSA. The monolayers were washed three times with Hanks balanced salt solution and treated with cold 2.5% glutaraldehyde for 30 min. The cells were then washed with Hanks balanced salt solution containing 0.5% BSA, removed by scraping, and centrifuged. The cell pellet was resuspended in 500 μl of DPBS containing 50 μl each of ascitic fluid from MAb E4D7 (an IgG), directed against pertactin, and MAb X4B (an IgM), which is reactive with FHA, and incubated for 2 h at 25°C. The cells were washed several times with DPBS and then incubated for 1 h with 100 μl of gold-conjugated goat anti-mouse IgG antibody (15-nm gold particles; Auroprobe; Amersham International, Amersham, United Kingdom) and 100 μl of gold-conjugated goat anti-mouse IgM antibody (5-nm gold particles). After being washed, the cells were fixed in 2.5% glutaraldehyde for 1.5 h, washed, and stored in PBS. The samples were embedded and stained as described previously (4) and viewed in a Hitachi HU 12A transmission electron microscope.

RESULTS

We have shown in earlier studies that the *B. pertussis* surface protein pertactin can promote the attachment of epithelial cells such as Chinese hamster ovary (CHO) cells (15) (Fig. 1A). As shown in Fig. 1B, CHO cells can also attach to another proposed bacterial attachment protein from *B. pertussis*, FHA. Other human epithelial cell lines such as HeLa and WiDr cells also specifically bind to both pertactin- and FHA-coated plastic wells (data not shown). Since both bacterial proteins contain RGD sequences, which are known to function as cell attachment sites in some adhesive proteins (20), we compared the effects of synthetic RGD peptides, corresponding to pertactin and FHA sequences (Table 1), on the attachment of mammalian cells to these purified bacterial proteins. In agreement with previous results (15), an RGD-containing peptide (PRN-RGD) synthesized from the predicted sequence of pertactin inhibited attachment of CHO cells to purified pertactin (Fig. 1A). The concentration of peptide used in these experiments, 1 mg/ml, is similar to that required for inhibition of the interaction of cells with eukaryotic cell attachment proteins such as fibronectin (16). This PRN-RGD peptide did not, however, inhibit attachment of cells to FHA (Fig. 1B). Moreover, neither the RGD-containing peptide corresponding to the FHA sequence (FHA-RGD) nor the control peptide (PRN-

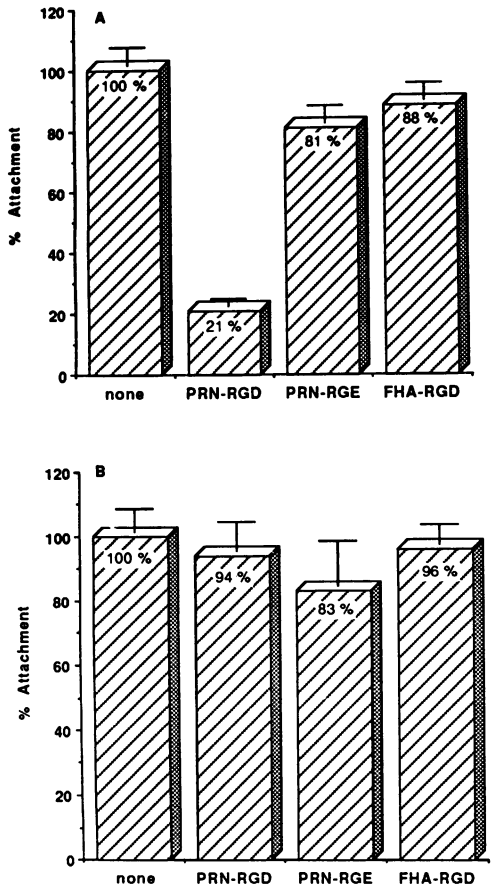

FIG. 1. Effects of peptides on CHO cell attachment to purified pertactin and FHA. Plastic wells were coated with purified pertactin (A) or FHA (B) at 30 μg/ml. Peptides and ^{3}H-CHO cells (5×10^5 cpm/ml) were diluted together to a final peptide concentration of 1 mg/ml and a cell concentration of 2.5×10^5 cells per ml. A total of 200 μl was then added to each well. Data represent an average of duplicate wells. Experiments were repeated at least two times. Standard error bars are shown. Inhibition of CHO cell attachment to pertactin was statistically significant for PRN-RGD ($P \leq 0.03$).

RGE) had a significant effect on CHO cell attachment to either pertactin or FHA (Fig. 1).

Recently, we have shown that *B. pertussis* can invade and survive within HeLa cells in culture (9). Electron microscopic examination of this interaction suggests an active phagocytic uptake of the bacteria by the HeLa cells (Fig. 2). Immunogold visualization of pertactin (large particles) and FHA (small particles) on the surface of the bacteria shows that both molecules are distributed over the surface of the bacteria. The MAbs seem to preferentially bind the distal hemisphere of the bacteria, which suggests that pertactin and FHA are occupied by receptors where they contact the HeLa cell surface.

TABLE 1. Synthetic RGD peptides

Protein	Sequence	Peptide
Pertactin	ATIRRGDALAGGAC	PRN-RGD
Pertactin[a]	ATIRRGEALAGGAC	PRN-RGE
FHA	VTVGRGDPHQGVLC	FHA-RGD

[a] The PRN-RGE peptide is a negative control peptide derived from the sequence of pertactin in which the aspartic acid residue (D) was changed to glutamic acid (E).

It is now apparent that *B. pertussis*, a microorganism previously described as noninvasive, can enter and survive within nonprofessional phagocytes (8, 9). Purified MAbs directed against pertactin (F6E5) or FHA (X3C) were assessed for their ability to inhibit this entry of *B. pertussis* into HeLa cells, using an assay that has been previously described (8; see also Materials and Methods). MAb F6E5 reduced the entry of *B. pertussis* into HeLa cells by 42% (Fig. 3). This MAb is directed against an amino-terminal epitope on pertactin which encompasses the RGD sequence as shown by epitope mapping with fusion proteins constructed from the pertactin gene (6). Other MAbs directed against the carboxy-terminal portion of pertactin have been shown to have no effect on invasion (8). The MAb specific for FHA (X3C) inhibited entry of *B. pertussis* by approximately 22%. This MAb has previously been shown to inhibit attachment of *B. pertussis* to mammalian cell monolayers by approximately 48% (14).

To further investigate the role of pertactin and FHA on the entry of *B. pertussis* into mammalian cells, we assayed the RGD-containing peptides for their ability to affect the invasion of HeLa cells by the bacteria. Peptides at a protein concentration of 10 μg/ml were incubated with HeLa cell monolayers for 1 h at 37°C. Bacteria were then added, and the invasion assay was performed as previously described (8). Only the RGD-containing peptide corresponding to the pertactin sequence, PRN-RGD, significantly inhibited the entry of *B. pertussis* into HeLa cells (Fig. 4). Neither the control RGE-containing peptide, PRN-RGE, nor the FHA-derived RGD peptide, FHA-RGD, had a significant effect on the entry of *B. pertussis*. These data suggest that the region of pertactin containing the RGD sequence has a more prominent role in promoting the entry of *B. pertussis* into HeLa cells than does FHA.

The ability of the *B. pertussis* proteins to directly promote bacterial entry was investigated by coating the gram-positive bacterium *S. aureus* with purified *B. pertussis* proteins and determining whether this treatment enhances entry of *S. aureus* into HeLa cells. *S. aureus* was used since this bacterium shows a low level of entry into HeLa cells under the conditions used here and the *B. pertussis* proteins bind well to its surface. *S. aureus* (2×10^9 bacteria per ml) was incubated with 50 μg of either pertactin or FHA per ml for 1 h. After thorough washing, 70% of the added pertactin and 97% of the FHA were found adsorbed to the surface of the bacteria as determined by Western blotting with MAbs specific for pertactin or FHA (Fig. 5) and by analysis with the NIH Image 1.43 gel analysis system. Compared with coating with BSA, FHA enhanced entry of the *S. aureus* cells by more than 10-fold while pertactin increased entry by approximately 4-fold (Fig. 6). To determine whether the RGD site on pertactin and FHA has a role in these enhanced bacterium-epithelial cell interactions, we also investigated the effect of the specific RGD-containing peptides on the *S. aureus* entry assay (Fig. 6). The PRN-RGD and FHA-RGD peptides had no inhibitory effect on the entry of the BSA-coated or the FHA-coated *S. aureus* cells into HeLa cells. However, the entry of the pertactin-coated *S. aureus* cells into HeLa cells was inhibited by approximately 75% in the presence of the RGD peptide homologous to the sequence of pertactin. No effect on the integrity of the HeLa cells was noted in these studies.

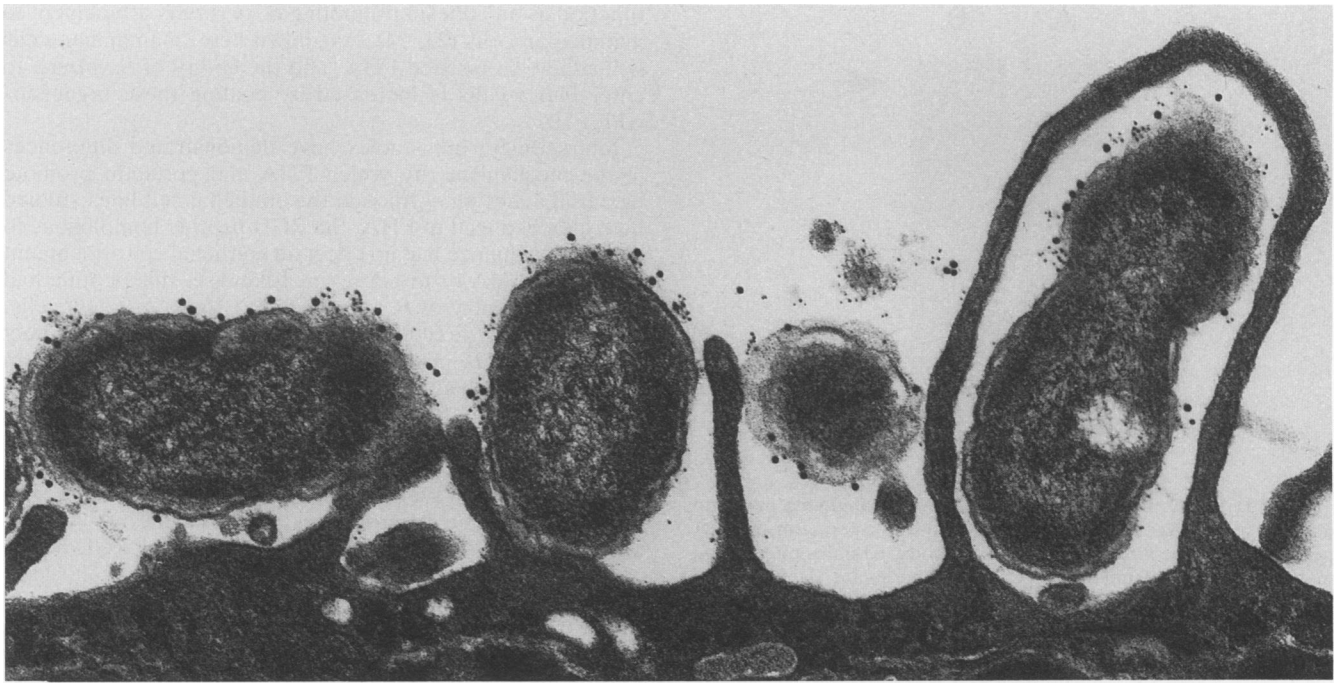

FIG. 2. Electron micrograph of *B. pertussis* interacting with HeLa cells. *B. pertussis* cells were incubated with HeLa cell monolayers for 4 h and reacted with MAbs E4D7 to pertactin and X4B to FHA and then with gold-conjugated antimouse Igs as described in Materials and Methods. The presence of pertactin is denoted by 15-nm gold particles, and the 5-nm particles designate the labeling of FHA (×60,000).

DISCUSSION

In this study, we showed that MAb F6E5, an antibody which binds to a region of pertactin containing the RGD sequence, inhibits internalization of *B. pertussis* into HeLa cells. Furthermore, an RGD-containing peptide with a sequence homologous to that found in pertactin can inhibit entry of virulent *B. pertussis* organisms into these human epithelial cells. Entry of the gram-positive organism *S. aureus*, which does not have pertactin on its surface, into HeLa cells can be increased more than fourfold by coating the surface of the bacteria with purified pertactin. The pertactin-enhanced entry of *S. aureus* into HeLa cells is also blocked efficiently and specifically (about 75% inhibition) by the pertactin-derived RGD peptide. Electron microscopy indicates that pertactin is localized around the surface of the bacteria, a prerequisite for invasin molecules which may

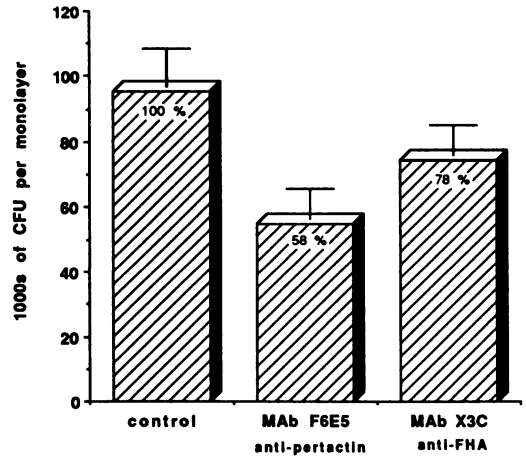

FIG. 3. Effects of MAbs on HeLa cell invasion by *B. pertussis* BP338. Bacteria were preincubated for 1 h with 50 μg of purified MAb per ml directed against either pertactin (F6E5) or FHA (X3C). The organisms were washed once and added to cell monolayers to assess invasiveness as described in an earlier publication (8). The bars represent the percent mean ± standard deviation of gentamicin-resistant CFU recovered from five independent experiments, except for MAb F6E5, for which $n = 3$. Inhibition by MAb F6E5 was statistically significant compared with the control ($P < 0.0005$), using the two-factor analysis of variance.

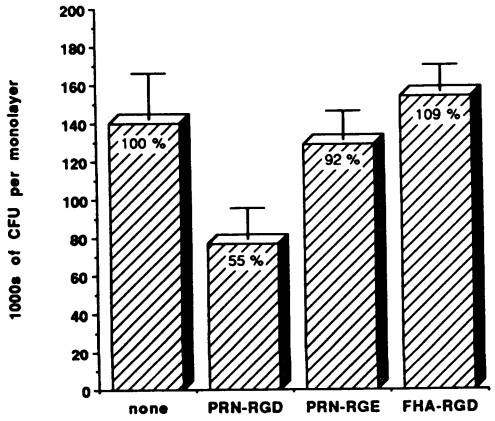

FIG. 4. Effects of peptides on *B. pertussis* BP338 entry into HeLa cells. Monolayers of HeLa cells were incubated with 10-μg/ml peptide solutions for 1 h at 37°C before the addition of the bacteria. Invasion was assessed as described in Materials and Methods. Results represent an average of triplicate determinations, and standard error bars are shown. Inhibition by PRN-RGD was statistically significant ($P \leq 0.005$) by Student's t test.

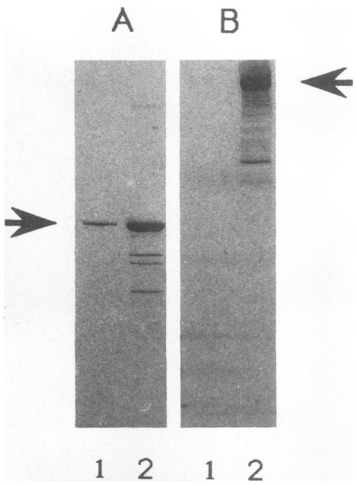

FIG. 5. Western blot analysis of *S. aureus* coated with pertactin or FHA. *S. aureus* was incubated with 50 μg of pertactin or FHA per ml as described in Materials and Methods. Samples of unbound material collected from the first wash supernatant (lanes 1) and samples of pelleted *S. aureus* after three buffer washes (lanes 2) were suspended in SDS sample buffer, separated on an SDS–4 to 20% polyacrylamide gradient gel, transferred to Immobilon-P, and probed with the antipertactin MAb BPE3 (A) or the anti-FHA MAb X3C (B). This analysis indicates that approximately 75% of the pertactin and almost all the FHA bound to the *S. aureus* cells. Arrows indicate the positions of the mature nondegraded forms of pertactin and FHA.

function via the zipper model of bacterial uptake (10, 11). Isberg and Leong (12) have provided evidence that the receptors for the invasin protein expressed by *Yersinia pseudotuberculosis* are integrins, a family of mammalian cell adhesin receptors that interact with RGD-containing proteins. We speculate that integrins may recognize the RGD region in pertactin, as suggested by our RGD inhibition studies, and subsequently promote *B. pertussis* internalization into mammalian cells.

There is also good evidence to indicate that FHA can function as an adhesin promoting *B. pertussis* attachment to mammalian cells (23, 24). As shown here, mammalian cells will adhere to purified FHA, and the ability of *S. aureus* to enter HeLa cells is increased by coating these organisms with FHA.

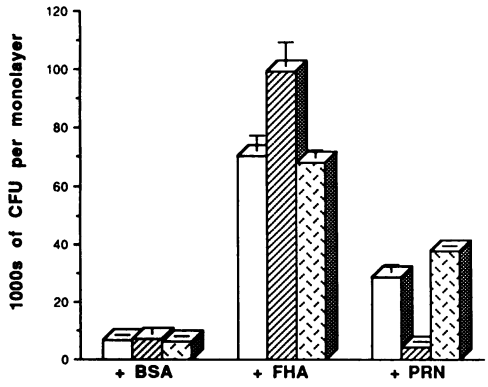

FIG. 6. Interaction of protein-coated *S. aureus* with HeLa cells. BSA-coated (+BSA), FHA-coated (+FHA), or pertactin-coated (+PRN) *S. aureus* cells were incubated with monolayers of HeLa cells for 4 h, and the gentamicin-resistant CFU were determined as described in Materials and Methods. Assays were performed with no peptide present (white bars) or in the presence of 0.1 mg of the pertactin-RGD peptide (striped bars) or the RGD peptide from FHA (stippled bars) per ml. Standard error bars are shown.

Interestingly, our studies have demonstrated differences in the mechanisms by which FHA and pertactin promote bacterial adhesion. Although the epithelial cell lines studied here attached well to FHA, the RGD peptide homologous to the FHA sequence had no effect on epithelial cell attachment to purified FHA or to pertactin. Likewise, this peptide had no effect on entry of *B. pertussis* into HeLa or CHO cells. The MAb X3C directed against FHA showed limited effect on invasion of *B. pertussis* into HeLa cells, although it can effectively inhibit attachment of the bacterium to mammalian cell monolayers (14). These observations suggest that the interaction of epithelial cells with FHA is not mediated through the RGD site on this bacterial adhesin. FHA has been shown, however, to interact with the CR3 integrin receptor found on macrophages (18), and the results presented here do not rule out a contribution of the RGD region of FHA in interactions of *B. pertussis* with other cell types. Note that the amino acids surrounding the RGD triplet found in pertactin and FHA are different and that sequences adjacent to RGD are believed to confer receptor specificity to proteins that attach to cells via an RGD-mediated process (20). The RGD region in FHA may specifically interact with CR3, an integrin found only on leukocytes, while, as the results presented here suggest, the RGD domain in pertactin may recognize integrins expressed by epithelial cells.

Our observations suggest that FHA is using other cell-binding sites to interact with different eukaryotic cells. Recently, a potential lectinlike site on FHA has been identified by sequence homology (7), and there is evidence that certain sugars can inhibit the attachment of *B. pertussis* to mammalian cells (3, 14, 22). It is therefore likely that FHA can recognize carbohydrate receptors on the surface of certain host cells and mediate bacterial attachment through a lectinlike interaction.

Studies with various *B. pertussis* mutants have also provided information about the adhesive and invasive properties of FHA and pertactin. Roberts et al. (19) have demonstrated that a *B. pertussis* Tohama I-derived mutant, which does not express FHA, is less efficient than the parent strain in invasion of HEp-2 cells. Another mutant, deficient in the expression of pertactin, does not show a significantly decreased internalization into these cells. They have also observed, however, that a mutant lacking both pertactin and FHA shows a further decrease in internalization, approaching the low levels seen with avirulent *B. pertussis* strains. There is also evidence showing that mutants derived from different *B. pertussis* strains (W28), deficient in pertactin, interact with CHO and HeLa cells less efficiently than their parent strain (15). We have also noticed that mutants lacking both pertactin and FHA attach to epithelial cells at a much lower level than mutants lacking either pertactin or FHA alone (2). These observations provide evidence that the two surface proteins may act in concert to promote efficient attachment of the pathogen to host cells, as has also been proposed for FHA and pertussis toxin double mutants (18).

It is always difficult to determine whether the measurement of entry of the bacterial ligand into host cells simply reflects the ability of these microorganisms to avidly interact with the surface of the host cell. In fact, the RGD peptides used for these studies do not effectively and/or reproducibly inhibit the total attachment (adhered and internalized) of *B.*

pertussis to epithelial cell monolayers (data not shown). This is most likely due to the presence of multiple adhesins on this pathogen which mediate bacterium-mammalian cell interactions via different pathways. We believe, however, that a specific effect of the RGD peptide homologous to the sequence of pertactin on entry of *B. pertussis* into epithelial cells has been shown in this report. At this time, a more complete molecular analysis of pertactin-mediated bacterial entry is required before pertactin can be defined as an invasin protein. The possibility that specific integrins actively promote the entry of *B. pertussis* into human cells after binding pertactin also warrants further study.

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