

# Cholesterol-dependent attachment of human respiratory cells by *Bordetella pertussis*

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## Keywords

*Bordetella pertussis*; attachment; cholesterol.

## Abstract

*Bordetella pertussis* is a re-emerging human respiratory pathogen whose infectious process is not fully understood, hampering the design of effective vaccines. The nature of bacterial attachment to host cells is a key event in the outcome of the infection. However, host cell receptors involved in *B. pertussis* colonization of the respiratory tract are still under investigation. Here, we report that cholesterol-rich domains are involved in *B. pertussis* adhesion to epithelial cells. Treatment of A549 cells with cholesterol-sequestering drugs such as methyl- $\beta$ -cyclodextrin, nystatin, or filipin resulted in a significant decrease of *B. pertussis* attachment. Confocal laser microscopy studies showed *B. pertussis* associated with cholesterol-rich domains. Accordingly, *B. pertussis* was found in detergent-resistant membrane domain fractions isolated from bacterial-infected A549 cells. Our results indicate a main role of filamentous hemagglutinin, an environmentally regulated virulence factor, in this interaction, and a specific affinity for cholesterol, one of the major components of traqueal secretions, which might additionally contribute to the effective colonization of the respiratory tract.

## Introduction

*Bordetella pertussis* is the etiologic agent of whooping cough, a re-emerging infectious disease. Upon entering the human host, the bacterium initiates the colonization of the respiratory tract by adhering to epithelial cells, which is a central phenomenon in the pathogenesis of *B. pertussis*. The interaction between virulent *B. pertussis* and the host involves several factors. Some of them are fairly well characterized, such as filamentous hemagglutinin (FHA), a major adhesin of this pathogen, and its docking molecules, namely lactosyl ceramides of ciliated respiratory cells (Prasad *et al.*, 1993), sulfated sugars at the surface of epithelial cells (Hannah *et al.*, 1994), and integrins in leukocytes (Relman *et al.*, 1990). However, much of this interaction is still unknown.

In recent years, it has been recognized that a variety of pathogens interact with microdomains in the plasma membrane known as lipid rafts, which eventually determines the outcome of the infection (Zaas *et al.*, 2005). Rafts are membrane microdomains enriched in cholesterol and glycosphingolipids. Lipid rafts are characterized by their insolubility in cold Triton X-100 and have thus been termed detergent-resistant membrane domains (DRMs). Because of their high lipid content, these detergent-insoluble do-

main float to a low density during gradient centrifugation (Brown & Rose, 1992). An important feature of lipid rafts is their highly dynamic nature, which allows the transient formation of membrane platforms that build up molecular machineries implicated in cell signaling (Shaul & Anderson, 1998) and endocytosis (Nichols, 2003). Several bacteria have been found to co-opt the mechanism of lipid rafts-mediated endocytosis in order to adhere, invade, and remain viable inside host cells (Zaas *et al.*, 2005). Likewise, we recently found plasma membrane cholesterol domains to play a crucial role in neutrophils (PMN) uptake of non-opsonized *B. pertussis* and bacterial intracellular survival (Lamberti *et al.*, 2008). Here we investigated whether cholesterol is also involved in *B. pertussis* interaction with epithelial respiratory cells. The results indicate that lipid rafts, and cholesterol itself, play a significant role in bacterial interaction with epithelial cells and FHA is a key factor in this process.

## Materials and methods

### Bacterial strains and growth conditions

*Bordetella pertussis* strain B213, a streptomycin-resistant derivative of Tohama I, was transformed with plasmid

pCW505 (Weingart *et al.*, 1999) (kindly supplied by Dr Weiss, Cincinnati, OH), which induces cytoplasmic expression of green fluorescent protein (GFP) without affecting growth or antigen expression (Weingart *et al.*, 1999). In some experiments, BPGR4, a Tohama-derived mutant lacking expression of FHA (Locht *et al.*, 1992) transformed with plasmid pCW505, was used. Bacteria were stored at  $-70^{\circ}\text{C}$  and recovered by growth on Bordet Gengou agar (BGA) plates supplemented with 15% defibrinated sheep blood (bBGA) at  $35^{\circ}\text{C}$  for 3 days. Virulent bacteria were subsequently plated on bBGA, cultured for 20 h at  $35^{\circ}\text{C}$  and used in adhesion assays.

In some experiments, *Escherichia coli* DH5 $\alpha$  transformed with plasmid PML2, a pSM10 (Selbitschka *et al.*, 1995) derivative, which induces cytoplasmic expression of GFP (a generous gift from Dr Lagares, Institute of Biochemistry and Molecular Biology, La Plata, Argentina), was used.

### Cells and growth conditions

The human alveolar epithelial cell line A549 (human lung type II pneumocyte) (ATCC CCL185, Rockville, MD) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (FBS),  $100\text{ U mL}^{-1}$  penicillin, and  $100\text{ mg mL}^{-1}$  streptomycin (Sigma, St. Louis, MO) at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . Routine subcultures for A549 pneumocytes were performed at 1:3 split ratios by incubation with 0.05% trypsin-0.02% EDTA for 5 min at  $37^{\circ}\text{C}$ . For the *Bordetella* adherence assay, epithelial cells were cultured to 80% confluence.

### Antibodies

The following antibodies were used: mouse monoclonal antibodies (mAbs) against human caveolin-1 (PharMingen, San Diego, CA), mouse mAb against the human transferrin receptor (PharMingen), and polyclonal rabbit antibody against human flotillin-1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunoglobulin G (IgG) fractions from pooled sera of pertussis patients with high titers against *B. pertussis*, as measured by enzyme-linked immunosorbent assay (Nagel *et al.*, 1985), were isolated as described previously (Rodriguez *et al.*, 2001). Polyclonal mouse anti-FHA *B. pertussis* antiserum was generated as described before (Sato & Sato, 1984).

### Treatment of A549 cells with cholesterol-affecting drugs

Cholesterol sequestration was achieved by incubating A549 cells with 10 mM of methyl- $\beta$ -cyclodextrin (M $\beta$ CD) (Sigma) (15 min at  $37^{\circ}\text{C}$ ), or  $35\text{ }\mu\text{g mL}^{-1}$  of nystatin (Sigma) (30 min at  $37^{\circ}\text{C}$ ), or  $5\text{ }\mu\text{g mL}^{-1}$  of filipin (Sigma) (30 min

at  $37^{\circ}\text{C}$ ) in serum-free DMEM medium plus bovine serum albumin (BSA) (0.2%) and lovastatin ( $5\text{ }\mu\text{g mL}^{-1}$ ) (Sigma) (DMEM-BSA-L). Cells were then washed, suspended in DMEM-BSA-L, and used immediately. No decrease in cell viability was detected after treatment as determined by trypan blue exclusion. In selected experiments, cholesterol replenishment was performed by incubation of M $\beta$ CD-treated A549 cells with  $8\text{ mg mL}^{-1}$  of water soluble cholesterol (Sigma) in DMEM-BSA for 30 min at  $37^{\circ}\text{C}$  or with FBS (20%) in DMEM-BSA for 3 h at  $37^{\circ}\text{C}$ .

Total cellular cholesterol in treated and untreated A549 cells was determined using the Amplex Red cholesterol assay kit (Molecular Probes). Under the experimental conditions used, the  $54 \pm 2\%$  of total cellular cholesterol was removed by M $\beta$ CD treatment. The cellular cholesterol content was found to be restored ( $98 \pm 1\%$ ) upon replenishment.

### Binding assays

Attachment assays were performed as described in Rodriguez *et al.* (2006), with minor modifications. Briefly, cells were seeded on glass coverslips in 24-well tissue culture plates (Nunc, Rockside, Denmark) and incubated for 18 h. Monolayers were washed twice with sterile phosphate-buffered saline (PBS) and treated with or without cholesterol-affecting drugs as described above. Cells were then incubated with either GFP-expressing *B. pertussis* at a multiplicity of infection (MOI) of 150 bacteria per cell for 2 h at  $37^{\circ}\text{C}$  or  $17\text{ }\mu\text{g mL}^{-1}$  of purified native FHA (Glaxo-SmithKline) in DMEM-BSA-L for 1 h at  $37^{\circ}\text{C}$ . To facilitate bacterial interaction with epithelial cells, plates were centrifuged for 5 min at 640 g. No changes in the number of viable A549 cells were detected after incubation with either bacteria or FHA. After incubation, the monolayers were washed with DMEM plus BSA (0.2%), before fixation with paraformaldehyde. The number of adherent bacteria per cell was estimated by fluorescence microscopy examination of 20 randomly selected fields showing a minimum of seven epithelial cells per field. The association of FHA with A549 cells was investigated both by fluorescence microscopy and by flow cytometry (Becton Dickinson, Lincoln Park, NJ). To this end, A549 cells treated with or without PBS-EDTA (0.2%) were incubated with anti-FHA mouse serum, followed by incubation with an fluorescein isothiocyanate-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse IgG (Jackson ImmunoResearch, West Grove). All experiments were performed at least three times in duplicate.

In some experiments, GFP-expressing *B. pertussis* ( $6 \times 10^6$ ) were incubated with  $1.8\text{ }\mu\text{g mL}^{-1}$  of cholesterol (Sigma) in DMEM medium plus BSA (0.2%) for 1 h at  $37^{\circ}\text{C}$ . After three washing steps, bacteria were incubated with A549 cells at a MOI of 150.

In control experiments, attachment of GFP-expressing *E. coli* DH5 $\alpha$  was evaluated as described above at a MOI of 500.

### Cholesterol-binding studies

Bacterial cholesterol-binding capacity was investigated as described elsewhere (Rostand & Esko, 1993) with some modifications. Briefly, 1  $\mu$ g of cholesterol diluted in ethanol was dried onto a microtiter plate at room temperature. Cholesterol-treated plates were then washed six times with PBS and blocked with 1% BSA in PBS for 1 h at 37 °C. Bacteria (wild type or BPGR4 strain) were added to the plates ( $2 \times 10^7$  bacteria per well) and incubated for 1 h at 37 °C. Non-adherent bacteria were removed by three washing steps with PBS. Human IgG anti-*B. pertussis* antibody was added to each well, and plates were incubated at 30 °C for 2 h. After three washing steps, anti-human IgG-peroxidase conjugate antibodies were added to each well. The plates were then incubated at 37 °C for 2 h. After three washing steps, the enzyme reaction was initiated by the addition of *O*-phenylene diamine (0.1 mg mL<sup>-1</sup>) and H<sub>2</sub>O<sub>2</sub> (30 volumes in 100 mL citrate-phosphate buffer, pH 5). After 15 min in the dark at room temperature, the reaction was stopped by the addition of H<sub>2</sub>SO<sub>4</sub> (4N). Bacteria attached to the wells were estimated by the A<sub>492nm</sub>. Wells coated with ethanol or blocking agent alone were used as control of nonspecific binding.

### Cell fractionation and isolation of DRMs

DRMs of A549 cells incubated either with *B. pertussis* or FHA were isolated as follows: A549 cells ( $1 \times 10^7$  cells) grown at 80% confluence were incubated for 2 h at 37 °C with *B. pertussis* at a MOI of 150 bacteria per cell, or for 1 h at 37 °C with 17  $\mu$ g mL<sup>-1</sup> of purified FHA. Triton-insoluble cell components were then isolated according to a previously published protocol (Chamberlain *et al.*, 2001). In brief, cells were washed twice with cold PBS and scraped from the dishes into PBS. The cell pellet was suspended in ice-cold Tris-buffered saline (TBS) (20 mM Tris, pH 7.4, 1 mM EDTA, and 140 mM NaCl) containing 1% Triton X-100, 1 mM phenylmethylsulfoxide, and 1% protease inhibitor cocktail (Sigma), and the cells were passaged 10 times through a 27G1/2 needle. The cell lysate was centrifuged at 800 g for 10 min at 4 °C, and the postnuclear supernatant was adjusted to 40% sucrose in TBS, placed at the bottom of an SW41 centrifuge tube (Beckman Instruments) and layered with 6 mL of 30% sucrose in TBS and 3 mL of 5% sucrose in TBS. After centrifugation at 247 000 g for 15 h at 4 °C in a swinging bucket rotor (model SW41, Beckman Instruments), 1-mL fractions were collected from the top of the gradient (fraction 1) to the bottom (fraction 12). Proteins from each fraction were precipitated with 10%

trichloroacetic acid in the presence of 0.02% sodium deoxycholate as a carrier. Samples were suspended in 50  $\mu$ L of PBS and stored at -20 °C until use.

In some experiments, cells were treated with 0.5% Triton X-100 plus 0.5% saponin (Sigma), a lipid raft-disrupting drug.

### Analysis of sucrose density-gradient fractions

Sucrose density-gradient fractions of cells incubated with or without *B. pertussis* were analyzed for the presence of bacteria, caveolin, and transferrin receptor by dot-blot assays as follows: equal volumes (10  $\mu$ L) of each fraction were spotted onto polyvinylidene fluoride (Immobilon PVDF, Millipore) and allowed to dry. Membranes were incubated for 2 h at room temperature in a blocking solution (PBS, 5% BSA), and then exposed for 2 h at room temperature to mouse monoclonal anti-caveolin-1 antibodies, mouse monoclonal anti-transferrin receptor antibodies, or human IgG anti-*B. pertussis* antibodies. After washing, specific binding of antibodies was visualized using alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, Baltimore Pike) or alkaline phosphatase-conjugated goat anti-human IgG (Jackson ImmunoResearch, Baltimore Pike) as secondary antibodies, and nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) (Bio-Rad) as a substrate.

Sucrose density-gradient fractions of cells incubated with or without FHA were analyzed by SDS-PAGE and immunoblotting. Fractions 1 and 2, 9 and 10, and 11 and 12 were pooled to allow immunoblot analysis of all gradient fractions on a single polyacrylamide gel. Samples were suspended in Laemmli sample buffer and run on 7% sodium dodecyl sulfate-polyacrylamide gel. Proteins were transferred to PVDF sheets (Towbin *et al.*, 1979) and incubated with mouse polyclonal anti-FHA, mouse monoclonal anti-caveolin-1, or mouse monoclonal anti-transferrin receptor antibodies. Immunochemical detection was performed using alkaline phosphatase-conjugated goat anti-mouse IgG and NBT/BCIP (Bio-Rad) as a substrate.

### Confocal microscopy

A549 cells were incubated with *B. pertussis* expressing GFP (MOI, 100) at 37 °C for 2 h. Nonadherent bacteria were removed by washing with PBS. Cells were then fixed using paraformaldehyde. After fixation, A549 cells were washed twice with PBS, and incubated for 10 min at room temperature with PBS containing 50 mM NH<sub>4</sub>Cl. After two washing steps, cells were permeabilized by incubation with PBS containing 0.1% saponin and 0.5% BSA for 30 min. Next, cells were incubated overnight at 4 °C with anti-flotillin antibodies in the presence of 0.1% saponin and 0.5%

BSA. After three washing steps, cells were incubated with a CY-3-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse IgG (Jackson ImmunoResearch, West Grove) for 30 min at 4 °C. To avoid cytoplasmic binding of antibodies, all incubations were performed in the presence of 25% heat-inactivated human serum. Microscopic analyses were performed using a confocal laser-scanning microscope (Olympus FV 300, Japan). The percentage of bacteria colocalizing with flotillin was calculated by analyzing at least 50 cells in randomly selected fields.

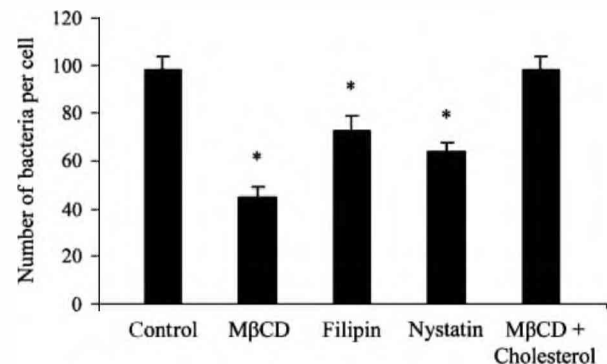
### Statistical analysis

Student's *t*-test (confidence level, 95%) or ANOVA was used for statistical data evaluation. The significance of the differences between the mean values of the data evaluated by ANOVA was determined using the least significant difference test at a confidence level of 95%. Results are shown as means and SD.

### Results and discussion

Adhesion is a central phenomenon in the pathogenesis of *B. pertussis*. Adhesins and their counterparts in host cells play a substantial role in determining the outcome of a prokaryotic–eukaryotic interaction. Recent observations highlighted the role of cholesterol-rich domains in microbial pathogenicity. Lipid rafts are involved in the attachment, cell entry, and intracellular survival of several microorganisms such as *Brucella suis* (Naroeni & Porte, 2002), *Chlamydia trachomatis* (Jutras et al., 2003), and *Mycobacterium bovis* (Gatfield & Pieters, 2000). A recent report of our group showed that cholesterol-rich domains are involved in *B. pertussis* interaction with human neutrophils and shape bacterial trafficking inside the immune cell, eventually leading to bacterial survival (Lamberti et al., 2008).

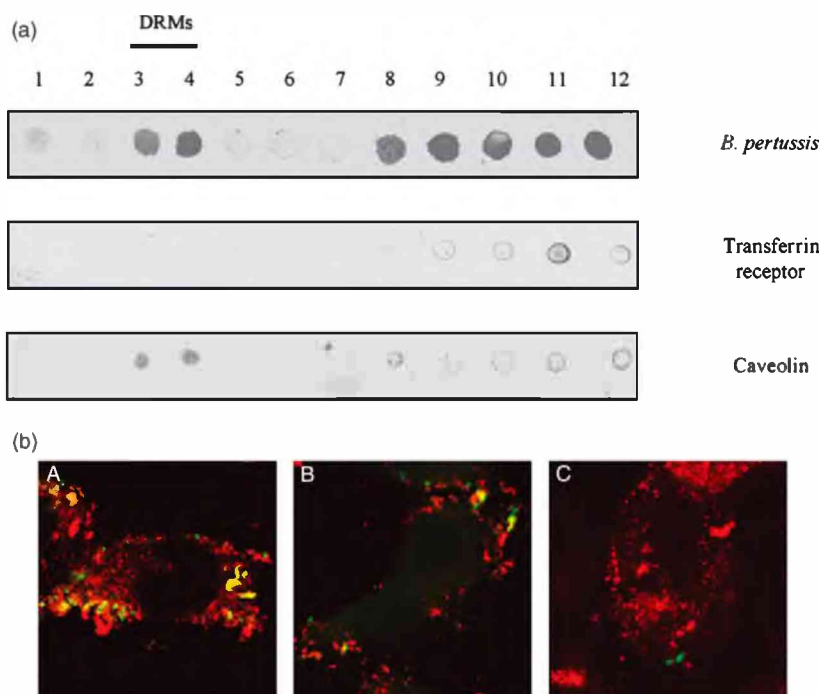
The ability of *B. pertussis* to adhere to the respiratory epithelium is pivotal in the subsequent development of infection. Here we investigated whether plasma membrane cholesterol is involved in this process. To this end, we used A549 cells, a human alveolar cell line previously used to study *B. pertussis* interaction with human respiratory epithelial cells (Ishibashi et al., 2001; Perez Vidakovic et al., 2006; Rodriguez et al., 2006). Cholesterol was depleted from cell plasma membrane by incubation with M $\beta$ CD. Because M $\beta$ CD does not interfere with cholesterol synthesis, the experiments described here were conducted in the presence of lovastatin, a drug that inhibits *de novo* cholesterol synthesis. As can be seen in Fig. 1, cholesterol depletion resulted in a significant decrease of virulent *B. pertussis* attachment (55%). The role of cholesterol in this process was further confirmed by incubation of A549 cells with cholesterol-binding drugs such as nystatin or filipin. Cells treated with any of the latter drugs showed a significant



**Fig. 1.** *Bordetella pertussis* attachment to A549 cells is dependent on cholesterol. A549 cells treated without (control) or with 10 mM of M $\beta$ CD, 5  $\mu$ g mL<sup>-1</sup> of filipin, or 35 mg mL<sup>-1</sup> of nystatin were incubated with GFP-expressing *B. pertussis* (MOI, 150) for 2 h at 37 °C. In selected experiments, A549 cells treated with M $\beta$ CD were further incubated with 8 mg mL<sup>-1</sup> of water-soluble cholesterol in DMEM–BSA (M $\beta$ CD+cholesterol) before incubation with *B. pertussis*. Bacterial attachment was evaluated by fluorescence microscopy. The data represent the mean  $\pm$  SD of four independent experiments. The number of *B. pertussis* attached to A549 cells treated with M $\beta$ CD, nystatin, or filipin differed significantly from the number of *B. pertussis* attached to either untreated cells or cells treated with M $\beta$ CD and further incubated with water-soluble cholesterol (\**P* < 0.05).

decrease in bacterial binding (Fig. 1). In control experiments, we studied the effect of M $\beta$ CD treatment of epithelial cells on the attachment of *E. coli* DH5 $\alpha$ , previously demonstrated to be independent of plasma membrane cholesterol (Pucadyil et al., 2004). As expected, the treatment of cells with M $\beta$ CD did not affect the binding capacity of *E. coli* (2.5  $\pm$  0.9 and 2.3  $\pm$  0.7 *E. coli* per cell in untreated and M $\beta$ CD-treated A549 cells, respectively). In order to determine whether the decrease in *B. pertussis* attachment was specifically due to cholesterol removal, we next replenished the cholesterol content of M $\beta$ CD-treated cells by incubation with either cholesterol water-soluble or FBS. The increase in the cellular cholesterol content led to a concomitant increase in the ability of the bacteria to attach to the cell surface (Fig. 1), underlining the specific requirement of cholesterol in the infection process.

Cholesterol is a major sterol of mammalian cells. More than 90% of cellular cholesterol is located at the plasma membrane. Cholesterol is not uniformly distributed in the membrane, but is preferentially confined to lipid rafts, membrane microdomains enriched in cholesterol, glycolipids, and glycosylphosphatidylinositol-anchored molecules. Lipid rafts form insoluble complexes (DRMs) upon membrane solubilization with cold Triton X-100 (Rietveld & Simons, 1998). We next investigated whether *B. pertussis* interacts with lipid rafts by subcellular fractionation of A549-infected cells on a sucrose gradient. DRMs are typically isolated in low-density fractions. Figure 2a shows that

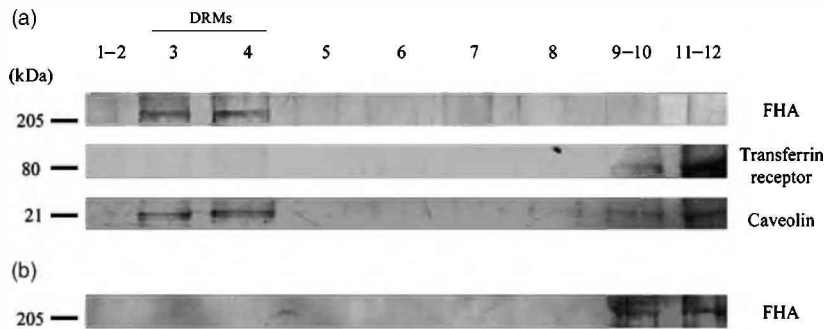


**Fig. 2.** Lipid rafts are implicated in *Bordetella pertussis* interaction with host cells. (a) A549 cells were incubated with *B. pertussis* for 2 h at 37 °C before subcellular fractionation. Equal volumes of each fraction were analyzed by a dot-blot analysis. Fractions were probed with IgG antibodies against *B. pertussis*, antibodies against the transferrin receptor and antibodies against caveolin. Representative results of one out of three independent experiments are shown. (b) A549 cells treated with (panel B) or without (panels A and C) MβCD were incubated for 2 h at 37 °C with GFP-expressing *B. pertussis* (panels A and B) or GFP-expressing *Escherichia coli* DH5α (panel C). After washing, A549 cells were fixed and permeabilized before incubation with mouse antibodies against flotillin-1, followed by CY-3-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse IgG. Panels A and B show green fluorescent *B. pertussis* colocalizing with red fluorescent-labeled flotillin, indicated by yellow areas (60% and 70% of colocalization, respectively). Panel C shows the lack of colocalization of green fluorescent *E. coli* DH5α with red fluorescent flotillin. The percentages of bacteria colocalizing with flotillin were calculated as described in Materials and methods. Representative panels of one out of three independent experiments are shown.

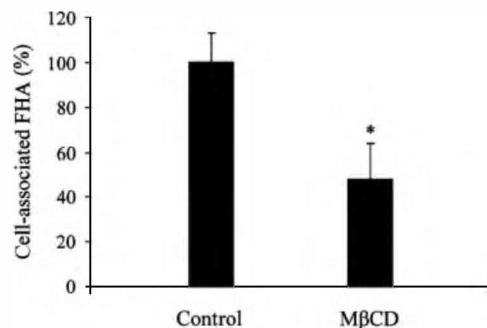
in our experimental setup DRMs were isolated in fractions 3 and 4, in which caveolin, a lipid raft marker, but not transferrin receptor, a cell surface protein typically not associated with lipid rafts, was detected. *Bordetella pertussis* was found in fractions 3 and 4, suggesting its association with DRMs (Fig. 2a). Immunoreactivity of *B. pertussis* was also observed in fractions 8–12, possibly indicating that *B. pertussis* also binds to sites that are not located in lipid rafts. Nevertheless, the distribution of *B. pertussis* in the different fractions and its relative enrichment in DRMs are similar to those found in other pathogens that have been shown to target cholesterol-rich domains (Jutras *et al.*, 2003). We further investigated this association by confocal microscopy using another lipid raft marker, flotillin. Around 60% of *B. pertussis* were found to be colocalizing with flotillin-enriched areas, confirming the involvement of lipid rafts in *B. pertussis* interaction with epithelial cells (Fig. 2b). Interestingly, Fig. 2b shows that, although less in amount, the characteristic lipid raft spots observed in control cells (panel A) are still present in MβCD-treated

cells (panel B), suggesting that lipid rafts were not fully disrupted by this treatment. Remarkably, bacteria attached to cells treated with MβCD, although less in number, were mostly found to be colocalizing with flotillin-positive areas.

The significant reduction in *B. pertussis* adhesion after pretreatment of the cells with cholesterol-sequestering drugs led us to speculate that FHA, reported to be critical for *B. pertussis* adhesion to A549 cells (Rodriguez *et al.*, 2006), could be involved in the interaction of this pathogen with cholesterol. A549 cells were incubated with purified FHA before Triton-insoluble cell components' separation by a sucrose density gradient. Most of the cell-associated FHA was found in low-density fractions, indicating its association with DRMs (Fig. 3a). Although from these results we cannot rule out that FHA also binds to other molecules or even cholesterol present in the plasma membrane outside DRMs, this figure shows that FHA preferentially targets lipid rafts in the plasma membrane. Importantly, when cell lysates were incubated with saponin, a lipid raft-disrupting drug (Chamberlain *et al.*, 2001), FHA was no longer detected in low-



**Fig. 3.** Interaction of FHA with lipid rafts. Purified FHA was incubated with A549 cells for 1 h at room temperature before subcellular fractionation. (a) The gradient fractions were analyzed by immunoblotting using anti-FHA antibodies, anti-transferrin receptor antibodies and anti-caveolin-1 antibodies. (b) FHA-treated cells were lysed using 0.5% Triton X-100 plus 0.5% saponin and analyzed by immune blotting as in (a). Representative results of one out of three independent experiments are shown.

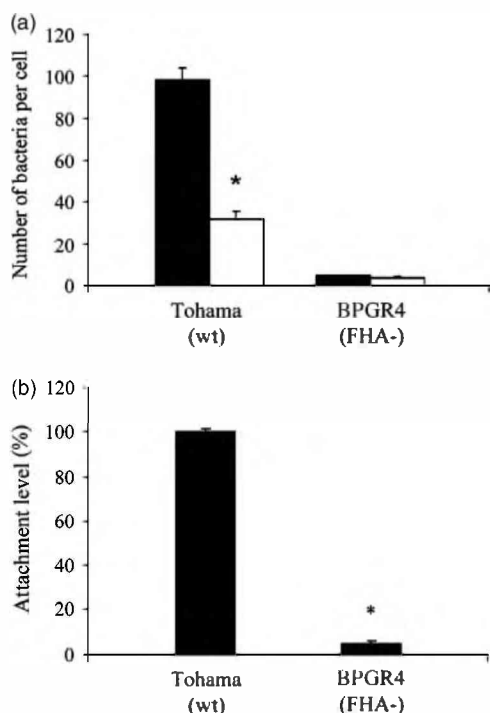


**Fig. 4.** Cholesterol sequestration affects FHA binding to A549 cells. A549 cells treated with or without 10 mM of MβCD for 15 min at 37 °C were incubated with 17 μg mL<sup>-1</sup> of FHA for 1 h at 37 °C. After washing, surface-bound FHA was detected by incubation with mouse anti-FHA antibodies, followed by incubation with a fluorescein isothiocyanate-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse IgG, and further analyzed by flow cytometry. Cell-associated FHA was determined by the geometric mean of cell-associated green fluorescence. FHA associated with MβCD-treated cells was expressed as a percentage of the level of FHA bound to untreated cells (control) arbitrarily set as 100%. The data represent the mean ± SD of three independent experiments. The level of FHA associated with A549 cells treated with MβCD differed significantly from the level of FHA associated with MβCD-treated cells (\**P* < 0.05).

density fractions, but at the bottom of the gradient (Fig. 3b). Control experiments showed that purified FHA analyzed directly on gradients is found solely in the bottom fractions of the gradient (data not shown). In parallel experiments, we studied whether FHA association with A549 cells was affected by MβCD treatment. In cells pretreated with MβCD, the total amount of cell-associated FHA was significantly reduced compared with the amount of cell-associated FHA in untreated cells (Fig. 4). These results, confirmed by fluorescence microscopy (data not shown), suggest that cholesterol-rich domains may function as platforms that cluster docking molecules for FHA contributing to effective bacterial binding. At least three binding domains have been identified in mature FHA (Locht *et al.*, 1993), including a heparin-binding domain that mediates attach-

ment to nonciliated cells, an arginine-glycine aspartate motif that mediates adherence to integrins in leukocytes and a carbohydrate recognition domain that mediates binding to lactosylceramides. Ceramides have been proposed to be involved in the reorganization of the membrane and/or membrane rafts (Holopainen *et al.*, 1998; Kolesnick *et al.*, 2000). We speculate that lipid rafts forming an operational attachment platform might ensure a threshold local concentration of lactosylceramides and glycolipids, such as asialo-GM1, that facilitates an efficient bacterial binding.

In order to gain a better insight into *B. pertussis* interaction with cholesterol-rich domains, we next explored whether *B. pertussis* directly interacts with cholesterol. Treatment of *B. pertussis* with cholesterol before incubation with A549 cells significantly reduced bacterial attachment (Fig. 5a), indicating that cholesterol, besides stabilizing host rafts, may function as a receptor for these bacteria. The binding of the *B. pertussis* FHA-deficient mutant (BPGR4) to A549 cells was not affected by cholesterol pretreatment of the bacteria, suggesting that the presence of FHA on the bacteria surface is critical for *B. pertussis* interaction with this molecule. This result was further confirmed by assessing bacterial adhesion to microtiter plates coated with cholesterol. As shown in Fig. 5b, the absence of FHA on the bacteria surface led to a drastic decrease in the cholesterol-binding activity of *B. pertussis*. Association with cholesterol may stabilize *B. pertussis* interaction with lipid rafts; however, we cannot exclude the possibility that cholesterol outside rafts also participates in *B. pertussis* attachment to epithelial cells. Furthermore, cholesterol is also a major component of tracheal secretions and its concentration increases under conditions of hypersecretion as during *B. pertussis* infection (Reid, 1986; Widdicombe, 1987). Therefore, apart from promoting bacterial attachment to epithelial cells, the cholesterol-binding capacity of *B. pertussis* might ensure bacterial colonization via other mechanisms, given the abundant reservoir of cholesterol this pathogen will encounter at the site of infection.



**Fig. 5.** Cholesterol binding of *Bordetella pertussis*. (a) Wild-type (wt) *B. pertussis* and a mutant strain deficient in the expression of FHA (BPGR4) were incubated with (white bars) or without (black bars)  $1.8 \mu\text{g mL}^{-1}$  of cholesterol in DMEM plus BSA for 1 h at  $37^\circ\text{C}$ . After two washing steps to remove nonbound cholesterol, bacteria were incubated with A549 cells for 2 h at  $37^\circ\text{C}$  (MOI, 150). Cells were washed and fixed. Bacterial attachment was evaluated by fluorescence microscopy. The data represent the mean  $\pm$  SD of four independent experiments. The attachment level of wt *B. pertussis* preincubated with cholesterol was significantly different from the attachment level of *B. pertussis* in the absence of cholesterol ( $*P < 0.05$ ). (b) Binding of wt *B. pertussis* and BPGR4 to cholesterol-coated plates. Non-specific binding, measured in wells coated with ethanol or blocking agent alone, was subtracted. Absorbance due to the attachment of wt was set as 100% of the attachment level. Data represent the mean  $\pm$  SD of three independent experiments. The binding of wt *B. pertussis* was significantly different from the binding of BPGR4 ( $*P < 0.05$ ).

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