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Gonococcal Opacity Protein Promotes Bacterial Entry-Associated Rearrangements of the Epithelial Cell Actin Cytoskeleton

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Neisseria gonorrhoeae **enters cultured human mucosal cells following binding of a distinct gonococcal opacity (Opa) outer membrane protein to cell surface proteoglycan receptors. We examined the route of internalization that is activated by Opa-expressing gonococci (strain VP1). Microscopy of infected Chang epithelial cells showed that gonococcal uptake was insensitive to monodansylcadaverine (150 μM), which interferes with clathrin-mediated endocytosis. Similarly, indirect immunofluorescence staining for clathrin in infected cells showed distribution of cellular clathrin unaltered from the distribution in noninfected cells. The microtubule inhibitors colchicine (50 μM) and nocodazole (20 μM) but not the microtubule-stabilizing agent taxol (10 μM) caused a moderate (30 to 50%) reduction in gonococcal entry without affecting bacterial adherence. The most dramatic effects were obtained with the microfilament-disrupting agent cytochalasin D (3 μM), which totally blocked bacterial entry into the cells. Double immunofluorescence staining of gonococci and actin filaments in infected cells demonstrated bacterium-associated accumulations of F-actin as an early signal of bacterial entry. This recruitment of F-actin was transient and disappeared once the bacteria were inside the cells. Cytochalasin D disrupted the actin cytoskeleton architecture but did not prevent the recruitment of F-actin by the bacteria. Adherent, noninvasive gonococcal Opa variants lacked the ability to mobilize F-actin. Recombinant *Escherichia coli* expressing the gonococcal invasion-promoting Opa of gonococcal strain MS11 (Opa_{50}) adhered to the epithelial cells in an Opa-dependent fashion but was not internalized and did not recruit detectable amounts of F-actin. Coinfection with the *E. coli* recombinant strain and gonococci resulted in specific entry of the diplococci, despite the presence of large numbers of adherent *E. coli* cells. Together, our results indicate that Opa-mediated gonococcal entry into Chang cells resembles phagocytosis rather than macropinocytosis reported for *Salmonella* spp. and sequentially involves gonococcal adherence to the cell surface, Opa-dependent and cytochalasin-insensitive recruitment of F-actin, and cytochalasin D-sensitive bacterial internalization.**

Many bacterial pathogens are able to penetrate the mucosal lining and gain access to the eukaryotic cell interior, thus escaping the hostile environment. This microbial invasion is achieved through a number of different bacterial strategies that exploit normal host cell functions. Grossly simplified, four different uptake pathways utilized by bacteria have been recognized. Some intracellular pathogens, including certain *Chlamydia* species (52), enter mucosal cells by triggering a phagocytosis-like event in these normally nonphagocytic cells. This process involves a sequential, circumferential contact between the bacteria and the host cell surface, leading to engulfment of the microorganisms and the formation of membrane-bound bacterium-containing vacuoles within the cytoplasm. This so-called zipper mechanism of uptake (14) requires rearrangements in the actin-based cytoskeleton, as illustrated by its sensitivity to cytochalasin D, which disrupts actin filaments. A second uptake mechanism, implicated in the penetration of *Salmonella* spp. (10, 24), can be described as macropinocytosis. *Salmonella* spp. and also the cytotoxic necrotizing factor type 1 (CNF1) produced by strains of pathogenic *Escherichia coli* (8) stimulate plasma membrane ruffling, which in turn facilitates ingestion of bacteria, as well as other large surface particles, in a pinocytotic fashion. This ruffling, which resembles the changes in the plasma membrane induced by growth factors and oncogenic stimuli (1), also requires a functional actin cytoskeleton, i.e., is sensitive to cytochalasin D (9). A third mechanism of bacterial "invasion" involves classical receptor-mediated endocytosis, which provides a normal route of entry for a variety of cell surface receptors. This pathway involves binding of the bacteria to receptors that carry on their cytoplasmic tails an internalization signal that is recognized by cytosolic adaptor proteins, which in turn serve as nucleation sites for clathrin, leading to the formation of coated pits. This receptor complex is then internalized to form clathrin-coated vesicles which ultimately deliver their content to endosomes (40). This clathrin-dependent pathway, which is believed not to involve actin filaments (cytochalasin D insensitive), is exploited by certain *Chlamydia* species (21, 52). Finally, a less-well-defined uptake mechanism involves the microtubule-dependent, cytochalasin D-insensitive internalization of bacteria. This pathway confers entry of certain strains of *Campylobacter jejuni* and *Citrobacter freundii* (36). Clearly, the different bacterial uptake pathways are not mutually exclusive, and pathogens have evolved strategies to utilize different routes of entry either simultaneously or in a manner dependent on the phenotype of the host cells (30, 41, 52).

Neisseria gonorrhoeae, the etiologic agent of the sexually transmitted disease gonorrhoea, also penetrates the mucosal barrier. In the natural infection, this bacterium is often found deeply embedded in the membrane of mucosal cells and, less frequently, inside epithelial cells (18). On the basis of studies with human fallopian tubes in organ culture, it is assumed that

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gonococci firmly adhere to the cell surface and then are ingested and transported toward the basolateral surface of the cells, where they may be released into the subepithelial tissue (33). The precise molecular mechanism(s) governing gonococcal entry into mucosal cells is yet to be defined. Experiments with cultured epithelial cells suggest that both actin microfilaments (2, 31, 42) and microtubuli (37) are required for gonococcal invasion, while electron microscopy demonstrated that membrane-bound bacteria were often surrounded by structures resembling coated pits (42). Thus, multiple uptake pathways may be activated by gonococci. One mechanism of gonococcal internalization by epithelial cells can be defined as Opa-mediated entry, referring to the apparent invasion-promoting properties of a distinct member of the opacity (Opa) outer membrane protein family (2, 4, 28, 31, 49). This invasion-associated protein binds cell surface proteoglycan receptors (46) and is thought to promote the formation of intimate contact between the bacteria and the surface of the host cells, which seems a prerequisite for bacterial entry in many cell types. In the present study, we examined the mechanism of Opa-dependent uptake of gonococci into mucosal cells by inhibition of different cellular uptake pathways, coinfection experiments with noninvasive bacteria, and fluorescence microscopy of infected cells. Our data indicate that in Chang conjunctiva epithelial cells, the prime mechanism of gonococcal entry is bacterium-specific microfilament-dependent phagocytosis. The uptake is accompanied by an opacity protein-dependent local rearrangement of the actin-based cytoskeleton which is manifested as a transient accumulation of polymerized actin surrounding invading bacteria. Invasive gonococci do not promote the uptake of other adherent, noninvasive bacteria.

MATERIALS AND METHODS

Bacterial strains. The source and the characteristics of *N. gonorrhoeae* VP1 (P.IA; P^- [nonpiliated]; Opa_{27}, $Opa_{27.5}$, Opa_{28}, Opa_{29}, Opa_{30}; lipopolysaccharide [LPS] type L1; auxotype Pro^-) and MS11-B2.1 (P.IB, P^-; Opa_{30}, LPS type A) have been described previously (31, 44). Variant VP1-b is a VP1 variant expressing a non-invasion-associated Opa (Opa_{29}) recognized by the monoclonal antibody 11CB8. Gonococcal strains were routinely grown on GC phosphate agar (composition per liter, 3.75 g of Trypticase peptone [BBL, Becton-Dickinson, Cockeysville, Md.]), 7.5 g of Thiotone E [BBL], 4 g of K_2HPO_4, 1 g of KH_2PO_4, 5 g of NaCl, 1 g of soluble starch [BBL], and 1% Bacto-agar [Difco, Detroit, Mich.] [pH 8.0]) containing 1% IsoVitaleX (BBL) at 37°C, 5% CO_2 for 16 h. When appropriate, the agar was supplemented with VCN (BBL) to promote selective growth of *N. gonorrhoeae*. For infection experiments, bacteria were grown to mid-logarithmic phase in 10 ml of GC broth in 50-ml Erlenmeyer flasks on a gyratory shaker (125 rpm, 37°C). *E. coli* DH5α carrying the *inv* gene of *Yersinia pseudotuberculosis* (N1423) was constructed essentially as described by Isberg et al. (23). The construction and characteristics of *E. coli* DH5α carrying the plasmid pTrc99A with or without the MS11 Opa_{50} gene have been described previously (28). The *E. coli* recombinants were kindly provided by T. F. Meyer (MPI für Biologie, Tübingen, Germany). *E. coli* strains were grown on Luria-Bertani (LB) agar with the appropriate antibiotics.

Cell culture. Chang human conjunctiva epithelial cells (ATCC CCL20.2) and ME-180 human endocervical epithelial cells (ATCC HTB33) were maintained in 5 ml of RPMI 1640 tissue culture medium (Gibco-BRL, Gaithersburg, Md.) supplemented with 5% fetal bovine serum (FBS) (HyClone, Logan, Utah) in 25-cm^2 tissue culture flasks (Corning Glass Works, Corning, N.Y.) at 37°C in a humidified 5% CO_2 atmosphere. For infection experiments, cells were seeded onto 12-mm circular glass coverslips in a 24-well tissue culture plate (1 ml of medium per well) 48 h before use.

Infection experiments. Infection experiments were routinely initiated by inoculating nearly confluent epithelial cell monolayers maintained in 1 ml of RPMI 1640 or phosphate-buffered saline (PBS) (140 mM NaCl, 2.5 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose [pH 7.4]) at a bacterium-host cell ratio of 100:1 (*N. gonorrhoeae*) or 250:1 (*E. coli* strains). At various time points of incubation (37°C, 5% CO_2), infection was stopped by rinsing the cells four times with PBS and then fixing them for at least 15 min in 2% paraformaldehyde in PBS at 24°C (44). Bacterial adherence and entry were scored by using light microscopy as described elsewhere (46, 47). When appropriate, cytochalasin D and taxol were added at 15 min prior to infection, monodansylcadaverine and cycloheximide were added at 1 to 5 h prior to infection, and ouabaine was added at 3 h prior to infection. Cells treated with colchicine and nocodazole were preincubated at 4°C (1 h) to depolymerize the microtubuli and then incubated at 37°C (1 h). All compounds except for nocodazole (Calbiochem, La Jolla, Calif.) were obtained from Sigma and were used throughout the assay. Control cells were treated with an equal amount of solvent lacking the active compound. Concentrations of solvent (dimethyl sulfoxide or ethanol) were always below 0.5%. Neither of the drugs used affected bacterial viability as estimated from bacterial growth during the assay and bacterial adherence to the host cells. Rapid depletion of intracellular potassium was performed by a 5-min hypotonic shock, followed by incubation in a K^+-free buffer as described by Larkin et al. (29), except that a 10 mM HEPES–140 mM NaCl buffer was used. Control cells underwent the same treatment except that 10 mM KCl was present in the buffer after the hypotonic shock. The bacterial adherence and uptake values shown represent the means ± standard errors (SE) for at least three different experiments. Data were analyzed by Student's *t* test for paired comparison.

Coinfection experiments. In the coinfection experiments, Chang cells were routinely inoculated simultaneously with 5×10^7 *E. coli* DH5α or Opa_{50}-expressing DH5α (DH5α-Opa_{50}) cells and 5×10^6, 5×10^7, or 5×10^8 *N. gonorrhoeae* VP1 cells for a period of 1 to 3 h. Next, the cells were either fixed and subjected to the immunostaining procedure (see below) with *E. coli*- and gonococcus-specific antibodies or washed and subjected to the gentamicin survival assay (31) to recover intracellular bacteria. In the latter procedure, the cells were incubated at the end of the infection period with gentamicin (100 μg/ml) for 90 min, washed, and lysed with 1% saponin to release the intracellular bacteria. The number of intracellular *E. coli* cells and gonococci was estimated by colony counting after overnight growth on selective media (LB agar for *E. coli* and GC-VCN for gonococci). In some experiments, DH5α-Opa_{50} was added to the Chang cells at 2 h prior to the addition of the gonococci to assure maximal adherence without gonococcal competition for the same binding sites. This procedure gave results essentially similar to those obtained after simultaneous addition of both types of bacteria.

Immunoreagents for cytochemistry. The specificity of the mouse monoclonal antibodies 11CB8 (final dilution, 1:100), 7D11E (1:125), and 4D6B (1:50), directed against Opa_{29}, Opa_{28}, and LPS of strain VP1, respectively, have been described previously (44, 49). The MS11-specific rabbit antiserum AK92 (1:150) was raised against whole cells of strain MS11. *E. coli* DH5α was visualized with rabbit antiserum PR1006H (1:50) raised against whole DH5α (kindly provided by P. A. Rosa, Rocky Mountain Laboratories, Hamilton, Mont.). The anticlathrin monoclonal antibodies X19 (1:100) and X22 (1:100) have been described previously (3) and were generously provided by F. M. Brodsky (University of California, San Francisco, Calif.). The secondary immunoreagents included fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) (1:100) (Sigma), tetramethyl rhodamine isocyanate (TRITC)-conjugated goat anti-mouse IgG (1:70)(Molecular Probes, Eugene, Oreg.), and FITC-conjugated sheep anti-rabbit immunoglobulins (1:300) (Sigma).

Immunofluorescence. For staining of bacteria and host cell elements, fixed cells on coverslips were permeabilized in 0.5% Triton X-100 in PBS (20 min), washed three times for 5 min each time with PBS plus 0.05% Tween 20 (U.S. Biochemical, Cleveland, Ohio), and incubated on 100-μl drops of the appropriate immunoreagent on Parafilm (47). All immunoreagents were diluted in PBS containing 5% FBS. For staining of F-actin, FITC-labelled (Sigma) or TRITC-labelled (Molecular Probes) phalloidin was diluted in PBS and used at concentrations of 0.5 to 1 μg/ml and 10 U/ml, respectively. After removal of the primary reagents, the coverslips were washed three times for 10 min each time in PBS–0.05% Tween 20 and, when appropriate, incubated with a secondary FITC- or TRITC-labelled immunoreagent, washed three times for 10 min each time in PBS plus 0.05% Tween 20 and three times for 2 min each time in PBS, and mounted in 50% glycerol in PBS. When double staining of bacteria and host cell structures was performed, labelling of bacteria preceded staining of the cytoskeletal components except that phalloidin was included in the secondary immunoreagent solution. All incubations were carried out for 30 min at 24°C. Cells were viewed with the Bio-Rad MRC 1000 confocal microscopy system coupled to a Zeiss Axiovert 35 microscope, and images were processed by using ADOBE Photoshop. Extensive control experiments in which either bacteria or host cell structures were stained showed that there was no detectable fluorescence signal overlap between the different fluorochrome detection channels under the conditions employed.

Radioactivity assays. The rate of eukaryotic protein synthesis was estimated from the incorporation of [^{35}S]methionine (60 μCi per well; Amersham, Arlington Heights, Ill.) into 2×10^5 Chang conjunctiva cells maintained in methionine-free tissue culture medium supplemented with 5% FBS over a 2-h incubation period. Next, the cells were washed with PBS to remove free label and incorporated label was quantitated with a Beckman liquid scintillation counter model LS 6000LL. Receptor-mediated endocytosis was measured by using ^{125}I-labelled human transferrin which was labelled by the chloramine T method to a specific activity of 30 μCi/μg. Label was added to 2×10^5 Chang cells maintained in 0.3 ml of tissue culture medium (without FBS) for a period of 1 to 3 h at 37°C. Next the cells were washed with PBS, and total radioactivity associated with the cells was measured in a Hewlett-Packard gamma counter. Internalization of transferrin was estimated by comparison of total cell-associated radioactivity and

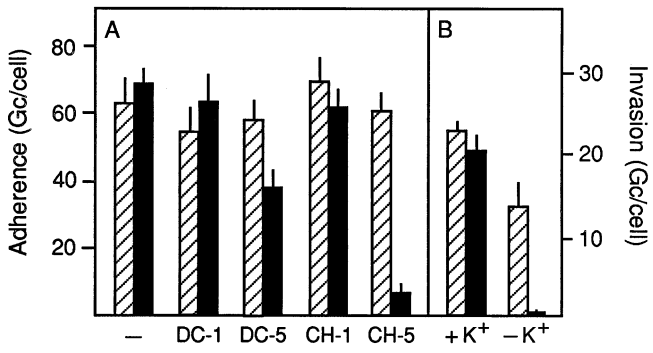

FIG. 1. Role of receptor-mediated endocytosis in gonococcal infection of Chang epithelial cells. Chang cells were preincubated with monodansylcadaverine (DC) (150 μM), cycloheximide (CH) (0.25 mM), or solvent (−) for 1 and 5 h (A) or subjected to the potassium depletion procedure and then incubated in buffer containing 10 mM KCl ($+K^+$) or no potassium ($-K^+$) for 1 h prior to infection (B). Infection with strain VP1 was stopped after 1 h, and bacterial adherence (hatched bars) and entry (filled bars) were determined as described elsewhere (46, 47). Values are the means ± SE for three to six experiments. Significant *P* values: invasion for DC-5 versus control, <0.05; invasion for CH-5 versus control, <0.001; invasion for $-K^+$ versus $+K^+$, <0.001. Gc, gonococci.

cell-associated radioactivity remaining after sodium acetate-induced dissociation of cell surface-bound transferrin (20 min, 20°C) (16). Similar experiments carried out at 15°C served as a control.

RESULTS

Role of clathrin-mediated endocytosis in the uptake of *N. gonorrhoeae* by human epithelial cells. In order to dissect the host cell structural elements that participate in the opacity protein-promoted internalization of *N. gonorrhoeae* by cultured epithelial cells, we determined first the contribution of receptor-mediated endocytosis, i.e., the clathrin-coated pit pathway, to the uptake process. For this purpose, Chang epithelial cells were infected with *N. gonorrhoeae* VP1 under conditions known to interfere with the clathrin-mediated uptake system. Bacterial entry was determined by light microscopy after 1 h of infection. In this approach, monodansylcadaverine (1 h preincubation), which blocks receptor-mediated endocytosis of various ligands (6), did not affect gonococcal entry into the host cells (Fig. 1A), although the compound did inhibit the internalization of ^{125}I-human transferrin by Chang cells (data not shown). It should be noted that prolonged exposure (6 h) to the drug decreased gonococcal entry in a dose-dependent fashion. Maximal inhibition (30%) occurred at a drug concentration of 150 μM (Fig. 1A). This effect, which was also observed for the uptake of an *E. coli* recombinant strain expressing the invasion of *Y. pseudotuberculosis* (data not shown), may, however, be nonspecific and due to altered cell metabolism caused by the drug. Measurements of the rate of incorporation of [^{35}S]methionine into monodansylcadaverine-treated (150 μM) cells showed a 60% reduction in eukaryotic protein synthesis in comparison with levels for untreated control during the final 2 h of the 6-h cells incubation period (data not shown). Additional infection experiments in the presence of cycloheximide (0.25 mM), an inhibitor of eukaryotic protein synthesis, showed that prolonged (6 h) inhibition of eukaryotic protein synthesis reduced gonococcal entry by up to 90% without affecting bacterial adherence (Fig. 1A).

In an alternative approach to investigate the role of receptor-mediated endocytosis in uptake of gonococci, we applied the cell potassium depletion procedure to arrest coated-pit formation. Lowering of the intracellular potassium concentration either with the Na^+/K^+ ATPase inhibitor ouabaine (200 μM, 3 h) or by the more rapid hypotonic-shock procedure (17, 29) arrests coated-pit formation by preventing binding of clathrin to receptor-associated adaptor proteins. As shown in Fig. 1B, rapid depletion of intracellular potassium completely inhibited gonococcal entry into Chang cells and caused a moderate (40%) decrease in gonococcal adherence. Control cells which underwent the same procedure except for inclusion of 10 mM KCl in the buffer showed normal uptake levels, suggesting that the inhibition was due to K^+ depletion. To address the apparently conflicting results obtained with monodansylcadaverine and K^+ depletion and to substantiate the possible role of clathrin in uptake of gonococci, we tried to visualize this key factor in receptor-mediated endocytosis in infected cells by microscopy using antibodies directed against the heavy chain of the clathrin molecule. Fluorescence microscopy of noninfected cells revealed a bright, typical punctate staining pattern, similar to that described for clathrin distribution in other cells types (Fig. 2A and B) (5, 17, 29). In VP1-infected (1 h) cells, no change in clathrin distribution was observed. Simultaneous visualization of clathrin and gonococci showed no detectable recruitment of clathrin at the sites of bacterial entry (Fig. 2C and D). These results were confirmed by immunoelectron microscopy (49a).

Role of the microtubule and actin filament network in the gonococcal invasion of Chang cells. The involvement of the microtubule network in the opacity protein-dependent gonococcal infection of Chang cells was examined by using the microtubule assembly inhibitors nocodazole (20 μM) (15) and colchicine (50 μM) (32) and the microtubule-stabilizing agent taxol (10 μM) (7). Confocal microscopy showed that both nocodazole and colchicine caused cellular retraction and a flattening of the cells, indicative of disruption of the microtubule network. This change in cell architecture did not interfere with the ability of gonococci (strain VP1) to adhere firmly to the surface of the cells. The number of adherent bacteria was comparable to that observed for untreated cells (Fig. 3). Microscopic measurements of the number of intracellular bacteria showed a moderate reduction of up to 30% (Fig. 3). It appeared that the reduced space between the plasma membrane and the nucleus hampered further bacterial penetration of the flattened cells. Taxol, which leaves microtubuli intact but prevents their rearrangement (7), had no visible effect on cell architecture and did not affect gonococcal adherence and entry into the host cells (Fig. 3).

The contribution of the actin-based cytoskeleton in the internalization of gonococci was assessed by the addition of various concentrations of the actin filament-disrupting agent cytochalasin D at 15 min prior to infection. At concentrations between 0.5 and 3 μM this drug rapidly changed the morphology of Chang cells, causing cell retraction and arborization but not cell detachment. This treatment prevented gonococcal invasion without affecting bacterial adherence. At a concentration of 3 μM, the uptake of gonococcal strain VP1 was reduced by more than 99% (Fig. 3). Similar results were obtained with the invasive gonococcal strain MS11-B2.1 or when ME-180 endocervical cells were used as an infection system (data not shown). These data are consistent with previous observations by a number of investigators (2, 31, 37, 42) and indicate that gonococci require a functional actin-based cytoskeleton to enter host cells but not to adhere to the cell surface. In contrast to monodansylcadaverine, cytochalasin D did not inhibit eukaryotic protein synthesis during a 1- to 5-h incubation period. Together, the data suggest that the internalization of gonococci by Chang cells primarily requires a functional microfilament network, perhaps in conjunction with intact microtubuli.

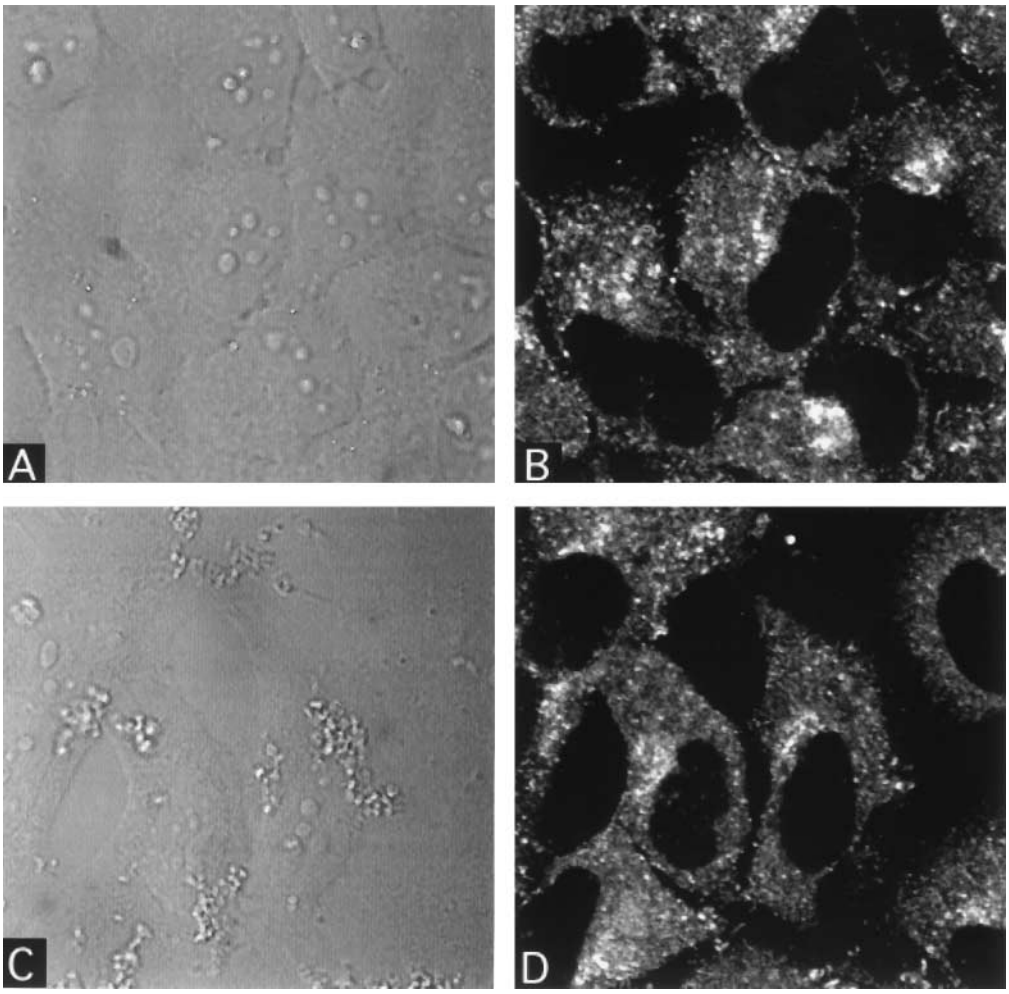

FIG. 2. Confocal micrographs showing the distribution of clathrin, appearing as bright punctate dots in noninfected (B) and infected (1 h; *N. gonorrhoeae* VP1) (D) Chang epithelial cells. Bacteria were visualized with Nomarski optics (A and C). Clathrin was stained by indirect immunofluorescence using FITC-conjugated antibodies (B and D).

Transient local accumulation of F-actin as a first sign of gonococcal entry. The role of the actin-based cytoskeleton in the uptake of gonococci was further established through direct observation of bacteria and microfilaments in infected monolayers of Chang cells by confocal laser microscopy. Polymerized actin was visualized by using FITC-phalloidin, and when appropriate, bacteria were made fluorescent by using bacterium-specific monoclonal antibodies and rhodamine-conjugated goat anti-mouse IgG. Microscopy of resting Chang cells showed bright staining of actin filaments forming thick, long bundles of filaments (stress fibers) at the base of the cells (Fig. 4A) and a fine network of thin filaments underneath the plasma membrane (Fig. 4B). Shortly (20 min) after infection with gonococcus strain VP1, however, first signs of a redistribution of microfilaments were apparent from the formation of small aggregates of F-actin that colocalized with some of the adherent bacteria. At 30 min of infection, the number of bacterium-associated condensations of F-actin had further increased and bacteria were ingested by the cells. The actin accumulations were of the same size and followed strictly the outline of adherent bacteria, giving a typical footprint-like appearance (Fig. 4C and D). Optical sectioning of the infected cells showed that the recruited actin was always located directly

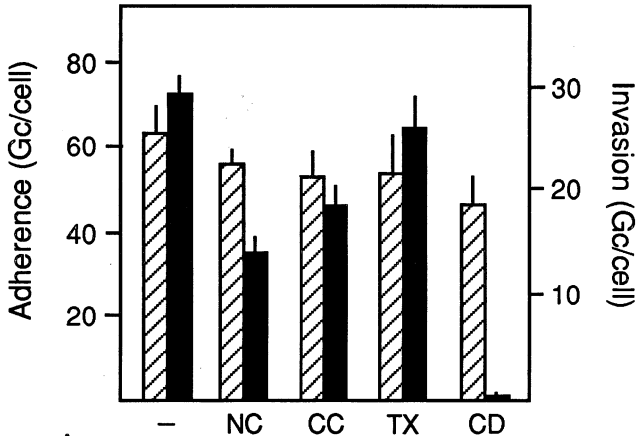

FIG. 3. *N. gonorrhoeae* VP1 adherence to (hatched bars) and entry into (filled bars) Chang epithelial cells in the absence (−) and presence of nocodazole (NC) (20 μM), colchicine (CC) (50 μM), taxol (TX) (10 μM), and cytochalasin D (CD) (3 μM). Epithelial cells were preincubated with the drugs as described in Materials and Methods. Infection was stopped after 1 h. Values represent the means ± SE for three to eight experiments. Significant *P* values: invasion for NC versus control, <0.005; invasion for CC versus control, <0.02; invasion for CD versus control, <0.001. Gc, gonococci.

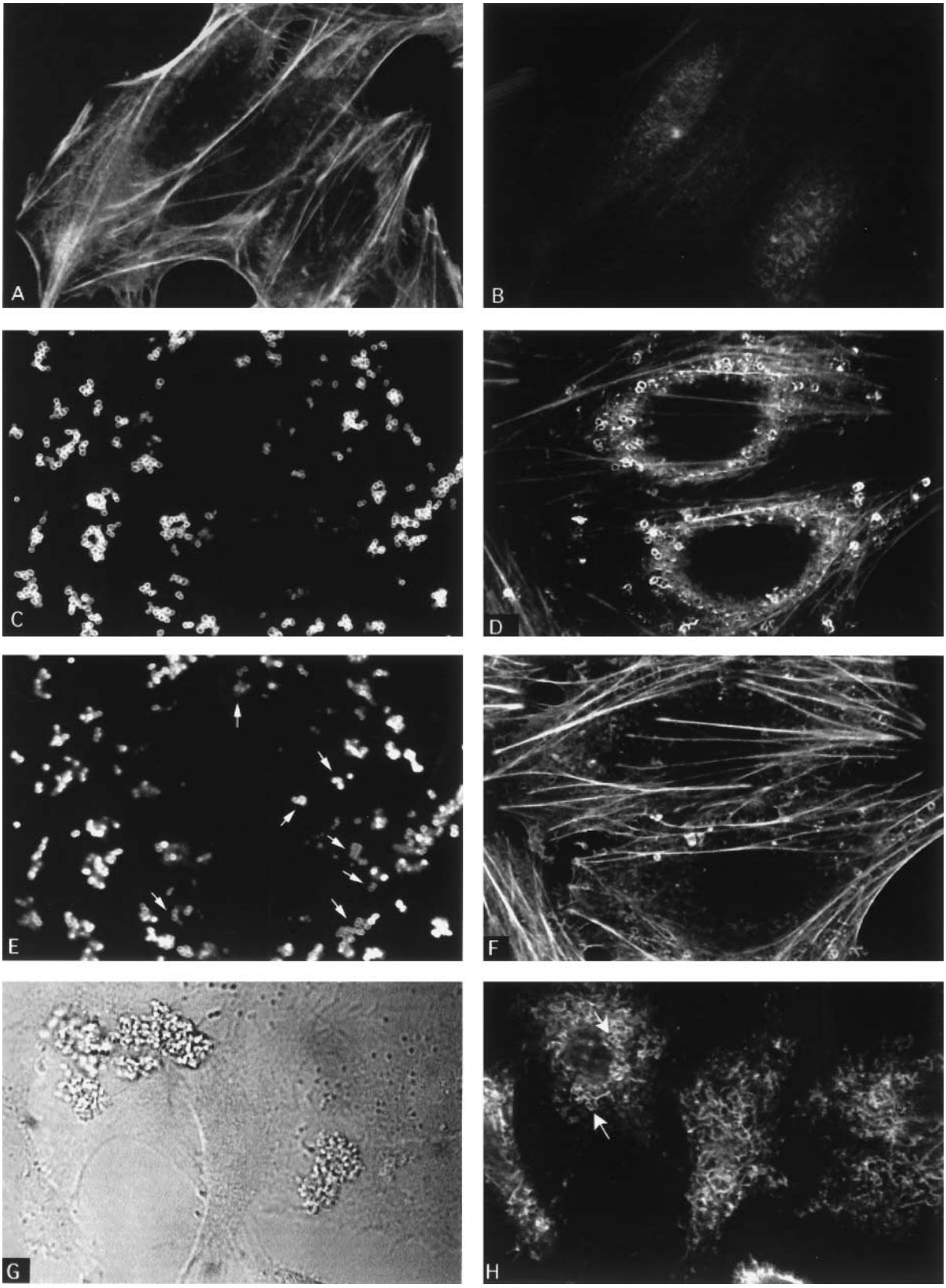

FIG. 4. Rearrangement of host cell F-actin during gonococcal infection (strain VP1, 30 min) of cultured Chang epithelial cells. Bacteria were visualized by using either specific antibodies and IgG-TRITC or differential contrast microscopy. F-actin was stained with FITC-phalloidin. (A and B) Optical sections of noninfected epithelial cells showing typical actin stress fibers (A) at the base and cortical actin (B) more apically in the cells. (C and D) Infected cells (30 min) showing the colocalization of invasive bacteria (C) and aggregates of F-actin giving a footprint-like appearance at the level of the plasma membrane (D). (E and F) The same cells as shown in panels C and D but sectioned at a lower plane, showing intracellular gonococci (E) (arrows) that are not surrounded by F-actin (F). (G and H) Infected (30 min) potassium-depleted Chang cells showing that some of the adherent bacteria (G) still induced footprints (H) (arrows), although bacteria are not internalized (Fig. 1). Note the different, disorganized microfilament architecture in the potassium-depleted cells (H) in comparison with that in the non-potassium-depleted cells (B and D).

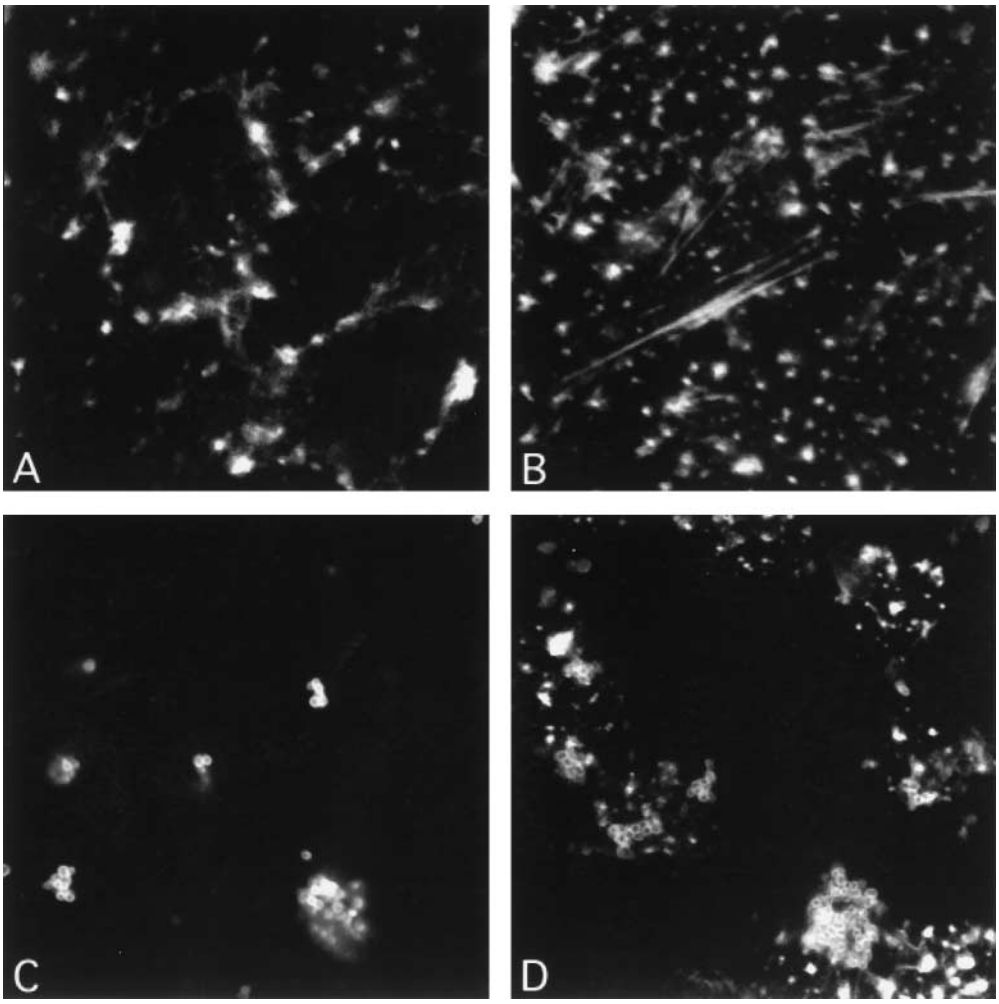

FIG. 5. Fluorescence micrographs illustrating the dramatic effect of cytochalasin D on the distribution of FITC-phalloidin-stained F-actin in uninfected cells and infected Chang epithelial cells (30 min of infection). (A and B) Noninfected Chang cells treated with 3 μM cytochalasin D. Note the aggregated state of F-actin at the cortical (A) and stress fiber (B) levels relative to the distribution in untreated Chang cells (Fig. 4A and B). (C and D) Cytochalasin D-treated Chang cells infected with gonococcus strain VP1. Note the spatial relationship between some of the adherent bacteria (C) and filamentous actin (D).

underneath and around invading bacteria. Once intracellular, the gonococci were not surrounded by polymerized actin (Fig. 4E and F), suggesting that F-actin was only transiently associated with the bacteria early in the cellular infection. At prolonged infection (5 h), the number of actin accumulations was considerably reduced, reflecting a decrease in bacterial uptake, and the actin cytoskeleton appeared to regain its original state despite the presence of large numbers of intracellular bacteria (data not shown). Mobilization of F-actin by gonococci also occurred in infected (5 h) dansylcadaverine (150 μM)- and cycloheximide (0.2 mM)-treated cells, despite the presence of much fewer and thinner actin bundles than in untreated cells. This apparent reduced actin content may explain the reduced bacterial invasiveness observed after prolonged exposure of cells to these compounds (Fig. 1). Phalloidin staining of K^+-depleted noninfected cells showed even more severe alterations in the actin-based cytoskeleton, with the formation of an irregular meshwork of thin actin filaments throughout the cell. Infected cells showed similar staining, although bacterial footprints of actin were occasionally observed (Fig. 4G and H). The dramatic impact of K^+ depletion on the organization of the actin cytoskeleton, as well as on other cell functions such as DNA synthesis, protein synthesis, and bulk-fluid pinocytosis (29), casts serious doubt about the value of this procedure in studies of bacterium-directed endocytosis.

Cytochalasin D uncouples actin accumulation and bacterial invasion. To further substantiate the apparent role of the actin cytoskeleton in the gonococcal entry process, we verified that the inhibitory effect of cytochalasin D on gonococcal uptake was related to changes in the actin filament network by fluorescence microscopy. FITC-phalloidin staining of noninfected cytochalasin D-treated (3 μM) cells showed a complete dissolution of stress fibers and the formation of a few aggregates of F-actin at the plasma membrane (Fig. 5A and B), as has been reported for other cell types (12, 39). In VP1-infected Chang cells (1 h of infection), a similar disruption of the actin filament architecture was observed, with the exception that in this case the remaining actin filaments colocalized with adherent bacteria, resulting in the typical footprint appearance observed during gonococcal entry in untreated cells (Fig. 5C and D). Intracellular bacteria were not observed (data not shown) (see also Fig. 3). Thus, despite the disruption of most of the microfilaments, the bacteria were still able to efficiently recruit F-actin. The uncoupling of actin recruitment and bacterial entry sug-

gests that these are discrete events and that cytochalasin D-sensitive factors are required to complete the entry process.

Recruitment of F-actin requires a distinct opacity outer membrane protein. The colocalization of F-actin with invasive bacteria raised the question of the nature of the bacterial ligand that triggers these events. Previously, the entry of nonpiliated gonococci into Chang cells has been associated with the expression of one distinct member of the opacity outer membrane protein family (4, 28, 31, 49). This protein confers attachment (and perhaps invasion) by recognition of cell surface proteoglycan receptors (46). In order to investigate whether the apparent requirement of a distinct opacity protein for bacterial internalization lies before or after the induced local condensation of F-actin, we tested several gonococcal variants differing in opacity protein expression for their ability to recruit F-actin. The parent strain VP1 expresses multiple opacity proteins, including the invasion-associated $Opa_{27.5}$. This strain was used in the above-described experiments. Variants that lacked the invasion-associated $Opa_{27.5}$, VP1-a (Opa_{30}), -b (Opa_{29}), and -c (Opa_{28}), showed moderate adherence to Chang cells, though they were unable to bind purified proteoglycan receptor (data not shown) and have proven to be not invasive (45). Fluorescence microscopy of VP1-b-infected cells stained for polymerized actin showed that this variant failed to accumulate cellular actin, in contrast to its invasive counterpart (Fig. 6A to D). Thus, actin accumulation appears to be associated with Opa-mediated entry and not with bacterial adherence.

In an attempt to further substantiate the association between opacity protein expression and the accumulation of F-actin, we evaluated the behavior of recombinant *E. coli* DH5α carrying the *opa* gene encoding the invasion-promoting Opa_{50} of strain MS11 but lacking other gonococcal surface components. Infection (2 to 3 h) of Chang cells with this strain but not with the control *E. coli* strain carrying the plasmid without an insert resulted in adherence of about 40 to 70 bacteria per cell (Fig. 6E). This adherence was inhibitable by heparin, indicative of its Opa dependence (46). Intracellular bacteria were not observed (Table 1), consistent with previous observations (46). Simultaneous detection of bacteria and F-actin revealed that the less than 0.1% of adherent *E. coli* Opa_{50}-expressing cells colocalized with phalloidin-positive structures (Fig. 6E and F), suggesting that these bacteria have a defect early in the bacterial uptake activation pathway.

Coinfection of *E. coli* Opa_{50}-expressing cells and invasive gonococci. The inability of DH5α-Opa_{50} to enter cultured Chang epithelial cells gave the opportunity to further dissect the mechanism of gonococcal entry through coinfection experiments with invasive gonococci. Coinfections with both types of bacteria may provide information about whether VP1 produces factors that specifically complement adherent *E. coli* and allow it to enter the host cells or perhaps activates a general host cell phagocytic response resulting in the ingestion of the noninvasive bacteria. For this purpose, gonococci and DH5α-Opa_{50} were added to epithelial cells at various ratios (VP1/DH5α-Opa_{50} ratios, 100/1, 1/1, and 1/100) and the fate of the bacteria was determined by light and fluorescence microscopy by using differential staining of the two types of bacteria. These experiments clearly demonstrated specific entry of gonococci only, despite the presence of large numbers of *opa*-expressing *E. coli* cells adherent to the cell surface under all the conditions employed (Table 1). Furthermore, even when the invasive gonococci were added 2 h after the start of the Opa_{50}-expressing *E. coli* infection, specific entry of the gonococci occurred within 30 to 60 min and did not alter the fate of the adherent *opa*-expressing *E. coli* cells. These results were confirmed by measurements of the number of intracellular bacteria by the gentamicin assay using bacterium-selective agar plates. Only gonococci were recovered from the intracellular compartment (Table 1). These data indicate that gonococci are unable to rescue *opa*-expressing *E. coli* entry and apparently do not to stimulate a general increase in cells' phagocytic behavior as reported for *Salmonella* spp. (10).

DISCUSSION

Bacterial pathogens have evolved multiple strategies to gain access to human mucosal cells, including the utilization of conventional phagocytosis, macropinocytosis, classical clathrin-dependent receptor-mediated endocytosis, and a microtubule-dependent uptake pathway (5, 10, 24, 36, 41, 52, 53). Our results indicate that the human mucosal pathogen *N. gonorrhoeae* invades cultured epithelial cells primarily through induction of a phagocytosis-like process in these normally nonphagocytic cells. The evidence that gonococcal entry involves a microfilament-dependent pathway is based on the observations that cytochalasin D inhibits internalization by more than 99% and that invasive gonococci induce a local bacterium-associated accumulation of polymerized actin early in the cellular infection. Once inside the cells, the gonococci were not surrounded by polymerized actin, indicating that the internalization event probably involves a sequential recruitment and depolymerization of F-actin, a feature which resembles the phagocytosis of large particles by macrophages (13) and of bacteria carrying the invasin of *Y. enterocolitica* by epithelial cells (53). The gonococcal uptake mechanism differs from the microfilament-dependent macropinocytosis utilized by *Salmonella* spp. (for a review, see reference 11). During gonococcal ingestion, the epithelial cells showed no signs of increased membrane ruffling (data not shown), and the size of the actin aggregates was much smaller than that observed during *Salmonella* entry (9). Furthermore, coinfection experiments with invasive gonococci and noninvasive, adherent recombinant *E. coli* showed selective uptake of gonococci, which contrasts with the passive entry of bacteria used in coinfection experiments with *Salmonella* spp. (10). Our data are consistent with immunoelectron microscopy observations pointing to a tight sequential circumferential contact of the bacteria and the host cell plasma membrane during bacterial entry (49). The apparent absence of cellular actin around intracellular gonococci may indicate that this bacterium (i.e., the strains used) does not possess the surface machinery to escape the vacuole and to synthesize actin-binding proteins to support its own movement and spread within and between cells as reported for *Shigella flexneri* (48), *Listeria monocytogenes* (43), and rickettsiae (19). Gonococci are generally believed to be contained in membrane-bound vacuoles inside the cytoplasm, perhaps as an intermediate step in a transcytosis pathway.

What is the signal that leads to the transient condensation of F-actin during gonococcal entry? In previous studies, gonococcal opacity outer membrane protein has been implicated as a major determinant of gonococcal internalization. It binds cell surface proteoglycans (46) and has been associated with the observed bacterial engulfment by the host cells (49). The current finding that gonococci recruit F-actin only when they express the invasion-associated opacity protein is consistent with the function proposed for this outer membrane protein. The presence of opacity protein, however, does not seem to be sufficient for recruitment of cellular actin, as demonstrated by the absence of actin underneath *E. coli* adhering via Opa. Here it should be noted that the level of Opa expression in the recombinant strain is less than that observed for gonococci

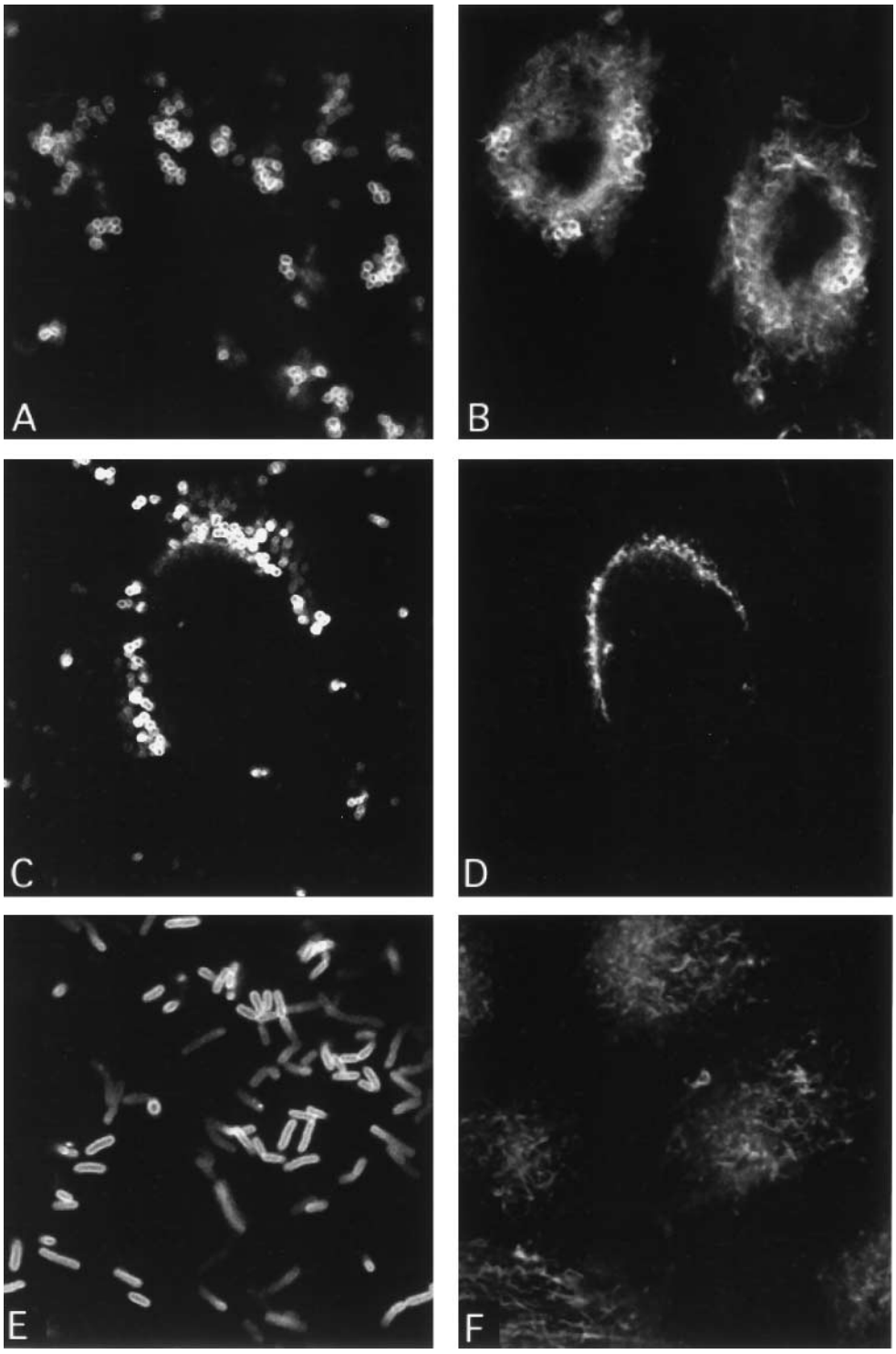

FIG. 6. Effect of opacity protein on the bacterium-induced recruitment of F-actin. (A and B) Chang cells infected with invasive gonococcus variant VP1 (30 min of infection). Note the colocalization of entering bacteria (A) and formed actin footprints (B). (C and D) Chang cells infected with the noninvasive opacity protein variant VP1-b illustrating unaltered distribution of F-actin (D) despite the presence of adherent bacteria (C). (E and F) Chang cells infected with Opa_{50}-expressing *E. coli* (2 h of infection) showing adherent bacteria (E) but no signs of recruitment of F-actin (F).

(28) and that the strain is relatively inefficient in binding purified cell surface heparan sulfate proteoglycan receptors (46). Thus, the possibility that higher levels of functional Opa (which appears nonpermissive for *E. coli*) may signal actin recruitment and initiate bacterial entry cannot be excluded. Alternatively, our findings may indicate that additional bacterial factors are required to establish opacity protein-mediated entry. This situation would contrast with that reported for

TABLE 1. Coinfection of Chang epithelial cells with Opa_{50}-expressing *E. coli* and gonococcus strain VP1[a]

Expt and strain	Bacteria/cell (mean ± SE)[b]		Intracellular survival (CFU/well)
	Adherence	Invasion	
A			
DH5α-Opa_{50}	63.20 ± 7.64	0.27 ± 0.19	2.6×10^2
VP1	100.0 ± 15.20	38.00 ± 2.08	5.2×10^4
B			
DH5α-Opa_{50}	47.00 ± 15.00	0.00	5.7×10^1
VP1	45.00 ± 6.45	22.25 ± 1.70	1.5×10^4

[a] The strains were added either to separate wells (A) or to the same well at an *E. coli*/VP1 ratio of 10:1 (B). After 2 h of incubation, the infection was stopped and cells were either fixed and stained to determine the number of adherent ("Adherence") and intracellular ("Invasion") bacteria or subjected to the gentamicin survival assay using selective agar plates for recovery of viable intracellular bacteria ("Intracellular survival").

[b] Data are the means for three to seven experiments. Significant *P* values: VP1 adherence for A versus B, <0.05; VP1 invasion for A versus B, <0.05.

Yersinia spp., where only a single outer membrane protein, invasin, is sufficient to promote adherence, actin polymerization, and internalization (53). This process involves binding to and clustering of β1-integrins, although the exact signaling events which accompany the actin accumulation and internalization of this bacterium remain to be elucidated (22). For *Shigella*, *Salmonella*, and enteropathogenic *E. coli* strains, bacterial uptake by host cells appears to be a multifactorial event involving several gene products (22, 26, 34). For enteropathogenic *E. coli*, the uptake of invasion-defective mutants can even be rescued by coinfection with wild-type cells (38). Invasive gonococci were unable to complement the lack of entry of the adherent Opa_{50}-expressing *E. coli* in our system, though both types of bacteria bound to the same type of host cell receptor.

Besides the recognition that gonococcal adherence and the recruitment of actin filaments are separate events, our data indicate that local condensation of F-actin is not sufficient to complete bacterial entry into the cells. Exposure to cytochalasin D clearly uncouples the bacterium-induced recruitment of polymerized actin and bacterial uptake; also, the inhibitory effects of monodansylcadaverine, cycloheximide, and K^+ depletion on bacterial entry but not on the actin accumulations may be explained by the observed secondary effects on the microfilament organization in the host cells. Furthermore, inhibitors of microtubule assembly reduce the number of intracellular bacteria, although they do not inhibit the accumulation of actin at the site of entry (data not shown). Whether the highly efficient recruitment by gonococci of the residual F-actin in the cytochalasin D-treated cells involves a clustering of cortical, perhaps receptor-linked cytochalasin D-insensitive actin filaments (12, 21) or results from incomplete inhibition of actin filament assembly remains to be determined. During phagocytosis of IgG-coated erythrocytes by macrophages, similar cytochalasin D-induced F-actin aggregates are observed to disappear at higher cytochalasin concentrations (50 versus 1 μM) (13). Such high concentrations could not be applied in our infection model since they caused detachment of the epithelial cells from the matrix (data not shown). Regardless of the mechanism, our results imply that actin may act not only in the anchoring of the bacteria to the host cell cytoskeleton but also in the internalization process itself. In this regard, the notion that microtubuli, perhaps in conjunction with actin-myosin interactions, regulate contractile processes in the cell (27) and that actin functions in the translocation to the plasma membrane of actin-binding protein factors as well as cytosolic signaling molecules (35) may be of interest. In *Salmonella* spp., translocation of some actin-binding proteins to the site of bacterial entry is disrupted by cytochalasin D, and this is associated with incomplete internalization (9). Drugs that disrupt microtubuli may inhibit transport of the membrane-bound bacteria from the plasma membrane area deeper into the cell either by preventing their movement along microtubules (25) or by inhibition of necessary actin-myosin-mediated contractile forces (27). Both events may result in a traffic jam at the site of entry and may explain the observed reduction in gonococcal uptake as well as the inhibition of complement-mediated phagocytosis by monocytes (51) caused by microtubule-disrupting agents.

On the basis of our observations, we propose that Opa-mediated entry of gonococci involves a series of discrete events, including Opa-dependent binding of the bacteria to cell surface proteoglycans, recruitment of F-actin, depolymerization of the aggregated actin, and internalization and intracellular transport of the bacteria via a microfilament- and perhaps microtubule-dependent process. The molecule(s) that links the bacteria to the actin-based cytoskeleton and that initiates the entry event is yet to be identified. A prime candidate antigen is the heparin sulfate-containing proteoglycan which was identified as the primary receptor for the invasion-promoting opacity protein (46). This molecule, which yields into the cytoskeleton-enriched cell fraction upon isolation (46), may either directly or via complexed molecules connect the adherent gonococci to the host cell cytoskeleton. Proteoglycans participate in the formation of focal adhesion plaques that connect cells to the extracellular matrix (50). Whether they also function in phagocytosis remains to be investigated.

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