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Escherichia coli expressing a *Neisseria gonorrhoeae* opacity-associated outer membrane protein invade human cervical and endometrial epithelial cell lines

(bacterial pathogenesis/bacterial adhesion/gonorrhea)

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ABSTRACT **Members of the opacity-associated (Opa) outer membrane protein family of *Neisseria gonorrhoeae* have been proposed to mediate adherence to and invasion of cultured human epithelial cells. We transformed *Escherichia coli* with a plasmid containing a gonococcal *opa* gene fused in-frame to the leader sequence of the β-lactamase gene as described by Palmer *et al.* [Palmer, L., Brooks, G. F. & Falkow, S. (1989) *Mol. Microbiol.* 3, 663–671]. These transformed *E. coli* [*E. coli* (*opa*)] expressed the heat-modifiable *opa* gene product (the Opa protein) in their outer membrane and adhered to and invaded ME-180 human endocervical epithelial cells. In a 2-h adherence assay, an average of 26.7 *E. coli* (*opa*) adhered per ME-180 cell, whereas the control *E. coli* carrying only the expression vector (pKT279) did not adhere at all (<0.15 bacterium per cell). We investigated the ability of the adherent *E. coli* (*opa*) to invade ME-180 epithelial cells by using a gentamicin selection assay. We recovered up to 1 × 10^6 gentamicin-resistant bacteria per monolayer when ME-180 cells were infected with *E. coli* (*opa*) compared to <10 bacteria when the epithelial cells were infected with the same number of control *E. coli* (pKT279). The kinetics and level of invasion by *E. coli* (*opa*) were similar to invasion by Opa$^+$ *N. gonorrhoeae*. Maximum invasion occurred 4 h after infection with 4 × 10^7 bacteria. Transmission electron microscopy studies confirmed that *E. coli* (*opa*) invaded ME-180 cells. In comparative studies, the number of *E. coli* (*opa*) that invaded HEC-1-B human endometrial epithelial cells was about an order of magnitude less than the number that invaded ME-180 cells, and *E. coli* (*opa*) did not invade Chang human conjunctival epithelial cells at all. The observations that early (<4 h) invasion by *E. coli* (*opa*) was dramatically inhibited, in a dose-responsive manner, by the actin-disrupting reagent cytochalasin D but later invasion (8–24 h) was not suggest that invasion mediated by Opa proteins may occur by two mechanisms, only one of which is dependent upon microfilament function. Transmission electron microscopy also revealed that infected epithelial cells had a dramatically increased amount of cytoplasmic fibrillar material surrounding the nucleus. The function and genesis of this material remain unclear. These studies indicate that at least one gonococcal Opa protein is an invasin.**

Neisseria gonorrhoeae causes several medically and economically important sexually transmitted diseases including gonorrhea, pelvic inflammatory disease, and disseminated gonococcal infection, as well as ophthalmia neonatorum. Adherence to host cells and tissue is the first and most important step of infection for the gonococcus. Gonococci possess several proteins that act as ligands for host tissue. Pili were the first such ligands shown to be important in the adherence of gonococci to epithelial cells in culture (1–6). Indeed, *in vivo* experiments have shown that gonococci possessing pili establish infection in male human volunteers inoculated intraurethrally, whereas mutant gonococci unable to make pili are avirulent (7). More recently a gonococcal gene encoding a glycolipid-binding component has been cloned into *Escherichia coli* (8). At least some members in the family of opacity-associated proteins (Opa, formerly called PII proteins) also appear to play an important role in adhesion to epithelial cells (2, 5, 9–12) and polymorphonuclear leukocytes (13–16). Very recently, at least one putative Opa protein has been reported to be essential for optimal gonococcal invasion of human epithelial cells in culture (17, 18).

Opas are a family of closely related proteins that are subject to rapid phase and antigenic variation. A gonococcal strain may carry as many as 11 or more different Opa genes (19, 20) that are independently switched on and off at a cumulative frequency of about 10^{-2} to 10^{-3} per cell per generation (21). Since a gonococcus can express from 0 to 3 or even more Opas, gonococcal cultures are always somewhat heterogeneous regarding their Opa protein content. Due to this complex array of Opa genes and gene expression, the study of the function of Opa proteins is very difficult. Recently, Palmer *et al.* (22) used *E. coli* to express a fusion protein comprising the signal peptide of β-lactamase and an Opa protein from *N. gonorrhoeae* F62SF (22). The mature fusion protein is 244 amino acids long with only the first 7 amino acids different from the gonococcal Opa protein. The Opa fusion protein exhibits the same physical and immunological properties as the gonococcal Opa and is expressed within or on the outer membrane of the transformed *E. coli* (22).

Since humans are the only host for *N. gonorrhoeae*, there is no definitive animal model for studies. Therefore, several organ and cell culture models have been used to study the interaction of gonococci with its host (23–25). In the present studies, we investigated whether *E. coli* expressing the Opa fusion protein described by Palmer *et al.* (22) adhered to or invaded human epithelial cells.

MATERIALS AND METHODS

Bacteria and Plasmids. *E. coli* DH5α was used throughout the studies. Although not discussed further in this manuscript, many of the experiments were also performed with *E. coli* HB101, with very similar results obtained. For adherence and invasion assays, bacteria were grown to midlogarithmic phase in 2 × YT broth (containing per liter of distilled water, 16 g of Bacto tryptone, 10 g of Bacto yeast extract, and 5 g of NaCl, pH 7.0). For plates, the medium was supplemented with 1.5% agar. Bacteria containing plasmids were grown in or on medium containing tetracycline (20 μg/ml; for pKT279 and pLPGC1) or ampicillin (100 μg/ml; for pGEM-3Z and pDS001). Classical molecular biology techniques were used, as described by Sambrook *et al.* (26).

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Plasmid pLPGC1 (22) (received from Lindy Palmer, University of California, San Francisco) is a derivative of pKT279 containing an *opa* gene from *N. gonorrhoeae* F62SF. Plasmid pKT279 (purchased from ATCC) is an expression vector that carries the leader sequence of β-lactamase for secretion or insertion into the outer membrane of cloned gene products (27). Plasmid pDS001 was derived from pLPGC1 and plasmid pGEM-3Z (Promega) derived as follows: *opa* was cleaved from pLPGC1 with *Hin*fI, which cuts upstream of *opa* (within the pKT279 vector to include the β-lactamase leader sequence) and at the *opa* stop codon, thus removing all nucleotides 3′ of *opa*. Ends of the gel-purified fragment containing *opa* were blunted with the Klenow fragment of DNA polymerase I and dNTPs, the blunted fragment was cut with *Eco*RI, and the fragment was ligated into pGEM-3Z, which had been cut with *Eco*RI and *Hin*cII.

Cell Culture. ME-180 epithelial cells (ATCC HTB 33), derived from a human cervical carcinoma, were maintained in McCoy 5A medium supplemented with 10% (vol/vol) fetal bovine serum and 20 mM Hepes (pH 7.3). HEC-1-B epithelial cells (ATCC HTB 113), derived from a human endometrial adenocarcinoma, were maintained in Dulbecco's modified Eagle medium/nutrient mixture F12 supplemented with 10% fetal bovine serum and 20 mM Hepes (pH 7.3). Chang epithelial cells (ATCC CCL 20.2), derived from human conjunctiva, were maintained in medium 199 with Hanks' balanced salt solution supplemented with 10% fetal bovine serum. All medium supplies were purchased from GIBCO. Cells were incubated with or without antibiotics (100 units of penicillin and 100 μg of streptomycin per ml) at 37°C in humidified 5% CO_2/95% air, were fed every third or fourth day, and were split every 7 days by using 0.025% trypsin and 1 mM EDTA in Hanks' balanced salt solution.

Bacterial Adherence to Epithelial Cells. Epithelial cell suspensions obtained from barely confluent logarithmic-phase monolayers were inoculated at 2×10^5 cells per well in 24-well tissue culture plates containing 15-mm Thermonox coverslips (Nunc) and incubated for 24 h. The medium was then removed and 500 μl of Dulbecco's phosphate-buffered saline (pH = 7.2; PBS) containing 4×10^6 colony-forming units of *E. coli* (*opa*) or *E. coli* (pKT279) was added and incubated for 2 h at 37°C. Nonadherent bacteria were removed by washing the monolayers three times with PBS. Monolayers were then stained with Wright stain and observed by light microscopy (×1000, oil immersion). Adherence was quantitated by counting adherent bacteria on 50–100 contiguous cells. Data are presented as the average number of bacteria per epithelial cell.

Bacterial Invasion of Epithelial Cells. The inability of gentamicin to kill bacteria residing within epithelial cells has been used to discriminate between intracellular and extracellular bacteria in bacterial invasion assays (28, 29). For invasion assays, suspensions of epithelial cells obtained from logarithmic-phase barely confluent monolayers were inoculated at 4×10^5 cells per well in 24-well tissue culture plates and incubated for 24 h. The confluent monolayers were then inoculated with bacteria and incubated for up to 24 h as indicated in the text. After appropriate incubation, monolayers were rinsed twice with 1 ml of tissue culture medium and 1 ml of fresh medium containing gentamicin (50 μg/ml) was added for 1 h to kill extracellular bacteria. The gentamicin-containing medium was then removed, the monolayer was lifted by incubation at 37°C for 5 min with 1 ml of PBS containing 5 mM EDTA, and the free cells were transferred to small tubes. Each well was rinsed with 1 ml of PBS, which was added to the lifted cells, and the cell suspension was lysed by a 15-s sonication in a bath sonicator (Heat Systems/Ultrasonics) at 40% power output to release internalized bacteria. Such sonication did not significantly alter *E. coli* viability (data not shown). Dilutions of the surviving bacteria were plated on 2 × YT agar containing tetracycline and incubated overnight, and colonies were counted.

Transmission Electron Microscopy. ME-180 cell monolayers infected overnight as above were rinsed with PBS to remove nonadherent bacteria, lifted with PBS containing 5 mM EDTA, pelleted, and fixed with 2.5% (vol/vol) glutaraldehyde in 0.2 M sodium phosphate, pH 7.4/1% $CaCl_2$ at 4°C. The fixed pellets were postfixed for 30 min in 1% osmium tetroxide in 0.1 M sodium phosphate (pH 7.4). Specimens were then dehydrated in a graded ethanol series and embedded in Spurr embedding medium (Electron Microscopy Sciences, Fort Washington, PA). Sections (60 nm thick) were cut on an LKB 8800 ultramicrotome III (LKB) with a diamond knife and stained for 20 min in 5% (wt/vol) uranyl acetate and for 5 min in Reynolds' lead citrate. Micrographs were taken on a Zeiss EM-9 electron microscope.

RESULTS

The plasmid content of the strains used was confirmed by restriction enzyme analysis (data not shown). *E. coli* (*opa*) (*E. coli* transformed with pLPGC1), which contains a gonococcal *opa* gene fused to the leader sequence of β-lactamase, expressed an additional heat-modifiable outer membrane protein not expressed by *E. coli* transformed with the expression vector alone [*E. coli* (pKT279)] (data not shown). The apparent molecular mass of this protein was 27 kDa when solubilized at 37°C and 32 kDa when solubilized at 100°C. This corresponds to what was observed by Palmer *et al.* (22), and we are assuming that it is a gonococcal Opa protein.

Bacterial Adherence to ME-180 Human Cervical Epithelial Cells. We measured adherence of *E. coli* (*opa*) and *E. coli* (pKT279) to ME-180 epithelial cells. *E. coli* (pKT279) adhered poorly or not at all (0.13 ± 0.1 bacterium per cell; n = 3; Fig. 1*A*), whereas *E. coli* (*opa*) adhered very well (26.7 ± 16.7 bacteria per cell; n = 3; Fig. 1*B*).

It is possible that a peptide encoded by the ≈500 base pairs of DNA downstream of *opa* in pLPGC1 could be affecting or mediating adherence. To eliminate this possibility, we constructed pDS001. pDS001 contains only the *opa* gene fused to the β-lactamase leader sequence, with no downstream insert DNA. In our standard adherence assay, *E. coli* (pDS001) avidly adhered to ME-180 epithelial cells, whereas *E. coli* (pGEM-3Z) did not adhere at all. These data indicate that only the *opa* gene product was mediating adherence. The Opa protein expressed by *E. coli* (pDS001) was identical to the Opa expressed by *E. coli* (pLPGC1), as determined by SDS/PAGE (data not shown).

Bacterial Invasion of Human Epithelial Cell Lines. The ability of *E. coli* containing either pKT279 or pLPGC1 to invade ME-180 cells was compared using a gentamicin invasion assay. *E. coli* (pKT279) did not invade ME-180 cells (data not shown). *E. coli* (*opa*) invaded ME-180 epithelial cells in a dose- and time-dependent manner (Fig. 2). Optimal invasion occurred with an inoculum of 4×10^7 *E. coli* (*opa*) allowed to invade for 4 h. At an inoculum of 4.1×10^8 *E. coli* (*opa*), the number of gentamicin-resistant bacteria recovered at later times decreased, perhaps due to damage to the epithelial cells by bacteria. *E. coli* (pDS001), the *E. coli* containing pGEM-3Z with only the *opa* gene and no downstream DNA, invaded ME-180 epithelial cells with kinetics essentially identical to those of *E. coli* (pLPGC1) (data not shown). In other experiments, internalized *E. coli* (*opa*) remained resistant to gentamicin for at least 6 h, at which time the experiments were terminated (data not shown). These results suggest that internalized *E. coli* (*opa*) remain viable within epithelial cells but do not multiply.

Cytochalasins have been used to demonstrate the involvement of the cytoskeletal system in bacterial invasion of human cells (30). We tested the ability of increasing concen-

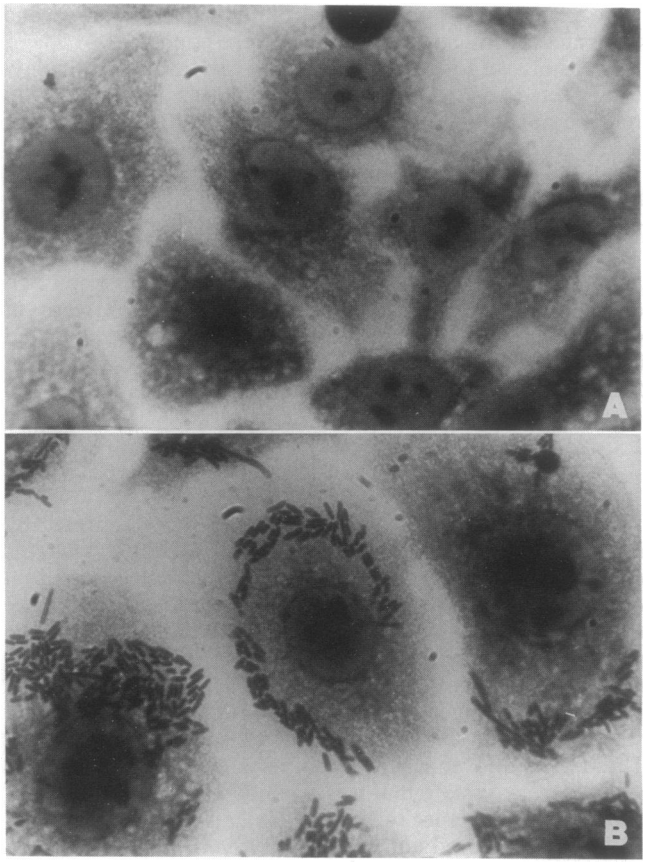

FIG. 1. Light micrographs of monolayers of ME-180 human endocervical epithelial cells inoculated for 2 h with *E. coli* DH5α transformed with plasmid pKT279 (*A*) or plasmid pLPGC1 [defined as *E. coli* (*opa*)] (*B*). Monolayers were stained with Wright stain. (×750.)

trations of cytochalasin D to prevent invasion of epithelial cells by *E. coli* (*opa*) (Fig. 3). Monolayers were treated with cytochalasin D for 1 h before the standard invasion assay was performed, and cytochalasin D was present throughout the assay. For up to 4 h after monolayers were infected with 4×10^7 *E. coli* (*opa*), cytochalasin D, in a dose-dependent manner, decreased the number of *E. coli* (*opa*) surviving gentamicin treatment by 50- to 250-fold; i.e., cytochalasin D dramatically reduced invasion at early times. After 4 h, inhibition of invasion by all concentrations of cytochalasin decreased by ≈10-fold. Cytochalasin D did not alter the

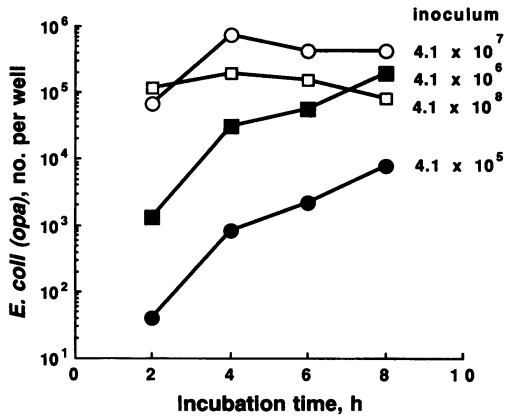

FIG. 2. Invasion by *E. coli* (*opa*) of ME-180 human cervical epithelial cells over time.

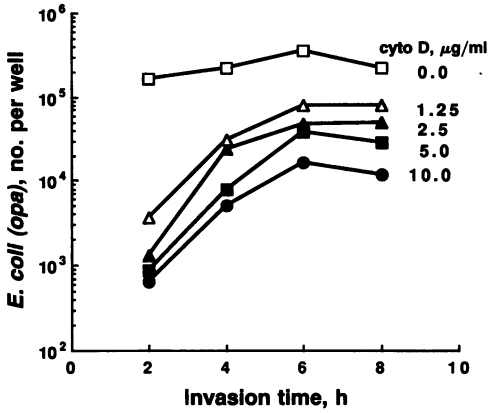

FIG. 3. Cytochalasin D (cyto D) inhibits, in a dose-responsive manner, invasion of ME-180 human cervical epithelial cells by *E. coli* (*opa*).

susceptibility of *E. coli* (*opa*) to gentamicin in the absence of epithelial cells (data not shown).

In the original paper describing pLPGC1, Palmer *et al.* (22) observed no adherence of *E. coli* (*opa*) to HeLa and HEC-1-B cells. Since we observed both adherence to and invasion of ME-180 cells (as described above), we performed comparative invasion assays with three epithelial cell lines, ME-180, HEC-1-B, and Chang (Fig. 4). In our hands, *E. coli* (*opa*) invaded HEC-1-B cells, albeit ≈10-fold less efficiently than they invaded ME-180 cells. *E. coli* (*opa*) did not invade Chang conjunctival cells at all; i.e., no bacteria (above a background of one to three colonies per plate in undiluted cell supernatants) were recovered from invasion assays. Thus, it appears that the expressed Opa protein mediates differential invasion of epithelial cells from different anatomic sites. It remains unclear to us why Palmer *et al.* (22) did not observe adherence of *E. coli* (*opa*) to HEC-1-B cells.

Electron Microscopy. The intracellular location of *E. coli* (*opa*) was confirmed by transmission electron microscopy studies. Fig. 5 shows events taking place during invasion of ME-180 cells by *E. coli* (*opa*). Although all these photographs were from a single sample, several levels of invasion were observed. Apparently, the bacteria first come into very close contact with the epithelial cell cytoplasmic membrane (Fig. 5*A*). Upon initial invasion of the epithelial cell, there is little question that the internalized *E. coli* (*opa*) are within mem-

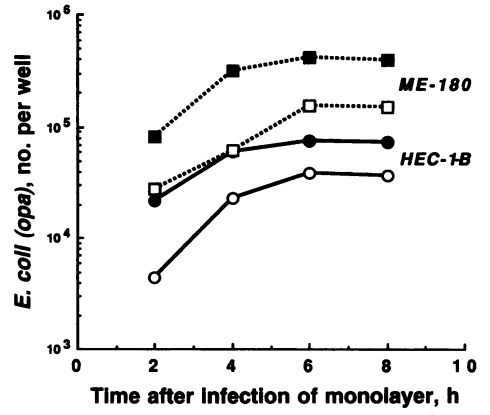

FIG. 4. Invasion by *E. coli* (*opa*) of ME-180 cervical (squares) or HEC-1-B endometrial (circles) human epithelial cells. Cell monolayers in 24-well plates were infected with 1×10^8 *E. coli* (*opa*) and invasion was measured over time. Results are from two experiments done in duplicate on different days (open vs. solid symbols). Invasion differences seen on the two days reflect as yet undefined daily variations.

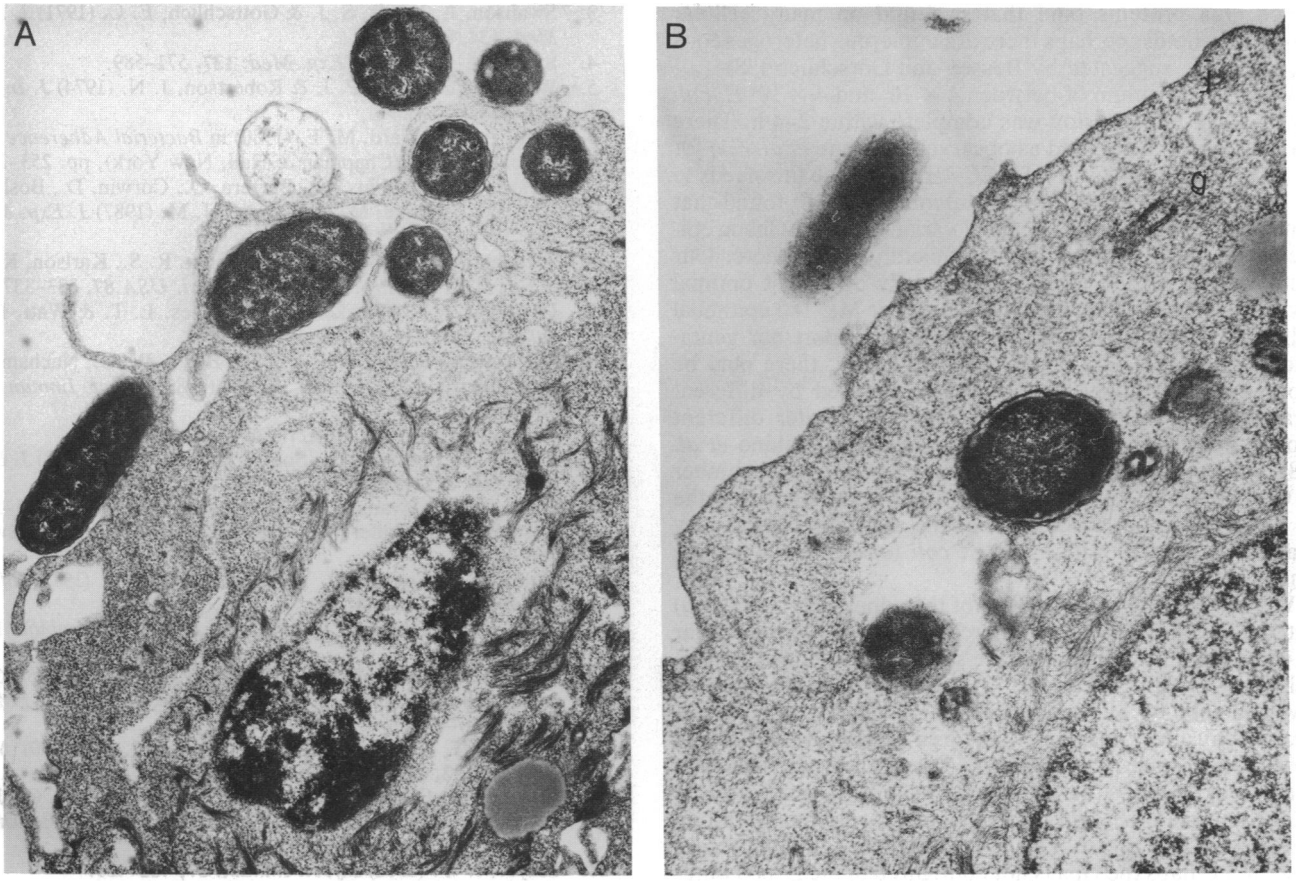

FIG. 5. Transmission electron micrographs of overnight invasion of ME-180 human cervical epithelial cells by *E. coli* (*opa*). (*A*, ×14,900; *B*, ×27,600.)

brane-bound vesicles (Fig. 5*A*). However, it is difficult to discern whether all bacteria remain within vesicles at later times after invasion (Fig. 5*B*).

It is also interesting to observe the epithelial cell during these interactions. In response to the bacterial adherence, there is an apparent increased activity in the epithelial cell membrane (Fig. 5*A*). At this time, some bacteria are engulfed whereas others remain on the epithelial cell surface. After invasion, the epithelial cell surface regains normal surface activity (Fig. 5*B*). In essentially every cell invaded by the *E. coli* (*opa*), we observed a striking increase in fibrillar material deposited or formed around the epithelial cell nucleus. The composition and genesis of this material remain unknown.

E. coli (pKT279) did not adhere to or invade ME-180 cells when observed by transmission electron microscopy (data not shown).

DISCUSSION

Our studies demonstrate that a gonococcal Opa protein expressed on the surface of *E. coli* mediates adherence to and invasion of ME-180 human cervical epithelial cells. This description of a biologically active gonococcal invasin expressed in *E. coli* clearly supports and extends the work of several investigators (refs. 17, 18, 22, 25, and 31, and S. T. Hingley and R.F.R., unpublished data).

Our work is based in good part on the studies of Palmer *et al.* (22) who were able to express relatively high amounts of Opa protein within the outer membrane of *E. coli*. They fused all but the first 7 amino acids of an Opa protein from *N. gonorrhoeae* F62SF with the leader sequence of *E. coli* β-lactamase. For reasons that remain unclear, Palmer *et al.* (22), using HeLa 229 and HEC-1-B cell lines, saw no biological activity of their *E. coli* (*opa*). The adherence conditions used by Palmer *et al.* (22) were slightly different (1 h vs. 2 h) than ours, and a different cell culture medium was used. In comparative studies with ME-180, HEC-1-B, and Chang cells, we found that *E. coli* (*opa*) did invade HEC-1-B cells, but ≈10 times less efficiently than they invaded ME-180 cells. It is possible that, with small variations in assay conditions, *E. coli* (*opa*) would not bind to HEC-1-B cells; perhaps those conditions were used by Palmer *et al.* (22).

We were surprised to find that *E. coli* (*opa*) did not invade Chang conjunctiva cells. At least some Opa-containing gonococci do invade Chang cells (ref. 17 and S. T. Hingley and R.F.R., unpublished observations). The results suggest that gonococci have more than one invasin or that different Opa proteins (or a combination of Opa proteins) are used to adhere to and invade different epithelial cell types. Of course these two possibilities are not mutually exclusive. Certain Opas appear to mediate adherence to epithelial cells, others mediate adherence to human neutrophils, and yet others mediate adherence to both (ref. 6 and S. T. Hingley and R.F.R., unpublished data). During gonorrhea, the variation of Opa adherence specificity expressed by a strain could enable it to adapt to different tissues or environments (32–35).

Although a receptor(s) for Opa proteins has not yet been identified, there is some evidence that it is a cellular glycoconjugate(s) (36, 37). Recently, Griffiss and colleagues (J. MacLeod Griffiss, personal communication) identified a carbohydrate binding domain in Opa proteins of gonococcal strain FA1090 that shares peptide sequence homology with the carbohydrate binding domain of the human asialoglycoprotein receptor. Whether this domain is involved in Opa-mediated gonococcal adherence to epithelial cells remains to be determined. Indeed, if there is a specific carbohydrate to

which Opa proteins bind that is found on many cellular proteins or lipids, perhaps there are numerous heterogeneous receptors, as suggested by Bessen and Gotschlich (38).

When an inoculum of between 4×10^7 and 4×10^8 *E. coli* (*opa*) was used, invasion was complete within 2–4 h. There was little or no lag period as observed by Chen *et al.* (31) for *N. gonorrhoeae* invasion of HEC-1-B, although this needs to be determined at times <2 h. Makino *et al.* (18) found that some Opa-containing gonococcal variants invade Chang epithelial cells ≈100 times more efficiently than others. Our studies with *E. coli* (*opa*) indicate only a modest optimal invasion, on the order of two bacteria per ME-180 epithelial cell. Electron microscopy observations support our gentamicin adherence data on this point. Clearly, there may be more than one adhesin or invasin expressed by different strains or variants of gonococci, perhaps under different growth conditions. It is very interesting that Makino *et al.* (18) found very high invasion only with a very limited number of Opa proteins. Apparently the F62SF Opa cloned into the *E. coli* used in our studies is not the same Opa defined by Makino *et al.* (18), since our *E. coli* (*opa*) did not invade Chang cells.

We observed that only ≈10% of the adherent *E. coli* (*opa*) entered the epithelial cells. Whereas this appears to be similar to what some investigators have seen for invasion of epithelial cells by gonococci (25, 31), it doesn't approach the invasion seen by Makino *et al.* (18). It remains to be investigated why all of the *E. coli* (*opa*) aren't internalized. Several possibilities exist. Perhaps only some epithelial cells are competent. Perhaps there is a threshold of Opa concentration on the surface of *E. coli* that is needed for optimal internalization, and that threshold is just barely met with the present system. Perhaps the Opa is not configured in the *E. coli* outer membrane for optimal biologic activity. Thus, it may mediate excellent adherence but poor invasion. This would be in accord with Isberg's hypothesis (39) that only very high-affinity binding of some cellular receptors leads to bacterial internalization. Finally, there seems to be mounting evidence that there is more than one surface component responsible for gonococcal host cell invasion; perhaps two or more components work in concert to induce optimal gonococcal invasion.

Electron microscopic observation of *E. coli* (*opa*) internalization by the ME-180 epithelial cells revealed that, at least initially, all bacteria were contained within a vesicle. We hesitate to call this vesicle a phagocytic or endocytic vesicle, because at this point, we do not know the molecular mechanism by which gonococci invade epithelial cells. At later times of invasion, however, it is difficult to discern a vesicle membrane surrounding the internalized bacteria. Whether it is there, but obscured by the fixing techniques or indeed by the *E. coli* (*opa*) themselves, remains to be resolved. Regardless of their exact intracellular compartment, the observation that *E. coli* (*opa*) remained viable (but did not grow) in the presence of extracellular gentamicin at 50 μg/ml for at least 6 h suggests a well-protected intracellular environment.

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