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Model for Invasion of Human Tissue Culture Cells by *Neisseria gonorrhoeae*

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A tissue culture model has been developed for studying the ability of *Neisseria gonorrhoeae* to invade eucaryotic cells. The cell line HecIB, a human adenocarcinoma endometrial cell line, was found to support gonococcal invasion. The bactericidal antibiotic gentamicin was used to kill those bacteria that had not entered the HecIB cells, allowing us to quantitate internalized bacteria. Kinetic studies showed an increase in the titer of gentamicin-protected gonococci at 4 h postinfection followed by a decrease; a second increase occurred after 6 h. The state of piliation did not affect the degree of invasion when the bacteria were spun down onto the monolayer. Gonococcal invasion was inhibited when the HecIB cells were preincubated with cytochalasin D before bacterial infection. *N. lactamica* was used as a negative control. No internalized *N. lactamica* cells were observed by electron microscopy. Electron microscopy documented the intracellular location of the gonococci in HecIB cells and the eventual destruction of the invaded HecIB cells. After 24 h, clusters of gonococci encased in a matrix of cell debris were observed.

Neisseria gonorrhoeae specifically infects humans; there is no appropriate small animal model for studying the full array of gonococcal pathogenetic mechanisms. We have limited understanding of how this bacterium interacts with its host or how gonococci cross mucosal barriers. In males the site of uncomplicated gonococcal infection is the urethra, and for females it is the endocervix (5). Complications arising from gonococcal infection include disseminated gonococcal infection (4) and pelvic inflammatory disease (3).

Examination of urethral scrapings from men with symptoms of early gonorrhea revealed that gonococci were present within the cytoplasm of epithelial cells (23). A number of investigators have focused upon gonococcal interaction with tissue culture monolayers. Depending on the tissue culture cell type, gonococci were found only to adhere (17) or to adhere or enter other cells (20, 22). Bessen and Gotschlich (1) found that PII variants of *N. gonorrhoeae* R10 differed in the ability to adhere to HeLa cells. After gonococci interact for 2 to 3 h with HeLa cell monolayers, a small percentage of these microorganisms are protected from the killing action of fresh rat serum or gentamicin (21), two bactericidal agents incapable of entering eucaryotic cells.

Studies with the human fallopian tube organ culture (FTOC) have demonstrated the ability of *N. gonorrhoeae* to enter and pass through mucosal cells (13, 14, 24). This process has been divided into four steps. (i) The gonococci attach to the microvilli of nonciliated cells, (ii) the bacteria are internalized by the mucosal cells, (iii) the gonococci are transported in vacuoles to the base of these cells, and (iv) the bacteria are released into the subepithelial tissue by exocytosis (14). Gonococci are not found within the mucosal cells until 24 h postinfection (14). It was demonstrated that piliated gonococci adhere in greater numbers than do nonpiliated gonococci; however, once attached the piliated and nonpiliated gonococci are internalized with equal efficiency (14). Gonococcal lipopolysaccharide causes the sloughing of ciliated cells from the mucosa of the fallopian tube (7, 15). *N. meningitidis* is also capable of invading mucosal cells in the FTOC system (14).

We present here our studies of gonococcal interaction with the human endometrial cell line HecIB, which we have found supports significant levels of invasion. In this model system, after internalization, the majority of the gonococci appear to be free within the cytoplasm of the HecIB cell.

MATERIALS AND METHODS

Bacterial strains and tissue culture cells. *N. gonorrhoeae* F62 and MS11 were used for most of the studies (12). *N. gonorrhoeae* 1498, a clinical isolate from a disseminated gonococcal infection, was obtained from Geoff Brooks (University of California at San Francisco). *N. lactamica* 30011 (2) was used as a negative control. *Neisseria* strains were passed daily on GC agar (BBL Microbiology Systems) supplemented with 1% IsoVitaleX (BBL). *Shigella flexneri* IA was from this laboratory (19). The human endometrial adenocarcinoma cell lines HecIB and AN3CA were obtained from Geoff Brooks and are available from the American Type Culture Collection. The HEp-2 cell line (human larynx) has previously been described (19). Tissue culture cells were maintained in RPMI medium (Irvine Scientific) supplemented with 10% fetal calf serum (MA Bioproducts)–2.0 mM glutamine (Irvine Scientific).

Invasion assay. HecIB cells (2×10^5) were seeded into each well of a 24-well microtiter plate (Becton Dickinson Labware) and incubated at 37°C in 5% CO_2 for 24 h. Bacterial cells (2×10^5 CFU/ml) were centrifuged onto the monolayer ($800 \times g$, 23°C) to initiate infection; however, centrifugation is not necessary (data not shown). The microtiter plates were incubated at 37°C (5% CO_2) for various times. After the desired incubation period, the infected monolayers were washed five times with phosphate-buffered saline (PBS), pH 7.0, to remove nonadherent bacteria. Fresh supplemented RPMI medium containing gentamicin (20 μg/ml) was added to the wells, and the plates were returned to the 37°C, 5% CO_2 incubator. After 90 min of incubation, the medium was removed and the monolayer was washed twice with PBS.

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To determine the number of intracellular enteric bacteria within a monolayer of tissue culture cells after gentamicin treatment, it has been possible to use the detergent Triton X-100 (1%) to lyse the monolayers and release the bacteria (10, 19). We observed that detergents which lysed the monolayers also killed gonococci. To overcome this problem, 0.5 mM EDTA in PBS along with agitation was used to release the monolayer from the plastic well. Appropriate dilutions were plated onto GC agar plates to assay the number of viable intracellular bacteria. EDTA at the concentration used does not affect the viability of the *Neisseria* cells (data not shown). To ensure that treatment of the monolayer with EDTA provided an accurate determination of internalized bacteria, a quantitation of intracellular *S. flexneri* was made by using EDTA or Triton X-100 to release the bacteria. Virtually equal numbers of internalized *S. flexneri* were seen with the two methods (data not shown). These data would indicate that treatment of the monolayer with EDTA is an effective means for quantitating intracellular bacteria.

Gonococcal invasion is sensitive to the particular lot of fetal calf serum being used for maintaining the tissue culture cells. All lots of fetal calf serum were heat inactivated (56°C for 30 min), but a range of interactions was observed between the gonococci and the HecIB cells. Lots of fetal calf serum were found that did not support gonococcal growth in the presence of the HecIB cells, whereas other lots supported growth but did not support gonococcal invasion. Gonococci did not increase in titer in supplemented RPMI unless a HecIB monolayer was present (data not shown). Supplementing the tissue culture medium with 0.1% IsoVitaleX (vol/vol) enabled the gonococci to invade in otherwise nonsupportive fetal calf serum. Iron appeared to be the necessary supplement (data not shown).

In some experiments, cytochalasin D (Sigma Chemical Co.) was added 30 min before addition of the bacteria.

Electron microscopy. Samples for electron microscopy were prepared as described previously (10). Samples prepared for direct embedding (25) were treated the same except the monolayers were grown and left intact in Contur Permanox tissue culture dishes (LUX) during the staining, dehydration, and embedding steps. All samples were embedded in Spurrs plastic (Polysciences, Inc.).

RESULTS

Bacterial interaction with tissue culture cells. In the experiments described below, invasive bacteria are defined as those bacteria that survived the following protocol: five PBS washes of the infected monolayer, 90-min exposure to 20 μg of gentamicin per ml in tissue culture medium, and two more PBS washes before disruption of the monolayer. Adherent and invasive bacteria are determined in the same way except the gentamicin treatment is omitted. The bactericidal antibiotic gentamicin was used because of its inability to enter eucaryotic cells; therefore, intracellular bacteria were protected. A minor proportion of noninvasive bacteria survive this treatment and may be protected by nonspecific interaction with the monolayer as the 20 μg of gentamicin per ml kills the *Neisseria* cells in the absence of a monolayer.

Presented in Table 1 are the results of a 20-h incubation of the bacteria with the HecIB monolayer. *N. lactamica* and both piliated and nonpiliated variants of *N. gonorrhoeae* F62 were adherent to the HecIB monolayer, but only the gonococci were invasive. After 20 h both piliated and nonpiliated strains of F62 were invasive at the same level. Similar results were observed when *N. gonorrhoeae* 1498, a clinical isolate from a disseminated gonococcal infection, was incubated with a monolayer for 24 h (data not shown). Our attempts to study gonococcal invasion by using monolayers of HEp-2 and AN3CA tissue culture cells resulted in similar levels of gentamicin survival for the gonococci and *N. lactamica* strains, indicating a lack of invasion.

TABLE 1. Binding to and invasion of HecIB cells by *Neisseria* spp.[a]

Organism	No. of bacteria
Adherent and internalized	
N. lactamica	$6.2 \times 10^5 \pm 0.8 \times 10^5$
N. gonorrhoeae F62 p^{+b}	$1.4 \times 10^6 \pm 0.5 \times 10^6$
N. gonorrhoeae F62 p^-	$6.9 \times 10^6 \pm 0.4 \times 10^6$
Internalized	
N. lactamica	$1.9 \times 10^3 \pm 0.3 \times 10^3$
N. gonorrhoeae F62 p^+	$6.5 \times 10^5 \pm 0.9 \times 10^5$
N. gonorrhoeae F62 p^-	$2.6 \times 10^5 \pm 0.8 \times 10^5$

[a] Results are the averages of three experiments. HecIB monolayers were infected with the bacteria for 20 h. The infection was initiated by centrifuging the bacterial cells onto HecIB monolayers.
[b] p^+, Piliated; p^-, nonpiliated.

The kinetics of gonococcal invasion are presented in Fig. 1. Initially the number of gentamicin-resistant gonococci

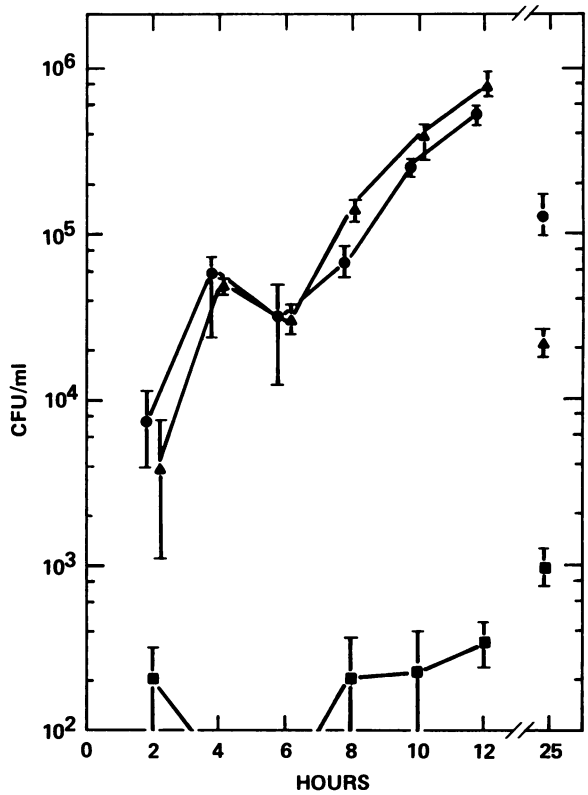

FIG. 1. Kinetics of *Neisseria* invasion. The graph shows the number of *Neisseria* spp. that survived 90 min of exposure to 20 μg of gentamicin per ml at the different time points. Time points indicate when the antibiotic was added. Input titers were: *N. lactamica*, 5.2×10^5; strain F62 p^+, 1.6×10^5; and strain MS11, 1.2×10^5. Results are based on three experiments. Key: ▲, MS11; ●, F62; ■, *N. lactamica*.

TABLE 2. Effect of cytochalasin D on gonococcal invasion

Bacterial species (duration of invasion)	Cytochalasin D treatment (μg/ml)	No. of internalized organisms[a]
N. gonorrhoeae F62	None	$2.4 \times 10^5 \pm 1.7 \times 10^5$
p^{+b} (6 h)	1.0	$1.6 \times 10^5 \pm 5.0 \times 10^3$
	5.0	$1.0 \times 10^5 \pm 9.0 \times 10^3$
N. gonorrhoeae F62	None	$3.8 \times 10^5 \pm 3.3 \times 10^5$
p^+ (14 h)	2.5	$4.0 \times 10^2 \pm 1.7 \times 10^2$
N. gonorrhoeae MS11	None	$2.5 \times 10^5 \pm 1.1 \times 10^5$
p^+ (14 h)	2.5	$1.7 \times 10^3 \pm 1.1 \times 10^3$
S. flexneri (4 h)	None	$3.5 \times 10^5 \pm 5.0 \times 10^4$
	2.5	$8.1 \times 10^3 \pm 2.7 \times 10^3$

[a] Results are the average of three experiments.
[b] p^+, Piliated.

increased in number for about 4 h. Both of the invasive gonococcal strains we examined reproducibly showed a decrease in the number of gentamicin survivors for the next 2 h. Maximum numbers of internalized bacteria were seen at the 12-h time point. Longer incubation periods (greater than 24 h) resulted in monolayer destruction and resulted in lower titers of gentamicin survivors at the 25-h time point.

Cytochalasin D inhibits the formation of microfilaments and is known to inhibit invasion by *S. flexneri*, *Salmonella typhimurium*, and *Yersinia enterocolitica* (6, 8, 11). HecIB cells were treated with cytochalasin D for 30 min before infection with the gonococci. Bacteria were added to these treated cells, and the cells were incubated for 6 and 18 h before the addition of gentamicin. Table 2 demonstrates that cytochalasin D inhibited gonococcal invasion after 18 h but did not prevent the protection from gentamicin seen after 6 h of incubation. The levels of cytochalasin D used in our experiments were also tested for *S. flexneri* infection of HecIB cells. Table 2 shows that this compound exhibited a strong inhibitory effect on *S. flexneri* invasion of HecIB cells.

Electron microscopy. Gonococcal invasion of HecIB cells was also examined by using electron microscopy. Infected monolayers were prepared in one of two ways for electron microscopy. Either monolayers were lifted off the plastic plates (by treatment with EDTA), pelleted, and then prepared for electron microscopy or the cells were directly fixed in situ on the tissue culture plates. Figure 2 shows a typical example of the interaction of *N. lactamica* with HecIB cells. *N. lactamica* was adherent to the HecIB cells (Table 1) but was never observed internalized within HecIB cells at any time point by electron microscopy.

Observations after 6 h of infection indicated that the gonococci interact with the HecIB cells at the cell surface (Fig. 3). The bacteria were often found adjacent to structures that appear to be coated pits (Fig. 4). Also visible in Fig. 4 is an unidentified, thin pililike structure which appears to be mediating contact between bacteria and the tissue culture cell. After 6 h of infection, gonococci were embedded in the membrane of the HecIB cells (Fig. 5). The bacteria can be observed interacting with the microvilli and at this time are being engulfed by the HecIB cells.

At 10 h after infection, many internalized gonococci were visible. Figures 6 and 7 illustrate our finding that the majority

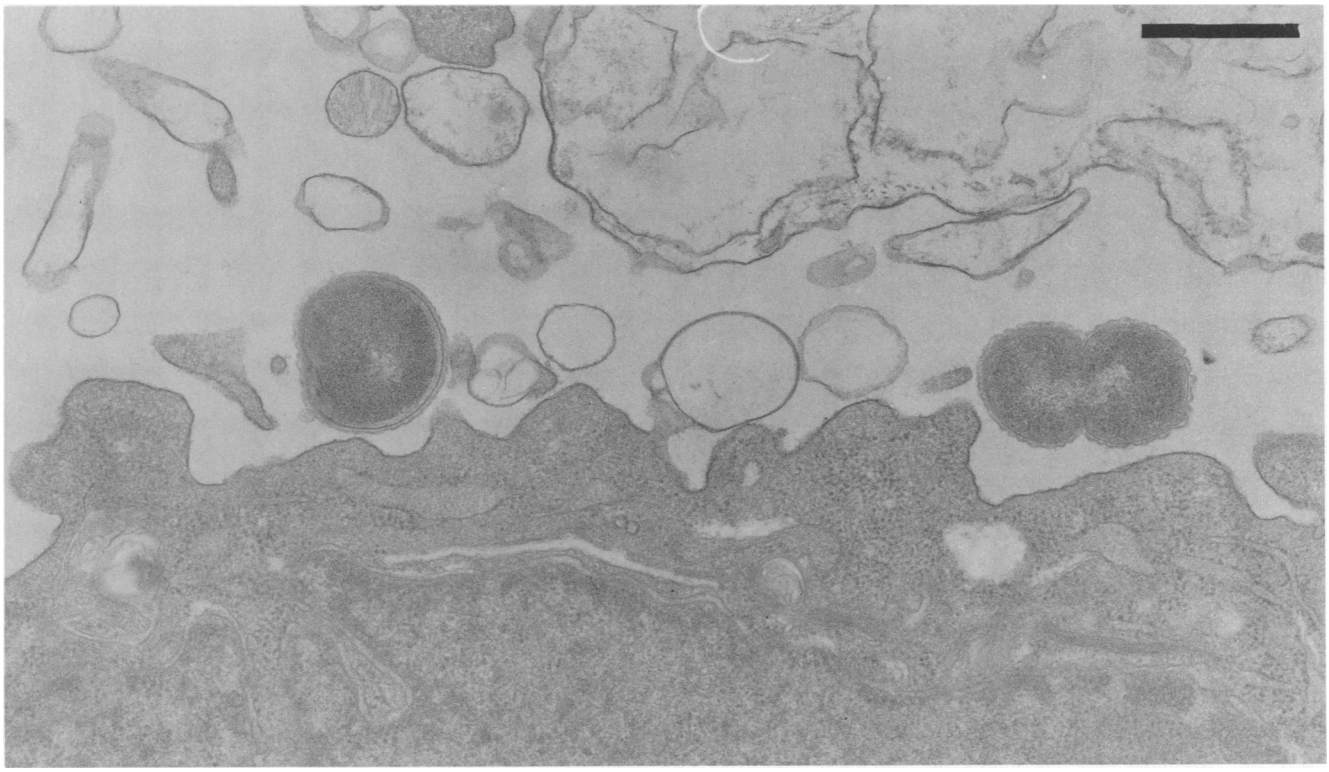

FIG. 2. *N. lactamica* interaction with HecIB cells at 12 h postinfection. Some material appears to bridge the gap between bacteria and HecIB cells. Bacteria remained with the monolayer even after extensive washing. Bar, 1 μm.

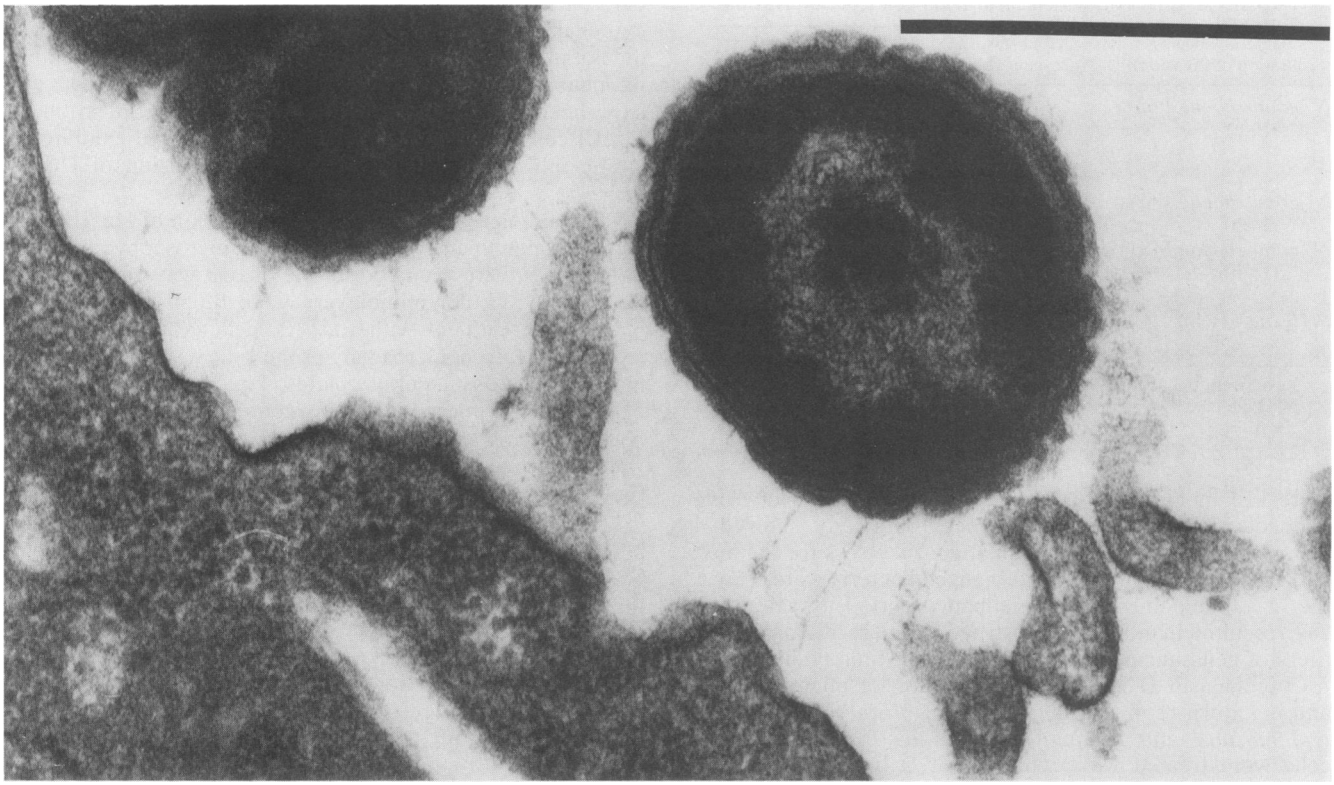

FIG. 3. *N. gonorrhoeae* F62 at the outer surface of a HecIB cell at 6 h postinfection. Gonococci are adherent because the monolayers were washed before fixation. Note fine structures that connect gonococci with the HecIB cell. Bar, 1 μm.

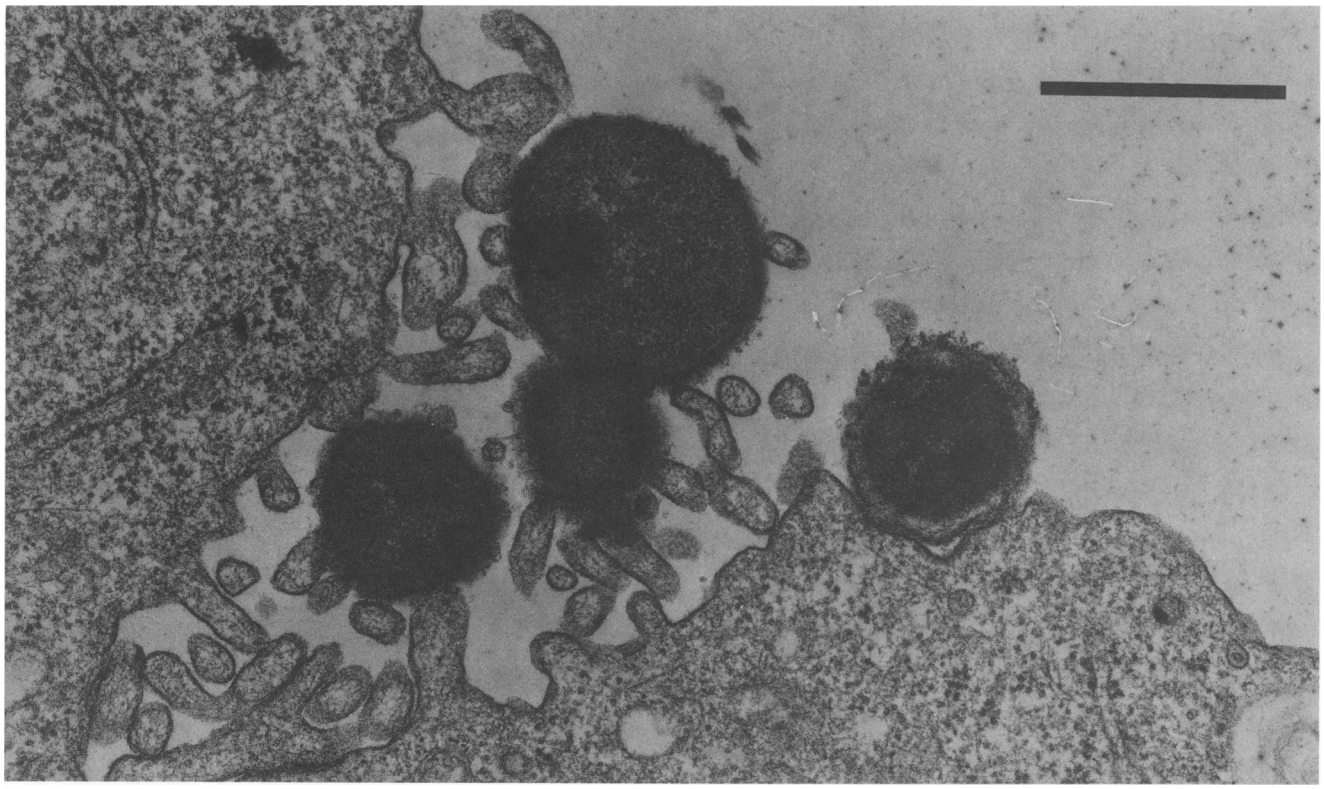

FIG. 4. *N. gonorrhoeae* F62 interacting with microvilli of HecIB cells at 6 h postinfection. Gonococci are attached to the microvilli, and the bacteria to the left are embedded in the membrane of the HecIB cell. Note the depression under the rightmost gonococcus which is reminiscent of a coated pit. Bar, 1 μm.

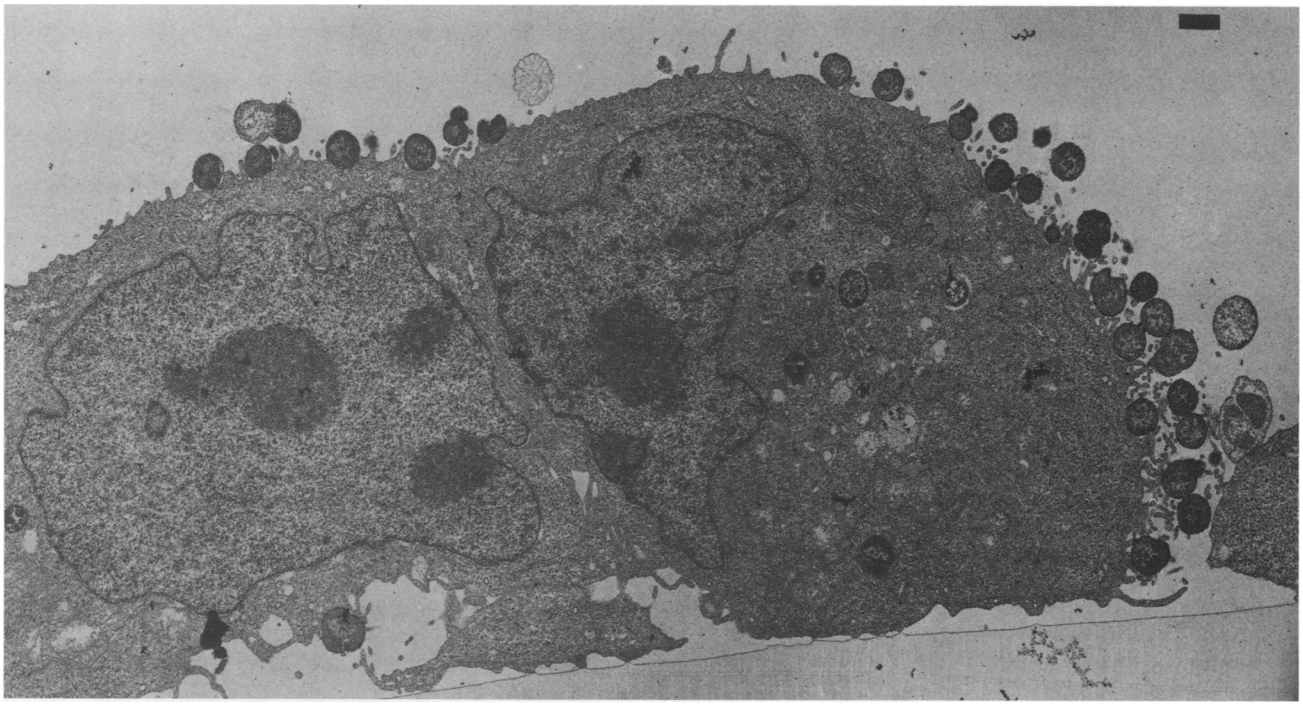

FIG. 5. *N. gonorrhoeae* F62 lining the membranes of HecIB cells. At 6 h postinfection, the gonococci are embedded in the HecIB cells and are being engulfed by the endometrial cells. Cells were prepared by the direct-embedding technique. Bar, 1 μm.

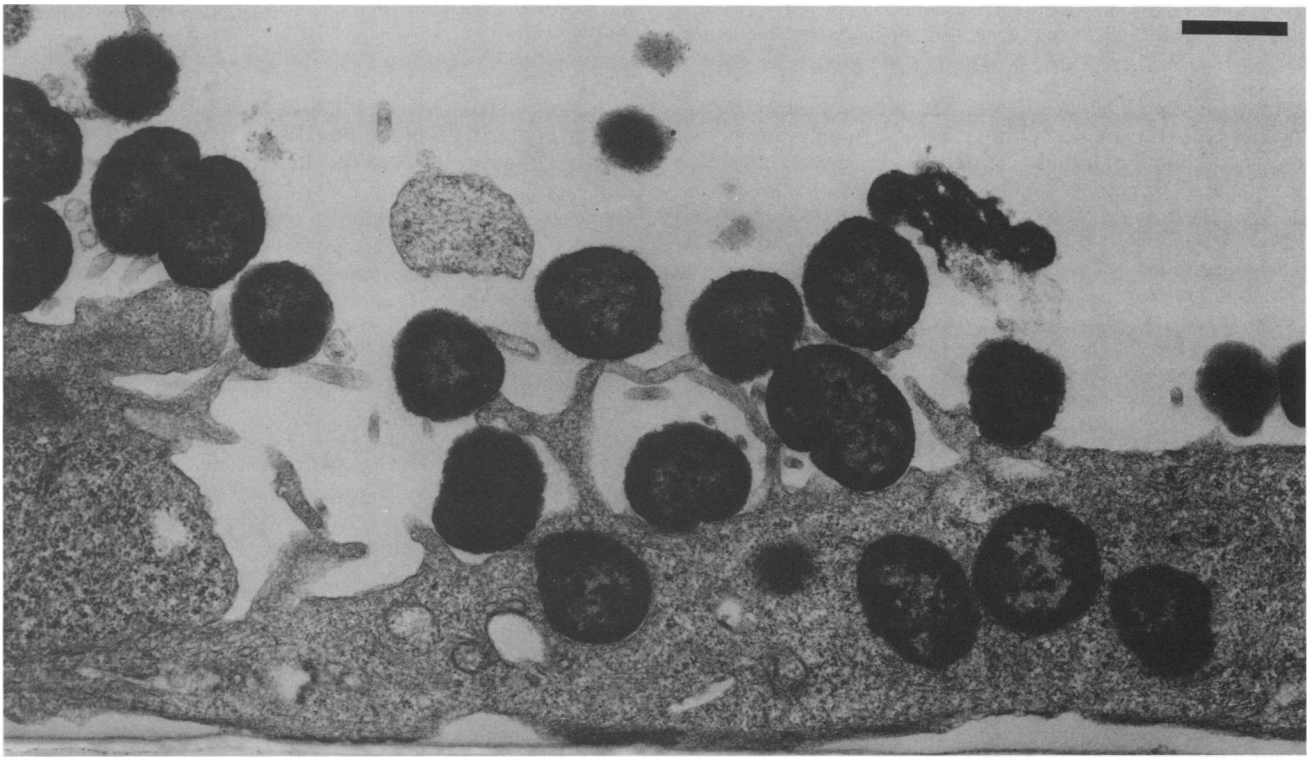

FIG. 6. At 10 h postinfection, *N. gonorrhoeae* F62 are clearly internalized by HecIB cells. The internalized gonococci do not appear to be bound by any type of vacuole. Other gonococci stud the HecIB membrane and appear to be invading the cell. Cells were prepared by the direct-embedding technique. Bar, 1 μm.

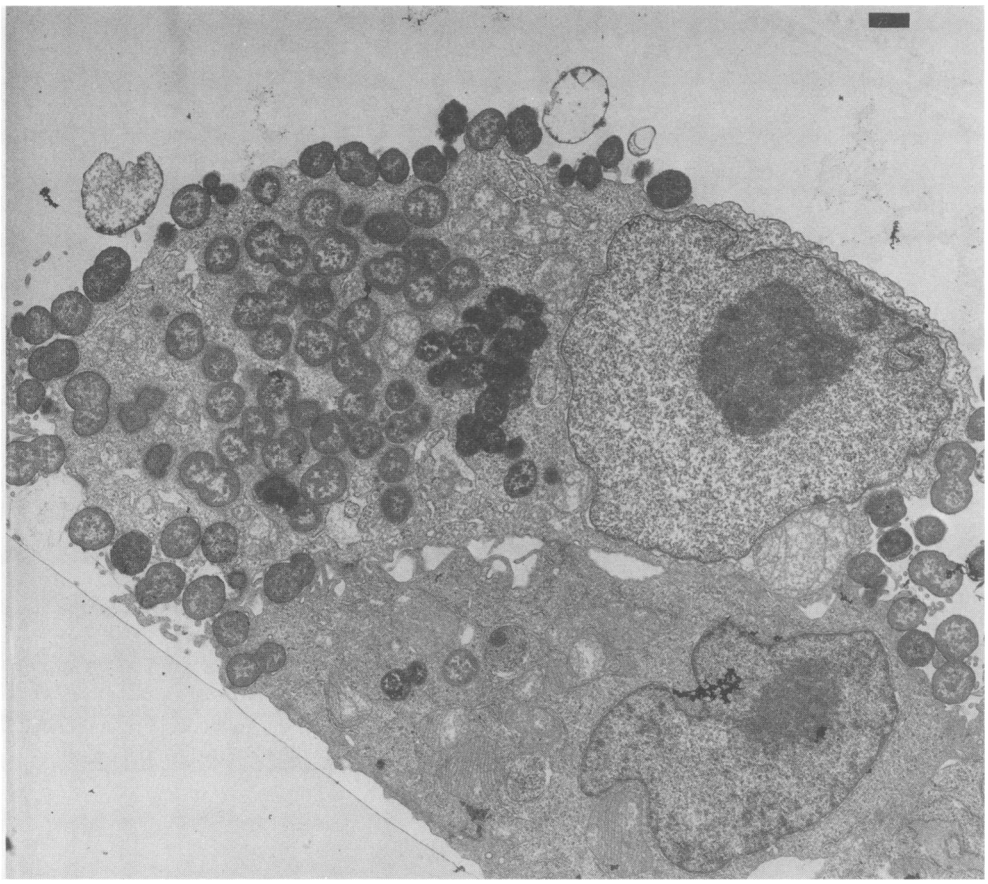

FIG. 7. At 12 h postinfection, HecIB cells are filled with gonococci. Internalized gonococci appear to be replicating within the HecIB cell. Most gonococci also appear to be free within the cytoplasm. The highly invaded HecIB cell has altered morphologic features in comparison to the other cell. Cells were prepared by the direct-embedding technique. Bar, 1 μm.

of internalized gonococci appear not to be contained within a well-defined vacuole, although some membrane-bound gonococci can be found. At the 12-h time point, large qnumbers of internalized gonococci can be observed (Fig. 7). After 24 h of infection, clusters of gonococci are found among normal HecIB cells (Fig. 8). Cellular debris, most likely the remains of a destroyed HecIB cell, surrounds the gonococci.

DISCUSSION

The factors governing the efficient entry of the gonococcus into specific tissue culture cell lines are still poorly understood. It is clearly dependent on the specific tissue culture cell line. Entry into HecIB cells requires a number of complicated processes in addition to simple adherence to the HecIB cell surface. In our studies, *N. lactamica* was as adherent as the gonococcus, yet no internalized *N. lactamica* cells were observed. In our assay of bacterial invasion we have not addressed the role of the well-studied cell surface molecule pili and PII on the invasion process, since we initiated infection by centrifuging the bacteria onto the monolayer. However, five PII variants of the gonococcal strain F62 did invade with equal efficiency in our assay (data not shown).

The studies with cytochalasin D indicated that the gonococci gain entry by a microfilament-dependent mechanism. Similar results were obtained with the invasive bacteria *S. flexneri*, which is in agreement with several other reports (6, 8, 11). Gonococcal entry into HecIB cells appears to occur in two distinct steps. For the first 6 h of bacteria-host cell interaction, a significant number of microorganisms are protected from the killing of gentamicin. Pretreatment of HecIB cells with cytochalasin D does not affect the titer of gentamicin-resistant gonococci at the 6-h time point. It is likely that the HecIB cells offer the adherent bacteria some form of protection from the antibiotic. Whether the bacteria are formally intracellular at this point is not clear, but the electron micrographs indicate that the gonococci are not internalized this early. Presumably the decrease in the titer of gonococcal survivors which occurs after the initial increase in gentamicin-resistant bacteria suggests either that there is a turnover of microorganisms at the HecIB cell surface back into the gentamicin culture medium or that host cell processes are initially effective in killing the invading bacteria. Other invasive bacteria such as *Shigella*, *Yersinia*, and *Salmonella* spp. enter tissue culture cells within 1 to 2 h (6, 8, 11) in comparison to the much slower entry of the gonococci. Attempts to preadapt the gonococci in tissue culture medium before infection did not accelerate the invasion process (data not shown).

The invasion process, as studied by electron microscopy, indicates that the gonococci make initial contact with the microvilli of the HecIB cell. The close proximity of the gonococci to structures resembling coated pits may be a

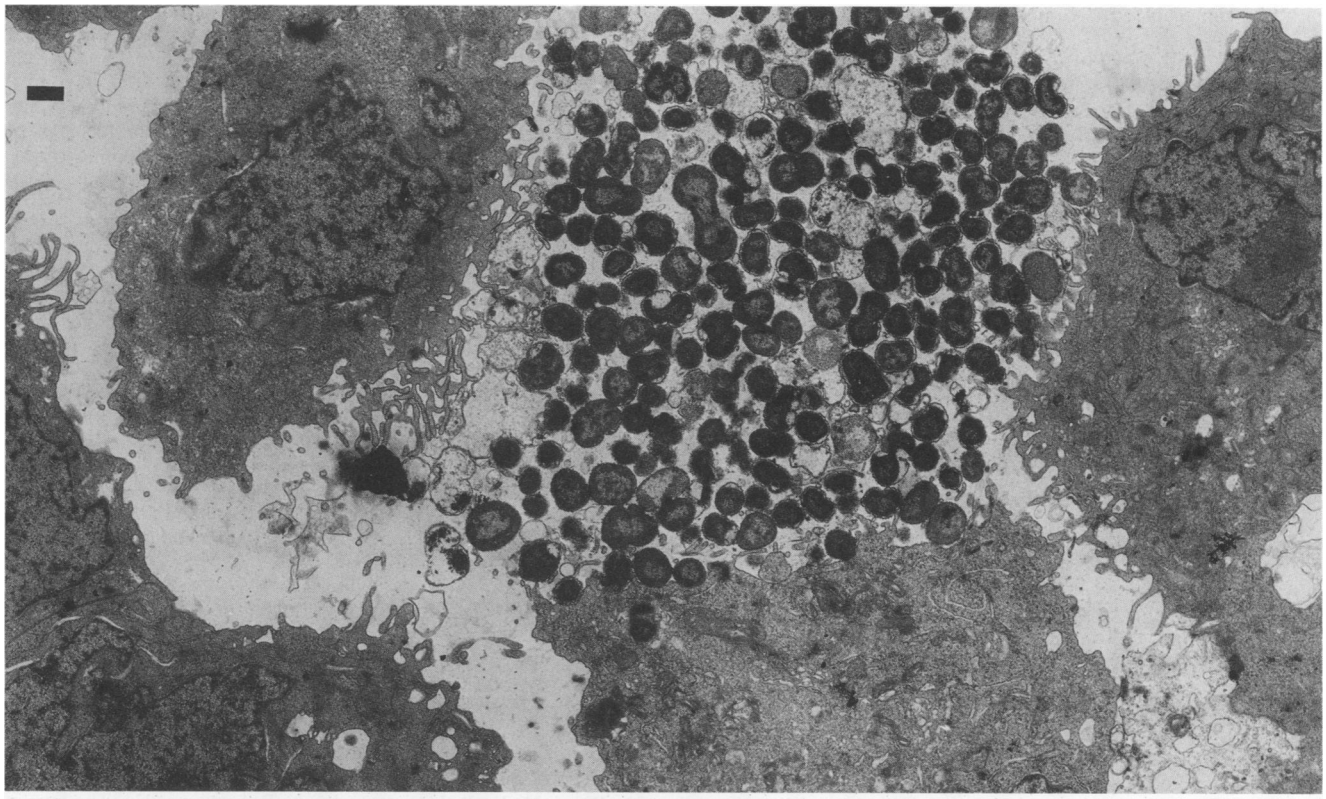

FIG. 8. *N. gonorrhoeae* F62 at 24 h postinfection appear to destroy the invaded HecIB cell. Debris surrounds gonococci and is most likely the remains of the invaded HecIB cell. Gonococci are probably embedded in this material, because monolayers were washed extensively before preparation for electron microscopy. Other HecIB cells untouched by the invading bacteria surround this cluster. Bar, 1 μm.

coincidence, but we observed numerous membrane-bound gonococci adjacent to such structures. Hodinka and Wyrick (9) demonstrated that *Chlamydia psittaci* cells were internalized in the same vacuole with another molecule that is known to be taken up by receptor-mediated endocytosis. This suggests that *C. psittaci* may be internalized by this mechanism.

After 12 h, HecIB cells containing large numbers of internalized bacteria were visible. Dividing gonococci within the cytoplasm were evident. By 24 h, some of the invaded HecIB cells appeared to have been destroyed by the invading gonococci. The bacterial cells were embedded within a matrix of eucaryotic cellular debris that resisted repeated washings and centrifugation and, by inference, protected the bacteria from added gentamicin.

Novotny and Short (16) examined urethral exudates from men and women with acute gonorrhea. Clusters of gonococci encased in a matrix were identified in the exudates, and these clusters were designated infectious units. These infectious units were proposed to offer protection from the host immune system and were suggested to be sites at which the bacteria could obtain nutrients. It was thought that these clusters arise from the entry and multiplication of gonococci in either macrophages or epithelial cells. Novotny and Short (16) believed that macrophages were the invaded cells. This was based on the appearance of the cell debris that was part of the matrix encasing the infectious unit. Electron micrographs taken at the 24-h time point in our study show structures quite similar to those seen by Novotny and Short (16).

Work done with the FTOC system suggests that a different interaction occurs between gonococci and these eucaryotic cells (14, 24). The initial steps of invasion appear to be similar to those we have described except that internalized gonococci are not observed until 24 h postinfection. In the FTOC system, the gonococci enter only nonciliated cells and remain within a vacuole (14, 24). This vacuole, which may contain many bacteria, is then transported to the basement membrane where the gonococci are exocytosed. In our studies, only a few internalized gonococci were clearly bounded by any eucaryotic membrane. We found that invasion of HecIB cells usually results in the death of the invaded cells, whereas the epithelial cells of the FTOC appear to act as a shuttle for transporting the gonococci into the subepithelial space.

The model system we describe here should permit us to more precisely examine the mechanism by which gonococci interact with and gain entry to eucaryotic cells. It is our hope that we can successfully identify the determinant(s) which allows the uptake of gonococci into eucaryotic cells.

ACKNOWLEDGMENT

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