INFECTION AND IMMUNITY, Oct. 1996, p. 4054–4059 Vol. 64, No. 10
0019-9567/96/$04.00+0
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Invasion of Respiratory Epithelial Cells by *Burkholderia* (*Pseudomonas*) *cepacia*

JANE L. BURNS,[1,2]* MECHTHILD JONAS,[3] EMIL Y. CHI,[3] DANA K. CLARK,[1] ANDREA BERGER,[1] AND ADAM GRIFFITH[1]

Division of Infectious Disease, Children's Hospital and Medical Center, Seattle, Washington 98105,[1] and Department of Pediatrics[2] and Department of Pathology,[3] University of Washington School of Medicine, Seattle, Washington 98195

Received 26 January 1996/Returned for modification 18 March 1996/Accepted 15 July 1996

Pulmonary infections caused by *Burkholderia* (*Pseudomonas*) *cepacia* are an important cause of morbidity and mortality in cystic fibrosis (CF) patients. Several features suggestive of cellular invasion and intracellular sequestration of *B. cepacia* in CF are persistence of infection in the face of antibiotic therapy to which the organism demonstrates in vitro susceptibility and a propensity to cause bacteremic infections in patients with CF. Epithelial cell invasion was demonstrated in vitro in A549 cells by a modified gentamicin protection assay. The kinetics of invasion appear to be saturable. Electron microscopy of invaded monolayers showed intracytoplasmic bacteria enclosed by membrane-bound vacuoles. No lysosomal fusion with these vacuoles was observed. Intraepithelial cell replication was suggested by electron microscopy and confirmed by both a quantitative assay and a visual assay. Cytochalasin D, but not colchicine, inhibited invasion, suggesting a role for microfilaments but not microtubules. The invasion phenotype in *B. cepacia* may be an important virulence factor for CF infections.

Burkholderia (*Pseudomonas*) *cepacia* is an important pulmonary pathogen in children and young adults with cystic fibrosis (CF). The organism is usually acquired late in the course of disease and may be associated with severe and sometimes fatal lung infections. The pathogenesis of *B. cepacia* infections in CF is largely unknown. Specific mechanisms by which *B. cepacia* evades the normal host defenses have not been identified. Although *B. cepacia* is known to produce virulence factors, including proteases (15), lipases (13, 16), and hemolysins (19, 26), none of these have been associated with disease production in CF patients (17).

Severe *B. cepacia* pulmonary infections may be associated with bacteremia (21), a distinctly unusual finding in patients with CF. Even *Pseudomonas aeruginosa*, which may be present in the lungs at a concentration as high as 10^{10} CFU/g of sputum, rarely causes bloodstream infections. An additional hallmark of *B. cepacia* infections in CF is the ability of the organism to persist even in the face of antibiotics which demonstrate in vitro activity (11). Once colonized with *B. cepacia*, a patient with CF rarely, if ever, eradicates the organism. Taken together, these findings suggest the possibility that *B. cepacia* invades respiratory epithelial cells and persists or replicates intracellularly.

Many species, including *Salmonella* spp. (6, 9, 25), *Campylobacter jejuni* (14), *Yersinia* spp. (12, 25), *Listeria monocytogenes* (10), *P. aeruginosa* (3, 8, 20), and group B streptococci (23) have demonstrated the ability to invade host epithelial cells. These organisms may simply be sequestered within the invaded host cells or may replicate (5, 10, 25). Some have demonstrated the ability to transcytose a polarized epithelial cell monolayer (6, 14).

Identification of intracellular *B. cepacia* in tracheal epithelial cells harvested at the time of autopsy from an adolescent female with severe pneumonia and signs of systemic sepsis suggested that, like other gram-negative pathogens, *B. cepacia* might have the ability to invade epithelial cells (1). Studies we performed to investigate this hypothesis include quantitative invasion studies using cultured respiratory epithelial cells in a modified gentamicin protection assay, electron microscopy to examine invaded cell monolayers, serial sampling from quantitative in vitro assays to examine intracellular replication, and examination of the effects of inhibitors of microtubule and microfilament formation on quantitative invasion.

MATERIALS AND METHODS

Bacterial strains. The *B. cepacia* strains used for invasion studies included 249-2, an antibiotic-susceptible laboratory strain provided by T. Lessie, Amherst University (2), and PC315, a clinical sputum isolate recovered at the time of autopsy from a patient with necrotizing pneumonia. DH5-α, a noninvasive *Escherichia coli* laboratory strain, was used as a negative control, and COH1, an invasive type III group B streptococcal clinical isolate (23), was used as a positive control. The *B. cepacia* and *E. coli* were grown in L broth for liquid culture and on L agar plates. Group B streptococci were grown in Todd-Hewitt broth and plated on Todd-Hewitt agar.

Epithelial cell invasion assay. The gentamicin protection assay described by Isberg and Falkow was used to examine cellular invasion in vitro (12). This was modified by the use of the A549 cell line (American Type Culture Collection, Rockville, Md.) and by changes in the incubation times and concentrations of antibiotics. The A549 line is a human alveolar epithelial carcinoma cell line. These cells are type II pneumocytes which are well differentiated, having microvilli and lamellar bodies and forming tight junctions. A549 cells were grown in RPMI 1640 tissue culture medium containing 10% fetal calf serum.

A549 cells were grown to confluence in monolayers in 24-well tissue culture plates and used for quantitative invasion assays. The number of eukaryotic cells per well was determined by quantitation in a counting chamber; the average number of cells per well was 1.68×10^6. Each well was inoculated with bacterial cells grown to mid-logarithmic phase (optical density at 600 nm, 0.6), washed in phosphate-buffered saline (pH 7.0) (PBS), and diluted 10^{-4} in tissue culture medium. Inocula ranged from 2.3×10^4 to 5.2×10^6. The infected cell monolayers were centrifuged at $800 \times g$ and incubated at 37°C in 5% CO_2.

After 2 h of incubation, the monolayers were washed free of nonadherent bacteria and tissue culture medium containing ceftazidime and gentamicin was added for a 1-h incubation to kill extracellular bacteria. Because *B. cepacia* is intrinsically gentamicin resistant, it was not possible to use 100 μg of gentamicin per ml for 2 h to kill extracellular organisms. Combinations of various concentrations of gentamicin and ceftazidime incubated with organisms for periods of time from 1/2 to 2 h were tested for bacterial killing in the assay system in the absence of eukaryotic cells. Although both gentamicin and the cephalosporins are thought to be inactive intracellularly (27), because such high concentrations of antibiotics were necessary for killing *B. cepacia*, we attempted to minimize the

* Corresponding author. Mailing address: 4800 Sand Point Way N.E., Seattle, WA 98105. Phone: (206) 526-2073. Fax: (206) 527-3890. Electronic mail address: jburns@u.washington.edu.

incubation period. The combination of ceftazidime (1 mg/ml) and gentamicin (500 μg/ml) incubated with bacteria for 1 h resulted in greater than 99.98% killing (with an initial inoculum of 6×10^4 CFU, less than 10 CFU were recovered).

After incubation with the antibiotics, the monolayers were washed, trypsinized, and lysed with 0.25% Triton X-100. The number of CFU recovered per well was determined by quantitative culture. All assays were performed in triplicate. The invasion frequency was calculated by using the following equation: [organisms recovered (CFU/ml)/organisms inoculated (CFU/ml)] × 100 = % invasion.

The same invasion assay was used to determine the relative importance of inoculum size and whether the uptake of organisms was saturable. A range of 10-fold dilutions of overnight cultures (10^{-1} to 10^{-6}) were examined for the total number of organisms recovered and calculated invasion frequency.

Assay of intracellular replication. To determine whether *B. cepacia* could survive or multiply within A549 cells, the standard invasion assay was modified by further incubation of the infected monolayers for up to 48 h. After the 1-h incubation with gentamicin and ceftazidime, the antibiotic-containing culture supernatants in the sample wells were replaced with antibiotic-free tissue culture medium because of the concern that bathing the extracellular fluid with high concentrations of antibiotics for prolonged periods might result in intracellular accumulation of drug and subsequent killing of intracellular bacteria. However, to ensure that any increase in intracellular bacteria was the result of replication and not the result of ongoing invasion by bacteria that might have survived the initial antibiotic treatment, a repeat 1-h incubation with ceftazidime and gentamicin was performed after 24 h of incubation. In addition to the initial sampling, both intracellular and extracellular organisms were quantitated over time. Culture supernatants were removed, colony counts were performed (extracellular organisms), sample wells were lysed, and intracellular bacteria were quantified at 2, 24, and 48 h, as described above.

A second, qualitative assay using acridine orange staining of invaded A549 monolayers was used to visually confirm intracellular replication. The technique of Miliotis (18) was modified to use A549 cells grown on 8-chambered slides and infected in quadruplicate with bacteria which were allowed to invade for 2 h and then treated with antibiotics as outlined above. Invaded A549 cells were washed with PBS and stained with 0.01% acridine for 45 s, rinsed with PBS, and counterstained with 0.05% crystal violet in 0.15 N NaCl. After removal of the chambers, coverslips were mounted with Cytoseal 60 (Stephens Scientific, Riverdale, N.J.) and viewed under a fluorescence microscope using 100× magnification with oil.

Inhibitor assays. Inhibitors of both microfilament and microtubule integrity were tested to examine the role of the cytoskeleton in the uptake of *B. cepacia*. The standard invasion assay was used, and inhibitors were incubated with the cell monolayer for 30 min prior to the addition of bacteria and left throughout the 2-h incubation period. Both cytochalasin D, an inhibitor of microfilament formation, and colchicine, a disrupter of microtubules, were examined in this model using strain PC315. Each inhibitor was tested in three experiments, each of which was performed in triplicate. Data are expressed as the percent invasion normalized to the control level (PC315 invasion in the absence of inhibitor = 100%). Cytochalasin D concentrations of 0.1, 0.2, 0.5, 1, and 2 μg/ml were used. Colchicine concentrations of 1, 2.5, 5, and 10 μg/ml were used. Viability of A549 cells was assessed by trypan blue exclusion.

Electron microscopy. To confirm that the observed invasion represented true intracellular parasitism, transmission electron microscopy was performed as previously described (3). After the initial 2-h incubation with bacteria, the A549 cells were washed gently in RPMI without fetal calf serum and then fixed immediately in the wells with 2.5% glutaraldehyde for 2 h, washed with 0.1 M sodium cacodylate buffer (pH 7.4), and postfixed with 1% osmium tetroxide in double-distilled water. The cells were dehydrated in a graded series of ethanol and embedded in Medcast (Ted Pella, Inc., Redding, Calif.). Following sectioning, the cells were stained with uranyl acetate and lead citrate and examined with a Philips EM420 electron microscope at 80 kV.

Electron microscopy experiments used live organisms as described above and organisms killed by either UV irradiation or formalin. Organisms killed by UV irradiation were grown as described above, pelleted and washed two times in PBS, resuspended in PBS, and placed in a sterile glass petri dish 15 cm below a UV light source for 5 min. Formalin-treated organisms were resuspended in 2% formalin in PBS for 20 min prior to inoculation. Both of these treatments resulted in a decrease in the number of viable organisms from 10^9 to <10 CFU/ml. After treatment, both the UV- and formalin-killed organisms were pelleted, washed in PBS, and resuspended in tissue culture medium for inoculation at the same density as the live organisms.

RESULTS

Epithelial cell invasion. The results of a representative experiment repeated in triplicate are displayed in Fig. 1. Although variability is seen from assay to assay, the degree of intra-assay variability is small. The mean invasion frequencies (± standard deviations) for a total of nine experiments were as follows: 249-2, 0.49% (± 0.24%); PC315, 0.97% (± 0.28%); and DH5-α, 0.07% (± 0.03%). A consistent difference between the laboratory strain, 249-2, and the clinical isolate, PC315, was seen, with the laboratory strain demonstrating an invasion frequency about one-half that of the clinical strain. COH1 under the same conditions demonstrated an invasion frequency of 2.29%.

The size of the infecting inoculum affected the number of recoverable intracellular organisms. Figure 2 shows the results of a representative experiment for strain PC315. As the inoculum size increased, the number of organisms recovered also increased, although the system appeared saturable. Similar numbers were obtained with strain 249-2 (data not shown). Because the calculated percent invasion was dependent upon

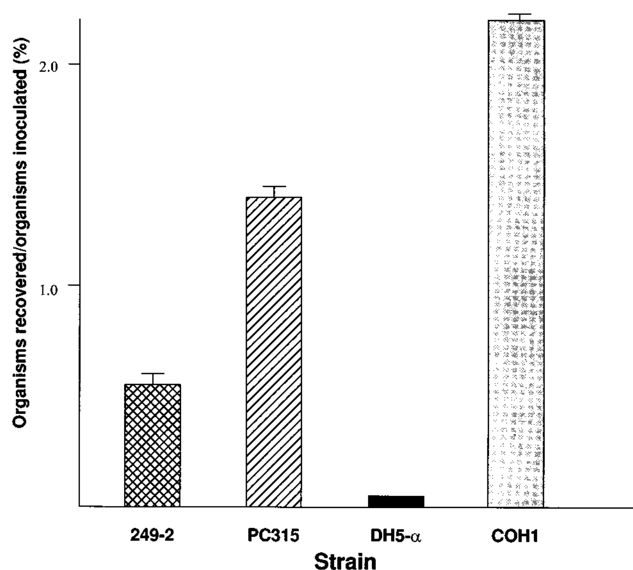

FIG. 1. Results of a representative invasion experiment performed with A549 cells in triplicate wells by using the modified gentamicin protection assay. The error bars represent the standard deviations (SD) of the three values. Mean invasion frequencies for this experiment (mean ± SD) were as follows: strain 249-2, 0.43% ± 0.17%; PC315, 1.31% ± 0.15%; DH5-α, 0.11% ± 0.009%; COH1, 2.20% ± 0.095%.

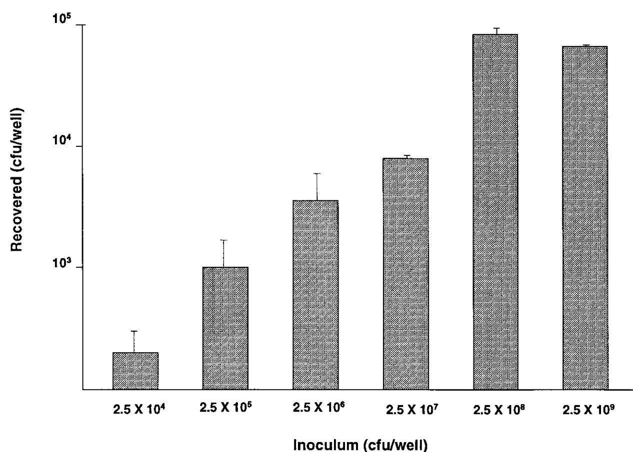

FIG. 2. Kinetics of invasion of A549 cells by PC315. Shown are the results of a representative experiment performed in triplicate by the modified gentamicin protection assay and comparing 10-fold dilutions of inocula ranging from 2.5×10^4 to 2.5×10^9. The error bars represent the standard deviations of the three values. Invasion of A549 cells by PC315 appears to be saturable.

the inoculum used, for subsequent experiments inocula were standardized to the range over which invasion was more linear ($\sim 10^5$ CFU).

Electron microscopy. Figure 3 illustrates evidence of intracellular invasion of A549 cells by *B. cepacia* 249-2. All electron micrographs depicted show the same set of sample wells following the initial 2-h incubation with organisms. Thickening of the cell membrane is visible during the initial contact between the bacterium and the epithelial cell, followed by apparent endocytosis by microvilli at the epithelial cell surface (Fig. 3A). At the cell surface, each bacterium appears to be phagocytized within its own membrane-bound vacuole (Fig. 3B). However, deeper within the cytoplasm, multiple organisms are visible within a single vacuole (Fig. 3C and D). Close attachment and fusion of lysosomes with phagocytic vacuoles containing bacteria were not observed. This suggests that either the vacuoles coalesce or the organisms are replicating intracellularly.

Invasion by killed organisms. Although it was not possible to quantitatively compare the intracellular invasion by killed organisms with that by live bacteria, electron microscopy of A549 cell monolayers inoculated with UV- and formalin-killed organisms enabled a qualitative comparison. Electron micrographs of monolayers inoculated with UV-irradiated PC315 demonstrated attached and unattached bacteria near the cell surface but no clearly intracellular organisms. Electron micrographs of monolayers inoculated with formalin-killed PC315 demonstrated no attachment of organisms to the A549 cell surface and no invasion (data not shown).

Intracellular replication. The results of a representative experiment examining intracellular replication are displayed in Fig. 4. A greater-than-2-log-unit increase in the number of organisms from cells incubated for 48 h suggests that organisms are replicating slowly intracellularly. In experiments to quantitate extracellular organisms, $<10^1$ CFU of extracellular organisms per ml at time zero and 2 and 24 h and 1.3×10^3 CFU/ml at 48 h were detected; this represented 0.13, 0.76, 0.55, and 1.3% of the total organisms recovered, respectively. This suggests that extracellular organisms remaining after incubation with antibiotics were not contributing significantly to invasion. There was no evidence of damage to the A549 cells by light microscopy at any of the time points.

Intracellular replication of *B. cepacia* was confirmed by acridine orange staining of invaded monolayers. Approximately 1 in 200 A549 cells contained intracellular organisms at each time point. After 2 h of incubation, 1 to 4 organisms were identified within each invaded A549 cell; at 24 h, approximately 10 to 20 organisms were identified in each invaded cell; and at 48 h, the intracellular organisms were too numerous to count. These results confirm those obtained in the gentamicin protection assay.

Cytoskeleton inhibitors. The lowest concentrations of cytochalasin D were not found to inhibit invasion, but at a drug concentration of 0.5 μg/ml invasion of PC315 was inhibited by 93% ± 6%. Colchicine had no effect on the invasion of A549 cells when the drug was used at concentrations up to 10 μg/ml. These results are comparable to those for group B streptococcus invasion of A549 cells (23). On the basis of cell quantitation and trypan blue exclusion, neither inhibitor killed A549 cells at the concentrations used.

DISCUSSION

The observation of intraepithelial *B. cepacia* in necropsy tissue from a child who died with an apparent systemic infection suggested that *B. cepacia* is capable of epithelial cell invasion (1). We have confirmed invasion of epithelial cells using a tissue culture assay and by electron microscopy. Experiments using killed organisms suggest that uptake is the result of parasite-mediated endocytosis and is not effected by nonprofessional phagocytes. On the basis of experiments using inhibitors of the eukaryotic cytoskeleton, microfilaments, but not microtubules, appear to be involved in the uptake of *B. cepacia* by A549 cells. Observations by electron microscopy were suggestive of intracellular replication. Thus, additional assays that confirmed an increase in the number of intracellular organisms during a 48-h incubation were performed.

There is no in vitro or in vivo evidence that *B. cepacia* causes destruction of respiratory epithelial cells. Rather, the organisms would appear to enter eukaryotic cells and be sequestered there, protected from antibiotics and host defenses. They may also transcytose the epithelium to cause bacteremia in a subpopulation of patients with CF.

Many bacterial species have demonstrated the ability to invade host epithelial cells. These organisms may simply be sequestered within the invaded host cells or may replicate (9, 10, 25); some have demonstrated the ability to transcytose a polarized epithelial cell monolayer (6, 10). For many of these organisms, invasiveness appears to be a virulence factor which permits them to evade host defenses, to aid in tissue destruction, or to spread into the systemic circulation of the host.

The level of invasiveness of A549 cells demonstrated by *B. cepacia* is of the same magnitude as that reported for group B streptococci in the same cell line (23). Direct comparison with well-characterized invasive gram-negative organisms such as *Salmonella* spp., *Yersinia* spp., and *E. coli* is not possible because these organisms do not invade respiratory epithelial cells. The frequency of invasion of other nonrespiratory epithelial cell lines such as HEp-2 cells by these organisms has been reported to be in the range of 1.6 to 23% (23). Because invasion appears to be cell line specific (5), these numbers cannot be directly compared.

Preliminary studies of the invasion of respiratory epithelial cells by another CF pathogen, *P. aeruginosa*, have been described. *P. aeruginosa* has been demonstrated to invade A549 cells with frequencies in the range of 1.6 to 12%, depending on the strain, but with evidence of poor intracellular survival (3). A recent report by Pier et al. (20) suggests that for *P. aeruginosa*, cellular invasion may actually be a pulmonary host defense mechanism which malfunctions in CF. Those authors found that *P. aeruginosa* invades transformed respiratory epithelial cells expressing wild-type CF transmembrane conductance regulator (CFTR) significantly better than cells expressing mutant CFTR. They hypothesize that CFTR-mediated epithelial cell uptake of *P. aeruginosa* may be an important respiratory tract clearance mechanism. Interestingly, they found no difference in the levels of invasion of *B. cepacia* into transformed respiratory epithelial cells whether they expressed wild-type or mutant CFTR.

Two paradigms have been proposed for the invasion of epithelial cells by microorganisms (28): the high-affinity interaction of bacterial ligands with host receptors, such as that mediated by the *Yersinia* invasin, and the signaling and subsequent modulation of the host cell cytoskeleton by the bacteria, such as exemplified by *Salmonella* spp. The experimental data presented here suggest that *B. cepacia* invasion more closely parallels that of *Salmonella* spp. For *Salmonella* spp., cytochalasin D, but not microtubule inhibitors, blocks invasion and intracellular replication (6, 7). Other pulmonary pathogens that have demonstrated intracellular invasiveness include *Bordetella* spp. (4) and group B streptococci (20, 21). Both organisms appear to utilize host cell microfilaments but not microtubules, similar to the situation with *B. cepacia*. However, neither dem-

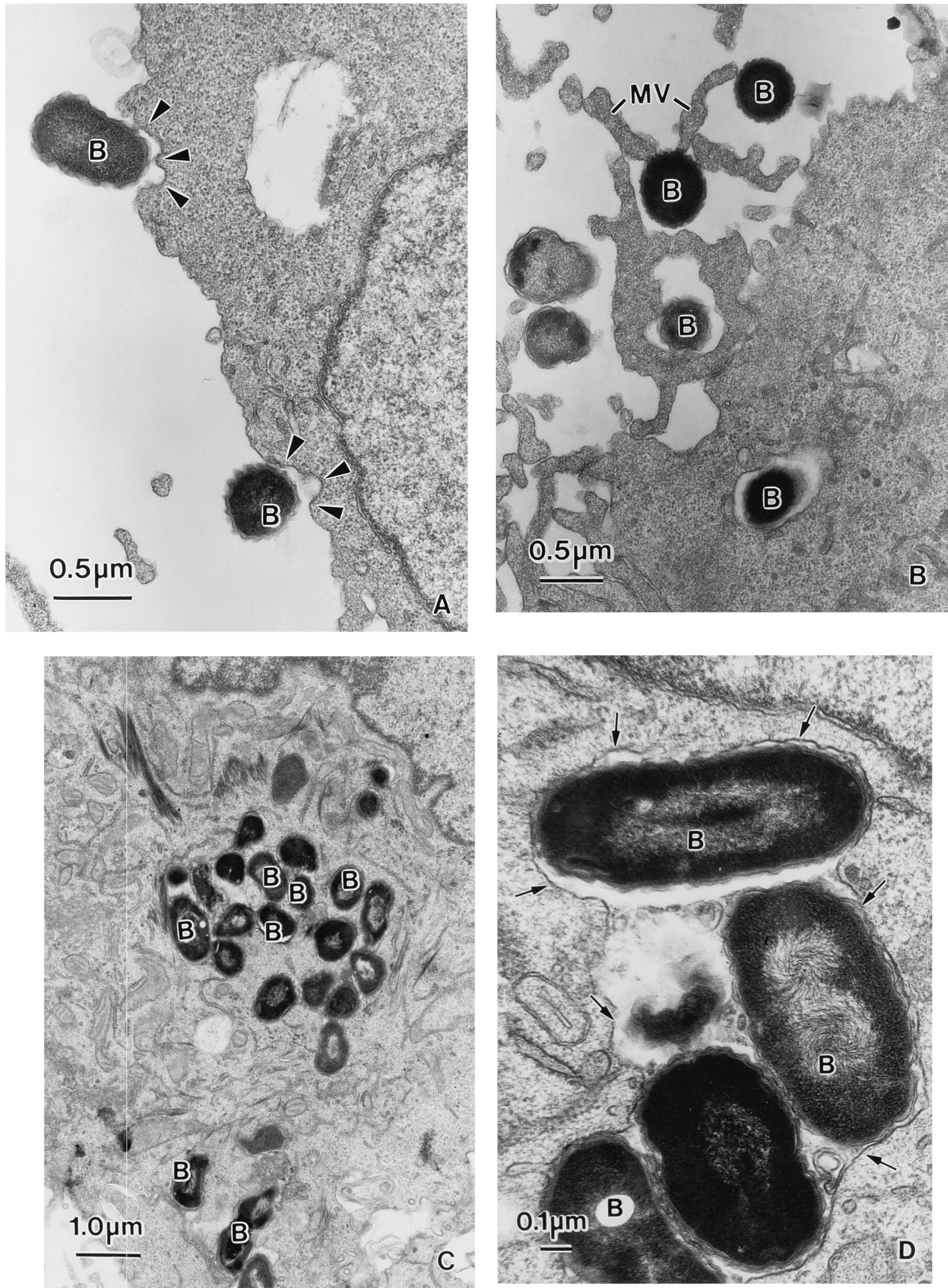

FIG. 3. Electron micrographs showing invasion of an A549 respiratory epithelial cell monolayer by *B. cepacia* 249-2. All four micrographs show the same epithelial cell monolayer following a 2-h incubation with the bacteria. (A) Initial contact of bacterial cells (B) with the surface of A549 cells. Thickening is seen at the site of contact (arrowheads). (B) Apparent endocytosis of bacteria in contact with microvilli (MV) at the cell surface. A single bacterium is present within each membrane-bound vacuole. (C) Lower-power view of bacteria present within the cell cytoplasm. Although apparently ingested singly, organisms appear in clumps within the cytoplasm. (D) Higher-power view of bacteria within the cytoplasm. The organisms are enclosed within membrane-bound vacuoles (arrows).

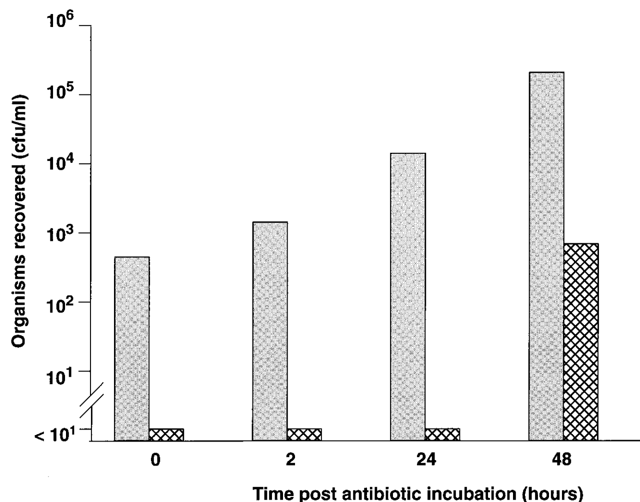

FIG. 4. Results of a representative experiment demonstrating intracellular replication of *B. cepacia* PC315. Both intracellular and extracellular organisms were quantitated 0, 2, 24, and 48 h following the standard 1-h incubation with gentamicin and ceftazidime (see text for details). Another 1-h incubation with ceftazidime and gentamicin was performed at 24 h for the wells harvested at 48 h. The mean number of intracellular organisms increased from 792 per well at time zero to 1.0×10^5 at 48 h. Extracellular organisms never numbered more than 1.3% of the number of intracellular organisms and thus were unlikely to have contributed to the apparent increase in intracellular organisms. ▨, intracellular organisms; ⊠, extracellular organisms.

onstrates the intracellular replication that we have identified for *B. cepacia*.

The finding that *B. cepacia* is able to invade and replicate within respiratory epithelial cells in vitro is consistent with the disease caused by the organism in CF patients. Both the persistence of the organism and its ability to cause bacteremic infections suggest the possibility that *B. cepacia* invades respiratory epithelial cells in vivo. Even the more common CF pathogen, *P. aeruginosa*, which colonizes lungs at a density as high as 10^{10} CFU/g of sputum, rarely causes bacteremic infections. This may reflect either that *P. aeruginosa* demonstrates poor intracellular replication (3) or that CF patients have a very active immune response to this organism. If *B. cepacia* organisms were sequestered and replicating within epithelial cells, bacteria could persist even in the presence of antimicrobial agents demonstrating in vitro activity. And if organisms were able to transcytose respiratory epithelial cells, bacteremic infections could result.

In our studies, the clinical CF isolate of *B. cepacia*, PC315, appeared more invasive than the laboratory strain, 249-2. The laboratory strain is well characterized and has been reported to have a 0.8-Mb deletion from one of the three large circular replicons present in *B. cepacia* (2). It is possible that the deleted DNA may encode genes responsible for the invasion phenotype.

Further studies should be performed with CF cell lines or primary cultures of respiratory epithelium from patients with CF, as well as polarized epithelial cell monolayers, in an attempt to confirm the finding of Pier et al. (20) that CF cell lines are no more or less susceptible to invasion than other cell lines and to determine whether transcytosis plays a role in bacteremic infections with *B. cepacia*. In addition, the role of the recently described cbl pilus (24) in invasiveness should be investigated.

ACKNOWLEDGMENTS

This research was supported by a grant to J.L.B. from the Cystic Fibrosis Foundation (G548).

We thank C. E. Rubens for critical review of the manuscript.

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Editor: B. I. Eisenstein