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Human Microvascular Endothelial Tissue Culture Cell Model for Studying Pathogenesis of Brazilian Purpuric Fever

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Brazilian purpuric fever (BPF) is a fulminant pediatric disease characterized by fever, with rapid progression to purpura, hypotensive shock, and death. All known BPF cases have been caused by three clones of *Haemophilus influenzae* biogroup aegyptius and have occurred in either Brazil or Australia. Using an immortalized line of human vascular endothelial cells, we developed an in vitro assay that identifies all known BPF-causing *H. influenzae* biogroup aegyptius strains (R. S. Weyant, F. D. Quinn, E. A. Utt, M. Worley, V. G. George, F. J. Candal, and E. W. Ades, J. Infect. Dis. 169:430–433, 1994). With multiplicities of infection (MOIs) as low as one bacterium per 1,000 tissue culture cells, BPF-associated strains produce a unique cytotoxic effect in which the tissue culture cells detach and aggregate in large floating masses after 48 h of incubation. In this study, using a BPF-associated strain and a non-BPF-associated control, we demonstrated that strains which produce the cytotoxic phenotype were able to replicate intracellularly whereas non-BPF-associated strains, with MOIs of ≥1,000 did not replicate and did not produce the phenotype. We also showed that this phenotype is not caused by the activity of an endotoxin or the release of some other compound from the bacterial cell, since neither gamma irradiation-killed whole BPF clone bacteria nor bacterial cell fractions at MOIs of >1,000 produced the cytotoxic effect. Furthermore, bacteria in numbers equal to MOIs of >1,000 treated with chloramphenicol did not produce the cytotoxic phenotype, suggesting a requirement for bacterial protein synthesis. In addition, viable bacteria separated from the tissue culture monolayer by a 0.2-μm-pore-size membrane also failed to produce the phenotype. The ability of the bacterium to invade, replicate, and produce the phenotype appears to be primarily parasite directed since phagocytosis, pinocytosis, and eukaryotic protein synthesis inhibitors, including cycloheximide, cytochalasin D, and methylamine, had no effect on the ability of the bacterium to invade and cause a cytotoxic response. Understanding the basic mechanisms involved in this tissue-destructive process should enhance our knowledge of the general pathogenesis of BPF.

Brazilian purpuric fever (BPF) is a fulminant infection of young children. This frequently fatal illness is usually characterized by a self-limiting purulent conjunctivitis followed 7 to 16 days later by an acute onset of fever, bacteremia, petechiae, purpura, and vascular collapse (4–6). The vascular destruction that occurs with this disease is a distinctive trait. *Haemophilus influenzae* biogroup aegyptius, a biogroup long associated only with conjunctivitis, has been recovered from the blood of patients with BPF (2, 4, 5, 17). Little is known about its bacterial virulence determinants, pathogenesis of infection, and host defense against infection.

Until recently, a nasopharyngeal organ culture system and an intraperitoneal infant rat model were the only systems which demonstrated utility in studying BPF pathogenesis (11, 23, 24). The organ culture system was useful for the study of HAE attachment and invasion of intact human tissues. However, in the nasopharyngeal system, both BPF-associated and non-BPF-associated organisms invaded nonciliated human nasopharyngeal cells and were found in membrane-bound vesicles within these cells (11). The intraperitoneal infant rat model developed by Rubin et al. (23) demonstrated that BPF clone strains caused bacteremia in the model system; this was the first experiment suggesting that the BPF clone strains possess a unique factor(s) that contributes to the disease. Unfortunately, several disadvantages are associated with the rat model, including its complexity and lack of physiological significance to the disease in humans. With the exception of these two published models that focus on the attachment, invasion, and other unknown mechanisms of virulence, virtually no analysis of the purpuric aspect of this disease has been performed.

The development of the HMEC-1 human vascular endothelial cell line (1) has provided an opportunity to compare *H. influenzae* biogroup aegyptius strains and other control *H. influenzae* strains in the HMEC-1 tissue culture model by using vascular cell destruction as the end point. In a previous study (28), we examined 97 strains of *H. influenzae* biogroup aegyptius for the presence of the BPF-specific phenotype. The assay was 100% sensitive and 87% specific for the detection of BPF-associated *H. influenzae* biogroup aegyptius. However, the mechanisms of cytotoxicity expressed by BPF-associated *H. influenzae* biogroup aegyptius were unknown. In the present study, we have examined some of the mechanisms suspected to be associated with this phenotype, including intracellular growth in human vascular endothelial cells and the requirement for intracellular growth to produce the cytotoxic effect. Understanding these mechanisms not only may allow for more-effective treatments of BPF but also may lead to a better

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understanding of the nature of other vascular destructive infectious diseases.

MATERIALS AND METHODS

Bacterial strains and culture media. *H. influenzae* biogroup aegyptius F3031 and F1947 were used in this study. The epidemiologic and clinical characteristics of these strains have been previously described (7). Strain F3031 was obtained from the blood of a child with BPF. Strain F1947 was isolated from a nonsterile site of a person who did not develop BPF; this strain has never been implicated in BPF disease, is considered avirulent in all model systems, and expresses none of the markers of the BPF clone (7, 24). Both strains were obtained from the Investigation and Surveillance Laboratory Section, Emerging Bacterial and Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Ga. Cultures were streaked for purity and maintenance on chocolate II agar plates (BBL Microbiology Systems, Cockeysville, Md.) and incubated overnight at 37°C in a water-jacketed incubator containing 5% CO_2. For stock cultures, overnight growth was transferred to 1.0 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 25% glycerol and was stored at −70°C.

Tissue culture. The HMEC-1 cell line is of human microvascular endothelial origin (1). These simian virus 40-transformed cells possess all of the characteristic markers of endothelial cells. In side-by-side comparisons, the HMEC-1 cells performed in a fashion identical to that of the primary endothelial cells (1, 30). Early confluent monolayers (approximately 5.0×10^4 per well) were grown in 24-well tissue culture dishes (Costar, Cambridge, Mass.) at 37°C with 5% CO_2. HMEC-1 cells were grown and maintained in, per well, 1 ml of endothelial cell basal medium (EBM; Clonetics, Santa Anna, Calif.) supplemented with 10 ng human epidermal growth factor per ml, 1 μg of hydrocortisone per ml, and 15% normal human serum (Gibco Laboratories, Grand Island, N.Y.).

HMEC-1 monolayer cytotoxicity assays. Inocula for infection of HMEC-1 cell cultures were prepared by growing frozen *H. influenzae* biogroup aegyptius stock suspensions on chocolate II agar plates for 18 h under the conditions described above and then suspending the bacterial cells in sterile phosphate-buffered saline (PBS; 0.01 M, pH 7.2) to an optical density at 600 nm of 0.5. A 10^{-7} dilution of the bacterial suspension was made in sterile PBS. Confluent monolayers of HMEC-1 cells in 24-well tissue culture dishes were washed with sterile PBS and overlaid with 900 μl of fresh tissue culture medium immediately prior to infection. An aliquot containing 100 μl of the bacterial suspension was then added to each tissue culture well. This method produced a multiplicity of infection (MOI) ranging from one bacterium per 1,000 to one bacterium per 10,000 tissue culture cells (1:1,000 to 1:10,000). After inoculation, the tissue cultures were incubated in a water-jacketed 35°C incubator with 5% CO_2. Some infected monolayers were centrifuged at $1,000 \times g$ in a Beckman tabletop clinical centrifuge for 10 min potentially to enhance attachment (9). However, this centrifugation did not seem to have any effect on attachment or uptake and therefore was not included in the routine protocol. Cytotoxicity was determined at 48 h of incubation by observing the tissue cultures with an inverted microscope and comparing infected wells with negative controls consisting of tissue cultures inoculated with 100 μl of sterile PBS.

Additionally, the effects of cytochalasin D (5 μg/ml), cycloheximide (5 μg/ml), colchicine (200 μg/ml), methylamine (100 mM), nondansylcadaverine (5 μM), chloramphenicol (50 μg/ml), concanavalin A (0.05 μM), *Bandeiraea simplicifolia* BS-I (0.05 μM), *B. simplicifolia* BS-II (0.05 μM), *Dolichos biflorus* DBA (0.04 μM), and *Lens culinaris* LcHA (0.10 μM) (Sigma Chemical Co., St. Louis, Mo.) on viable counts and the cytotoxic phenotype were examined by using infected monolayers to which the inhibitors were added 30 min before infection and maintained throughout the experiment. To be assured of potency of the nonlectin phagocytosis and pinocytosis inhibitors, control experiments were performed with other intracellular bacteria, including *Legionella pneumophila*, known to be inhibited by these compounds and concentrations (3, 15, 21, 26). The potency of cycloheximide was demonstrated by the inability of tissue culture cells to incorporate [^{35}S]methionine.

In order to study secreted soluble cytotoxins a Transwell-COL insert (Costar) with 0.2-μm-diameter pores was placed in each well of a six-well tissue culture cluster plate containing the confluent HMEC-1 monolayers. Approximately 10^8 viable F3031 cells suspended in EBM tissue culture medium were added to the upper chambers (equaling an MOI of 1,000 bacteria per tissue culture cell). At 24-h intervals up to 96 h, the tissue culture monolayers were examined for the development of the cytotoxic phenotype.

Additionally, tissue culture monolayers were exposed directly to 10^8 lysed bacterial cells (disrupted by using a French pressure cell two times at 20,000 lb/in^2), to gamma irradiation-killed bacteria (maximum exposure in a gamma cell for 5 min), and to spent filtered (2-μm-pore-size filter) tissue culture medium obtained from previously infected monolayers that displayed a positive phenotype after 48 h of infection.

Measure of intracellular growth. To study *H. influenzae* biogroup aegyptius intracellular growth and associated attachment and invasion, monolayers in T25 flasks were washed with PBS, overlaid with supplemented EBM medium, and infected with either strain F3031 at an MOI of one bacterium per 1,000 tissue culture cells or strain F1947 at an MOI of 1,000 bacteria per tissue culture cell. Controls included the inoculum in the absence of the tissue cell monolayer. After 3 h of incubation at 37°C in 5% CO_2, the medium was removed from each flask; the monolayers were washed three times with PBS, fresh EBM medium was added, and the monolayers were reincubated. At various time points the medium was removed, and the monolayers were washed with PBS, scraped, mixed with distilled water to lyse the eukaryotic cells, diluted in PBS, and plated for colony counts.

To examine the specific aspects of intracellular growth, HMEC-1 monolayers were infected with strains F3031 and F1947 as described above. After 3 h of incubation, the tissue culture medium was removed, the monolayers were washed three times to remove loosely adherent bacteria, and EBM containing 100 μg of gentamicin per ml was added and maintained throughout the remainder of the assay. At regular time intervals the monolayers were examined for the presence of the cytotoxic phenotype, washed three times with PBS, and, as described above, scraped, lysed in distilled water, diluted, and plated for viable counts. Gentamicin used in low concentrations (40 to 100 μg/ml) inhibits the growth of extracellular bacteria since aminoglycosides are theoretically unable to enter eukaryotic cells, inactive intracellularly, or at a concentration within the eukaryotic cell phagocytic vaculoles and cytoplasm low enough to not affect intracellular bacterial viability (10, 14).

Electron microscopy. Infected cells in suspension or scraped from tissue culture wells were prepared for electron microscopy by pelleting at $1,000 \times g$ for 5 min, washing the cell pellets with PBS, and suspending the pellets in 2% glutaraldehyde fixative. The cells were held at 4°C for 1 h before being recentrifuged, washed, and resuspended in ice-cold PBS. The cells were postfixed in 2% osmium tetroxide, dehydrated in graded solutions of ethanol, passed through propylene oxide, and embedded in epon resin. Sections, 1 μm thick, were stained with methylene blue for examination by light microscopy. Sections, 40 to 60 nm thick, were stained with uranyl acetate and lead citrate for examination in a transmission electron microscope (Philips 410; Holland).

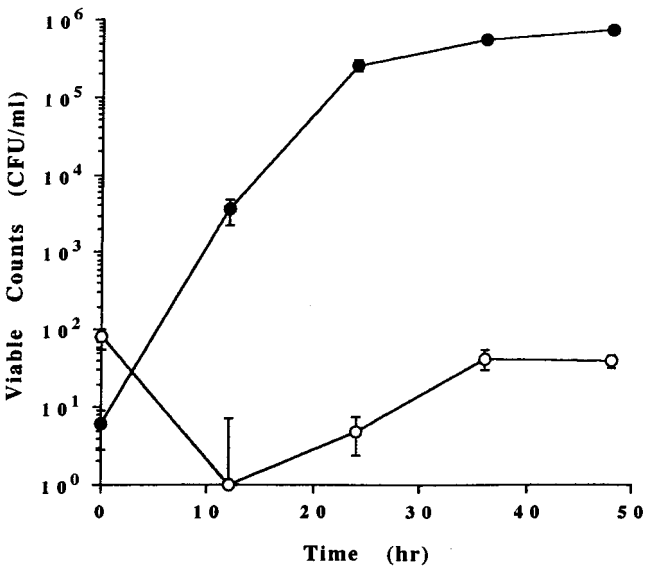


FIG. 1. Growth of *H. influenzae* biogroup aegyptius F3031 in HMEC-1 cells alone (●) and in HMEC-1 cells with 100 μg of gentamicin added to the culture 3 h after infection and maintained throughout the experiment (○). Viable counts were determined for these cultures and control experiments at selected time points. Each datum point is the mean of four independent trials; error bars indicate standard errors.

RESULTS

The result of a typical HMEC-1 cytotoxicity experiment using monolayers was demonstrated previously (28). Three hours after the initial infection with an MOI of one F3031 bacterium per 1,000 tissue culture cells, the monolayers were washed, and fresh medium containing gentamicin (100 μg/ml) was added and maintained throughout the 48-h assay. Viable counts were determined at regular time points for these monolayers as well as controls without antibiotics. As shown in Fig. 1, without antibiotics bacterial growth of strain F3031 increased over time. With the addition of gentamicin to the tissue culture medium, the viable count curve reflecting the number of in-

tracellular bacteria remained static, indicating no net increase in growth. When monolayers were infected with strain F3031, the cytotoxic phenotype was observed with or without the addition of antibiotics. Strain F1947 did not grow or produce the phenotype under any conditions, and neither of the strains grew in the tissue culture medium alone (data not shown).

Electron micrographs of several infected cell cultures clearly showed bacterial attachment and phagocytosis of strain F3031 (Fig. 2A and B). Attachment, but no invasion, was observed under the same conditions with strain F1947. Once the tissue culture cells had been invaded, the bacteria appeared to replicate within the phagocytic vacuoles (Fig. 2C). These phagosomes then fused to form larger vacuoles, sometimes encompassing the total cytoplasmic cell volume (Fig. 2D). Eventually, the host cell membrane was degraded, and the bacteria were released into the extracellular environment (Fig. 2E). Although large numbers of intracellular bacteria were observed (Fig. 2C and D) beginning 6 h after infection, the large numbers of bacteria were far more prevalent between 24 and 48 h after infection. This increase in prevalence does correlate with the upward slope in the viable count curve shown in Fig. 1.

The addition of cycloheximide, an inhibitor of eukaryotic protein synthesis (21); cytochalasin D and colchicine, inhibitors of phagocytosis (3, 26); and methylamine and nondansylcadaverine, inhibitors of pinocytosis (26), to the tissue culture 1 h before infection had no effect on invasion, intracellular growth, or the production of the cytotoxic phenotype. Additionally, lectin inhibition assays were performed with five common lectins (18): concanavalin A, which has specificity for α-D-mannosyl residues; BS-I and BS-II, specific for *N*-acetyl-α-D-galactosamyl and *N*-acetyl-D-glucosamyl residues, respectively; DBA, specific for the sugar *N*-acetyl-α-D-galactosamyl; and LcHA, specific for α-D-mannosyl residues. As observed above, none of these lectins prevented attachment, invasion, intracellular growth, or the production of the cytotoxic phenotype, compared with normal controls.

To determine if endotoxin or some other constitutively produced compound was being secreted and/or released by the bacteria, 10^8 bacteria in medium containing 50 μg of gentamicin per ml, 10^8 lysed F3031 bacteria, or gamma-irradiation-killed bacteria were added to the monolayer system; no cytotoxic phenotype was observed up to 96 h after the addition of these preparations. Culture supernatants from HMEC-1 cells previously infected for 48 h likewise did not produce the cytotoxic phenotype, compared with control supernatants from uninfected monolayers.

In experiments using the Transwell-COL insert to detect soluble cytotoxins, it was observed that the bacteria, unable to pass through the membrane and interact with the monolayer, did not replicate in the chamber and during a 96-h period did not release any products which could diffuse through the membrane to produce the cytotoxic phenotype.

DISCUSSION

Almost a decade has passed since BPF first appeared in the Brazilian state of Sao Paulo (4–6, 29). So far, no bacterial virulence factors have been conclusively identified in the causative organism, and little is known concerning the contribution of host factors in the pathogenesis of this disease (8, 11, 25).

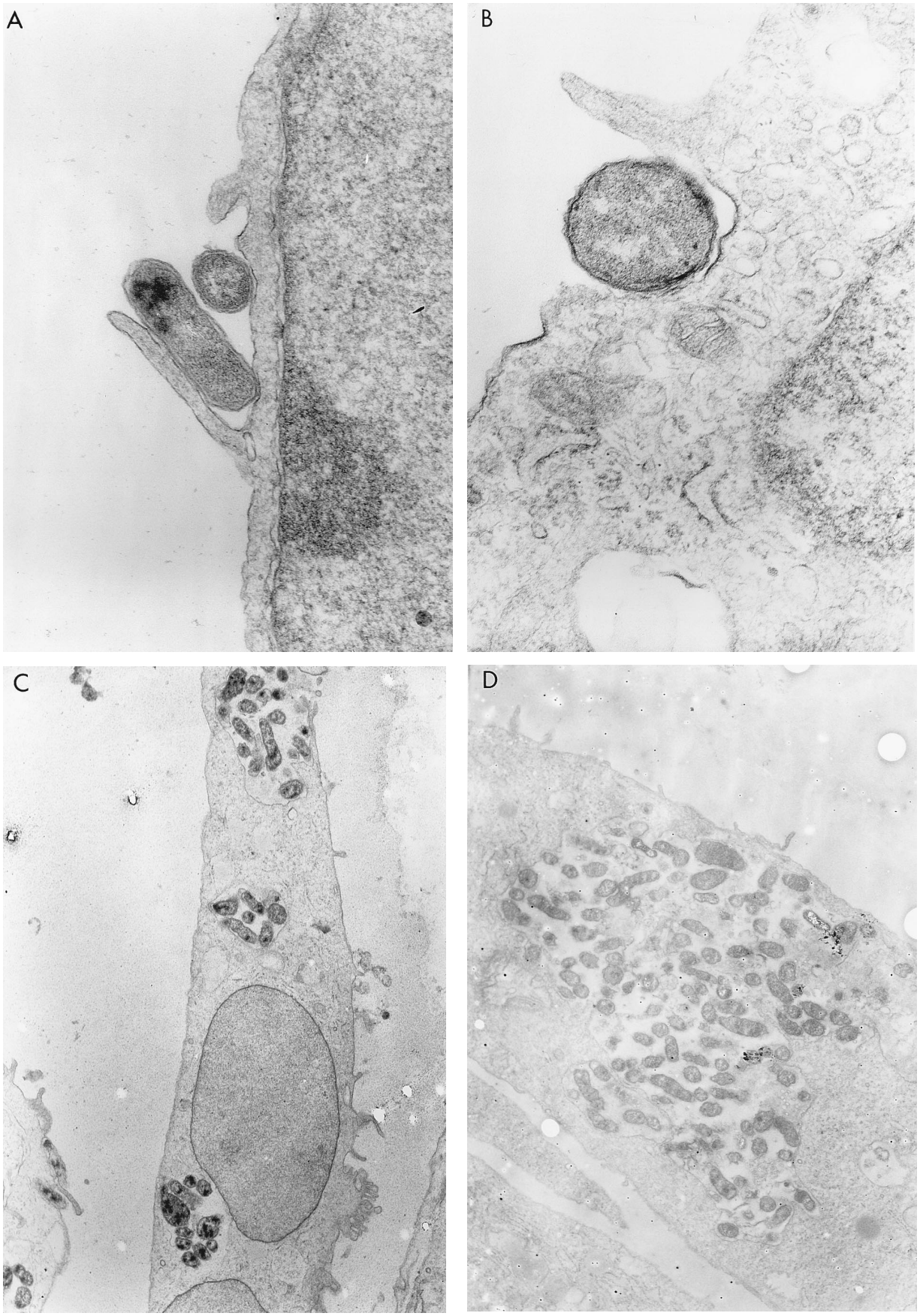
BPF pathogenesis was first studied with an in vivo intraperitoneal infant rat model of bacteremia developed by Rubin et al. (24). Using this model, Rubin et al. demonstrated that both Brazilian and Australian case strains caused a bacteremia in infant rats while control *H. influenzae* biogroup aegyptius strains were readily cleared. The HMEC-1 cytotoxicity model is the first step in studying the vascular destruction and purpura associated with BPF. Our earlier findings indicated that HMEC-1 cell monolayers (28) and capillary-like microtubules (22) are sensitive and specific to the cytotoxic effects of BPF-associated *H. influenzae* biogroup aegyptius strains. Since these cells are derived from the same tissues that appear to be a major target in the pathogenesis of BPF, the HMEC-1 model should make possible the study of host factors as well as bacterial factors contributing to this disease.

The experiments presented here were designed to elucidate the mechanism of toxicity associated with BPF-causing strains in HMEC-1 cells. The data suggest that BPF case strains of *H. influenzae* biogroup aegyptius are able to attach, invade, and multiply intracellularly in HMEC-1 cells. This intracellular multiplication of the virulent strains is required as a prerequisite for the cytotoxic phenotype. Because attachment, invasion, and intracellular growth are important to the development of the cytotoxic phenotype, we examined several aspects of these processes.

Attachment to epithelial cells of the conjunctiva and perhaps the upper respiratory tract is a preliminary step to gaining entry into the bloodstream during the initial stages of BPF. This process has been studied in vitro by others using human epithelial cells, Chang conjunctival cells, and a nasopharyngeal organ culture system (11, 25). Our results with the HMEC-1 endothelial cell line agreed with those of previous BPF attachment studies in that we observed attachment by both the BPF-associated strain and the BPF-negative control strain. Our findings suggest that attachment results from the recognition of preexisting structures on the host cell surface, since pretreatment of HMEC-1 monolayers with cycloheximide does not inhibit the process.

Of the bacterial structures that may be involved in the attachment process, the role of receptors for mannose and other carbohydrates as requirements for successful attachment and ultimate disease production by several bacterial pathogens (18) was examined. Lectin-blocking studies were not reported for the conjunctival and organ culture models. In the present study, a variety of carbohydrate lectins were examined for their ability to block attachment to HMEC-1 cells. None of these lectins successfully blocked attachment, invasion, intracellular growth, or production of the cytotoxic phenotype. Our findings also suggest that the receptor(s) recognized by the *H. influenzae* biogroup aegyptius cells may be either a preexisting protein or a carbohydrate moiety not recognized by the lectins that were examined. Because attachment to particular host receptors likely dictates correct trafficking through the host vacuole system, we will continue to investigate attachment factors required by BPF-causing *H. influenzae* biogroup aegyptius strains.

When examining the mechanisms of invasion and intracellular growth within the HMEC-1 cell line, we found that inhibitors of neither phagocytosis nor pinocytosis prevented invasion by strain F3031. Therefore, the mechanism of invasion must be totally parasite directed. Once bacteria are intracellular, replication apparently occurs within the phagocytic vacuoles. These phagosomes then fuse to form larger vacuoles, sometimes encompassing one-half of the cell volume. It is unknown whether lysosomal fusion is inhibited, as in the cases of *L. pneumophila* and *Chlamydia psittaci*, or whether the bacteria survive the infusion of lysosomal enzymes, as in the cases of *Yersinia pestis* and *Coxiella burnetii* (12, 19). The replicating bacteria eventually destroy the integrity of the host cell membrane and are released to the extracellular environment. The number of heavily infected and degenerating tissue culture cells increased with time and reached a maximum 24 to 48 h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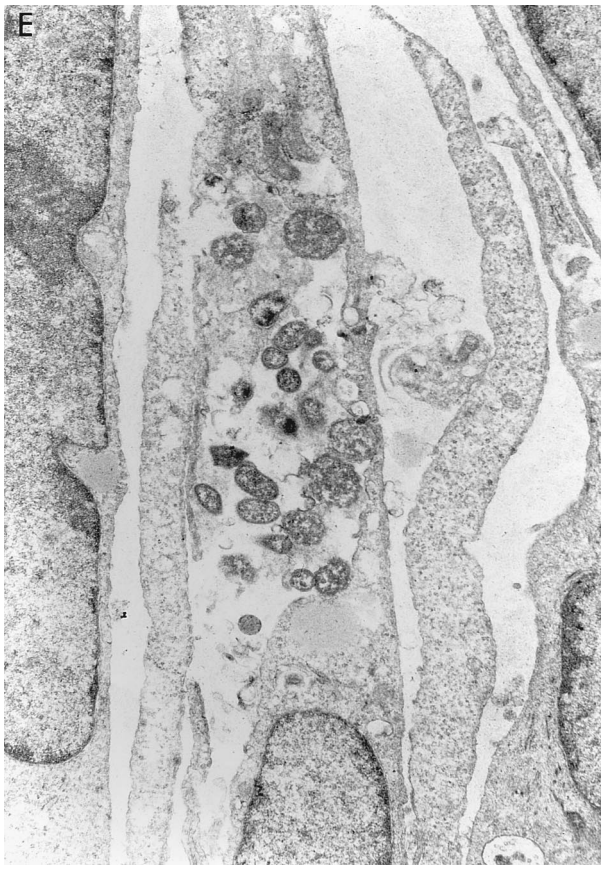

FIG. 2. Electron micrographs of HMEC-1 cells infected with *H. influenzae* biogroup aegyptius F3031. (A) Attachment of bacterium to the host cell; (B) phagocytosis of bacterium by host cell (note coated pits [arrow]); (C) bacteria residing in a phagocytic vacuole; (D) phagocytic vacuoles may converge into one large vacuole, sometimes encompassing most of cytoplasmic region; (E) degradation of host cell membrane and release of bacteria. Magnification, ×47,250 (A and B) and ×17,500 (C to E).

after infection. Coincidentally, this time period correlates with the increase in the cytotoxic phenotype.

As the infection progresses, released host cell components likely supply essential nutrients for extracellular bacterial growth. The addition of gentamicin to the medium after several hours of bacterial infection with the case-associated F3031 strain prevented extracellular replication of the organism. The lack of net growth after the addition of the antibiotic, as evidenced by the growth curve, is likely due to intracellular growth balanced by extracellular death following bacterial release from the HMEC-1 cells. Likely, some of the released bacteria manage to infect remaining monolayer tissue culture cells, but a majority of the bacteria are killed by the antibiotic after release from the host cells.

Tissue culture studies of *Salmonella* (13), *Yersinia* (14), and *Chlamydia* (21) species have examined the mechanisms of adherence and invasion and detected the synthesis of proteins specific to these processes and to survival within the host cell. We have begun to examine differences between the *H. influenzae* biogroup aegyptius strains that invade and cause disease and those that do not in order to identify putative virulence factors (27). More specifically, in this study we observed that tissue culture supernatants from F3031-infected HMEC-1 cells, as well as radiation-killed F3031 bacteria or lysed bacteria, did not produce the cytotoxic effect on fresh HMEC-1 monolayers. Moreover, the addition of chloramphenicol to the HMEC-1 system infected with strain F3031 also prevented the production of the cytotoxic phenotype. These observations indicate either the labile nature of the toxin or the requirement for intracellular growth of the bacteria. Likewise, the presence of viable bacteria separated from the monolayer by a small-pore membrane did not permit either the production of the appropriate toxins or access to the correct host cell targets. Likely, several proteins that are required for intracellular replication and cytotoxin production are produced during the infectious process.

Several potential virulence factors have been described for *H. influenzae* biogroup aegyptius (4, 8, 16, 20, 24, 25). However, with the exception of endotoxin, none of these compounds has been shown to be associated with vascular cell cytotoxicity. Our present research suggests that endotoxin is not the compound responsible for the HMEC-1 cell cytotoxicity since cell wall preparations from BPF-causing strains and culture supernatants from tissue culture infections did not produce the observed cytotoxic phenotype. One possible explanation is that a cytotoxin is produced only during intracellular growth and that the bacterium is selective of the cell lines in which it can grow intracellularly.

Destruction due to specific replication in endothelial cells, and not in epithelial cells, may be a vital aspect of this disease and needs to be examined in greater detail. The bacterium can attach to several cell types and invade to a limited extent in several examined cell lines, although it appears that the bacterium is ultimately cleared in these lines (28). Therefore, the mechanism of intracellular growth may be limited to only this particular cell type.

Vascular destruction and purpura can result from infections with other infectious agents, such as *Neisseria meningitidis*, that have a well-documented worldwide distribution. Insights gained from HMEC-1 cytotoxicity studies with BPF-associated strains may provide novel approaches to the study of these other infectious agents.

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