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Pseudomonas aeruginosa Selective Adherence to and Entry into Human Endothelial Cells

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The pathogenesis of *Pseudomonas aeruginosa* disseminated infections depends on bacterial interaction with blood vessels. We have hypothesized that in order to traverse the endothelial barrier, bacteria would have to adhere to and damage endothelial cells. To test this hypothesis, we studied the adherence to human endothelial cells in primary culture of the piliated *P. aeruginosa* strain PAK and of two isogenic nonpiliated strains: PAK/p−, which carries a mutation in the pilin structural gene, and PAK-N1, a mutant defective in the regulatory *rpoN* gene. PAK adhered significantly more than did the pilus-lacking strains. *P. aeruginosa* was also taken up by endothelial cells, as determined by quantitative bacteriologic assays and by transmission electron microscopy. This internalization of *P. aeruginosa* seems to be a selective process, since the piliated strain was taken up significantly more than the nonpiliated bacteria and the avirulent *Escherichia coli* DH5α, even following bacterial centrifugation onto the cell monolayers. A significant fraction of the internalized *P. aeruginosa* PAK was recovered in a viable form after 6 h of residence within endothelial cells. Progressive endothelial cell damage resulted from PAK intracellular harboring, as indicated by the release of lactate dehydrogenase. An increasing concentration of PAK cells was recovered from the extracellular medium with time, suggesting that ingested bacteria were released from endothelial cells and multiplied freely. We speculate that in vivo the ability of some *P. aeruginosa* strains to resist intracellular residence would afford protection from host defenses and antibiotics and that the release of viable bacteria into bloodstream may represent a central feature of the pathogenesis of bacteremia in compromised patients.

Pseudomonas aeruginosa remains one of the most important causal agents of bacteremia among the gram-negative microorganisms (1). Attention has largely been focused on bacteremia in selected groups of patients, especially those with neoplastic or hematologic diseases. However, *P. aeruginosa* bacteremia has also been associated with burns, endocarditis, pneumonia, urological problems, and newborn infants (1, 9). It may also occur, albeit infrequently, in the absence of predisposing disease or immunocompromised status and outside the nosocomial environment (10, 24).

P. aeruginosa may invade and destroy blood vessels without the aid of inflammatory cells (9, 47), leading to necrosis of infected tissues. Scattered infectious necrotizing vasculitis involving many organs is a common consequence of the bacterial hematogenic dissemination in neutropenic patients. The broad range of proteolytic enzymes produced by *P. aeruginosa* is thought to favor the spread of the organism throughout the body (31). However, no relationship between the production of proteases and the course of infection in neutropenic rabbits has been established. Following inoculation with different clinical *P. aeruginosa* isolates, the protease-negative strains were just as invasive as the protease producers and some elastase-negative strains were associated with a high incidence of vasculitis (47). Therefore, the mechanism by which bacteria gain access to the intravascular space and escape from it remains unclear.

Adherence of bacteria to host cells is usually quite specific and is regarded as a prerequisite for tissue invasion. This specificity is important because the availability of suitable receptors will often determine which body site will be infected. *P. aeruginosa* chronically colonizes the airways of cystic fibrosis patients. However, apart from the respiratory infections in these patients, *P. aeruginosa* does not present the tissue specificity shown by other human pathogens but rather infects tissues of different origins previously damaged in some way.

Like many other microorganisms, *P. aeruginosa* has several products that may function as adhesins (33). The pilus was one of the first *P. aeruginosa* gene products to be associated with pathogenicity, because of its ability to allow bacterial adherence to human epithelial cells (36, 45). Recently, *P. aeruginosa* pili were shown to allow also bacterial entry into A549 pneumocyte cells (3). Although the expression of pili mediates bacterial attachment to and colonization of epithelial surfaces, paradoxically it can also be detrimental to the pathogen since direct attachment to receptors on a phagocytic cell can facilitate killing of the microorganisms (20, 41). Bacteria have therefore evolved regulatory mechanisms that limit the expression of pili. For example, the transcription of the *P. aeruginosa* pilin gene requires a functional alternative sigma subunit of RNA polymerase (*rpoN*) (17). In addition, regulatory *rpoN* also appears to control the expression of the nonpilus adhesin(s) responsible for *P. aeruginosa* adherence to both cells and mucin (3, 35).

In the attempt to understand the process involved in the establishment of intravascular *P. aeruginosa* infections, we studied the early interaction of a piliated and two pilus-lacking isogenic *P. aeruginosa* strains with endothelial cells, a cellular barrier which bacteria must presumably come in contact with and traverse to initiate disseminated infections.

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MATERIALS AND METHODS

Bacteria. Piliated *P. aeruginosa* PAK and pilus-lacking isogenic PAK/p− constructed by gene replacement were kindly provided by W. Paranchych, from the Department of Microbiology, University of Alberta, Alberta, Canada. *P. aeruginosa* PAK-N1, carrying a mutation in the *rpoN* gene required for the expression of the pilin structural gene (17), was a generous contribution of S. Lory, from the Department of Microbiology, University of Washington, Seattle. *Escherichia coli* DH5α was included in this study as a nonadherent avirulent negative control. Bacteria were grown overnight at 37°C in Trypticase soy broth (Difco Laboratories, Detroit, Mich.), harvested by centrifugation, and resuspended in RPMI 1640 medium containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma Chemical Co., St. Louis, Mo.) to an A_{640} of 0.1, corresponding to 1×10^8 to 2×10^8 CFU/ml. The bacterial concentration was confirmed by quantitative culture on Trypticase soy agar (TSA; Difco).

Culture of human endothelial cells. Cells were isolated from human umbilical cord veins by a method modified from that of Jaffe et al. (19) and grown in RPMI 1640 HEPES medium supplemented with 20% fetal bovine serum, 50 μg of gentamicin at 50 μg/ml, and amphotericin at 250 μg/ml. Their endothelial nature was ensured by demonstrating their reactivity with a rabbit anti-human von Willebrand antibody (Dako S.A.) (18) by indirect immunofluorescence.

Adherence assay. Five hundred microliters of the bacterial suspensions was added to endothelial cells cultured on 13-mm plastic coverslips (Thermanox; Nunc Inc., Naperville, Ill.) placed in tissue culture plate wells. Following incubation for 1 h, the monolayers with attached bacteria were washed three times with phosphate-buffered saline (PBS) and fixed in 2.5% glutaraldehyde in PBS. Cells were then either stained with May-Grunwald-Giemsa stain for light microscopic observation or processed for transmission electron microscopy (TEM).

Uptake of *P. aeruginosa*. Five hundred microliters of the PAK and PAK/p− suspensions at 2×10^7 CFU/ml was added to confluent endothelial cell monolayers. The supernatants were then removed after incubation for different time periods. The monolayers were rinsed three times with RPMI 1640 HEPES medium and incubated for 1 h with medium containing gentamicin sulfate at 300 μg/ml, to kill extracellular bacteria. Gentamicin at 100 μg/ml was sufficient to kill *P. aeruginosa* at 10^8 CFU/ml in 1 h (data not shown). Control wells were incubated with medium without antibiotics. After removal of the antibiotic-containing medium, the cells were washed and treated with sterile PBS containing 0.025% trypsin and 1% Tween 20 (Sigma) for 30 min at 37°C. Aliquots of the cell lysates were diluted and plated on TSA to quantify the number of viable intracellular bacteria. In all experiments, aliquots of the antibiotic-containing media were plated in TSA, to ascertain whether the antibiotics present in the postinfection medium were efficient in killing extracellular *P. aeruginosa*. Following the gentamicin treatment, in some uptake assays, cells were fixed and processed for TEM to further confirm the presence of intracellular *P. aeruginosa*.

The extinction of acridine orange fluorescence by crystal violet has been largely used to distinguish internalized from surface-bound microorganisms in phagocytosis assays (13, 26, 28, 30). Accordingly, a modification of the fluorescence-quenching technique (15, 34) was used to demonstrate further the uptake of *P. aeruginosa* PAK by endothelial cells. Briefly, cell monolayers were grown on glass coverslips, exposed to bacterial suspensions at 2×10^7 CFU/ml for 15 min, and rinsed with Hanks balanced salt solution (HBSS) to eliminate nonadherent microorganisms. Thereafter, cells were incubated with culture medium without antibiotics for additional 30-, 60-, and 120-min periods, to allow bacterial entry. Cells were then rinsed, stained with 0.001% acridine orange (Sigma) in HBSS for 45 s, rinsed with HBSS, and quenched with 0.05% crystal violet in 0.15 M NaCl for 45 s. Finally, coverslips were washed, mounted in glycerol-PBS, and examined with a Nikon Labophot microscope equipped with quartz iodine illumination. The number of fluorescent bacteria in at least 300 different cells was counted, and the average number of intracellular bacteria per cell was determined.

Selectivity of *P. aeruginosa* uptake. To ascertain whether there was some specificity in *P. aeruginosa* internalization by endothelial cells, we evaluated the uptake of a nonadherent *rpoN* mutant of strain PAK (3, 35) and of an avirulent, noninvasive *E. coli* DH5α strain (12). Since adherence to host cells is a necessary initial step for bacterial internalization, the handicap of nonadherent microorganisms was circumvented by bacterial centrifugation (1,000 × *g* for 10 min) onto the endothelial cell monolayers. After 1 h at 37°C, cells were rinsed and treated with gentamicin-containing medium to kill extracellular microorganisms, as described above.

Effect of cytochalasin D on endothelial cell uptake of *P. aeruginosa*. Since many bacteria enter eucaryotic cells via a microfilament-dependent mechanism, we examined the effects of cytochalasin D, in concentrations ranging from 0.01 to 0.1 μg/ml, on the process of *P. aeruginosa* internalization by endothelial cells. Higher cytochalasin D concentrations resulted in cell detachment from the monolayers (data not shown). To assess the effect of cytochalasin D on actin filaments, endothelial cells cultured on glass coverslips and treated for 1 h with different concentrations of the drug were fixed for 10 min in 5% formaldehyde in PBS, rinsed, permeabilized with 0.01% Triton X-100 in PBS for 4 min, rinsed, and treated for 30 min with a 5-μg/ml solution of fluorescein isothiocyanate-phalloidin (Sigma), to specifically stain filamentous actin (42, 46). Thereafter, coverslips were rinsed, mounted in glycerol-PBS, and observed under a fluorescence microscope. Cytochalasin D stock solution (at 1 mg/ml) was prepared in dimethyl sulfoxide at 10% (42). Therefore, control cells were exposed to dimethyl sulfoxide at 10 μg/ml, fixed, and treated as described above.

Fate of intracellular *P. aeruginosa*. To determine whether *P. aeruginosa* could survive intracellularly, we modified the uptake assay described above. After incubation with gentamicin-containing medium for 1 h, cells from at least three culture wells were lysed with trypsin-Tween 20 solution and plated on TSA. In parallel, cells from the other wells were incubated with antibiotic-containing medium for additional 1-, 2-, 3-, and 5-h periods and then subjected to the lysing treatment and to quantitative bacteriology. The efficiency of gentamicin in killing extracellular bacteria was systematically analyzed by plating the postinfection media in TSA.

In other experiments, following the incubation of the infected cells with gentamicin, monolayers were incubated in medium without antibiotics. Aliquots of the antibiotic-free medium from each culture well were diluted and plated on TSA at different intervals to determine whether intracellular *P. aeruginosa* would regain the extracellular medium following prolonged incubation (because of either endothelial cell lysis or active release).

Assay for intracellular killing of *P. aeruginosa*. The killing of intracellular *P. aeruginosa* by endothelial cells was investigated by a modification of the test described by Smith and Rommel (40), which distinguishes between viable and dead microorganisms by their green and red-yellow fluorescence, respectively

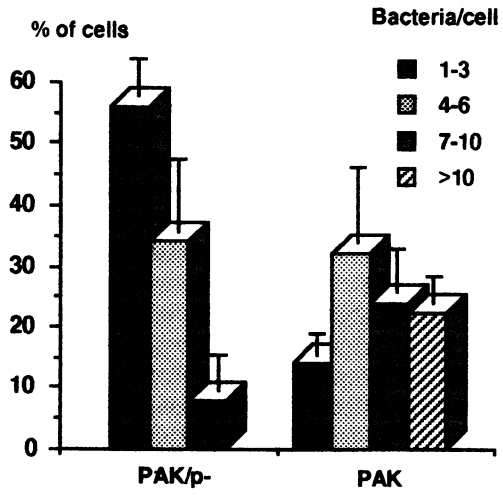

FIG. 1. Range of adherent piliated PAK and nonpiliated PAK/p− *P. aeruginosa* bacteria per human umbilical vein endothelial cell in primary culture, following an incubation period of 1 h.

(2, 6, 13, 23, 26, 30, 34, 40). Briefly, suspensions of *P. aeruginosa* PAK containing 10^8 CFU/ml were added to endothelial cells grown on glass coverslips. After 1 h at 37°C, cells were rinsed, incubated with medium containing gentamicin for different periods, rinsed, and exposed to 0.01% acridine orange (Sigma) in HBSS for 1 min. Coverslips were mounted in glycerol-PBS and observed under a fluorescence microscope. The sensitivity of vital acridine orange staining has previously been tested (25): *P. aeruginosa* suspensions were subjected to different treatments (2) and then to concurrent acridine orange staining and plating in TSA to assess bacterial viability.

Assay for endothelial cell damage. To evaluate whether the presence of intracellular bacteria would damage endothelial cells, monolayers were incubated with *P. aeruginosa* PAK at 10^7 CFU/ml for 1 h, rinsed, and exposed to gentamicin at 300 μg/ml for different periods. The supernatants of the culture wells were recovered, and their lactate dehydrogenase (LDH) activity was determined by a colorimetric assay (Roche). Control wells were also treated with gentamicin but not exposed to bacteria. LDH activity of control cells was measured in the cell supernatant and in cell lysates obtained by exposing cells to distilled water. LDH released in each condition was expressed as a ratio of the LDH in supernatants of experimental wells to the LDH in control cell lysates plus LDH in the supernatant of control cells times 100 (12).

TEM. Endothelial cells exposed to *P. aeruginosa* suspensions were rinsed, fixed in 2.5% glutaraldehyde in PBS, rinsed, postfixed in 1% OsO_4, and dehydrated through a graded ethanol series. They were then embedded in Epon, thin sectioned, and examined with a EM 10C Zeiss transmission electron microscope.

Statistical analysis. All experiments were repeated at least twice. Results are presented as the mean ± standard deviation. A Student's *t* test was used to compare means, and a *P* value of <0.05 was considered significant.

RESULTS

***P. aeruginosa* adherence to endothelial cells.** Most of the cells incubated with *P. aeruginosa* PAK (92.1% ± 10.2%) showed attached microorganisms after an incubation period of 1 h. The percentage of cells with attached PAK strain was

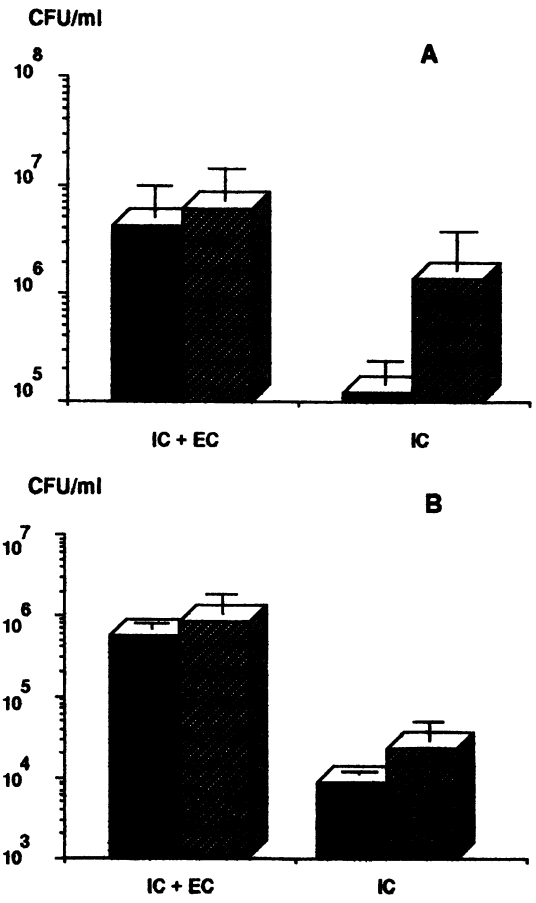

FIG. 2. Concentration of intracellular (IC) piliated PAK (A) and nonpiliated PAK/p− (B) *P. aeruginosa* CFU in cell lysates of endothelial cells treated with gentamicin, following bacterium-endothelial cell interaction for 15 min (■) and 60 min ( ), as well as in cell lysates of untreated cultures (intracellular and extracellular bacteria [IC + EC]).

significantly higher than that with PAK/p− (72.3% ± 0.3%) or with PAK-N1 *P. aeruginosa* (3.3% ± 1.7%). There was also a difference between piliated and nonpiliated *P. aeruginosa* regarding the number of adherent bacteria per endothelial cell: while 56.0% ± 6.6% of the cells exposed to the PAK/p− strain presented from one to three microorganisms, 78.9% ± 10.3% of the cells incubated with the PAK strain had more than four attached bacteria (Fig. 1). Most of the cells incubated with *P. aeruginosa* PAK-N1 (87.5% ± 17.7%) presented only one adherent bacterium per cell.

***P. aeruginosa* uptake by endothelial cells.** The PAK and PAK/p− strains were isolated from the lysates of endothelial cells treated with gentamicin to kill extracellular microorganisms. As shown in Fig. 2, both the total number of bacteria associated with endothelial cells (intracellular and extracellular attached bacteria) and the number of bacteria in the intracellular compartment were significantly higher for PAK than for PAK/p−. A greater number of bacteria attached to endothelial cells than invaded them. After a 15-min incubation period, 2.8% of PAK and 1.7% of PAK/p− endothelial cell-associated *P. aeruginosa* were in the intracellular compartment. After 1 h, the percentages of intracellular bacteria were 22.4 and 2.9%, respectively.

The fluorescence quenching assay confirmed the presence of

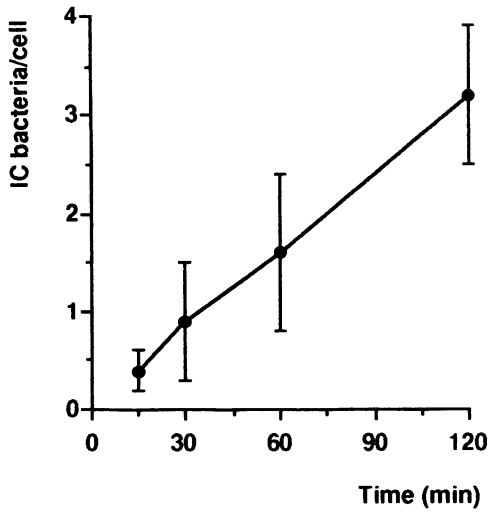

FIG. 3. Time course of *P. aeruginosa* PAK uptake by endothelial cells, as assessed by the fluorescence-quenching technique. Data are expressed as means ± standard deviations from two experiments carried out in triplicate.

intracellular microorganisms and demonstrated the kinetics of *P. aeruginosa* PAK internalization by endothelial cells. As shown in Fig. 3, the number of intracellular bacteria increased linearly with time during the course of the experiment.

Figure 4 shows the effect of bacterial density on uptake by endothelial cells. Up to an input of approximately 3×10^7 bacteria, the internalization was linear, whereas a decreasing fraction of bacteria was internalized when higher concentrations were added to cell monolayers. These data suggest an approaching saturation of the host cell sites available for bacterial adherence and ulterior internalization.

TEM. TEM was performed on monolayers from a 1-h uptake assay. As shown in Fig. 5A, extracellular bacteria were

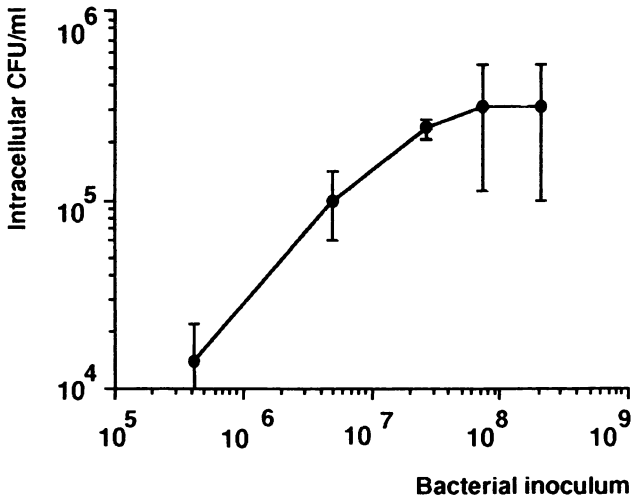

FIG. 4. Dose-response curve of *P. aeruginosa* PAK uptake by endothelial cells. Cells were incubated with 4.2×10^5 to 2.1×10^8 bacteria for 1 h, rinsed, and treated with gentamicin to kill extracellular microorganisms. Each point represents the mean ± standard deviation from three experiments carried out in triplicate.

in close association with surface extensions of endothelial cells. Intracellular *P. aeruginosa* was seen in membrane-bound vesicles (Fig. 5D and E); however, the microorganisms appeared to be mostly free in the cell cytoplasm (Fig. 5B and C). Occasionally, multiple bacteria were observed in the same vesicle (Fig. 5C), suggesting that cell division occurred while they were internalized.

Selectivity of *P. aeruginosa* uptake. To assess the specificity of *P. aeruginosa* internalization by endothelial cells, we compared the uptake of two different isogenic nonpiliated mutants of the PAK strain with that of their wild-type parent. PAK cells were taken up significantly more the other bacterial strains. Intracellular PAK/p−, PAK-N1, and *E. coli* DH5α, used as a negative control, represented approximately 15.0, 3.7, and 0.1% of the number of intracellular *P. aeruginosa* PAK cells, respectively (Table 1).

Effect of cytochalasin D on the number of intracellular *P. aeruginosa* cells. The effect of cytochalasin D on the arrangement of endothelial cell cytoskeletal actin was visualized by fluorescence staining with a phallotoxin. The fluorescence of control untreated cells was predominantly localized at the cell periphery and in stress fibers. In contrast, in cells treated with different concentrations of cytochalasin D, stress fibers were replaced by masses of aggregated actin-containing material (not shown), as previously described (42). Cytochalasin D caused a significant inhibition of *P. aeruginosa* PAK penetration within endothelial cells at doses as low as 0.01 μg/ml (Table 2), suggesting an active role of cell microfilaments in the entry of bacteria.

Fate of *P. aeruginosa* following internalization by endothelial cells. The ability of *P. aeruginosa* to survive within endothelial cells was tested by a modification of the invasion assay. As shown in Fig. 6A, the number of viable intracellular PAK strain cells increased from 1 to 2 h postinfection but decreased later, although not significantly ($P > 0.05$). In parallel to this decline, we detected a significant ($P < 0.05$) increase in the number of extracellular *P. aeruginosa* cells. To evaluate whether the slight decrease in the CFU of intracellular bacteria could have resulted from endothelial cell killing of *P. aeruginosa*, cells at different stages of infection were exposed to acridine orange. As shown in Fig. 6B, endothelial cells could kill intracellular *P. aeruginosa*, although not efficiently. The percentage of green fluorescent viable intracellular bacteria decreased significantly with the incubation period but still remained elevated after a 6-h incubation period.

Damage of infected endothelial cells. As shown in Table 3, the LDH release by endothelial cells increased with the infection period, suggesting that cell injury had occurred as a consequence of *P. aeruginosa* infection.

DISCUSSION

P. aeruginosa is perhaps unique among gram-negative bacilli in its predilection for certain subpopulations of patients: those with burns, malignancies, immunosuppression, and traumatic wounds. Secondary bacteremia is a common event in these patients, but the means by which bacteria proceed from skin or mucous membranes to the circulation and from the circulation to tissue parenchyma remains unknown. In this study, we looked for an in vitro parallel of *P. aeruginosa* dissemination. *Candida albicans*, another opportunistic microorganism causing systemic disease in compromised hosts, penetrates the endothelial barrier by disrupting cell surfaces soon after attachment, possibly via a process dependent on tissue enzymatic degradation (21). Since *P. aeruginosa* produces metalloproteases with broad substrate specificities that potentially can

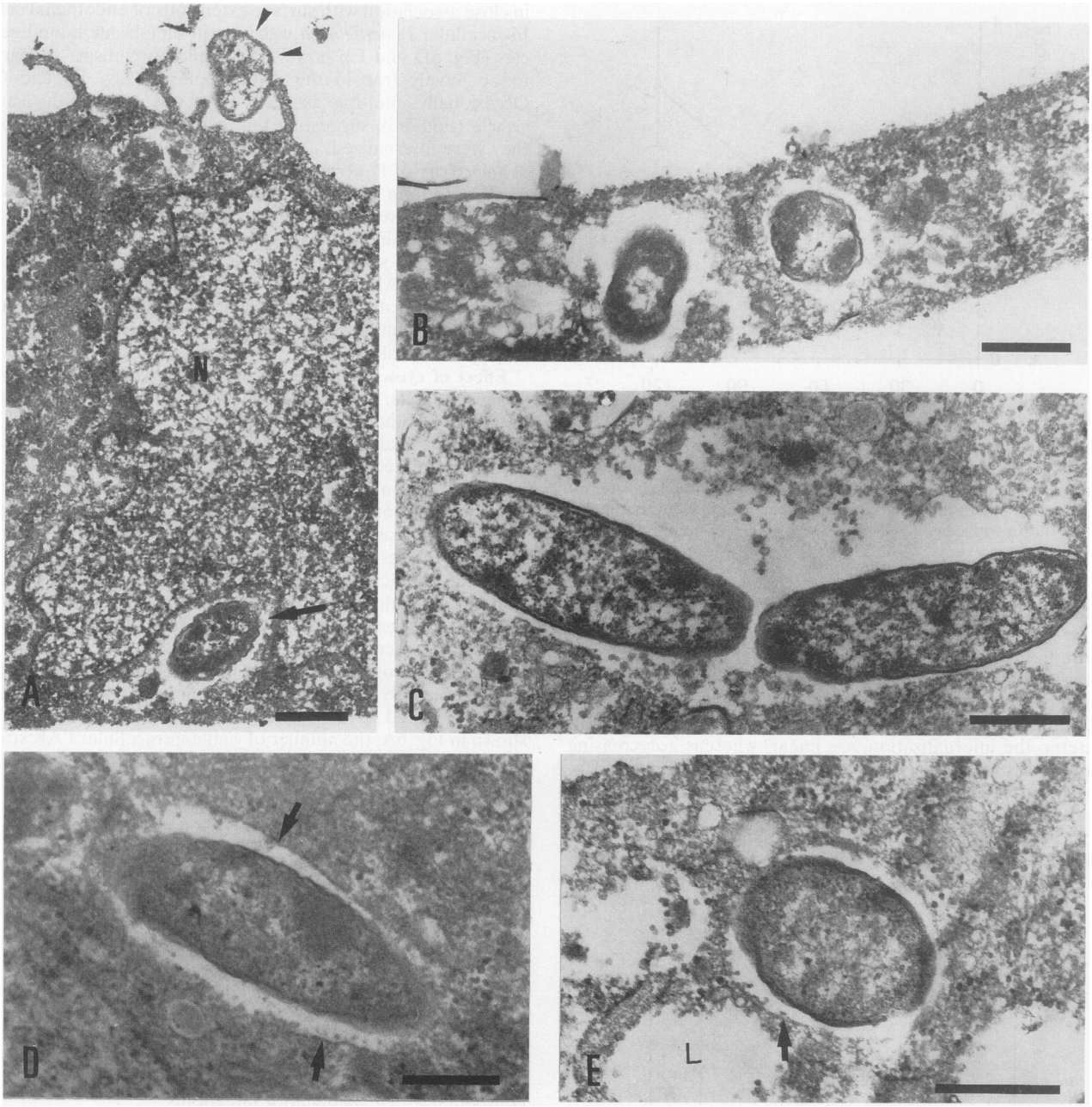

FIG. 5. Transmission electron micrographs of human umbilical vein endothelial cells exposed to a suspension of piliated PAK *P. aeruginosa* for 1 h. (A) Note the significant morphological changes of the extracellular bacterium (arrowheads), indicating that it has been affected by the antibiotic, in contrast with the well-preserved morphology of the intracellular microorganism (arrow and panels B to E). (B and C) *P. aeruginosa* apparently free in the cell cytoplasm, since no membrane surrounding the bacterium-containing vacuole is seen. (D and E) The membranes limiting the vacuoles are still partially present (arrows). N, nucleus; L, lysosome. Bars represent 0.5 μm.

induce substantial tissue damage (31), we hypothesized that *P. aeruginosa* could make its way through host bodies by attaching to endothelial cells and injuring them by secreting toxic products.

Pilus is the major adhesin by which *P. aeruginosa* adheres to host tissues (16, 20, 36). In this study, we evaluated the role of pili in *P. aeruginosa* interaction with human endothelial cells in primary culture. The piliated PAK strain adhered significantly more to endothelial cells than the isogenic pilus-lacking PAK/p− strain; however, the nonpiliated bacteria also adhered substantially. Therefore, many of the other adhesins produced by *P. aeruginosa* may participate in bacterium-endothelial cell interactions, as reported for other human tissues (32, 38, 39). TEM observation of endothelial cells exposed to *P. aeruginosa* showed the presence of bacteria closely associated with cell membranes. More significantly from the standpoint of the physiopathology of infection, the microorganisms were also found inside endothelial cells. The acridine orange-crystal violet quenching technique, largely used to differentiate microorganisms attached to from those

TABLE 1. Bacterial uptake by endothelial cells following microbial centrifugation onto cell monolayers[a]

Bacterial strain	CFU of intracellular bacteria	% of the bacterial inoculum
P. aeruginosa PAK	$(1.6 \pm 1.4) \times 10^4$	0.690 ± 0.7900
P. aeruginosa PAK/p−	$(2.4 \pm 1.4) \times 10^3$	0.160 ± 0.2200[b]
P. aeruginosa PAK-N1	$(5.9 \pm 0.1) \times 10^2$	0.029 ± 0.0060[c]
E. coli DH5α	$(0.2 \pm 0.1) \times 10^2$	0.004 ± 0.0001[c]

[a] Bacteria (1×10^6 to 2×10^6 CFU/ml) were centrifuged onto cell monolayers. Data are means ± standard deviations from at least three different assays carried out in triplicate.
[b] $P < 0.05$.
[c] $P < 0.001$.

ingested by professional phagocytes (2, 6, 13, 23, 26, 30, 34), revealed the presence of green-fluorescing intracellular *P. aeruginosa*. Finally, the uptake of *P. aeruginosa* by endothelial cells was confirmed by exposing infected cells to gentamicin, which selectively kills extracellular bacteria, and by releasing the internalized microorganisms by detergent-induced lysis of endothelial cells. The gentamicin method has recently been used to assess the uptake of *P. aeruginosa* by human epithelial cells (3), and although some *P. aeruginosa* strains may be resistant to therapeutic doses of the antibiotics, this does not invalidate the use of this method. However, it is necessary that the final concentration of gentamicin used be higher than the MIC for the bacterial strains under the particular conditions of the assay. In addition, appropriate controls should also be performed to confirm the efficiency of gentamicin in killing extracellular microorganisms.

Piliated *P. aeruginosa* was taken up by endothelial cells at a higher rate than the nonpiliated PAK/p− strain. This was not a very surprising result, since adherence is the first necessary step in the process of cell endocytosis. This has been clearly demonstrated in studies of *E. coli* invasion of epithelial cells (22) and in studies of *Shigella flexneri* entry into HeLa cells (4). However, the entry advantage presented by *P. aeruginosa* PAK did not seem to be dependent only on its higher adhesiveness. We compared the uptake of piliated PAK with that of two nonpiliated isogenic bacteria, in experimental conditions in which the adherence advantage conferred by pili was circumvented by centrifugation of the microorganisms onto the cell monolayers. Under these conditions, the internalization of *P. aeruginosa* PAK was still significantly higher. This observation led us to speculate that the genes coding for pilin synthesis might concurrently code for the synthesis of still unknown virulence factors that might confer entry facilities to piliated bacteria.

The heterogeneity in the internalization of different *P. aeruginosa* strains studied and the very low uptake of the avirulent *E. coli* DH5α suggest that bacterial entry into endothelial cells is a selective phenomenon in which microbial factors may be also concerned.

A few parasites actively invade eucaryotic cells by making

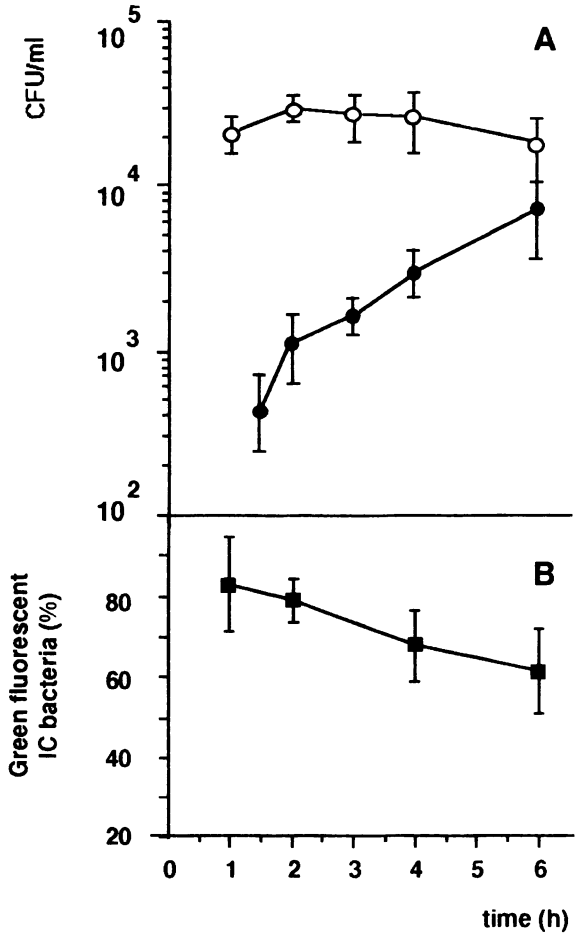

FIG. 6. (A) Time course of the concentration of viable piliated *P. aeruginosa* PAK in the intracellular compartment of infected endothelial cells (○) and in extracellular media (●). Data are expressed as means ± standard deviations from two independent determinations performed at least in triplicate. (B) Percentage of green fluorescent intracellular *P. aeruginosa* PAK bacteria, as determined by the acridine orange test, at timed intervals.

TABLE 2. Effect of cytochalasin D on *P. aeruginosa* PAK entry into endothelial cells

Cytochalasin D concn (μg/ml)	CFU of intracellular bacteria[a]	Inhibition (%)
None (control)	$(24.4 \pm 5.0) \times 10^4$	—[b]
0.01	$(9.6 \pm 7.7) \times 10^4$	60.7[c]
0.05	$(12.4 \pm 6.8) \times 10^4$	49.2[c]
0.1	$(5.2 \pm 1.5) \times 10^4$	78.7[c]

[a] Data are means ± standard deviations from a typical experiment carried out with at least five different wells for each drug concentration.
[b] —, not applicable.
[c] $P < 0.05$.

TABLE 3. Time course of the release of LDH by PAK-infected human endothelial cells in primary culture[a]

Infection time (h)	LDH activity (U)	% LDH release[b]
None (control)	69.00 ± 24.65	100
1	1.33 ± 1.53	1.93 ± 2.21
2	3.00 ± 2.45	4.35 ± 2.21
3	3.25 ± 0.50	4.71 ± 0.82
4	5.20 ± 4.30	7.53 ± 6.26
6	7.80 ± 7.39	11.30 ± 10.71

[a] Data are means ± standard deviations for a typical experiment carried out in triplicate.
[b] Data represent the ratios of LDH in supernatant of infected cells to LDH in control cell lysates plus LDH in control cell supernatants times 100.

holes in their envelopes and are never enclosed in a host-derived membrane. However, most parasites invade host cells by exploiting existing eucaryotic internalization pathways (8, 27). The exposure of endothelial cells to cytochalasin D at 0.1 μg/ml prior to and during the incubation with *P. aeruginosa* suspension resulted in a 78.7% reduction of intracellular bacteria. This finding suggests the involvement of a microfilament-dependent, phagocytosis-like bacterial entry into endothelial cells. Moreover, ultrastructural analysis by TEM provided corroborative evidence that an event analogous to phagocytosis was involved in internalization of *P. aeruginosa*, since bacteria were observed within membrane-limiting vacuoles resembling endosomes.

Although endothelial cells are not considered professional phagocytes, the uptake of inert particles such as polystyrene microspheres (37) and of a wide variety of organisms has been previously described (5, 11, 12, 14, 29, 43, 44). However, limited data concerning post-bacterial uptake events are available. In the present study, the issue of *P. aeruginosa*-endothelial cell interaction was addressed with different emphases: experiments were carried out to investigate whether *P. aeruginosa* multiplies within endothelial cells, whether endothelial cells are capable of killing *P. aeruginosa*, and whether the harboring of intracellular bacteria results in endothelial cell damage.

Bacterial killing by endothelial cells was assessed by using the acridine orange vital stain (2, 6, 13, 30, 34, 40). This was appropriate since an excellent correlation has been reported between green fluorescent staining by acridine orange and bacterial viability and between red staining and bacterial death (2).

Quantitative bacteriological assays on lysates of infected endothelial cells showed a slight increase in the number of intracellular *P. aeruginosa* bacteria during the first hour of infection followed by a progressive although similarly slight decline. After 6 h of infection, there was a decrease of approximately 14% in the viable counts of intracellular bacteria. This decline was much less extensive than that observed following *P. aeruginosa* uptake by professional phagocytes. After 2 h of phagocytosis, between 68.3 and 86.5% of the ingested nonmucoid *P. aeruginosa* bacteria were killed by human polymorphonuclear leukocytes (41). Our findings are thus in agreement with those of Ryan (37), who demonstrated that endothelial cells can phagocytose bacteria and harbor them intracellularly. However, despite the generation of reactive oxygen compounds, endothelial cells were not efficient at killing intracellular bacteria. Accordingly, at least for some bacterial species, endothelial cells may provide an environment protected from antibiotics and host defense mechanisms and thus contribute to the pathogenesis or the progression of the disease.

The decline in the counts of viable intracellular *P. aeruginosa* bacteria was also detected by the acridine orange assay. In addition, this test revealed the relative inefficacy of endothelial cells in killing intracellular bacteria. After 6 h of infection, approximately 60% of intracellular *P. aeruginosa* bacteria were still viable.

The orthochromatic shift of acridine orange from green to red is known to be pH dependent. Acidification of endocytic vacuoles may occur independently of phagosome-lysosome fusion (7) but more often depends on the transfer of lysosomal acid hydrolytic enzymes, creating a degradative compartment where internalized particles are digested. In our present study we speculate that the discharge of lysosomal acid enzymes into *P. aeruginosa*-containing endosomes may have accounted for the decline of the green fluorescent intracellular bacteria. However, further studies are required to ascertain whether phagosome-lysosome fusion occurs following microbial uptake by endothelial cells. The killing activity of these cells also warrants further investigation.

The increase in the number of bacteria in the cell culture supernatant suggested that *P. aeruginosa* became extracellular and multiplied. Similar results have been obtained in studies of *Staphylococcus aureus* ingestion by bovine endothelial cells (43). The mechanism by which *S. aureus* and *P. aeruginosa* may escape from endothelial cells is largely unknown. However, since polystyrene microspheres taken up by endothelial cells in culture may eventually be returned to the culture medium without apparent reuptake (37), release of endocytosed particles may be a constitutive pathway of endothelial cells. On the other hand, *P. aeruginosa*, like *S. aureus* (43), was observed to cause progressive cell injury with increasing duration of intracellular residence. We thus speculate that intracellular bacteria may damage endothelial cells, resulting in the release of microorganisms to the culture medium. The in vivo release of viable *P. aeruginosa* into the bloodstream may therefore represent a central feature of the pathogenesis of bacteremia in compromised patients.

To our knowledge, there has been no report regarding invasion of endothelial cells in tissues from patients heavily colonized by *P. aeruginosa* or from bacteremic patients. Since in our study only a minority of adherent bacteria appeared to be internalized, the lack of histological evidence may well reflect the relatively few endothelial cells containing bacteria at any time. Further experiments using the neutropenic rabbit model (47), which mimics compromised patients, and careful research on intracellular bacteria in endothelial cells may validate the relevance of our study.

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