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Invasion of Epithelial Cells by *Actinobacillus actinomycetemcomitans*: a Dynamic, Multistep Process

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The invasion process of *Actinobacillus actinomycetemcomitans*, a periodontopathogen, was studied with microscopy and viable quantitative assays using both KB and Madin-Darby canine kidney (MDCK) epithelial cells. Microscopy revealed that the events associated with the *A. actinomycetemcomitans* invasion process occurred rapidly. Scanning electron micrographs revealed *A. actinomycetemcomitans* associated with craters on the KB cell surface and others entering the KB cells through apertures with lip-like rims within 30 min of infection. Both transmission electron and immunofluorescence micrographs demonstrated that by this time some bacteria had, in fact, already entered, replicated, and exited host cells. Scanning electron micrographs revealed that infected KB cells exhibited fibrillar protrusions which contained bulges with the conformation of bacteria. Some protrusions formed intercellular connections between KB cells. Immunofluorescence micrographs revealed protrusions which harbored *A. actinomycetemcomitans*. The spread of internalized *A. actinomycetemcomitans* from one MDCK epithelial cell monolayer to another was demonstrated using a sandwich assay developed in our laboratory. Transcytosis of *A. actinomycetemcomitans* through polarized MDCK cells was also demonstrated. This study indicates that soon after entry of *A. actinomycetemcomitans* bacteria into epithelial cells, they undergo rapid multiplication and may subsequently be found in protrusions which sometimes extend between neighboring epithelial cells. The protrusions are thought to mediate the cell-to-cell spread of *A. actinomycetemcomitans*. Cell-to-cell spread may also occur by the endocytosis of *A. actinomycetemcomitans* bacteria which have been released into the medium via rudimentary protrusions which do not interconnect epithelial cells. The finding that the *A. actinomycetemcomitans* invasion process is so dynamic sheds significant new light on the interaction of this periodontopathogen with mammalian cells.

Periodontitis is the inflammatory response in gingival and connective tissue elicited by bacteria which accumulate on adjoining teeth. *Actinobacillus actinomycetemcomitans* is strongly implicated as an etiologic agent of this destructive disease (25, 26, 33). Bacterial invasion of epithelial cells is associated with the initiation of infection by many bacteria. Clinical studies clearly demonstrate that *A. actinomycetemcomitans* has the ability to infiltrate the oral epithelium (2, 22). In an attempt to understand the *A. actinomycetemcomitans* tissue infiltration process, we have used model systems to study the interaction of *A. actinomycetemcomitans* with oral epithelial cells. Using a quantitative in vitro invasion assay we determined that *A. actinomycetemcomitans* can invade epithelial cells (16). Invasion of human primary gingival cells and the human KB oral epidermoid cell line is greater than that of other commonly used cell lines (5, 16). All *A. actinomycetemcomitans* strains do not invade to the same extent. In general, those strains which exhibit a smooth colonial morphology are more invasive than those with a rough morphotype (16). Invasion occurs by receptor-mediated endocytosis, as evidenced by inhibition of invasion by cytochalasin D (16). Other requirements for *A. actinomycetemcomitans* invasion include active metabolism and de novo protein synthesis by both the bacterium and mammalian cell and attachment of *A. actinomycetemcomitans* to the host cell (29). Microscopic analyses of KB cells infected with *A. actinomycetemcomitans* have revealed the following. Scanning electron microscopy (SEM) showed *A. actinomycetemcomitans* attached to KB cells via microvilli (29). Transmission electron microscopy (TEM) indicated that intracellular *A. actinomycetemcomitans* bacteria are initially present in endocytic vacuoles (16). Immunofluorescence microscopy showed internalized *A. actinomycetemcomitans* surrounded by foci of actin, which suggests an interaction of *A. actinomycetemcomitans* with the KB cell cytoskeleton (5). Taken together these studies reveal that the invasion process of *A. actinomycetemcomitans* shares commonalities with those of the much-studied enteric pathogens.

The survival strategies of the enteroinvasive pathogens vary (3, 8, 10, 11, 17, 27). Some, such as *Salmonella typhimurium* and yersiniae remain localized in the vacuole, where they multiply (3, 17). *Listeria monocytogenes* and *Shigella flexneri* escape from the vacuole into the cytoplasm, where they undergo rapid multiplication (7, 24, 30). The multiplication is coupled with the polymerization of cytoplasmic actin, which enables the bacterium to move both within and between host epithelial cells (1, 18, 23). In cell-to-cell spread, the bacterium forms a finger-like protrusion from the surface of the infected cell which is engulfed by an adjacent cell (14, 30). Thus, bacterial invasion can be a multistep process which, in addition to entry, may involve escape from the vacuole, multiplication, and both intracellular and intercellular spread.

It is our hypothesis that *A. actinomycetemcomitans* uses invasion of the epithelial cell as a mechanism to migrate to deeper tissues by cell-to-cell spread. The goal of the present study was to elucidate further the nature of *A. actinomycetemcomitans* internalization and to determine the fate of intracellular *A. actinomycetemcomitans*. Can *A. actinomycetemcomitans* bacteria survive in epithelial cells, and if so, can they proliferate and spread to adjacent epithelial cells?

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MATERIALS AND METHODS

Bacterial strains and growth conditions. SUNY 465, the *A. actinomycetemcomitans* invasion prototype (5, 16, 29), originated from the laboratory of J. J. Zambon, State University of New York, Buffalo. *A. actinomycetemcomitans* 652 originated from the laboratory of A. Tanner, Forsyth Dental School, Boston, Mass. *Haemophilus aprophilus* ATCC 19415, *Escherichia coli* DH5α, *Listeria monocytogenes*, and *Salmonella typhimurium* were from the culture collection retained in our laboratory. Cells were maintained frozen at −70°C in 10% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.). *A. actinomycetemcomitans*, *H. aprophilus*, *L. monocytogenes*, and *S. typhimurium* were grown in trypticase soy broth (TSB) (Difco Laboratories, Detroit, Mich.) supplemented with 0.6% yeast extract (YE) (Difco). *E. coli* DH5α was grown in L broth. Solid medium was prepared by adding agar (Difco) to liquid medium to a final concentration of 1.5% (wt/vol). Bacteria were cultivated at 37°C; *A. actinomycetemcomitans* and *H. aprophilus* were in a humidified atmosphere which contained 10% CO_2 in air.

Mammalian cell culture. KB cells were used in the majority of experiments in this study. However, in some experiments, as noted, KBR2A, RPMI-4788 (16), HeLa, and/or MDCK cells (provided by J. Moerhing of the University of Vermont) were used. All cell lines were maintained in RPMI 1640 medium (Sigma) supplemented with 5% fetal bovine serum (GIBCO, Grand Island, N.Y.) and 50 μg of gentamicin (Sigma) per ml. Cells were cultured in 75-cm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2 in air and split by treatment with 0.5 mM EDTA (GIBCO), followed by treatment with trypsin (GIBCO) to detach cells.

Standard quantitative invasion assay. Approximately 10^5 mammalian cells in antibiotic-free medium were seeded onto glass coverslips in wells of 24-well tissue culture plates and incubated for 16 to 18 h. Bacteria from overnight cultures were diluted in fresh broth and harvested during early exponential growth. Bacteria were pelleted by centrifugation and suspended in antibiotic-free medium, and the semiconfluent cell monolayers were inoculated with bacterial suspensions adjusted to obtain a multiplicity of infection of 1,000 bacteria to 1 epithelial cell. Bacteria were centrifuged onto the monolayer at 900 × *g* for 10 min at room temperature and incubated at 37°C for 2 h. Extracellular, unattached bacteria were removed by washing monolayers two times with phosphate-buffered saline which contained 1.0 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS). Monolayers were then incubated for 1 h in the presence of cell culture medium which contained gentamicin (100 μg/ml) to kill extracellular bacteria. The medium was removed, and the monolayers were washed twice with PBS. A 0.1-ml volume of a 0.5% Triton X-100 (Sigma) solution in PBS was added to lyse the mammalian cells and release internalized bacteria. After lysis, 2.0 ml of PBS was added to each well to dilute the detergent. Appropriate dilutions were spread onto TSB-YE plates, and CFU were enumerated. Quadruplicate measurements were carried out for each invasion determination.

Quantitative assay for persistence of internalized *A. actinomycetemcomitans*. Persistence of internalized *A. actinomycetemcomitans* in epithelial cells was investigated as follows. Standard invasion assays were carried out using KB and the other cell lines. After incubation with gentamicin to kill extracellular bacteria, epithelial cells from one set of wells were lysed with 0.5% Triton X-100 and plated on TSB-YE. In parallel, cells in other sets of wells were incubated further (up to 24 h), and then they were lysed and plated. All determinations were carried out in quadruplicate.

Assessment of epithelial cell integrity. Standard invasion assays were carried out using KB cells. After removal of the medium and washing, the monolayers were not lysed. Instead, the cells were detached from the glass coverslips by EDTA-trypsin treatment, suspended in 0.16% trypan blue dye solution, applied to a hemocytometer, and evaluated for cell number and viability by light microscopy. Trypan blue dye stains nonviable, but not viable, cells. Epithelial cell viability was also determined using the LIVE/DEAD EukoLight viability/cytotoxicity assay (Molecular Probes, Inc., Eugene, Oreg.), which distinguishes viable and dead cells, respectively, by green and red fluorescence. Coverslip mounting and microscopy are described below.

Cell-to-cell spread. Suny 465 cell-to-cell spread was studied as follows using a sandwich assay developed in our laboratory. MDCK cells were seeded in replicate sets onto glass coverslips at a density of ~10^5 cells per well in 24-well plates. Standard invasion assays were carried out in some wells. After gentamicin treatment, the monolayers were washed with PBS and suspended in fresh tissue culture medium. The medium was removed from wells which contained the untreated monolayers, and coverslips were removed from wells, inverted, and placed onto the medium over the infected monolayer. Uninfected monolayers immediately floated through the medium and became sandwiched with the infected monolayer. After incubation for 1 h, the top coverslips were removed and placed monolayer up in wells with fresh medium and the monolayers were washed. The total number of SUNY 465 bacteria associated with the bottom monolayer (infected) and the top monolayer (initially uninfected) were determined by lysing the cells, plating, and enumerating CFU. To determine the number of intracellular SUNY 465 bacteria in the top monolayer (initially uninfected), plates were centrifuged at 900 × *g* and incubated for 1 h, and monolayers were treated with gentamicin prior to cell lysis and CFU enumeration. Experiments were also carried out in which monolayers were examined by fluorescence microscopy (12, 32). In those studies, monolayers were not treated with Triton X-100 and plated. Instead, they were washed and fixed in 3.7% formaldehyde (in PBS). After being washed, they were incubated in SUNY 465 antiserum (29) diluted 1:2,500 for 15 min. Monolayers were washed and incubated in tetramethylrhodamine isothiocyanate (TRITC) (Sigma, #T-5268)-conjugated anti-rabbit immunoglobulin G (IgG) (1:100 dilution) for 15 min. After being washed, monolayers were permeabilized by incubation in 0.1% Triton X-100 for 15 min. Monolayers were washed and incubated again in SUNY 465 immune serum and then in fluorescein isothiocyanate (FITC) (Sigma, #F-0382)-labeled anti-rabbit IgG (1:100 dilution) and in rhodamine-phalloidin (Molecular Probes), which stains actin. Cells were washed, and coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, Calif.) and sealed with nail polish. Indirect immunofluorescence was examined under oil immersion using an Olympus BMAX 50 microscope and photographed with an Olympus PM-30 automatic photomicrographic system using Kodak Ektachrome professional slide film with an ASA of 400.

Transcytosis of SUNY 465 through polarized MDCK monolayers. Transwell filter units with 3-μm-diameter pores (Costar, Cambridge, Mass.) were seeded with 3.5 × 10^5 MDCK cells and grown for 4 days in RPMI 1640 medium supplemented with 5% fetal bovine serum. Transmonolayer electrical resistance measurements were performed with a Millicell-ERS apparatus (Millipore Corporation, Bedford, Mass.). The area resistance (Ωcm^2) was calculated by multiplying the measured resistance by the area of the filter (0.33 cm^2). Monolayers were infected by adding ~10^7 *A. actinomycetemcomitans* SUNY 465 or *H. aprophilus* ATCC 19415 bacteria to the apical surface. *E. coli* DH5α does not transcytose through polarized MDCK cells (4); thus, ~10^7 DH5α bacteria were added together with the SUNY 465 or ATCC 19415 as a control. At various intervals after infection, basolateral medium was plated to determine the number of bacteria which had transcytosed the monolayer. *A. actinomycetemcomitans* and *H. aprophilus* are easily distinguished from DH5α because of their distinctly different colonial morphology and growth characteristics.

Immunofluorescence microscopy. Immunofluorescence microscopy was used to identify intracellular and extracellular SUNY 465 (12). Invasion assays were carried out using KB cells at a multiplicity of infection of 1,000:1. Thirty minutes after infection, the medium was removed from wells and monolayers were washed with PBS and fixed in 3.7% formaldehyde for 20 min at room temperature. Subsequent treatments were carried out at room temperature in the dark. Extracellular *A. actinomycetemcomitans* bacteria were localized by treatment with SUNY 465 antiserum for 20 min and then labeled with TRITC-conjugated goat anti-rabbit IgG for 20 min. After being washed, monolayers were permeabilized by treatment with 0.1% Triton X-100 for 15 min. Monolayers were washed and incubated in SUNY 465 antiserum to localize internalized *A. actinomycetemcomitans*. Internalized *A. actinomycetemcomitans* and actin microfilaments were labeled simultaneously by treatment with FITC-conjugated goat anti-rabbit IgG and rhodamine-phalloidin (32). Coverslips were washed, mounted, and examined by immunofluorescence microscopy.

Identification of transient SUNY 465. A modification of the double immunofluorescence assay (12) was used to identify SUNY 465 which transited through KB cells. By using the standard invasion protocol, KB cells were infected with SUNY 465, and the 24-well plates were centrifuged. After incubation for 10 min, SUNY 465 antiserum diluted 1:2,500 was added to the infected monolayers. SUNY 465 invasion is decreased 99% by this dilution of antiserum (29). After incubation for 15 min in the presence of antiserum, infected monolayers were washed and incubated in the presence of TRITC-conjugated anti-rabbit IgG for 15 min. After removal of TRITC and washes, fresh, antibiotic-free RPMI 1640 medium was added and the plates were incubated for 30 min. The medium was removed, cells were washed, and monolayers were fixed in 3.7% formaldehyde in PBS. After being washed, monolayers were incubated in the presence of SUNY 465 antiserum (1:2,500 dilution) for 15 min. Monolayers were washed and incubated in the presence of FITC-labeled anti-rabbit IgG (1:100 dilution) for 15 min. After being washed, coverslips were mounted and examined by immunofluorescence microscopy.

Electron microscopy. Examination of *A. actinomycetemcomitans*-infected KB cell monolayers under the electron microscope was achieved as follows. Standard invasion assays were carried out in which infection was for 30, 45, or 75 min. Medium was removed, monolayers were washed twice with PBS, and cells were fixed in the culture dishes by adding 2.5% glutaraldehyde (Electron Microscopy Sciences [EMS], Fort Washington, Pa.) in phosphate buffer dropwise to monolayers. After 10 min, the solution was removed and fresh glutaraldehyde was added. After 30 min at room temperature the fixative was removed and monolayers were washed three times with PBS. Cells were postfixed with osmium tetroxide (Bio-Rad, Richmond, Calif.) for 20 min at room temperature. The cells were washed in PBS and dehydrated through a graded series of ethanol to 100% ethanol (Quantum Chemical Co., Cincinnati, Ohio). One duplicate set of cell-fixed coverslips was critical-point dried in a CO_2 system and coated with 20-nm-diameter Au-Pd (Bio-Rad) for SEM analysis. For TEM, other sets of monolayers were infiltrated with epoxy resin (Embed 812/Araldite 502; EMS), embedded by inverting the coverslips over resin-filled flat embedding molds (Pelco 105; EMS), and polymerized overnight at 70°C. After polymerization, coverslips were removed from the resin blocks by brief immersion in liquid nitrogen. The resin blocks, which contained the cell monolayers, were sectioned transversely with respect to their culture subsubstrata. Sections were stained with lead citrate and uranyl acetate (EMS). Microscopy was carried out on a JEOL 100CX TEM-SCAN.

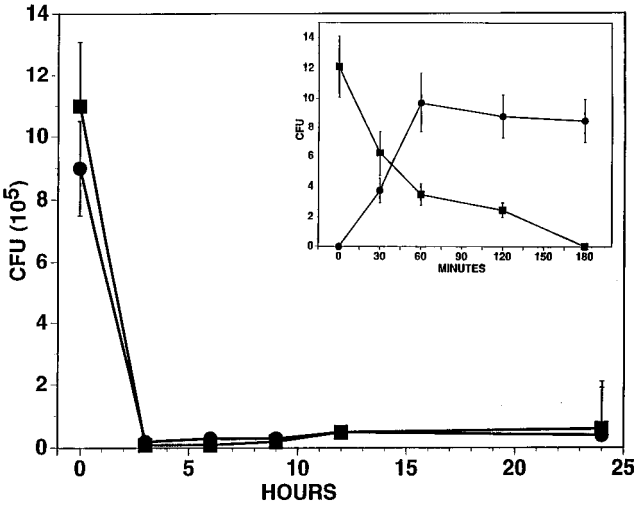


FIG. 1. Kinetics of SUNY 465 persistence within KB (■) and KBR2A (●) cells. Time zero represents the CFU recovered after a 2-h infection period followed by a 1-h gentamicin treatment. Points represent the means and standard deviations of four experiments carried out in quadruplicate. (Insert) Time course of SUNY 465 loss from KB cells (■) and gain in the assay culture medium (●). Time zero represents CFU recovered after a 2-h infection period followed by a 1-h gentamicin treatment. Points represent the means and standard deviations of a typical experiment carried out in quadruplicate. ■, 10^5 CFU; ●, 10^3 CFU.

RESULTS

Internalized *A. actinomycetemcomitans* bacteria do not persist in epithelial cells. The ability of *A. actinomycetemcomitans* to persist intracellularly after invasion was measured using a modification of the quantitative invasion assay. The number of viable intracellular SUNY 465 recovered from KB monolayers decreased rapidly and dramatically with time. As early as 5 h after initiation of infection, only 1% of theretofore intracellular *A. actinomycetemcomitans* remained intracellular (Fig. 1). In general there was a continuous, sharp decrease in the number of CFU recovered after internalization (2-h infection followed by a 1-h gentamicin treatment) (Fig. 1), but occasionally there was an initial (at 1 h), brief, moderate increase in the number of CFU recovered prior to the sharp decline (data not shown). Although small numbers of viable bacteria were recovered as long as 24 h postinfection, no increase in the number of intracellular organisms ever occurred between 5 and 24 h.

Fate of internalized *A. actinomycetemcomitans*. *A. actinomycetemcomitans* SUNY 465 bacteria were internalized, but they did not persist intracellularly. What happened to them? Were internalized SUNY 465 being killed, or were the SUNY 465 damaging the KB cells? To answer these questions the following experiments were carried out.

(i) Assessment of possible killing of internalized *A. actinomycetemcomitans* by gentamicin. One possibility was that the gentamicin was getting into the KB cells and killing the internalized SUNY 465. To test this we used different gentamicin treatment regimens (25 or 50 μg/ml for 2 h or 50 or 500 μg/ml for 1 h), as well additional cell lines known to be impervious to gentamicin. The different gentamicin treatment regimens did not change the kinetics of persistence of SUNY 465 in KB cells (data not shown). Furthermore, association of SUNY 465 with HeLa, RPMI, and MDCK cells was the same as with KB cells; i.e., bacteria were internalized, but they did not persist intracellularly in significant numbers (data not shown). Both *L. monocytogenes* and *S. typhimurium* invaded and persisted intracellularly in KB cells during the period of time under consideration (data not shown). Thus, the inability of *A. actinomycetemcomitans* to persist intracellularly was not a result of killing of internalized bacteria by gentamicin or a phenomenon peculiar to the KB cell line.

(ii) Endosomal acidification. Previously, we reported that endosomal acidification did not affect invasion of *A. actinomycetemcomitans* SUNY 465 (29). However, it is known that *A. actinomycetemcomitans* is killed in vitro between pH 4.5 and 5.0 (28). To determine if endosomal acidification could account for the inability of *A. actinomycetemcomitans* to persist after entry into epithelial cells, studies were carried out with (i) KB-R2A cells (KB cells which are defective in endosomal acidification) and (ii) KB cells in which endosomal acidification was inhibited by pretreatment of the cells with ammonium chloride, a lysosomotropic agent (29). The time course of SUNY 465 persistence in KB-R2A cells was the same as that in KB cells, i.e., at 5 h postinfection only 1% of those organisms which were initially internalized remained intracellular (Fig. 1). Furthermore, internalized SUNY 465 did not persist at invasion levels in KB cells which had been pretreated with ammonium chloride to inhibit endosomal acidification (data not shown).

(iii) Assessment of integrity of the KB monolayer. Was SUNY 465, a leukotoxic strain, causing damage to the KB epithelial cells? To test this we used strain 652, a nonleukotoxic *A. actinomycetemcomitans* strain. The persistence time course exhibited by strain 652 was identical to that of SUNY 465. KB monolayers were also evaluated to determine if the presence of intracellular *A. actinomycetemcomitans* caused damage to and/or loss of KB cells. Persistence assays were set up and carried out as described above. KB monolayers which were manipulated in the same way, but which were not infected with *A. actinomycetemcomitans*, served as controls. Three and 5 h after infection was initiated (zero time and 2 h, respectively) the medium was removed and the cell monolayers were washed. Cells in some wells were detached by treatment with EDTA-trypsin, suspended in trypan blue dye, and analyzed in a hemocytometer under a light microscope. Cells in other wells were fixed in formaldehyde, treated with reagent stains as described for the LIVE/DEAD EukoLight Viability/Cytotoxicity assay, and examined under a fluorescence microscope. Trypan blue dye exclusion studies revealed no differences between infected and uninfected KB cells. Under all experimental conditions, approximately 0.5×10^5 to 0.6×10^5 KB cells, which exhibited 95% viability, were recovered. The LIVE/DEAD fluorescence assay also revealed no differences between the viability of infected and uninfected KB cell monolayers, which confirmed the trypan blue dye exclusion. These studies indicate that the reduction in the number of intracellular SUNY 465 after entry did not result from impaired KB cells which had sloughed off into the culture medium.

Recovery of viable SUNY 465 from the culture medium. Since we determined that SUNY 465 bacteria were not being killed and that the KB monolayer remained viable, the only possible explanation was that SUNY 465 bacteria were getting out of the epithelial cells. Thus, experiments were carried out to determine if internalized SUNY 465 bacteria were released from KB cells into the culture medium. Standard quantitative invasion assays were carried out. After the gentamicin treatment step, the medium was removed, the monolayer was washed with PBS, fresh antibiotic-free medium was added, and the plates were incubated at 37°C. The medium was removed at different intervals, aliquots were plated, and CFU were enumerated. The medium was also applied to a hemocytometer to determine if KB cells were being sloughed off into the

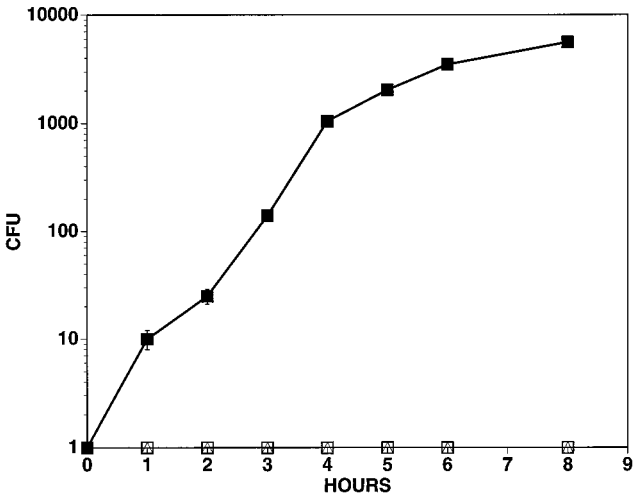


FIG. 2. SUNY 465 penetration through polarized MDCK monolayers. Equal numbers ($\sim 10^7$) of SUNY 465 (■) and *E. coli* DH5α (□) or *H. aprophilus* (△) and *E. coli* DH5α were added together to the apical surface, and the number of bacteria (CFU) recovered in the basolateral medium after passing through the monolayer was enumerated. Note: since no *E. coli* DH5α or *H. aprophilus* bacteria were recovered, the symbols and lines representing these bacteria run along the *x* axis.

medium. The KB monolayers from those wells were lysed with 0.5% Triton X-100, released bacteria were plated, and CFU were enumerated. A significant fraction of the internalized SUNY 465 was recovered from the extracellular medium (Fig. 1, insert). The majority (85%) were recovered during the first 2 h (Fig. 1, insert), but some were recovered even after 19 h (data not shown). No KB cells were detected in the medium for at least 6 h; thus, the sloughing off of KB could not account for the decrease of SUNY 465 in the cell monolayer or its increase in the medium.

***A. actinomycetemcomitans* penetration through polarized MDCK monolayers.** Polarized MDCK cells have been used as a model to study the ability of bacteria to transit through epithelial cells (4). Having established that internalized *A. actinomycetemcomitans* bacteria were getting out of KB cells into the medium, we asked if they could penetrate through epithelial cells. We determined that as early as 1 h after inoculation *A. actinomycetemcomitans* had penetrated through polarized cells into the medium. The number which were recovered in the basolateral medium during the 8 h period after infection is depicted in Fig. 2. The number of bacteria detected includes those which passed through the monolayer as well as those which divided after reaching the opposite side. The generation time of *A. actinomycetemcomitans* in RPMI 1640 medium is 150 min (28); thus, the numbers shown in Fig. 2 could represent more than twice the number of *A. actinomycetemcomitans* which actually passed through the monolayer. Transcytosis experiments utilized MDCK cells (KB cells do not polarize well) grown on Transwell filter units with 3-μm-diameter pores. Electrical resistance measurements were carried out on successive days after seeding to establish the presence of tight junctions in the monolayers. Fully confluent monolayers with tight junctions exhibited an electrical resistance of $\sim 110 \pm 10$ Ωcm^2. As long as the monolayer exhibited this level of resistance we were assured that the monolayer contained tight junctions. Bacteria were added to the medium at the apical surface, and medium was withdrawn over time from the basolateral surface and examined for the presence of bacteria. *E. coli* DH5α, the standard control organism, was not able to pass through the MDCK monolayers as long as the tight junctions were intact (Fig. 2). In general, 10 to 12 h after inoculation with *E. coli* DH5α, organisms were recovered in the medium and a concurrent drop in electrical resistance occurred. However, the use of *E. coli* as a control concerned us because it is significantly larger than *A. actinomycetemcomitans*. Consequently, we tested the transcytosis capability of *H. aprophilus*, a noninvasive oral bacterium similar in size and properties to *A. actinomycetemcomitans*. As long as tight junctions remained intact, no *H. aprophilus* bacteria were recovered in the basolateral medium (Fig. 2). Since we were now confident that DH5α permitted the detection of minute numbers of exposed pores in the monolayer and that it would serve as a proper control, it was added together with SUNY 465 as a sensitive, internal criterion of monolayer integrity.

Spread of *A. actinomycetemcomitans* from one monolayer to another. We also determined that *A. actinomycetemcomitans* SUNY 465 could spread intercellularly between adjoining monolayers. These studies were carried out using confluent MDCK monolayers grown on glass coverslips which were adjoined sandwich style. One of a pair of monolayers was infected with SUNY 465. The uninfected monolayer of the pair was removed from the well, inverted, and placed on top of the infected monolayer. After 1 h, monolayer pairs were analyzed for both total associated and intracellular SUNY 465. In a typical experiment, the total number of SUNY 465 associated with monolayer pairs was 10^4 cells. Of the total, 1.5×10^3 (15% of the total) were associated with the initially uninfected (top) monolayer. The majority of these organisms were adherent, but about 2% were intracellular. Fluorescence microscopy confirmed the quantitative results and revealed that the assay manipulations had not resulted in damage to the MDCK monolayer (data not shown). Compared with most cell lines, MDCK monolayers bind extremely tightly to plastic and glass. Attempts to use KB cells in sandwich assays were not successful because the integrity of the monolayer was disrupted by the manipulations. For these reasons we chose to use MDCK cells even though invasion of MDCK cells by *A. actinomycetemcomitans* is substantially lower than invasion of KB cells.

Electron microscopy. To complement quantitative studies, SUNY 465 invasion and intracellular persistence were also monitored by electron microscopy.

(i) SEM. SEM revealed a number of significant features of *A. actinomycetemcomitans* infection of KB cells. As early as 30 min after infection bacteria were associated with KB microvilli (Fig. 3A). Bacteria were also observed in indentations or craters on the KB cell surface (Fig. 3B). Other bacteria could be seen in the process of passing through apertures which were rimmed with lip-like folds (Fig. 3C). At later times, similar apertures which were not associated with bacteria were observed (Fig. 3D). We assume that these apertures represented ones through which bacteria had already passed, since we never saw apertures on uninfected KB cells. Besides craters and apertures, KB cells which had been infected with *A. actinomycetemcomitans* also exhibited fibrillar protrusions which were distinctly different from the microvilli (Fig. 4). The protrusions had larger diameters than the microvilli, and they varied in length (>20 μm). Some of the protrusions extended from one KB cell to another (Fig. 4C and D). Others, termed rudimentary protrusions, were associated with only one cell (Fig. 4A and 4B). Many protrusions contained bulges which conformed to the structure of bacteria (Fig. 4B to D). The distal ends of some rudimentary protrusions were associated with bacteria (Fig. 4A).

(ii) TEM. To characterize the invasion process in more detail, infected cells were examined by TEM. As can be seen in

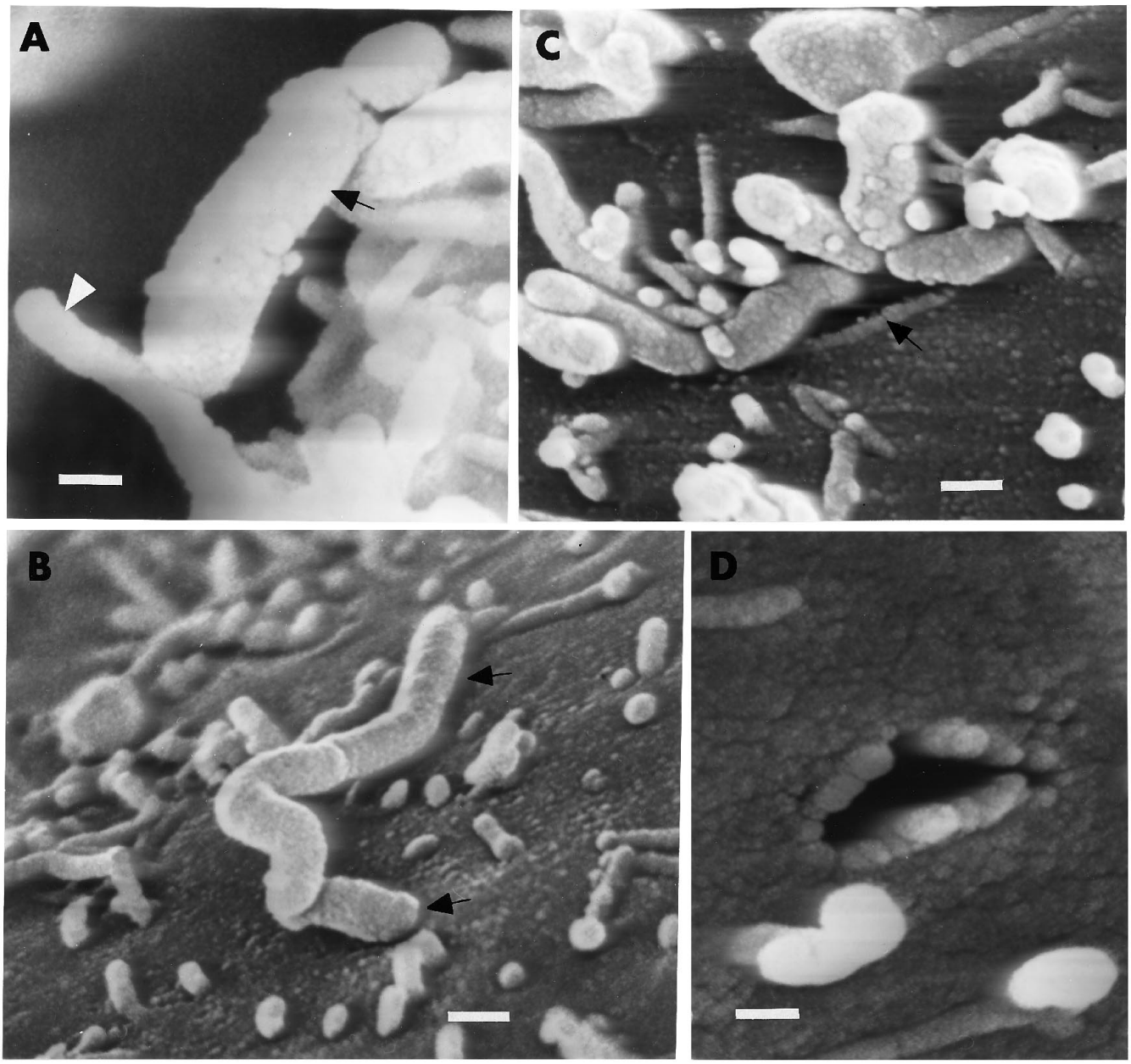


FIG. 3. Scanning electron micrographs of SUNY 465-infected KB cells. (A) SUNY 465 (black arrow) interacting with KB microvillus (white arrowhead) 30 min postinfection. (B) SUNY 465 in indentations (arrows) on the KB cell surface 30 min postinfection. (C) SUNY 465 passing through lip-rimmed aperture (arrow) 30 min postinfection. (D) Aperture which is not associated with SUNY 465, 75 min postinfection. Samples were prepared as described in Materials and Methods. Bars: 0.2 μm (A and D) and 0.5 μm (B and C).

Fig. 5, *A. actinomycetemcomitans* bacteria which appear to have more than one morphotype were present. Thirty minutes after infection, most extracellular bacteria appeared somewhat diffuse (i.e., less dense) with poorly defined membranes and they were rarely dividing or in pairs (Fig. 5A). By contrast, the intracellular bacteria were much more dense and had membranes which were somewhat better defined than those of the extracellular bacteria (Fig. 5A). Thirty minutes after infection some intracellular *A. actinomycetemcomitans* bacteria appeared extremely dense with well-defined membranes (Fig. 5B). Most of these were present in pairs which had recently divided, and they were in the process of dividing once again (Fig. 5B). The intracellular bacteria which exhibited intermediate density (Fig. 5A) appeared to be in transition between the diffuse and extremely dense types. Paired and/or dividing, extremely dense appearing bacteria were also observed extracellularly, usually in association with KB cell protrusions (Fig. 5C). Some protrusions, such as that in Fig. 5C, appeared to be intercellular fibrillar protrusions which were not totally intact, perhaps a consequence of the electron microscopy technique.

Transit of SUNY 465 through KB cells. Immunofluorescence labelling of infected monolayers was carried out to analyze further the nature of the transit of SUNY through KB cells. In these studies invasion assays were carried out in the presence of SUNY 465 antiserum and TRITC to differentially label extracellular and transient organisms over time. SUNY

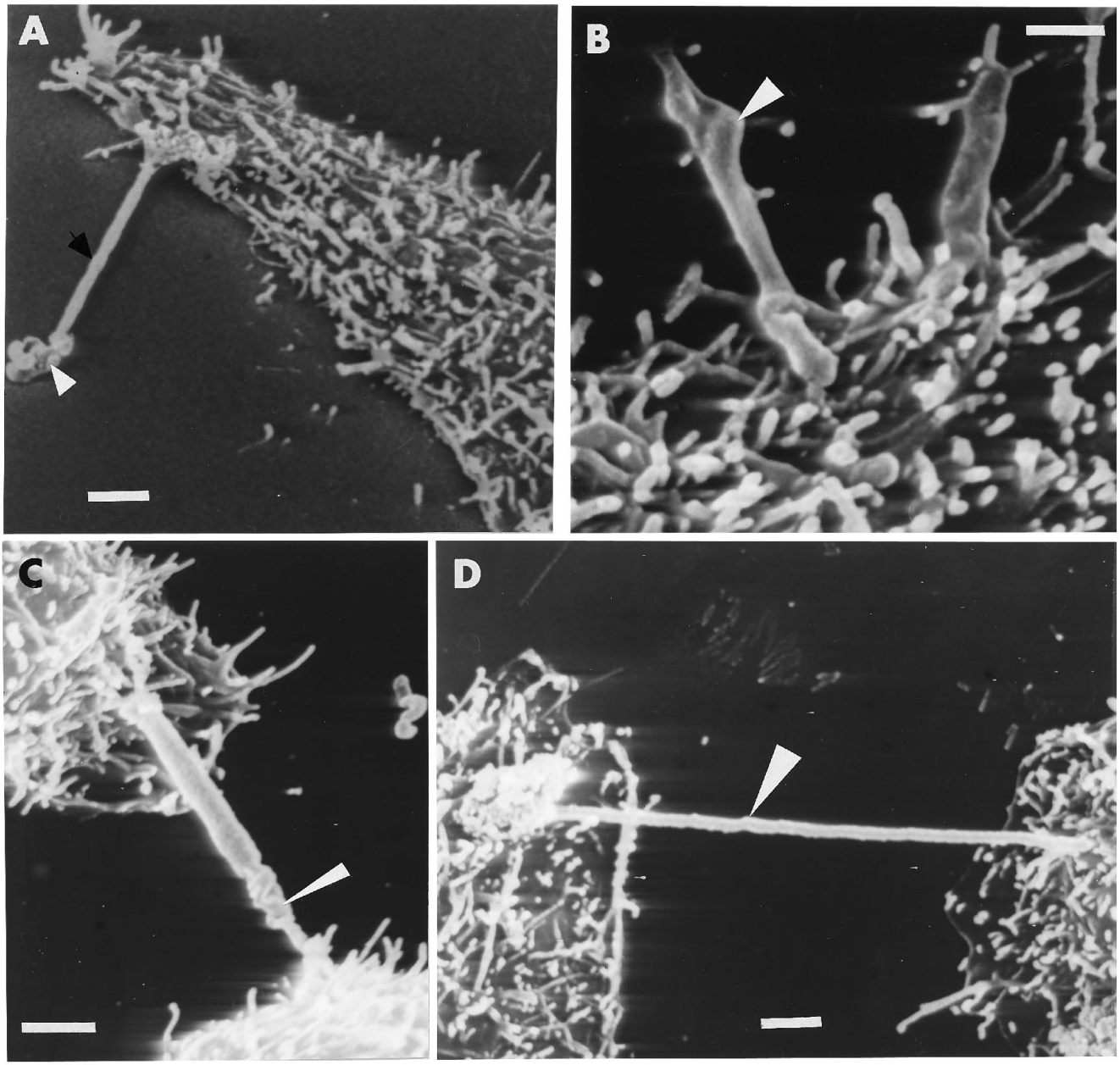
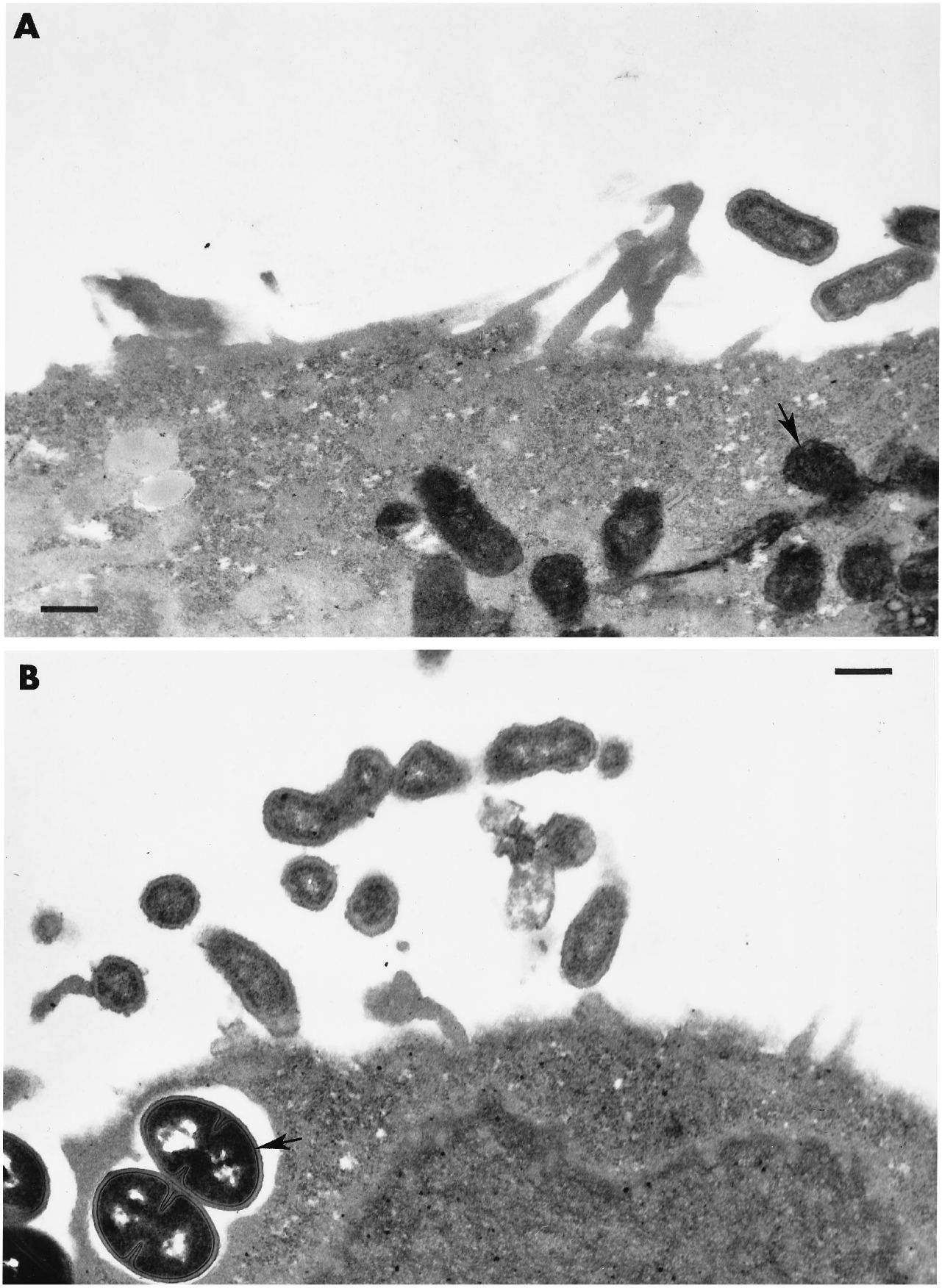


FIG. 4. Scanning electron micrographs showing fibrillar protrusions on KB cells after infection with SUNY 465. Protrusions with bulges with the conformation of bacteria (black arrow) and bacteria associated with its distal end (white arrowhead) 30 min postinfection. (B) Protrusions with the conformation of bacteria (white arrowhead) 75 min postinfection. (C and D) Protrusions interconnecting neighboring KB cells 75 min postinfection. Bulges with the conformation of bacteria (white arrowheads) are also present in these protrusions.

465 was allowed to invade KB cells for 10 min, and then SUNY 465 antiserum, which inhibits invasion, was added to the infected monolayer and the incubation was continued for 15 min. TRITC (red fluorescence) was then added, and the incubation was continued for an additional 15 min. Monolayers were washed, the invasion plates were incubated for 30 min, and the fixed monolayers were treated with FITC (green fluorescence). Bacteria which were extracellular at the time of TRITC staining would remain extracellular because of the presence of SUNY 465 antiserum. Extracellular organisms were also labelled with FITC after fixation; thus, they would exhibit both red and green fluorescence. By contrast, SUNY 465 organisms which were intracellular at the time the monolayer was treated with TRITC and thereafter became extracellular would be exposed only to the postfixation FITC label and thus they would exhibit only green fluorescence. KB cells were not permeabilized; thus, only extracellular bacteria were labelled. Fluorescence staining of the same SUNY 465-infected monolayer field examined under conditions which identify bacteria stained green and red is shown in Fig. 6. A number of bacteria in the field exhibited only green fluorescence (shown by arrows in Fig. 6A), which indicated that they had transited through the KB cells. This study confirms the quantitative studies. Furthermore, it provides evidence that *A. actinomycetemcomitans*


A
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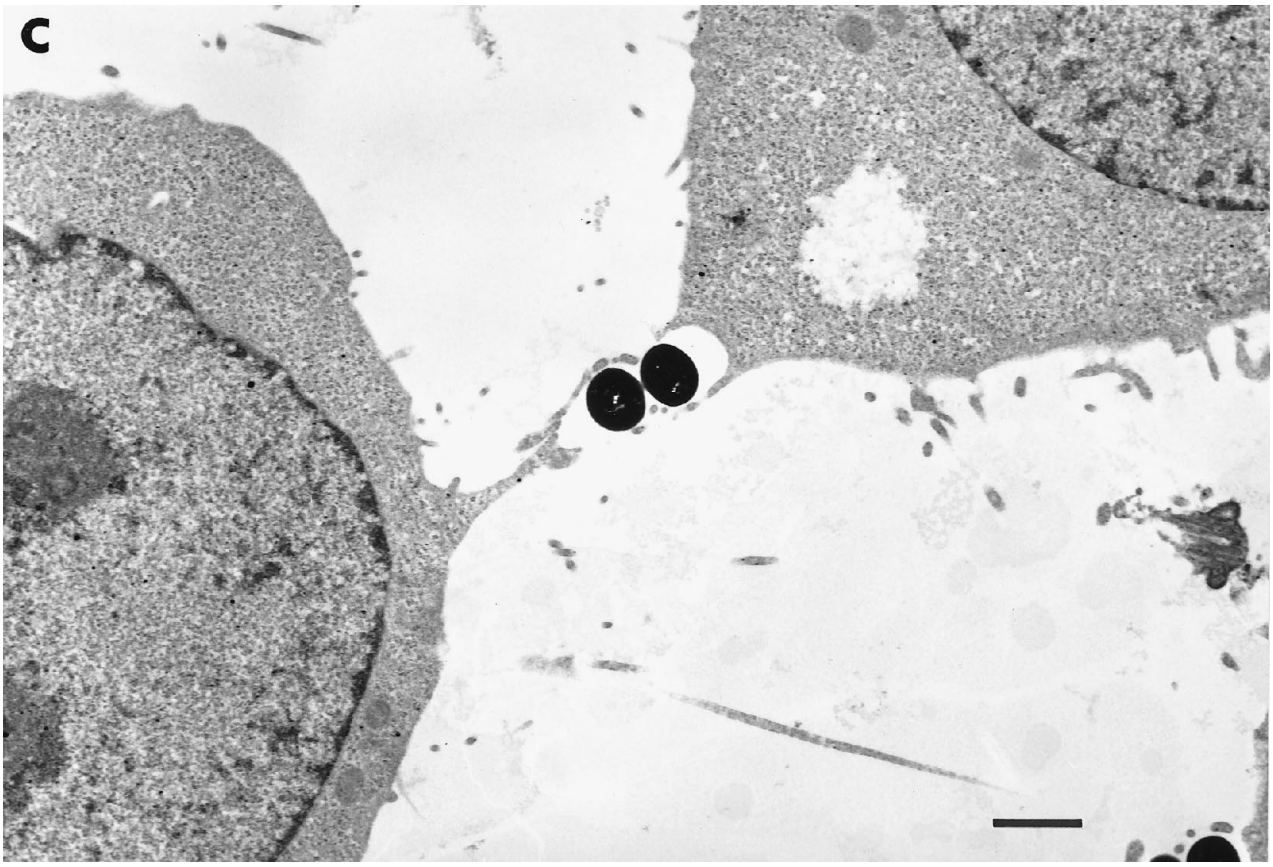

FIG. 5. Transmission electron micrographs of KB cells infected with SUNY 465. (A) KB cell associated with both extracellular and intracellular SUNY 465 30 min postinfection. Note that the internalized bacteria appear more dense (one of many is indicated by an arrow) than those which are extracellular. (B) Thirty minutes after infection rapidly dividing bacteria, which appear extremely dense and have well-defined membranes (arrow), are present both intra- and extracellularly. Bacteria which appear less dense only occur extracellularly. The intracellular bacteria in panel A appear to be in transition. (C) Paired, recently divided, extremely dense bacteria in association with appurtenances between two KB cells 30 min postinfection. Samples were prepared as described in Materials and Methods. Bars: 0.4 μm (A), 0.3 μm (B), and 1.0 μm (C).

SUNY 465 bacteria enter KB cells soon after infection and that as early as 45 min after infection, some of those bacteria which had entered the KB cells had already exited.

Intercellular protrusions harbor SUNY 465. Immunofluorescence labeling of *A. actinomycetemcomitans*-infected monolayers confirmed that some protrusions harbored bacteria. A single SUNY 465 bacterium can be seen on the surface of a protrusion connecting two KB cells (Fig. 7A). A lower focus of the same field reveals two SUNY 465 cells within the protrusion (Fig. 7B).

DISCUSSION

This work represents an important advance in understanding *A. actinomycetemcomitans* invasion. It provides evidence that *A. actinomycetemcomitans* invasion is part of a dynamic, multistep process which includes cell-to-cell spread. Scanning electron micrographs showed that the process of SUNY 465 entry is operative within 30 min of infection. The micrographs revealed SUNY 465 in craters on the KB cell surface. These craters appear similar to those with which *Salmonella choleraesuis* is associated during infection of MDCK cells (4). SUNY 465 bacteria were also observed entering the KB cells through lip-rimmed apertures which resembled the membrane bulges which surround *S. typhimurium* during its internalization (9). Effacement of microvilli, a process associated with enteropathogenic infection (4), occurred in areas in which KB cells had either bacterium-associated craters or lip-rimmed apertures. Apertures which were not associated with *A. actinomycetemcomitans* were also observed. We believe that these apertures, which did not occur in uninfected KB cells, may represent openings through which bacteria already had entered.

Bacteria present in scanning electron micrographs all exhibited the same morphotype. Colonies recovered from quantitative assays carried out at the same time as those assays used for electron microscopy also exhibited a single morphotype. However, when viewed by TEM they appeared to exhibit two distinct morphotypes. Bacteria which were intracellular were almost all of the morphotype which was very dense and had distinct membranes. This type occurred in vacuoles, and the bacteria were usually in the process of dividing or had just divided; thus, they occurred in pairs. Occasionally, remnants of membranes were observed in association with vacuoles. Whereas some extracellular bacteria exhibited the less-dense morphotype, others had the dense, dividing morphotype. These bacteria were usually associated with KB cellular material, which suggested that they had been internalized and released.

To date only a few bacterial species which can gain access to and multiply in the cytoplasm have been identified. Both *L. monocytogenes* and *S. flexneri* escape from the vacuole and

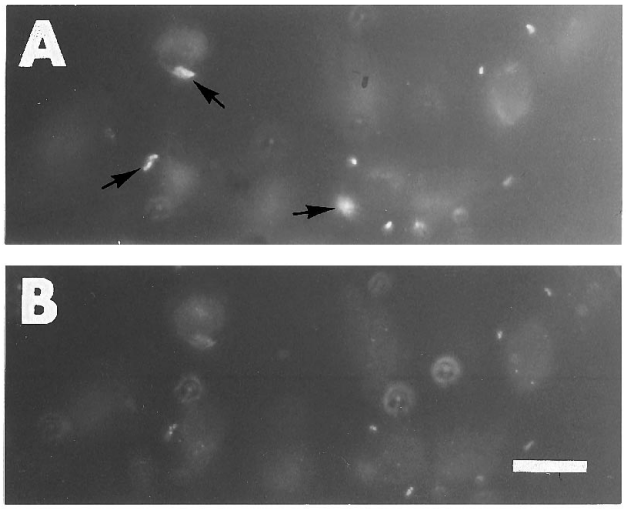

FIG. 6. Identification of transient SUNY 465 by double immunofluorescent labeling. (A) SUNY 465-infected KB cells were incubated in the presence of SUNY 465 immune serum and then in TRITC-conjugated IgG. After a washing, the incubation was continued for 30 min and the coverslips were incubated in formaldehyde and then in SUNY 465 immune serum and FITC-conjugated IgG. SUNY 465 bacteria which were intracellular during staining with TRITC are stained only with FITC, whereas those which were extracellular are stained with both FITC and TRITC. (A) Extracellular SUNY 465 stained with FITC; (B) the corresponding field showing bacteria stained with TRITC. A number of SUNY 465 bacteria stained only with FITC are identified in panel A by arrows. Experimental details are described in Materials and Methods. Bar, 16 μm.

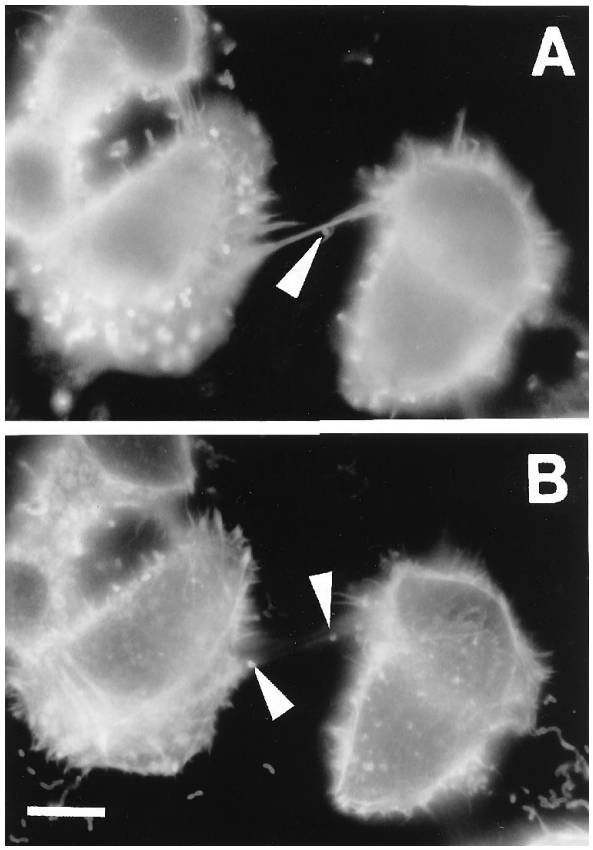

FIG. 7. Identification by immunofluorescence of *A. actinomycetemcomitans* SUNY 465 within an intercellular protrusion. (A) A single SUNY 465 bacterium (arrowhead) on the surface of a protrusion connecting two KB cells. (B) A lower focus of the same field which reveals two SUNY 465 bacteria (arrowheads) within the intercellular protrusion. Experimental details are described in Materials and Methods. Bar, 6 μm.

undergo rapid multiplication soon after internalization (14, 30). The multiplication is associated with their intracellular spread, which involves the polymerization of actin (21). Our studies indicate that *A. actinomycetemcomitans*, like *S. flexneri* and *L. monocytogenes* (20, 24), also escape from the vacuole and undergo rapid multiplication soon after entry. The limiting factor in this process for many intracellular bacteria is the inability to escape from the vacuole and not the inability of epithelial cell cytoplasm to promote bacterial growth (3). Our data suggest that the cytoplasmic environment is highly conducive to *A. actinomycetemcomitans* growth. The in vitro doubling time of SUNY 465 in TSB-YE or RPMI 1640 cell culture medium is 150 min (28). Yet this study indicates that as early as 30 min after infection SUNY 465 bacteria have entered KB cells, undergone several divisions, and passed from the cell. *L. monocytogenes* uses the hemolytic toxin listeriolysin to lyse and escape the vacuole (20). By contrast, *S. flexneri* escapes the vacuole by contact hemolysis, an activity which is under the control of the same gene product that triggers entry (13). Our results suggest that *A. actinomycetemcomitans* is extremely proficient at lysing the vacuole. However, *A. actinomycetemcomitans* is not hemolytic, nor does it exhibit contact hemolytic activity (unpublished observation); thus, the mechanism it uses to escape from the vacuole is different from that of either *L. monocytogenes* or *S. flexneri.*

In order to attain *A. actinomycetemcomitans* invasion efficiencies comparable to those of enteroinvasive organisms, high infection inoculum must be employed. Prior to these studies we had no explanation for this difference. We now know that internalized *A. actinomycetemcomitans* bacteria exit KB cells and are found in the external environment soon after entry. We feel that this phenomenon explains the need for such a high infecting inoculum for *A. actinomycetemcomitans*. Clearly, bacteria released into the external environment would be killed during the gentamicin treatment step. Both *Staphylococcus aureus* and *Pseudomonas aeruginosa* escape from cultured endothial cells (19, 31). The mechanism of escape is not known, but escape by *S. aureus*, like that by *A. actinomycetemcomitans*, occurs soon after internalization (19, 31).

The bacterium-ladened protrusions which we observed in association with SUNY 465-infected KB cells appear somewhat similar to the protrusions used by *S. flexneri* to spread from one host cell to another (14). We propose that the cell-to-cell spread of *A. actinomycetemcomitans* is mediated by the intercellular epithelial cell protrusions. However, the mechanism of cell-to-cell spread via the protrusions does not appear to be the same as that used by *L. monocytogenes* and *S. flexneri*. For both *L. monocytogenes* and *S. flexneri* cell-to-cell spread is mediated by the engulfment of protrusions by contiguous epithelial cells (14, 30). *A. actinomycetemcomitans* cell-to-cell spread seems to involve movement and transfer through protrusions, rather than engulfment of protrusions. *A. actinomycetemcomitans* bacteria, like *S. flexneri* (14), were found at the distal tips of protrusions, where they appeared to have been released from the protrusions. Thus, some *A. actinomycetemcomitans* intercellular spread could also involve the endocytosis of SUNY 465 bacteria which have been internalized and released from rudimentary protrusions which do not interconnect epithelial cells.

In this study we have also demonstrated that SUNY 465 can transcytose through polarized MDCK cells, confirming that *A.*

actinomycetemcomitans can spread intracellularly. The mechanism of intracellular spread is not known, but it appears that, like *L. monocytogenes* and *S. flexneri* (21, 30), it may be linked to bacterial division. Preliminary studies suggest that microtubules may play a role in the exit of *A. actinomycetemcomitans* from the epithelial cell (6, 15). To our knowledge, microtubules have not been shown to be involved in the cell-to-cell spread of either *L. monocytogenes* or *S. flexneri.*

In summary, this work reveals that *A. actinomycetemcomitans* invasion of epithelial cells is a multistep process which involves entry, escape from the vacuole, rapid multiplication, and intracellular and intercellular spread. Whereas the invasion processes of both *L. monocytogenes* and *S. flexneri* encompass similar events, the mechanisms used by *A. actinomycetemcomitans* appear different. We postulate that the events described are involved in the ability of *A. actinomycetemcomitans* to spread to the gingival and connective tissue and cause destruction. The finding that *A. actinomycetemcomitans* invasion is dynamic is of considerable significance. It points out that the infection strategies of bacteria which cause chronic diseases, such as periodontitis, are similar in nature to those of bacteria which cause acute diseases, such as dysentery.

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