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Intercellular Spreading of *Porphyromonas gingivalis* Infection in Primary Gingival Epithelial Cells

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***Porphyromonas gingivalis*, an important periodontal pathogen, is an effective colonizer of oral tissues. The organism successfully invades, multiplies in, and survives for extended periods in primary gingival epithelial cells (GECs). It is unknown whether *P. gingivalis* resides in the cytoplasm of infected cells throughout the infection or can spread to adjacent cells over time. We developed a technique based on flow cytofluorometry and fluorescence microscopy to study propagation of the organism at different stages of infection of GECs. Results showed that *P. gingivalis* spreads cell to cell and that the amount of spreading increases gradually over time. There was a very low level of propagation of bacteria to uninfected cells early in the infection (3 h postinfection), but there were 20-fold and 45-fold increases in the propagation rate after 24 h and 48 h, respectively, of infection. Immunofluorescence microscopy of infected cells suggested that intercellular translocation of *P. gingivalis* may be mediated through actin-based membrane protrusions, bypassing the need for release of bacteria into extracellular medium. Consistent with these observations, cytochalasin D treatment of infected cells resulted in significant inhibition of bacterial spreading. This study shows for the first time that *P. gingivalis* disseminates from cell to cell without passing through the extracellular space. This mechanism of spreading may allow *P. gingivalis* to colonize oral tissues without exposure to the humoral immune response.**

The gram-negative bacterium *Porphyromonas gingivalis* is an important component of oral microbiota and is widely acknowledged to be a primary etiological agent of chronic periodontal disease in humans (34, 43, 44). This successful colonizer of oral tissues invades and survives in primary gingival epithelial cells (GECs) (26, 27, 33, 45). Entry of *P. gingivalis* into GECs, which are nonprofessional phagocytic cells, is rapid and requires the bacteria and host cells to be metabolically active. It has been demonstrated that adherence of *P. gingivalis* to GECs is multimodal, and yet the trigger event for subsequent invasion is mediated primarily by the binding of major fimbriae to β1 integrin receptors (46). Invasion of *P. gingivalis* is accompanied by the phosphorylation and activation of putative integrin-signaling and structural proteins, i.e., FAK (focal adhesion kinase) and paxillin, and by significant remodeling of the actin cytoskeleton, suggesting that rearrangements of the host cell signaling/cytoskeleton proteins permit bacterial entry into GECs (46, 47).

P. gingivalis is nonmotile while in the extracellular space, invades GECs in high numbers, and is then localized in the cytoplasm without being constrained by membranous vacuoles (5). The organism is capable of intracellular replication and remains viable for extended periods in GECs cultured in vitro. Despite the burden of large numbers of intracellular bacteria, infected GECs do not die and are resistant to staurosporine-induced apoptosis. The phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B) pathway appears to be one of several signaling pathways that could promote the survival of GECs during *P. gingivalis* infection (45). Evidently, transcriptomic analysis of gingival cells (human immortalized gingival keratinocytes) shows that a variety of antiapoptotic pathways are also activated by the organism (16). Moreover, recent proteomics and genomics studies have revealed that *P. gingivalis* infection induces regulation of a large number of distinctive *P. gingivalis* proteins and genes that could be important for the adaptation and survival of the microorganism in epithelial cells (19, 35, 48). Hence, the interaction between bacteria and GECs leads to the modulation of a wide array of biological functions in both host and pathogen, most of which remain to be characterized.

Several invasive bacterial species have been shown to rely on the host cell actin cytoskeleton in order to complete their infection cycles (2, 6, 10, 29, 42). For example, *Shigella flexneri* and *Listeria monocytogenes* multiply in the host cell cytoplasm, move intracellularly, and spread to adjacent epithelial cells. *Shigella flexneri* and *Listeria monocytogenes* cells move through the cytosol of the infected cells and spread to neighboring cells by the assembly of a propulsive actin tail at one end of the bacteria (38, 40). *Rickettsia* spp., which are gram negative and obligate intracellular bacteria, induce the formation of actin-based, long, twisted protrusions bundled around the microorganisms' outer membrane that allow the organisms to exit the infected cell and enter adjoining uninfected cells (14). Exploitation of the host cell actin cytoskeleton thus appears to be a common mechanism for the spread of invasive bacteria (6, 15, 39, 42). Direct cell-to-cell movement of bacteria has been postulated to facilitate local and systemic spread and avoidance of the host humoral immune response (18).

Molecular and cellular changes associated with *P. gingivalis* invasion have been subject to extensive studies during the last

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decade (17, 29). Surprisingly, it is unknown whether the pathogen resides in the cytoplasm of the same infected cell throughout the infection, whether it needs to be released into the extracellular space in order to initiate infection of new host cells, or whether it can spread directly to adjacent cells over time. Furthermore, the consequences of prolonged infection by *P. gingivalis* in GECs, in the absence of host cell death, for the dissemination of the organism within deeper layers of the oral epithelium and colonization of adjacent tissues remain to be investigated.

The present study represents the first report that *P. gingivalis* can spread from cell to cell without passing through the extracellular space, and that spreading is dependent on the host cell actin cytoskeleton. Little cell-to-cell spreading can be detected at early times of infection, but the spreading process gains momentum after 24 h of infection. These findings suggest a novel mechanism for *P. gingivalis* colonization of host tissues and evasion of the humoral immune response.

MATERIALS AND METHODS

Bacteria and culture conditions. *P. gingivalis* ATCC 33277 was cultured anaerobically for 24 h at 37°C in Trypticase soy broth supplemented with yeast extract (1 mg/ml), hemin (5 μg/ml), and menadione (1 μg/ml). Bacteria were harvested by centrifugation at 6,000 × *g* and 4°C for 10 min, washed, and resuspended in Dulbecco's phosphate-buffered saline (PBS; Sigma, St. Louis, MO), pH 7.3. The number of bacteria was determined using a Klett-Summerson photometer (26).

Culture of GECs. Primary cultures of GECs were generated as described previously (28). Briefly, healthy gingival tissue was obtained after oral surgery, and surface epithelium was separated by overnight incubation with 0.4% dispase. Cells were cultured as monolayers in serum-free keratinocyte growth medium (KGM) (Cambrex, New Jersey) at 37°C in 5% CO_2. GECs were used for experimentation at ~75% confluence and reacted with bacterial cells or other test reagents in KGM.

Fluorescent labeling and coculturing of GECs for examination of bacterial spreading. GECs, grown in six-well plates, were labeled with CellTracker Blue CMAC (7-amino-4-chloromethylcoumarin) (Molecular Probes, Inc., Eugene, OR). CellTracker dyes are fluorescent chloromethyl derivatives that freely diffuse through the membrane of live cells. Once inside the cells, these mildly thiol-reactive probes can react with thiols on proteins and peptides and thus become membrane impermeant. The labeling allows subsequent fixation and probing with fluorescence-labeled antibodies (12). Briefly, the cells are incubated in a warm, fresh KGM medium containing 2.5 μM CellTracker Blue for 30 min and then rinsed three times with Dulbecco's PBS (Sigma). The cells were then incubated with fresh KGM medium, and the monolayers were infected with *P. gingivalis* ATCC 33277 at a multiplicity of infection of 100 for the indicated time periods at 37°C in 5% CO_2. At the end of each experimental condition, the cells were dissociated by mild trypsinization using 0.05% trypsin-0.53 mM EDTA (Gibco BRL, California) and collected by centrifugation. New sets of uninfected GECs were similarly labeled with CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) (Molecular Probes), mixed with approximately equal numbers of previously collected infected blue-labeled cells, and coplated onto the new plates. The growth medium was supplemented with metronidazole (200 μg/ml) and gentamicin (300 μg/ml) for each condition. Uninfected blue-labeled cells, uninfected green-labeled cells, uninfected unlabeled cells, and the infected blue-labeled cells were used separately as controls. Cytochalasin D (1 μg/ml) (Sigma) was added to control cocultured samples and maintained throughout the coculturing period.

Quantitation of bacterial spreading by flow cytofluorimetry. Following coculturing of the initially infected and uninfected cells for the indicated time periods, samples were collected and centrifuged. Pellets were fixed in 10% neutral buffered formalin and permeabilized using 0.1% Triton X-100. Samples were then incubated with anti-*P. gingivalis* ATCC 33277 rabbit polyclonal antibody (1:2,000) followed by red fluorescence (Alexa-Fluor 633)-conjugated goat anti-rabbit immunoglobulin G (IgG) (heavy plus light chains [H+L]) highly cross-absorbed antibody (1:500) (Molecular Probes). Samples were washed in Dulbecco's PBS twice. Measurements were taken and analyzed by flow cytometry with excitation at 350 nm and 450 nm using a band-pass filter for Blue CMAC detection, excitation at 488 nm and 525 nm using a band-pass filter for Green CMFDA detection, and excitation at 638 nm using a 645-nm long-pass filter for Alexa-Fluor 633 (red fluorescence) detection. For each dye, appropriate electronic compensation of the instrument was performed to avoid overlapping of the three emission spectra. Samples were calibrated for light scattering and fluorescence properties using controls (uninfected blue- or green-labeled cells or uninfected unlabeled cells treated with or without anti-*P. gingivalis* plus red fluorescence antibodies). Samples were then analyzed by setting gates for forward scatter, side scatter, and red fluorescence staining. The total percentage of events that produced red staining (cells that are infected by bacteria) was further analyzed, and the cells were separated according to their green and blue fluorescence intensities. The percentage of the blue and green cells that contain *P. gingivalis* was then quantitated. Uninfected cells, incubated with anti-*P. gingivalis* ATCC 33277 rabbit polyclonal antibody and Alexa-Fluor 633-conjugated goat anti-rabbit IgG (H+L) antibody, were used as controls to determine the red staining threshold level. Positive events for the infected cells were then gated based on the determined threshold.

Imaging of bacterial spreading by fluorescence microscopy. GECs labeled with CellTracker Blue CMAC and preinfected with *P. gingivalis* were mixed with the uninfected CellTracker Green CMFDA-labeled cells and grown on four-well chambered slides (Nalge-Nunc International, Rochester, NY) under conditions similar to those described above. The growth medium was supplemented with metronidazole (200 μg/ml) and gentamicin (300 μg/ml) for each condition. After incubation, the slides were washed with ice-cold PBS and fixed in 10% neutral buffered formalin for 20 min. The cells were permeabilized for 10 min with 0.1% Triton X-100, and the slides were incubated with anti-*P. gingivalis* ATCC 33277 rabbit polyclonal antibody (1:2,000) followed by red-orange fluorescence (Alexa-Fluor 546)-conjugated goat anti-rabbit IgG (H+L) highly cross-absorbed antibody (1:500) (Molecular Probes) for 1 h at room temperature. After consecutive washes, the slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined with an epifluorescence microscope (Zeiss Axioskop) equipped with band-pass optical filter sets appropriate for imaging of the dyes. The images were captured by multiple exposures with a cooled charge-coupled-device camera controlled by QCAPTURE software, version 1394.

Staining of bacteria and the actin cytoskeleton. Labeling of intracellular bacteria and organization of actin filaments was performed employing an immunofluorescence technique described previously (47). Briefly, the GECs grown on coverslides were incubated with anti-*P. gingivalis* ATCC 33277 antibody and then reacted simultaneously with Oregon Green 488 secondary antibody, phalloidin-tetramethylrhodamine B isothiocyanate, and 4,6-diamidino-2-phenylindole (DAPI) (1 μg/ml) (Sigma). This was followed by visualization with the epifluorescence microscope described above. The images are representative of three independent experiments.

RESULTS

Cell-to-cell spreading of *P. gingivalis* in GECs and potential role of the actin cytoskeleton. In preliminary studies, we examined the effects of early and prolonged incubation of *P. gingivalis* with GECs by fluorescence microscopy, which suggested that *P. gingivalis* could be transferred between the host cells through protrusions containing tightly packed actin filaments after 24 h of infection. The localization of intracellular bacteria and the organization of actin filaments were visualized through immunofluorescence microscopy (Fig. 1). The nuclei were also labeled with DAPI in order to confirm the subcellular cytoplasmic location of *P. gingivalis*. The results demonstrate that *P. gingivalis* is often localized within actin-based membrane protrusions, which could facilitate the organism's spreading to neighboring cells after 24 h of infection. The protrusions frequently demonstrated a long cylindrical appearance, as shown in Fig. 1A and B. In addition, the infected cells sometimes assembled multiple filaments into larger-scale protrusions appearing as bundles where the filaments (red) align in parallel with colocalized *P. gingivalis* cells (green) merging to the adjacent cell (Fig. 1C).

Time/actin-dependent kinetics of intercellular *P. gingivalis* spreading analyzed by flow cytofluorometry. In order to quan-

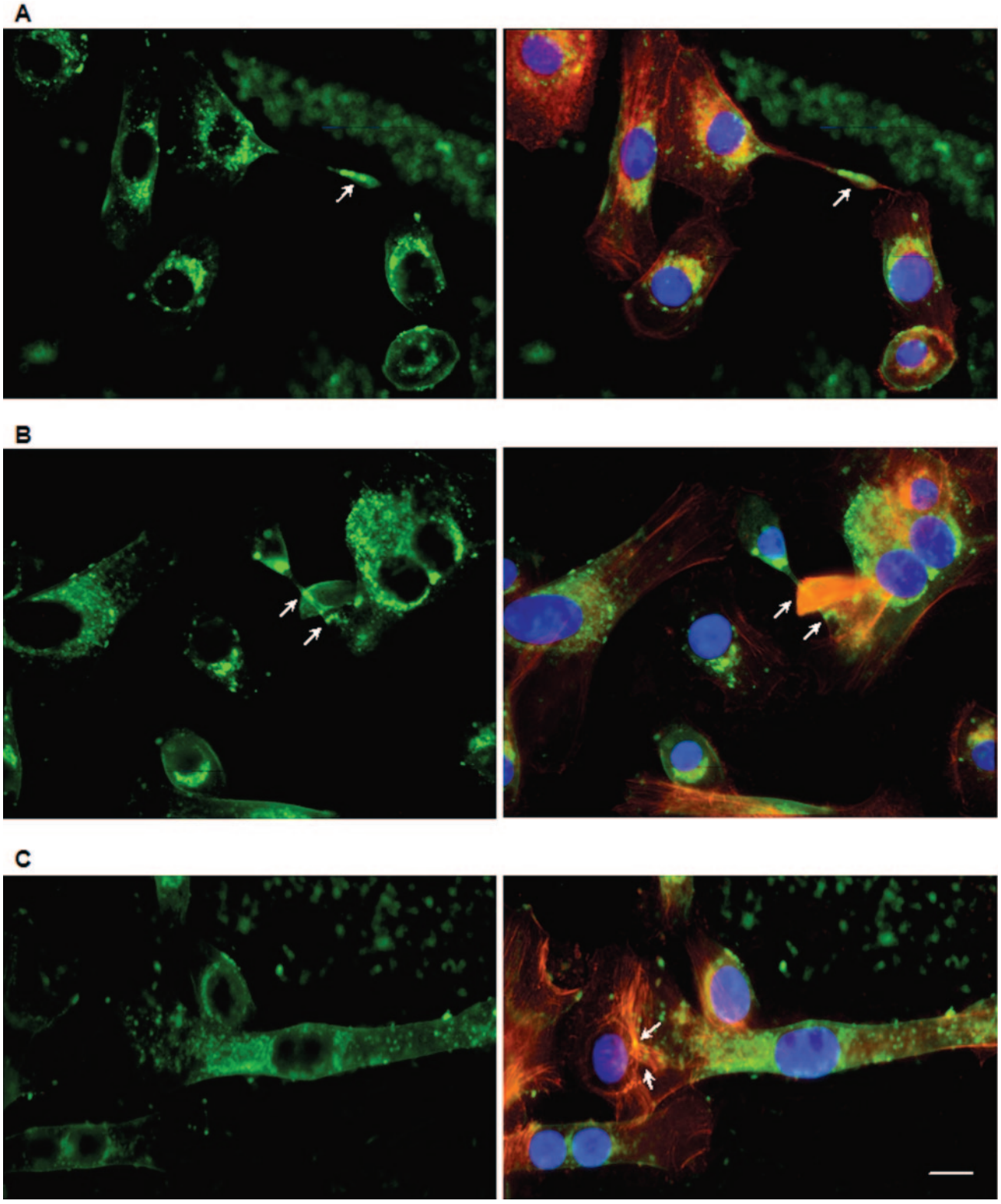
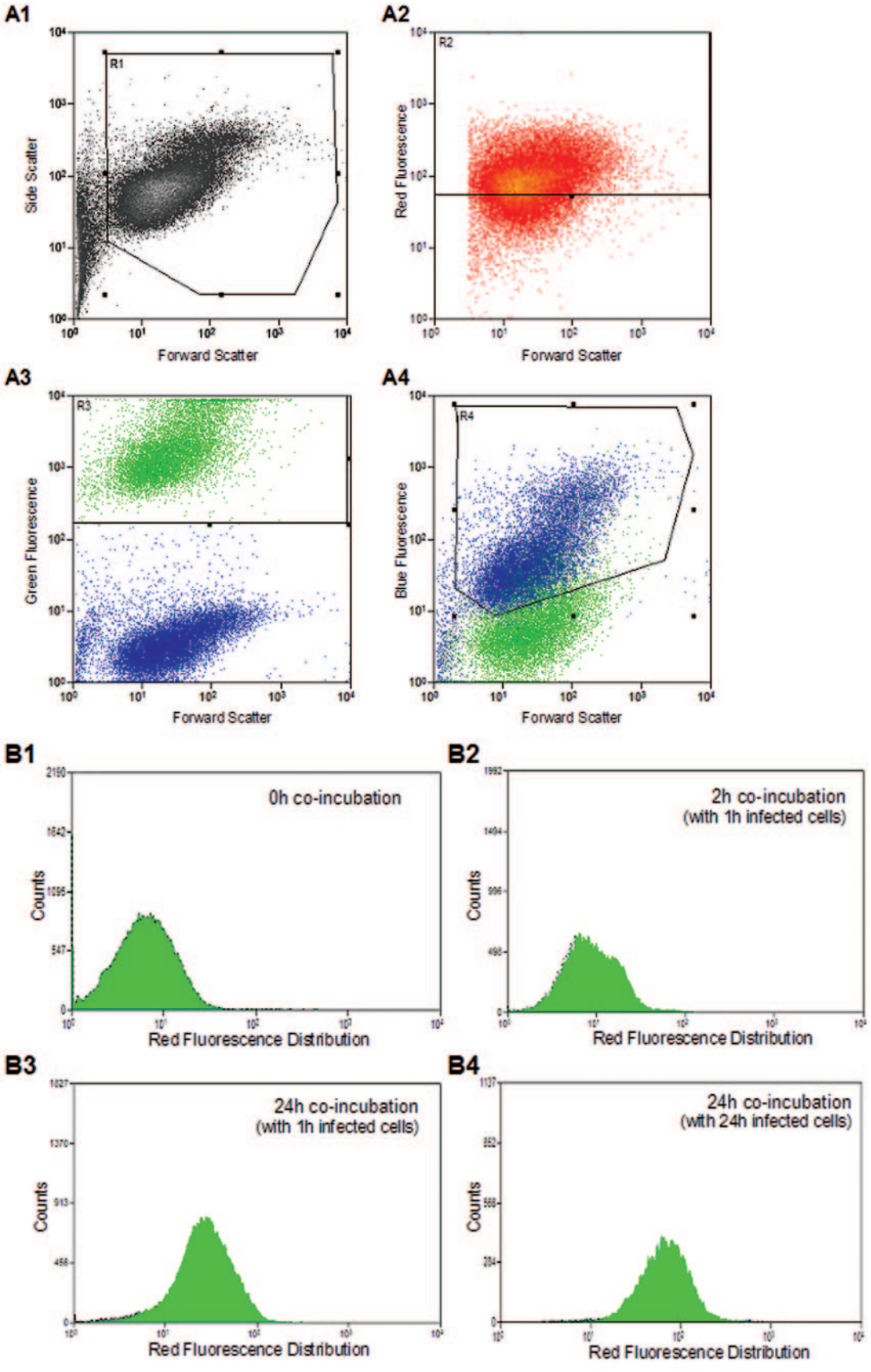


FIG. 1. Intercellular translocation of *P. gingivalis* through actin fibers at 24 h postinfection. Image sets (A to C) denote three independent experiments to visualize the localization of *P. gingivalis* ATCC 33277 and the organization of actin filaments using immunofluorescence microscopy. GECs were fixed and stained with phalloidin-tetramethylrhodamine B isothiocyanate (red) and anti-*P. gingivalis* antibody plus Oregon Green 488-conjugated secondary antibody to visualize the intracellular *P. gingivalis* (green) (left columns). DAPI (blue) was used in staining the nucleus to determine the localization of *P. gingivalis* in the cytoplasm (images in the right columns represent overlay combinations of three different experiments). White arrows indicate actin projections and bacterial translocation between the host cells. Bar, 10 μm.



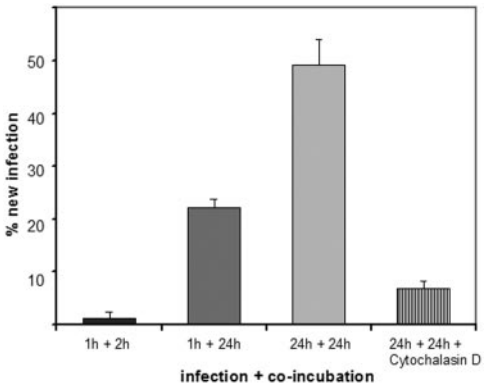


FIG. 3. Cells exhibiting bright blue fluorescence in the cytoplasm were infected for 1 h or 24 h and then coplated with uninfected green-labeled cells for 2 h or 24 h, as indicated in the histogram. Cytochalasin D was used to determine whether the actin cytoskeleton was required for spreading. (Cytochalasin D was added to cocultured control samples and maintained throughout the coculturing period.) The bars show the percentages of GECs stained for *P. gingivalis* infection. Error bars represent standard deviations of at least three independent measurements.

titatively measure the spreading of *P. gingivalis* cells to adjacent cells and begin examining the cellular basis for spreading, we followed the time course of bacterial spreading and evaluated whether actin participates in this process. For the purpose of quantification, we developed a novel technique based on cytofluorimetry, which is described in Materials and Methods. GECs were labeled with CellTracker Blue, which exhibits bright blue fluorescence in the cytoplasm, and the fluorescent cells were infected with *P. gingivalis* and then cocultured with uninfected CellTracker Green-labeled GECs for the indicated time periods. Finally, the samples were probed for *P. gingivalis* infection by immunostaining (red fluorescence) and analyzed by flow cytofluorometry (Fig. 2A). Cells were gated based on morphological profiles (size and light refraction properties) determined by side scatter-versus-forward scatter measurements (Fig. 2A1). Bacterium-containing cells within the whole population were quantitated by setting a gate for red fluorescence staining (Fig. 2A2). The total percentage of bacterium-containing cells (Fig. 2A2, gated region R2) was then further analyzed, and the cells were separated according to their green (gated region R3 in Fig. 2A3) and blue (Fig. 2A4, gated region R4) intensities to measure the percentage of newly infected cells.

To rule out the possibility that externalized bacteria may contribute to cell-to-cell spreading, high levels of gentamicin and metronidazole were included in the growth medium throughout the coculturing periods. Therefore, any bacteria present in the extracellular medium will be rendered nonviable and thus unable to invade.

The red fluorescence distribution analysis of blue and green cells over time showed increasing infection of green cells after incubation with blue cells (Fig. 2B1 to B4). Initially, bacterial levels for green cells cocultured for 2 h with 1-h-infected blue cells did not indicate a significant amount of infection (Fig. 2B2) compared with that for green cells that had not been incubated with infected blue cells (Fig. 2B1). After coculturing of green cells for 24 h with 1-h-infected blue cells, the bacteria levels in the green cells increased by almost an order of magnitude (Fig. 2B3), as indicated by the rightward shift of the red fluorescence distribution peak. The red fluorescence peak for green cells cocultured for 24 h with 24-h-infected blue cells shifted even further to the right on the logarithmic *x* axis scale, implying an even higher level of infection in the green cells (Fig. 2B4).

Quantitative analysis of the percentage of initially infected cells (blue) with initially uninfected cells (green) by flow cytofluorometry (Fig. 2A1 to A4 and 3) demonstrated that coculturing of cells that had been infected with *P. gingivalis* for 1 h with the uninfected cells for 24 h resulted in 22 ($\pm$ 1.6)% newly infected cells. In contrast, a 2-h coculture with the same uninfected cells failed to promote new infection (1.1 [$\pm$ 0.4]%), as seen in Fig. 3. Furthermore, the cells that had been infected for 24 h and then cocultured with uninfected cells for another 24 h produced a 49 ($\pm$ 4.8)% infection rate for the initially uninfected cells (Fig. 3). The latter infection rate is approximately 2.5-fold higher than that for the cells infected for 1 h and then cocultured for an additional 24 h and approximately 45-fold higher than that for the cells infected for 1 h and then cocultured for an additional 2 h. These results reinforce the interpretation that *P. gingivalis* can spread directly from cell to cell over the time course of infection.

Cytochalasin D, a fungal toxin that disrupts actin filaments and inhibits actin polymerization, was used to evaluate the role played by the actin cytoskeleton in the propagation of *P. gingivalis* through GECs. Cytochalasin D was employed only during the period when the two groups of host cells were cocultured and not during the primary infection of the CellTracker Blue-stained cells (cytochalasin D was maintained in the medium throughout the coculturing period).

Treatment of the cocultured cells with cytochalasin D revealed significant inhibition of the level of new infection (6.8 [$\pm$ 1.4]%) (Fig. 3), confirming that the stimulation of actin polymerization is an underlying factor for dissemination of *P. gingivalis* within GECs.

FIG. 2. Intercellular spreading analyzed and quantified by flow cytofluorometry. The CellTracker Blue fluorescence-labeled cells infected with *P. gingivalis* ATCC 33277 were cocultured with uninfected CellTracker Green fluorescence-labeled cells, and samples were probed with anti-*P. gingivalis* antibody plus Alexa-Fluor 633-conjugated secondary antibody (red fluorescence). (A) Samples were analyzed by setting gates for forward scatter, side scatter, and red fluorescence staining (A1 and A2), with cells in the R2 window being defined as infected (A2). The total percentage of cells stained with red (bacterium-containing cells in gate R2) were further analyzed, and the cells were separated according to their green (A3) and blue (A4) intensities to measure the percentage of newly infected cells. (B) Histograms of red fluorescence distributions in blue and green cells over time show increasing infection of green cells. Green cells at 0 h of coincubation with 1-h-infected blue cells (B1), green cells after a 2-h coincubation with 1-h-infected blue cells (B2), green cells after a 24-h coincubation with 1-h-infected blue cells (B3), and green cells after a 24-h coincubation with 24-h-infected blue cells (B4) are shown.

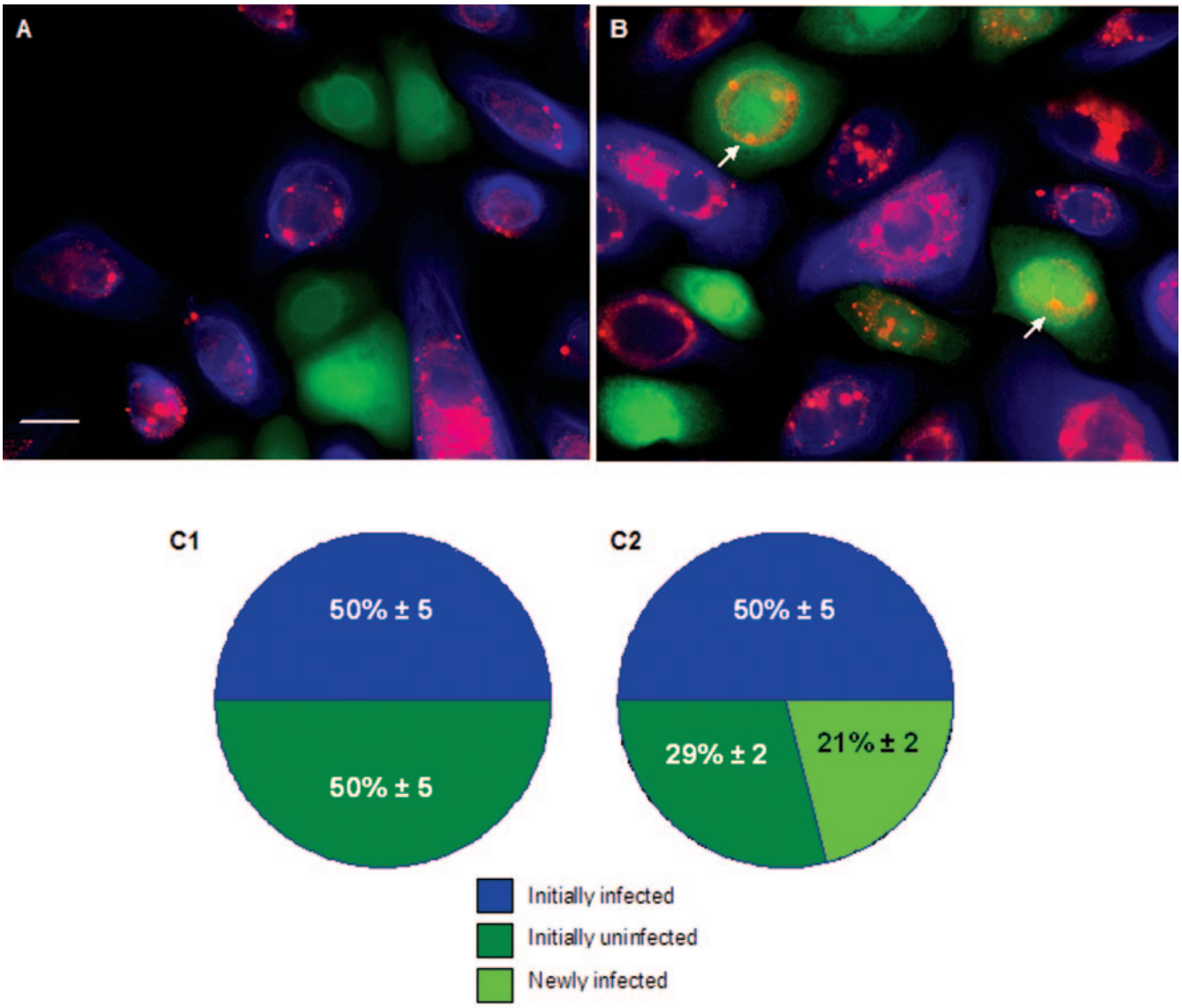


FIG. 4. *P. gingivalis* spreading visualized by fluorescent microscopy. The images of cocultured cells confirmed the flow cytofluorometry results. (A) Blue cells that were infected with *P. gingivalis* ATCC 33277 for 1 h and incubated with uninfected green cells for 2 h displayed no visible transmission of bacteria to green cells. (B) Coculturing (24 h) of cells initially infected for 24 h showed transmission of bacteria to newly infected cells (green), which displayed red-labeled *P. gingivalis* in their cytosol. White arrows indicate newly infected cells. Bar, 10 μm. (C) The conceptual diagram illustrates the quantitative distributions of initially infected, initially uninfected, and newly infected cells as detected by fluorescence microscopy. At the outset, infected and uninfected GECs were present in approximately equal amounts (C1). Coculturing for 24 h of uninfected cells with 24-h-infected cells resulted in approximately 21% newly infected cells (C2). The ratio of newly infected cells translates to an approximately 42% infection rate among initially uninfected cells (green). This infection rate is similar to that detected by flow cytofluorometry. At least 10 separate fields containing an average of 25 GECs were studied in each of two independent experiments performed in duplicate.

Examination of intercellular *P. gingivalis* infection by fluorescence microscopy. We next employed the labeling strategy used in the flow cytofluorometry analysis to visualize the distribution of *P. gingivalis* dissemination in GECs by immunofluorescence microscopy. Figure 4A shows 1-h-infected blue cells after a 2-h coincubation with uninfected green cells. After 24 h of coincubation, many of the green cells acquired red-labeled bacteria (Fig. 4B). This technique allowed us not only to distinguish the newly infected cells but also to quantify their numbers (Fig. 4C1 and C2). In agreement with the flow cytofluorometry data, the results of the fluorescence microscopy exhibited similar infection rates for early and later stages of invasion, as shown in Fig. 4A and B. A count of the newly infected green cells revealed a 42 (± 2)% infection rate among previously uninfected cells (21 [± 2]% infection rate in the whole population of blue and green cells), as shown in the conceptual diagram (4C1 and C2). Treatment with gentamicin and metronidazole was used to inactivate the bacteria that may have escaped outside the cells, and fluorescence immunostaining verified the absence of extracellular bacteria (Fig. 4A and B). It is also noteworthy that the newly infected cells (green-labeled cells infected with red-labeled bacteria) displayed

much lower levels of intracellular *P. gingivalis* than the cells that had been previously infected for 48 h (blue cells) (Fig. 4B). Indeed, quantitative analysis of intracellular bacterial staining by NIH ImageJ analysis software indicated that initially infected cells (blue) harbor at least four times more bacteria than the newly infected cells (green) (data not shown). This is consistent with the ability of *P. gingivalis* to successfully replicate in GECs over time (26). Since the main focus of this initial study was to characterize intercellular spreading of *P. gingivalis*, the comprehensive examination of the replication will be undertaken in future follow-up studies. Overall, the data demonstrate that *P. gingivalis* can efficiently spread within GECs. More importantly, the capacity to disseminate intercellularly appears to be acquired later in the intracellular infectious process.

DISCUSSION

Epithelial cells in mucosal membranes act as an initial barrier to bacterial infection. They produce a variety of innate antimicrobial defenses, cytokines, and other factors integrating signals from microorganisms to the host immune system (1, 13, 24). Therefore, the challenge for bacterial pathogens is to devise strategies that enable them to breach that barrier, enter the host cells, and colonize host tissues. Extensive bacterial host invasion studies have revealed that the internalization of invasive bacteria into epithelial tissues involves a concerted interplay of many bacterial and epithelial cell proteins (10, 22, 36). While various bacterial pathogens can invade epithelial cells, their ability to disseminate and colonize host tissues depends on their ability to survive and replicate in epithelial cells (2, 30, 40).

P. gingivalis, an important pathogen of severe/chronic cases of periodontal disease, successfully invades, multiplies in, and survives for extended periods in primary GECs, which are the lining cells of the gingival crevice and natural targets of this subgingival organism. It has been documented that the organism can also invade dendritic cells, fibroblasts, and heart or aortic endothelial cells (3, 11, 20, 37). Although the virulence mechanisms of *P. gingivalis* and invasion by the organism of epithelial cells and other cell types are under intense scrutiny (17, 25, 29, 41), it is unknown whether the intracellular bacteria reside exclusively in the cytosol of the same cell throughout the infection or spread to neighboring cells to propagate.

The findings of this study demonstrate that intracellular *P. gingivalis* can spread directly from cell to cell, and that the intercellular translocation of the organism is facilitated by an actin network-dependent process, where the cortical actin filaments are assembled to form membranous projections to transmit the bacteria to adjacent host cells. The levels of the spreading increase markedly around the first 24 h postinfection (~22%) compared to the levels at early stages of infection (a total of 3 h postinfection), which showed a transfer rate of ~1.5%. Longer incubations (a total of 48 h postinfection) exhibited a significant increase in the overall new-infection rate (~49%). The results suggest that the intercellular spreading of *P. gingivalis* is potentially associated with the organism's stage of infection cycle and/or the bacterial load carried by the GECs. The utilization of cytochalasin D confirmed that polymerization (elongation) of actin filaments is required for the promotion of *P. gingivalis* infection cell to cell, since disruption of actin polymerization by toxin treatment had a significant inhibitory effect on the amount of spreading. The exploitation of the host actin cytoskeleton is customary for other bacterial pathogens. A typical example is seen with *Listeria*, which promotes the appearance of an actin tail that projects the bacterium through the cytoplasm and moves through host cells rapidly (38). Although *P. gingivalis* invasion resembles that in the *Listeria* model, in which the organism does not remain in a membrane vacuole, the utilization of an actin cytoskeleton for translocation of the bacteria intercellularly differs noticeably. *P. gingivalis* does not use an actin tail but instead takes advantage of actin-dependent protrusions to reach new host cells. It is tempting to speculate that the association of *P. gingivalis* with the actin cytoskeleton may enable the bacterium to evade the host immune system response and propagate successfully.

A common theme that has been observed for an increasing number of bacteria is for them to invade epithelial cells, survive, replicate, and eventually spread to adjacent host cells. For example, *Actinobacillus actinomycetemcomitans*, an important periodontopathogen, invades epithelial cells, undergoes rapid multiplication, and subsequently propagates intercellularly (31). *Salmonella enterica* serovar Typhimurium also survives and begins to replicate after a 3- to 5-h lag period and continues to do so up to 24 h, when the organism exits the host cell (8, 30). Interestingly, *Salmonella* infection is known to cause apoptosis in macrophages but not in epithelial cells (23, 32), and direct cell-to-cell spread has not been observed for *Salmonella* in macrophages. Similarly, invasion of GECs by *P. gingivalis* does not induce apoptosis and instead inhibits apoptosis in these cells (33, 45). Our previous studies indicate that the organism exerts this effect at the level of mitochondria (e.g., blocking of mitochondrial membrane potential depolarization and cytochrome *c* release), and that *P. gingivalis*-dependent inhibition of apoptosis is likely mediated by the PI3K/Akt pathway (45). Accordingly, the resistance to apoptosis could represent a coordinated plan utilized by intracellular bacteria to acquire adequate time to adapt and to express necessary effector molecules to disseminate infection (7).

To date, several mechanisms have been proposed for the organism's ability to invade into deeper layers of gingival epithelium and gain access to subepithelial tissues, such as through transcellular or paracellular pathways (4). *P. gingivalis* proteases may contribute to the disintegration of the epithelial transmembrane proteins, E-cadherin, β_1 integrin, and occludin, which are essential components of the epithelial function as a mechanical barrier against microbes (9, 21). This suggests that, in addition to intercellular spreading, *P. gingivalis* may employ other complementary dissemination mechanisms.

The observations of intercellular spreading of *P. gingivalis* in later phases of infection may result from the bacterium's ability to diminish the host apoptotic response. Indeed, this observation presents a logical link to the bacterium's capacity to multiply successfully and suppress host cell death. The delay or interruption of apoptosis may enable the bacterium to shift from intracellular multiplication to intercellular dissemination. It will be intriguing to examine whether the same pathways involved in suppression of host cell apoptosis also play a role in determining the timing of cell-to-cell spread.

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