INFECTION AND IMMUNITY, Sept. 2001, p. 5698–5708 Vol. 69, No. 9
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.9.5698–5708.2001

Porphyromonas gingivalis Traffics to Autophagosomes in Human Coronary Artery Endothelial Cells

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Received 18 January 2001/Returned for modification 21 March 2001/Accepted 4 June 2001

***Porphyromonas gingivalis* is a periodontal pathogen that also localizes to atherosclerotic plaques. Our previous studies demonstrated that *P. gingivalis* is capable of invading endothelial cells and that intracellular bacteria are contained in vacuoles that resemble autophagosomes. In this study, we have examined the trafficking of *P. gingivalis* 381 to the autophagic pathway. *P. gingivalis* 381 internalized by human coronary artery endothelial (HCAE) cells is located within vacuoles morphologically identical to autophagosomes. The progression of *P. gingivalis* 381 through intracellular vacuoles was analyzed by immunofluorescence microscopy. Vacuoles containing *P. gingivalis* colocalize with Rab5 and HsGsa7p early after internalization. At later times, *P. gingivalis* colocalizes with BiP and then progresses to a vacuole that contains BiP and lysosomal glycoprotein 120. Late endosomal markers and the lysosomal cathepsin L do not colocalize with *P. gingivalis* 381. The intracellular survival of *P. gingivalis* 381 decreases over 8 h in HCAE cells pretreated with the autophagy inhibitors 3-methyladenine and wortmannin. In addition, the vacuole containing *P. gingivalis* 381 lacks BiP but contains cathepsin L in the presence of wortmannin. These results suggest that *P. gingivalis* 381 evades the endocytic pathway to lysosomes and instead traffics to the autophagosome.**

Porphyromonas gingivalis is a gram-negative, anaerobic rod that is considered to be among the major pathogens associated with adult periodontitis (64). A possible mechanism of pathogenesis may be cellular invasion. *P. gingivalis* has been demonstrated to be internalized within gingival epithelial cells in vitro (19, 34, 53) and buccal epithelial cells in vivo (51). Recent epidemiological studies have demonstrated a strong relationship between periodontal disease and coronary heart disease (2, 3, 17, 38, 39). Oral bacteria have a direct route to the circulatory system in periodontitis patients due to transient bacteremias produced by flossing, mastication, and toothbrushing (11, 57, 62). *P. gingivalis* localizes to atherosclerotic plaques (12, 30) and is capable of invasion of coronary artery cells in vitro (16, 18). Therefore, invasion and intracellular parasitism of endothelial cells by *P. gingivalis* in vivo may exacerbate the inflammatory response of atherosclerosis.

Invasion of nonphagocytic cells is a common strategy of evading the immune system for many pathogens (23). Once within the cell, these pathogens have developed various mechanisms for survival (28, 40). *Legionella pneumophila* and virulent *Brucella abortus* gain access to and replicate in vacuoles that resemble autophagosomes and are associated with endoplasmic reticulum proteins (46, 47). Autophagosomes, multimembranous vacuoles formed from invaginations of ribosome-free regions of the rough endoplasmic reticulum (RER) (20), are the organelles of the autophagic process. Autophagy is a process whereby cytosol and organelles are sequestered for lysosome degradation in response to nutrient deprivation (20). Under normal conditions, the autophagosome matures into an autolysosome, where the contents are degraded. The autophagosome-like vacuoles containing these bacterial species do not acquire lysosomal hydrolases (46, 66). Bacterial trafficking to the autophagic pathway has been proposed to be a mechanism of increasing the concentration of free amino acids to be utilized by the bacteria for their biochemical pathways and/or to inhibit host cell protein synthesis in order to reduce the cellular response to the pathogen (63).

In a previous study, we demonstrated that the vacuoles containing *P. gingivalis* in human coronary artery endothelial (HCAE) cells morphologically resembled autophagosomes, similar to the vacuoles containing *L. pneumophila* in macrophages and virulent *B. abortus* in HeLa cells (18). The first goal of this study was to characterize and delineate the trafficking of *P. gingivalis* within endothelial cells using strain 381. The second goal was to determine whether the autophagosome-like vacuole was the intracellular niche for *P. gingivalis* in the HCAE cell or whether the HCAE cell utilized the autophagic pathway to rid itself of this intracellular intruder.

MATERIALS AND METHODS

Bacterial and cell culture conditions. *P. gingivalis* strain 381 was subcultured on tryptic soy agar (Difco Laboratories, Detroit, Mich.) supplemented with 5.0% sheep blood (Lampire Biological Laboratories, Pipersville, Pa.), 0.5% yeast extract (Difco), hemin (5 μg/ml), and vitamin K (5 μg/ml). Liquid cultures were grown in brain heart infusion broth (Difco) supplemented with 0.5% yeast extract, 0.1% cysteine (Sigma), hemin (5 μg/ml), and vitamin K (5 μg/ml) under anaerobic conditions. These strains were grown at 37°C in a Coy (Ann Arbor, Mich.) anaerobic chamber with an atmosphere of 5% CO_2, 10% H_2, and 85% N_2. *Escherichia coli* MC1061was subcultured at 37°C aerobically on Luria-Bertani (LB) plates consisting of Bacto Agar (15 g/liter; Difco), Bacto Tryptone (10 g/liter; Difco), yeast extract (5 g/liter), and sodium chloride (10 g/liter; Fisher Scientific, Springfield, N.J.) and was also grown in LB broth media. The HCAE cells are a primary cell culture line purchased from Clonetics Inc. (San Diego, Calif.), cryopreserved on third passage, and were passaged an additional two or three times before use. The HCAE cells were maintained in endothelial growth medium-2 (EGM-2), which consisted of endothelial basal medium-2 supplemented with fetal bovine serum, hydrocortisone, human recombinant fibroblast growth factor, vascular endothelial growth factor, recombinant insulin growth

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factor-1, ascorbic acid, human recombinant epidermal growth factor, gentamicin, and amphotericin (Clonetics).

Antibodies. Rabbit polyclonal anti-BiP (provided by S. Frost); rabbit polyclonal anti-cathepsin L (C. Gabel); rabbit polyclonal anti-HsGSA7p (human-specific Gsa7p); 61BG1.3, a mouse monoclonal anti-*P. gingivalis* hemagglutinin A (R. Gmür) (7); rabbit polyclonal antilysosomal glycoprotein 120 (LGP120) (22); rabbit polyclonal anti-mannose 6-phosphate receptor (MPR) (P. Nissley); rabbit polyclonal anti-Rab5 (Stressgen Biotechnologies Corp., Vancouver, British Columbia, Canada); rabbit polyclonal anti-Rap1 (Stressgen); and rabbit polyclonal anti-2,6 Gal β1,4-GlcNAc sialyltransferase (G. Hart) antibodies were used in this study. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Sigma Chemical Co., St. Louis, Mo.) and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit antibody (Sigma) were used as secondary antibodies.

Persistence assay. The numbers of intracellular bacteria at various times and in the absence or presence of autophagy inhibitors were quantitated. Approximately 10^5 HCAE cells per well in a 24-well tissue culture plate were washed three times with phosphate-buffered saline (PBS). For inhibition of autophagy, both 10 mM 3-methyladenine (Sigma) and 10 nM wortmannin (Sigma) in EGM-2 were preincubated with HCAE cells for 60 min prior to the start of the invasion assay. For wells not receiving the autophagy inhibitors, media were replaced with fresh antibiotic-free EGM-2. Following preincubation, broth-grown bacteria were centrifuged at low speed and resuspended in antibiotic-free medium to a concentration of 10^7 cells/ml as determined spectrophotometrically (Shimadzu UV-1201; VWR, Marietta, Ga.). Then 1.0 ml of the bacterial suspension was added to each well and the HCAE cells plus bacteria were incubated at 37°C in 5% CO_2 for 90 min. The autophagy inhibitors were present during the time of infection in the appropriate wells. The media were removed from infected cells after 90 min, and the cells were washed three times with PBS. Medium containing gentamicin (300 μg/ml) and metronidazole (200 μg/ml) was then added to each well to kill any extracellular bacteria, and the plates were incubated for an additional 60 min in 5% CO_2 at 37°C. The autophagy inhibitors were also present during the antibiotic incubation in the appropriate wells. The media were removed, and the cells were washed three times with PBS. The HCAE cells from one set of wells were then lysed by a 20-min incubation with 1.0 ml of sterile distilled water at 37°C. This was the 2.5-h time point. For the wells to be assessed at 4, 6, and 8 h postinfection, the media were replaced with antibiotic-free EGM-2 without autophagy inhibitors and further incubated at 37°C in 5% CO_2. At the appropriate time points, the HCAE cells were lysed by the aforementioned protocol. Dilutions of the lysates of cells infected with *P. gingivalis* were plated in triplicate on tryptic soy agar (Difco) plates supplemented with 5.0% sheep blood, 0.5% yeast extract, hemin (5 μg/ml), and vitamin K (5 μg/ml) and were cultured anaerobically. The dilutions of the lysates of *E. coli* MC1061, the negative control, were cultured on LB plates at 37°C aerobically. The CFU of invasive bacteria were then enumerated. Each condition was performed in duplicate, and each assay was performed three times independently. All of the inhibitors were also tested at the appropriate concentration for adverse effects on the viability of the bacteria by plate count. The viability of the HCAE cells were tested by trypan blue exclusion and by examining the confluency of the monolayer.

Transmission electron microscopy. The bacteria were incubated with the HCAE cells for 30, 60, and 90 min and were subsequently fixed in 2% glutaraldehyde in PBS at room temperature for 1 h. For inhibition studies, the HCAE cells were preincubated with 10 nM wortmannin and 10 mM 3-methyladenine in EGM-2 for 1 h, and the wortmannin was also present during the infection. After the cells were centrifuged and the pellet was washed with PBS (pH 7.3), 3 drops of 3% low-gelling-point agarose was added, and the pellet was solidified at 4°C for 10 min. The agarose-embedded pellet was then washed twice for 10 min in PBS, postfixed in 1% osmium tetroxide for 1 h, and washed three times in distilled H_2O for 10 min. The specimens were then dehydrated in a graded series of ethanol and stained overnight en bloc in 2% uranyl acetate. Following the last ethanol treatment, the specimens were infiltrated and embedded in EM Bed-812 (Electron Microscopy Sciences, Fort Washington, Pa.). Thin sections were cut, poststained with uranyl acetate and lead citrate, and examined on a Hitachi 7000 transmission electron microscope.

Immunofluorescence microscopy. HCAE cells were grown on glass coverslips in a six-well tissue culture plate. The HCAE cells were then washed three times with PBS prior to infection with *P. gingivalis* 381 at different time points at a multiplicity of infection of approximately 100. The media were removed, and the HCAE cells were washed vigorously three times with PBS. Wortmannin (10 nM) was added 1 h before infection and was present during infection. The infected HCAE cells were then fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. This was followed by washing twice in PBS and quenching in 50 mM NH_4Cl–0.3% Tween 20–PBS for 10 min at room temperature. After quenching, the HCAE cells were washed two times in PBS. The primary antibodies, 1/50 dilution in PBS–5% normal goat serum–0.3% Tween 20, were applied for 2 h at room temperature. The HCAE cells were then washed four times in PBS for 5 min each wash. In all cases, the bacteria were detected and organelle and host protein markers were detected with FITC-conjugated goat anti-mouse antibody and TRITC-conjugated goat anti-rabbit antibody, respectively, as the secondary antibodies. The secondary antibodies (1/200 dilution in PBS–5% normal goat serum–0.3% Tween 20) were applied for 1 h at room temperature. The HCAE cells were then washed twice with PBS before mounting with Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, Ala.) onto glass microscope slides.

Images were viewed using a Zeiss Axiophot fluorescence photomicroscope with a spot camera and Adobe Photoshop imaging software. Each time point was analyzed by examining at least 100 internalized bacteria (entire fields of view under the microscope were counted, so totals exceeded 100) except for 15 min postinfection, at which time too few bacteria had invaded to view 100 internalized *P. gingivalis*. All immunofluorescence tests were performed at least twice. These same colocalization experiments were also examined by deconvolution microscopy using an Olympus IX70 microscope and Deltavision software (Applied Precision, Inc., Wepahah, Wash.). Raw images were captured by a charge-coupled device camera in both color channels in a series of 10- to 0.2-μm increments. The Deltavision software converted the stack of images into a computational three-dimensional view using a constrained iterative deconvolution algorithm.

Statistical analysis. Student's *t* test (Microsoft Excel; Microsoft, Redmond, Wash.) was used to compare differences in colocalization data between untreated and wortmannin-treated time points. Due to similar comparisons over multiple time points, Bonferroni's correction was utilized, and statistical significance was achieved when P was <0.01 (0.05/5). The percentages of colocalization from each individual field of view were compared for this analysis.

RESULTS

Morphological analysis of vacuoles containing *P. gingivalis*. Internalized *P. gingivalis* was found within structures resembling autophagosomes at 90 min postinfection (Fig. 1A). These vacuoles were bound by one or two membranes and contained undegraded cytoplasm and vesicles of cytoplasmic origin (Fig. 1C and D). The presence of cytoplasmic components in these vacuoles suggests that the vacuoles arose via autophagic events. Profiles of RER were routinely observed in close association with these vacuoles. The morphological data suggest that the internalized *P. gingivalis* 381 cells reside in autophagosomes, which have been derived from the RER. We next examined the intracellular fate of *P. gingivalis* when 3-methyladenine or wortmannin suppresses autophagy. Internalized *P. gingivalis* was observed within vacuoles in wortmannin-treated HCAE cells at 30 min postinfection (Fig. 1B). However, these vacuoles were morphologically distinct from autophagosomes. They were bounded by only a single membrane and lacked undegraded cytoplasmic ground substance. These inhibitors appeared to have no effect on bacterial internalization. At later time points only what appeared to be bacterial fragments were observed in HCAE cells preincubated with 3-methyladenine and wortmannin.

Trafficking through the autophagic pathway. Autophagy is responsible for the sequestration and delivery of cytoplasmic proteins and organelles to the autolysosome, where they are degraded. Our data suggest that internalized *P. gingivalis* enters the autophagic pathway. Therefore, we utilized protein markers of autophagy to investigate the intracellular trafficking of *P. gingivalis* in HCAE cells. We found that *P. gingivalis* resided in a vacuole that contained HsGsa7p but lacked cathepsin L (Fig. 2A and C). We next examined the trafficking of *P. gingivalis* in HCAE cells treated with 10 nM wortmannin, an

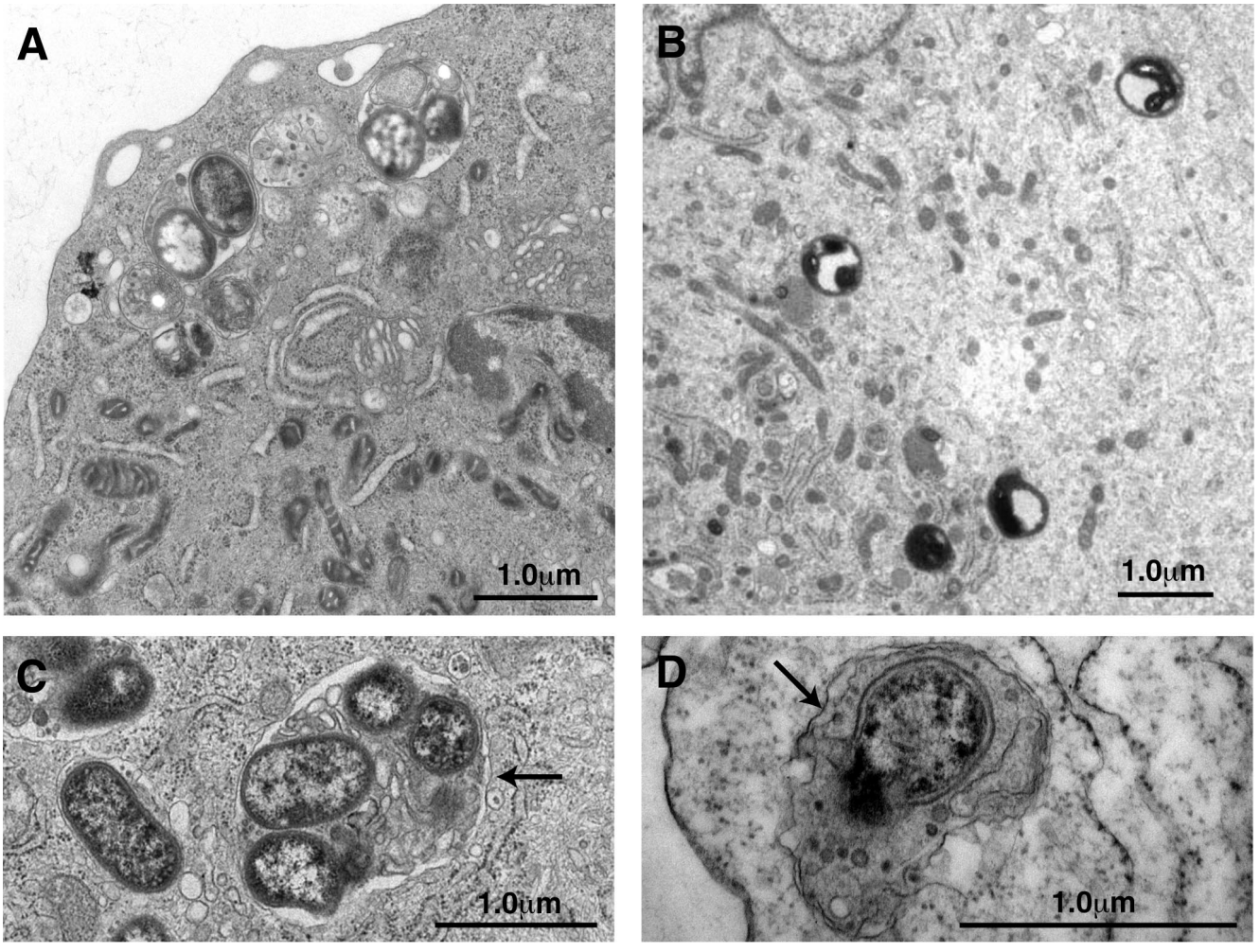
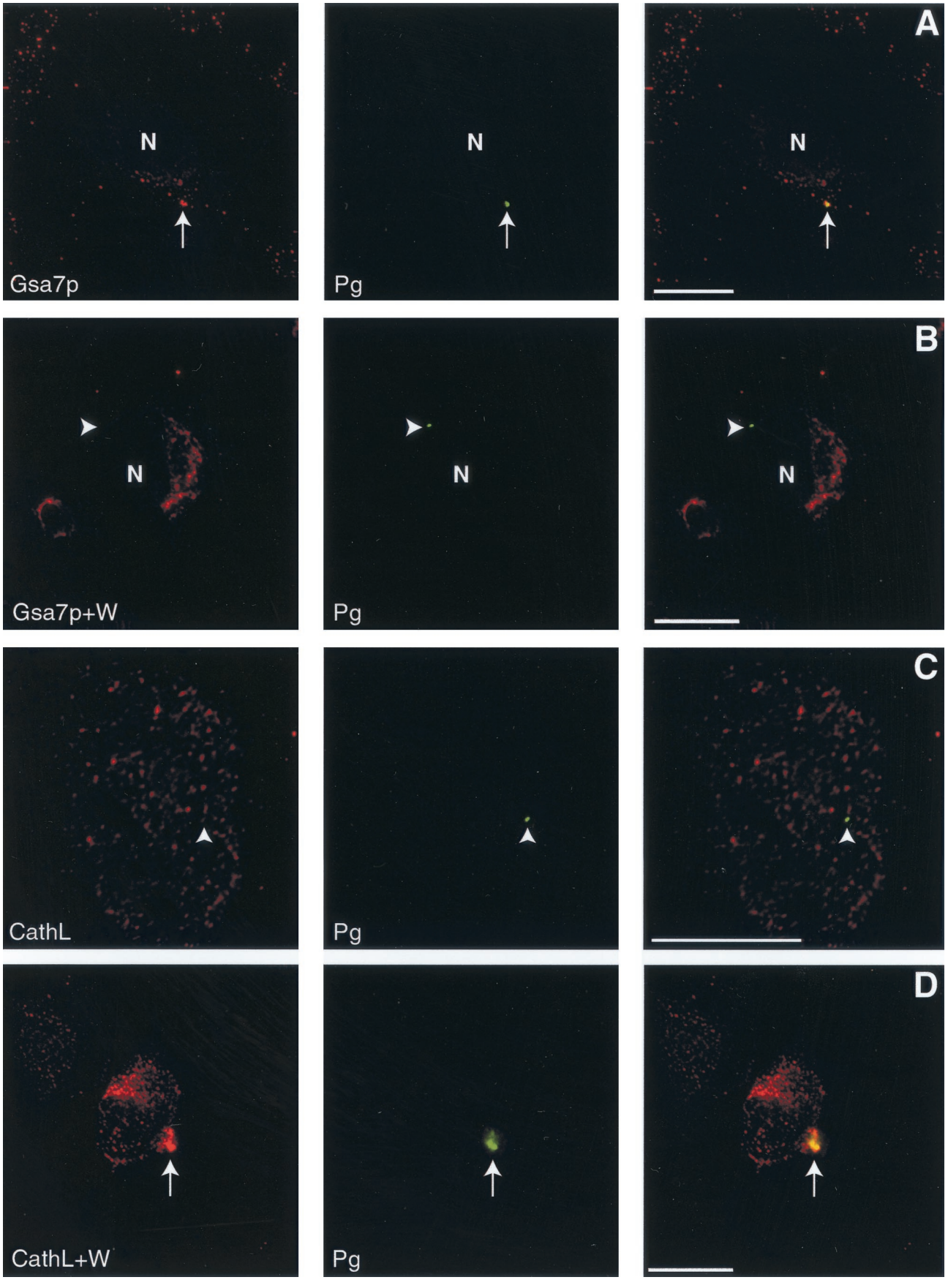


FIG. 1. Internalized *P. gingivalis* in HCAE cells. HCAE cells were infected with *P. gingivalis* in the presence (A, C, and D) and absence (B) of wortmannin. After 90 min of infection, *P. gingivalis* could be observed in vacuoles within the cytoplasm of HCAE cells (A). These vacuoles were bound by one or two membranes (arrows) and contained undegraded vesicles and cytoplasmic ground substance (C and D). After 30 min of infection of wortmannin-treated HCAE cells, *P. gingivalis* appears to be in the process of degradation and is within vacuoles that resemble lysosomes (B).

inhibitor of autophagy. Under these conditions, the bacterial vacuole contained cathepsin L (Fig. 2D) but not HsGsa7p (Fig. 2B). We also observed that the cellular distribution of HsGsa7p differed in the absence and presence of wortmannin (Fig. 2). HsGsa7p resided in a Golgi-like vacuole when autophagy was suppressed by wortmannin (Fig 2B). During bacterial invasion in the absence of wortmannin, the HsGsa7p was found in small unknown organelles distributed throughout the cell. We believe that this alteration in the cellular distribution of HsGsa7p reflects the onset of autophagy. Indeed, we found HsGsa7p colocalized with Golgi markers in fed HuH7 cells when autophagy is suppressed but distributed to round organelles in starved HuH7 cells when autophagy is enhanced (data not shown).

At 90 min postinfection, we observed that HsGsa7p, BiP (Fig. 3A), and LGP120 (Fig. 3B) colocalized with the *P. gingivalis* vacuoles. We next quantified the colocalization between the bacteria and cellular markers over a time course to delineate the intracellular trafficking of *P. gingivalis*. Within 15 min of infection, 42% of the internalized bacteria colocalized with BiP, an RER luminal protein (Fig. 4A) (71). At 30 to 90 min, 68 to 80% of the *P. gingivalis* was found in BiP-positive compartments. Even at 120 min, 52% of the bacteria were localized to the BiP compartment. In contrast, *P. gingivalis* was not

FIG. 2. Localization of HsGsa7p and cathepsin L to vacuoles containing *P. gingivalis* in HCAE cells using deconvolution microscopy. (A) *P. gingivalis* colocalized with HsGsa7p (arrow). (B) In the presence of wortmannin, *P. gingivalis* did not colocalize with HsGsa7p (arrowhead). HsGsa7p resided in a Golgi-like vacuole in the wortmannin-treated HCAE cells (arrowhead). (C) *P. gingivalis* did not colocalize with cathepsin L (arrowhead). (D) *P. gingivalis* colocalized with cathepsin L (arrow) in the presence of wortmannin. Abbreviations: Gsa7p, HsGsa7p; W, wortmannin; CathL, cathepsin L; Pg, *P. gingivalis*; N, nucleus. Bar, 15 μm.


A
N
N
N
Gsa7p
Pg
B
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N
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Gsa7p+W
Pg
C
CathL
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CathL+W
Pg

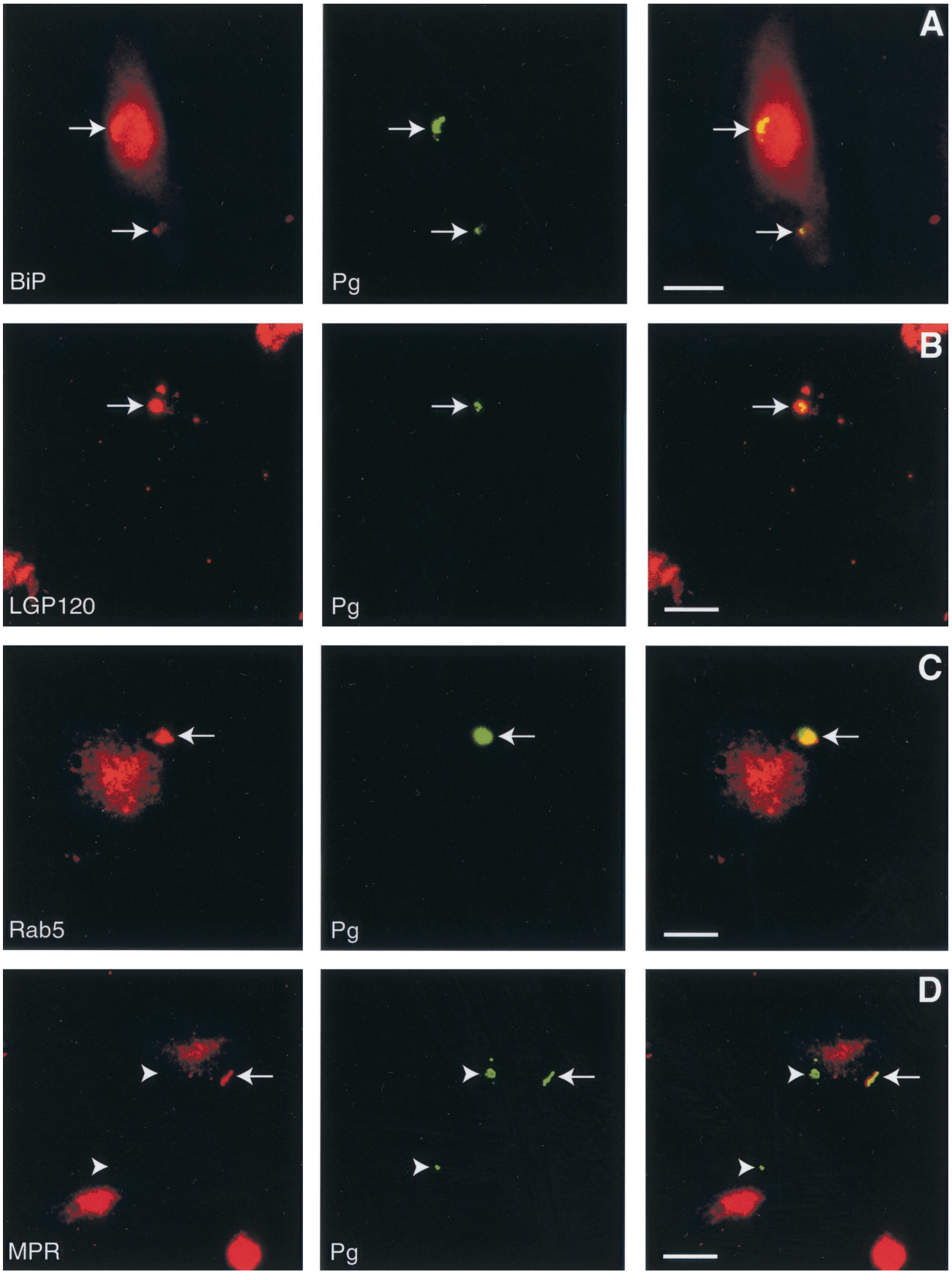
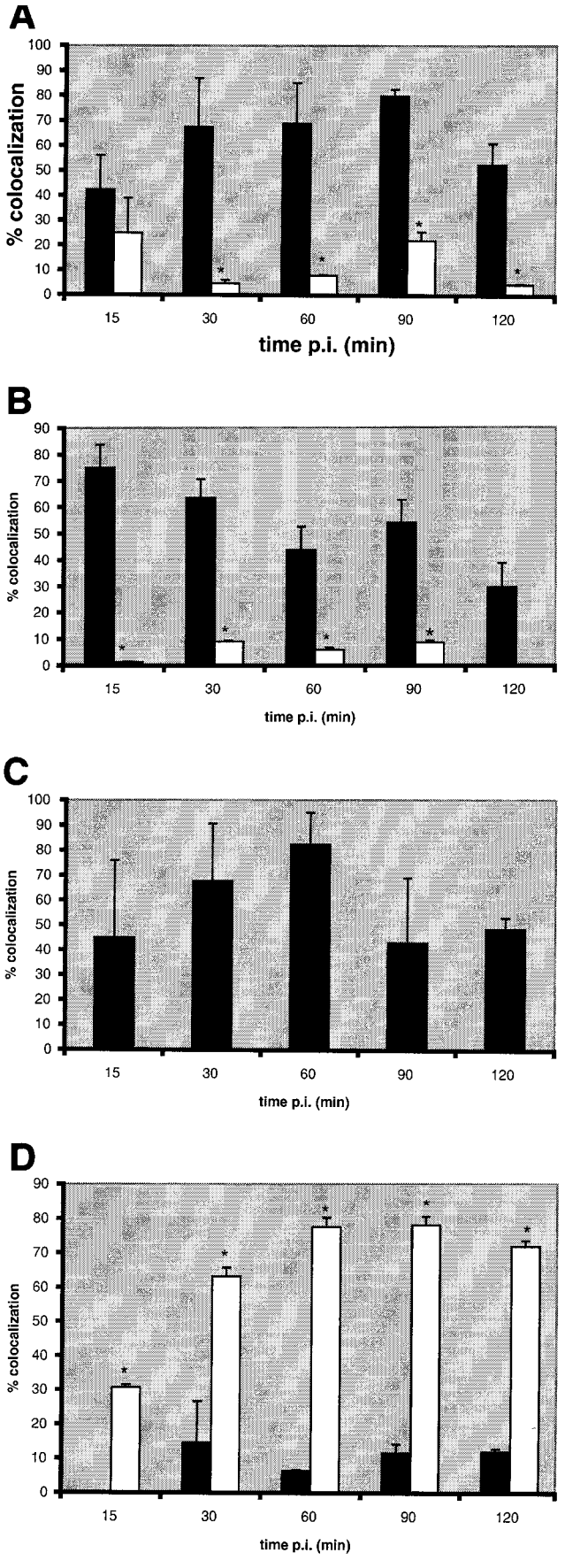


FIG. 3. Localization of BiP, LGP120, Rab5, and MPR to vacuoles containing *P. gingivalis* in HCAE cells. At 90 min postinfection, the *P. gingivalis* (Pg) vacuoles contained BiP (A) and LGP120 (B) (arrows). (C) At 35 min after infection, *P. gingivalis* colocalized with Rab5 (arrow). (D) MPR was absent from a majority of the vacuoles observed up to 2 h postinfection (arrowheads indicate colocalization). Bar, 15 μm.



found in this BiP-positive compartment when the cells were treated with wortmannin. We also examined the distribution of HsGsa7p in the infected HCAE cells in the absence and presence of wortmannin (Fig. 4B). Within 15 min, 75% of the internalized *P. gingivalis* cells were localized to vacuoles containing HsGsa7p, and this value decreased over time to 30 at 120 min. However, in the presence of wortmannin, less than 9% of the internalized *P. gingivalis* localized to the HsGsa7p vacuole. The data suggest that soon after invasion *P. gingivalis* becomes associated with an early autophagosome containing BiP and HsGsa7p but over time loses HsGsa7p.

The above data suggest that *P. gingivalis* is sequestered into the early autophagosome after invasion. We next examined whether *P. gingivalis* trafficked to the late autophagosome and eventually to the autolysosome for degradation. LGP120 is a membrane protein present in late autophagosomes, autolysosomes, and lysosomes (22, 36, 58). Cathepsin L is a thiol-proteinase present in autolysosomes and lysosomes. *P. gingivalis* was observed in vacuoles that contained LGP120 but not cathepsin L. Within 60 min of invasion, 82% of the *P. gingivalis* cells were in an LGP120-positive vacuole (Fig. 4C). At this same time point, only 6% of the bacteria were in an autolysosome or lysosome as determined by the presence of cathepsin L (Fig. 4D). In contrast, 78% of the internalized *P. gingivalis* cells were localized to a cathepsin L-positive vacuole when the cells were treated with wortmannin. These data suggest that *P. gingivalis* traffics to a late autophagosome that fails to mature to an autolysosome, but the bacterium is transported to phagolysosomes when autophagy is inhibited by wortmannin.

Trafficking along the endocytic pathway. We have demonstrated that *P. gingivalis* is internalized and sequestered into a late autophagosome containing BiP and LGP120 but lacking cathepsin L. *P. gingivalis* must either promote its entry into the autophagic pathway or block its entry into the endocytic pathway, thereby channeling itself into the autophagic pathway. When autophagy was inhibited by wortmannin, the bacteria were found in a cathepsin L-positive, lysosome-like vacuole. Hence, we propose that *P. gingivalis* does not block the endocytic pathway but rather promotes its entry into the autophagosome. Therefore, we suggest that bacteria are trafficked through the endocytic pathway to lysosomes during autophagy inhibition. To test this hypothesis, we traced the trafficking of *P. gingivalis* through the endocytic pathway in the presence and absence of wortmannin using antibody markers to endosomal vacuoles.

A majority of the *P. gingivalis* vacuoles contained Rab5, a

FIG. 4. Colocalization of *P. gingivalis* with protein markers of the autophagic pathway. HCAE cells were incubated with *P. gingivalis* 381 in the absence (solid bars) and presence (open bars) of 10 nM wortmannin for 15 to 120 min. The data represent the percentage of *P. gingivalis* vacuoles that contained BiP (A), HsGsa7p (B), LGP120 (C), and cathepsin L (D), expressed as the mean + the standard deviation (error bars). The data suggest that the *P. gingivalis* vacuole first acquires HsGsa7p and then BiP and LGP120; however, the vacuole fails to acquire cathepsin L. In the presence of wortmannin, the vacuole does not acquire HsGsa7p or BiP but rather acquires cathepsin L rapidly. The effects of wortmannin at each time point were evaluated by Student's *t* test utilizing Bonferroni's correction. Statistical significance (*) was achieved when P was <0.01.

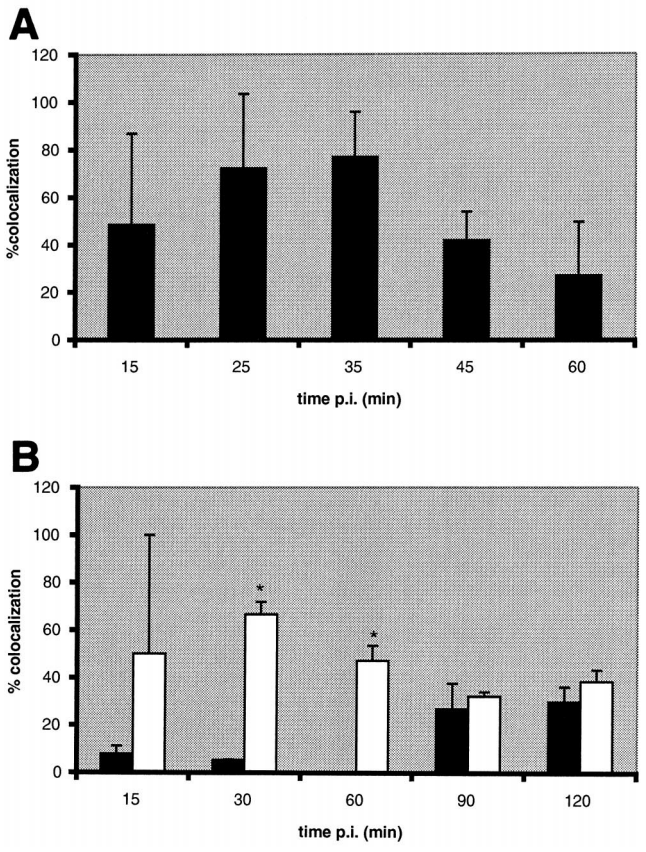


FIG. 5. Colocalization of *P. gingivalis* with protein markers of the endocytic pathway. HCAE cells were incubated with *P. gingivalis* 381 in the absence (solid bars) and presence (open bars) of 10 nM wortmannin for 15 to 120 min. The data represent the percentage of *P. gingivalis* vacuoles that contained Rab5 (A) and MPR (B), expressed as the mean + the standard deviation (error bars). The data suggest that the *P. gingivalis* vacuole acquires Rab5 early but does not acquire the late endocytic marker MPR. In the presence of wortmannin, a higher percentage of *P. gingivalis* vacuoles acquire MPR. The effects of wortmannin at each time point were evaluated by Student's *t* test utilizing Bonferroni's correction. Statistical significance (*) was achieved when P was <0.01.

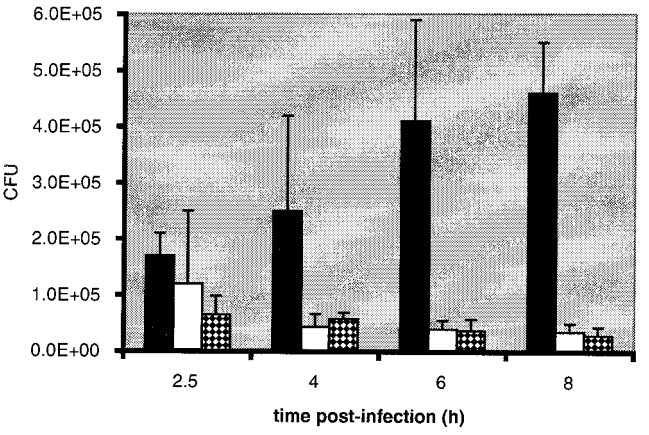


FIG. 6. Persistence of *P. gingivalis* within HCAE cells over 8 h. HCAE cells were preincubated for 1 h at 37°C with either fresh antibiotic-free EGM-2 (solid bars), 10 mM 3-methyladenine (open bars), or 10 nM wortmannin (checkered bars) ($n = 3$). *P. gingivalis* 381 cells were incubated with EGM-2 with or without the appropriate inhibitor. The number of CFU of HCAE cells infected with *P. gingivalis* in the absence of autophagy inhibitors grew over the 8 h, whereas the number of CFU of HCAE cells infected with *P. gingivalis* in the presence of either autophagy inhibitor declined over the 8 h. The number of CFU for *E. coli* MC1061, the negative control, at 2.5 h postinfection was <200 ($n = 3$).

GTP-binding protein that is a marker of early endosomes (26) (Fig. 3C), but few contained the late endosomal marker MPR (Fig. 3D). Within 15 min of invasion, 49% of the *P. gingivalis* vacuoles contained Rab5 (Fig. 5A). This value increased to >70% at 25 and 35 min postinfection and then decreased to 27% at 60 min. A majority of these vacuoles did not acquire the endosome marker MPR (Fig. 5B). This receptor is normally found in the Golgi apparatus and late endosomes or prelysosomes but not in autophagosomes (21, 25, 27, 35, 65). However, 46% of the bacteria colocalized with this receptor at the final 120-min time point in wortmannin-treated HCAE cells. This labeling is probably not due to its distribution to the Golgi apparatus, since α2,6 Gal β1,4-GlcNAc sialyltransferase did not colocalize to these vacuoles (data not shown). Similar results were obtained with Rap1, a Ras-related protein localized to late endosomes (48). At 90 min postinfection, 27% of the vacuoles contained Rap1, while as much as 67% (30 min postinfection) of the vacuoles contained Rap1 when the cells were treated with wortmannin (data not shown). These data suggest that the majority of *P. gingivalis* initially traffic to a Rab5 vacuole and then to the autophagosome. However, a few of the *P. gingivalis* cells reach a Rap1 vacuole. This is consistent with our observations that a minority of the internalized bacteria ultimately traffic to a cathepsin L-positive lysosome (Fig. 4D). When the autophagic pathway is blocked, the majority of the bacteria traffic from a Rab5 vacuole to a MPR vacuole and then to a cathepsin L vacuole.

Bacterial persistence. Since autophagy is a degradative pathway, endothelial cells could use this pathway as a defensive mechanism against invading bacterial aggregates. However, if the autophagosome is the intracellular niche of *P. gingivalis* (at least initially), then inhibition of autophagy should result in a decrease in the number of bacteria able to survive. A modification of the antibiotic protection assay was used to test the effects of the autophagy inhibitors 3-methyladenine and wortmannin on the persistence of *P. gingivalis* within HCAE cells.

The number of CFU of *P. gingivalis* recovered from untreated HCAE cells at 2.5 h postinfection was $(1.7 \pm 0.4) \times 10^5$ (Fig. 6). The number of CFU continued to increase from 4 to 8 h postinfection. At 8 h postinfection, the number of CFU was $(4.6 \pm 0.9) \times 10^5$, 2.7-fold higher than that seen at 2.5 h postinfection. When the HCAE cells were treated with 3-methyladenine or wortmannin, bacterial persistence decreased dramatically. At 8 h postinfection, the number of CFU from wortmannin-treated cells was 6.3% of that from untreated infected HCAE cells. The decreased persistence in the presence of these drugs is consistent with our immunofluorescence data that reveal the bacteria colocalizing with cathepsin L (Fig. 4D). This is also consistent with the ultrastructural analyses that showed degraded bacteria at later time points in 3-methyladenine- or wortmannin-treated HCAE cells. The

data suggest that these autophagy inhibitors prevented the establishment of the intracellular niche for the replication of *P. gingivalis*.

It is possible that 3-methyladenine or wortmannin may affect bacterial viability, adhesion, and/or invasion. Neither 3-methyladenine nor wortmannin inhibited the growth of *P. gingivalis* on blood agar plates (data not shown). The data presented here and reported by Sandros et al. show that internalization of *P. gingivalis* proceeds via endocytosis (52). Araki et al. and Reaves et al. have also demonstrated that the phosphoinositide 3-kinase (PI3-kinase) targeted by these drugs is not required for endocytosis (1, 49). Nevertheless, to allay concerns that these drugs may affect adherence and/or invasion, the HCAE cells were preincubated for 60 min with the inhibitors, at which time these drugs were withdrawn during the 90-min infection. The drugs were then reintroduced during the antibiotic incubation and were also present during the remainder of the incubation time (up to 6.5 h). These changes in the protocol did not appreciably alter the results in Fig. 6 (data not shown).

DISCUSSION

P. gingivalis is invasive of a variety of cells in vitro. The intracellular location of *P. gingivalis* has not been singularly defined within the variety of cells tested for invasion. Deshpande et al. have demonstrated that *P. gingivalis* resides within uncharacterized vacuoles, which may or may not be autophagosomes, in fetal bovine heart endothelial cells, bovine aortic endothelial cells, and human umbilical vein endothelial cells (16). *P. gingivalis* was reported free in the cytoplasm in gingival epithelial cells (33). Sandros et al. also found evidence of *P. gingivalis* free in the cytoplasm and within endosomes in KB cells, human pocket epithelium, and human junctional epithelium (44, 53, 54). The data suggest that the intracellular niche of *P. gingivalis* may be host cell specific. We have examined the trafficking of *P. gingivalis* in primary HCAE cells to better understand the proposed relationship between periodontal disease and coronary heart disease.

***P. gingivalis* traffics to the late autophagosome.** The data presented here are consistent with *P. gingivalis* being targeted to autophagosomes shortly after invasion of HCAE cells. We have shown that *P. gingivalis* cells are internalized into Rab5-positive vacuoles that rapidly acquire HsGsa7p. This likely represents the first step in the autophagic sequestration of *P. gingivalis*. The bacteria eventually traffic to a vacuole that contains both the RER protein BiP and the lysosomal protein LGP120 but is devoid of cathepsin L. This vacuole has been previously defined as a late autophagosome that contains RER and lysosomal membrane proteins but lacks hydrolytic enzymes (22). Then the late autophagosome containing *P. gingivalis* does not appear to acquire cathepsin L and thereby become an autolysosome, at least during the 2-h time course. The data suggest that *P. gingivalis* is sequestered into an early autophagosome which then matures into a late autophagosome, where the bacteria presumably replicate (Fig. 7A). These observations are substantiated by our ultrastructural observations. We observed the bacteria in a double-membrane-bound vacuole containing undegraded ribosomes and cytoplasmic ground substance. Profiles of dividing *P. gingivalis* can be observed in these vacuoles.

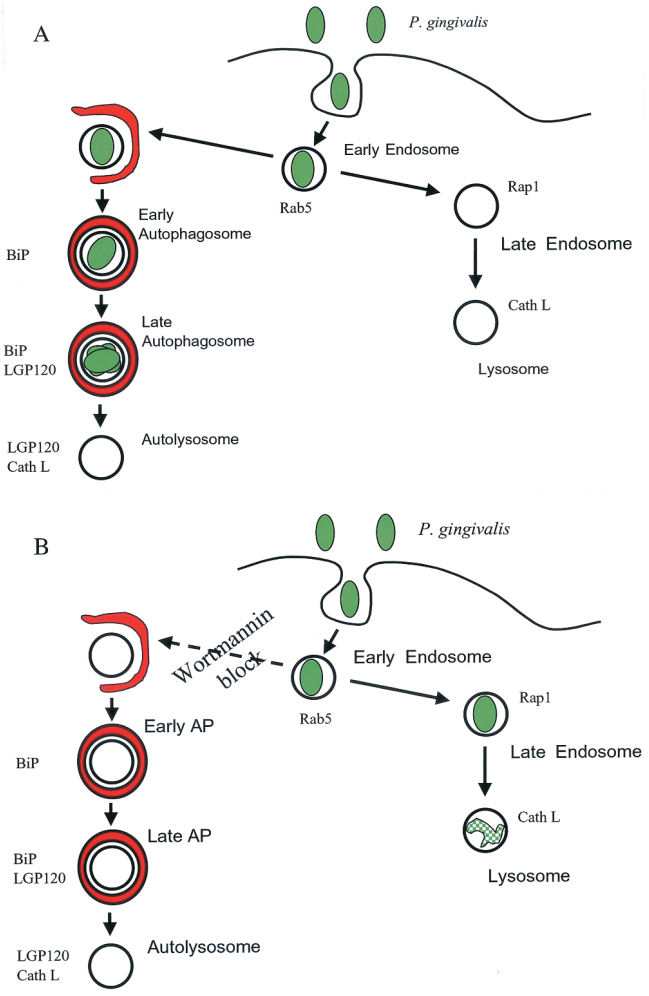


FIG. 7. Model of *P. gingivalis* trafficking in HCAE cells. *P. gingivalis* is initially found within an early endosome after internalization. (A) The bacteria promote their own entry into the autophagic pathway. The *P. gingivalis* 381-containing vacuole acquires BiP and later LGP120. However, this vacuole does not acquire cathepsin L. (B) Upon inhibition of autophagy with wortmannin, *P. gingivalis* enters the endocytic pathway. The vacuole matures into a late endosome and then a lysosome, as characterized by the presence of Rap1 and cathepsin L.

In contrast, when 3-methyladenine or wortmannin suppresses autophagy, internalized *P. gingivalis* cells transit a population of single-membrane-bound vacuoles that lack HsGsa7p and BiP and contain Rap1, MPR, and cathepsin L (Fig. 7B). The localization of *P. gingivalis* in cathepsin L-positive vacuoles is consistent with the profiles of degraded bacteria observed in these cells as well as the decrease in bacterial persistence within these cells. The ability of the HCAE cells to enter the endocytic pathway when autophagy is inhibited further suggests that this bacterium does not suppress the endocytic pathway but rather promotes its sequestration into a late autophagosome that provides a beneficial environment for its survival and growth.

Comparison to *L. pneumophila* and virulent *B. abortus*. Studies of *B. abortus* and *L. pneumophila* trafficking demonstrate several similarities to *P. gingivalis* intracellular trafficking. All

three bacterial species reside in vacuoles bound by multiple membranes surrounded by ribosomes (47, 66). The data are consistent with all three of these microorganisms evading the endocytic pathway and entering the autophagic pathway. Additionally, both virulent *B. abortus*- and *L. pneumophila*-containing vacuoles do not acquire Rab7, which is characteristic of late endosomes (46, 50). Both *P. gingivalis* and *B. abortus* are present in early endosome-like vacuoles as assessed by Rab5 and EEA1, respectively (46). There does exist a rapid convergence between autophagosomes and early endosomes (37, 69). However, *L. pneumophila* completely avoids the endocytic system and does not colocalize with Rab5 (13). Additionally, virulent *B. abortus* does not colocalize with MPR (46). Each of the three wild-type, bacterial species also fails to traffic to vesicles that fuse with lysosomal hydrolases, but vesicles containing attenuated *B. abortus* or growth mutants of *L. pneumophila* fuse with lysosomes (47, 72).

Although very similar, there are some differences among these three species in colocalization with components of the autophagic pathway. All three bacteria are found in vacuoles surrounded by RER, but *B. abortus* does not colocalize with BiP or ribophorin (46). However, *B. abortus* colocalizes with two other RER proteins, s61β and the protein disulfide isomerase. Both *L. pneumophila* and *P. gingivalis* are present in vacuoles that colocalize with BiP (66). Vacuoles containing *P. gingivalis* acquire LGP120, and virulent *B. abortus*-containing vacuoles acquire both LAMP1 and LAMP2 (46). However, the majority of *L. pneumophila* phagosomes are negative for LGP120 during its interaction with the autophagic pathway (50, 67). From the data presented elsewhere and here, we propose that vacuoles containing *P. gingivalis* and virulent *B. abortus* mature into late autophagosomes whereas the vacuoles containing *L. pneumophila* remain early autophagosomes. Although these three bacteria have evolved similar strategies to survive in the host, there appear to be some differences in the exact mechanism of trafficking through the autophagic pathway.

Bacterial survival. The specific genes of *P. gingivalis* required for its intracellular survival have not yet been identified. However, studies have indicated specific mechanisms by which the other two species survive intracellularly. For example, the functional secretion systems Icm-Dot and VirB in *L. pneumophila* and *B. abortus*, respectively, are necessary for their intracellular replication (8, 61). When the replication vacuole and host cell are depleted of amino acids, *L. pneumophila* converts to a virulent phenotype (29). The virulence traits, e.g., motility and cytotoxicity, allow the bacteria to escape the host cell and infect adjacent cells to start this cycle anew. Similar to *P. gingivalis*, *L. pneumophila* uses short peptides and amino acids as its carbon and energy source (24). We expect that *P. gingivalis* has evolved similar mechanisms to survive, which we are just beginning to elucidate.

Regulation of autophagy. Our data suggest that *P. gingivalis* promotes its entry into the autophagosome. At this time, we are uncertain whether this is directly mediated by the bacterium or a consequence of cellular signals transduced during invasion. A functional secretion system in both *L. pneumophila* and *B. abortus* is necessary for the establishment of an intracellular niche divergent from the endocytic pathway (14, 15). Once established within this replication vacuole, the Icm-Dot secretion system is no longer expressed (9). This suggests that a secreted protein interacts with the host cell to induce autophagy and is essential for the biogenesis of the replication vacuole. However, the precise mechanism for the sequestration of the bacterium into the autophagosome-like vacuole is unknown.

A bacterial protein may regulate autophagy at several different points. For example, it could activate the class III PI 3-kinase or cause the redistribution of HsGsa7p. In mammalian cells, the class I PI 3-kinase has been shown to negatively regulate autophagy, whereas the class III PI-3 kinase is a positive regulator (45). Wortmannin and 3-methyladenine inhibit autophagy by suppressing the class III PI 3-kinases (5, 59, 70). In addition to the kinase regulation, an amide linkage between the C terminus of Apg12p and an ε-amino group of Apg5p has been shown to be required for autophagosome formation in *Saccharomyces cerevisiae* (60). An ubiquitin E1-like activating enzyme that has been identified in *S. cerevisiae* (Apg7p), *Pichia pastoris* (Gsa7p), and humans (HsGsa7p [also known as hAPG7]) activates Apg12p (32, 68, 73). The conjugation of the activated Apg12p to Apg5p is performed by Apg10p, a ubiquitin E2-like conjugation enzyme (60). The human homologues for Apg10p and Apg5p have been identified (55). Thus, this conjugation process appears to be conserved throughout eukaryotes, including humans (41, 42).

Another possible mechanism could be the inactivation of TOR (target of rapamycin) or the class I PI-3 kinase. TOR, a phosphatidylinositol kinase, is a negative regulator of autophagy in *S. cerevisiae* (43). It is believed that rapamycin stimulates autophagy by inhibiting TOR in yeast and RAFT (rapamycin and FKBP12 target) or FRAP (FKBP12 and rapamycin-associated protein) in mammalian cells, thereby suppressing phosphorylation of the ribosomal protein S6 (6, 56). TOR stimulates the expression of some genes and represses the expression of others under conditions of nutrient deprivation by controlling the repressor Ure2 and the transcription factor Gln3 (10). TOR phosphorylation of Gln3 enhances its binding to Ure2, thereby preventing transcription (4). Gln3 phosphorylation is also dependent on Tap42, which is phosphorylated by TOR (31). A bacterial protein could also interact at one or more of these steps in the autophagic pathway.

In summary, this study demonstrates that *P. gingivalis* traffics to late autophagosomes in primary HCAE cells. The ability to persist within HCAE cells and thus establish a chronic infection could exacerbate the immune response at sites along the vascular tree. Whether the *P. gingivalis* at the sites of atherosclerosis contributes to disease or constitutes a "bystander effect" has yet to be determined. This work is the beginning of a series of studies to investigate the cellular interactions between *P. gingivalis* and HCAE cells that could provide molecular explanations for the association between periodontal disease and cardiovascular disease.

ACKNOWLEDGMENTS

We are very grateful to and thank all of the people mentioned in Materials and Methods for their generous gifts of antibodies. We also thank Paul Gulig, Martin Handfield, Jeffrey Hillman, and William McArthur for useful discussion; Jacob Burks and Todd Barnash for assistance; Amy Perwien for consultations on statistics; the Optical Microscopy Facility of the Center for Structural Biology at the University of Florida Brain Institute for the use of the deconvolution

microscope; and R. Davis, S. Whittaker, and the University of Florida Electron Microscopy Core Laboratory of the Interdisciplinary Center for Biotechnology Research for the transmission electron microscopy.

This study was supported by NIDCR grant DE 07496 (A.P.-F.) and NSF grant MCB 9817002 (W.A.D.).

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Editor: B. B. Finlay