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Desulfovibrio spp. survive within KB cells and modulate inflammatory responses

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SUMMARY

Desulfovibrio **are sulfate-reducing anaerobic gram-negative rods that have been proposed as potential periodontopathogens. We investigated the capacity of *Desulfovibrio* to invade epithelial cells and induce cytokine secretion from these cells. *Desulfovibrio* strains were co-cultured with KB cells and counts of intracellular bacteria evaluated up to 3 days after infection. *Desulfovibrio desulfuricans* and *Desulfovibrio fairfieldensis* were able to survive within epithelial cells. Intracytoplasmic location of both bacterial species was confirmed by confocal laser scanning microscopy and transmission electron microscopy. Invasion was sensitive to nocodazole, an inhibitor of microtubule polymerization, but not to cytochalasin D, a microfilament inhibitor, suggesting that microtubule rearrangements were involved in the internalization of *Desulfovibrio* strains by KB cells. Infection by *Desulfovibrio* resulted in increased production of IL-6 and IL-8 by KB cells. The ability of *D. desulfuricans* and *D. fairfieldensis* to survive within oral epithelial cells and to modulate the epithelial immune response may contribute to the initiation and progression of periodontal diseases.**

INTRODUCTION

Periodontitis is a multifactorial disease involving complex interactions between various anaerobic bacteria and host cells, which may lead to periodontal tissue destruction. Epithelial cell invasion has been suggested to play an important role in the pathogenesis of periodontitis (Lamont & Yilmaz, 2002; Colombo *et al.*, 2007; Johnson *et al.*, 2008). Cell invasion may allow periodontopathogens to persist within oral epithelial cells, to evade the immune system, and possibly to disseminate through underlying tissues (Andrian *et al.*, 2004). So far, invasion potential has been shown *in vitro* and/or *in vivo* for several bacteria associated with periodontal diseases, including *Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Porphyromonas gingivalis*, '*Tannerella forsythia*', and *Treponema denticola* (Meyer *et al.*, 1991; Duncan *et al.*, 1993; Dorn *et al.*, 1998; Han *et al.*, 2000; Colombo *et al.*, 2007). While the interaction of all these pathogens with epithelial cells may contribute to the release of inflammatory mediators, it has been shown that the expression of various cytokines, including interleukin-1β (IL-1β), IL-6, and IL-8, by epithelial cells infected with *P. gingivalis* may be positively correlated with its invasive capacity (Eick *et al.*, 2006). Consequently, epithelial invasion may also have a direct impact on disease progression and the inflammatory processes.

Sulfate-reducing bacteria (SRB) are a diverse group of organisms that are found in the environment

but may also colonize the digestive tract of animals and humans (Gibson, 1990; Willis *et al.*, 1995; Zinkevich & Beech, 2000; Boopathy *et al.*, 2002). These organisms have been reported to be the causes of human infections including bacteremia and brain and liver abscesses, and may play a role in inflammatory bowel diseases (Watanabe *et al.*, 2007; Rowan *et al.*, 2009). Recent findings suggest that SRB may also be involved in periodontitis (Langendijk *et al.*, 1999, 2000; Langendijk-Genevaux *et al.*, 2001; Boopathy *et al.*, 2002; Loubinoux *et al.*, 2002; Vianna *et al.*, 2008). Different isolates of SRB have been isolated from the oral cavity, generally belonging to the genus *Desulfovibrio* (Langendijk *et al.*, 2001; Boopathy *et al.*, 2002; Loubinoux *et al.*, 2002). So far, *Desulfovibrio fairfieldensis* and, to a lesser extent, *Desulfovibrio desulfuricans*, are the two *Desulfovibrio* species that have been isolated from the oral cavity (Beerens & Tahon-Castel, 1965; Loubinoux *et al.*, 2002). However, their role in the pathogenesis of periodontitis remains unclear. To further investigate the potential pathogenic role of *D. fairfieldensis* and *D. desulfuricans*, we studied their potential ability to invade non-professional phagocytic cells and their capacity to induce inflammatory responses from these cells.

METHODS

Bacterial strains and tissue culture conditions

Strains used in this study were obtained from the American Type Culture Collection (ATCC, Rockville, MD): *D. desulfuricans* ATCC 29577, *D. desulfuricans* ATCC 27774, *D. fairfieldensis* ATCC 700045, and *P. gingivalis* ATCC 33277. All strains were stored at −80°C in Brucella broth containing 15% glycerol (weight/volume). Before testing, bacteria were grown anaerobically at 37°C on Brucella agar supplemented with 5% defibrinated sheep blood, for 24 h for *P. gingivalis* ATCC 33277 and 72 h for the *Desulfovibrio* strains.

The KB cell line ATCC CCL-17 (HeLa cells) was routinely cultured in flasks under a humidified atmosphere containing 5% CO_2 at 37°C in RPMI-1640 medium (Gibco, Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum, 1% l-glutamine (Gibco), 16 μg ml^{-1} amikacin, and 128 μg ml^{-1} amoxicillin.

Invasion assay

Invasion of epithelial cells was determined using an antibiotic protection and invasion assay as previously described by Han *et al.* (2000). For each strain, plate cultures were harvested, washed twice in phosphate-buffered saline (PBS) and suspended in unsupplemented cell culture medium at a concentration of 5×10^7 cells ml^{-1} determined by optical density. Bacterial suspensions (1 ml) were added to confluent KB monolayers (multiplicity of infection 100 bacteria per cell) in 24-well plates. After 2 h of incubation at 37°C, unattached bacteria were removed by three washes with unsupplemented RPMI-1640 medium. External adherent cells were killed by incubating the infected monolayers with fresh medium containing metronidazole (200 μg ml^{-1}) and gentamicin (300 μg ml^{-1}) for 1 h. The cell culture medium was removed and cells were washed three times with PBS. Intracellular bacteria were then released by lysis of the cells in sterile distilled water for 20 min. To assess intracellular persistence and multiplication of *Desulfovibrio*, monolayers infected with *D. fairfieldensis* ATCC 700045 and *D. desulfuricans* ATCC 29577 were further incubated in antibiotic-free and fetal calf serum-free medium for 24, 48, and 72 h. Before cell lysis, 0.1-ml aliquots of the supernatant were sampled and examined for the presence of extracellular bacteria. Serial dilutions of the lysates were performed in sterile 0.9% NaCl, and 0.1-ml aliquots of the dilutions were plated onto Brucella agar. Bacterial counts were expressed as colony-forming units (CFU) per well.

Inhibitor assays of internalization

Inhibitor assays were performed using cytochalasin D (Sigma, Saint Quentin Fallavier, France), an actin microfilament polymerization inhibitor, and nocodazole (Sigma), a microtubule polymerization inhibitor. Stock solutions of inhibitors were prepared in dimethylsulfoxide and frozen at −20°C before assays. These solutions were diluted in unsupplemented RPMI-1640 to obtain final assay concentrations of 1 μg ml^{-1} nocodazole and 10 μg ml^{-1} cytochalasin D. The KB cells were incubated with cytochalasin D for 30 min before bacterial contamination. Nocodazole was added to the monolayers for 1 h on ice, and then warmed to 37°C for 30 min before the addition of bacteria. Each inhibitor was present during the assay.

The invasion assay was conducted as previously described. For each *Desulfovibrio* strain tested, intracellular viable counts were determined at 2 h postinfection. At the concentrations used, inhibitors affected neither the viability of epithelial cells, as determined by examining the confluency of the monolayers and by trypan blue exclusion, nor the viability of bacterial strains as assessed by viable cell counting.

Electron microscopy

For each *Desulfovibrio* strain tested, ultrastructural studies of KB cells were performed at 2 h postinfection using transmission electron microscopy. Infected cell monolayers were fixed with 2.5% glutaraldehyde in 0.1 m cacodylate buffer (pH 7.4). Following rinses with cacodylate buffer, the samples were postfixed for 1 h in 0.1 m cacodylate buffer containing 1% osmium tetroxide. The monolayers were dehydrated using increasing concentrations of ethanol [30, 50, 70, 80, and 90% (5 min for each step), 100% (20 min, three times)], and three changes of propylene oxide (20 min for each step). The samples were placed in a propylene oxide-epoxy resin (Epikote 812; Consortium International Pharmaceutique et Chimique, Paris, France) for 1 h at room temperature before being placed in 100% epoxy resin overnight as described by Luft *et al.* (1961). Then, they were embedded in fresh epoxy resin for 3 d at 56°C. Ultrathin sections were cut on a Reichert OMU_3 ultramicrotome using a diamond knife, collected on copper grids, and stained with uranium acetate and Reynold's lead citrate. Sections were observed using a Philips CM12 M at 80 kV.

Generation of antibodies

Desulfovibrio fairfieldensis ATCC 700045 was cultured as previously described on supplemented Brucella blood agar for 72 h. Bacterial cells were washed three times with PBS, resuspended in sterile 0.9% NaCl at a final concentration of 10^8 cells ml^{-1} and finally inactivated using ultraviolet light. Inactivated bacteria were sent to Covalab Laboratory (Lyon, France) for the generation of polyclonal antibodies in rabbits. Specificity of the immune serum was evaluated by indirect immunofluorescence on *D. fairfieldensis* ATCC 700045, *D. desulfuricans* ATCC 29577, *P. gingivalis* ATCC 33277, *F. nucleatum* Roger, *Prevotella intermedia* ATCC 25611, and *Prevotella buccae* ATCC 33574. Briefly, a 50-μl sample of cell suspension of each strain was spotted onto a slide for immunofluorescence tests, air dried, and fixed in cold ethanol. Smears were incubated with antiserum used at dilutions of 1 : 100, 1 : 200, or 1 : 500 for 30 min at 37°C in a moist chamber. After three washes in PBS, fluorescein isothiocyanate (FITC) -conjugated goat anti-rabbit immunoglobulin G antibody (Sigma) was added and incubated for another 30 min. After being washed with PBS, slides were examined with an Olympus fluorescence microscope at × 400 magnification. Specific reactivity was observed up to a dilution of 1 : 500 for both *Desulfovibrio* strains. No cross-reactivity was found with any of the other strains tested.

Confocal scanning laser microscopy

KB cells were seeded in eight-well Labteck II chambers (Nunc, Dutscher, France) at a concentration of 5×10^4 cells $well^{-1}$ and incubated for 48 h. Before infection, KB cells were stained with 2 μm 1′-di-octadecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate ($DiIC_{16}$) (Molecular Probe, Invitrogen, Cergy Pontoise, France) in RPMI-1640 for 2 min. $DiIC_{16}$ is a lipophilic carbocyanine membrane probe suitable for labeling cell surface membranes as well as intracytoplasmic membranes (Mukherjee *et al.*, 1999). Invasion assays were performed with *D. fairfieldensis* ATCC 700045 and *D. desulfuricans* ATCC 29577 as described above. At 2 h postinfection, cells were washed three times with unsupplemented RPMI-1640 medium and fixed in 2% paraformaldehyde at 4°C for 15 min. After three washes with PBS, cell monolayers were permeabilized with 0.1% Triton X-100 for 5 min. Cells were washed twice and incubated in PBS containing 5% bovine serum albumin (Sigma) for 20 min at room temperature (Kuehnel *et al.*, 2001). After removing the supernatant, cells were incubated overnight with rabbit anti-*Desulfovibrio* serum diluted at 1 : 200. Cells were then incubated with an FITC-conjugated goat anti-rabbit immunoglobulin G (1 : 100) (Sigma) for 45 min followed by Alexa Fluor 488 anti-FITC-conjugated rabbit polyclonal immunoglobulin G (1 : 100) (Molecular Probe) for 45 min. Samples were observed with a confocal scanning laser

microscope (CLSM) SP2-AOBS (Leica Microsystems, Wetzlar, Germany), constituted by an inverted epifluorescence microscope equipped with an argon laser source at 488 nm and a helium/neon laser at 543 nm. The intensity of the fluorescence signal was measured between 500 and 520 nm for Alexa 488 (coded in green) and between 520 and 580 nm for DiI (coded in red) with a $\times$ 63 objective (water immersion/1.32 NA). For each frame, the collection of fluorescence signal was adjusted spectrally to avoid the overlapping of the two emission dyes with a sequential mode. Different controls were achieved to eliminate non-specific detections. Uninfected cells with antibodies I, II, and III, and infected cells with antibodies II, III were used as negative controls to determine the threshold value of positive detection. Images were achieved with a z-step of 0.2 μm with a format of 512 $\times$ 512 pixels, 400 Hz, and analysed with Leica software lcs. Images were pseudocolored according to their respective emission and overloaded with Leica/Metamorph software.

Cytokine induction assay

Culture media from 24, 48, and 72 h infected or uninfected KB cells were collected at the end of the experiments and stored at −20°C until assayed using enzyme-linked immunosorbent assay kits for IL-1β, IL-8 (RayBio, Norcross, GA), and IL-6 (Bender Medsystems, Vienna, Austria). Samples were processed according to the manufacturer's instructions. Plates were read at 450 nm using a microplate reader (Sunrise, Tecan, Lyon, France). The detection levels were 3, 8, and 0.98 pg ml^{-1} for IL-1β, IL-8, and IL-6, respectively. Cytokine concentrations were determined in triplicate by comparison with a standard curve prepared for each kit. Data were presented as means ± SD. Cytokine levels were compared using the unpaired Student's *t*-test (Graph Pad Software, San Diego, CA). Significances were defined as having $P < 0.05$.

RESULTS

Entry and survival of *Desulfovibrio* in KB cells

Two hours after infection, all three *Desulfovibrio* strains tested exhibited similar invasion efficiencies as assessed by the antibiotic protection assay (Table 1). However, the invasion efficiency of these strains (0.05–0.13% of the initial inoculum) was lower than those observed with *P. gingivalis* ATCC 33277 (0.69% of the initial inoculum), which was used as a positive control (Houalet-Jeanne *et al.*, 2001). For *D. fairfieldensis* ATCC 700045, the mean number of internalized viable bacteria was 3.5 times higher ($P < 0.01$) at 24 h postinfection than at 2 h postinfection, suggesting intracellular replication (Fig. 1). Thereafter, the viable intracellular counts gradually decreased ($P < 0.01$) down to about 57% of the initial intracellular count at 72 h postinfection. Similar results were found, as expected (Houalet-Jeanne *et al.*, 2001), for *P. gingivalis* ATCC 33277. For *D. desulfuricans* ATCC 29577, a statistically significant twofold increase ($P < 0.01$) of the mean number of viable intracellular bacteria occurred at 48 h. Thereafter, the intracellular counts decreased but remained slightly higher than those found at 2 h postinfection. The numbers provide evidence of an intracellular replication for *D. fairfieldensis* ATCC 700045 and a similar pattern is apparent for *D. desulfuricans* ATCC 29577 although the bacterial proliferation starts somewhat later. At no time, did the supernatant counts exceed 7% of the intracellularly recovered bacteria.

Table 1 Comparison of invasion of KB cells by *Desulfovibrio* spp. and *Porphyromonas gingivalis*

Strain	CFU well^{-1} recovered after antimicrobial treatment[1]
D. fairfieldensis ATCC 700045	(1.53 ± 0.6) $\times$ 10^4
D. desulfuricans ATCC 29577	(6.5 ± 2) $\times$ 10^4
D. desulfuricans ATCC 27774	(3.81 ± 0.44) $\times$ 10^4
P. gingivalis ATCC 33277	(3.86 ± 0.17) $\times$ 10^5

CFU, colony-formig units.

[1]Values represent the means ± standard deviations from quadruplicate independent determination 2 h postinfection.

Tubulin-dependent KB cells invasion by *Desulfovibrio* strains

Entry of bacteria into host cells requires microtubule or microfilament rearrangements (Meyer *et al.*, 1997). To assess whether internalization of *Desulfovibrio* strains is actin- and/or tubulin-dependent, we evaluated the effects of cytochalasin D and nocodazole

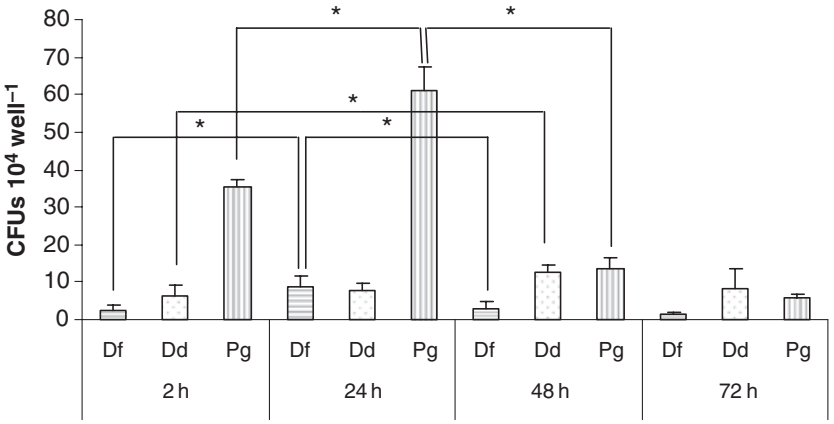

Figure 1 Intracellular viability of *Desulfovibrio desulfuricans* ATCC 29577, *Desulfovibrio fairfieldensis*, and *Porphyromonas gingivalis* ATCC 33277. Values represent the means ± SD from triplicate independent determinations at 2, 24, 48, and 72 h postinfection. Differences are statistically significant between connected values (*$P < 0.01$).

Table 2 Effects of metabolic inhibitors on invasion of KB cells by *Desulfovibrio desulfuricans* and *Desulfovibrio fairfieldensis*

	Percentage of invasion after treatment by[1]	
Strains	Nocodazole	Cytochalasin D
D. fairfieldensis ATCC 700045	23.3 ± 3.8	237.5 ± 39.2
D. desulfuricans ATCC 29774	52.6 ± 1.9	138.3 ± 11.6
D. desulfuricans ATCC 27774	10.7 ± 1.8	139.6 ± 11

Values represent the means ± standard deviations from triplicate independent determination 2 h postinfection.
[1]The level of invasion was expressed as a percentage of the control level without any inhibitor.

pretreatment of KB cells on the internalization of the three *Desulfovibrio* strains tested. Treatment of the KB cells with cytochalasin D resulted in increased numbers of internalized organisms at 2 h postinfection (Table 2). In contrast, for all three *Desulfovibrio* strains tested, the counts of internalized viable bacteria at 2 h postinfection were lower in nocodazole-pretreated KB cells than in control cells, indicating that microtubule formation was required for cell invasion.

Intracytoplasmic localization of *Desulfovibrio* in KB cells

Once inside host cells, invasive bacteria replicate within endosomal organelles or escape from the endosomal pathway to the cytoplasm. At 2 h postinfection, TEM examination of KB cells infected with either *D. fairfieldensis* or *D. desulfuricans* showed that numerous bacteria adhered to the cell surface

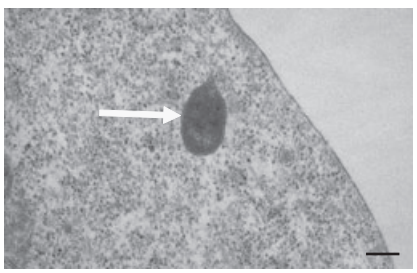

Figure 2 Transmission electron micrograph of *Desulfovibrio desulfuricans* ATCC 29577 (arrow) inside the cytoplasm of a KB cell, at 2 h postinfection. Bar: 1 μm.

while all observed intracellular bacteria appeared to be free in the cytoplasm without surrounding membranes (Fig. 2). The intracytoplasmic localization of both organisms tested was further confirmed by using confocal microscopy. Indeed, while numerous immunolabeled bacteria were shown to adhere to the epithelial cell surface 2 h postinfection, a few bacteria could be visualized within the cytoplasm of KB cells (Fig. 3). Internalized bacteria could be clearly differentiated from adherent bacteria by superimposing FITC-Alexia 488 and DiI images of serial sections (0.2 μm). No colocalization of intracellular immunolabeled bacteria with intracytoplasmic DiI-stained vesicles was observed suggesting that the observed intracellular bacteria were free in the cytoplasm.

Production of cytokines by infected KB cells

The levels of IL-1β, IL-6, and IL-8 secreted by KB cells after infection with *D. desulfuricans* 29577 ATCC and *D. fairfieldensis* ATCC 700045 were also

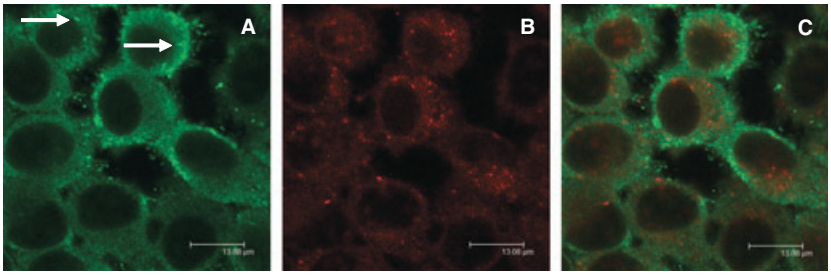

Figure 3 Invasion of KB cell by *Desulfovibrio fairfieldensis* ATCC 700045 visualized by CSLM. (A) *D. fairfieldensis* were stained green (arrow); (B) intracytoplasmic vesicles stained with DiI appeared red; (C) digital overlapping of images (A) and (B). No vesicles were colocalized with intracytoplasmic bacteria.

measured. KB cells secreted significantly higher amounts of IL-8 at 2 h after infection with *D. fairfieldensis* ATCC 700045 (642 ± 65 pg ml^{-1}) or *D. desulfuricans* 29577 ATCC (744 ± 121 pg ml^{-1}) than uninfected KB cells (356 ± 73 pg ml^{-1}) (Fig. 4). This difference was no longer observed at 24, 48, and 72 h after infection with any of the strains tested. No IL-6 was detected in the supernatants of uninfected or infected cells at the 2-h time-point. KB cells constitutively expressed low levels of IL-6 at 24 h (2 ± 0.6 pg ml^{-1}), 48 h (2.4 ± 0.9 pg ml^{-1}), and 72 h (4.48 ± 1.9 pg ml^{-1}). In contrast, the amount of IL-6 released from KB cells infected with *D. desulfuricans* ATCC 29577 was significantly increased compared with the control cells at 24 h (4.1 ± 0.4 pg ml^{-1}) and 48 h (5.7 ± 0.7 pg ml^{-1}) (Fig. 5). When compared with uninfected cells, an increase, although not statistically significant, of IL-6 production was also observed for KB cells at 24 h (4.2 ± 1.5 pg ml^{-1}) and 48 h (4.7 ± 1.2 pg ml^{-1}) ($P = 0.07$) after infection with *D. fairfieldensis* ATCC 700045. For both strains, IL-1β concentrations in supernatants from infected KB cells were found to be similar at each time-point studied to those measured in supernatants from control uninfected cells.

DISCUSSION

In the present study, we have shown that two putative periodontopathogens, *D. fairfieldensis* and *D. desulfuricans*, have the ability to invade oral epithelial cells. The intracellular location of *D. fairfieldensis* ATCC 700045 and *D. desulfuricans* ATCC 29577 was confirmed by both TEM and CSLM examination of infected monolayers. For these strains, 0.05–0.13% of the total number of bacteria initially added were recovered intracellularly at 2 h postinfection. For *P. gingivalis* ATCC 33277, 0.69% of the initial inoculum was recovered after lysis of the infected KB cells, which is very close to the 0.6% obtained by

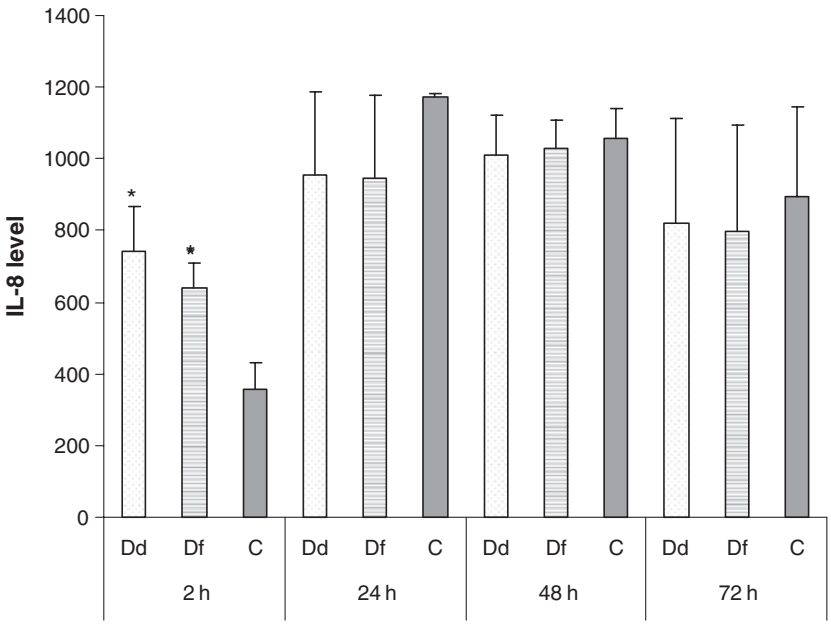

Figure 4 Secretion of interleukin-8 (IL-8) by KB cells at 2, 24, 48, and 72 h after infection with *Desulfovibrio desulfuricans* ATCC 29577 (Dd) and *Desulfovibrio fairfieldensis* ATCC 700045 (Df). The bars indicate the means and the standard deviations from three independent experiments. Statistically significant ($P < 0.01$) induction of cytokine production compared with control epithelial cells (C).

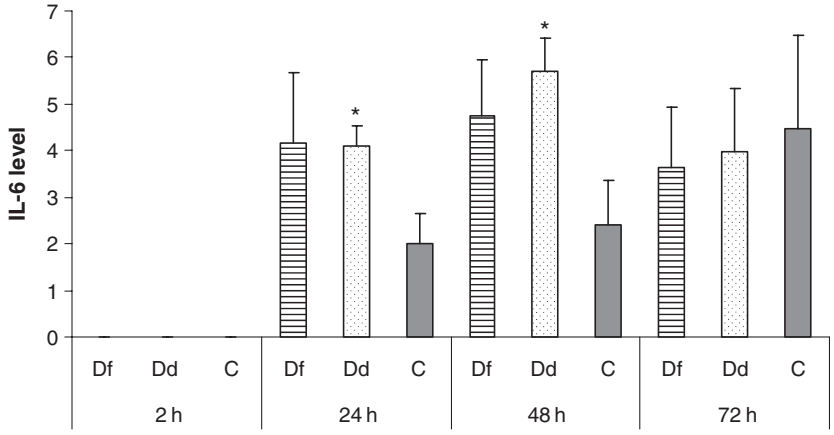

Figure 5 Secretion of interleukin-6 (IL-6) by KB cells at 2, 24, 48, and 72 h after infection with *Desulfovibrio desulfuricans* ATCC 29577 (Dd) and *Desulfovibrio fairfieldensis* ATCC 700045 (Df). The bars indicate the means and the standard deviations from three independent experiments. Statistically significant ($P < 0.01$) induction of cytokine production compared with control epithelial cells (C).

Houalet-Jeanne *et al.* (2001) who examined the invasive potential of this isolate using similar experimental conditions. In our study, early internalization levels observed with *D. fairfieldensis* and *D. desulfuricans* were 5- to 15-fold lower than those observed with *P. gingivalis*. However, to investigate the possibility that invasion profiles are strain-specific more strains need to be evaluated. Similar levels of internalization were reported by Han *et al.* (2000) for another putative periodontopathogen, *P. intermedia*, the invasion level of which has been reported as being 1/20 of that of *P. gingivalis* 381 and *F. nucleatum* 12230. Consequently, the *Desulfovibrio* spp. strains tested appeared to be weakly invasive. However, it cannot be ruled out that other more invasive *Desulfovibrio* strains may exist or that interactions with other oral bacteria may enhance the invasion potential of *Desulfovibrio* as shown for '*T. forsythia*' interacting with *P. gingivalis* (Inagaki *et al.*, 2006). Also, it must be stressed that KB cells, which are transformed cells, are less efficient than epithelial cells from primary gingival tissue cultures (Belton *et al.*, 2004).

As previously reported by Houalet-Jeanne *et al.* (2001), we also observed that for *P. gingivalis* ATCC 33277, the number of intracellular viable bacteria increased about 1.5-fold at 24 h postinfection in infected KB cells. Quite similar results were also reported with another *P. gingivalis* strain (*P. gingivalis* FDC381) by Madianos *et al.* (1996), suggesting that *P. gingivalis* has the ability to multiply within KB cells. This could be of great clinical importance because Johnson *et al.* (2008) have demonstrated the presence of *P. gingivalis* within buccal epithelial cells. This suggests a mechanism by which bacteria might be protected from the host response and antimicrobial therapy, enabling them to re-colonize debrided sites and perhaps contribute to the onset of refractory disease. Likewise, host cells could constitute a reservoir of *Desulfovibrio*. In the present study, the average number of intracellular viable bacteria increased during the first 24 h for *D. fairfieldensis* ATCC 700045 and during the first 48 h for *D. desulfuricans* ATCC 29577. These findings suggest that *Desulfovibrio* is able to multiply within KB cells. Preliminary experiments permitted us to ensure that the antibiotic concentrations used were effective in killing the entire bacterial inoculum within 1 h and that supernatant bacterial counts remained low (≤7% of the intracellular counts) during the whole study period. Therefore, the possibility of an invasion artefact is unlikely. However, the fact that some intracellular organisms may exit the initially infected cells and then enter and multiply in new cells, as shown for other periodontopathogens invading oral epithelial cells such as *P. gingivalis* and *A. actinomycetemcomitans* (Meyer *et al.*, 1999; Yilmaz *et al.*, 2006), cannot be ruled out and needs further study.

In our study, TEM examination revealed that, at 2 h postinfection, all intracellular *Desulfovibrio* organisms appeared free within the cell cytoplasm. Moreover, colocalization of intracellular bacteria within intracytoplasmic vesicles was not found, suggesting that *Desulfovibrio* may escape the vacuole in early stages of invasion as described for other pathogens (Meyer *et al.*, 1997; Houalet-Jeanne *et al.*, 2001).

For many bacterial pathogens, internalization by epithelial cells is dependent on rearrangements of the actin skeleton. Additionally, some pathogenic bacteria enter epithelial cells via a microtubule-

dependent mechanism (Yoshida & Sasakawa, 2003). Among periodontopathogens, *P. gingivalis* and *F. nucleatum* have been shown to enter epithelial cells via both a microtubule-dependent and actin-dependent mechanism (Lamont *et al.*, 1995; Han *et al.*, 2000). It has also been reported that a few strains of *A. actinomycetemcomitans* utilize microtubule-dependent mode of invasion whereas most strains use an actin-dependent mode of entry into epithelial cells (Brissette & Fives-Taylor, 1999; Meyer *et al.*, 1999). In the present study, treatment with nocodazole, a microtubule-destabilizing agent, resulted in a significant inhibition of bacterial invasion whereas internalization was enhanced in cells pretreated with cytochalasin D. These data suggest that internalization of all three *Desulfovibrio* strains tested was microtubule-dependent. It is noteworthy that invasion of these strains was increased by cytochalasin D. Similar observations were reported for other organisms that require microtubule rearrangement for invasion. This may be related to the fact that cytochalasin D enhances lysosomal movement to the cell periphery and hence invasion because of the lysosome recruitment and fusion at the plasma membrane (Tardieux *et al.*, 1992).

Besides bacterial internalization, inflammation appears to be another important factor that may be involved in the progression of periodontal infections and local tissue destruction. It has been shown that the production of various cytokines, including IL-6 and IL-8, by oral epithelial cells following stimulation by periodontopathogens is an early event which participates in the cascade of events leading to inflammation and tissue destruction, and hence is critical for the progression of periodontitis (Graves, 2008). *In vitro* studies have suggested that cytokine production by epithelial cells may be triggered by their invasion by various bacterial pathogens including *P. gingivalis* (Sandros *et al.*, 2000; Eick *et al.*, 2006). Weglarz *et al.* (2007) demonstrated that *D. desulfuricans* has the ability to induce the secretion of IL-6 and IL-8 from the human colon cell line Caco-2. In our study, both *Desulfovibrio* strains tested stimulated the production of these cytokines by KB cells. A significant increase in IL-8 secretion by epithelial cells was observed at 2 h post-infection. This effect may be the result of various mechanisms triggered by bacterial adhesion or invasion and may play a role in the local immune response as IL-8 acts as a chemokine directing neutrophil migration to the infectious site. It cannot be excluded that soluble products from *Desulfovibrio* strains directly stimulated epithelial cells and induced upregulation of IL-8, as described for *E. corrodens* (Yumoto *et al.*, 2007). In contrast, increased production of IL-6 was only detected at 24 and 48 h after infection with *D. desulfuricans* ATCC 29577 and, to a lesser extent, with *D. fairfieldensis* ATCC 700045, suggesting that this epithelial response might be more predominantly associated with the presence of intracellular bacteria. IL-6 has also been identified as a periodontal disease marker and its release by host cells may participate in the inflammatory reaction as well as in tissue destruction via the activation of osteoclasts (Moreira *et al.*, 2007; Herman *et al.*, 2008).

In conclusion, this study provides the first evidence that *D. fairfieldensis* and *D. desulfuricans*, two putative periodontopathogens, may invade oral epithelial cells via a microtubule-dependent process. We have also shown that, once inside the cells, these bacteria had the capacity to remain viable for prolonged periods and were able to elicit a minor host cell response that may contribute to the development of periodontal disease. Further studies of these bacterium–host cell interactions are necessary to increase our insight into the role of sulfate-reducing bacteria in periodontitis.

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