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Porphyromonas gingivalis survives within KB cells and modulates inflammatory response

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Eick S, Reißmann A, Rödel J, Schmidt K-H, Pfister W. Porphyromonas gingivalis *survives within KB cells and modulates inflammatory response.*
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Background/aims: The purpose of the study was to investigate the intracellular survival of *Porphyromonas gingivalis* as a possible mechanism for maintaining periodontitis.
Methods: *P. gingivalis* strains, the strain ATCC 33277 and seven clinical isolates, were co-cultured with KB cells. The number of intracellular bacteria was determined up to 3 days after infection. In addition, the numbers of KB cells per well, the concentrations of the cytokines interleukin-1β (IL-1β), IL-6, IL-8 and tumour necrosis factor-α (TNF-α) and the arginine-specific amidolytic activity were measured. The 16S rRNA of *P. gingivalis* and the mRNA expression of IL-1β, IL-6, IL-8, TNF-α and *rgpA* were also determined.
Results: All the *P. gingivalis* strains studied were able to survive within KB cells. In contrast to the reduced values of colony-forming units at day 3, equal and higher levels of 16S rRNA were seen in comparison to day 0. Arginine-specific amidolytic activity declined in all samples during infection. Expression of mRNA for *rgpA* was not found after infection of KB cells by *P. gingivalis* strains. IL-8 was detectable in all samples 2 days after infection with *P. gingivalis* strains. Principal components analysis underlined a correlation between the arginine-specific amidolytic activity 1 h after infection and both the released IL-8 and the mRNA expression of IL-8. Associations were found between the cultivable numbers of intracellular *P. gingivalis* and the mRNAs of IL-1, IL-6 and TNF-α at the day of infection.
Conclusion: The results indicate survival of *P. gingivalis* within epithelial cells, possibly in a non-cultivable stage. Invasion into cells modulates the virulence properties of *P. gingivalis* as well as the inflammatory response of the cells.

Key words: cytokines; epithelial cells; intracellular survival; *Porphyromonas gingivalis*

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Strong evidence has been accumulated to implicate *Porphyromonas gingivalis* in the pathogenesis of periodontitis (9, 12). Most of the patients are colonized by a single genotype, detectable in healthy and diseased sites and distinct from other subjects (33). Even after treatment the patient remains colonized by his own genotype (36), indicating bacterial persistence. *P. gingivalis* possesses at least three major virulence factors – fimbria (1), lipopolysaccharide (2) and gingipains (15). Gingipains, extracellular cysteine proteinases, are the products of three genes, two encode arg-specific proteinases (RgpA and RgpB) and one encodes lysine-specific proteinases (Kgp) (25). Lipopolysaccharides and fimbriae stimulate secretion of inflammatory mediators, such as interleukin-1β (IL-1β), IL-6, IL-8 and tumour necrosis factor-α (TNF-α), whereas gingipains can cleave IL-1β, IL-6, IL-8 (3, 11) and their receptors are essential for activation (22, 32).

Adhesion to and invasion of epithelial cells are essential steps in pathogenesis. Studies *in vitro* clearly demonstrated *P. gingivalis* invasion into transformed and primary epithelial cells (6, 29). This invasion is a rapid and efficient process,

reaching completion after 20 min. Only viable bacteria are capable of invasion, and intracellular bacteria congregate in the perinuclear region of epithelial cells (4). Invasion of epithelial cells by *P. gingivalis* is correlated with activation of $[Ca^{2+}]$-dependent host signalling systems (16). Examinations of tissue samples confirmed an intracellular location of the species within buccal and gingival epithelial cells *in vivo* (17, 27). On the one hand, our studies using the reference strain *P. gingivalis* ATCC 33277 and clinical isolates obtained from patients with severe periodontitis showed differences in their ability to adhere to and to invade KB cells. The low levels of the inflammatory interleukins IL-6 and IL-8 and the undetectable cytotoxicity are indicators of the bacterial ability to evade the local immune response and of survival within epithelial cells (8).

Therefore, the purpose of the present study was to investigate the intracellular survival of *P. gingivalis* within epithelial cells as a possible mechanism for maintaining periodontal disease. Moreover, the influence of the uptake and survival of different *P. gingivalis* strains in KB cells on mRNA expression and on the release of proinflammatory cytokines over 72 h was determined.

Materials and methods

Eight *P. gingivalis* strains, the reference strain ATCC 33277 and seven clinical isolates, were included in the study. The genetic diversity had been checked by random amplified polymorphic DNA analysis (Ready-To-Go RAPD Analysis beads; Amersham Biosciences, Freiburg, Germany), using a single short oligonucleotide primer of arbitrary sequence in a polymerase chain reaction (PCR) for each strain. The clinical isolates were obtained from patients with severe chronic periodontitis and maintained at −80°C until used. Identification was made by colony morphology and biochemistry and was confirmed by PCR. The strains were subcultivated for 16 h on Schaedler agar enriched with 10% sheep blood and vitamin K, harvested, washed twice with phosphate-buffered saline (PBS) and resuspended in Medium 199 (Gibco Life Technologies Ltd, Paisley, UK) to an optical density of 0.1 at 660 nm, which is equivalent to 10^8 bacteria/ml.

KB cells (epithelial cell line, ATCC CCL 17) were seeded into two six-well tissue culture plates at a density of about 5×10^5 cells/well, the cells were grown to a just confluent monolayer in Eagles' modified essential medium (EMEM; Bio-Whitacker, Verviers, Belgium) enriched with 10% fetal calf serum (Gibco Life Technologies; FCS). Then the cells were washed twice with PBS. The bacterial suspension in Medium 199 was mixed with EMEM 1 : 9 and each well was inoculated with 3 ml of this final suspension (about 20 bacteria per KB cell). Bacteria were co-cultured with KB cells at 37°C for 1 h. After incubation the monolayers were washed five times with PBS. EMEM supplemented with 10 μg/ml metronidazole (Braun, Melsungen, Germany) was added to the other plate for 1.5 h to kill the extracellular adherent bacteria. In preliminary experiments this antibiotic concentration and time of exposure were proven to be sufficient to kill most of extracellular, but not intracellular, bacteria. After that, this medium was removed and the cells were washed three times with PBS. From one line the number of intracellular bacteria was determined by cell lysis and subsequent cultivation. EMEM with 10% FCS was added to the other lines. The procedure was repeated on days 1, 2 and 3. In addition, suspension was taken, centrifuged at 400 **g** for 4 min to separate detached cells and subsequently samples of the supernatants were subcultivated to determine the number of free *P. gingivalis*.

Determination of the number of KB cells

After three-fold washing and removal of all floating cell components, 100 μl tryp-

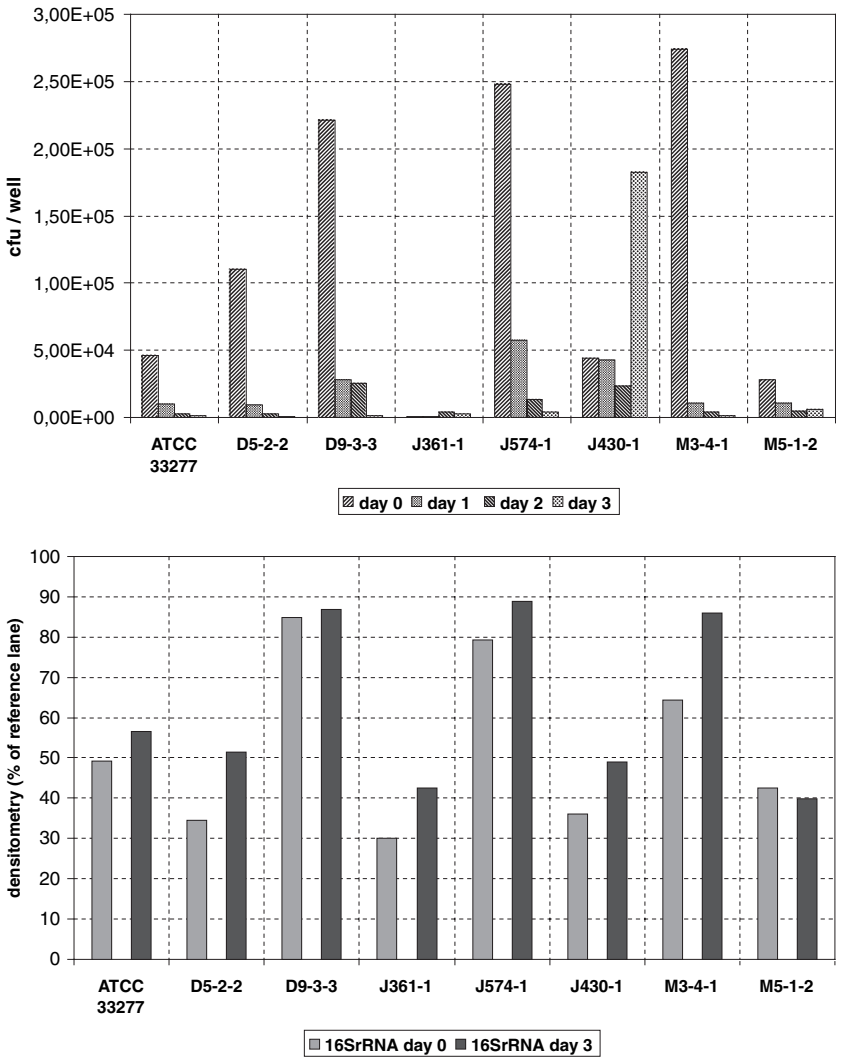

Fig. 1. Number of intracellularly cultivable bacteria and densitometry values of 16S rRNA of *Porphyromonas gingivalis* ATCC 33277 and different clinical isolates immediately, 1 day, 2 days and 3 days after infection. (16S rRNA immediately and 3 days after infection).

sin/EDTA (BioChrom AG, Berlin, Germany) was added to each well to detach the cells. Then, 250 µl EMEM supplemented with 10% FCS was added to stop the trypsin activity. The suspension was centrifuged at 400 **g** for 4 min and the supernatant was replaced by 250 µl fresh EMEM with 10% FCS. Finally, after spinning, the numbers of cells were determined on a Casy model TT cell counter (Schärfe System GmbH, Reutlingen, Germany) according to the manufacturer's instruction.

Cytokine assays

Six-well tissue plates with KB cell monolayers were infected by *P. gingivalis* as described above. First, immediately after the 1-h infection period the culture medium was collected and centrifuged at 10,000 **g**, and the supernatant was stored at −20°C until assayed. Each day the FCS-containing medium was removed and replaced by EMEM without FCS to avoid side effects caused by FCS. After an incubation of 6 h the media were obtained and handled in the same way. The concentrations of the cytokines IL-1β, IL-6, IL-8 and TNF-α were determined using commercially available enzyme-linked immunosorbent assay kits (Biosource, Ratingen, Germany) as described in the manufacturer's instructions. The detection level of all kits was about 2 pg/ml.

Determination of arginine-specific cysteine protease activity

Six-well tissue plates with KB cell monolayers were infected with *P. gingivalis*. The arginine-specific amidolytic activities of *P. gingivalis* proteases were assayed at 37°C with 0.5 mM *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA; Sigma-Aldrich, Taufkirchen, Germany) in 1.0 ml of 0.2 M Tris–HCl, 0.1 M NaCl, 5 mM $CaCl_2$, 10 mM cysteine, pH 7.6 (24). For this, both bacterial suspensions before addition to the cells and cell cultivation media obtained after incubation times of 1 and 6 h were screened. Additionally after 24, 48 and 72 h the cell cultivation medium containing FCS was replaced by EMEM without FCS for 6 h. After that time, the medium was also used for analysis.

Non-infected cells (negative control) and 10 nM high-molecular-mass RgpA (HRgpA; positive control) served as controls. The purified HRgpA was a gift from J. Potempa (Jagiellonian University of Krakow, Poland).

RNA extraction and RT-PCR

Total RNA from approximately 2×10^6 KB cells was purified using the RNeasy kit (Quiagen GmbH, Hilden, Germany) and cDNA was synthesized from 1 µg of total RNA using the Omniscript kit (Quiagen GmbH) according to the manufacturer's instruction. For PCR analysis, 2.5 µl cDNA in a total volume of 25 µl was used. Oligonucleotide primers were used at a final concentration of 5 nM. The oligonucleotide primers were as follows: IL-1β (10) 5′- TAC GAA TCT CCG ACC ACC ACT ACA G-3′ (forward), 5′-TGG AGG TGG AGA GCT TTC GTT CAT ATG-3′ (reverse); IL-6 (10) 5′-AGC TCA GCT ATG AAC TCC TTC TC-3′ (forward), 5′-GTC TCC TCA TTG AAT CCA GAT TGG-3′ (reverse); TNF-α (10) 5′-CGG GAC GTG GAG CTG GCC GAG GAG (forward), 5′-CAC CAG CTG GTT ATC TCT CAG CTC-3′ (reverse); β-actin (10) 5′-ATT GCC GAC AGG ATG CAG AA-3′ (forward), 5′-GCT GAT CCA CAT CTG CTG GAA-3′ (reverse); IL-8 (37) 5′-CTT GGC AGC CTT CCT GAT TT-3′ (forward), 5′-CAG CCC TCT TCA AAA

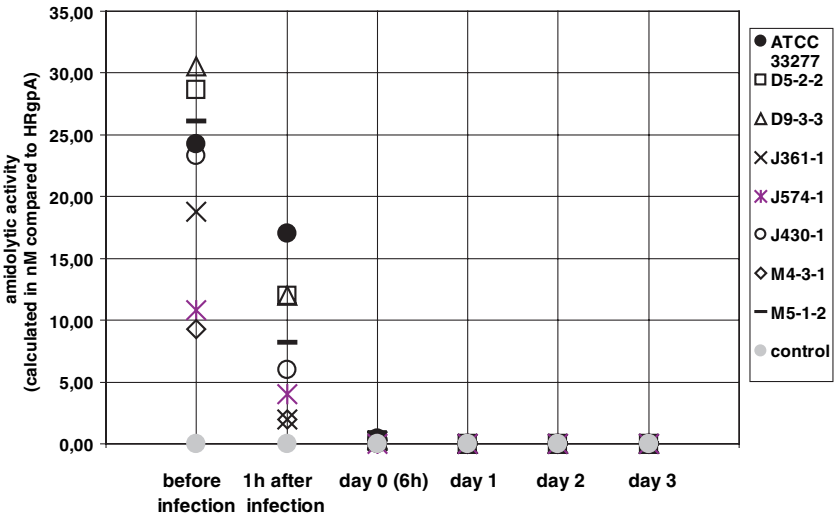

Fig. 2. Levels of released, non-cell-bound arginine-specific cysteine proteases immediately before and after infection (1 h after infection, after replacing the media after 6 h on the day of infection (day 0), and 1, 2 and 3 days after infection) with *Porphyromonas gingivalis* ATCC 33277 and different clinical isolates. Amidolytic activity was determined by BA*p*NA and calculated in comparison with 10 nM HRgpA. Controls resemble uninfected cells.

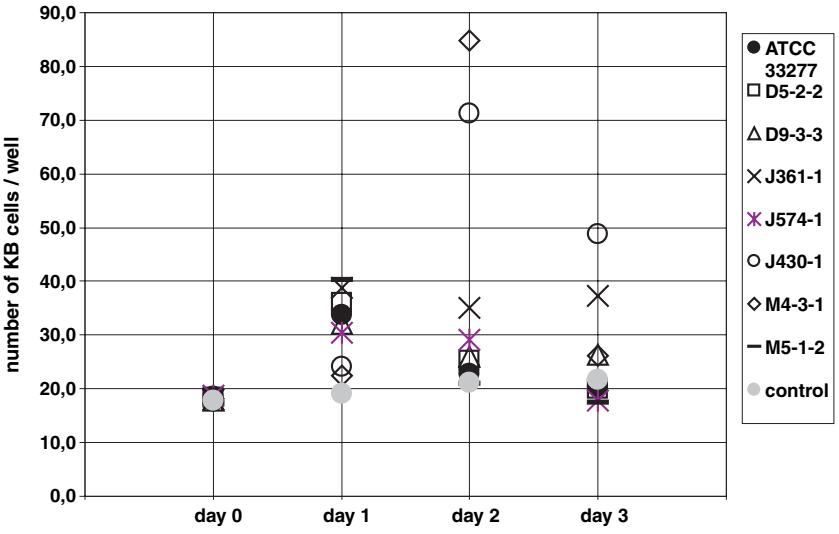

Fig. 3. Number of adherent KB cells immediately, 1 day, 2 days and 3 days after infection by *Porphyromonas gingivalis* ATCC 33277 and different clinical isolates. Controls resemble uninfected cells.

ACT TC-3′ (reverse); *P. gingivalis* 16S rRNA (31): 5′-CAT AGA TAT CAC GAG GAA CTC CGA TT-3′ (forward), 5′-AAA CTG TTA GCA ACT ACC GAT GTG G-3′ (reverse); *rgpA* (23): 5′-TCA ACA CCG GTA GAG GAA AA-3′ (forward), 5′-GCG AAG AAG TTC GGG GGC AT-3′ (reverse). The *sodB* gene served as a positive control for the *P. gingivalis* gene *rgpA*. Primers according to the appropriate regions of sodB (accession: NC_002950), had been selected by the program DNASIS: 5′-AAT TCC ACC ACG GTA AGC AC-3′ (forward), 5′-TTC TCG ATG GAC AGT TTG CC-3′ (reverse).

The PCR was performed for 30 cycles, with one cycle consisting of denaturation at 94°C for 1 min, annealing at 60°C (IL-8, *rgpA* at 55°C) for 1 min, and polymerization at 72°C for 2 min. The amplified PCR products were then analysed by electrophoresis on 1% agarose gel. The gels were scanned to quantify density using IMAGEMASTER software (Amersham Biosciences). The deviation of β-actin was never more than ±10%.

Principal components analysis

All experiments were performed in at least triplicate. The medians were used for each analysis. Principal components analysis is a multivariate statistical method to find hidden complex, and possibly causally determined, relationships between features in a data set. Correlated features are converted by means of the principal axes transformation into new features, the so-called principal components, which are themselves uncorrelated. The plot of the transformed objects in the plane of the first two principal components, which describe a maximum of the variance of the data set, can be used as a display technique for a better visualization of the multivariate relationship between the objects.

Results

Intracellular survival

All *P. gingivalis* strains were able to survive within KB cells but in different quantities. In median 7.8×10^4 colony-forming units (c.f.u.) were counted 1 h after infection. A good correlation was seen between these c.f.u. and the 16S rRNA expression at day 0 calculated by densitometry. Twenty-four hours after infection (day 1) the number of c.f.u. decreased to 1.1×10^4 (range 5.4×10^2 to 5.8×10^4). On day 2 (48 h after infection) the c.f.u. remained at a level of about 4.4×10^3 (range 2.6×10^3 to 2.6×10^4) and by day 3 (72 h after infection) c.f.u. were 2×10^3 (range 8.4×10^2 to 8.3×10^4). However, higher counts were found in one strain at day 2 compared to day 1 and two strains at day 3 compared to day 2. In contrast to the generally reduced c.f.u. values at day 3, equal (±10%) densitometry values for 16S rRNA were seen in three strains (including the ATCC strain) and increased densitometry values were seen in five strains in comparison to day 0 values (Fig. 1). The median supernatant count was 8.5% (range 0–24.2%) of the intracellularly recovered bacteria, but the c.f.u. of free bacteria did not correlate with numbers of intracellular c.f.u. (data not shown).

Arginine-specific cysteine amidolytic activity

Before infection in culture supernatants an arginine-specific amidolytic activity was measured equivalent to the median 24 nM for HRgpA, the range of the strains was between 9.26 and 30.56 nM, indicating large individual differences. These levels declined in all samples during infection. One hour after infection the highest level of amidolytic activity was measured for the ATCC strain (Fig. 2). With 6-hourly repeated media exchanges only in some cases (12%), extremely low levels of BApNA activity (corresponding to 0.1 nM HRgpA) have been found from 24 to 72 hours after infection.

We did not find any mRNA expression of *rgpA* after infection of KB cells with *P. gingivalis* strains. Controls made from bacterial suspensions before contact with KB cells revealed a positive signal and confirmed our method. In contrast, in all samples taken from infected KB cells a strong signal for mRNA expression of *sodB* was found.

KB cells and interleukins

One day after infection the numbers of KB cells in infected cultures were always higher than the numbers of uninfected controls ($P < 0.05$, Mann–Whitney *U*-test). Similar results were obtained 2 days after infection (Fig. 3). Microscopic controls confirmed confluent, thick monolayers and the viability of the cells was always more than 95%, as determined by trypan blue exclusion test. The number of KB cells had dropped by day 3 when infected with *P. gingivalis* ATCC 33277 and five of the clinical isolates.

At all time-points IL-1β release was detected after infection with one strain, M3-4-1. The supernatants after infection with another strain (D5-2-2) were positive for IL-1β on days 2 and 3. Release of IL-6 and TNF-α was always below the detection level. Immediately after infection, IL-8 was only found in the supernatant of the *P. gingivalis* ATCC 33277 infected cells (2.1 pg/ml). By day 2, IL-8 was detectable in all samples after infection with *P. gingivalis* strains, but in different quantities. The highest values were measured for the *P. gingivalis* ATCC 33277 strain (133 pg/ml at day 2 and 112 pg/ml at day 3) but the clinical isolates also increased the release of IL-8 up to 119 pg/ml. Non-infected KB cells released IL-8 (medians 5.4 pg/ml at day 0 and 10.4 pg/ml at day 3; Fig. 4).

Signals of mRNA expression encoding IL-1β, IL-6, and TNF-α in uninfected KB cells were not detectable or very

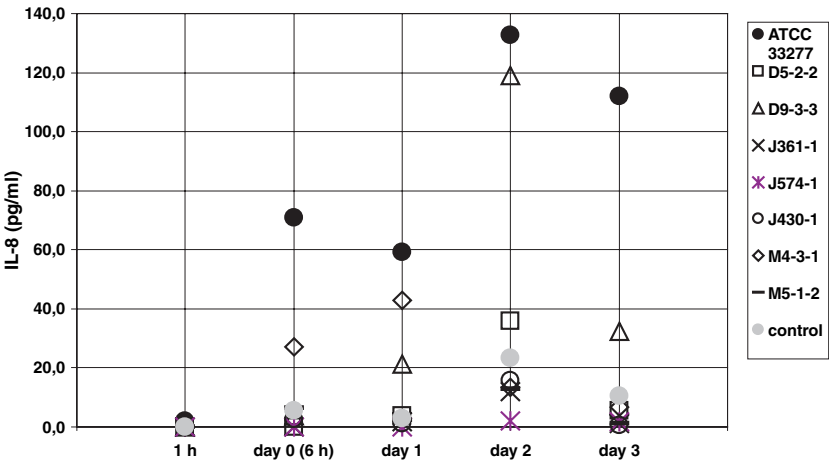

Fig. 4. Levels of IL-8 released by KB cells infected by *Porphyromonas gingivalis* ATCC 33277 and different clinical isolates at different time-points: 1 h after infection, and following replacement of the media at 6 h on the day of infection (day 0), and 1, 2 and 3 days after infection.

weak. In all the KB cells infected with *P. gingivalis* higher densitometry values for IL-1β mRNA expression were found at day 0 compared to day 3. Strain-dependent differences were observed in particular at day 0. The results for IL-6 mRNA expression were similar. Signals of mRNA expression of TNF-α were seen in some cases at day 0, but at a relatively low level. Uninfected KB cells expressed IL-8 mRNA, but expression strongly increased after infection with all *P. gingivalis* strains (Fig. 5).

Principal components analysis

The two main principal components with a variance of more than 25% are presented in Table 1 and Fig. 6. Factor 1 underlines a correlation between the arginine-specific amidolytic activity 1 h and 6 h (day 0) after infection, the released IL-8 at days 1, 2 and 3 and mRNA expression of IL-8 at days 0 and 3. In factor 2, associations were found between the cultivable numbers of intracellular *P. gingivalis* at days 0, 1 and 2; the 16S rRNA at days 0 and 3, and the mRNAs of IL-1, IL-6 and TNF-α at day 0.

Discussion

In this report the KB cell line derived from the oral epithelium, which is an established model for the investigation of adherence and internalization of oral pathogens (6, 8, 13, 19), was applied to characterize the intracellular survival of several *P. gingivalis* strains.

Immediately after infection a drop in the arginine-specific amidolytic activity was found in cell supernatants. Proteolytic activities of extracellular protein preparations gradually decreased during the first 2 h of KB cell incubation in Dulbecco's modified Eagle's minimal essental medium (5). A possible explanation might be that HRgpA, a heterodimer derived from RgpA, rapidly enters epithelial cells and localizes in and around the nucleus within 30 min; the adhesion component, and not the proteolytic activity, appeared to be important in nuclear localization (30). Furthermore, contact with epithelial cells induces *P. gingivalis* ATCC 33277 to repress secretion of Arg-gingipains and Lys-gingipains (23). Otherwise, Arg-gingipains play a role in the colonization of the gingival margins, so Arg-gingipain mutants were deficient in attachment (34) and invasion (23). We did not find any *rgpA* mRNA expression either immediately after infection or 3 days later, indicating a down-regulation of the *rgpA* gene by intracellular *P. gingivalis*.

One topic of our study was to observe the number of intracellular bacteria over a time period of 3 days. The number of c.f.u. in the medium was 0.32% of the inoculum, which is within the range of Houalet-Jeanne et al. (13). In contrast, we did not confirm their described increase of c.f.u. after 24 h. In two cases, after 2 and 3 days higher c.f.u. were enumerated supporting the results about persistence and multiplying of intracellular bacteria (18). We found bacteria in the supernatants, indicating the possibility that some of the bacteria can leave and re-enter cells. At the beginning of the infection a good correlation between 16S rRNA and the cultivable number of

Table 1. Principal components analysis (loadings of the main components)

	Principal components	
Variable	1	2
c.f.u. 1 h		0.769
c.f.u. day 0		0.825
c.f.u. day 2		0.718
16S rRNA day 0		0.932
16S rRNA day 3		0.936
BA*p*NA 1 h	0.741	
BA*p*NA day 0	0.793	
mRNA IL-1 day 0		0.739
mRNA IL-6 day 0		0.764
IL-8 day 1	0.758	
IL-8 day 2	0.869	
IL-8 day 3	0.720	
mRNA IL-8 day 0	0.737	
mRNA IL-8 day 3	0.908	
mRNA TNF-α day 0		0.765
% variance	28.06	26.88

Only loadings of the principal components >|0.7| are mentioned.

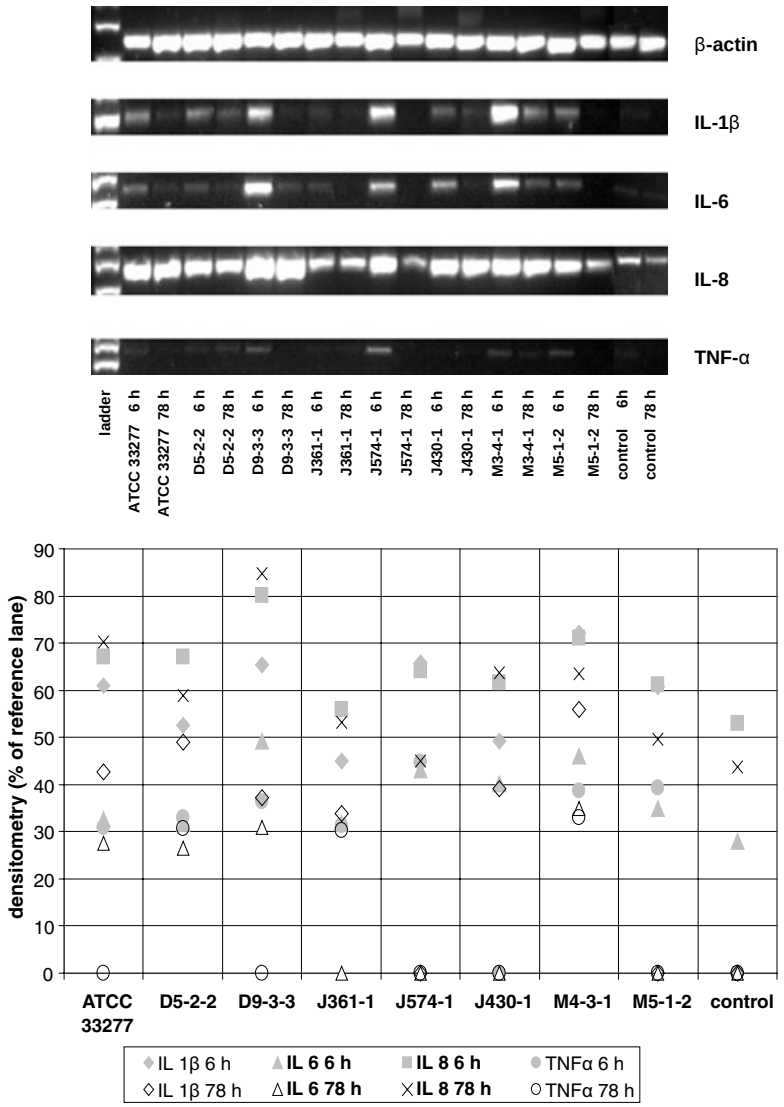

Fig. 5. Analysis of IL-1β, IL-6, IL-8 and TNF-α mRNA levels in KB cells in response to *Porphyromonas gingivalis* 6 h (day 0) and 3 days after infection. Controls resemble uninfected cells at the same time-points.

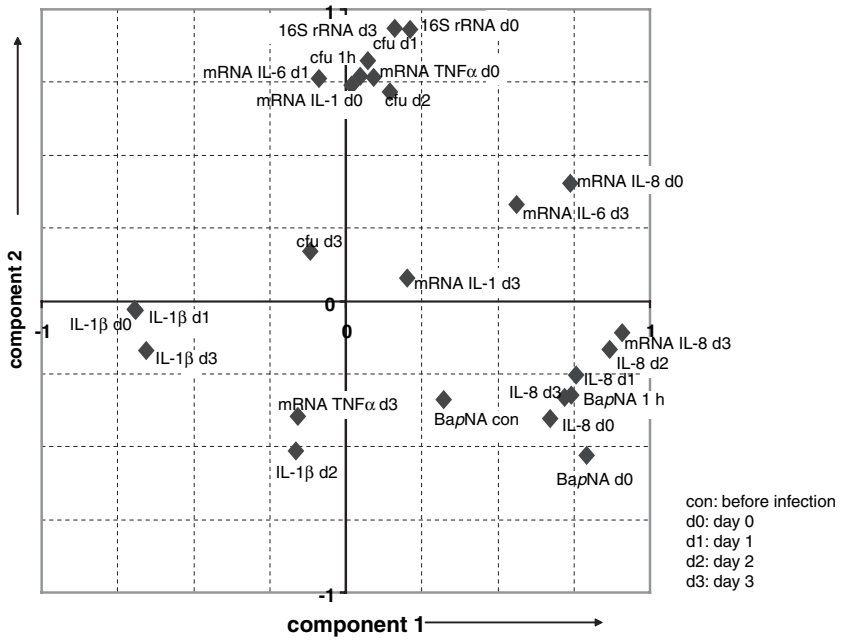

Fig. 6. Loadings of the variables in association with the first two principal components.

bacteria was found, later the levels of 16S rRNA often increased, in contrast to the numbers of c.f.u., which declined. Further studies should focus on mRNA expression of several house-keeping genes of intracellular *P. gingivalis* that indicate the possible survival of *P. gingivalis* in a non-cultivable stage. We are already tempted to speculate that the insufficient effectiveness of antibiotics against intracellular *P. gingivalis* (7) is caused by changes in the phenotype of the bacterium after its entry into the cells.

An elevated number of KB cells was found 1 and 2 days after infection with *P. gingivalis* strains compared with the non-infected control. Lipopolysaccharide of *P. gingivalis* may induce proliferation of epithelial cells by up-regulating keratinocyte growth factor 1 expression in fibroblasts (26). In our study, which used only KB cells as an epithelial cell line, the proliferation stimulating effect might be caused by the low levels of Arg-gingipains immediately after contamination. HrgpA in a low concentration stimulates mitotic activity, whereas high concentrations suppress mitotic activity (30). Extracellular proteases, especially HrgpA, induce loss of cell adhesion and apoptosis in human epithelial KB cells (5, 30). This might be also a concentration-dependent effect, *P. gingivalis* infection itself blocks apoptosis and enhances survival of epithelial cells (21, 38). In our study, after 48 h the number of KB cells decreased in some cases, indicating cell distress.

Interaction of *P. gingivalis* with epithelial cells modulates the expression and release of cytokines. We also found out, consistent with the report of Sandros et al. (28), that infection by *P. gingivalis* initially induces IL-1β, IL-6, IL-8 and TNF-α mRNA expression, positively correlated with invasive capacity. The observed decline in mRNA expression of IL-1β, IL-6 and TNF-α 3 days after infection suggests that intracellular bacteria down-regulate the initial inflammatory response to survive within a silent stage. However, in contrast to the report by Huang et al. (14), we did not observe a decrease in mRNA expression of IL-8 compared to baseline.

In general, IL-8 was the only detectable cytokine and we found an association between the levels of arginine-specific amidolytic activity and of detected IL-8 from the second day of infection. Addition and removal of serum is always a critical point in such experiments. Without any addition of serum, KB cells will not survive for several days. In the oral cavity the components of serum are found in gingival pockets. We focused on this fact in another study that is not yet published. There, we clearly showed that serum increases the release of interleukins, nevertheless the ATCC strain induced the strongest release of IL-8 after addition of all the serum concentrations used.

Cysteine proteases in a soluble form are able to initially convert IL-8 (20). However, it is apparent that most bacterial strains release relatively low levels of gingipains in the soluble form, and they retain the enzymes on the cell surface (25). These enzymes, when associated with bacterial outer membrane blebs, instantly degrade IL-8, (20, 39), whereas cells still express IL-8 mRNA (38). It is probable that the increased levels of released IL-8 both 2 and 3 days after infection resulted from intracellular location of bacteria; cell-surface bound proteinases cannot act on released IL-8 and finally mRNA expression, at least of *rgpA*, is down-regulated. An influence of lipopolysaccharide, normally a stimulant of cytokine expression, can be excluded. Lipopolysaccharide does not react with gingival epithelial cells and KB cells (35).

The invasion and survival of *P. gingivalis* into epithelial cells might contribute to the maintenance of a chronic infection by modulating inflammatory response, e.g. by release of the chemokine IL-8. Strain-specific effects may support an individual innate response of the host.

Acknowledgments

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