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Colonization of hard and soft surfaces by *Aggregatibacter actinomycetemcomitans* under hydrodynamic conditions

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Introduction: Oral bacteria must attach to hard and soft tissues to colonize the oral cavity in the presence of a variety of forces caused by shear and flow. *In vitro* models mimicking this dynamic process are indispensable to study factors that might interfere with the first step towards infection. For extrapolation purposes the comparability between the dynamics of colonization on hard vs. soft surfaces needs to be evaluated.
Methods: The colonization of glass and epithelial cell surfaces by the periodontal pathogen *Aggregatibacter actinomycetemcomitans* was followed in time with two flow cell models: a modified Robbins device (MRD) and an *in situ* image analysis system.
Results: The number of *A. actinomycetemcomitans* recovered from the soft surfaces in the MRD experiments was higher than on glass. The amount of bacteria on the hard surfaces kept increasing with time, while on soft surfaces saturation was reached. The microscope-mounted flow cell allowed real-time *in situ* monitoring of the colonization process of both surfaces.
Conclusion: These experimental models may have a great contribution to make in the development of new treatment approaches for periodontal diseases. Colonization by *A. actinomycetemcomitans* could be studied under flow conditions and its dynamics showed important surface-dependent characteristics.

Key words: *Aggregatibacter actinomycetemcomitans*; colonization; flow cell; modified Robbins device

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Periodontal infections and tooth decay are arguably the most common infectious diseases affecting mankind (7). The periodontal pathogen *Aggregatibacter actinomycetemcomitans* is a gram-negative capnophilic coccobacillus that is responsible for an aggressive periodontal disease known as localized juvenile periodontitis. It can also cause systemic infections such as endocarditis (2). This organism uses several virulence factors to colonize the oral cavity and overcome the host defenses (18). One of the first challenges *A. actinomycetemcomitans* faces when attempting to colonize the oral cavity is the adhesion to oral tissues in an environment were eating, drinking, talking, and salivary flow induce many shear forces. Additionally, microorganisms that are not firmly attached are quickly cleared (7).

Attachment of periodontal pathogens to oral surfaces obviously plays a significant role in the development, maintenance, and recurrence of periodontal diseases. Bacterial adhesion is in general mediated by surface components collectively known as adhesins. Adhesins are typically protein structures found on the surface of the bacterium that interact with host receptors in saliva, on the surface of the tooth, on extracellular matrix proteins, and on epithelial cells. Multiple adhesins, including lipopolysaccharide, fimbriae (pili), and fimbria-associated peptides, have been described and single or multiple adhesion systems can be expressed on the same bacterium (18).

Different *in vitro* studies have shown that *A. actinomycetemcomitans* is capable

of adhering to hydroxyapatite (13), saliva-coated hydroxyapatite (21), glass (13), plastic (21), epithelial cells (23), and human gingival fibroblasts (9). For the evaluation of current and new therapeutic strategies against periodontal pathogens, *in vitro* models that mimic the *in vivo* situation are indispensable. While using such models, it is necessary to know whether data obtained on hard surfaces can be extrapolated to the soft tissue situation, and vice versa. Therefore, the characteristics of the colonization processes on both types of tissue surface, need to be evaluated.

The aim of the current study was to compare the dynamics of hard and soft tissue colonization by *A. actinomycetemcomitans* using two *in vitro* models under flow conditions, a modified Robbins device (MRD) and a parallel plate flow chamber combined with real-time visualization.

Material and methods

Bacterial strains and growth conditions

Smooth, non-fimbriated laboratory strains of *A. actinomycetemcomitans* serotype b [American Type Culture Collection (ATCC) 43718 and ATCC 29522] were grown in brain–heart infusion (BHI, Oxoid, Basingstoke, UK). The latter strain contained pNP3M, a mutated version of the green fluorescent protein (GFP) expression vector pNP3 (20). Cultures were incubated at 37°C in a 5% CO_2 environment. After harvesting by centrifugation (7970 ***g*** for 10 min), the bacterial pellets were resuspended in fresh medium at a concentration of 1×10^8 colony-forming units (CFU)/ml. For the visual experiments, colorless Dulbecco's modified Eagles medium (Gibco Life Technologies Ltd., Paisley, UK) was used. For the MRD experiments on glass, BHI was used, and for the MRD experiments on epithelial cells a mixture was made consisting of 50% BHI and 50% keratinocyte growth medium (Gibco).

Epithelial cell culture

HOK-18A is an immortalized oral keratinocyte cell line derived from normal human oral keratinocytes (12). Cells were grown in tissue culture flasks with keratinocyte growth medium, supplemented as indicated by the manufacturer (Gibco), in a humidified atmosphere containing 5% CO_2 at 37°C. Cell medium was refreshed twice a week until confluent monolayers were observed. After trypsinization (19) cells were used to seed 24-well tissue culture plates (Iwaki microplate, Scitech, Diu, Japan) containing glass discs (2 mm thick, 7–8 mm diameter) for the MRD experiments. For the parallel plate flow chamber assays, cells were plated on 22 × 22 mm glass slides in six-well tissue culture plates (Iwaki microplate).

Bacterial colonization evaluated using a modified Robbins device

Clean glass discs and glass discs covered with monolayers of epithelial cells were placed in the stainless steel flow chambers of an MRD (Dentaid, Barcelona, Spain). These chambers were connected by tubes to a bioreactor containing a continuous culture of *A. actinomycetemcomitans*. Peristaltic pumps brought the bacterial culture from the bioreactor to the flow chambers at a rate of 200 μl/min (10). A gas mixture containing 10% CO_2 was continuously supplied to the system. Discs were taken out of the MRD at specific time-points after the start of the experiment. Samples were vortexed for 1 min and sonicated for 15 min at 100 W in 1 ml 0.1% Triton-X-100 (Sigma, St Louis, MO) in phosphate-buffered saline. Serial dilutions were plated onto blood agar and incubated for 3 days at 37°C in a 5% CO_2 environment. Colony counts were used to calculate the number of *A. actinomycetemcomitans* in CFU/ml. DNA was extracted (Instagene Matrix, Bio-Rad, Hercules, CA) and used for quantitative polymerase chain reaction (QPCR) to determine both the number of *A. actinomycetemcomitans* bacteria (3) and the epithelial cell number. The quantification of the epithelial cells was performed using a single copy gene of the human epithelial cells, the human B defensin-1 gene (15). Human β-defensin is an antimicrobial peptide implicated in the resistance of epithelial surfaces to microbial colonization. The TaqMan primers and probe sequences, the composition of the reaction mixture, and the reaction conditions were similar to those described elsewhere (25). Quantification was, for both assays, performed using plasmid standard curves, based on known quantities of plasmid DNA with the target DNA sequences.

A fluorescent staining (Live/Dead BacLight; Molecular Probes, Leiden, the Netherlands) was applied to the epithelial cells to differentiate between dead and living cells (19). The observation was performed using a fluorescence microscope with a dual fluorescent filter (fluorescein/Texas red) at a magnification of ×400.

Other samples were fixed upon removal for scanning electron microscopy (SEM). Discs were first placed for 12 h at 4°C in 2.5% glutaraldehyde (Sigma) in 0.1 M sodium cacodylate buffer (pH 7.4). Then, the specimens were rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h with three changes and rinsed with distilled water for 1 min. Next, samples were dehydrated by placing the discs in an ascending ethanol series (25, 50, 75, 95, and 100%) each time for 10 min, followed by a 5-min bath in hexamethyldisilazan (Sigma). Finally, the samples were air-dried, mounted on aluminum stubs with silver paint, sputter coated with gold, and examined by SEM (Philips XL20 Fe-SEM; Philips Co., Eindhoven, the Netherlands) (1).

Modified Robbins device experiments on hard and soft surfaces were both repeated eight times independently on separate days.

Bacterial colonization *in situ*

A parallel plate flow cell (Warner Instruments, Hamden, CT) with a diamond-shaped imaging chamber was mounted on a confocal laser scanning microscope (CLSM, Olympus IX 70; Olympus, Aartselaar, Belgium). Glass coverslips, either clean or covered by a monolayer of epithelial cells, served as top and bottom plates (2.5 mm interdistance). Tygon perfusion lines were attached to the inlet and exit ports of the chamber and the chamber and tubes were perfused with phosphate-buffered saline to remove air bubbles (8). Subsequently, a suspension of green fluorescent *A. actinomycetemcomitans* was pumped through the system for 3 h at a velocity of 200 μl/min (10). Z-scans were made after every 30 min. A series of confocal images from various Z-axis planes of the sample (also known as Z-stacks) was made, which contained the image information from the whole specimen. For the experiments on hard surfaces, Z-stacks consisted of 10 slices with an interdistance of 1 μm. For the experiments on epithelial cells 20 slices were made. The focus was maintained in the centre of the chamber during the entire experiment. GFP was excited at a wavelength of 488 nm. The average fluorescence intensity of the Z-stacks was determined using imaging software (FLUOVIEW 500; Olympus, Aartselaar, Belgium). The epithelial cells were also monitored throughout the course of the assay by using the transmitted-light channel in parallel with the fluorescent channel. Data were obtained

from at least five independent experiments for each surface.

Statistical analysis

A log-transformation was conducted on the quantitative data obtained with the MRD set-up. Data from both flow cell models were analysed using analysis of variance tests. The level of significance was set at $P < 0.05$.

Results

Bacterial colonization in the MRD

The colonization of hard and soft tissue surfaces by *A. actinomycetemcomitans* in the MRD under hydrodynamic conditions is shown in Fig. 1. Bacterial colonization was analysed using conventional culturing (Fig. 1A) and QPCR (Fig. 1B). The latter was used to enumerate the total *A. actinomycetemcomitans* biomass (viable and non-viable bacteria).

Conventional culturing showed that 30 min after the start of the experiment, *A. actinomycetemcomitans* had colonized hard and soft tissue surfaces at mean levels of 1.08×10^5 CFU/ml and 6.08×10^5 CFU/ml, respectively. The number of colonizing bacteria, determined by QPCR, was on average 2–3 log higher than the number determined by conventional culturing. On hard tissue surfaces the number of bacteria after 30 min was 1.20×10^7 CFU/ml and on soft tissue surfaces was 1.61×10^8 CFU/ml. However, for both techniques, *A. actinomycetemcomitans* colonization was higher on soft tissue surfaces than on hard tissue surfaces at all time-points. The number of cultivable *A. actinomycetemcomitans* on epithelial cells was statistically significantly different from the number on glass at 1 h and 2 h ($P < 0.05$). For the QPCR results, these differences were statistically significant at all times ($P < 0.05$). Both analysis techniques showed that the increase in colonization over time was more prominent on glass surfaces than on epithelial surfaces. The colonization of hard surfaces showed a linear increase in contrast to the colonization of epithelial cells, which showed saturation after 1 h.

Scanning electron micrographs of the soft and hard tissue colonization under hydrodynamic conditions 3 h after the start of the experiments are shown in Fig. 2. Both the epithelial monolayers and the glass surfaces were covered with a bacterial population. Using high magnification, the epithelial and bacterial cell morphology could be clearly seen. The epithelial cells showed an irregular cell surface and tubular intercellular cell-to-cell connecting structures were prominent. These cells appeared healthy and well formed with distinct cell boundaries and were on average 10 μm in size. The bacterial cells were cocci-rods of approximately 1 μm diameter. On the epithelial cell surfaces it was difficult to differentiate between extracellular vesicles and attached bacterial cells. In a field of view of the same size with the same magnification, it looked as if more bacteria could be detected on the soft tissue surface compared to the hard tissue surface. On the hard tissue surfaces, a more homogeneously spread community seemed to be present compared to on the soft tissue surfaces.

The integrity of the epithelial monolayers was determined by QPCR-based cell counts. The number of epithelial cells present on the discs in the MRD at the different time-points during the experiment showed no statistically significant differences ($P > 0.05$) (data not shown). The structural integrity of the cellular monolayer and the host cell vitality were also evaluated using a Live/Dead staining (Fig. 3). No major changes in the proportion of living (green) vs. dead (red) cells took place during the 3-h colonization experiment.

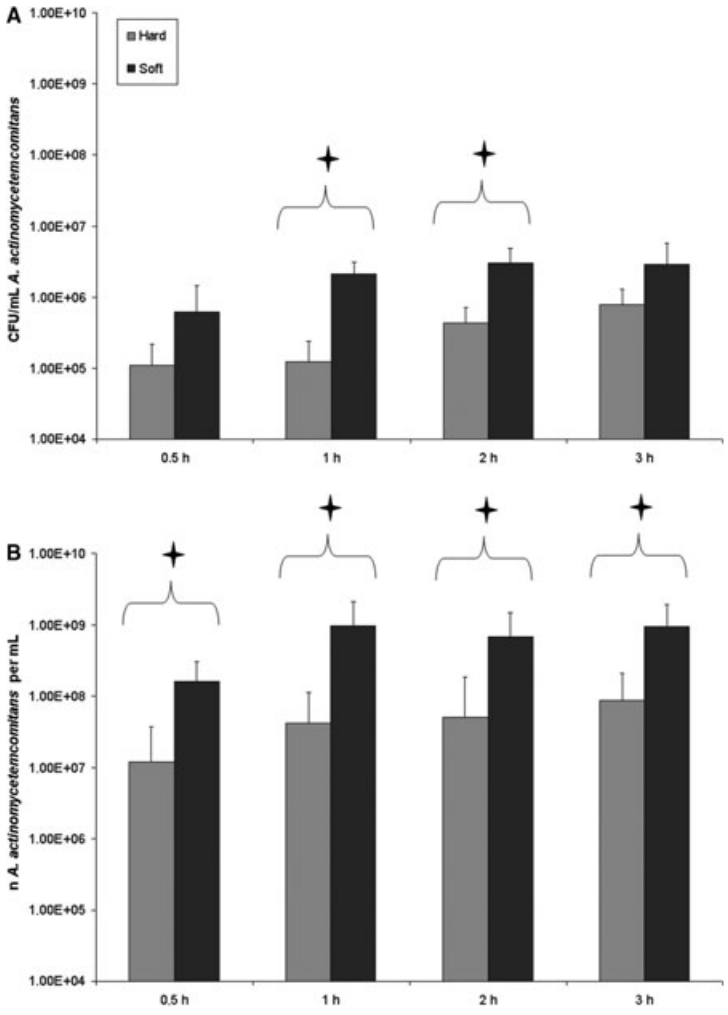

Fig. 1. Recovery of *Aggregatibacter actinomycetemcomitans* from hard and soft tissue surfaces in a modified Robbins device. Bacteria were quantified by microbial culture (A) and quantitative polymerase chain reaction (B) at different time-points. Bars represent standard deviations; +indicates $P < 0.05$.

Parallel plate flow chamber assay

The colonization of GFP-labeled *A. actinomycetemcomitans* could be followed in real time using a CLSM mounted parallel plate flow chamber. Z-stacks of confocal

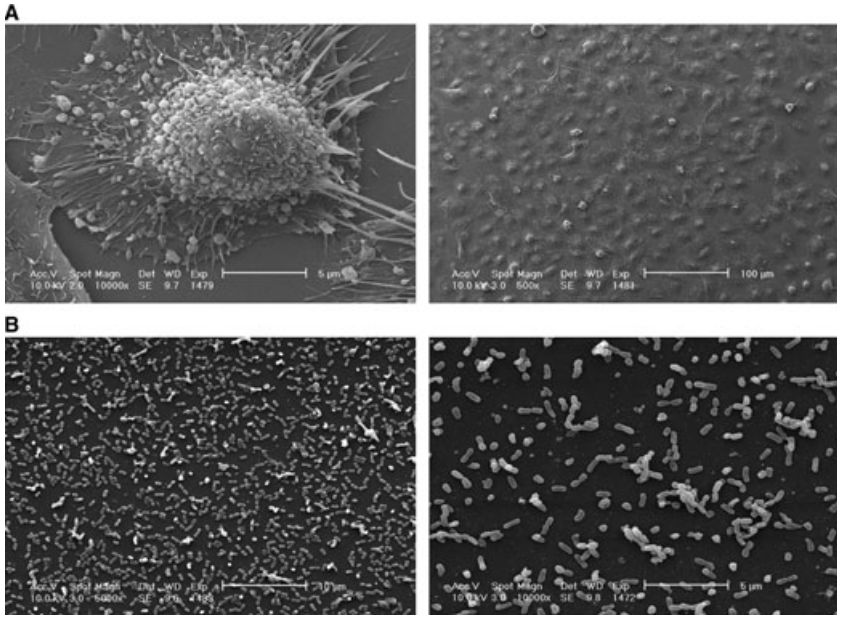

Fig. 2. Scanning electron micrographs of soft (A) and hard (B) surfaces removed from the modified Robbins device after perfusion for 3 h with an *Aggregatibacter actinomycetemcomitans* solution. Bars represent 5 μm (left) and 100 μm (right) for A, and 10 μm (left) and 5 μm (right) for B.

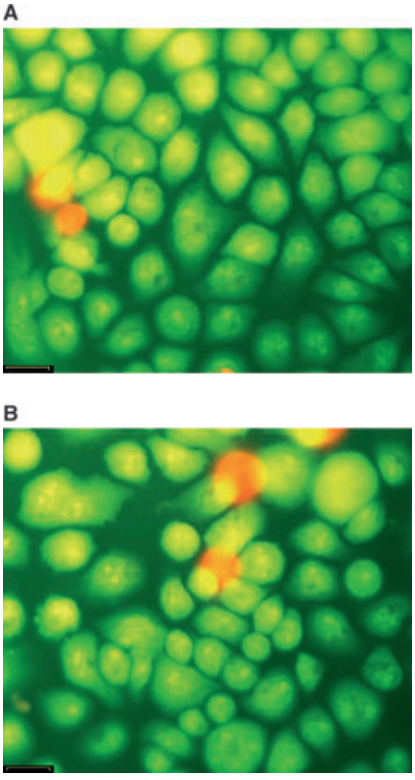

Fig. 3. Epithelial monolayers (HOK-18a cells) before (A) and after (B) a colonization experiment under flow conditions. Samples were removed from the modified Robbins device for examination after 3 h. Staining was performed with BacLight Live (green)/Dead (red). Bars represent 10 μm.

images, taken at the same spot during an adhesion assay, are depicted in Fig. 4A. These fluorescent images show a considerable increase in the number of green fluorescent bacteria colonizing hard tissue surfaces and soft tissue surfaces. For both types of surfaces, rapid colonization of planktonic aggregates was observed in the early stages, minutes after the flow was initiated. No initial lag phase in colonization was observed. On hard tissue surfaces, bacteria were rather spread over the surface whereas on soft tissue surfaces, more autoaggregation was present among the bacterial cells.

The integrity of the epithelial monolayer and epithelial cell morphology could be followed *in situ* by taking transmitted light images at the same time as the fluorescent images (Fig. 4B). Overlays of the fluorescent channel and the transmitted light channel were made to visualize the interaction between bacteria and eukaryotic cells. GFP enabled the differentiation of bacteria from cellular granules and vesicles of approximately the same size.

The average fluorescence intensity of the Z-stacks was measured in function of time (Fig. 5). Thirty minutes after the start of the experiment the average fluorescence intensity was identical for both types of surfaces. Three hours after the start of the experiment the average fluorescence intensity on glass surfaces was 4.5 times higher than the average fluorescence intensity on epithelial cells ($P < 0.05$). The increase in fluorescence intensity on epithelial cells leveled after 2 h. On glass, the fluorescence intensity kept increasing over the entire experiment. On epithelial cells a 1.52-fold increase between 30 min and 3 h was recorded, whereas on glass surfaces there was a 6.85-fold increase.

Discussion

Bacterial colonization by certain pathogenic species is a first and critical step in the development of periodontal diseases. Factors that interfere or inhibit this process are promising for the development of new treatment approaches. Evaluation should be performed with realistic experimental models that mimic the oral cavity. In the present study we compared the colonization of the periodontal pathogen *A. actinomycetemcomitans* over time, on both hard and soft tissue surfaces *in vitro*, and under hydrodynamic conditions. The results obtained from the MRD set-up showed that the numbers of cultivable bacteria and the total biomass of *A. actinomycetemcomitans* were higher on epithelial surfaces than on glass. There was a linear increase in bacterial cell numbers over time on hard tissue surfaces. In contrast, soft tissue surfaces became saturated after approximately 1 h. There was a minor loss, if any, in epithelial cell numbers. This ensured that the saturation effect was not an artifact caused by detachment of cells over time after infection under hydrodynamic conditions. The average number of epithelial cells showed no correlation with the amount of bacteria on these surfaces. The MRD flow cell model has the major advantage that multiple samples can be analysed under identical conditions at the same time (14). Samples can be analysed using a wide array of techniques. The CLSM-mounted parallel plate flow chamber model enabled *in situ*, real-time monitoring of the colonization of fluorescently marked *A. actinomycetemcomitans* without disturbing the process. Removal of the biofilm from the system might result in its disruption and the loss of structural integrity (4).

A. actinomycetemcomitans strains of serotype b were used. This serotype is strongly associated with periodontal disease (28) and is the most common serotype found in patients with aggressive periodontitis (27). Bacteria were transformed by electroporation (24) with a GFP expression vector (20) to allow differentiation in the presence of other bacterial species and/or epithelial vesicles or other similar

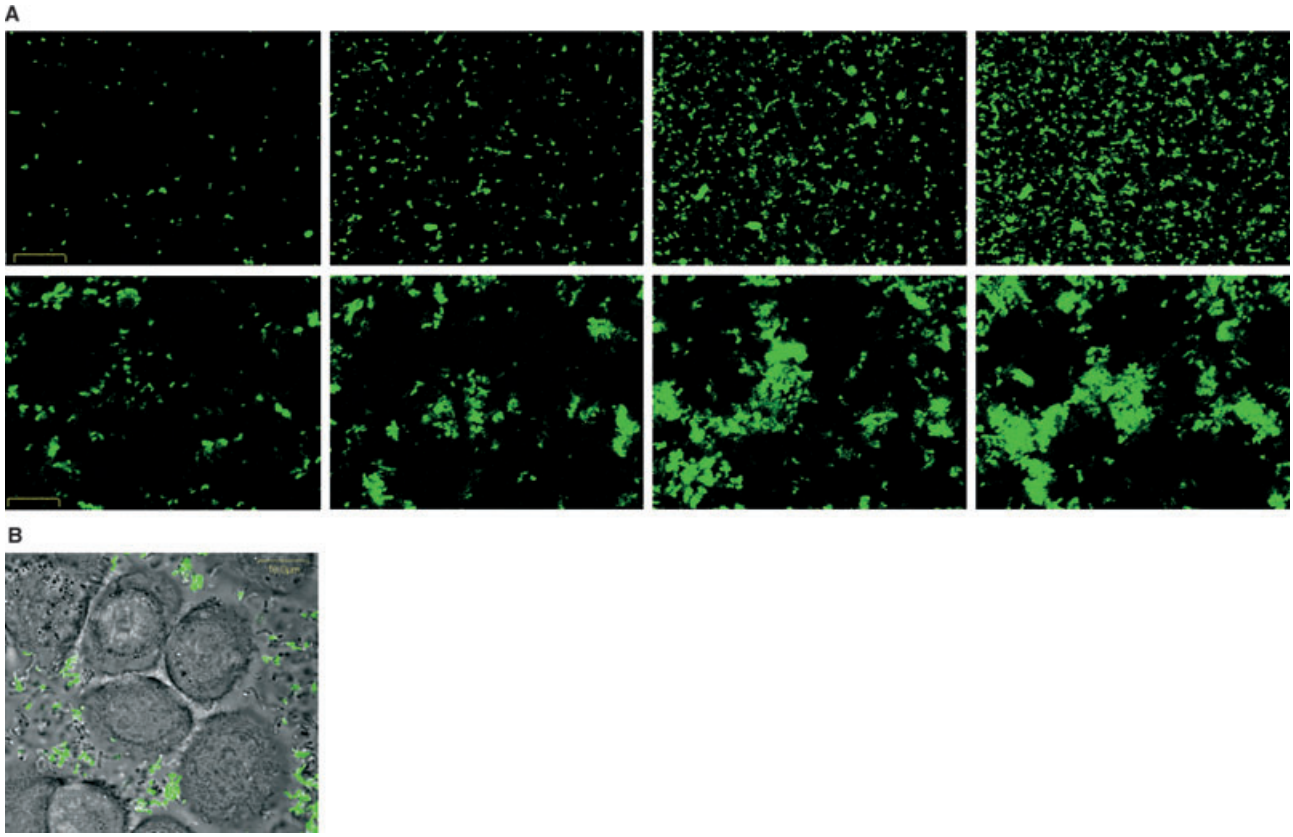

Fig. 4. (A) Confocal laser scanning microscopic images during colonization of green fluorescent protein-labeled *Aggregatibacter actinomycetemcomitans* in a parallel plate flow chamber on glass (upper row) and on epithelial cells (bottom row). Z-stacks of confocal images were shown after 0.5 h, 1 h, 2 h, and 3 h. Bars represent 10 μm. (B) Confocal microscopic image of epithelial cells and attached fluorescent *A. actinomycetemcomitans* after 3 h in the parallel plate flow chamber. The overlay of the fluorescent channel and the transmitted light channel is shown for one Z-slice. Bar represents 10 μm.

structures. Average fluorescence intensity was used as a marker for biomass accumulation because the greater the accumulation, the higher the intensity (4). The medium used for the *in situ* colonization experiments was colorless cell medium, containing a buffer, to ensure minimal artifacts. Combined 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, trypan blue, and culture assays were performed in advance to ensure ideal conditions for the bacterial and epithelial cells during the experiments (data not shown).

Previous studies of the colonization of *A. actinomycetemcomitans in vitro* were mostly performed under static conditions. Colonization of hard surfaces or epithelial monolayers was, for example, investigated using a quantitative cell culture assay; by direct examination with light, electron, and fluorescence microscopy (19); or by using epithelial monolayers in microtiter plates and detecting adherent bacteria with an enzyme-linked immunosorbent assay (18). Recently, models incorporating hydrodynamic forces have been applied in the field of colonization research (11). For the periodontal pathogen *A. actinomycetemcomitans*, a recent study describes the use of a flow cell, combined with a CLSM, to measure the dynamics of biofilm formation (10). Other researchers examined the role of autoinducer-2 in biofilm growth of *A. actinomycetemcomitans*. The adherent growth was studied on a coverglass in a flow chamber (22). In both studies, the flow cells were sacrificed for staining and microscopic observation.

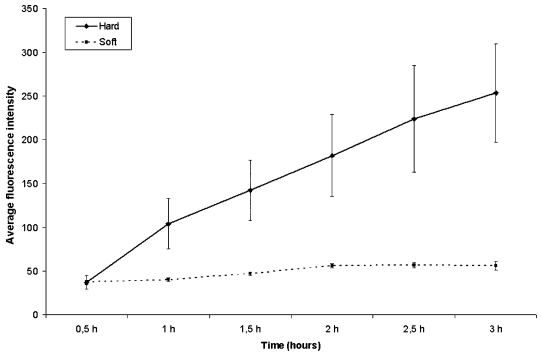

Fig. 5. Average fluorescence intensity of confocal Z-stacks. Measurements were performed on confocal images taken during adhesion experiments with green fluorescent protein-labeled *Aggregatibacter actinomycetemcomitans* in a parallel plate flow chamber. Bars represent standard deviations.

Colonization and persistence of bacteria within the oral cavity depend on their ability to adhere to several oral surfaces. *A. actinomycetemcomitans* can adhere to all components of the oral cavity (the tooth surface, other oral bacteria, epithelial cells,

or the extracellular matrix). The adherence is mediated by a number of distinct adhesins that are elements of the cell surface, including: outer membrane-proteins, microvesicles, fimbriae, and extracellular amorphous material (26). On hard surfaces *A. actinomycetemcomitans* adheres in a non-specific way (2). It is known that the binding of *A. actinomycetemcomitans* to host cells occurs in a completely different manner (6). This process is multifactorial with several mechanisms playing a role; it also shows host range specificity. Binding occurs rapidly and reaches a type of saturation phase (5). Colonization by *A. actinomycetemcomitans* of human epithelial cells in microtiter plate assays, for example, was first detected 10 min after the addition of the bacteria to the monolayers and saturation was reached within 1 h (18). This phenomenon was also observed in the current study. The number of *A. actinomycetemcomitans* attached to the glass surface, however, increased during the entire experiment. This evolution in bacterial colonization to the surfaces was clear, both in the quantitative data obtained with the MRD, and in the images and fluorescence intensity measurements recorded with the parallel plate flow chamber set-up.

Whether *A. actinomycetemcomitans* has a higher affinity for hard or soft surfaces is not described in the literature. Data obtained with the MRD gave, for all observations, a higher bacterial cell number on the soft surface than on the hard surface. This difference was, however, much smaller for the culture results compared to the QPCR data. The reason for this discrepancy is probably autoaggregation. The microscopic observations showed more autoaggregation between bacteria during colonization on epithelial cells compared to on the hard surface. Culture is a technique that is sensitive to artifacts caused by aggregation. The bacterial number detected can be lower than the actual amount of bacteria present. QPCR is based on the detection of a specific gene sequence and is therefore insensitive to aggregation. This autoaggregation issue could also explain partly why QPCR results are higher in absolute values than the culture results. Another reason for this is that the DNA-based technique counts all the cells, both dead and alive. Culture only recovers the cultivable, viable ones. The results of the fluorescence intensity measurements with the second model are not in line with the idea that *A. actinomycetemcomitans* adheres better to soft than hard tissue. Care should be taken, however, before comparing the fluorescence intensity data of both surfaces. The discrepancy might be attributed to the fact that measurements were not performed in exactly the same way for both surfaces in the visual model. For the epithelial cells more slices were made because the bacteria were spread over a larger area in Z. Another explanation for the different results obtained with the models might be that *A. actinomycetemcomitans* invaded the epithelial cells and that the internal *A. actinomycetemcomitans* emitted no or a weaker fluorescent signal. The confocal images showed that the fluorescent *A. actinomycetemcomitans* were mainly present on the epithelial cell surfaces and between the cells. It was not clear on the images whether internalized, but non-fluorescent *A. actinomycetemcomitans* were present inside the epithelial cells. A standard quantitative invasion assay was therefore also performed (17). The results of this assay proved that the fluorescent *A. actinomycetemcomitans* strain used in the present study was able to invade the HOK cells (data not shown). Many clinical and laboratory isolates of *A. actinomycetemcomitans* are capable of invading a variety of cell lines and invasion occurs quickly after adhesion (16). Therefore, this effect might have already been present at the first time-point, 30 min after the start of the experiment. An interesting characteristic of the intracellular existence of *A. actinomycetemcomitans* is its rapid rate of replication. The normal doubling time of *A. actinomycetemcomitans in vitro* in rich broth is 150 min. However, internalized bacteria multiply much more rapidly, about every 20 min (7). This event might cause loss of the normally segregationally stable vector, which might cause in its turn a lower fluorescent signal. The even slight decrease in fluorescence intensity that was sometimes seen near the end of the experiment was probably the result of photobleaching. This irreversible event also causes the fluorescence signal to fade. Another possible explanation for the differences could be because different strains were used. However, both were reference strains of serotype b with similar characteristics.

Colonization by periodontopathogenic bacteria could be a key target for new therapeutic approaches. *In vitro* models presented in this study allowed the follow-up of bacterial colonization under flow conditions, approximating the *in vivo* situation. The data revealed important differences between the dynamics of *A. actinomycetemcomitans* colonization under hydrodynamic conditions on hard compared with soft tissue surfaces.

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