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Pathogenesis and toxins

Mixture of periodontopathogenic bacteria influences interaction with KB cells

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ABSTRACT

Introduction: The purpose of this study was to investigate the adhesion and invasion of periodontopathogenic bacteria in varied mixed infections and the release of interleukins from an epithelial cell line (KB cells).
Methods: KB cells were co-cultured with *Porphyromonas gingivalis* ATCC 33277 and M5-1-2, *Tannerella forsythia* ATCC 43037, *Treponema denticola* ATCC 35405 and *Fusobacterium nucleatum* ATCC 25586 in single and mixed infections. The numbers of adherent and internalized bacteria were determined up to 18 h after bacterial exposure. Additionally, the mRNA expression and concentrations of released interleukin (IL)-6 and IL-8 were measured.
Results: All periodontopathogenic bacteria adhered and internalized in different numbers to KB cells, but individually without any evidence of co-aggregation also to *F. nucleatum*. High levels of epithelial mRNA of IL-6 and IL-8 were detectable after all bacterial challenges. After the mixed infection of *P. gingivalis* ATCC 33277 and *F. nucleatum* ATCC 25586 the highest levels of released interleukins were found. No IL-6 and IL-8 were detectable after the mixed infection of *P. gingivalis* M5-1-2 and *F. nucleatum* ATCC 25586 and the fourfold infection of *P. gingivalis* ATCC 33277, *T. denticola* ATCC 35405, *T. forsythia* ATCC 43037 and *F. nucleatum* ATCC 25586.
Conclusion: Anaerobic periodontopathogenic bacteria promote the release of IL-6 and IL-8 by epithelial cells. Despite a continuous epithelial expression of IL-8 mRNA by all bacterial infections these effects are temporary because of the time-dependent degradation of cytokines by bacterial proteases. Mixed infections have a stronger virulence potential than single bacteria. Further research is necessary to evaluate the role of mixed infections and biofilms in the pathogenesis of periodontitis.

1. Introduction

Periodontitis which is one of the most frequent infectious diseases affecting humans is the main reason for tooth loss in industrial nations [1]. Periodontitis results from the inflammatory response of the host tissues, e.g. epithelial cells, to mixed infections of periodontopathogenic bacteria in the periodontal pocket [2]. Oral and gingival epithelial cells prevent microbes from invading the periodontal tissues. They form an integral part of the innate immune system and play an active role in inflammatory responses [3,4]. Epithelial cells produce interleukin (IL)-8 [5] which is considered to be responsible for migration and accumulation of neutrophils [6,7]. Periodontitis patients have higher levels of IL-8 and also of IL-6 within gingival crevicular fluid [8]. IL-6 plays an important role in regulating the immune response to periodontal pathogens and is notably responsible for the differentiation of activated B cells into immunoglobulin-secreting plasma cells as well as contributing to osteoclastogenesis [9]. Although the human oral cavity harbors about 500–600 bacterial species [10,11], only very few anaerobic species are considered to be responsible for the development and maintenance of periodontal diseases [12]. Among them the anaerobes *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* which are the members of the so-called "red complex" – play the major role [13,14]. Periodontopathogenic bacteria express a variety of virulence factors, among them proteases are considered to be the most important ones [15]. Bacteria of the oral cavity are usually located in a biofilm called dental plaque [16,17]. Biofilms are multi-species communities consisting of a whole range of different micro-organisms covering surfaces irreversibly, embedding in an extracellular polymeric substance (EPS) matrix and being surrounded by a wet milieu [16,17].

Regardless of the complexity of microbial composition involved in pathogenesis of periodontitis, most studies focusing on the interaction of periodontal bacteria and oral tissues have often used only single infections for very short time periods.

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The purpose of our study was to investigate the adhesion and invasion of periodontopathogenic bacteria in mixed infections. The mRNA expressions of interleukin (IL)-6 and IL-8 as well as the release of these cytokines were measured as variables of the inflammatory response of an epithelial cell line.

2. Material and methods

2.1. Bacterial strains

In the present study the following bacteria were obtained from the German strain collection DSMZ Braunschweig: *Fusobacterium nucleatum* ATCC 25586, *P. gingivalis* ATCC 33277, *T. forsythia* ATCC 43037 and *T. denticola* ATCC 35405. In addition *P. gingivalis* M5-1-2, a clinical isolate, sampled from a patient with a severe chronic periodontitis, was included. *P. gingivalis* M5-1-2 is characterized by a thick polysaccharide capsule. All strains were maintained on Schaedler agar (Oxoid, Basingstoke, UK) enriched with 10% sheep blood and vitamin K, harvested, washed twice with phosphate-buffered saline (PBS; Braun, Melsungen, Germany) and resuspended in Medium 199 (Gibco, Life Technologies Ltd, Paisley, UK) to an optical density of 0.1 at 640 nm (10^8 bacteria/ml).

2.2. Adherence and internalization assay

KB cells (epithelial cell line, HeLa derivative, ATCC CCL 17) were seeded into 24-well tissue culture plates at a density of about 1×10^4 cells per well. The cells were grown to confluent monolayers in Eagles' Modified Essential Medium (EMEM, Bio Whittaker, Verviers, Belgium) enriched with 10% fetal calf serum (FCS; Gibco Life Technologies) at 37 °C and 5% CO_2 over a period of 24 h. The cells were washed twice with PBS. Afterwards, 800 μl of EMEM (without the addition of FCS) was placed to a well. Then, each 50 μl of the bacterial suspension was given to the wells and added by medium 199 to made up to a total volume of 1000 μl. Medium 199 without bacteria served as a control for non-infected KB cells (Table 1).

As proposed in the design of this study the cell culture plates and bacteria were co-cultured for 1, 6 and 18 h. After incubation the supernatants were collected, centrifuged at 10,000 *g* and stored at −20 °C for the subsequent cytokine assays. After washing the monolayers five times with PBS, KB cells were lysed by the addition of dH_2O and the numbers of adherent bacteria were counted as colony forming units (cfu) from Schaedler agar culture plates after incubation for 7 days at 37 °C in an anaerobic milieu. Selective cultivation and counting of bacteria in mixed infections was enabled by different colony morphology and by addition of different antibiotics to the Schaedler agar (e.g. *F. nucleatum* ATCC 25586: 7.5 mg vancomycin/l; *P. gingivalis* ATCC 33277 and M5-1-2: 100 mg kanamicin/l; *T. forsythia* ATCC 43037: 1 mg metronidazole/l Schaedler agar). *T. denticola* could only be cultivated in a semi-liquid medium, so cfu counts were not available.

Table 1
Different single and mixed infections used in the present study.

Single infections	*F. nucleatum* ATCC 25586
	P. gingivalis ATCC 33277
	P. gingivalis M5-1-2
	T. forsythia ATCC 43037
	T. denticola ATCC 35405
Double infections	*P. gingivalis* ATCC 33277 + *F. nucleatum* ATCC 25586
	P. gingivalis M5-1-2 + *F. nucleatum* ATCC 25586
	T. forsythia ATCC 43037 + *F. nucleatum* ATCC 25586
Triple infection	*P. gingivalis* ATCC 33277 + *T. forsythia* ATCC 43037 + *F. nucleatum* ATCC 25586
Fourfold infection	*P. gingivalis* ATCC 33277 + *T. forsythia* ATCC 43037 + *T. denticola* ATCC 35405 + *F. nucleatum* ATCC 25586

For internalization assays, EMEM supplemented with 10 IU penicillin/ml (Jenapharm®, Jena, Germany) was added to the wells for 1 h to kill the extracellular adherent bacteria. In preliminary experiments, this antibiotic concentration and time of exposure had been proven to be sufficient to kill 99% of extracellular, but not intracellular bacteria.

After washing three times with PBS, the numbers of internalized bacteria were determined by cell lysis and subsequent cultivation. The number of adherent bacteria was calculated as the difference between the number of adherent plus internalized bacteria and the number of internalized bacteria.

2.3. Cytokine assays

The collected cell cultivation media of the KB cells were used to determine the concentrations of IL-6 and IL-8 using commercially available ELISA kits (Biosource, Ratingen, Germany) as described in the manufacturer's instructions. The lowest detection level of the interleukins in both kits was about 1 pg/mL.

2.4. mRNA assays

To determine the mRNA expression, KB cells had been infected with *P. gingivalis* M5-1-2 and *P. gingivalis* ATCC 33277 as single strains as well as *P. gingivalis* ATCC 33277 in combination with *F. nucleatum* ATCC 25586, *T. forsythia* ATCC 43037 and *T. denticola* ATCC 35405 for 6 h.

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 instructions. 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-6 [18] 5′-AGC TCA GCT ATG AAC TCC TTC TC-3′ (forward), 5′-GTC TCC TCA TTG AAT CCA GAT TGG-3′ (reverse); IL-8 [19] 5′-CTT GGC AGC CTT CCT GAT TT-3′ (forward), 5′-CAG CCC TCT TCA AAA ACT TC-3′ (reverse); β-actin [18] 5′-ATT GCC GAC AGG ATG CAG AA-3′ (forward), 5′-GCT GAT CCA CAT CTG CTG GAA-3′ (reverse); ACG GTA AGC AC-3′ (forward), 5′-TTC TCG ATG GAC AGT TTG CC-3′ (reverse). β-actin served as a control to ensure equal loading of cells.

The PCR was performed for 40 cycles, with one cycle consisting of denaturation at 94 °C for 30 s, annealing at 60 °C (IL-8 at 55 °C) for 30 s, and polymerization at 72 °C for 1 min. The specificity of the amplification was always assayed with the use of melting curves. Amplification, detection and data analysis were performed with the Rotorgene 2000 cycler system (Corbett Research, Sydney, NSW, Australia).

2.5. SEM photographs

SEM photographs of the double infection of *P. gingivalis* M5-1-2 and *F. nucleatum* ATCC 25586 were taken. Before seeding KB cells into 24-well culture plates, a cover slip was given per well, the bacterial suspension was added and co-cultured for 5, 15 and 60 min at 37 °C and 5% CO_2. After removing the supernatants, KB cells were washed three times with PBS, fixed with 2% glutare aldehyde, dehydrated by ethanol up to 100%, critical point dried and covered with gold. The photographs were taken by means of LEO 1450 VP (Carl Zeiss Company, Germany).