Clindamycin promotes phagocytosis and intracellular killing of periodontopathogenic bacteria by crevicular granulocytes: an in vitro study

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Phagocytosis of periodontopathogenic bacteria by crevicular polymorphonuclear neutrophil granulocytes (PMNs) plays a key role in the aetiology of periodontitis. Antimicrobials such as clindamycin have been proven to be effective in treating progressive forms of this disease. Therefore, the purpose of this study was to determine the effect of clindamycin on the phagocytosing properties of gingival crevicular PMNs obtained from 16 patients with rapidly progressive periodontitis (RPP), eight with localized juvenile periodontitis (LJP), 12 with adult periodontitis (AP) and 13 periodontally healthy controls. The phagocytosis assay was performed with the two strains Porphyromonas gingivalis ATCC 33277 and Actinobacillus actinomycetemcomitans Tanner FDC 44 on a slide. Phagocytosis and intracellular killing were assessed by fluorescence microscopy after staining with acridine orange. The addition of clindamycin elevated the percentage of phagocytosing PMNs in periodontitis patients and controls regardless of whether P. gingivalis or A. actinomycetemcomitans was used as test strain. In granulocytes of healthy controls an enhancement of the intracellular killing of both strains was observed if clindamycin was added. Besides the antimicrobial effect, the enhancement of the phagocytosis might be an additional indication for treatment of periodontitis patients with clindamycin.

Introduction

Porphyromonas gingivalis and Actinobacillus actinomycetemcomitans are the most important species in subgingival plaque samples obtained from patients with progressive forms of periodontitis. Rapidly progressive periodontitis is associated with P. gingivalis, which produces connective tissue destructive enzymes. A. actinomycetemcomitans is often seen in localized juvenile periodontitis. In cases of periodontitis it usually produces a leucotoxin which is able to impair polymorphonuclear granulocytes. Polymorphonuclear neutrophil granulocytes (PMNs) play a key role in defence against bacterial infections. The ability of specific bacterial pathogens to initiate periodontal diseases depends on their evasion of PMN defences or the presence of host neutrophils that are dysfunctional.

In dentistry, antimicrobial chemotherapy, e.g. metronidazole, doxycycline and clindamycin, is an effective adjunct in the treatment of patients with progressive periodontal disease. Besides their bacteriostatic or bactericidal effects antibiotics can interact directly with cells of the immune system. Clindamycin has very low MICs for obligate anaerobes such as P. gingivalis, Prevotella intermedia and Fusobacterium nucleatum. In contrast, the capnophilic species A. actinomycetemcomitans exhibits resistance to this drug. In general clindamycin has a positive immuno-modulating effect. Moreover, an enhancement of phagocytosis has been reported. However, there have not been studies concerning the influence of clindamycin on phagocytosis by gingival crevicular PMNs in cases of periodontitis, in which the function of the granulocytes often seems to be altered.

In this study the in vitro effect of subinhibitory concentrations of clindamycin on the phagocytosis and intracellular killing of P. gingivalis and A. actinomycetemcomitans by granulocytes obtained from the gingival sulci has been assessed.

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Material and methods

Patients

Twenty-four patients with progressive forms of periodontitis [16 with rapidly progressive periodontitis (RPP), eight with localized juvenile periodontitis (LJP) and 12 with non-progressive adult periodontitis (AP)] participated in the trial. Thirteen periodontally healthy subjects served as controls. All subjects selected for the study were systematically healthy. None of them had received antibiotics in the previous 6 months. The diseased sites were characterized by assessment of clinical and radiographic parameters. In addition this diagnosis was confirmed by micro-biological cultivation of subgingival plaque samples.

Preparation of crevicular granulocytes

Leucocytes of the sulcus were obtained from the periodontal pockets or gingival sulci through 15-fold washing with phosphate-buffered saline (PBS) using a 0.005 mL Eppendorf pipette, as described previously. In each patient two periodontal pockets with a probing depth of 6–9 mm were selected in each quadrant. The 0.075 mL cell preparation was centrifuged at 150g for 10 min, washed twice and resuspended in 0.2 mL PBS. Finally the pellet was divided into two parts. One part was resuspended in 0.1 mL PBS, and the other one in 0.1 mL PBS containing 0.1 mg/L clindamycin. The viability of the granulocytes was determined by the trypan blue exclusion test.

Bacterial strains

The species P. gingivalis ATCC 33277 and A. actinomyctemcomitans Tanner FDC 44 (known to be a leucotoxin producing strain) were used in the phagocytosis assay. The minimal inhibitory concentration (MIC) of clindamycin (Upjohn, Heppenheim, Germany) had been determined by agar dilution technique as 0.15 mg/L for the P. gingivalis strain and >16 mg/L for A. actinomyctemcomitans FDC 44. The strains were subcultured to log phase on Schaedler agar in the appropriate anaerobic or CO2 atmosphere 24 h before the assay.

The bacterial suspensions were adjusted photometrically with PBS to 10⁹ bacteria per mL. Each 0.2 mL of suspension was mixed with 0.2 mL anti-AB serum for opsonization for 30 min. These mixtures were centrifuged at 1000g for 10 min and then the pellets were washed and resuspended in 1 mL PBS. Finally 0.2 mL of fetal bovine serum was added.

In vitro phagocytosis assay

In vitro phagocytosis was performed on a glass slide according to Smith & Rommel and Pantazis & Kniker. After addition of a 0.05 mL cell suspension the cells were allowed to adhere in a CO2 incubator at 37°C for 30 min. Subsequently, 0.01 mL of a bacterial suspension was transferred to a slide and the phagocytosis assay was incubated in 5% CO2 at a temperature of 37°C for 30 min. The slides were stained with acridine orange (2.5 mg/7.5 mL PBS) and a total of 100 PMNs were immediately examined by fluorescence microscopy. Viable and killed bacteria in granulocytes were distinguished by their uptake of acridine orange, viable bacteria appeared green and dead bacteria were red. The number of PMNs containing bacteria was counted. These phagocytosing cells were separated into two groups by the following criteria: cells with less than 10 ingested bacteria and cells with more than 10 ingested bacteria. Additionally, the number of granulocytes containing viable bacteria (granulocytes with viable bacteria/phagocytosing granulocytes) was documented.

Statistical analysis

The Wilcoxon test was used to assess the significance of the results between paired samples. Differences between the periodontitis groups were analysed by the Mann–Whitney U test.

Results

General findings

P. gingivalis was found at higher percentages in subgingival plaque samples of patients with the two progressive forms of periodontitis (RPP, 10.0% ± 7.3%; LJP, 16.7% ± 13.6%) than in controls (0%, both P < 0.05) or AP patients (3.2% ± 2.4%). In patients belonging to the LJP group subgingival plaque contained a high number of A. actinomyctemcomitans (LJP, 17.9% ± 12.3%; controls, 0%; RPP, 1.0% ± 1.0%; AP, 0.22% ± 0.36%).

The granulocytes obtained from the gingival sulci showed sufficient viability with at least 80% viable in each case.

Phagocytosis of P. gingivalis

In all groups more than 90% of the crevicular PMNs were found to contain P. gingivalis. A higher percentage of phagocytosing granulocytes was found in RPP patients (95.0% ± 2.0%) compared with healthy subjects (90.2% ± 3.8%, P < 0.05). This result was due to the number of granulocytes with less than 10 ingested bacteria (RPP, 34.9% ± 15.7%; controls, 29.6 ± 13.3%; P < 0.05). Comparing the cells with viable bacteria/phagocytosing PMNs, there were no significant differences between the two groups without the addition of the antibiotic.

Treatment of neutrophils with clindamycin resulted in a statistically significant increase in the number of phagocytosing PMNs in each group (Figure 1). Moreover, clindamycin elevated the percentage of the granulocytes with more than 10 internalized bacteria (Figure 2). These differences were significant in AP patients (P < 0.01) and controls (P < 0.05). The PMNs with less than 10 P. gingivalis...
Clindamycin promotes phagocytosis

Figure 1. Crevicular PMNs phagocytosing *Porphyromonas gingivalis* or *Actinobacillus actinomycetemcomitans* in different forms of periodontitis with and without the addition of 1 mg/L clindamycin (mean and standard deviation). *P. gingivalis*: □, without clindamycin; ▮, with clindamycin; *A. actinomycetemcomitans*: □, without clindamycin; ▮, with clindamycin; ○, *P* < 0.05; *, *P* < 0.01.

Figure 2. Crevicular PMNs phagocytosing more than 10 *Porphyromonas gingivalis* or *Actinobacillus actinomycetemcomitans* in different forms of periodontitis with and without the addition of 1 mg/L clindamycin (mean and standard deviation). *P. gingivalis*: □, without clindamycin; ▮, with clindamycin; *A. actinomycetemcomitans*: □, without clindamycin; ▮, with clindamycin; ○, *P* < 0.05; *, *P* < 0.01.

were reduced, the result was statistically confirmed in the AP group (*P* < 0.05, Figure 3). In control subjects clindamycin significantly promoted intracellular killing (PMNs with viable *P. gingivalis*: without clindamycin, 20.7% ± 6.9%; with clindamycin, 14.0% ± 6.1%; *P* < 0.01). The reduced number of granulocytes with viable bacteria in the LJP group was not statistically significant (Figure 4).

**Phagocytosis of *A. actinomycetemcomitans***

The observations were similar when using *A. actinomyctemcomitans* as test strain. Without addition of an antibiotic more phagocytosing PMNs were seen in RPP patients (94.3% ± 4.7%) than in control subjects (91.8% ± 6.8%, *P* < 0.05). Also there was a significant difference (*P* < 0.05) in PMNs with less than 10 phagocytosed *A. actinomyctemcomitans* if comparing RPP group (39.3% ± 16.1%) and controls (30.0% ± 10.1%).

By treating the PMNs with clindamycin significant increases both in the percentage of phagocytosing PMNs (Figure 1) and the percentage of PMNs with more than 10 ingested *A. actinomyctemcomitans* (Figure 2) were observed in each periodontitis and control group.

In contrast to the result mentioned above, the number of
cells with less than 10 phagocytosed bacteria was significantly reduced in all groups (Figure 3). Also, the addition of clindamycin significantly depressed the percentage of viable bacteria/phagocytosing PMNs only in controls ($P < 0.05$), while the difference in LJP patients was not significant (Figure 4).

**Discussion**

Patients selected for the study suffered from different forms of periodontitis. The microbiological results showed that *P. gingivalis* comprised a high percentage of cfu in subgingival plaque samples collected from patients with both progressive forms of periodontitis (RPP and LJP), while *A. actinomycetemcomitans* was detected in a high number only in LJP patients. These microbiological findings confirm that relevant species were used for the phagocytosis assay.

The fluorochrome PMN phagocytosis and killing assay described by Smith & Rommel\textsuperscript{12} and Pantazis & Kniker\textsuperscript{13} was used to determine the effect of a subinhibitory concentration of clindamycin on the phagocytosing and killing properties of granulocytes obtained from the gingival sulci. The endpoint concentration of clindamycin in the cell sus-
pension chosen in our test system was near the MIC for \textit{P. gingivalis} and much lower than the MIC for the \textit{A. actinomycetemcomitans} strain tested. The bactericidal effect of the antibiotic can be excluded as the time of exposure to bacteria was only 30 min and, in the periodontitis group, the number of granulocytes with viable \textit{P. gingivalis} after addition of clindamycin was not significantly reduced.

Comparisons between each periodontitis group and the controls showed an increased phagocytosis in RPP patients as response to the inflammation. This result was due to the higher number of phagocytes containing \textit{P. gingivalis} and \textit{A. actinomycetemcomitans}. However, significant differences between the controls and the periodontitis groups were observed when comparing the granulocytes with a high phagocytosing and intracellular killing capacity.

Observations clearly confirmed a positive immunomodulating effect of clindamycin on phagocytosis by crevicular PMNs collected from both patients with periodontitis and periodontally healthy subjects. Treatment of neutrophils with clindamycin resulted in an increased number of phagocytosing cells in both periodontitis and control groups, regardless of whether \textit{P. gingivalis} or \textit{A. actinomycetemcomitans} was the test strain. Moreover, the percentage of granulocytes with more than 10 ingested bacteria (both for \textit{P. gingivalis} and \textit{A. actinomycetemcomitans}) was enhanced in all groups studied.

Nevertheless, differences were observed between the periodontitis patients and periodontally healthy controls. An enhancement of the intracellular killing of \textit{P. gingivalis} or \textit{A. actinomycetemcomitans} after the addition of clindamycin was found only in controls. The lack of any enhancement in bactericidal activity of crevicular PMNs from periodontitis patients suggests that these cells have an intrinsic deficiency.

The enhancement of opsonophagocytosis by clindamycin is known.\(^\text{10}\) Incubation of granulocytes with clindamycin caused an increase in the proportion of granulocytes bearing Fc receptors,\(^\text{14}\) but the adhesion of non-opsonized \textit{Staphylococcus aureus} was decreased after addition of this antibiotic.\(^\text{15}\) Clindamycin is highly concentrated in the cytoplasm of the granulocytes.\(^\text{16}\) This high concentration in PMNs may promote the bactericidal effect on susceptible species in the cells even if the serum concentration is subinhibitory. Differing effects on intracellular killing have been described. Some authors\(^\text{17,18}\) found an enhancement of intracellular killing, also of resistant species, while other studies have reported that there was no influence\(^\text{19}\) or an inhibition of the respiratory burst.\(^\text{20}\)

Clindamycin therapy is an effective means of treating periodontal disease due to obligate anaerobic bacilli such as \textit{P. gingivalis} and \textit{P. intermedia}. These species are sufficiently susceptible to this antibiotic.\(^\text{8,9}\) The use of locally applied clindamycin gel inserted into periodontal pockets was beneficial in the treatment of advanced periodontitis by eliminating and preventing early recolonization of periodontopathogenic species and might avoid known side effects of systemic administration, such as antibiotic-associated pseudomembranous enterocolitis.\(^\text{21,22}\) The enhancement of phagocytosis against periodontopathogenic species is a useful side effect in periodontitis patients, although a promoting effect on intracellular killing was found only in healthy subjects. Other positive effects of clindamycin are the ability to penetrate into bone and the negative influence on the formation of biofilms.\(^\text{23}\) However, \textit{A. actinomycetemcomitans} is also an important species in periodontitis, especially in LJP and exhibits resistance to clindamycin.\(^\text{9}\) Further \textit{in vivo} studies are therefore necessary to find out if the improvement in phagocytosis alone justifies the use of clindamycin in such cases.

**Acknowledgements**

We are grateful to K. Abendroth, B. Sigusch and G. Klinger for the possibility to perform the clinical examinations and the obtaining of subgingival plaque samples and sulcular granulocytes from their patients in the Department of Periodontology of the University Hospital, Jena.

**References**


Received 21 October 1999; returned 22 February 2000; revised 5 April 2000; accepted 23 May 2000