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Anaerobes and short-chain fatty acids in crevicular fluid from adults with chronic periodontitis

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SUMMARY

Pathogeny of adult chronic periodontitis is still unclear. Bacteriological and chemical analysis of crevicular fluid have shown, in active sites of the disease, a simultaneous presence of anaerobes and their major by-product: short-chain fatty acids. The last can decrease «in vitro» the neutrophil intracellular pH, whenever these cells are incubated in an acid medium. Clinical investigations are scarce which hold out data useful to attempt verifying this possible physiopathological mechanism. This work shows the presence of anaerobes in the active periodontal pockets, together with the presence of short-chain fatty acids likely to reach a concentration level comparable to that used for inhibiting neutrophils «in vitro». Forthcoming studies should investigate about a possible intracellular pH drop in the neutrophils and other cells of the inflamed periodontium.

KEY WORDS:

Periodontitis - microbiology

RÉSUMÉ

La pathogénie des parodontites chroniques de l'adulte n'est pas encore bien comprise. Des analyses bactériologiques et chimiques du liquide créviculaire ont permis de mettre en évidence, dans des sites actifs de la maladie, des germes anaérobies et leurs principaux produits cataboliques: les acides gras à courte chaîne. Ceux-ci peuvent réduire «in vitro» le pH intracellulaire des neutrophiles en suspension dans un tampon acide. Peu d'études présentent des données cliniques permettant de vérifier «in vivo» cet éventuel mécanisme physiopathologique. Ce travail montre la présence simultanée, dans des poches parodontales, de germes anaérobies et d'acides gras à courte chaîne à des concentrations similaires à celles utilisées pour inhiber «in vitro» des neutrophiles. D'autres travaux devront étudier la chute éventuelle du pH intracellulaire des cellules du parodonte en état d'inflammation chronique.

MOTS-CLES

Parodontite - microbiologie

INTRODUCTION

Short-chain fatty acids (e.g. acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproïc, caproïc and succinic acids) are known to inhibit «in vitro» specific activities of the neutrophil granulocytes (PMN), especially their chemotaxis [1], protein kinase C activity [2], superoxide generation and biochemiluminescence [1]. PMN inhibition is even more conspicuous whenever the extra-cellular medium gets more acid [3]. This acidification likely couldn't be instrumental by itself, but rather by secondarily lowering of intra-cellular pH levels [3].

Short-chain fatty acids (SCFA) are found among by-products of fermenting anaerobic microorganisms (e.g. Bacteroïdes sp, Capnocytophaga sp, Fusobacterium sp, Wolinella sp,...) [4]. Their ability to inhibit both phagocytosis and chemotaxis, was especially investigated in the case of experimental abcesses of the abdominal wall [1]. Their presence in the pus of these abcesses has been demonstrated as well as a low acid pH there, down to 5.50 (3,5). Inhibition of phagocytosis might facilitate the proliferation of a second microorganism, giving way to a so called «mixed infection».

In these experiments [1], the inoculum of anaerobes, had been prepared from dental plaques. These data suggest that some microorganisms of the buccal flora, could actually produce an «acid micro-environnement», which would consecutively disable the PMN and accordingly allow the formation of periodontal abcesses and suppuration during the course of periodontitis.

CLINICAL DATA AND METHODS

Crevicular fluid was collected from 7 adult patients (4 males and 3 females with a mean age of 45.3, ranging from 34 to 61 years), exhibiting obvious signs of chronic evolutive periodontitis. All patients had suffered loss of one or several teeth in consequence of their periodontal condition; they had experienced periodontal abscesses, exhibited a resorption of more than 25% of the alveolar bone support and displayed abnormal tooth mobility, gingival pockets more than 4 mm deep as well as important gingival bleeding. Active pockets, deeper than 4 mm, were selected for fluid sampling. Aspiration was performed after the crevicular area had been washed by water spray, then dried using gauze pads and finally isolated to avoid contaminating saliva. Capillary microtubes were introduced at mid depth of the pockets. Enough capillary segments were used to drain 2 to 3 $\times$ 10^{-5} ml of fluid at each site.

Centrifugation (1000 g during 10 minutes) then allowed to separate the exsudate from the contaminating subgingival microflora. This residue was used for bacteriological examination together with additional scaling of deep subgingival plaque. Cultivation and identification of anaerobes, identification of short-chain fatty acids by gas liquid chromatography (Packard model 419 Gas Chromatograph with a Hewlett Packard Integrator 3380 A) were achieved according to the methods described by Moore [4]. Gas liquid chromatography was again used to detect a possible dilution of SCFA in the serum of peripheral blood from 7 other patients also suffering from evolutive chronic periodontitis and 3 healthy adults without active periodontal pathology.

RESULTS

The bacteriological and the chemical data of this study are collected in Table I. Five to eleven different types of anaerobes could be isolated from 7 different samples of crevicular fluid plus adherent subgingival plaque. Microorganisms that are known to secrete succinic acid, are given a «S» mark (indeed, succinic acid was the first SCFA reported to inhibit PMN functions). Different SCFA species were found in each of the 7 crevicular fluids; their concentrations varied from one sample to the other and sometimes went beyond (up to times 8) the critical 5 mM threshold (which is the lowest inhibiting dilution of PMN functions). Total concentration of all SCFA varied from 4.3 up to 881.5 mM (in coming order: 7.3; 4.3; 537.7; 436.7; 881.5; 272.8; 6.8 for the 7 patients successively). This exceeds the critical 5 mM value in 6 instances. Acetic and succinic acids appeared to be the most abounding SCFA. There was no correlation between the succinic acid strength and the presence of succinic acid secreting anaerobes.

The peripheral blood-serum that was investigated in 3 healthy adults and in 7 instances of periodontal disease, was always found to be free of succinic acid.

Figure n° 1 shows the GLC graph of the crevicular fluid for patient n° 6. This typical curve exemplifies the different pikes corresponding to the volatile SCFA.

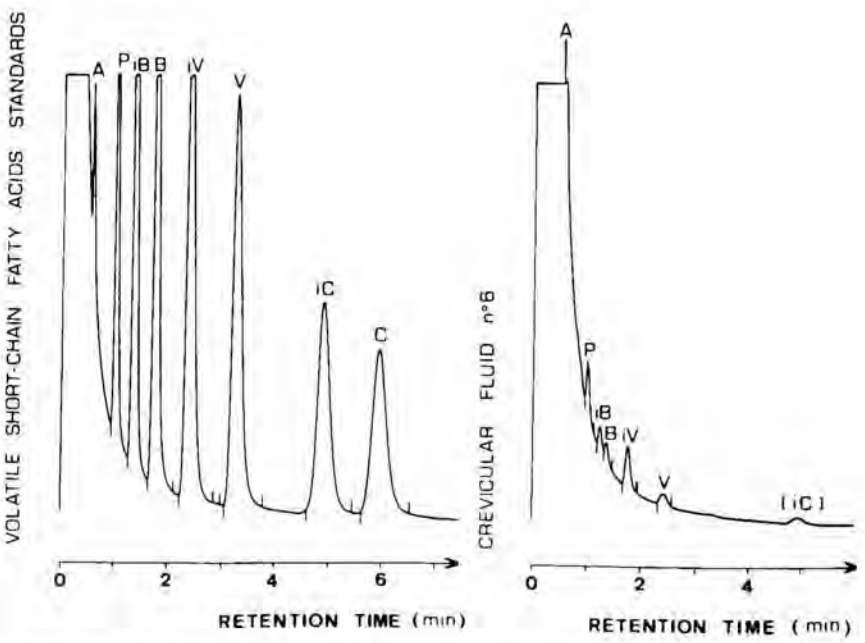

Fig. 1: Volatile SCFA in the crevicular fluid n° 6 (GLC graph).
A: acetic acid; P: propionic acid; iB: isobutyric acid;
B: butyric acid; iV: isovaleric acid; V: valeric acid;
iC: isocaproïc acid; C: caproïc acid.

TABLE I:
Anaerobes and short-chain fatty acids in crevicular fluid from 7 adults with chronic periodontitis.

patient	isolated anaerobes	fatty acids concentration
1.	Bacteroïdes loescheii (S) Capnocytophaga ochracea (S) Leptotrichia buccalis Treponema sp (S) Veillonella parvula (s) Wolinella recta (S)	acetic acid 5.8 mM propionic acid 0.5 mM isobutyric acid 0.1 mM butyric acid 0.5 mM isovaleric acid 0.2 mM isocaproïc acid 0.2 mM
2.	Bacteroïdes intermedius (S) Bacteroïdes oris (S) Bacteroïdes loescheii (S) Fusobacterium nucleatum (S) Selenomonas sputigena (S)	acetic acid 2.6 mM butyric acid 0.2 mM isovaleric acid 0.3 mM valeric acid 0.5 mM caproïc acid 0.7 mM
3.	Actinomyces odontolyticus (S) Capnocytophaga ochracea (S) Fusobacterium nucleatum (s) Leptotrichia buccalis Treponema sp (S) Veillonella parvula (s) Wolinella recta (S)	acetic acid 527 mM propionic acid 3 mM isobutyric acid 1 mM butyric acid 3.7 mM isovaleric acid 2 mM caproïc acid 1 mM
4.	Actinomyces israelii (S) Actinocymes naeslundii (S) Actinomyces odontolyticus (S) Bacteroïdes intermedius (S) Bacteroïdes loescheii (S) Peptostreptococcus sp (s) Veillonella parvula (s)	acetic acid 424 mM propionic acid 0.8 mM butyric acid 0.3 mM succinic acid 11.6 mM
5.	Actinomyces odontolyticus (S) Bacteroïdes gingivalis (S) Capnocytophaga ochracea (S) Eubacterium saburreum Lactobacillus sp Selenomonas sputigena (s) Streptococcus sp	acetic acid 872 mM propionic acid 2.5 mM butyric acid 0.7 mM succinic acid 6.3 mM
6.	Actinomyces odontolyticus (S) Actinocymes naeslundii (S) Bacteroïdes intermedius (S) Bacteroïdes asaccharolyticus (S) Capnocytophaga ochracea (S) Leptotrichia buccalis Peptostreptococcus sp (s) Propionibacterium acnes (s) Streptococcus sp Treponema sp Wolinella recta (S)	acetic acid 202 mM propionic acid 0.8 mM isobutyric acid 0.2 mM butyric acid 0.6 mM isovaleric acid 0.2 mM succinic acid 69 mM
7.	Bacteroïdes intermedius (S) Capnocytophaga ochracea (S) Centipeda periodontii (S) Eubacterium nodatum (S) Peptostreptococcus sp (s) Fusobacterium nucleatum (s) Leptotrichia buccalis Selenomonas sputigena (s) Streptococcus sp Treponema sp (S)	acetic acid 4 mM propionic acid 0.8 mM isobutyric acid 0.1 mM butyric acid 0.3 mM isovaleric acid 0.1 mM succinic acid 1.5 mM

S: high succinic acid secretor; s: low succinic acid secretor

Figure n° 2 describes the changes of pH values in relationship with the variations of succinic acid concentration in the supernatant of a Capnocytophaga ochracea broth culture. After a 4 days period, pH went down from 7.00 to approximately 5.00, while succinic acid grew from 0 to more than 20 mM.

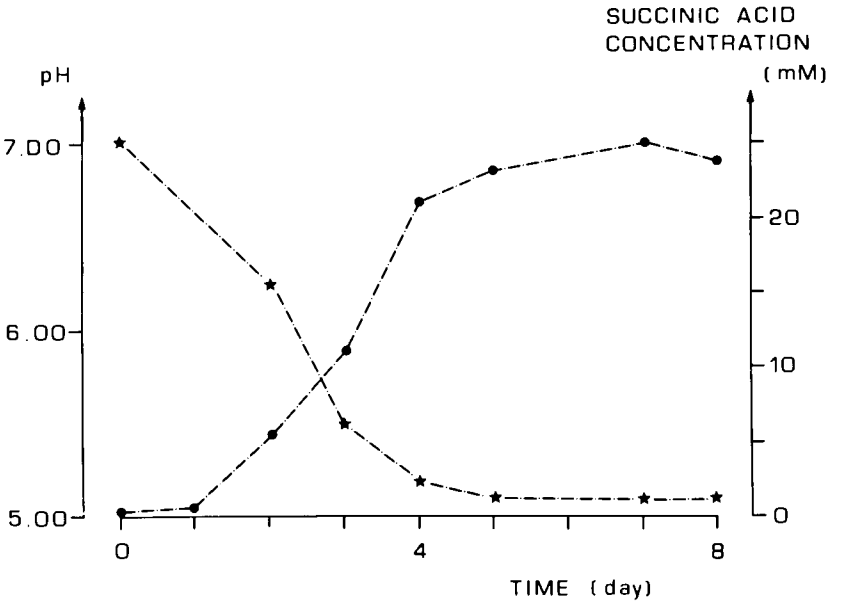

Fig. 2: Evolution of pH and succinic acid concentration in the supernatant of a Capnocytophaga ochracea culture.

Figure n° 3 shows the GLC graph of the same supernatant of Capnocytophaga ochracea culture medium after a 4 days incubation.

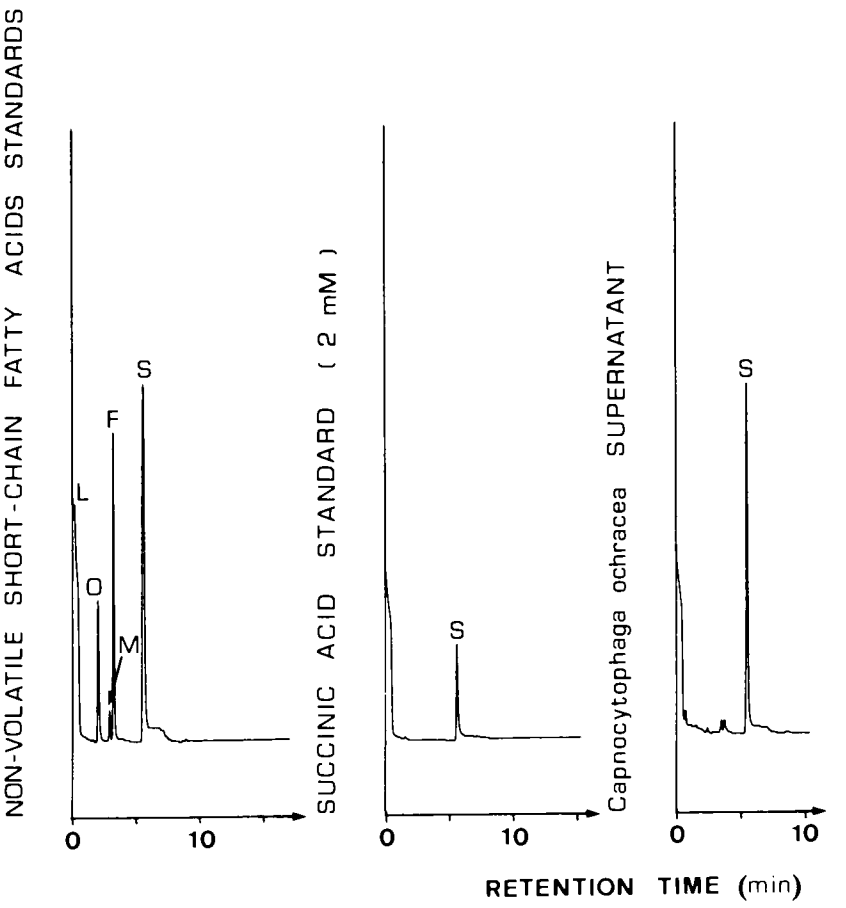

Fig. 3: Non-volatile SCFA in a Capnocytophaga ochracea supernatant after a 4 days culture (GLC graph).
L: lactic acid; O: oxalic acid; M: malonic acid;
F: fumaric acid; S: succinic acid.

This typical standard curve exemplifies the different pikes corresponding to the non-volatile SCFA. The supernatant curve shows an obvious pike corresponding to succinic acid standards.

DISCUSSION

Previous investigations have shown that SCFA (volatile or not volatile) can inhibit «in vitro» both phagocytosis and chemotaxis of PMN when these cells are placed in an acid medium which secondarily yield an intracellular pH drop [3].

Few studies have been carried out for evaluating the simultaneous presence of low pH and high SCFA concentration, in clinical conditions. The SCFA are the major metabolic by-products of anaerobic bacteria in such a way that they can be used as chemical criteria for bacteriological identification [4]. A large number of these anaerobes have been isolated from oral cavity where they could be implicated in human periodontal disease [6]. The observation reported here shows that anaerobic bacteria could be found together with SCFA in the crevicular fluid in 7 different instances of adult periodontitis. Nearly all strains identified in crevicular fluid of patients n° 1, 2 and 3 are mentionned for secreting succinic acid; nevertheless, no concentration of this acid was found. This is not necessary conflicting: on the one hand the identified germs were not quantified, on the other hand the succinic acid clearance conditions out the crevicular fluid are not known. Since several microorganisms (from 5 up to 11) were isolated and identified in each instance, it was not possible to relate the SCFA with their putative producer. But it is interesting to notice that the same concentration of SCFA which induced PMN inhibition «in vitro» [7] can be reached in periodontal pockets ((that is more than 5 mM). Experimental «in vitro» inhibition becomes obvious after a twenty minutes incubation. The situation «in vivo» is indeed different since the inhibitors then are simultaneously and unceasingly present. One could argue that, under these circumstances, lower SCFA concentration might still be inhibiting the PMN activity. However, there are no experimental data to corroborate this assumption. Moreover, the mere presence of SCFA in crevicular fluid is not enough to assess their inhibitory role on PMN, since we ignore their influence on the intracellular pH. Observation of an intracellular pH drop in the leukocytes and other cell, is a necessary condition to accept this putative mechanism.

Actually, such observations are not available. Forthcoming works should deal not only with PMN but also with other cells that play an important role in the inflamed periodontium: lymphocytes, platelets, endothelial cells and fibroblasts. Indeed the protein kinase C [8], a key enzyme in the transduction of extracellular signals into cellular responses, can be inhibited by low pH incubation [2]. And this enzyme can be found in all these cell types.

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