RESEARCH REPORTS
Biological

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Amplified Crevicular Leukocyte Activity in Aggressive Periodontal Disease

ABSTRACT

Exaggerated neutrophil responses are a critical component in the pathogenesis of periodontal disease. We investigated whether leukocyte activity in aggressive periodontitis (AP) is increased compared with that in chronic periodontitis (CP) by gingival crevicular fluid (GCF) analysis of myeloperoxidase (MPO), beta-N-acetyl-hexosaminidase (beta-NAH), cathepsin D (CD), and elastase-alpha-1-proteinase inhibitor complex (alpha-1-EPI) before and 6 months after therapy. Initial AP neutrophil responses were significantly amplified compared with those in CP (MPO, 3.2-fold; beta-NAH, 37.5-fold; CD, 2.2-fold; alpha-1-EPI, 1.4-fold; $p < 0.05$). Surgical therapy resulted in a significant reduction of GCF markers compared with non-surgical treatment. However, the changes in clinical parameters were not different between AP and CP ($P > 0.05$). Analysis of the results suggests that the local inflammatory response in AP is characterized by increased release of inflammatory mediators of neutrophil origin into the GCF. Analysis of the data further suggests that surgical therapy is a more predictable method for removal of the pro-inflammatory etiology.

KEY WORDS: aggressive periodontitis, inflammation, gingival crevicular fluid, PMN enzymes, protease-complexed elastase, host defense mechanisms.

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INTRODUCTION

Periodontal inflammation may affect and worsen systemic conditions such as cardiovascular disease, pre-term labor, diabetes mellitus, and other clinical syndromes associated with leukocyte-mediated pathology. Several prominent neutrophil-specific molecules have been implicated as amplifiers of the inflammatory response (Battino *et al.*, 1999; Yamalik *et al.*, 2000). The myeloperoxidase-hydrogen peroxide-chloride system of the neutrophil, as part of innate host defense, possesses potent antimicrobial activity (Ihalin *et al.*, 1998). The liberation of the 150-kDa-protein myeloperoxidase, a potent oxidative enzyme from the azurophilic granules of neutrophils, in combination with reactive oxygen species, such as O_2^-, gives rise to hypochlorous acid (HOCl). In homeostasis, inflammation, and during phagocytosis, glycoside sulphatases and acid hydrolases secreted by neutrophils (Shikhman *et al.*, 2000) may be released from terminal lysosomes serving as storage bodies (Tjelle *et al.*, 1996). The lysosomal acid hydrolase, beta-N-acetyl-hexosaminidase, is released into gingival crevicular fluid during neutrophil-mediated inflammatory events in the gingiva, subsequent to phagocytosis by the neutrophil. Salivary beta-NAH-glycosidase activity correlates with the degree of inflammation, and follows resolution of inflammation after treatment (Nieminen *et al.*, 1993). The 52-kDa lysosomal aspartate-endopeptidase, cathepsin D, is released from the primary azurophilic granules of neutrophils into the extracellular compartment. Cathepsin D is functional in a wide variety of tissues during remodeling or regression, and in apoptosis. Cathepsin D is found in fibroblasts and macrophages of the subepithelial connective tissue, and in desquamating junctional epithelial cells of the gingival crevice (Ayasaka *et al.*, 1993). These molecules in GCF have been used as an endpoint marker of tissue degradation and cell lysis (Lamster, 1991; Hasilik, 1992). In rheumatoid arthritis (Momohara *et al.*, 1997) and acute exacerbation of respiratory tract infections (Groeneveld *et al.*, 1997), the elastase-alpha-1-proteinase inhibitor complex, a 53-kDa-serine proteinase, has been shown to be an early inflammatory marker.

Recently, aggressive periodontitis was redefined as a complex disease exhibiting microbial alteration and cellular dysfunction that differentiate the underlying molecular mechanisms of its pathogenesis from chronic periodontal disease (Armitage, 1999). The release of granule components from infiltrating leukocytes, such as lysosomal enzymes and reactive oxygen species, which are normally intended to degrade ingested microbes, can also lead to tissue degradation and amplification of the inflammatory response, with continued recruitment of new leukocytes. In localized aggressive periodontitis in particular, it has been demonstrated that uncontrolled neutrophil recruitment and activation can lead to the aberrant release of an array of noxious agents intended to fight the bacteria, with the potential for causing further tissue damage (Pouliot *et al.*, 2000). In aggressive periodontitis, most sites respond following therapy, but certain sites fail to resolve, and actually continue to experience active disease (American Academy of Periodontology, 2000), suggesting incomplete removal of etiology.

With this background, the null hypothesis tested was that leukocyte activity in periodontal tissues of aggressive periodontal disease is not significantly different from that in chronic periodontitis.

MATERIALS & METHODS

Patients

Twenty-five patients with aggressive periodontitis (AP) (Armitage, 1999) and nine subjects with chronic periodontitis (CP) were recruited from the Department of Periodontology, University of Münster. AP patients were younger (AP, 39.7 ± 8.6 yrs *vs.* CP, 49.2 ± 7.1 yrs) and exhibited more severe disease radiographically, with evidence of intrabony defects exceeding 50% (AP) and 30% (CP), respectively, of the root length. Another inclusion criterion was probing depths > 5 mm in at least 8 sites within each quadrant. AP subjects displayed generalized severe periodontal tissue destruction, loss of periodontal support inconsistent with age, persistent gingival inflammation, increasing probing depths, and progressing tooth mobility (American Academy of Periodontology, 2000). Exclusion criteria included pregnancy, periodontal therapy or antibiotics in the previous six months, any systemic condition that might have affected the progression or treatment of periodontitis, and the need for antibiotic pre-medication before dental therapy. A written informed consent was obtained from each volunteer. The study protocol was approved by the Institutional Review Board of the Medical Faculty of the University of Münster.

Biochemical Analyses

Myeloperoxidase

GCF sampling, analysis, and recovery and validation protocols have been described previously (Buchmann *et al*., 2002). A 10-μL quantity of the diluted GCF sample was added to 0.1 M citric acid buffer, pH 5.5, containing 0.125% Triton-X-100 solution and 0.1 mM hydrogen peroxide. The oxidation of H_2O_2 was performed with 0.8 mM ó-Dianisidin as substrate. After 7 min of incubation at 21°C, the samples turned brown, and the reaction was stopped by the addition of glycine/NaOH, pH 10.4. The myeloperoxidase activity of the GCF samples was determined spectrophotometrically at a wavelength (wv) of 405 nm (reference wv 650 nm) on uncoated 96-well microtiter plates with the use of a microplate reader. The total MPO activity was calculated in duplicate and expressed in μU/GCF sample.

Beta-N-acetyl-hexosaminidase

A 10-μL quantity of the diluted GCF sample was eluted in 25 μL of 0.9% NaCl and 0.1 M citric acid buffer, pH 4.6, and a 25-μL quantity of p-nitrophenyl-N-acetyl-beta-D-glusoaminide (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) was added. The reaction was allowed to proceed at 37°C for 30 min and then was stopped with 225 μL of 0.4 M Glycine/NaOH stopping buffer, pH 10.4. The concentration of *p*-nitrophenolate was assessed at a wv of 405 nm (reference wv 492 nm). The total beta-NAH activity was calculated in duplicate and expressed in μU/GCF sample (Hasilik, 1992).

Cathepsin D

Cathepsin D was determined with [^{14}C] hemoglobin used as the substrate. The diluted sample (10 μL) was incubated in a total volume of 200 μL in 0.1 M Na-acetate buffer, pH 3.65, with 10 μg [^{14}C] hemoglobin (specific activity = 240 pg/cpm). After 6 hrs of incubation at 37°C, the reaction was stopped by the addition of 0.5 mL 1% (w/v) casein solution and 0.5 mL 25% (w/v) trichloroacetic acid. The samples were centrifuged for 5 min at 14,000 x *g*, and the radioactivity of the supernatant was quantified in a liquid scintillation counter. The total cathepsin D activity was determined in duplicate, and average values were calculated in ng.

Elastase-alpha-1-proteinase inhibitor complex

The total amount of complexed, functionally inactive, PMN elastase was determined with the Merck-Immunoassay (Merck KgaA, Darmstadt, Germany). In 2 x 5 μL of the diluted samples, the alpha-1-EPI-complex was bound to 20 μL of the solid-phase fixed anti-PMN-elastase antibody. After 10 min of incubation at 37°C, a 20-μL quantity of the alpha-1-proteinase-antibody conjugate was added. The alpha-1-EPI complex was determined at a wv of 492 nm by the addition of 50 μL 4-p-nitrophenylphosphate as substrate, and 150 μL of 2 N NaOH as stopping reagent. The total amount of elastase-alpha-1-proteinase inhibitor complex was computed from 492-nm absorbance readings with the use of a calibration curve of 4 different standards and expressed in ng.

Periodontal Intervention

All patients were monitored at baseline and 6 mos after periodontal treatment. The clinical examination included measurement of probing depth at 6 sites *per* tooth (PD), clinical attachment level at 6 sites *per* tooth (CAL), the gingival index (GI) and the plaque index (PI) (Löe, 1963), **AQ** bleeding on probing (BOP), and gingival crevicular fluid volume (GCFv) quantified by means of a Periotron 8000. The periodontal treatment protocols have been previously reported (Buchmann *et al*., 2002). In AP patients, 3 x 500 mg amoxicillin (Ratiopharm GmbH, Blaubeuren, Germany) and 3 x 250 mg metronidazole (Artesan GmbH, Lüchow, Germany) were administered systemically as an adjunct to therapy, while in CP subjects, no antibiotics were prescribed.

Statistical Analysis

Data analysis and statistical tests were performed on the patient level. A site-level analysis was applied to differentiate surgical from non-surgical treatment outcomes. Significant changes of the GCF parameters and clinical parameters after therapy were analyzed by the Wilcoxon Signed-rank test. Differences between AP and CP were analyzed by the Mann-Whitney U-test. The GCF parameters were computed as the total amount collected onto the paper strips in 30 sec and plotted for both visits as medians, with the Q1-Q3 quartiles the minima and the maxima. Statistical significance was defined as $P < 0.05$.

RESULTS

Periodontal Parameters

Both of the patient population groups exhibited a significant treatment effect, as evidenced by changes in PD, CAL, GI, PI, BOP, and GCFv ($P = 0.08$ to 0.0001, inter-group comparisons, Table 1). AP patients demonstrated more severe disease, in general, as evidenced by deeper pockets and more attachment loss. However, at 6 mos, there were no differences between groups for any parameter except GI ($P = 0.0001$). The significant change of GCF volumes in both treatment groups (AP, $P = 0.0001$; CP, $P = 0.008$) indicated a marked decrement of inflammatory exude after therapy.

Table 1. The Differences in Clinical Parameters between Untreated (baseline) and Treated Periodontal Disease (6 mos) were Statistically Significant (P < 0.05)

Parameter	Group	Time	Median	Mean	Outset (6 mos)	Range	Change	AP $_{vs}$. CP (after 6 mos)[e]
PD	AP	1[a]	6.1	6.1 (0.9)[b]		4.4 - 7.6		
		2	3.1	3.3 (0.7)	2.8 (1.0)[b]	2.4 - 5.3	0.0001[c]	
	CP	1	5.9	5.4 (2.1)		2.6 - 8		
		2	3.1	2.9 (1.0)	2.5 (1.0)	1.4 - 4.3	0.005	0.553[d]
CAL	AP	1	7.3	7.1 (1.0)		5.4 - 8.9		
		2	5.1	5.1 (1.4)	2.0 (1.2)	3.0 - 9	0.0001	
	CP	1	6.8	6.7 (2.1)		3.7 - 9.3		
		2	4.5	4.4 (1.3)	2.3 (0.8)	2.7 - 6.1	0.005	0.706
GI	AP	1	2.4	2.4 (0.3)		1.8 - 2.9		
		2	0.8	0.8 (0.6)	1.6 (0.6)	0.1 - 2.5	0.0001	
	CP	1	1.8	1.7 (0.8)		0.4 - 2.8		
		2	1.1	1.1 (0.4)	0.6 (0.6)	0.4 - 1.8	0.008	0.0001
PI	AP	1	0.8	0.8 (0.5)		0 - 2		
		2	0.4	0.5 (0.4)	0.3 (0.6)	0 - 1.9	0.02	
	CP	1	0.9	1.3 (0.9)		0.3 - 2.6		
		2	0.8	0.9 (0.4)	0.4 (0.6)	0.3 - 1.6	0.08	0.928
BOP	AP	1	0.9	0.8 (0.2)		0.5 - 1		
		2	0.4	0.4 (0.2)	0.4 (0.3)	0 - 0.9	0.0001	
	CP	1	0.6	0.6 (0.2)		0.2 - 0.9		
		2	0.3	0.2 (0.2)	0.4 (0.3)	0 - 0.6	0.01	0.577
GCFv	AP	1	143.1	142.0 (25.7)		83.5- 184.8		
		2	92.5	96.4 (28.2)	45.6 (34.1)	51 - 142	0.0001	
	CP	1	136.1	129.8 (39.4)		52.6- 183.9		
		2	72	68.6 (20.8)	61.2 (18.6)	27.9 - 97.1	0.008	0.225

Abbreviations; PD = Probing depths (mm); CAL = Clinical attachment loss (mm); GI = Gingival index; PI = Plaque index; BOP = Bleeding on Probing; GCVv = Gingival crevicular fluid volume.

[a] 1 = Baseline, 2 = 6 months.

[b] Standard deviation.

[c] Wilcoxon Signed-rank test, exact P-value.

[d] Mann-Whitney test, P-value.

[e] Treatment-induced changes after 6 mos were not different between AP and CP (except GI).

Gingival Crevicular Fluid Markers

Neutrophil enzyme release into GCF was significantly elevated in AP at baseline (MPO, beta-NAH, CD, P = 0.001; alpha-1-EPI, P = 0.007). The post-treatment levels remained significantly different between the groups for MPO and beta-NAH (P = 0.001). CD (P = 0.324) and alpha-1-EPI scores (P = 0.225) did not differ statistically (Fig.).

Myeloperoxidase

At baseline, AP MPO levels were 3.2-fold increased compared with CP (6.4 *vs*. 2.0 ìU). There was a significant reduction in both AP and CP after therapy, but the difference between the groups remained statistically significant (P = 0.003) (Table 2).

Beta-N-acetyl-hexosaminidase

At baseline, AP beta-NAH was elevated 37.5-fold compared with CP (1.5 [AP] *vs*. 0.04 ìU [CP]). The reduction of beta-NAH after therapy was significant for both AP and CP, but AP beta-NAH scores remained significantly elevated over those of CP (P = 0.002) (Table 2).

Cathepsin D

Baseline CD was 2.2-fold higher in AP than in CP (3.1 in AP *vs*. 1.4 ng in CP). Periodontal treatment significantly lowered CD in both AP and CP, but AP CD levels remained elevated compared with those in CP (P = 0.004) (Table 2).

Elastase-alpha-1-proteinase inhibitor complex

Baseline alpha-1-EPI was 1.4-fold higher in AP (94.0 in AP, and 68.2 ng in CP). The decrease of alpha-1-EPI after therapy was significant in both AP (54 ng) and CP (21.2 ng). The reduction of GCF markers was significantly different between AP and CP (P = 0.002) (Table 2).

Non-surgical *vs*. Surgical Therapy

One hundred thirty-two periodontal sites were treated surgically in the AP group and 40 sites in the CP group, while 68 sites were treated by scaling and root planing in the AP group and 32 sites in the CP group. At sites with PD ³ 6 mm treated by surgery, significantly greater reduction of leukocyte markers was observed for both AP and CP compared with

scaling and root planing alone. In AP, the reduction of MPO (P = 0.0001), CD (P = 0.002), and alpha-1-EPI (P = 0.001) significantly differed between the surgical and non-surgical groups, whereas no differences were noted for beta-NAH (P = 0.027) and GCFv (P = 0.426). In CP, the changes were more pronounced (Table 3).

DISCUSSION

In this report, we describe an elevation of neutrophil-derived gingival crevicular fluid enzymes of aggressive periodontitis patients compared with chronic periodontitis subjects. Treatment of the periodontal condition reduced these markers of neutrophil-mediated inflammation in the GCF of both patient groups; however, the AP group values remained higher than those of the CP group after therapy. We also describe a difference in the responses of sites treated surgically and non-surgically, where the surgically treated sites revealed a greater reduction in concentration of inflammatory markers in GCF.

Hypochlorous acid (HOCl), the product of an MPO-catalyzed reaction, inactivates alpha-1-proteinase inhibitor by oxidizing a methionine residue that is essential for its biological activity (Travis *et al.*, 1994). Due to the inactivation of the antiproteinase shield by HOCl, MPO provides appropriate conditions for the action of latent proteases (Yamalik *et al.*, 2000). Our findings support the concept that severe periodontal inflammation, as seen in AP, is due in part to an abnormally high PMN response. Since the inflammatory exudates (GCF volume) were similar in untreated AP and CP, our findings suggest that the oxygen-dependent metabolism in neutrophils may serve as a qualitative marker for the differentiations of periodontal inflammation.

The profile of the lysosomal glycosidase beta-NAH that participates in the matrix degradation process is characterized by an up-regulation (37.5-fold) in AP compared with CP. These differences cannot be explained by altered signal transduction events governing the PMN responses in AP. Beta-NAH is considered to be an outer-membrane-associated lipoprotein of *P. gingivalis* (Lovatt and Roberts, 1994). Unwanted contributions of microbial-origin beta-NAH from the periodontal environment may accelerate neutrophil-generated tissue damage. Resolution of inflammation and clinical healing induced a concomitant down-regulation of beta-NAH significant for both disease groups. The striking differences for beta-NAH between AP and CP after therapy, where the microbial impact on inflammation is

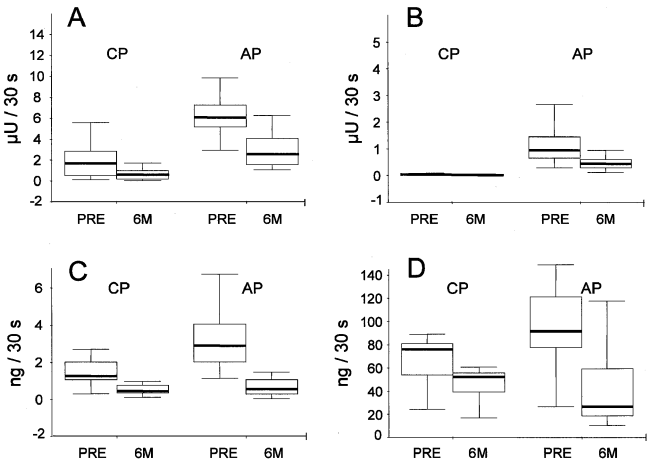

Figure. Initial GCF neutrophil responses in AP were significantly amplified compared with those in CP. **(A)** Myeloperoxidase activity (MPO); **(B)** beta-N-acetyl-hexosaminidase activity (Beta-NAH); **(C)** cathepsin D (CD); and **(D)** elastase-alpha-1-proteinase inhibitor complex (Alpha-1-EPI). PRE = outset. 6 M = 6 mos post-therapy. The post-treatment levels remained significantly different between AP and CP (not CD and Alpha-1-EPI).

Table 2. Six Months after Periodontal Therapy, the Reduction in GCF Leukocyte Patient Levels of Myeloperoxidase (MPO), Beta-N-acetyl-hexosaminidase (Beta-NAH), Cathepsin D (CD), and Elastase-alpha-1-proteinase Inhibitor Complex (Alpha-1-EPI) was Statistically Significant in AP and CP

GCF Marker		MPO (µU/30 sec)		Beta-NAH (µU/30 sec)		CD (ng/30 sec)		Alpha-1-EPI (ng/30 sec)	
Period		Baseline	6 mos	Baseline	6 mos	Baseline	6 mos	Baseline	6 mos
AP	Means	6.4	2.9	1.5	0.5	3.1	0.7	94.0	40.1
	SD	(2.5)[a]	(1.6)	(1.3)	(0.4)	(1.6)	(0.5)	(31.3)	(29.8)
	Change (6 mos)		3.5		0.9		2.4		54.0
	SD		(2.7)		(1.3)		(1.7)		(33.9)
	P-value		0.001[b]		0.001		0.001		0.001
CP	Means	2.0	0.7	0.04	0.03	1.4	0.5	68.2	47.0
	SD	(1.7)	(0.5)	(0.03)	(0.02)	(0.7)	(0.3)	(20.0)	(13.3)
	Change (6 mos)		1.3		0.01		0.9		21.2
	SD		(1.2)		(0.01)		(0.4)		(7.2)
	P-value		0.005		0.017		0.005		0.005
						AP *vs.* CP (change after 6 mos)		MPO	0.003[c]
								Beta-NAH	0.002
								CD	0.004
								Alpha-1-EPI	0.002

[a] Standard deviation.
[b] Wilcoxon Signed-rank test.
[c] Mann-Whitney test, P-value. The reduction of GCF markers was statistically different between AP and CP.

Table 3. Surgical Therapy Resulted in a Significant Reduction in GCF Site Levels of Myeloperoxidase (MPO), Beta-N-acetyl-hexosaminidase (Beta-NAH), Cathepsin D (CD), and Elastase-alpha-1-proteinase Inhibitor Complex (Alpha-1-EPI) after 6 Months Compared with Non-surgical Treatment

Treatment	Non-surgical Therapy (NS)[a]					Surgery (S)[b]				
	GCFv	MPO (μU/30 sec)	Beta-NAH (μU/30 sec)	CD (ng/30 sec)	Alpha-1-EPI (ng/30 sec)	GCFv	MPO (μU/30 sec)	Beta-NAH (μU/30 sec)	CD (ng/30 sec)	Alpha-1-EPI (ng/30 sec)
AP sites	68[c]	68	67	68	66	132	129	132	132	131
Baseline	126.6[d]	4.9	1.3	3.8	71.9	149.0	8.5	1.3	2.5	102.7
6 mos	86.1[d]	2.8	0.5	0.9	38.6	100.9	2.9	0.6	0.8	39.9
Change	40.5	2.1	0.8	2.9	33.3	48.1	5.6	0.7	1.7	62.8
SD[e]	(53.6)	(4.9)	(3.3)	(2.7)	(66.6)	(56.1)	(8.3)	(3.0)	(2.1)	(65.0)
				NS *vs.* S (change after 6 mos)		0.426[f]	0.0001¨	0.027	0.002¨	0.001¨
CP sites	32	31	32	29	32	40	40	36	40	40
Baseline	104.5	1.4	0.03	1.0	63.2	150.5	2.9	0.06	1.7	77.2
6 mos	55.2	0.4	0.02	0.4	44.1	79.6	0.9	0.03	0.7	53.0
Change	49.3	1.0	0.01	0.6	19.1	70.9	2.0	0.03	1.0	24.2
SD	(25.0)	(1.1)	(0.01)	(0.6)	(7.2)	(18.2)	(1.2)	(0.01)	(0.6)	(4.1)
				NS *vs.* S (change after 6 mos)		0.0001¨	0.0001¨	0.008¨	0.0001¨	0.017¨

[a] Initial probing depths < 6 mm.
[b] Initial probing depths ³ 6 mm.
[c] Number of sites.
[d] Means.
[e] Standard deviation.
[f] ¨ Significant after adjustment to Bonferroni **AQ**
[g] Wilcoxon Signed-rank test.

less pronounced, support the hypothesis of a pivotal role of neutrophil action in severe inflammation.

CD is linked to PMN secretion at the endpoint of inflammation (Lamster, 1991), as the lesion begins to resolve. We found a 2.2-fold increase of CD in AP compared with CP. The release of CD during acute inflammation simultaneously inactivates endogenous proteinase inhibitors, allowing uncomplexed, functionally active CD to contribute to ongoing tissue damage (Tervahartiala *et al.*, 1996). CD concentrations decrease with resolution of inflammation. The differences between AP and CP disappeared after completion of periodontal therapy.

The functional stability of the alpha-1-EPI complex depends on oxidation by free radicals and the action of proteolytic enzymes destroying the reactive alpha-1-PI loop. We demonstrate here significantly different alpha-1-EPI levels at sites from AP or CP patients. These observations provide evidence of potent leukocyte actions in excessive inflammation. The decrement of the alpha-1-EPI complex in AP following therapy is consistent with previous findings in CP (Flemmig *et al.*, 1996), where exudating neutrophils were reduced by additional topical application of antimicrobials during therapy.

Probing depths were found to be associated with leukocyte activity in the gingival crevice. Regardless of the type of disease, observed changes in GCF markers at sites with PD ³ 6 mm treated by surgery were more pronounced compared with non-surgically-treated sites. The relationship of alpha-1-EPI to PD has recently been confirmed (Meyer *et al.*, 1997). In contrast, the location of sample sites in the oral cavity does not affect leukocyte activity and the resulting GCF parameters (data not shown). The need for surgical intervention in the treatment of AP is controversial. It remains unclear why surgical access would yield better results in the resolution of inflammatory markers, but it is likely related to better access for the removal of etiologic factors in the treatment of the disease.

In conclusion, analysis of the data presented here supports the concept that effective treatment of periodontal disease can be measured with the use of biochemical markers of PMN activity. Our results stress the apparent tight regulation of PMN activity during periodontal inflammation, wound healing, and resolution, until homeostasis is achieved.

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