Original Article

Assessment of Neutrophil Chemotaxis, Phagocytosis and Specific Granule Release in Aggressive and Chronic Periodontitis Patients : An In-Vitro Study

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ABSTRACT

Introduction: Neutrophils are responsible for immunologically induced tissue injury. When normal regulatory mechanisms fail, following activation by bacterial by-products or other immune stimuli, neutrophils execute several specialized functions that include chemotaxis, phagocytosis, generation of reactive oxygen metabolites and specific granule release. The important role polymorphonuclear neutrophils (PMNs) play in optimal functioning of the immune defense system has led to speculation that a partly compromised system could severely weaken the defense mounted against a bacterial insult and permit the occurrence and progression of infections. The study was aimed to assess neutrophil function i.e. chemotaxis, phagocytosis and specific granule release in patients with aggressive and chronic periodontitis in Indian population.

Methodology: 60 patients with age group varying between 13-60 years from both sex were selected and were divided into three groups: Group I – 20 Generalized Aggressive Periodontitis subjects (GAgP), Group II – 20 Generalized Chronic Periodontitis subjects (CP), Group III – 20 Control Group of periodontally healthy subjects. Venous blood samples were collected and various neutrophil functions viz. chemotaxis, phagocytosis and specific granule release were carried out using different assays such as N-Formylmethionyl-leucyl-phenylalanine (FMLP), Candidia albicans suspension, Nitroblue tetrazolium test (NBT).

Results: The results of the study suggest that chemotaxis was significantly reduced in generalised aggressive periodontitis patients as compared to chronic periodontitis and periodontally healthy subjects. GAgP and CP patients showed no significant difference in phagocytosis rate as compared to periodontally healthy control group. Unstimulated NBT reduction is increased in neutrophils from patients with infection and the intracellular killing capacity of neutrophils is reduced in GAgP and CP patients.

Conclusion: The assessment of neutrophil functions may be important and useful tool for explaining various types of periodontal pathologies.

INTRODUCTION

Periodontal disease is an infectious inflammatory process of multifactorial nature that involves the interplay between bacteria, host, and environmental factors.[1] The dynamics between the host immune responses and oral bacteria is essential to understand the pathogenesis of periodontal diseases.[2] Periodontitis occurs in more than one form: aggressive and chronic. When normal regulatory mechanisms fail, the neutrophils are responsible for immunologically induced tissue injury. Following activation by bacterial byproducts or other immune stimuli, neutrophils execute several specialized functions that include chemotaxis, phagocytosis, generation of reactive oxygen metabolites and specific granule release. All of these processes are required for the

elimination of invading microorganisms. Disturbances in these processes results in increase in susceptibility to bacterial infection.[3] Periodontium, in particular reflect the effects of an over exuberant neutrophil mediated immune response. Evidence of polymorphonuclear neutrophils (PMNs) in periodontal disease comes from the observations that individuals with severe periodontitis exhibit qualitative and/or quantitative defects in their peripheral PMNs.[1] The important role PMNs play in optimal functioning of the immune defense system has led to speculation that a partly compromised system could severely weaken the defense mounted against a bacterial insult and permit the occurrence and progression of infections. Since there are few studies been done in this context and very little data available in Indian population the present study was undertaken to assess in vitro neutrophil chemotaxis, phagocytosis and specific granule release in aggressive and chronic periodontitis and also to compare these neutrophil functions with the control group of periodontally healthy subjects.

METHODS

The present study was carried out on 60 patients selected from the outpatient department of Periodontology, Dr. D.Y Patil Dental College and Hospital, Pune. Patients of either sex willing to comply with study related procedure and having good general systemic health within the age group ranging from 13-60 years were included in the study. Peripheral venous blood samples from patients were collected and sent to laboratory for various assays. A written informed consent was signed by the patients selected for the study. Patients with a history of any systemic disorder or if undergone radiotherapy/ chemotherapy or any kind of periodontal treatment in last six months were excluded from the study. Patient having habit of tobacco use, alcohol consumption within 48 hrs prior to study were excluded from the study. Lactating mothers and pregnant females were also excluded from participating in the study. Female patients on contraceptives and also, patients who had used any kind of medication 48 hours prior to study were excluded from the study. Subjects were divided into three groups: - Group I – 20 Generalized Aggressive Periodontitis subjects (GAgP), Group II – 20 Generalized Chronic Periodontitis subjects (CP), Group III – 20 Control Group of periodontally healthy subjects. The following clinical parameters were recorded: Gingival Index (GI), Plaque Index (PlI), Periodontal Index (PI), probing depth recorded by using UNC-15 periodontal probe, Clinical Attachment Level (CAL), Radiographs- Intraoral Periapical (IOPA)/ Orthopantomograph (OPG) was taken as diagnostic aid.

Neutrophil Function Tests

5 ml of venous blood was drawn from the antecubital vein under aseptic condition which was transferred into a vial containing EDTA and transported to the laboratory for test. The neutrophil function tests done were chemotaxis, phagocytosis and specific granule release estimation.[4,5,6,7]

Neutrophil Chemotaxis Assay: - (Agarose Technique): Chemotaxis was measured as linear distance (in cm) that white blood cells have migrated from margin of well toward FMLP chemoattractant.

Phagocytosis Assay: Number of ingested candida associated with each cell was counted for phagocytosis assay.

Specific Granule Release Assay: Specific granule release from stimulated human neutrophils was done using qualitative test by Nitroblue Tetrazolium (NBT) test.

The data were analyzed using Student 't' test for paired and unpaired observations to assess changes obtained within the group. One way ANOVA test was used to assess changes obtained between the groups. p values <0.05 were considered as singnificant and < 0.001 were considered as highly significant. All analysis was performed using SPSS software.

RESULTS

Neutrophil chemotaxis: Neutrophil chemotaxis was measured using N-Formylmethionyl-leucyl-phenylalanine assay FMLP as chemoattractant. Neutrophil chemotaxis FMLP between all the three groups was measured using one way ANOVA test. The mean chemotaxis between FMLP of group I , group II and group III with $p<0.001$ was found to be statistically significant (Table 1).

Table 1: Comparison of mean chemotaxis assay between generalized aggressive, chronic periodontitis and healthy control subjects

CHEMOTAXIS ASSAY	Mean ±SD	ANOVA
GAgP	2.805±0.8513	79.9 (HS)
CP	0.845±0.1261	
Control	0.770±0.2523	

HS=Highly Significant
GAgP: Generalized aggressive periodontitis subjects;
CP: Generalized chronic perioodontitis subjects

Table 2: Intergroup comparison of mean phagocytosis of generalized aggressive, chronic periodontitis and healthy control subjects

PHAGOCYTOSIS	GENERALIZED AGGRESSIVE PERIODONTITIS		CHRONIC PERIODONTITIS		HEALTHY CONTROL	
	Mean ± SD	t-teset	Mean ± SD	t-teset	Mean ± SD	t-teset
Control	2.85 ±0.447	1.83 (NS)	2.75 ±0.503	1.83 (NS)	2.73 ±0.510	1.71 (NS)
Test	2.90±0.394		2.70±0.394		2.65±0.639	

NS=Not Significant

Phagocytosis :The mean phagocytosis in group I, group II and group III in control and in test with $p>0.05$ was found to be statistically non significant (Table 2).

Phagocytosis assay was calculated between all the three groups using one way ANOVA test. The mean phagocytosis between controls of group I, group II and group III with $p<0.001$ was found to be statistically significant (Table 3).

Table 3: Comparison of mean phagocytosis assay between generalized aggressive, chronic periodontitis and healthy control subjects

CHEMOTAXIS ASSAY	Mean ±SD	ANOVA
GAgP	2.85±0.447	16.08 (HS)
CP	2.75±0.587	
Control	2.73±0.510	

HS=Highly Significant
GAgP: Generalized aggressive periodontitis subjects
CP:Generalized chronic perioodontitis subjects

Table 4 : Comparison of mean specific granule release assay using nitroblue tetrazolium (NBT) reduction test between generalized aggressive, chronic periodontitis and healthy control subjects

	NBT REDUCTION TEST			
	Stimulated		Unstimulated	
Groups	Mean ±SD	ANOVA	Mean ±SD	ANOVA
GAgP	77.05±5.04%	0.054 (NS)	38.35±11.73%	22.297 (HS)
CP	76.60±8.55%		38.40±10.22%	
Control	76.40±4.91%		21.85±1.66%	

NS=Not Significant, HS=Highly Significant
GAgP: Generalized aggressive periodontitis subjects;
CP:Generalized chronic perioodontitis subjects

Specific granule release assay using Nitroblue Tetrazolium Reduction Test (NBT):

Stimulated and unstimulated tests of all the three groups were calculated using one way ANOVA test. The NBT stimulated test for group I, group II and group III with $p>0.05$ was found to be statistically non significant. The NBT unstimulated test for group I, group II was and group III with $p<0.001$ was found to be statistically significant (Table 4).

DISCUSSION

In the present study, result indicate that GAgP subjects exhibit defective chemotaxis towards FMLP chemoattaractant as compared to CP and periodontally healthy control subject (Figure 1). Results of the present study also show that in CP subjects, there was moderate chemotactic movement towards an FMLP chemoattractant as compared to control group (Figure 2). These results extend and confirm earlier studies[4, 5, 6] in which defective PMNL chemotaxis was reported in subjects with GAgP. Vandyke et al[7] showed that two of 23 patients with adult periodontitis exhibited chemotaxis defect. Tufano et al 1992[8] showed that neutrophil chemotaxis response in adult periodontitis group was comparable to those of a healthy control group.

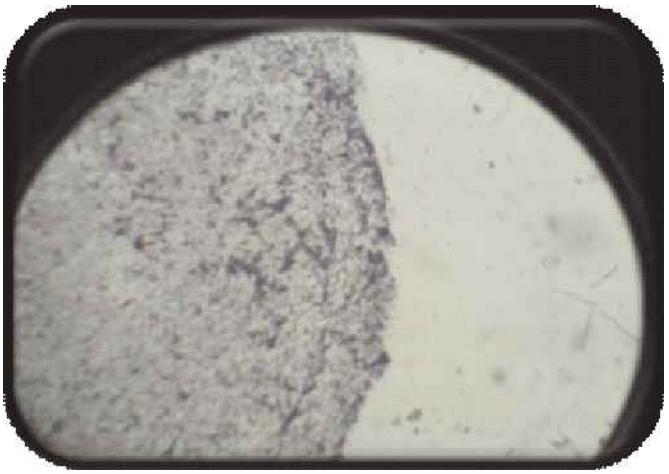

Figure 1: GAgP subject showing no chemotaxis towards an FMLP Chemoattractant.

Figure 2: CP subject showing minimal chemotaxis towards FMLP chemoattractan.

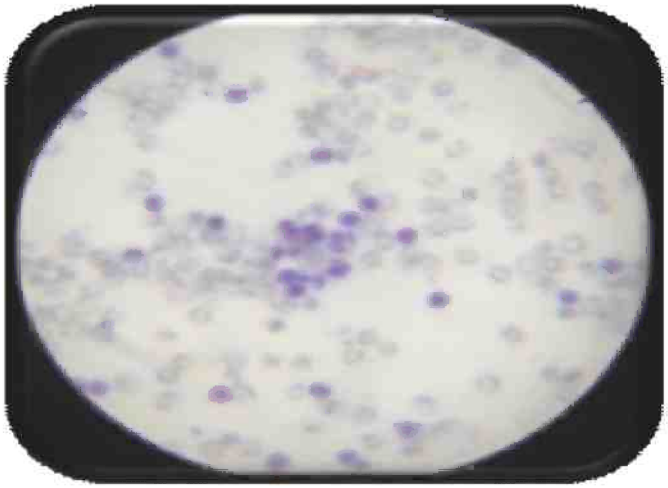

Figure 3: Phagocytosis showing three candida albicans in GAgP subject.

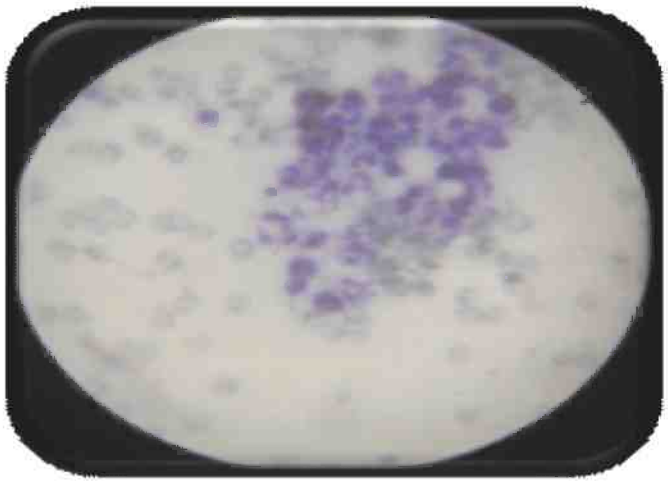

Figure 4 : Phagocytosis showing multiple candida albicans in CP subject.

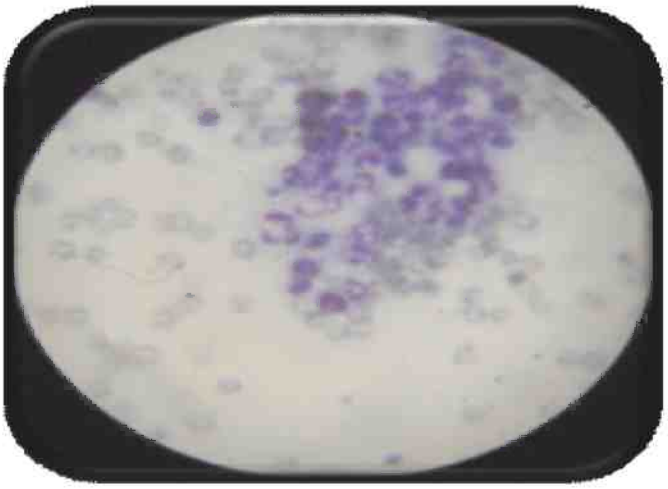

Figure 5 : Phagocytosis showing multiple candida albicans in Control subject.

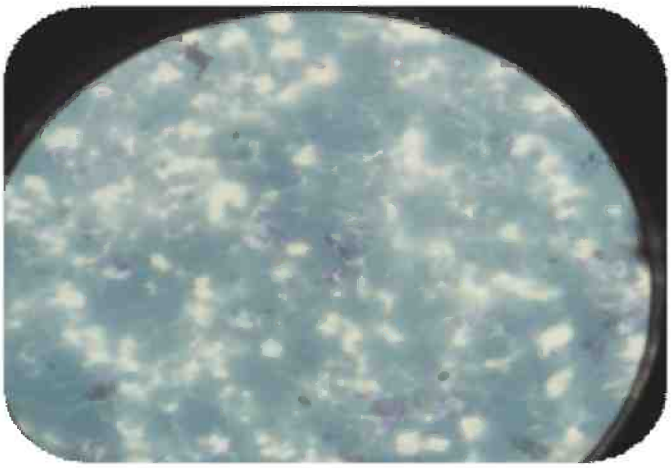

Figure 6: NBT showing stimulated cells after taking up the dye in control subject.

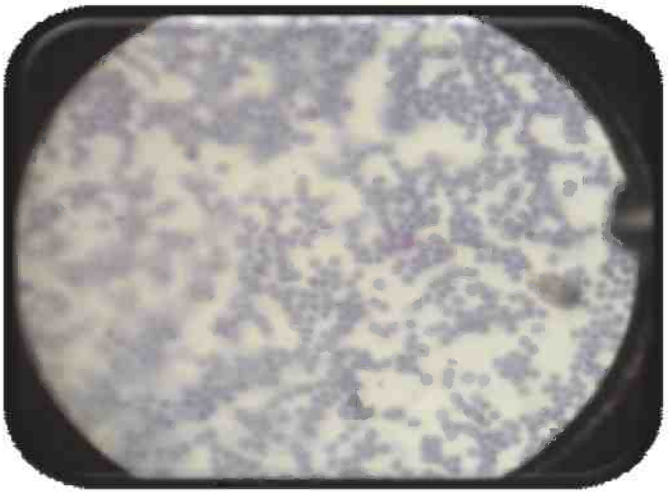

Figure 7: NBT showing unstimulated cells which did not take up the dye in GAgP subject.

Correlation between peripheral PMN phagocytosis among GAgP, CP and periodontally healthy control group showed no significant difference in phagocytosis rate as compared to periodontally healthy control group (Figures 3, 4 and 5).

Similar findings have been reported by Takahashi et al 2001[9] who studied 50 GAgP subjects 43 CP subjects and 36 periodontally healthy subjects and reported no significant PMN phagocytic dysfunctions between GAgP, CP and control subjects. Okada et al 2002[10] also reported normal phagocyte function levels and a remarkable level of depressed neutrophil chemotaxis to N-formyl-methionyl-leucyl-phenylalanine in all subjects in comparison to healthy control subjects. On the contrary, results of few other studies[4, 11, 12, 13] have showed that GAgP patients presented a significantly diminished phagocytosis rate of opsonised bacteria compared with periodontally healthy subjects.

When specific granule release was assayed, it was found that the intensity of the blue color was less in unstimulated cells in GAgP and CP group as compared to the periodontally healthy control group subjects. Stimulated cells from all the three groups showed that the dye was taken up into phagosomes and intracellular reduction of dye converted it to an insoluble blue crystalline form which was visible in light microscope (Figure 6). This suggested that unstimulated NBT reduction is increased in neutrophils from patients with periodontal infection and the intracellular killing capacity of neutrophils is reduced in GAgP and CP patients (Figure 7) GAgP and CP subjects were less able to reduce NBT as compared to healthy controls. Lavine et al 1979[15] reported abnormal NBT reduction in only one of eight localised juvenile periodontitis (LJP) subjects. Conflicting data among our findings and other studies may have resulted from differences in study population, assays for chemotaxis, phagocytosis, and specific granule release detection. One possible limitation of our study may have been the small size of the sample population and diagnosis of GAgP based on clinical and radiographic investigations excluding more conclusive microbiological diagnosis.

CONCLUSION

Within the limitations of the present study, it can be suggested that the assessment of neutrophil functions viz, chemotaxis, phagocytosis, specific granule release may be important and useful tool for explaining various types of periodontal pathologies. Defective neutrophil function may play an important role in the induction and pathogenesis of severe periodontitis in young individuals. Diseases resulting in destruction of periodontal support may be due to decreased capabilities of selected aspects of neutrophil function.

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REFERENCES

1. Page RC, Kornman KS. The pathogenesis of human periodontitis: An Introduction. Periodontol 2000 1997; 14:9-11.
2. HiromasaYoshie, Kobayashi T. The role of genetic polymorphisms in periodontitis. Periodontol 2000 2007; 43: 102-132.
3. Page RC, Sims TM, F Gaissler, Altman and Baab. Defective neutrophil and monocyte motility in patients with early onset periodontitis. Infect Immun 1985; 47 (Jan): 169-175.
4. Van Dyke TE, Horoszewicz HU, Cianciola LJ, and Genco RJ. Neutrophil chemotaxis dysfunction in human periodontitis. Infection and Immunity1980; Jan : 27(1): 124-132.
5. Clark RA, Page RC and Wilde G. Defective Neutrophil Chemotaxis in Juvenile Periodontitis. Infect Immun 1977; Dec (18): 694-700.
6. Katsuragi Y, Matsuda N, Nakamura M. and Murayama Y. Neutrophil Functions in Patients with Severe Periodontal Disease. Adv Dent Res 1988; 2: 359-363.
7. Van Dyke TE, Zinney W, Winkel K, Taufiq A, Offenbacher S and Arnold RR. Neutrophil function in localized juvenile periodontitis phagocytosis, superoxide production and specific granule release. J Periodontol 1986; 57(Nov):703-708.
8. Tufano MA, Ianniello R, Sanges MR, Rossano F. Neutrophil function in rapidly progressive and adult periodontitis. 1992 :Jan; 8(1): 67-73.
9. Takahashi K, Ohyama H, Kitnaka M et al. Heterogeneity of host immunological risk factors in patients with aggressive periodontitis. J Periodontol 2001; 72: 425-437.

10. Okada M, Awane S, Suzuki J et al. Microbiological, immunological and genetic factors in family members with periodontitis as manifestation of systemic disease, associated with haematological disorders. J Periodontal Res 2002; 37: 307-315.
11. Van Dyke TE, Horoszewicz HE, Cianciola LJ, and Genco RJ. Neutrophil chemotaxis dysfunction in human periodontitis. Infect Immun 1980; 27(1): 124-132.
12. Kimura S, Yonemura T, Hiraga T, Okada H. Flow cytometric evaluations of phagocytosis by peripheral blood polymorphonuclear leukocytes in human periodontal diseases. Arch Oral Biol 1992; 37: 495-501.
13. Eick S, Pfister W, Sigusch B, Straube E. Phagocytosis of periodontopathogenic bacteria by crevicular granulocytes is depressed in progressive periodontitis. Infection 2000; 28: 301-304.
14. Lavine WS, Madezaro, EG, Stolman J, Ward PA, Cogan RB, Greenblatt I, Robertson PB. Impaired neutrophil chemotaxis in human periodontitis. J Periodont Res 1979; 14:10.

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