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SALIVARY PLATELET ACTIVATING FACTOR LEVELS IN PERIODONTAL DISEASE

A THESIS

Presented to the Faculty of

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at San Antonio

in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

By

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San Antonio, Texas

May 1991

SALIVARY PLATELET ACTIVATING FACTOR LEVELS IN PERIODONTAL DISEASE

Martha Laura Garito

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Supervising Professor

April 8, 1991
Date

APPROVED:

Dean

DEDICATION

To my family, without whose love and support this endeavor would not have been possible.

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SALIVARY PAF LEVELS IN PERIODONTAL DISEASE

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Platelet activating factor (PAF), a potent phospholipid inflammatory mediator, has been found to be present in normal human saliva; however, its contribution to oral pathobiology remains unknown. The purpose of this study was to evaluate possible relationships between salivary PAF levels and periodontal disease. One ml of mixed saliva was collected from 69 untreated subjects presenting for evaluation at the UTHSC dental hygiene or periodontal clinic. After phospholipid extraction and fractionation by thin layer chromatography, salivary PAF activity was

determined by platelet bioassay. PAF activity was estimated relative to that of authentic PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine [C16:0-AGEPC]), and was expressed in C16:0-AGEPC fmole equivalents/ml saliva; tracer amounts of ^{3}H-AGEPC were included in all samples prior to initial extraction and used to calculate PAF recovery. Subjects were subdivided into 6 similarly sized groups according to disease severity (based on probing depths). The healthiest group, Group 1, had $\leq$4 mm probing depths throughout, while the most severely affected group, Group 6, averaged >4 mm probing depths in 50% of the sites. No significant differences were noted between the groups for age, sex or number of teeth. A correlation was found between the number of bleeding sites and the 6 groups. PAF levels generally increased from Group 1 to Group 6, with Group 1 levels (2,365 $\pm$ 900 C16:0-AGEPC fmole equivalents/ml; mean $\pm$ SE) being significantly lower than Groups 5 and 6 (10,489 $\pm$ 1,775 and 10,251 $\pm$ 3,075, respectively). These findings indicate that salivary PAF levels correlate with periodontal status and suggest that this phospholipid inflammatory mediator may play a role in the pathogenesis of periodontal disease.

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I. INTRODUCTION

Platelet-activating factor (PAF) represents a lipid autocoid family of acetylated phosphoglycerides which have been shown to induce inflammatory reactions (Braquet, *et al*, 1987). PAF is rapidly synthesized by a variety of inflammatory cells following stimulation (*c.f.*. Pinckard, *et al*, 1988). In 1981, Cox *et al* identified the presence of PAF in normal human saliva. More recently, McManus, Marze and Sc' 'ess (1990) demonstrated a significant decrease in the levels of PAF in edentulous patients. Although the source of salivary PAF is not known, characterization studies originally described by Cox (1981) and later substantiated by Smith, Schiess and McManus (1986) have found salivary PAF to be indistinguishable from the PAF produced by activated inflammatory cells. The relation of salivary PAF to periodontal disease has not been established. Thus, the purpose of this investigation was to determine whether a significant relationship exists between salivary PAF levels and periodontal disease.

II. LITERATURE REVIEW

A. Role of Inflammation in Periodontal Disease

Inflammation occurs as a response to tissue injury. The clinical presentation of this response was first reported by Cornelius Celsus (30 BC- 38 AD) as redness, swelling, heat and pain (Weissmann, 1988). These are referred to as the cardinal signs of inflammation, with loss of function later added by Galen (Walter, 1987). On a cellular level, one may see a varied inflammatory response dependent on the quantity and quality of the irritant and the nature of the host (Bhaskar, 1986). Overall, the purpose of the inflammatory response is to eliminate or wall off the injurious agent.

Following injury, alterations occur in the local vasculature. The initial response is that of vasoconstriction and develops as a result of nerve reflexes and muscle spasm (Hurley, 1983). This response is enhanced by the subsequent release of serotonin and thromboxane A_2 (Hurley, 1983). This initial vasoconstrictive response is transient due to the release of vasodilators such as histamine and prostacyclin (White and Kaliner, 1988). Increased blood flow and increased vascular permeability then follow (Hurley, 1983). While one may expect the increase in permeability to be a result of vasodilation, these two processes may occur independently and are therefore distinct entities. The increase in vascular permeability is due to the contraction of endothelial cells lining post-capillary venules. When these cells contract, small gaps occur which

allow the extravasation of plasma fluids and proteins with or without polymorphonuclear leukocytes (PMN). While histamine produces vasodilation and increased vascular permeability (Hurley, 1983), other mediators are more specific in their effect. For example, bradykinin has been shown to produce immediate and massive increases in vascular permeability, yet it has only slight effects on vasodilation and hyperemia (Hay, *et al*, 1977). Conversely, prostaglandins E_1 and E_2 have strong hyperemic effects but no significant effects on permeability (Hay, *et al*, 1977). The exact effects of these mediators in the natural process of inflammation are difficult to determine as they tend to interact with one another. For example, when bradykinin and prostaglandin E_1 are injected simultaneously, a synergistic response develops and results in increased vascular permeability which is one-hundred fold greater than when bradykinin is injected alone (Williams and Morley, 1973). Thus, the vascular response during inflammation is multifarious and is activated through multiple mediators.

The inflammatory process can be subdivided into acute and chronic inflammation. Stedman's Medical Dictionary (1990) defines acute inflammation as, "any inflammation that has a fairly rapid onset, quickly becomes severe, and has a relatively clear and distinct termination...." It is characterized by the local vascular response described above. Exudation of plasma follows and acts to dilute whatever toxins may be present (Madri, 1990). In addition, endogenous mediators, such as C5a, may attract polymorphonuclear leukocytes (PMN) to the site of injury (Madri, 1990). This most likely explains why the PMN is the predominant cell in the acute inflammatory process (Walter, 1987).

While a major function of these cells is associated with phagocytosis, PMN have been shown to secrete enzymes and other biologically relevant mediators into the extracellular fluid as well (Van Dyke, *et al*, 1985). These include such enzymes as collagenase, elastase and lysozyme as well as reactive oxygen species and arachidonic acid metabolites (Van Dyke, *et al*, 1985). As outlined above, the objective of the inflammatory response is to destroy or inactivate the injurious agent. The acute inflammatory phase may last from several days to several weeks, and, depending on whether the injuring agent is eliminated or not, will either cease or progress into a chronic inflammatory state (Madri, 1990).

In contrast to acute inflammation, chronic inflammation tends to be a slow, prolonged process which tends to involve different cells and mediators. It may begin as a progression of an acute inflammatory process or as an initially chronic lesion (Madri, 1990). In chronic inflammation, tissue damage may be widespread with an abundance of lymphocytes, plasma cells and macrophages (Hurley, 1983). Connective tissue proliferation and scar formation develop concomitantly (Madri, 1990). The resolution of chronic inflammation, as with acute inflammation, centers around elimination of the irritant. Unfortunately, this is not always possible, as evidenced by the number of chronic inflammatory diseases.

Periodontal diseases tend to be inflammatory in nature, with the same characteristic inflammatory patterns that are noted elsewhere in the body. Some structural and functional features are unique to the periodontium and influence the progression and magnitude of the inflammatory response. Grant, Stern and Listgarten (1988) describe

these as: 1) intimate contact of the soft tissue with the tooth on which plaque accumulates, 2) nonkeratinized junctional epithelium with wide intercellular spaces, 3) accessibility of plaque products to extensive microvasculature (subjacent to the sulcus) resulting in increased permeability and gingival exudate and 5) gingival crevicular fluid containing inflammatory cells and mediators.

Page and Schroeder (1976) examined the pathogenesis of inflammatory periodontal disease and described four histopathologic stages. The "initial lesion" occurs 2-4 days following plaque accumulation and manifests as an acute exudative inflammatory response. Vessels subjacent to the junctional epithelium are engorged and dilated, PMN are increased in the junctional epithelium and sulcus, and perivascular collagen is lost. Fluid and serum proteins (including immunoglobulins and complement) also accumulate in the periodontal tissues. The "early lesion" appears 4-7 days after plaque accumulation, and is characterized by a dense lymphocytic infiltrate. Cellular hypersensitivity has been suggested as a component of this stage (Page and Schroeder, 1976). Crevicular fluid flow and crevicular PMN accumulation reach a peak at 6-12 days and are accompanied by loss of up to 70% of the collagen at the reaction site. The "established lesion" develops within 2-3 weeks following plaque accumulation and is distinguished by the predominance of IgG (and IgA to a lesser extent) producing plasma cells. Where pocket epithelium is present, blood vessels loop high within the epithelium. Immunoglobulins, complement and antigen-antibody complexes are present especially around the blood vessels. The "advanced lesion" constitutes a continuation of the established lesion, but also includes

surface ulceration and suppuration, loss of alveolar bone, loss of collagen subjacent to the pocket epithelium with fibrosis at more distant sites and conversion of the bone marrow (distant from the lesion) into fibrous connective tissue (Page and Schroeder, 1976). The histological appearance of the lesion is dominated by a dense infiltrate of plasma cells, lymphocytes and macrophages, with cellular degeneration noted in many of the plasma cells. In the midst of this chronic fibrotic inflammation, an acute exudative vasculitis persists. Thus, the histopathologic features of the advanced lesion resemble those of long term chronic inflammatory disease.

The initial, early and established lesions of inflammatory periodontal disease are similar in that they can remain stable without progression into an advanced lesion or functional impairment for unlimited periods of time (Suomi, *et al*, 1980). In these stages, the host defense is successful in providing a barrier which separates the oral environment from the deeper tissues. The mechanism for progression into the advanced stage is not known. It may be due to changes in the oral flora resulting in the establishment of periodontopathogens, or may be the result of a host response in which the damage created outweighs the defense achieved (Genco, *et al*, 1974).

Several studies suggest that periodontal tissue damage may occur due to inflammatory cells and mediators. Taichman *et al* (1966) evaluated the response of normal and neutropenic rabbits to intradermal injections of plaque. They found less tissue damage in the neutropenic rabbits than in those with normal PMN function. Similar results were described by Attstrom & Schroeder (1979) who observed a decrease in

clinical gingivitis in dogs following reduction of circulating PMN. Tissue damage is speculated to be due, at least in part, to PMN degranulation or release of lysosomal enzymes (Miller, *et al*, 1984). PMN degranulation has been observed in chronically inflammed (Freedman, *et al*, 1968) and elevated levels of collagenase, elastase and other hydrolases have been noted as well (Taichman, *et al*, 1984). Other inflammatory mediators have also been implicated in the destruction of periodontal tissues. For example, Offenbacher *et al* (1986) found increased levels of PGE_2 in sites with attachment loss versus the contralateral sites exhibiting no attachment loss. Further, Jadinski and co-workers (1991) reported a three-fold increase in interleukin-1β producing cells. Just recently, inflammed human gingival tissues were analyzed and found to contain PAF (Noguchi, *et al*, 1989). Thus, multiple components of the inflammatory system have been shown to be present in periodontally diseased sites.

In summary, periodontal disease is bacterial in nature (Kahnberg, *et al*, 1976). Clearly, gingivitis occurs routinely following plaque accumulation (Loe, *et al*, 1965). Unfortunately, the determinants of periodontal disease progression are not known, and may involve the host response. While the inflammatory process is protective to the host as a whole, the potential exists to severely damage the local tissues.

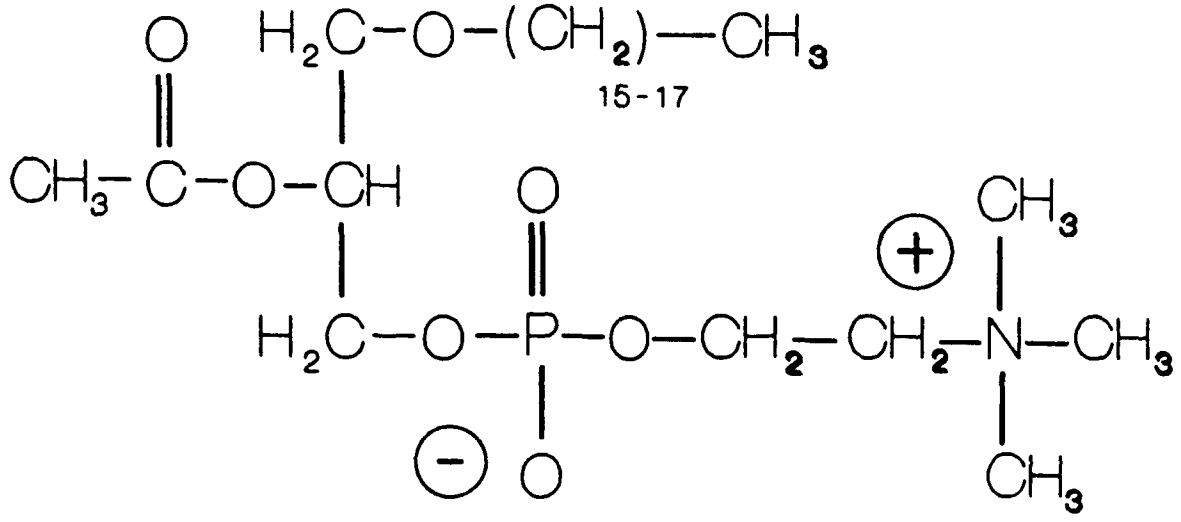
B. Platelet Activating Factor (PAF)

Platelet activating factor was originally identified during a characterization study of IgE sensitized rabbit leukocytes following antigen stimulation (Siraganian and Osler, 1971). During this reaction, a fluid-phase mediator was released which was capable of aggregating

platelets and causing them to release histamine. In view of its biologic activity, and because its structure was not known, this mediator was given the name "platelet activating factor" or PAF (Benveniste, *et al*, 1972).

Studies to determine the chemical structure of PAF were simultaneously accomplished by Demopoulos *et al* , Blank *et al*, and Benveniste *et al* in 1979. The chemical structure of PAF is represented in Figure 1. PAF was originally found to be extractable in ethanol and chloroform, indicating that it was a lipid-like molecule. When subjected to thin layer chromatography (TLC), it was found to migrate between lysolecithin and sphingomyelin. It was resistant to acid treatment and heating in the presence of atmospheric oxygen indicating that no vinyl ether groups or unsaturation in aliphatic side chains were present. Vicinal glycols and unmodified hydroxyls as essential structural components were ruled out by the inability of periodate, acetic acid anhydride and sodium nitrate to modify PAF activity. An essential ester linkage was required, as the activity of PAF was quickly destroyed following base-catalyzed methanolysis with methanolic sodium hydroxide. The base-catalyzed degradation product was found to be soluble in 1:1:0.9 ratio of chloroform, methanol and water, which indicated that it was not a diacyl phosphoglyceride, but that it probably contained an alkyl linkage. Reacylation of this inactivated molecule with acetic, proprionic, or butyric acid anhydrides restored the biological activity. When this acetylated molecule was subjected to TLC, it was found to have the same migration pattern as PAF.

Figure 1. Chemical structure of C16:0-C18:0-AGEPC



1-O-hexadecyl/octadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine

Ultimately, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (acetyl glyceryl ether phosphorylcholine or AGEPC) was synthetically produced and determined to have the same biological activity and indeed, the same structural identity as rabbit basophil (Hanahan, et al., 1980) and human neutrophil PAF (Pinckard, et al., 1984). However, it is also recognized that a variety of related phospholipid molecules (see below) are produced by these cells (Pinckard, et al., 1984). For the purpose of this paper, structures which remain unknown will be referred to as PAF, while those that are known will be referred to by their chemical name.

A variety of cells produce PAF including neutrophils, monocytes, macrophage, eosinophils, mast cells, basophils, platelets, natural killer cells, mesangial cells and vascular endothelial cells (Chilton, 1989). It can be isolated from a variety of tissues including the embryo, myocardium, lung, retina, skin, blood, kidney, brain, liver, uterus and saliva (Pinckard, et al., 1988). Its action appears to involve not only the regulation of inflammation but also may mediate physiological processes associated with reproduction, fetal development, parturition and kidney function (Snyder, 1990).

PAF Biosynthesis

Two enzymatic pathways have been described for the biosynthesis of PAF: the deacylation-acetylation remodeling pathway and the *de novo* pathway (Chilton, 1989). The deacylation-acetylation pathway was described by Wykle et al. (1980) and involves the modification of a structural phospholipid component of cell membranes. This appears to be the predominant pathway by which inflammatory cells synthesize PAF (Chilton, 1989). The *de novo* pathway involves the action of a

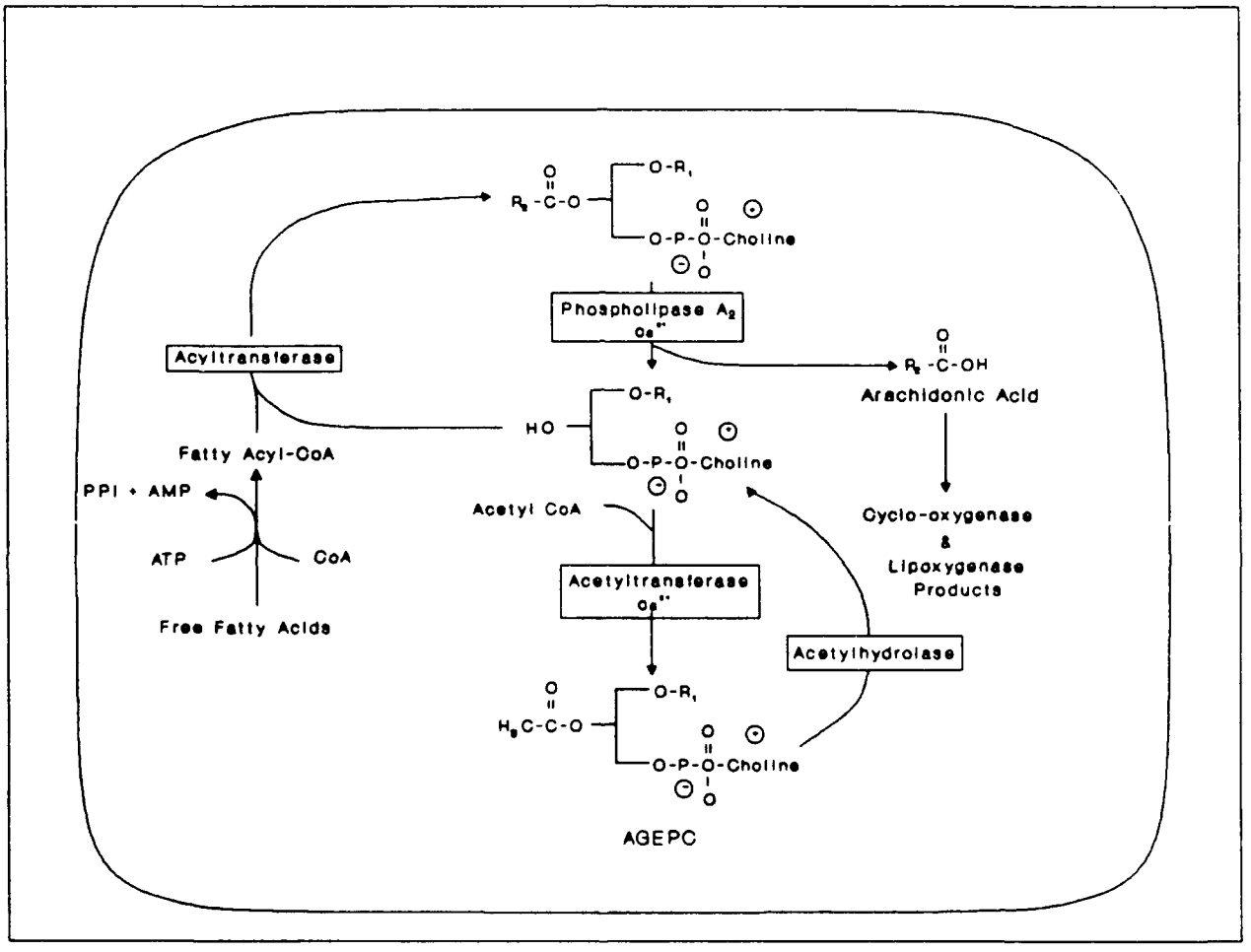
choline phosphotransferase and appears to play a more significant role in non-inflammatory cells (Renooij and Snyder, 1981).

Deacylation-acetylation pathway

Within the cell membrane, there is a precursor pool of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine. This molecule differs from AGEPC by having long chain fatty acids (consisting in part of arachidonic acid) in the number 2 position (see Figure 2). Cell stimulation causes the activation of phospholipase A_2, which acts on this precursor to cleave the long chain fatty acids, thereby releasing arachidonic acid and producing 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lyso-GEPC). This biologically inactive molecule is the immediate precursor of AGEPC. Acetyltransferase with acetyl-CoA acetylates the lyso-GEPC to produce 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC).

Thus, two principal enzymes are involved in the deacylation-acetylation pathway: phospholipase A_2 and acetyltransferase. The role of phospholipase A_2 (PLA_2) is major as its action may result in the formation of both AGEPC and arachidonic acid metabolites (Snyder, 1990). A PLA_2 which hydrolyzes both 1-alkyl and 1-acyl linked phosphatidylcholine has been isolated from human PMN (Alonso, et al., 1986). In addition, PLA_2 inhibitors such as mepacrine, EDTA, bromophenacyl bromide and hydrocortisone inhibit synthesis of lyso-GEPC and AGEPC (Braquet, 1988). Therefore, PLA_2 appears to modulate the availability of lyso-GEPC for acetylation to AGEPC.

Figure 2. PAF biosynthesis by the deacylation-acetylation pathway. Within the cell membrane, there is a PAF precursor pool of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine. Cell stimulation causes the activation of phospholipase A_2 which acts on this precursor to cleave the long chain fatty acid thereby releasing arachidonic acid and producing 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lyso-GEPC). Acetyltransferase with acetyl-CoA then acetylates lyso-GEPC to 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC). Degradation of PAF occurs through the action of acetylhydrolase. Further enzymatic activity by acyltransferase quickly restores this molecule to 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine, the PAF precursor molecule in the cell membrane.


Phospholipase A2
Ca++
Acyltransferase
Arachidonic Acid
Cyclo-oxygenase
&
Lipoxygenase
Products
Fatty Acyl-CoA
PPI + AMP
ATP
CoA
Free Fatty Acids
Acetyl CoA
Acetyltransferase
Ca++
Acetylhydrolase
AGEPC

The other principal enzyme involved in the deacylation-acetylation pathway is acetyltransferase. The activity of this enzyme is be elevated in stimulated inflammatory cells (Wykle, et al., 1986), and it now appears that its activation may occur through phosphorylation by a protein kinase (Lenihan and Lee, 1984). The substrate specificity of this enzyme in humans is not yet known. However, rat spleen acetyltransferase can acetylate different lysophospholipids including those with polar head groups other than choline (Pinckard, et al., 1988). Therefore, the substrate specificity may well determine the types of PAF synthesized by particular cell types.

De novo pathway

The synthesis of AGEPC via the *de novo* pathway involves three steps. First, the number 2 carbon in alkyl-lysoglycerophosphate is acetylated. In the second step, dephosphorylation of alkyl-acetyl-glycerophosphate takes place. This results in formation of alkyl-acetyl-glycerol. Phosphorylation of this molecule with CDP-choline is the third and final step leading to formation of AGEPC (Snyder, 1990).

As was mentioned above, the major pathway for biosynthesis of AGEPC in inflammatory cells appears to be the deacylation-acetylation or remodeling pathway. High proportions of alkyl linked phospholipids are present in inflammatory cells; thus providing the large precursor pools needed to support that pathway (Chilton, 1989). Moreover, the activity of acetyltransferase and not that of cholinephosphotransferase is increased significantly following inflammatory cell stimulation (Alonso, et al., 1982). In contrast, the *de novo* pathway for AGEPC synthesis may play a role in maintaining levels of PAF for the regulation of blood

pressure. The kidney, for example, has been reported to have increased levels of cholinephosphotransferase and little or no acetyltransferase (Chilton, 1989). Therefore, while the function of these pathways is the same, it appears that each may preferentially act in differing cells and tissues.

PAF Degradation

Inactivation of AGEPC occurs through a highly specific, soluble enzyme, acetylhydrolase (Farr, et al., 1983). Its mechanism of action involves the hydrolysis of the acetyl group from AGEPC. The product, lyso-GEPC, is rapidly reacylated with unsaturated fatty acids at the *sn*-2 position and returned to the membrane compartment (Snyder, 1990).

Acetylhydrolase differs from phospholipase A_2 in that it lacks calcium dependence (Wardlow, 1985). Moreover, it is specific for short-chain fatty acyl residues at the 2 position. Enzymatic activity has been detected both intra- and extracellularly (Blank, et al., 1983). Extracellular acetylhydrolase from human plasma has been isolated and characterized (Stafforini, et al., 1987) and found to be present in the circulation in association with high and low-density lipoproteins (Stafforini, et al., 1987; Wardlow, 1985). Interestingly, the low-density lipoproteins contain approximately 70% of the total plasma acetylhydrolase content and are associated with the active form of the enzyme. The high-density lipoproteins, on the other hand, contain only 30% of the total plasma acetylhydrolase content and are associated with an inactive form of this enzyme (Stafforini, et al., 1989). Plasma acetylhydrolase has been shown not only to inactivate AGEPC but other PAF analogs as well (Stafforini, et al., 1987).

Acetylhydrolase has a rapid onset of action, with almost complete AGEPC (10^{-9} M) hydrolysis after 20 minutes of exposure to human blood (Stafforini, et al., 1987). The half-life of AGEPC in whole blood was approximately 8 seconds.

In summary, the action of acetylhydrolase is swift; by hydrolyzing the acetate group, PAF is rapidly rendered inactive. Therefore, a critical role is suggested for this enzyme in the bioregulation of PAF.

PAF Heterogeneity

In view of the PAF biosynthetic pathways described above, it is now known that even within the same cell, different analogs and homologs of PAF are synthesized (Mueller, et al., 1984; Oda, et al., 1985; Pinckard, et al., 1984; Weintraub, et al., 1985). For example, the human PMN is known to synthesize at least 16 different molecular species of choline-containing PAF (Ludwig, et al., 1986). Mueller and co-workers reported the presence of differing alkyl chain length AGEPC molecules (Mueller, et al., 1984). They found that C16:0-AGEPC accounted for 40% of the total PAF produced, while C18:1-, C18:0- and C17:0- accounted for 18%, 16% and 13%, respectively. Moreover, the remaining 13% of the PAF had an acyl linkage in the number 1 position rather than the alkyl moiety more commonly reported. Studies by Tessner and Wykle have additionally revealed the presence of non-choline containing PAF molecules in human PMN (Tessner and Wykle, 1987). Therefore, molecules with differing chain lengths, extent of saturation in the number 1 position, varying linkages at the number 1 position and polar head groups other than choline have been identified (Pinckard, et al., 1988). However, the biological activity of these PAF molecules have not been fully explored as

most research has focused on C16:O- or C18:0-AGEPC. Nevertheless, each of these structural modifications significantly alter the platelet stimulating activity of the molecule. Therefore, it is conceivable that all of these PAF molecules may participate in biological reactions *in vivo*.

C. ROLE OF PLATELET ACTIVATING FACTOR IN INFLAMMATION

In inflammatory cells, AGEPC is rapidly generated after cell stimulation or perturbation and released extracellularly (Ludwig *et al*, 1986). When intravenously infused into experimental animals, the effects of PAF are seen within one minute (McManus, et al., 1980). Its role in inflammation appears to be widespread, with known effects on neutrophils, monocytes/macrophage, eosinophils, platelets, vascular endothelial cells and vascular smooth muscle cells (Chilton, 1989).

In vitro effects of PAF.

In vitro experiments utilizing human inflammatory cells have revealed activity in response to PAF stimulation. This discussion will focus on the effects of PAF on platelets, PMN, monocytes and eosinophils.

Platelets

In isolated washed human platelets, low concentrations of AGEPC (0.2-10 nM) result in a primary wave of platelet aggregation; while higher concentrations (10-100 nM) produce a primary and secondary wave of aggregation which is followed by secretion (Kloprogge, et al., 1983). Platelets in plasma respond similarly to washed platelets, and although they require 10-100 times more AGEPC, the range is within the realm of what can be produced *in vivo* (Pinckard, et al., 1988). It is

interesting to note that response levels differ among different species. For example, rat platelets are unresponsive to AGEPC stimulation due to lack of PAF receptors (Sanchez-Crespo, et al., 1982). Conversely, rabbit platelets respond to minimal quantities of AGEPC, with 0.1 nM concentration resulting in irreversible aggregation and 50% secretion (Demopoulos, et al., 1979). Because of their responsiveness and ease of preparation, rabbit platelets are often used in bioassays to quantify PAF activity.

PMN

The response of isolated human PMN to AGEPC includes aggregation, chemotaxis, chemokinesis, priming, superoxide production and release of lysozyme and β-glucuronidase (Braquet, et al., 1987). Further, AGEPC-stimulated neutrophils have been shown to release arachidonic acid (Lin, et al., 1982). AGEPC-stimulated aggregation of human PMN appears to be dependent, in part, on the lipoxygenase pathway of arachidonic acid metabolism; blockers of the lipoxygenase pathway produce a dose-dependent reduction in AGEPC-induced neutrophil aggregation, while cyclooxygenase pathway blockers fail to have an effect (Lin, et al., 1982). Leukotriene B_4 (LTB_4), a lipoxygenase pathway biproduct, is ten to one hundred times more potent at stimulating PMN aggregation than AGEPC (Lin, et al., 1982). PMN desensitization to LTB_4 stimulation results in a significant reduction in AGEPC-stimulated aggregation, strongly implicating this metabolite as one of the mediators in AGEPC-stimulated PMN aggregation (O'Flaherty, et al., 1981). Another factor contributing to AGEPC-stimulated aggregation is the decrease in electrophoretic mobility; when PMN are stimulated with AGEPC, a decrease in the net negative

charge of cell surfaces occurs (Ingraham, et al., 1982). Thus, AGEPC mediated PMN aggregation appears to work in various ways.

AGEPC modulates PMN adherence to the vascular endothelium; this adherence is an early step in PMN migration into tissues. AGEPC-induced PMN adherence to the vascular endothelium is dependent on the expression of CD11/CD18 on the PMN cell surface which suggests that AGEPC is acting on the PMN. The maximal response occurs rapidly, *i.e.*, within 2 minutes (Tonnesen, 1989). By modulating PMN adherence to the vascular endothelium, AGEPC is, at least in part, responsible for PMN migration into the tissues.

The release of lysosomal enzymes from PMN seem also to be related to the lipoxygenase pathway since lipoxygenase pathway blockers inhibit secretion in a dose dependent manner (Smith and Bowman, 1982). However, LTB_4 does not appear to be involved (O'Flaherty, et al., 1981). While lysozyme, lactoferrin, β-glucuronidase, myeloperoxidase, gelatinase and vitamin B-binding protein are released in response to AGEPC stimulation, the release of all but gelatinase and vitamin B-binding protein require the presence of cytochalasin B or 1 μM or greater concentration of AGEPC (O'Flaherty, et al., 1981). While extracellular calcium is not required for AGEPC-induced lysosomal enzyme secretion from human PMN, it will enhance this response (Smith and Bowman, 1982).

Monocytes

Human monocytes are also stimulated by AGEPC. Monocyte aggregation occurs within 8 seconds following addition of AGEPC, and is dependent on Ca^{2+}, Mg^{2+} and on glycolysis (Pinckard, et al., 1988).

Monocytes also exhibit a weak chemotactic response to AGEPC. This response is heightened in patients with certain inflammatory dermatoses (Czarnetzki, 1983). In the presence of LPS or muramyl dipeptide, AGEPC (1nM-5μM) has also been found to modulate interleukin-1 (IL-1) production in a positive fashion (Salem, et al., 1990). Monocyte phagocytosis of damaged red blood cells is also enhanced by AGEPC (Bussolino, et al., 1989). These researchers found that AGEPC induced the translocation of protein kinase C to the monocyte membrane compartment, stimulated phosphorylation of complement receptor type 1 (CR-1), and activated the receptor capable of mediating phagocytosis. Monocyte production of superoxide anion or secretion of lysozyme do not appear to be affected by AGEPC. Accumulation of PAF produced by monocytes decreases by approximately 90% as they differentiate into macrophage. This is due to a 260-fold increase in the catabolic enzyme, PAF acetylhydrolase (Elstad, et al., 1989).

Eosinophils

Cultured human eosinophils are also affected by AGEPC. Walsh and co-workers (1989), showed greater dose and time dependent increases in IgE binding with AGEPC than with histamine or LTB_4. Degranulation of cultured human eosinophils has also been evaluated. In a study by Kroegel et al.(1989), approximately 1.5 nM AGEPC produced 17.9% release of peroxidase, 20.6% release of β-glucuronidase, 22.4% release of alkaline phosphatase and 28.8% release of aryl sulfatase. At higher concentrations (1-30 μM), AGEPC also resulted in superoxide production (Kroegel, et al., 1989). Eosinophil binding to human vascular endothelial cells, a critical step in eosinophil infiltration, has also been found to

increase in the presence of PAF (Lamas, et al., 1988). AGEPC also mediates eosinophil chemotaxis (Kay, et al., 1989). Indeed, Kay referred to PAF as "the most potent chemotactic mediator for eosinophils so far described."

Other

Other human cell lines have also been shown to have a pro-inflammatory effect when stimulated with AGEPC. Cultured human keratinocytes, when treated with AGEPC, release inositol phosphates (Fisher, et al., 1989). Human vascular endothelial cells have also been shown to respond to AGEPC stimulation (Bussolino, et al., 1985). In cultured human umbilical vein endothelial cells, Ca^{2+} influx occurs resulting in an increase of intracellular Ca^{2+} (Bussolino, et al., 1985).

***In vivo* effects of PAF**

The most dramatic effects of PAF are seen when 0.1-0.9 μg/kg is administered to experimental animals intravenously (Mathias and Behrens, 1990). The results are indistinguishable to those observed during IgE anaphylactic shock with the same intravascular, pulmonary and cardiac manifestations (Halonen, et al., 1981; McManus, et al., 1980). Thirty seconds after PAF infusion, pulmonary vascular constriction and platelet sequestration are noted, followed by increased pulmonary resistance. This is most likely a result of histamine and thromboxane release from PAF stimulated platelets. Myocardial depression, peripheral and coronary vasoconstriction and neutrophil stimulation leading to reversible neutropenia are also noted. While the pulmonary mechanical

effects are dependent on platelet stimulation, the cardiovascular effects are not (Vargaftig, et al., 1980; Halonen, et al., 1981).

In vivo human studies also reveal the inflammatory effects of PAF. Intradermal injection of AGEPC results in immediate blanching, indicative of acute vasoconstriction; this is followed by the development of a wheal and flare reaction. Burning pain and pruritus were also reported by the subjects. This response heightened in intensity at 10 minutes and tapered off after 60 minutes (Pinckard *et al*, 1980; Pinckard *et al*, 1988). In rat skin, AGEPC was found to be 1000 times more vasoactive than histamine (Humphrey, et al., 1982).

Eosinophil response to AGEPC has also been evaluated *in vivo* (Henocq and Vargaftig, 1986). Intracutaneous injection of AGEPC into atopic subjects resulted in a significant accumulation of eosinophils. This finding further supports *in vitro* studies showing the potent chemotactic properties of this inflammatory mediator (Kay, et al., 1989).

Inhalation of PAF has also been found to produce profound effects (Cuss, et al., 1986). All of the normal human volunteers who inhaled micro-molar concentrations of AGEPC had a dose-dependent bronchoconstriction of two hours duration. An increase in non-specific bronchial responsiveness similar to that seen in asthmatics following antigen challenge was also noted. These effects were maximal for several days, then gradually decreased over several weeks. Some patients additionally experienced facial flushing and increased heart rate, but these effects were short lived, lasting approximately ten minutes (Cuss, et al., 1986).

Role of PAF as a Mediator of Endotoxin-Related Diseases

Endotoxin is a lipopolysaccharide component present in the outer membrane of gram-negative bacteria (Stedman's Medical Dictionary, 1990). It has been associated with many diseases including adult respiratory distress syndrome, pulmonary hypertension, necrotizing enterocolitis, renal failure and septic shock (Handley, et al., 1990). Moreover, the presence of endotoxin in periodontally diseased root surfaces has long been verified (Aleo, 1974; Daly, 1982). Interestingly, endotoxin itself is relatively harmless to many cells *in vitro* and *in vivo* with most pathology resulting from release of endogenous mediators from inflammatory cells (Beutler and Cerami, 1987).

One such mediator is PAF. *In vitro* studies have revealed endotoxin-induced synthesis of PAF in PMN, and PAF synthesis and release in macrophage (Rylander and Beijer, 1987; Worthen, et al., 1988). PAF has also been detected *in vivo* in endotoxin-injected rats (Chang, et al., 1987).

Perhaps the most convincing evidence for a role for PAF in endotoxin-related diseases is the effect of PAF-antagonists on endotoxin-induced animal reactions. In these instances, PAF antagonists have been shown to 1) inhibit increases in pulmonary permeability (Chang, et al., 1987), 2) prevent the development of necrotizing enterocolitis (Hsueh, et al., 1987), 3) inhibit increases in ocular permeability (Rubin, et al., 1987) and 4) suppress or reverse the decrease in platelets and fibrinogen, prolonged PT and PTT values associated with endotoxic shock (Imura, et al., 1985).

In summary, PAF has been shown to have pro-inflammatory effects in humans. *In vitro* and *in vivo* studies consistently demonstrate the

powerful properties of this mediator. Because of the diversity of biological activity associated with PAF, it has been suggested to play a key role in numerous inflammatory diseases (Chilton, 1989).

D. SALIVARY AND CREVICULAR PAF

Few published details regarding PAF activity in normal human mixed saliva are available. Cox and co-workers (1981) were first to describe the presence of PAF in human saliva. This PAF was structurally and biologically similar to AGEPC and was found to be present in all 24 individuals sampled. The biological activity, determined by adding the PAF to rabbit platelets and measuring the percent release of serotonin, ranged from 10 to 99%. Cox attributed this broad range to "real differences among individual saliva donors." Samples of pure parotid fluid (from donors with salivary PAF in their mixed saliva) lacked any PAF activity, suggesting the possibility of a source other than salivary gland tissue. In 1984, Wardlow used high performance liquid chromatography to further characterize human salivary PAF. He found that at least two species of PAF were present and that their retention times were very similar to that of AGEPC. More recently, studies by Smith, Schiess and McManus (1986) have also found salivary PAF to be indistinguishable from the PAF produced by inflammatory cells. Neutrophils, mast cells and macrophages are known to be present in periodontally involved tissues, and all of these cells are known to generate PAF. Therefore, the contribution of PAF from the periodontium cannot be ignored. The study by McManus, Marze and Schiess (1990) showing a significant decrease in the levels of PAF in edentulous patients

adds supports to this theory. Further studies, however, are needed to answer questions regarding the source and function of salivary PAF.

STATEMENT OF PURPOSE

Platelet-activating factor is a potent acetylated phosphoglyceride with important biological activities. PAF is not only synthesized by inflammatory cells, but also stimulates them. Thus, the role of PAF in a variety of inflammatory diseases has been suggested. Normal human, mixed saliva contains PAF. In addition, edentulous subjects have lower levels of salivary PAF than dentate subjects. These findings suggest that salivary PAF originates from the periodontium and may play a role in periodontal inflammation, However, the relation of salivary PAF to periodontal disease has not been established. Thus, the purpose of this investigation was to determine whether a significant relationship exists between salivary PAF and clinical estimates of periodontal disease.

III. MATERIALS & METHODS

A. Clinical Aspect

Ninety untreated patients presenting for treatment at the University of Texas Health Science Center dental hygiene or periodontal clinic were asked to participate in the study. The following information was recorded for each patient: age, sex, time elapsed since last cleaning, presence of oral lesions, medications taken in the last 6 weeks and systemic diseases in the past year. Patients were called prior to the appointment and asked to refrain from any oral intake (including eating, drinking, chewing gum, smoking, brushing or flossing) for one hour prior to the appointment. One milliliter (ml) of unstimulated mixed saliva was collected into a 16 x 100 disposable glass test tube. Patients then received a comprehensive periodontal examination which included probing depth measurements, taken along six sites/tooth, and bleeding on probing, annotated as present or absent. This data was then used to calculate the percentage of sites with >4 mm probing depths from which the statistical analyses were performed. Patients with oral lesions or severe xerostomia were excluded from the study. In addition, those who had any oral intake within one hour of sampling, or were currently taking antibiotics or nonsteroidal anti-inflammatory drugs were also excluded. Informed consent was obtained prior to all saliva collections. This protocol was approved by the Institutional Review Board (#856-8000-118).

B. Laboratory Procedures

Saliva collection and lipid extraction

Chloroform and methanol were added to 16 x 100 mm disposable glass tubes in preparation for saliva collection (final ratio, chloroform:methanol:saliva, 1:2:0.8, v/v/v, respectively). Markings were placed at the level of the tube to indicate where 1 additional ml (of saliva) would be. Patients expectorated unstimulated, mixed saliva directly into each tube, attempting to get as close to the mark as possible (Corrections for excess or inadequate saliva fluid volume were subsequently made). Tubes were capped and vigorously shaken immediately after saliva collection. Trace amounts (approximately 2000 dpm) of tritiated-AGEPC (^{3}H-1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, ^{3}H-C16:0-AGEPC, 50 Ci/mmole New England Nuclear, Boston, MA) were added to each sample and used to calculate PAF recovery after lipid extraction and phospholipid fractionation. Within one week of collection, Bligh and Dyer lipid extraction was performed (Bligh and Dyer, 1959). In brief, samples were extracted at room temperature for 1 hr, then centrifuged (1000 xg, 10 min, room temperature) to remove bacterial and cellular debris, cells and precipitated protein. The supernatant was decanted into phasing tubes containing a final ratio of 1:1:0.9, chloroform:methanol:water-saliva, v/v/v, respectively. The methanol layer was removed leaving the chloroform-rich phase containing the lipids of the saliva samples.

Thin layer chromatography and PAF bioassay

Thin layer chromatography (TLC) was then accomplished to fractionate salivary lipids and thus isolate the PAF. Prewashed, heat-inactivated, silica gel G plates (Analtech, Inc., Newark, DE) were used with a solvent system of chloroform:methanol:acetic acid:water, 50:28:6:4, v/v/v/v, respectively). Lipids were recovered from each TLC lane in serial fractions of silica gel *via* Bligh and yer lipid extraction as previously described. These extracts were stored at -20°C. Prior to bioassay, samples were allowed to come to room temperature, the methanol layer removed, chloroform extracts evaporated to dryness, and lipids dissolved in pyrogen-free saline containing 0.25% bovine serum albumin (BSA; Miles Laboratories, Elkhart, IN).

Rabbit platelets were obtained from blood removed from the central ear artery. ^{3}H-5-hydroxytryptamine (serotonin, New England Nuclear) labeling was accomplished as described by Demopoulos and co-workers (1979). Two hundred µl of platelets (250,000/µl) were pre-warmed (37°C) and added to tubes containing 4 µl of standard dilutions of C16:0-AGEPC or the unknown samples (at 37°C). The reaction was terminated sixty seconds later by the addition of 20 µl of 1.5 M formalin and immediate cooling to 0°C. The tubes were then centrifuged (1,000 xg) for 10 min at 4°C. Serotonin release was measured by quantifying the amount of ^{3}H-serotonin present in the cell-free supernatant. This was compared with total ^{3}H-serotonin release as determined by tubes processed in parallel containing 0.1% Triton X-100, final concentration. Salivary PAF activity was estimated in comparison to the biological activity of authentic C16:0-AGEPC (Bachem Fine Chemicals, Torrance, CA);

in every assay, using known amounts of C16:0-AGEPC (5 to 50 fmoles), a standard curve of ^{3}H-serotonin release was generated. The sensitivity of this assay was 3-5 fmoles. PAF activity in TLC fractions of saliva-lipids was determined directly from this curve and expressed as C16:0-AGEPC fmole equivalents/ml of saliva. Loss of PAF occurring through the extraction, TLC, and bioassay procedures was estimated for each saliva sample by determining the final amount of ^{3}H-AGEPC tracer present in each fraction; 60 to 80% ^{3}H-AGEPC recovery was routinely obtained. This information was used to correct the PAF values obtained from the bioassay for each unknown saliva sample.

Histology

During the initial lipid extraction of saliva, pellets containing protein, cells and cellular debris were obtained. They were stored in 10% neutral buffered formalin (NBF) until they were prepared for histologic processing. For light microscopic examination, the pellets were placed in polypropylene microcentrifuge tubes (Beckman Instruments Inc., Palo Alto, CA) containing pre-warmed (60^{o}C), 0.5% gelatin (Sigma Chemical Co., St. Louis, MO). These tubes were immediately centrifuged for 5 min (10,000 xg, room temperature) and placed in an ice bath for 10-15 min to allow the gelatin to solidify. The plastic tubes were then bisected and the gelatin embedded specimen removed and placed into tissue casettes. Specimens were submerged in 10% NBF and were subsequently embedded in paraffin, sectioned (6 μm) and stained with hematoxylin and eosin (H&E) by the Special Histology section of the Department of Pathology. Sections were viewed under a light microscope with a 10X eyepiece and a 40X objective A grid was utilized to calculate the number

of PMN within each section. Three representative fields per specimen were examined to determine the mean number of PMN/mm^2. Magnification error was determined by placing a millimeter ruler on the microscope stage and comparing it to the grid on the eyepiece. Thus, all numerical values were divided by the correction factor 0.0625. All tissue specimens were quantified in a blinded manner; the specimens were coded so that neither the sample identity nor donor status were known until after PMN enumeration.

C. Statistical Evaluation of Data:

Data are presented as the mean $\pm$ the standard error. Pearson's correlation coefficients were used to determine the relation between any two variables. Tukey's and Duncan's multiple range tests for analysis of covariance were used to compare means adjusted for variables such as sex, smoking, medications, number of teeth and time of sample collection. Point biserial correlation coefficients were used to correlate "yes", "no" type variables with continuous variables. The Statistical Analysis Systems (SAS) software was used for statistical analyses of all data. P values of less than 0.05 were considered significant. Residuals from the analysis of covariance were plotted to determine that they followed the bell-shaped curve.

IV. RESULTS

A. Clinical Findings and Laboratory Data

Of the ninety samples that were collected, 69 were evaluated. Reasons for excluding samples were as follows: 1) the information sheet containing patient data was not turned in for seven of the samples and precluded sample identity, 2) one sample was lost due to breakage of the test tube during processing and 3) periodontal charting was not accomplished for two of the patients. In addition, 11 samples were excluded because they did not meet our initial criteria; that is, the patients sampled were currently taking antibiotics or nonsteroidal anti-inflammatory drugs, had oral lesions, severe xerostomia, or had oral intake within one hour of sampling. Thus, a total of 69 samples from 41 males and 28 females were evaluated. The mean age was 45, with a range of 14-84. Patients had an average of 26 teeth, with a minimum of 6 and a maximum of 32 teeth. Eleven of the 69 patients smoked tobacco products. Bleeding on probing was present in an average of 36 sites per patient, with a range of 0 to 167 sites. An average of 17.5% of sites per patient had greater than 4 mm probing depths, with a range of 0-75%.

All salivary PAF co-migrated with authentic PAF in thin layer chromatography. PAF activity was detected in all but 1 sample. The amount of PAF present per milliliter of saliva varied from 0 to 26,059 C16:0-AGEPC fmoles equivalents, with an average of 6,349. The PMN count was found to average 352/mm^2, with a range of 16 to 1,536.

B. Statistical Findings

Pearson's correlation analysis revealed no significant relations between salivary PAF levels and age (r=0.04, p=0.75) or number of teeth (r=0.19, p=0.11). Point biserial correlation coefficients revealed no significant relations between salivary PAF levels and sex of patient (r_{pb}=-0.11, p=0.37), occasional use of non-steroidal anti-inflammatory drugs (r_{pb}=-0.75, p=1.54), smoking (r_{pb}=0.16, p=0.19) diabetes (r_{pb}=-0.189, p=0.12) or medications causing decreased salivary flow (r_{pb}=0.17, p=0.16).

In contrast to the above values, significant relations were found between salivary PAF and probing depths (r=0.46, p=0.0001) (Figure 3), number of bleeding sites (r=0.33, p=0.0059) (Figure 4) and PMN count (r=0.45, p=0.0002) (Figure 5). A small, but significant correlation was also noted between bleeding on probing and probing depths >4 mm (r=0.41, p=0.0006)

Further analysis of these data were performed in an effort to determine if salivary PAF levels were related to periodontal status. Thus, the percentage of sites with probing depths greater than 4 mm was calculated and this data were used to subdivide patients into 6 groups with 10-13 patients per group (See Table 1). The healthiest group, Group 1, had only 1-4 mm probing depths throughout The percentage of involved sites increased from group to group with 37-75% of the sites in Group 6 having greater than 4 mm probing depths. While this grouping appears to reflect only the incidence of disease, the severity of disease progressed with each group as well. Figure 6 illustrates the percentage of sites with greater than 4 mm probing depths as well

Figure 3. Comparison of salivary PAF levels and percentage of sites with probing depths >4 mm for all patients included in data analysis (n = 69).

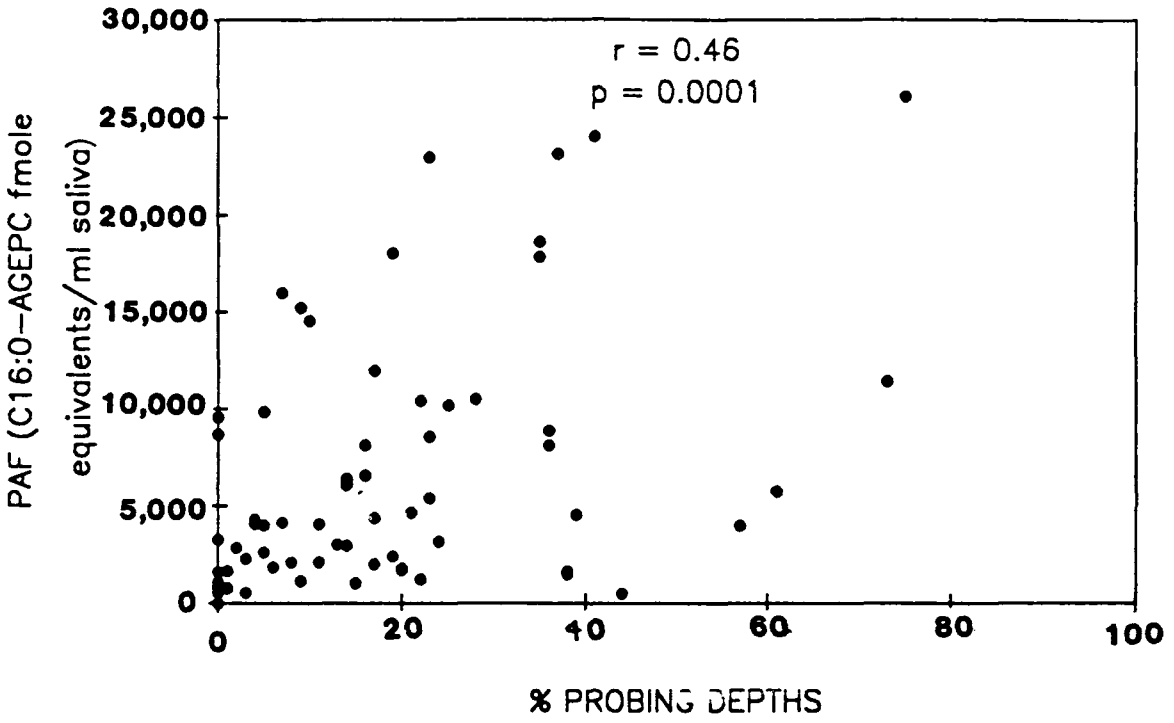

r = 0.46
p = 0.0001
PAF (C16:0–AGEPC fmole equivalents/ml saliva)
30,000
25,000
20,000
15,000
10,000
5,000
0
0
20
40
60
80
100
% PROBING DEPTHS

Figure 4. Comparison of salivary PAF levels and number of bleeding sites for all patients included in data analysis (n = 69).

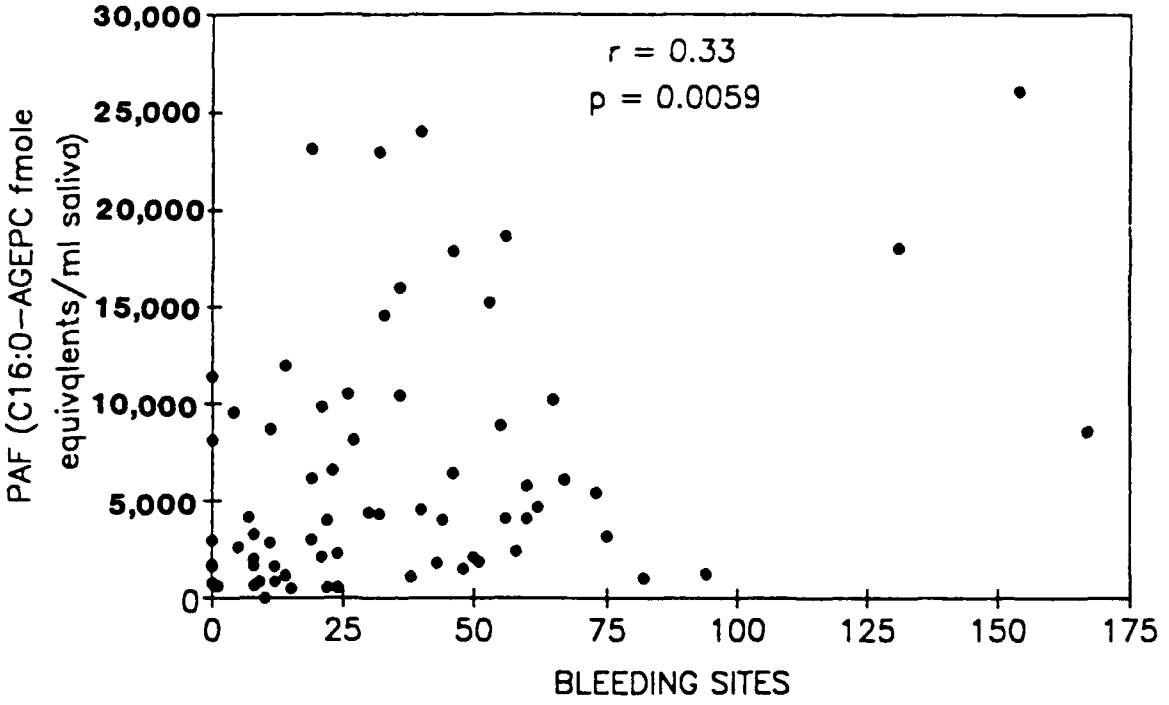

r = 0.33
p = 0.0059
PAF (C16:0–AGEPC fmole equivqlents/ml saliva)
30,000
25,000
20,000
15,000
10,000
5,000
0
0
25
50
75
100
125
150
175
BLEEDING SITES

Figure 5. Comparison of salivary PAF and PMN levels for all patients included in data analysis (n = 69).

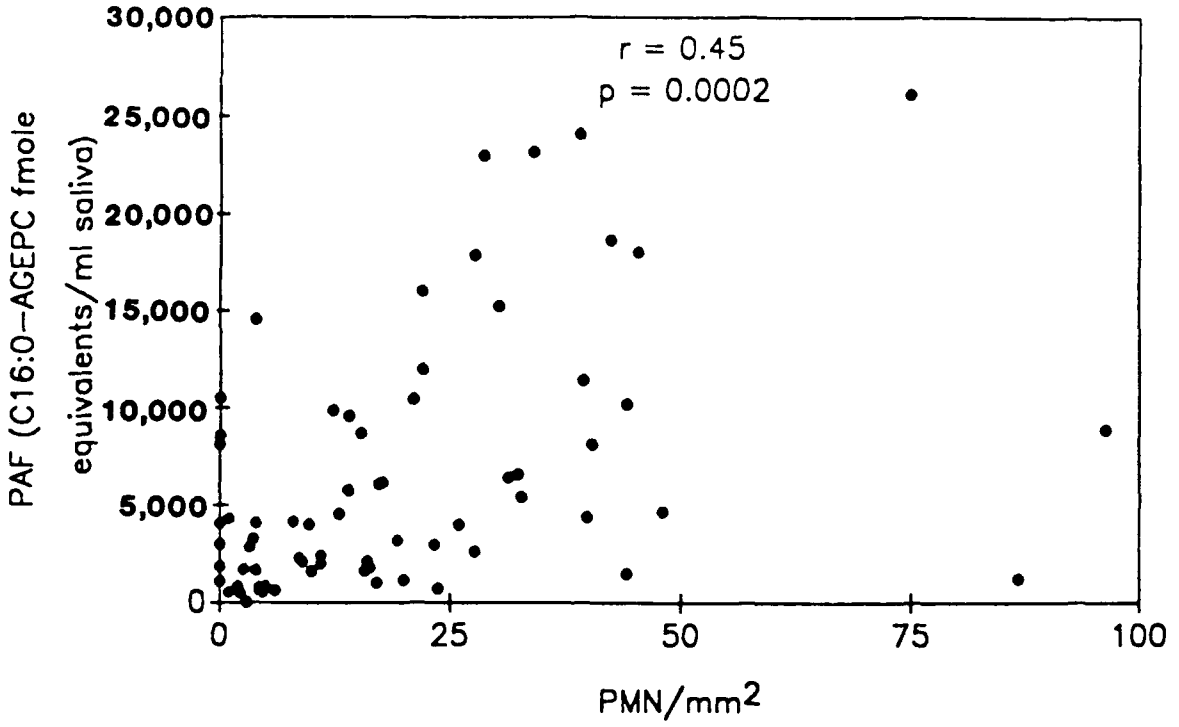
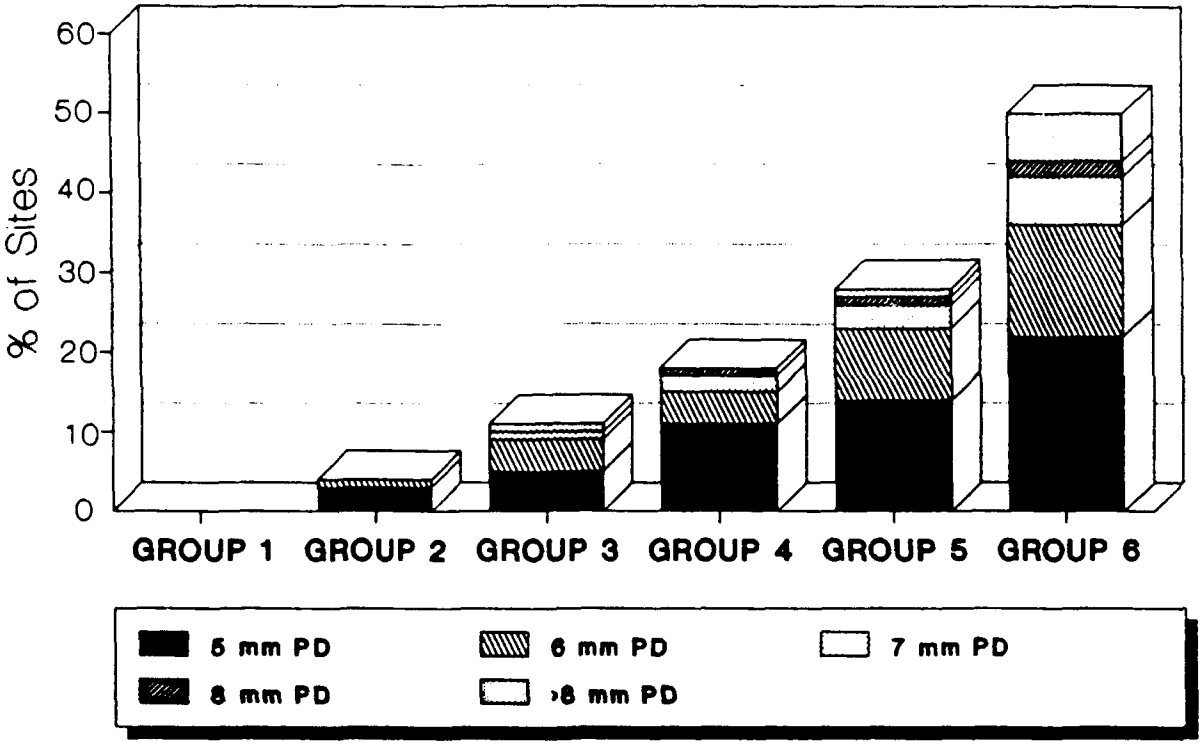

r = 0.45
p = 0.0002
30,000
25,000
20,000
15,000
10,000
5,000
0
0
25
50
75
100
PAF (C16:0–AGEPC fmole equivalents/ml saliva)
PMN/mm2

Table 1. Subdivision of Patient Population on the Basis of Frequency of Sites With Probing Depths > 4 mm.

	% PD > 4 mm	n*
Group 1	0	12
Group 2	1 - 6	11
Group 3	7-14	13
Group 4	15 - 21	11
Group 5	22-36	12
Group 6	37-75	10

n = number of patients

Figure 6. Disease severity with patient groups. Disease severity as measured by the percentage of sites with >4 mm probing depths, were calculated for each group. In addition, within each group, the frequency of deeper probing depths (*e.g.*, 5 mm) are also indicated. As illustrated, disease severity progressed from Group 1 to Group 6.

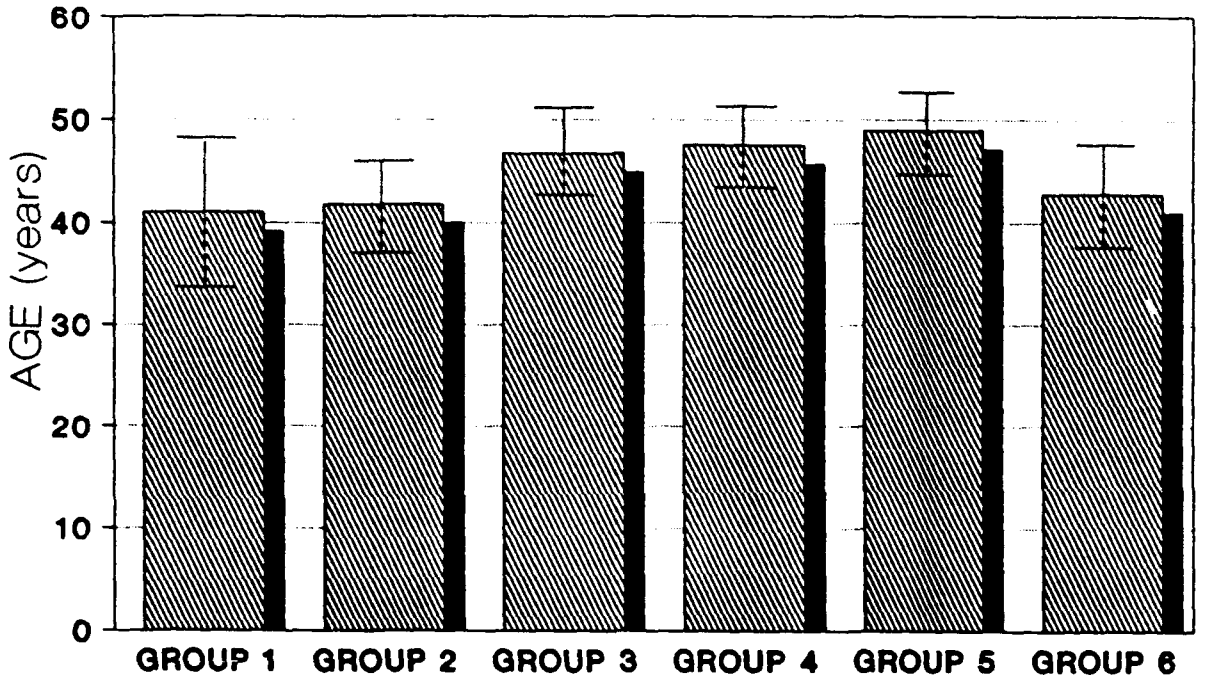

60
50
40
30
20
10
0
% of Sites
GROUP 1
GROUP 2
GROUP 3
GROUP 4
GROUP 5
GROUP 6
5 mm PD
6 mm PD
7 mm PD
8 mm PD
>8 mm PD

as the percentage of sites for individual probing depths. For example, Group 2 has greater than 4 mm probing depths in 4% of the sites, with 3% having 5 mm probing depths and 1% having 6 mm depths. Conversely, in Group 6, 50% of the sites had greater than 4 mm probing depths; with 22% having 5 mm probing depths, 14% 6 mm depths, 6% 7 mm depths, 2% 8 mm depths and 6% greater than 8 mm probing depths. Thus, the disease severity progressed from Group 1 to Group 6 with a significant increase in the number of 6, 7, 8 and greater than 8 mm probing depths (Figure 6).

Comparisons were made to assess differences between these 6 groups for age, number of teeth, number of bleeding points, salivary PMN count and salivary PAF levels. The mean age for each group varied between 41 and 49 years (Figure 7). The average number of teeth per patient was found to range from 23-28 (Figure 8). No significant correlations were noted between the six groups for age ($r=0.10$, $p=0.41$) or number of teeth ($r=0.03$, $p=0.80$). Conversely, the number of sites with bleeding on probing was correlated with disease severity and is depicted in Figure 9, with Group 1 having 12 ± 3 bleeding sites versus 44 ± 14 for Group 6. Although Group 5 had more sites (60 ± 12) which bled on probing than Group 6, the difference between the two was not statistically significant.

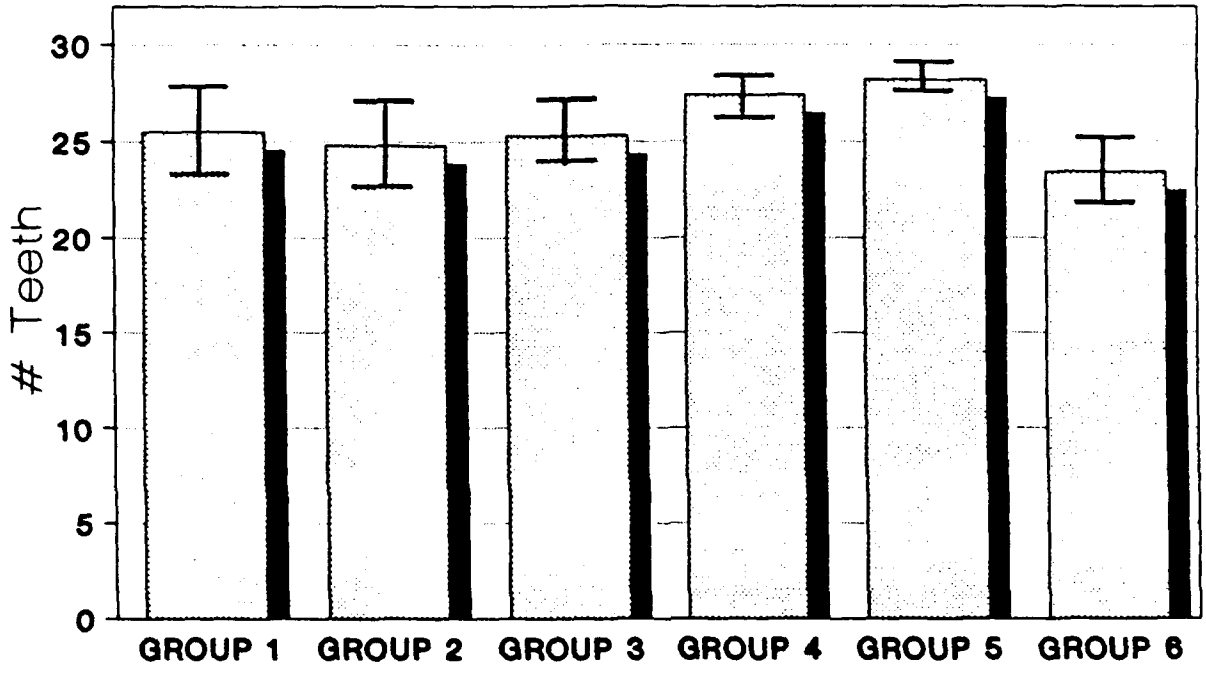
The salivary PMN count was also found to progressively increase with disease severity (Figure 10) with a significant correlation between the salivary PMN count and the six groups ($r=0.55$, $p=0.0001$).

Figure 7. Age distribution of subjects within each group. No significant differences were observed between these groups. Values represent the mean $\pm$ SE for data derived from all subjects within the respective group.


60
50
40
30
20
10
0
AGE (years)
GROUP 1
GROUP 2
GROUP 3
GROUP 4
GROUP 5
GROUP 6

Mean ± S.E.

Figure 8. Number of teeth present for each group. No significant differences were observed between these groups. Values represent the mean $\pm$ SE for data derived from all subjects within the respective groups.


Teeth
30
25
20
15
10
5
0
GROUP 1
GROUP 2
GROUP 3
GROUP 4
GROUP 5
GROUP 6

Mean ± S.E.

Figure 9. Frequency of bleeding sites for each group. A significant ($p = 0.005$) correlation ($r = 0.34$) was observed between the number of bleeding sites and increasing disease severity. Values represent the mean $\pm$ SE for data derived from all subjects with the respective groups.

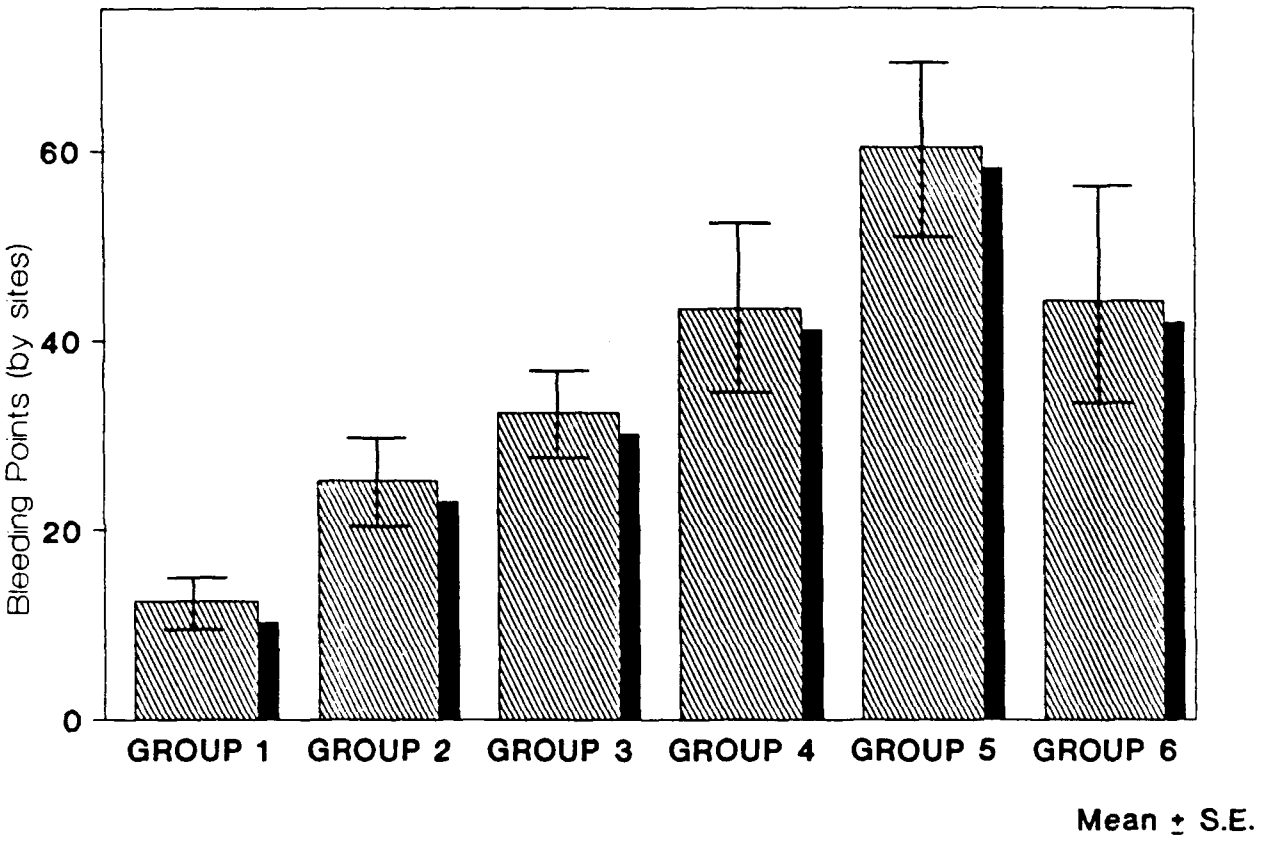
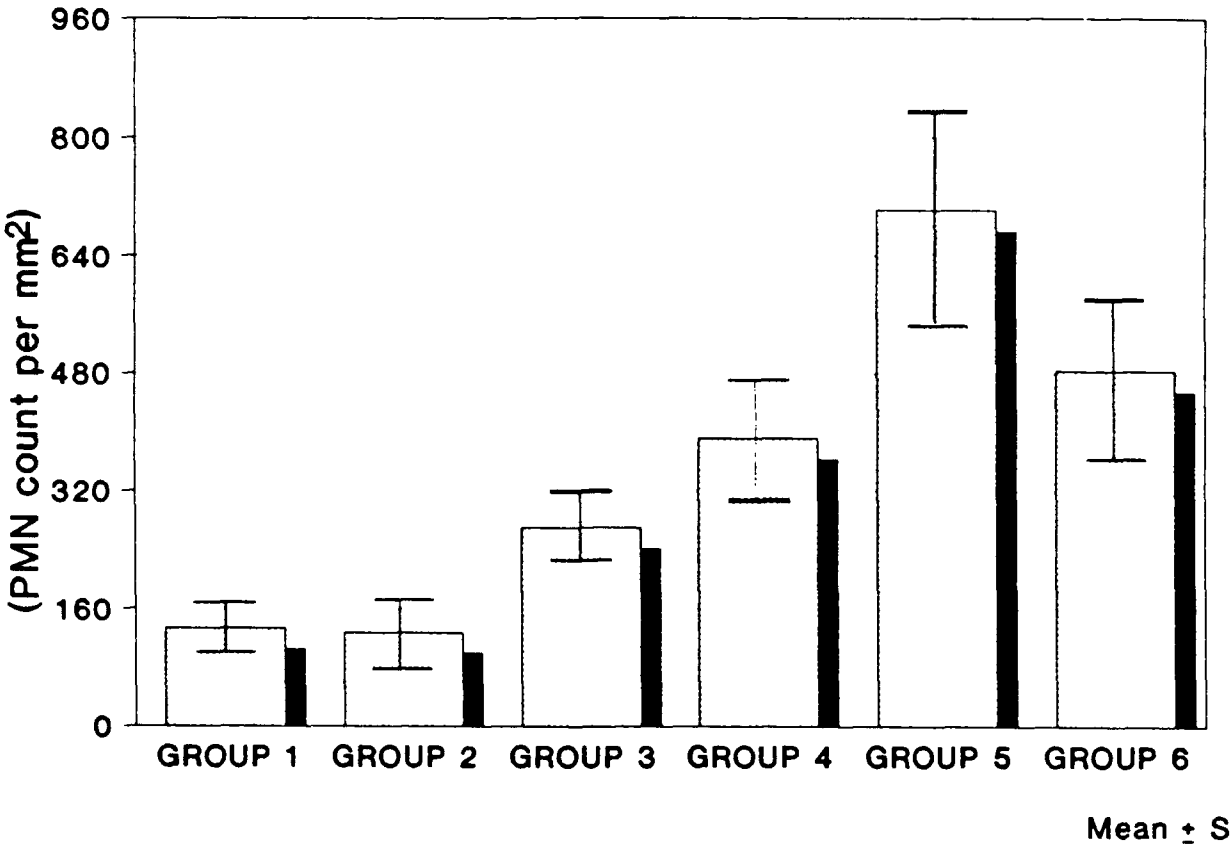

Bleeding Points (by sites)
60
40
20
0
GROUP 1
GROUP 2
GROUP 3
GROUP 4
GROUP 5
GROUP 6
Mean ± S.E.

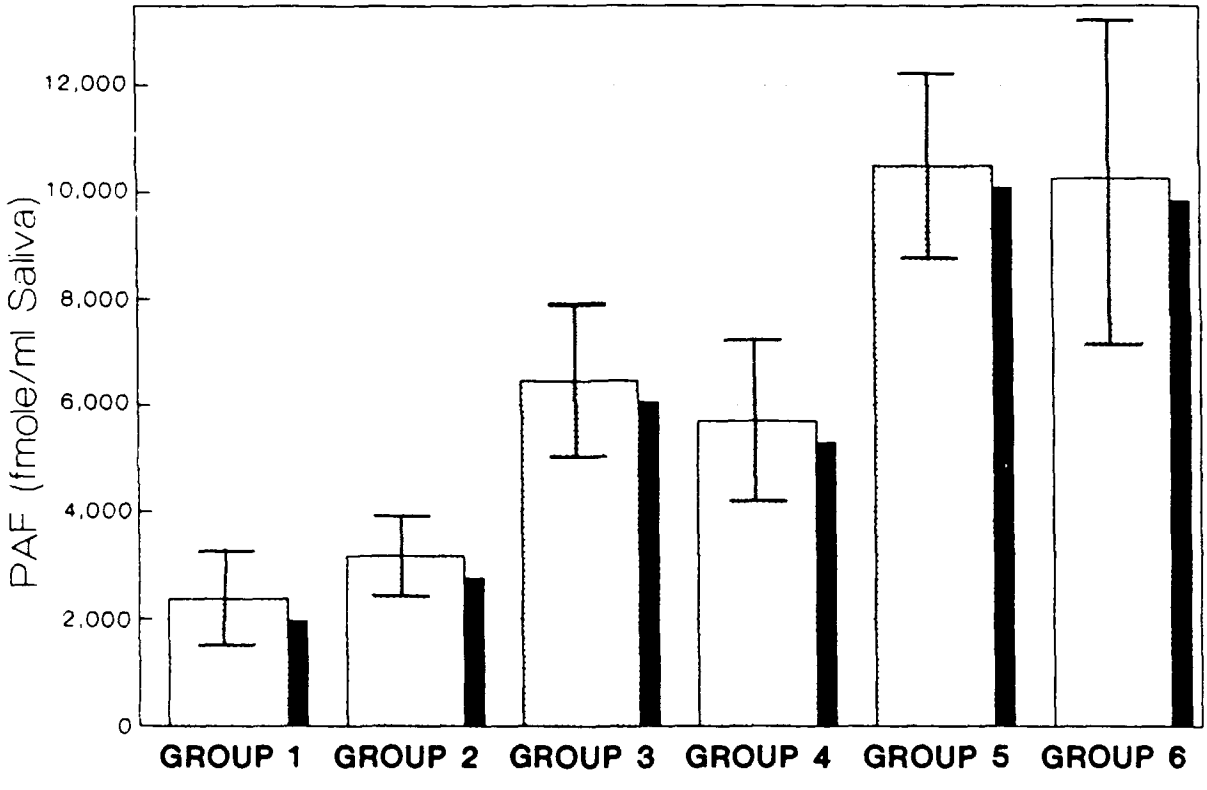
Figure 10. Salivary PMN count for each group. A significant ($p = 0.005$) correlation ($r = 0.34$) was observed between the number of PMN/mm^2 of the saliva pellet and increasing disease severity. Values represent the mean $\pm$ SE for data derived from all subjects with the respective groups.


960
800
640
480
320
160
0
(PMN count per mm2)
GROUP 1
GROUP 2
GROUP 3
GROUP 4
GROUP 5
GROUP 6
Mean ± S.E.

Salivary PAF levels generally increased from Group 1 to Group 6 (Figure 11). Low levels of PAF, averaging slightly above 2,000 C16:0-AGEPC fmole equivalents/ml saliva, were noted for Group 1. This was in contrast to groups with increased probing depths (Groups 5 and 6) which had PAF levels above 10,000 C16:0-AGEPC fmoles equivalents/ml saliva. A significant correlation was present between PAF and the six periodontal status groups ($r=0.46$, $p=0.0001$).

In addition to the above analyses, Duncan's and Tukey's multiple comparisons tests for analysis of covariance were performed for these variables as well as for sex, smoking, time of sampling, occasional use of non-steroidal anti-inflammatory drugs, or medications which could interfere with salivary flow. No significant relation was noted for most of these variables between the six groups (Table 2-4). The relationship between the number of teeth and number of bleeding sites and between the non-steroidal anti-inflammatory drugs and salivary PMN levels did approach significance ($p=0.062$ & $p=0.053$), and significant relations were noted between the patient's sex and number of bleeding sites ($p=0.02$), between smoking and salivary PAF levels ($p=0.03$) and between smoking and salivary PMN levels ($p=0.04$). PAF levels for smokers averaged 3,968 $\pm$ 1,301 C16:0-AGEPC fmole equivalents/ml saliva, versus 6,801 $\pm$ 887 for non-smokers. When these means were corrected for disease severity, the difference between the two became even greater (2,757 $\pm$ 1,814 for smokers and 7,118 $\pm$ 767 for non-smokers). A test of interaction was performed to determine if the difference in salivary PAF levels between the six groups was attributable to smoking, and no significant relation was found ($p=0.266$). Thus, while smoking

Figure 11. Salivary PAF levels for each group. A significant ($p = 0.005$) correlation ($r = 0.34$) was observed between the levels of PAF in saliva and increasing disease severity. Values represent the mean $\pm$ SE for data derived from all subjects with the respective groups. The group means for Groups 5 & 6 were significantly different from that of Group 1.


PAF (fmole/ml Saliva)
12,000
10,000
8,000
6,000
4,000
2,000
0
GROUP 1
GROUP 2
GROUP 3
GROUP 4
GROUP 5
GROUP 6

Mean ± S.E.

Table 2. Multivariate analyses of the effects of confounding variables on salivary PAF levels

Variable	Probability* (p Values)
Age	0.7017
Teeth	0.5375
Sex	0.2708
↓ Salivary Flow**	0.5348
Diabetes	0.8274
NSAID***	0.7453
Time of Sampling	0.5097
Smoking	0.0335

*Probability values were determined using Duncan's & Tukey's multiple comparisons.

**Medications causing decreased salivary flow

***Non-steroidal anti-inflammatory drugs

Table 3. Multivariate analyses of the effects of confounding variables on bleeding sites

Variable	Probability* (p Values)
Age	0.5512
Teeth	0.0618
Sex	0.0234
↓ Salivary Flow**	0.5851
Diabetes	0.4127
NSAID***	0.1772
Time of Sampling	0.7298
Smoking	0.9314

*Probability values were determined using Duncan's & Tukey's multiple comparisons.

**Medications causing decreased salivary flow

***Non-steroidal anti-inflammatory drugs

Table 4. Multivariate analyses of the effects of confounding variables on PMN count

Variable	Probability* (p Values)
Age	0.2848
Teeth	0.3007
Sex	0.8065
↓ Salivary Flow**	0.2604
Diabetes	0.1790
NSAID***	0.0532
Time of Sampling	0.7803
Smoking	0.0357

*Probability values were determined using Duncan's & Tukey's multiple comparisons.

**Medications causing decreased salivary flow

***Non-steroidal anti-inflammatory drugs

significantly affects salivary PAF levels, it did not affect the differences in salivary PAF levels between the six groups.

Although Group 5 and 6 were found to be significantly different from Group 1 for number of bleeding sites and salivary PMN and PAF levels, Group 5 had higher (although not significant) values than Group 6 in each instance. However, when corrected (by analysis of covariance) for differences in sex, medications causing decreased salivary flow, diabetes, use of non-steroidal anti-inflammatory drugs, smoking, time of sampling, age and disease status, these means were reversed for PAF levels. In fact, the corrected means increased successively for each group. This phenomena was not noted for bleeding on probing or PMN count, as, for both of these variables, Group 5 continued to have higher values than Group 6. Because Group 5 had the greatest number of teeth and Group 6 had the least number of teeth of all the groups, an analysis of covariance was performed to adjust for the number of teeth in each group. After performing these analyses, the corrected means for PAF and for number of bleeding sites decreased for Group 5 and increased for Group 6. However, the mean bleeding on probing values for Group 5 was still higher than for Group 6. The mean salivary PMN count was not changed following these analyses.

The residuals from all analyses of variance and covariance were charted as a normal probability plot and appeared very close to a straight line so that the assumptions for the analyses were valid.

V. DISCUSSION AND SUMMARY

In this investigation, the relation of salivary PAF levels and periodontal disease status was evaluated. The findings of this study indicate that salivary PAF levels do correlate with periodontal status, *i.e.*, higher PAF levels are noted in patients with increased probing depths and bleeding on probing. While the source of this phospholipid mediator in saliva remains to be determined, these findings suggest that salivary PAF originates, at least in part, from the periodontium. The high significance levels between salivary PAF and PMN counts also imply involvement of PMN in salivary PAF biosynthesis. Dentate subjects were found to have greater number of salivary PMN than edentulous subjects (McManus, et al., 1990) suggesting a major source of salivary PMN from tooth bearing areas. In addition, PMN have been shown to be the main cell type present in periodontally diseased gingival crevices (Saito, et al., 1987). Since the crevicular fluid is continually being replaced, it is not surprising to see both PMN and PAF in the salivary fluid. Thus, it seems likely that salivary PAF may be derived from crevicular PAF and that increases of this inflammatory mediator are associated with increases in disease severity.

Salivary PAF levels were also significantly different between smokers and non-smokers. While it was not the purpose of this study to determine the difference in salivary PAF levels between smokers and non-smokers, a trend toward lower salivary PAF levels was observed in individuals who smoked ($p=0.03$). Further research to determine the extent of significance between salivary PAF levels and smoking is, therefore, warranted.

No significant relations were found to exist between salivary PAF levels and age, sex of patient, time of sampling, diabetes, occasional use of non-steroidal anti-inflammatory drugs, or medications causing decreased salivary flow. These were simple correlations, however, and did not take into account the relation between salivary PAF levels and disease severity. When an analysis of covariance was performed to take periodontal disease severity into account, the results were also negative. However, these latter findings may also be questioned. Emrich (1990) alluded to problems with making inferences from negative results, stating that statistical tests are often not "powerful" enough to detect meaningful differences when evaluating small sample sizes. This may well be the case in the current study. Therefore, these negative findings must be interpreted with caution.

Because the above analyses did not adequately evaluate the relation between salivary PAF levels and different grades of disease severity, further analyses were performed. Based on the percentage of probing depths >4 mm, six similarly sized groups were created. It was felt that by having similarly sized groups, better inter-group comparisons could be made. This grouping not only made it possible to compare the healthiest group (Group 1) with the most severely diseased group (Group 6), but allowed for comparisons between all six levels of disease severity.

No significant differences in age, number of teeth, sex of patient, smoking, time of sampling, occasional use of non-steroidal anti-inflammatory drugs, or medications which could interfere with salivary flow were observed between the 6 groups. Although salivary PAF levels were affected by the patient's smoking status ($p = 0.03$), disease severity

was not. Analysis of covariance revealed that the effect of smoking on PAF levels did not in any way affect the relation between salivary PAF levels and the six groupings of disease severity ($p = 0.48$). In other words, the difference in PAF levels due to smoking was similar across all levels of disease.

The number of bleeding sites, salivary PAF and PMN levels were found to differ significantly between the six groupings of disease severity ($p = 0.0004$, 0.0001 and 0.0001, respectively). As the disease severity increased, the number of bleeding sites and salivary PAF and PMN levels increased. Interestingly, Group 5 had consistently higher values than Group 6 for each of these variables. However, the differences between Groups 5 and 6 were not statistically significant. In an effort to understand this trend, Groups 5 and 6 were compared as outlined below.

Prior to performing an analysis of covariance with number of bleeding sites as the dependent variable, the trend toward higher values in Group 5 was thought to be attributable to two different factors. The first centered around the number of teeth in these groups. Group 5 had the greatest number of teeth (n=28), while Group 6 had the least (n=23). Because six sites were evaluated for each tooth, a 30 site difference could be accounted for from this variable alone. Another reason for this trend could be related to the amount of inflammation present in each group. Bleeding on probing has been associated with increased inflammation as assessed histologically (Davenport, *et al.*, 1982; Greenstein, *et al.*, 1981) and has been shown to be a weak predictor of periodontal disease activity (Lang, *et al.*, 1986). Thus, it could be that the group with

increased bleeding on probing (Group 5) had increased inflammation. The similar trend toward increased salivary PMN and PAF levels in Group 5 add support to this working hypothesis, as both PMN and PAF are strongly associated with the inflammatory response (Chilton, 1989; Madri, 1990). However, it was not until analysis of covariance was performed, using the number of bleeding sites as the dependent variable that the role of the above mentioned factors were clarified. The relationship between the number of teeth and the number of bleeding sites approached significance ($p = 0.06$). In addition, the patient's sex also had a significant effect the number of bleeding sites ($p = 0.02$). Correcting for these variables reversed the relation between Groups 5 and 6. Thus, while there was greater inflammation in Group 5 patients, the increase was a function of the number of teeth and the sex of the patient, not the amount of inflammation/tooth. Interestingly, these findings were not the same for salivary PAF or PMN levels.

Analysis of covariance with salivary PAF as the dependent variable revealed only the smoking status to have an effect on salivary PAF levels. When the group means for salivary PAF were corrected for smoking, the relation between Groups 5 and 6 were reversed, with Group 6 having the highest salivary PAF levels. No other variables discussed thus far significantly affected salivary PAF levels. Thus, it appears that smoking accounted for the small, non-significant reversal in salivary PAF levels between these two groups.

When salivary PMN count was evaluated as the dependent variable, the differences between Group 5 and Group 6 were found to approach significance ($p = 0.09$). Variables affecting the PMN count included

smoking and use of non-steroidal anti-inflammatory drugs ($p = 0.04$ and 0.05, respectively). This finding showing decreased salivary PMN levels in smokers is in agreement with Kenney, *et al.* (1975), who noted decreased mobility and phagocytosis in crevicular PMN. While the role of smoking in periodontal disease is controversial, it does appear to decrease both salivary PAF and PMN levels.

The relation between salivary PAF levels and disease severity was highly significant ($p<0.0001$). However, the correlation value was not very high ($r = 0.46$). Several possibilities will be considered in the following paragraphs which may account for this finding.

First, it must be acknowledged that saliva represents a compilation of fluids from salivary gland, gingival crevicular fluid and sputum. Thus, contribution from areas other than the periodontium is possible. In an attempt to minimize the effect of these contributors, this study excluded all patients with recent systemic disease. Nevertheless, the possibility exists that not all of the salivary PAF was of crevicular fluid origin.

Another reason for low correlation values may be due to differences in salivary flow amongst patients. Perhaps it would have been better to measure salivary PAF levels relative to mg of protein instead of saliva volume. Because the saliva was collected into an organic solvent, however, protein quantification was not possible. Thus, the benefits of measuring the salivary PAF relative to mg of protein was outweighed by the need to use a second (and possibly different) sample.

Correlation values may also have been affected by the type of PAF measured. That is to say, both bound and free PAF were recovered and measured in this study. By removing the cellular content prior to the

extraction process, it would have been possible to measure only the free PAF. Because it was not possible to differentiate between PAF which was bound to the inflammatory cell as part of cell stimulation, and between PAF which was produced by the cell and being retained, it was decided to measure the total PAF concentration.

Lastly, the low correlation between salivary PAF levels and periodontal disease may suggest that other factors are involved in periodontal disease severity as well. This is in agreement with our current concept of periodontal disease. While the primary etiology is bacterial, the interaction between the host and the pathogenic organisms determine the extent of tissue destruction, and thus, disease progression (Lindhe, 1988). Low correlation values are undesirable in agents used for diagnostic purposes, as there would increased numbers of false readings. On the basis of the results in the current study, it would appear that salivary PAF would not make a good diagnostic tool for determining disease severity. However, it may be that a baseline would need to be established for each patient with subsequent sampling evaluated relative to that baseline value. Longitudinal studies are required to answer this important question. In addition, future research should be directed in such a way as to answer the following three questions.

Does PAF play a protective or destructive role in periodontal disease or is it merely a bystander producing no harm or benefit? The fact that PAF acts to amplify the inflammatory response could lead us to suggest that it plays either a protective *or* destructive role. It is yet to be determined what causes tissue destruction during periodontitis: the

virulence of the organism or the host response. If it is the virulence of the organism as suggested by the periodontal breakdown common in patients with neutrophil defects (Van Dyke, *et al.*, 1985), then the role of PAF is likely to be protective in nature. However, it is also possible that an excessive inflammatory response could result in increased tissue destruction. The list of potentially toxic substances that inflammatory cells can release is lengthy and includes collagenase, superoxide ion, hydroxyl radicals, interleukin 1, arachidonic acid metabolites and PAF (Van Dyke, *et al.*, 1985). The ability of PAF to stimulate inflammatory cells to release some of these substances (Chilton, 1989) could suggest a destructive role. Thus, blockade of PAF may result in less tissue damage without total suppression of the inflammatory response. In this regard, a variety of specific PAF receptor antagonists have been tested *in vitro*, and are currently being evaluated for clinical use in asthmatic patients (Guinot, *et al.*, 1987). Would topical application of one of these specific PAF receptor antagonists be effective in treating periodontal disease?

What is the relation between PAF and periodontal disease activity? Currently, the gold standard for evaluating whether periodontal disease activity has occurred is by determination of loss of attachment or bone loss (Haffajee, *et al.*, 1983). Either of these methods require longitudinal monitoring of patients. Longitudinal studies looking at salivary and crevicular PAF levels are currently underway, and should provide insight as to its relation with periodontal disease activity. If a relation is found to exist, then PAF could possibly be used to detect active disease episodes.

Are high levels of PAF released as a result of disease, or does disease occur due to an overresponsive inflammatory process? To answer this question, one would need to follow two groups of subjects: those with high PAF levels and those with low levels. Because PAF does tend to amplify the inflammatory response (Chilton, 1989), it could well be that "hyper-responders", releasing increased amounts of PAF, have increased tissue destruction. If this is the case, then PAF may possibly be of use in the detection of disease susceptible individuals.

While a lot remains to be established about the action of PAF in the periodontal disease process, the current study demonstrated that a definite relation does exist between salivary PAF levels and periodontal disease severity. In addition, a positive relation was also found to exist between salivary PAF and bleeding on probing and PMN count. These findings suggest that this potent phospholipid inflammatory mediator may play a role in the pathogenesis of periodontal disease warranting future research in this area.

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