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A THESIS

Presented to the Faculty of

The University of Texas Graduate School of Biomedical Sciences

at San Antonio

in Partial Fulfillment of the Requirements for the Degree of

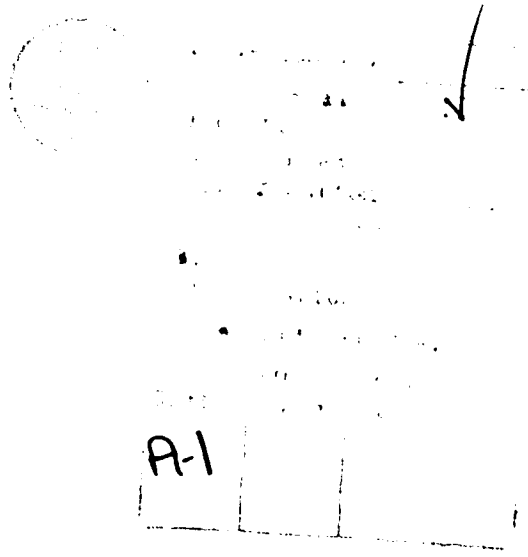
MASTER OF SCIENCE

By

Ricardo Diaz D.D.S.

San Antonio, Texas

May 1991



RELATION OF SALIVARY PAF TO PERIODONTAL DISEASE IN PERIODONTAL MAINTENANCE AND REFRACTORY PERIODONTITIS PATIENTS

Ricardo Diaz D.D.S.

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Date

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DEDICATION

I dedicate this thesis to my family for all the encouragement and support they've given me over the years. I especially want to thank my wife Anna for her tremendous patience and understanding during the course of this project. Her support through these last three years was invaluable to me. I would finally like to thank God for bringing Jacqueline Olivia to Anna and me. This will truly be a year to remember in my life.

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RELATION OF SALIVARY PAF TO PERIODONTAL DISEASE IN PERIODONTAL MAINTENANCE AND REFRACTORY PERIODONTITIS PATIENTS

Publication No. _____

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Platelet activating factor (PAF), a potent phospholipid inflammatory mediator present in normal human mixed saliva, may play a role in periodontal inflammation. The purpose of this study was to evaluate the level of salivary PAF in patients previously treated for periodontal disease and seen on a regular basis in a periodontal maintenance program. Thirteen of the patients were refractory to treatment, whereas the remaining 63 responded to conventional periodontal therapy. Salivary lipids were fractionated by thin layer chromatography and assessed for PAF activity by platelet bioassay as compared to authentic PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; C16:O-AGEPC). For the refractory periodontitis

group, salivary PAF levels were 7897 ± 1941.1 C16:0-AGEPC fmole equivalents/ml saliva (mean ± SEM). For this group, salivary PAF levels correlated to the number of sites with probing depths greater than 3mm ($r = 0.697$, $p<0.01$) and number of probing depths greater than 5mm ($r = 0.768$, $p<0.003$). Significant correlations were also found between salivary PAF levels and the number of total teeth ($r = 0.572$, $p<0.04$), histologic salivary neutrophil counts ($r = 0.570$, $p<0.04$), and the number of teeth with at least one site with a probing depth of greater than 3mm ($r = 0.722$, $p<0.005$). For the 63 treatment responsive, maintenance subjects, the salivary PAF level was significantly less than the refractory group, *i.e.*, 3,414 ± 554.5 C16:0-AGEPC fmole equivalents/ml saliva. Within this group, salivary PAF correlated weakly to the number of probing depths greater than 5mm ($r = 0.253$, $p<0.04$) and the histologic neutrophil count ($r = 0.351$, $p<0.006$).

The clinical presentation of these two patient groups at time of saliva sampling indicated that these patients represented distinct groups relative to level of periodontal disease. The refractory periodontitis group had significantly higher number of diseased teeth, bleeding sites, bleeding scores, and probing depths greater than 5mm. The higher number of diseased teeth and probing depths greater than 5mm in the refractory group are consistent with a history of more severe periodontal disease.

The results of the current study showed a significant difference in the level of salivary PAF in two groups of patients with different levels of periodontal inflammatory disease. These results support the hypothesis that PAF may play a role as an important inflammatory mediator in periodontal disease.

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I. INTRODUCTION AND LITERATURE REVIEW

A. THE INFLAMMATORY PROCESS

Diseases characterized by inflammation are an important cause of morbidity and mortality in humans. Information concerning the mechanisms whereby inflammatory cells accumulate in tissues, as well as the mechanisms whereby such cells are stimulated to damage tissues, should provide better insights into the pathogenesis of human disease and also should provide clues for developing more rational forms of therapy.

Inflammation can be defined as a localized physiologic, protective response elicited by injury or destruction of tissue which serves to destroy, dilute, or wall-off both the injurious agent and the injured tissue (Gallin, *et al.*, 1988). It is characterized in the acute form by the classical signs of pain, heat, redness, swelling, and loss of function. Microscopically, it involves a complex series of events including (a) dilation of arterioles, capillaries, and venules, with increasing permeability and blood flow; (b) exudation of fluids including plasma protein; and (c) leukocyte migration into the inflammatory focus (Casley-Smith, 1980; Issekutz, 1984). These microscopic changes result in the classical clinical signs of inflammation.

Inflammation can be divided into two types: acute and chronic. This division is primarily based on the duration, severity, degree, and type of cellular proliferation present (Hurley, 1983). It is important to note that there is a great deal of overlap between acute and chronic inflammation, but they will be considered separately for the sake of simplicity. The two main features of the acute inflammatory response

are described as vascular alterations and cellular emigration. Vascular alterations include a transient vasoconstriction followed by vasodilation, increased blood flow, and increased vascular permeability (Movat, 1985). The latter three vascular effects result from the release of endogenous vasoactive mediators within the injured tissues (Wilhelm, 1969). Some of the prominent mediators released include histamine, bradykinin, arachidonic acid metabolites (prostaglandins, leukotrienes, thromboxane, etc.), interleukins, tumor necrosis factor, complement, and platelet activating factor (PAF) (See Table 1). The exact sequence of the appearance of these mediators, their dependence on, or independence of each other is still largely speculative. The increased vascular permeability of the early phase of acute inflammation is due to the contraction of venular endothelial cells in response to vasoactive mediators (Butcher, *et al.*, 1986). The contraction leads to endothelial gaps which facilitate the extravasation of plasma fluids and proteins and permits diapedesis of leukocytes into the injured tissue (Grega and Adamski, 1987).

As indicated in Table 1, some vasoactive mediators also have pro-inflammatory chemotactic effects on leukocytes. The polymorphonuclear leukocyte (PMN) is the predominant leukocyte observed in acute inflammation. With the continuous presence of a stimulant or irritant, the acute inflammatory response may progress into chronic inflammation.

Chronic inflammation represents a persistent ongoing process characterized by increased cellular influx and widespread tissue damage which is usually the result of a reaction of the host rather than

Table 1. Pro-inflammatory Effects of Various Mediators of Inflammation.

INFLAMMATORY EVENT	INFLAMMATORY MEDIATOR
VASODILATION	Bradykinin, Prostaglandins (PGI_2, PGE_2, PGE_2), Leukotrienes (LTD, LTE), PAF
INCREASED VASCULAR PERMEABILITY	Histamine, Complement (C_{3a}, C_{5a}), PGE_1, PGE_2, LTD, LTE, PAF, TxA_2
LEUKOCYTE FUNCTION (CHEMOTAXIS, PHAGOCYTOSIS)	Bradykinin, C_{5a}, HETE, HPETE, Interleukins (IL-1, IL-3), Tumor necrosis factor, PAF
PLATELET STIMULATION	PAF, Thromboxane A_2

any direct action of an invading pathogen (Gallin, *et al.*, 1988). Vascular changes seen in the acute phase of inflammation persist or increase during chronic inflammation and most of the inflammatory mediators seen in acute inflammation participate in the development and maintenance of chronic inflammation. The predominant cells present during chronic inflammation are mononuclear cells such as macrophages, lymphocytes, and plasma cells (Dannenburg, 1975). The factors determining whether an inflammatory process will be acute or chronic in some cases seem to be dependent upon the nature of the invading pathogen or local irritant and the resultant reactivity of the host defenses to the noxious agent.

Many forms of acute and chronic inflammation are initiated, amplified and propagated as a result of the recruitment of humoral and cellular components of the immune system (Fagan, *et al.*, 1982; Ward, 1983). Immune-mediated elimination of foreign bodies proceeds through a series of integrated steps. First, the foreign antigen is recognized as being "foreign" by cellular recognition components of the immune system. The antigen is subsequently bound and destroyed or eliminated. The binding of antigen by immune system recognition cells leads to the production of pro-inflammatory mediators (Ferreri, *et al.*, 1986). These mediators alter blood flow, increase vascular permeability, augment adherence of circulating leukocytes to vascular endothelium, promote migration of leukocytes into tissues, and stimulate leukocytes to destroy the foreign agent (Spiegelberg, 1984). Thus, the immune system can promote events that are pro-inflammatory in nature.

Acute and chronic inflammation are generally considered as protective host responses, however, certain aspects of acute and chronic inflammation are not necessarily of benefit. An example is the sequelae related to the development of hypersensitivity reactions. Hypersensitivity reactions represent host responses which produce injury to the host. Examples of these injurious reactions include hemolytic anemia, organ transplant rejection, asthma, and autoimmune disease (Male, 1986). A majority of the most crippling diseases of humans involve chronic inflammatory reactions. Rheumatoid arthritis, tuberculosis, syphilis, liver cirrhosis, and periodontal disease are all examples of chronic inflammatory disease (Gallin, *et al.*, 1988). Studies on the regulation of these acute and chronic reactions that may damage host tissue are required.

B. INFLAMMATORY ORAL BACTERIAL PRODUCTS

Oral bacteria and their metabolites have been implicated as the primary etiologic factors in the initiation of human periodontal disease (Dzink, *et al.*, 1985; Loe, *et al.*, 1965; Socransky, 1977). There is extensive literature which indicates that bacterial products in dental plaque may be important in producing inflammatory reactions and/or cause cytotoxicity. One group of bacterial products that have been studied in depth is bacterial endotoxin.

Endotoxins are large molecular weight lipopolysaccharides which are major constituents of the outer membrane of the gram-negative bacterial cell wall. Most of the prominent periodontopathic bacteria are gram-negative organisms ((Gold, 1985; Socransky, 1977). Endotoxins

possess several biologic activities, including pyrogenicity, the general and local Shwartzman reactions, complement activation, stimulation of bone resorption, and induction of hypersensitivity (Mergenhagen, 1970). There is evidence that endotoxin is present in dental plaque and may play a role in oral inflammation. Endotoxin activity in subgingival dental plaque has been correlated with the degree of gingival inflammation (Shapiro, *et al.*, 1972). The activation of complement by oral bacterial endotoxin has been proposed as being one of the major mechanisms involved in periodontal inflammation (Mergenhagen, 1970). This reaction may result in the production of the vasoactive, chemotactic, and opsonic products of complement in the periodontium. Together, these endotoxin-induced reactions are the basis for the *potential* pro-inflammatory role of bacterial endotoxins.

Endotoxin may also effect inflammatory oral processes by other non-immunologically mediated processes. Aleo *et al.* (Aleo, 1974; Aleo, 1975) demonstrated that cementum bound endotoxins, obtained from periodontally diseased teeth, reduced the growth of fibroblasts and reduced fibroblast attachment to the root surface. These effects may have important implications in initial healing response and long term repair of periodontally diseased tissues.

The structural analog to the lipopolysaccharide complex of gram-negative bacteria is the lipoteichoic acid (LTA) complex present in the cell membrane of a wide variety of gram-positive bacteria. The LTA complex is composed of a glycerol teichoic acid moiety covalently joined to a glycolipid or phosphatidyl glycolipid. LTA may be found intracellularly as part of the cell membrane and also extracellularly as a

result of cell lysis, active secretion, or loss of this moiety during cell growth and division (Joseph and Shockman, 1975). LTA exhibits several endotoxin-like activities and it has been demonstrated that a glycolipid moiety is important in determining immunogenicity, induction of local and general Shwartzman reactions, stimulation of bone resorption, and complement activation (Hausmann, 1975; Wicken and Knox, 1974). Studies have demonstrated the presence of streptococci and lactobacilli throughout the oral cavity, as well as in developing dental plaque (Socransky, 1977). Thus, considering the numerous potential biologic properties of LTA and the large number of LTA-producing microorganisms in the oral cavity, it is apparent that these compounds have the potential to play a role in the initiation and progression of inflammatory oral disease.

Bacterial enzymes also may be involved in oral inflammatory diseases. Some of the enzymes found in dental plaque include hyaluronidase, collagenase, chondroitin sulfatase, elastase, beta-glucuronidase, protease, and lipase (Caffesse and Nasjleti, 1976; Lamster, *et al.*, 1988; Lesanti, 1960; Loesche, *et al.*, 1974). Tissue destruction could result from a direct tissue damaging effect of these enzymes or as the result of enzymatic activation of endogenous inflammatory mediators (Schwartz and Dibblee, 1975). For example, bacterial enzyme activation of the complement system has been described (Page and Schroeder, 1981). Further, collagenase activity may result in the loss of collagen fibers in the connective tissue of the periodontium (Loesche, *et al.*, 1974). Hyaluronidase may penetrate the intact sulcular epithelium and result in hydrolysis of the intercellular

cementing substance of the epithelium and connective tissue and widening of the intercellular epithelial spaces and disorganization of the connective ground substance (Caffesse and Nasjleti, 1976). Chondroitin sulfatase may have direct tissue effects by hydrolyzing chondroitin sulfate present in connective tissue. Beta-glucuronidase in gingival crevicular fluid has been shown to increase during periods of loss of periodontal attachment (Lamster, *et al.*, 1988). It is apparent that bacterial enzymes form one of the components of a group of potentially pro-inflammatory and tissue destructive bacterial by-products.

Other potentially injurious products of oral bacteria include a variety of cytotoxic metabolites that may impair the integrity of the periodontium. These cytotoxic substances include ammonia, hydrogen sulfide and indole, toxic amines, and formic, acetic, propionic, and lactic acids (Rizzo, 1967; Socransky, *et al.*, 1964). These substances are not only cytotoxic in themselves, but could aid gingival penetration of macromolecules such as LTAs and peptidoglycans. Thus, direct and indirect effects of these cytotoxic metabolites may play a role in periodontal tissue injury.

In summary, bacteria and their products have been implicated as etiologic factors in oral inflammatory disease. Many organisms associated with oral inflammatory disease can produce multiple products or factors which either directly damage tissue or elicit host responses which may be destructive rather than protective in nature.

C. THE PATHOGENESIS OF INFLAMMATORY PERIODONTAL DISEASE

It is generally accepted that the inflammatory response of the host to bacterial plaque plays a major role in the pathogenesis of gingivitis and periodontitis (Page and Schroeder, 1976; Page and Schroeder, 1982). This reaction involves inflammatory and immunologic defense mechanisms which operate at both a local and systemic level. The observation (Page, 1989) that the pathogenic potential of similar levels and types of plaque varies between individuals underscores the importance of host defense mechanisms and has been the subject of intense investigation.

Attention has been increasingly focused upon the host and upon the potential of host defense mechanisms participating in destructive aspects of disease (Pennel and Keagle, 1977; Schroeder, 1986; Socransky, *et al.*, 1984). A large volume of data has been accumulated from experimental animal models and from experimentally-induced and spontaneously-occurring gingivitis and periodontitis in humans (Loe, *et al.*, 1965; Page and Schroeder, 1981; Page and Schroeder, 1982; Schroeder, *et al.*, 1975). A useful framework for organization and consideration of these data has been devised on the basis of histopathologic, radiographic, and ultrastructural features and biochemical measurements (Page and Schroeder, 1976; Page and Schroeder, 1982). The sequence of events culminating in clinically apparent gingivitis and periodontitis has been separated into the initial, early, established, and advanced stages.

The initial lesion manifests the characteristics of classic acute inflammation. In experimental animals and humans exposed to plaque accumulation, an acute exudative inflammatory response occurs (Schroeder, *et al.*, 1975). This response is manifested by an increased flow of gingival fluid and enhanced migration of granulocytes, especially PMN, from the vessels of the subgingival plexus through the gingival connective tissue and junctional epithelium and into the gingival sulcus and oral cavity. The perivascular connective tissue matrix becomes altered most likely by collagenase and other enzymes released by migrating neutrophils (Attstrom and Schroeder, 1979). There is exudation and deposition of fibrin in the affected area. The infiltrated area comprises 5% to 10% of the marginal gingival connective tissue and, in this zone, much of the collagen is destroyed. In humans, the initial lesion is usually seen within about four days after the beginning of plaque accumulation (Page and Schroeder, 1982). The early lesion may then develop.

The early lesion evolves from the initial lesion within about one week following the beginning of plaque accumulation (Page and Schroeder, 1976). Clinically, the early lesion may appear as gingivitis. It is characterized by a cellular infiltrate in which small, medium, and large lymphocytes and macrophages predominate together with small numbers of plasma cells located at the periphery of the infiltrate. Lymphocytes account for 75% of the total inflammatory cell population. Five to 15% of the marginal connective tissue may be occupied, with collagen loss in the affected area reaching 60-70%. Acute inflammation persists as evidenced by vasculitis and presence of PMN, especially in

the junctional epithelium. Overall, however the morphologic features of the early lesion are consistent with those of delayed hypersensitivity (Wilde, *et al.*, 1977).

The duration of the early periodontal lesion has not been definitely determined. In studies of human experimental gingivitis, Seymour *et al.* found the initial infiltrate to consist mostly of lymphocytes, 70% of which were T cells; the composition of the infiltrate did not change during the 21 day experimental period (Seymour, *et al.*, 1983). Therefore, the early lesion may persist longer than the previously suspected period of 1-2 weeks (Page and Schroeder, 1976).

With the passage of time, the early lesion progresses into the established lesion which is characterized histologically by a predominance of plasma cells and B lymphocytes (Page and Schroeder, 1982). A small gingival pocket lined with a pocket epithelium may be created but without significant bone loss (Schroeder, *et al.*, 1975). Large numbers of PMN appear in the junctional and pocket epithelium. Macrophages are present in the pocket wall. This established lesion may persist or may progress to an advanced lesion.

Established periodontal lesions of two types appear to exist. Some lesions remain stable and do not progress for months or years (Suomi, *et al.*, 1971). However, other lesions may become more active and convert to progressive, destructive lesions. The factors which determine whether or not a lesion progresses to an active lesion or remains a stable lesion are not understood. The conversion to an active lesion may relate to activation of immunopathologic or other destructive inflammatory mechanisms of the host in which the tissue damage

created overshadows the defense achieved (Genco, *et al.*, 1974; Horton, *et al.*, 1974). An uncontrolled release of lymphokines, inflammatory mediators or hydrolytic enzymes likely are involved (Goodson, *et al.*, 1974; Horton, *et al.*, 1974; Offenbacher, *et al.*, 1984).

The advanced lesion of periodontitis occurs following the conversion of a stable established lesion to an active lesion (Page, 1986). It is characterized by the formation of a periodontal pocket with destruction of alveolar bone. Signs of acute exudative vasculitis persist. A dense infiltrate of plasma cells, lymphocytes, and macrophage are present (Page, 1986). Periods of acute exacerbation, with pus and abscess formation, and quiescence may occur.

In summary, there is extensive evidence that bacteria cause *periodontal disease.* The disease begins as acute inflammation of the marginal gingiva and progresses to the formation of a periodontal pocket with the loss of alveolar bone. Host defense factors play an important role in the progression of disease by attempting to control and regulate microbial colonization and infection. The factors determining the conversion of a stable lesion to an aggressive, destructive lesion are not well understood. Further study is required to clearly delineate the role of individual host defense mechanisms during the course of inflammation and periodontal disease. Identification of these host defense mechanisms could be used in attempts to modulate mechanisms responsible for the progression of periodontal disease.

D. REFRACTORY PERIODONTITIS

1. DEFINITION

The word refractory is defined in the American Academy of Periodontology (AAP) Glossary of Periodontic Terms (1986) as: "persistent, not readily responding to treatment". Although the AAP recognized refractory periodontitis as a classification of periodontal disease in 1986, it failed to define it in the Glossary of Terms. In the literature, refractory has been used as a broad generalized descriptor of any form of periodontitis that has not responded favorably to treatment (Lindhe, *et al.*, 1984; Rosling, *et al.*, 1976). Refractory periodontitis "includes patients who are unresponsive to any treatment provided whatever the thoroughness of frequency as well as patients with recurrent disease at *a few or many sites*" (Proceedings of the World Workshop in Clinical Periodontics, 1989). Periodontal relapse can manifest in persistence or re-occurrence of treated periodontal pockets. No diagnostic parameters exist to establish a diagnosis of refractory periodontitis beyond the definition of non-responsiveness to treatment. Refractory cases have been reported in adult periodontitis, localized juvenile periodontitis, prepubertal periodontitis, and rapidly progressive periodontitis. Additional descriptive terms include recurrent, relapse, intractable, recalcitrant, unstable, downhill, and refractory progressive periodontitis. A diagnosis of refractory disease is a retrospective one, which further exemplifies our inability to predict outcome of treatment in many cases.

2. INCIDENCE

A number of retrospective studies have categorized patients *via* their response to treatment. Hirschfield and Wasserman categorized 600 patients as either well-maintained, downhill, or extreme downhill on the basis of tooth loss subsequent to periodontal therapy over an average maintenance period of 22 years (range 15-53 years) (Hirschfield and Wasserman, 1978). The downhill and extreme downhill groups accounted for 13% and 4% of the 600 patients, respectively. McFall (McFall, 1982) identified 15% and 8% as downhill and extreme downhill groups, respectively, in 100 patients receiving treatment over a period of 15 to 29 years (average 19 years). Goldman *et al.* (Goldman, *et al.*, 1986) treated 211 patients over 15 to 34 years (average 22.2 years) and found 28% and 10% of cases to be downhill and extreme downhill, respectively. Meador *et al.* (Meador, *et al.*, 1985) retrospectively examined 60 cases with a full range of treatment modalities over a 22 year period. Overall, 28% of the patients were classified as unstable and 72% were stable. These categories were based on overall response to treatment and indicated that the overall incidence of refractory periodontitis is low.

It is difficult to speculate on the prevalence of refractory periodontal disease based on these and other studies, as all groups identified as well-maintained, downhill, extreme downhill, stable, or unstable were comprised, in part, of patients with localized areas of periodontal breakdown despite treatment. Fortunately, the incidence of refractory patients with generalized destruction is low, but most studies of any size seem to contain some refractory sites. Recurrent

periodontitis has been related by Lindhe *et al.* (Lindhe, *et al.*, 1984), Rosling *et al.* (Rosling, *et al.*, 1976) and Nyman *et al.* (Nyman, *et al.*, 1977) to inadequate oral hygiene or inadequate maintenance treatment. These patients may be termed "pseudorefractory" patients and may be confused with patients that receive proper care and that maintain adequate oral hygiene. Truly refractory sites would represent sites with progression of disease despite adequate conventional initial therapy and follow-up (Lindhe, *et al.*, 1984; Philstrom, *et al.*, 1988; Philstrom, *et al.*, 1981).

3. CLINICAL FEATURES

No established clinical criteria have been established for the diagnoses of refractory periodontal disease. By definition (AAP Glossary of Terms, 1986), continued loss of attachment in spite of treatment is the gold standard in identifying refractory disease. The studies described above (Goldman, *et al.*, 1986; Hirschfield and Wasserman, 1978; McFall, 1982) identified patient response based on tooth loss and thus loss of attachment and, thus, retrospectively identified areas that were non-responsive to treatment.

Clinical indices such as bleeding on probing have proven to lack specificity in predicting loss of attachment (Goodson, 1986; Haffajee, *et al.*, 1983). Similarly, Baderstein *et al.* (Baderstein, *et al.*, 1985) found that other clinical indices, *i.e.*, plaque, bleeding on probing, suppuration, and probing depth, were also not highly predictive of subsequent attachment loss. Loss of attachment, unfortunately is a retrospective indicator of destruction. Parameters associated with attachment loss such as increased probing depth, mobility, recession, radiographic

evidence of bone loss, and tooth loss will manifest prospective to the extent of attachment loss. Thus, none of these parameters can be used to predict sites of future loss of attachment. Until indicators are developed that prove to be diagnostic for refractory periodontitis, we must continue to rely on clinical indices as the best indicators of recurrent disease available.

4. ETIOLOGIC FACTORS OF REFRACTORY PERIODONTITIS

Various potential etiologic factors have been examined in efforts to identify patients or specific sites that will be refractory to treatment. Included in this consideration are a variety of diverse factors such as anatomic variation, diverse microbiota, and host factors. Specific sites such as furcations may have a poorer prognosis due to anatomic variables. The percentage of furcated teeth lost in the previously mentioned retrospective studies was much larger in the downhill and extreme downhill groups. The anatomical variable present in multirooted teeth may complicate treatment and oral hygiene measures. Specific microbiota have been evaluated to determine association of specific bacteria to sites exhibiting loss of attachment (Haffajee and Socransky, 1986; Listgarten and Hellden, 1978; Socransky, 1985). Methods of bacterial evaluation have included dark field microscopy, culturing, DNA probes, and serum and local antibody responses. A high degree of variability between patients and diseased sites has been found and making it difficult to identify specific etiologic microorganisms in refractory periodontal disease.

Host factors have also been evaluated as contributing factors in refractory disease. Periodontitis refractory to nonsurgical, surgical, and

antimicrobial therapy has been reported in patients with defective or dysfunctional PMN (Van Dyke, *et al.*, 1984; Waldrop, *et al.*, 1987). However, studies examining defective PMN function in refractory periodontitis patients are few. As with other potential etiologic factors, there seems to be variability between patients.

In summary, current treatment approaches to periodontal disease are based on the concept that all cases in adults respond equally to conventional periodontal therapy with the only modifications in treatment generally based on the anatomic factors and severity of destruction which may limit access to subgingival instrumentation. In spite of these concepts and the longitudinal studies which have demonstrated that the majority of patients respond predictably to treatment, a small percentage of adult periodontitis cases fail to respond in the same predictable manner as other cases with similar signs and symptoms of disease. This group of patients has been referred to clinically and in the literature as refractory, downhill, extreme downhill or unstable.

Although the term refractory periodontitis has appeared repeatedly in the literature, there has not been a consensus concerning an objective definition of this term. In many cases, the term refractory has been used in reference to subjects with localized areas of poor treatment response and/or disease recurrence in cases which have otherwise responded well. In other instances, the response to treatment is often defined in reference to the average of sites in the mouth. It is apparent that further studies are needed to discriminate generalized non-responding disease, from apparently unique host

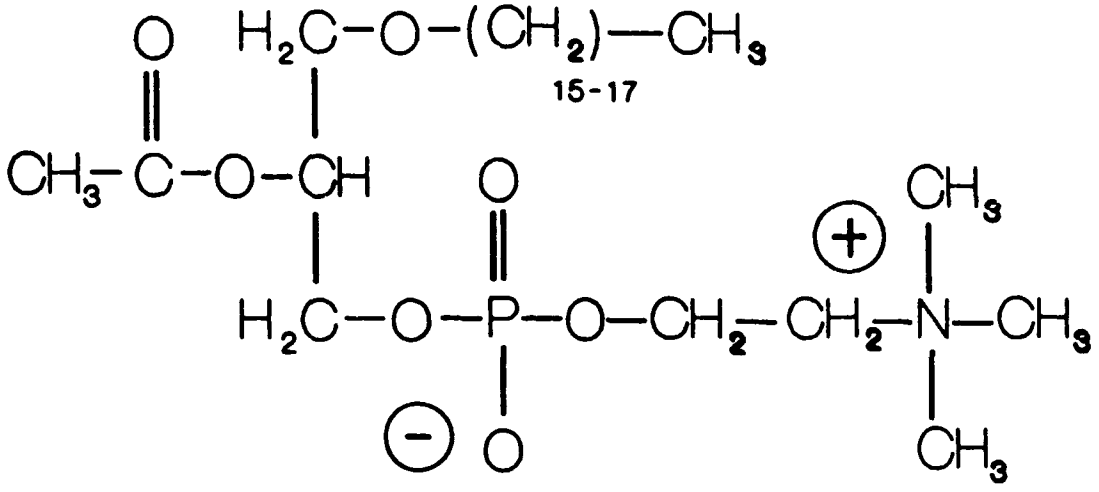
characteristics and from localized site specific factors which may influence response to treatment.

E. PLATELET ACTIVATING FACTOR (PAF)

The term platelet activating factor was originally proposed by Benveniste *et al.* (Benveniste, *et al.*, 1972) and indicated the primary biologic effect of a substance released from basophils. This factor was released from antigen-stimulated basophils and activated platelets to release histamine and caused the platelets to aggregate (Siraganian and Osler, 1971). During the next few years, studies to determine the chemical structure of rabbit basophil PAF were accomplished (Hanahan, *et al.*, 1980). Initially, Demopoulos, Hanahan, and Pinckard (Demopoulus, *et al.*, 1979), Benveniste *et al.* (Benveniste, *et al.*, 1979), and Blank, *et al.* (Blank, *et al.*, 1979) synthesized 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC) and showed that its biologic activity was indistinguishable from naturally occurring PAF (See Figure 1). The acetyl moiety at the *sn*-2 position was shown to be important since both AGEPC and naturally occuring PAF were deactivated by base catalyzed-hydrolysis and their activities restored by a reaction with acetic anhydride (Demopoulus, *et al.*, 1979). Hog leukocyte derived PAF was reported to be lipid-like and found to be inactivated by phospholipase A_2 and thus had characteristics of a phospholipid (Benveniste, 1974; Benveniste, *et al.*, 1977).

A number of recent studies have further characterized the structure of PAF by elucidating the length and degree of unsaturation of the 1-*O*-alkyl chain (Clay, *et al.*, 1984; Mueller, *et al.*, 1984; Pinckard, *et al.*, 1988). From these studies, different analogs and homologs of AGEPC

Figure 1. Structure of 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine or AGEPC.



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

have been identified as being synthesized within the same cell. Molecules with differing chain lengths, extent of saturation in the number 1 position, linkage changes in the number 1 position, and polar head groups other than choline have been identified. The biological activity of these PAF molecules have not been fully explored; most research has focused on AGEPC.

Certain structural features have proven to be necessary for PAF to have biologic activity. An ether linkage at the *sn*-1 position and an acetyl group at the *sn*-2 position are required for maximal activity (Demopoulus, *et al.*, 1979). A choline head group at the *sn*-3 position is also necessary for optimal biologic activity, although ethanolamine head groups have some activity (Satouchi, *et al.*, 1981).

In addition to being released from basophils, a diverse group of cells and tissues synthesize PAF in response to a variety of stimuli (Arnoux, *et al.*, 1981; Camussi, *et al.*, 1980; Clark, *et al.*, 1980; Lotner, *et al.*, 1980; McIntyre, *et al.*, 1985; Pinckard, *et al.*, 1988). Many of these cell and tissue sources of PAF are listed in Table 2. The action of PAF appears to involve not only the regulation of inflammation, but the modulation of cardiovascular, pulmonary, uterine, renal, and hepatic pathophysiological function as well (Pinckard, 1983; Pinckard, *et al.*, 1988).

PAF is not stored in a preformed state within inflammatory cells, but is rapidly generated after cell stimulation or perturbation and then released extracellularly (*c.f.* Snyder, 1989). Two pathways for the synthesis of PAF are believed to occur. These pathways are referred to as the deacylation-reacetylation (remodeling) pathway and the *de novo*

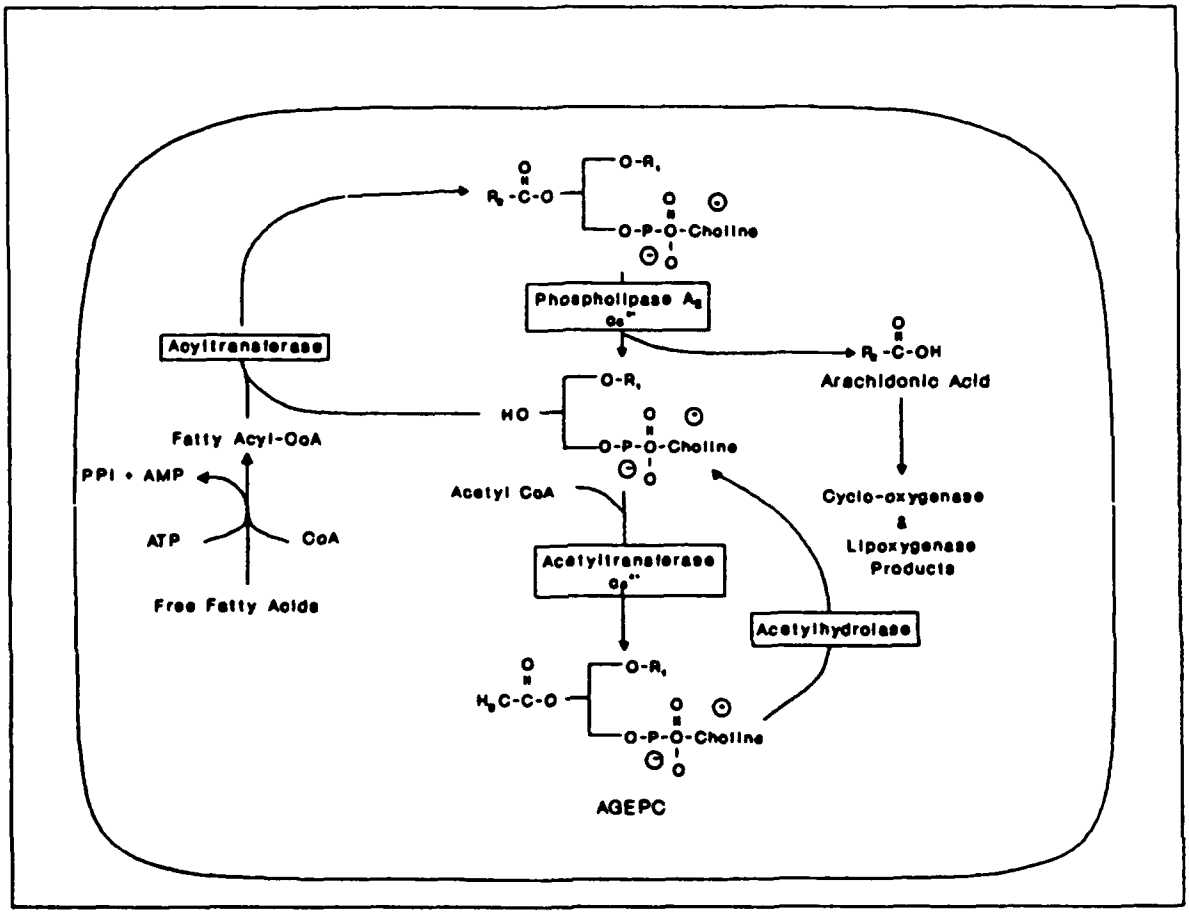
Table 2. Cellular and Tissue Sources of PAF.

CELLULAR SOURCES	TISSUE SOURCES
Platelet	Kidney
Basophil	Blood
Neutrophil	Skin
Mast Cell	Lung
Eosinophil	Myocardium
Monocyte/Macrophage	Embryo
Vascular Endothelium	Uterus
Natural Killer Cell	Liver
Mesangial Cell	Saliva
	Brain

pathway. In the deacylation-reacetylation pathway, PAF is derived from cellular pools of a precursor molecule 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine which is widely distributed in cell membranes. This molecule differs from PAF by having long chain fatty acids in the number 2 position consisting in part of arachidonic acid. Cell stimulation causes the activation of phospholipase A_2 which cleaves the long chain fatty acids at the number 2 position to release arachidonic acid and produce the immediate precursor of PAF, a lysophospholipid, 1-0-alkyl-*sn*-glycero-3-phosphocholine (lyso-GEPC). This is then acetylated by acetyltranferase and acetyl-CoA to produce 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC) (Wykle, *et al.*, 1980) (See Figure 2). The substrate specificity of acetyltranferase is not yet known, but is important since PAF derived from inflammatory cells does exhibit molecular heterogeneity. If this specificity can be defined, the ultimate types and proportions of PAF molecules that are synthesized by stimulated cells may be better determined.

A second enzymatic pathway for PAF biosynthesis, the *de novo* pathway has been described (Renooji and Snyder, 1981). This pathway involves the transfer of phosphocholine to 1-*O*-alkyl-2-acetylglycerol by CDP choline:1-*O*-alkyl-2-acetylglycerol phosphocholine transferase. This pathway has been shown to be important in a number of non-inflammatory cells and certain tissues (Renooji and Snyder, 1981). However, the deacylation-reacetylation pathway for PAF biosynthesis described above likely predominates in stimulated inflammatory cells, inasmuch as the activity of actetyltranferase significantly increases after cell stimulation while the activity of the enzymes in the *de novo*

Figure 2. Deacylation-reacetylation pathway for the biosynthesis of PAF.


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

pathway remains unchanged (Alonso, *et al.*, 1982; Renooji and Snyder, 1981).

A pathway for the catabolism of PAF in various tissues and cells involves the enzyme acetylhydrolase (Figure 2). An intracellular form and a plasma form of this enzyme have been identified (Farr, *et al.*, 1983; Pinckard, *et al.*, 1979; Wardlow, 1986). Farr *et al.* (Farr, *et al.*, 1983) and Wardlow *et al.* (Wardlow, 1986) established the enzyme to be selective for PAF, independent of calcium, and present in low density lipoproteins. Acetylhydrolase catalyzes the hydrolysis of the acetyl group from the *sn*-2 position of AGEPC and generates the inactive metabolite, lyso-GEPC. The removal of the acetate by acetylhydrolase has been found to be the first step in PAF degradation in inflammatory cells and, thus, this enzyme plays an important role in the catabolism of PAF.

F. ROLE OF PAF IN INFLAMMATION

PAF is one of the most potent lipid mediators known. It stimulates a wide span of biological responses ranging from aggregation and degranulation of platelets to a variety of cellular agonistic effects involving the stimulation of chemotaxis, chemokinesis, superoxide production, and activation of arachidonic metabolites in PMN. PAF has also been shown to have potent effects on vascular endothelial cells, vascular smooth muscle cells, mesangial cells, and renal epithelial cells. With such a diversity of biological activities, PAF has been considered to be a key component in numerous diseases related to inflammatory

responses. Important *in vivo* and *in vitro* effects of PAF will be highlighted in this review.

1. *IN VITRO* EFFECTS

PAF is recognized as a potent agonist for rabbit and human platelets although it has agonistic effects on platelets derived from other species (Billah and Lapetina, 1983; McManus, *et al.*, 1981). AGEPC induces platelet aggregation, secretion of serotonin and histamine, and thromboxane B_2 production (Demopoulus, *et al.*, 1979; McManus, *et al.*, 1983). These PAF effects occur almost instantaneously and are complete within 60 seconds after PAF stimulation (Pinckard, *et al.*, 1988). In isolated washed human platelets, AGEPC (0.2-10nM concentration) results in a primary wave of platelet aggregation, while higher concentrations (10-100 nM) produce a primary and secondary wave of aggregation, followed by secretion (Pinckard, *et al.*, 1988). Similar responses are seen in platelets in plasma, although 10-100 times higher PAF concentrations (10-100 nM) are required (Kloprogge, *et al.*, 1983). The higher required concentration likely reflects the presence of plasma acetylhydrolase and plasma albumin. Albumin binds PAF and interferes with its receptor interaction (Ludwig, *et al.*, 1985) and would therefore possibly reduce the effect of PAF within plasma.

In vitro pro-inflammatory effects of PAF on other cells have also been shown. PAF stimulation of PMN results in aggregation, chemotaxis, chemokinesis, superoxide production, and release of lysozyme and β-glucuronidase (Goetzl, *et al.*, 1980; O'Flaherty, *et al.*, 1981; Shaw, *et al.*, 1981). The lipooxygenase product, LTB_4, is formed by PMN upon PAF-

induced activation and it has been suggested that LTB_4 may mediate PAF-induced aggregation responses (Chilton, *et al.*, 1982). Some evidence for this possible mediation involves the dose-dependent decrease in PMN activation by PAF in the presence of lipooxygenase inhibitors such as eicosatetraynoic (Smith and Bowman, 1982). Cyclooxygenase inhibitors such as indomethacin and ibuprofen do not produce similar inhibition indicating cyclooxygenase products may not mediate PAF induced aggregation (Smith and Bowman, 1982).

Eosinophils are induced to migrate after stimulation by PAF. When compared to other eosinophil chemotactic mediators such as LTB_4, histamine, FMLP, and eosinophilic chemotactic factor of anaphylaxis, eosinophils have been shown to be much more sensitive to activation by PAF (Bruijnzeel, *et al.*, 1986; Tamura, *et al.*, 1987). In agreement with these *in vitro* studies, PAF has been shown to induce the accumulation of eosinophils in the skin of allergic patients (Henocq and Vargaftig, 1986).

Monocytes and macrophage are also affected by PAF. PAF initiates aggregation of human blood monocytes in a dose dependent manner (Yasaka, *et al.*, 1982). On a molar basis, PAF is slightly more active than FMLP in producing aggregation and the response is also initiated in a shorter period of time (Pinckard, *et al.*, 1988). AGEPC also has a weak chemotactic effect on monocytes (Czarnetzki, 1983). Salem (Salem, *et al.*, 1990) found IL-1 production by monocytes to be modulated by AGEPC (1 nM-5μM concentration). Phagocytosis of damaged red blood cells by monocytes is also enhanced by AGEPC (Bussolino, 1989). AGEPC does not initiate the production of superoxide

anion or secretion of lysozyme by monocytes. Macrophage chemotaxis induced by PAF decreases by approximately 90% as monocytes differentiate into macrophage; cellular acetylhydrolase appears to increase by 260 times within macrophages and may be responsible for the reduced effects of PAF on these cells (Elstad, *et al.*, 1989)

2. *IN VIVO* EFFECTS

Intravenous administration of PAF (0.1-0.9 μg/kg) in experimental animals has indicated that this phospholipid has potent effects. In rabbits, PAF produces a reaction almost identical to an IgE anaphylactic shock with the same intravascular, pulmonary and cardiac manifestations (Halonen, *et al.*, 1981; McManus, *et al.*, 1980). Platelet sequestration in the pulmonary microvasculature is seen soon after PAF infusion producing pulmonary vascular constriction and increased pulmonary resistance. Histamine and thromboxane release from stimulated platelets occurs and is accompanied by thrombocytopenia, neutropenia, and basopenia. Concurrent with platelet stimulation, myocardial depression occurs and the peripheral and coronary vasculature constricts (Braquet, 1988; Pinckard, *et al.*, 1988) producing the characteristic signs of an anaphylactic shock.

Extravascular PMN migration is modulated by PAF. PAF has been shown to have effects on PMN adherence to vascular endothelium (Tonnesen, 1989). Injection of PAF into rabbits intracutaneously induces PMN margination and plugging within venules (Humphrey, *et al.*, 1982). This response appears to be dependent on cell surface receptors on the PMN, suggesting PAF is acting on the PMN versus the endothelium (Tonnesen, 1989).

Other *in vivo* animal studies have shown the pro-inflammatory effects of PAF. PAF has been shown to induce edema and hyperalgesia in the rat paw (Vargaftig and Ferreira, 1981). The intrathoracic injection of PAF induces pleurisy and neutropenia in the rat (Martins, *et al.*, 1989). Intradermal injections of PAF increase vascular permeability in rabbits, rats, hamsters, and man (Humphrey, *et al.*, 1982; Wedmore and Williams, 1981). Together, these *in vivo* animal studies are consistent with the known *in vitro* effects of PAF.

Intracutaneous injection of AGEPC in humans results in potent inflammatory reactions. After injection with 0.1 pmole PAF, profound vasoactive effects develop in human skin and are characterized by blanching of the skin, pain, and pruritus (Pinckard, *et al.*, 1980). These skin reactions subside within 60 minutes. Histamine was subsequently shown not to be responsible for the erythema and pruritus (Fjellner and Hagermark, 1985). Similar AGEPC-induced vasoactive effects have been shown in other species. In all species tested, AGEPC appears to be from 1000 to 10,000 times more potent than histamine (Pinckard, *et al.*, 1988). In rats, these potent vasoactive properties of AGEPC appear to result from AGEPC-induced contraction of endothelial cells in postcapillary venules (Humphrey, *et al.*, 1984). This endothelial cell contraction results in increased vascular permeability allowing extravasculion of intravascular fluids and cells.

3. PAF INTERACTION WITH OTHER INFLAMMATORY MEDIATORS

A biologically important synergism exists between AGEPC and other inflammatory mediators. Several platelet agonists have been

shown to significantly enhance the release reaction and aggregation of human platelets in response to AGEPC. Examples of these include ADP, arachidonic acid, epinephrine, collagen, and thrombin (Altman and Scazziota, 1986; Ostermann, *et al.*, 1983; Valone, 1986). The mechanism of synergism between AGEPC and these agents, however, remains to be established.

Synergism between the lipooxygenase product, 5-L-HETE, and AGEPC potentiates leukocyte activation (O'Flaherty, 1985). 5-L-HETE alone induces no secretory response in human PMNs, but significantly augments AGEPC-induced lysosomal enzyme secretion (O'Flaherty, *et al.*, 1983). This synergistic relationship between these two mediators appears to be unique since 5-L-HETE does not synergistically interact with other inflammatory mediators. This unique relationship to 5-L-HETE exemplifies the potentially potent inflammatory effects of PAF.

Through synergistic interactions with other inflammatory mediators, AGEPC more effectively initiates the respiratory burst in human PMN. For example, prior exposure of human PMN to AGEPC significantly enhances the rate and total production of superoxide induced by FMLP (Gay, *et al.*, 1986; Ingraham, *et al.*, 1982). Reverse synergism also occurs, since pretreatment of the human PMN with FMLP increases superoxide production in response to AGEPC (Ingraham, *et al.*, 1982).

AGEPC potency for increasing vascular permeability is significantly enhanced by the vasodilatory prostaglandins PGE_1 and PGE_2 (Brain and Williams, 1985; Hwang, *et al.*, 1985). For example, co-injection of AGEPC and PGE_2 produces vasoactive effects on postcapillary

crevicular PAF, which ultimately could provide the source for salivary PAF. A recent study by Marze, Schiess, and McManus (McManus, *et al.*, 1990) showing a significant decrease in the levels of salivary PAF in edentulous patients supports this theory. Further studies, however, are needed to determine the source of salivary PAF and its possible relation to inflammatory periodontal disease.

H. PAF AND PERIODONTAL DISEASE

Studies relating PAF and the periodontal disease state are limited. The presence of PAF was shown within gingival crevicular fluid in a preliminary study by Rasheed (Rasheed and McManus, 1990). Gingival crevicular fluid samples from representative sites of shallowest and deepest probing depths were obtained from subjects with slight to severe signs of periodontitis. Gingival crevicular fluid PAF was found to be significantly higher in periodontal sites with probing depths >3mm compared to sites with probing depth $\leq$3mm. These increased levels of PAF associated with increased disease state suggest local tissue production.

Krause (Krause, *et al.*, 1990) studied blood granulocyte aggregation behavior in patients with periodontal disease. Venous blood granulocytes from 17 periodontitis and 16 control subjects were challenged by the chemotactic peptide, formyl-methionyl-leucyl-phenylamine (FMLP) and PAF *in vitro*. Aggregation induced by PAF was found to be significantly enhanced (50%) in the periodontitis patients, while no significant difference was observed between the periodontitis patients and control patients when aggregation was

induced by FMLP. If similar responsiveness occurs *in vivo*, this enhanced granulocyte response to PAF could serve to amplify local inflammatory reactions within periodontal tissues and, therefore, contribute to periodontal disease.

Noguchi (Noguchi, *et al.*, 1989) found PAF to be present within inflamed human gingival tissue; in this study, PAF within periodontally diseased sites was assessed in comparison to levels in healthy tissues. Diseased sites (probing depth >4mm with bleeding on probing) proved to have significantly higher PAF values. This study indicated local production of PAF within the gingival tissues and suggested that this lipid mediator of inflammation could play a role in inflammatory events at this location.

I. STATEMENT OF PROBLEM

Previous investigations have elucidated certain host mechanisms that may be involved in the initiation and progression of periodontal disease. Considerable progress is continuing to be made in determining how immune and inflammatory systems mediate the disease process. The current investigation has focused upon a more recently identified family of phospholipid inflammatory mediators known as PAF. PAF possesses many potent biologic activities including PMN activation (chemotaxis, chemokinesis, degranulation). Additionally, PAF has potent pro-inflammatory effects on other inflammatory cells such as monocytes, macrophage, and eosinophils and platelets. Vascular permeability is also increased through the effects of PAF on vascular

endothelium. This spectrum of biologic effects of PAF has implicated PAF as an important component in inflammatory diseases.

PAF has been isolated and identified in normal human saliva, gingival crevicular fluid, and inflamed gingival tissue. However, the relationship of salivary PAF to periodontal disease has not been clearly established.

The purpose of the present investigation was to determine the relative levels of salivary PAF within two groups of periodontally diseased patients. These patient groups represent patients with relatively different levels of periodontal inflammation. The hypothesis to be evaluated was: a significant difference in the level of salivary PAF would be present between the two groups. This difference would, thus, parallel clinical differences between the groups and provide further evidence to support the hypothesis that PAF is an important inflammatory mediator in the pathogenesis of periodontal disease.

II. MATERIALS AND METHODS

A. HUMAN SUBJECTS

The patient population consisted of 76 individuals of a local private practice periodontist (Dr. Hal Meador). Sixty-three of the patients had been effectively treated, *i.e.*, responded positively (improved) with active periodontitis treatment prior to being placed on a conventional periodontal maintenance program carried out at 3-4 month intervals. These 63 patients had been diagnosed as suffering from chronic adult periodontitis. In contrast, 13 of the patients had been given a diagnosis of refractory periodontitis (RP) based on a poor or negative response to similar previous periodontal therapy.

B. SALIVA COLLECTION AND CLINICAL EXAM

One milliliter of unstimulated, whole mixed saliva was collected between 8:00 AM and 5:00 PM from all patients. Patients expectorated directly into disposable glass tubes (16 X 100 mm) containing chloroform and methanol (final ratio, chloroform:methanol:saliva, 1:2:0.8, v/v/v, respectively). All patients were free of overt clinical signs of oral inflammation other than plaque-associated gingivitis or periodontitis. This protocol had been approved by the Institutional Review Board (#856-8000-118) and informed consent was obtained prior to all saliva collections.

After saliva sample collection, the clinical parameters of number of remaining teeth, number of diseased teeth, number of bleeding sites, bleeding score, probing depths, and plaque index were derived. Diseased teeth were defined as any tooth with at least one site with a

probing depth of 4 mm or greater. Bleeding sites were those sites that bled on probing, while the bleeding score was calculated by dividing the number of bleeding sites by the total number of teeth multiplied by six and then multiplied by 100. The plaque index was determined by dividing the number of tooth plaque free surfaces by the total number of teeth multiplied by four and multiplied by 100. Probing depths were determined to the nearest millimeter manually with a periodontal probe in the maintenance patients. A pressure-sensitive electronic probe (Florida probe) was used to determine probing depths to the nearest millimeter in the refractory group. Probing force for the electronic probe was 25 grams.

C. SALIVA LIPID EXTRACTION

Tracer quantities of ^{3}H-C16:0-AGEPC (^{3}H-1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, New England Nuclear, Boston, MA) were added to every sample for the subsequent determination of PAF recovery. Phospholipid extraction of each sample was completed as described by Bligh and Dyer (Bligh and Dyer, 1959). In brief, each sample was incubated for 1 hour at room temperature with mixing at 10 minute intervals. Samples were then centrifuged (1000 xg, 10 minutes, room temperature) to remove cells, precipitated cellular and bacterial debris and protein. Phase separation was effected in the decanted supernatant by the addition of chloroform and water (final ratio, chloroform:methanol:water/saliva, 1:1:0.9, v/v/v, respectively). PAF activity was present only in the lower, chloroform-rich phase.

D. THIN LAYER CHROMATOGRAPHY (TLC) AND PAF BIOASSAY

Lipids in saliva extracts were fractionated by TLC on prewashed, heat-inactivated, silica gel G plates (Analtech, Inc., Newark, DE) using a solvent system of chloroform:methanol:acetic acid:water, 50:25:8:6, v/v/v/v, respectively. Lipids were recovered from each TLC lane in 1 cm sequential fractions of silica gel *via* extraction into chloroform according to the extraction procedure of Bligh and Dyer (See above). With these TLC procedures, normal human mixed saliva-derived PAF always co-migrated with authentic PAF, synthetic C16:0-AGEPC. Chloroform extracts of TLC fractions were stored at -20°C prior to PAF bioassay. Bioassay for the detection and quantification of PAF consisted of the assessment of sample to induce ^{3}H-serotonin release from washed rabbit platelets (see below).

E. PAF BIOASSAY

Washed rabbit platelets were prepared as previously described (Pinckard, *et al.*, 1979). In brief, 50 milliliters of arterial blood was collected from the central ear artery of rabbits into 7 ml of acid citrate dextrose. Centrifugation (550 x g, 15 min, room temperature) of the blood yielded the upper platelet-rich plasma (PRP) which was removed and incubated for 15 minutes at 37°C with ^{3}H-5-hydroxytryptamine (1 μCi/ml PRP, New England Nuclear). The labeled platelets were then layered onto Ficoll (2ml Ficoll to 10 ml of PRP). Following centrifugation (100 x g, 20 min, room temperature), the platelet layer (at the plasma-Ficoll interface) was transferred, resuspended in Tyrode's buffer (NaCl,

8.0 g/l; KCl. 0.195 g/l; $NaHCO_3$, 1.02 g/l; $MgCl_2$ $6H_2O$, 0.213 g/l; D-glucose, 1.0 g/l; and gelatin, 2.5 g/l) containing 0.1mM EGTA, pH 6.5, and layered again onto Ficoll. After centrifugation (1000 x g, 15 min, room temperature), the platelet layer was removed, resuspended in Tyrode's buffer, pH 6.5, and centrifuged (1000 x g, 15 min, room temperature). The washed rabbit platelet pellet was then resuspended in Tyrode's buffer, pH 6.5, to a concentration of 1.25 x 10^9 cells/ml. Ten minutes prior to bioassay, the platelet suspension was diluted in Tyrode's buffer, pH 7.2, containing 1.3 mM Ca^{++} (1:5, platelet:buffer) and warmed at 37°C.

For bioassay, 200 µl of the pre-warmed platelets (250,000/µl) were added to tubes containing 4 µl of various known and unknown samples at 37°C. Sixty seconds later, the reaction was terminated by the addition of 200 µl of 1.5 M formalin and immediate cooling to 0°C. Following centrifugation (1000 xg, 10 min, 4°C), the amount of ^3H-serotonin present in the cell-free supernatant was determined by liquid scintillation spectrometry. One hundred percent ^3H-serotonin was assessed in parallel tubes containing 0.02% Triton X-100, final concentration. Specific release for PAF samples was corrected for non-specific release after the addition of BSA-saline (the PAF carrier; see below) to similarly processed platelet samples.

Prior to bioassay, all chloroform extracts were evaporated to dryness and redissolved in pyrogen-free saline containing 0.25% bovine serum albumin (BSA-saline; Miles Laboratories, Elkhart, IN.). When necessary, dilutions were prepared in this same solvent. Salivary PAF activity was estimated in comparison to the biologic activity of

authentic C16:0-AGEPC (Bachem Fine Chemicals, Torrence, CA) assayed in parallel. In every bioassay, a standard curve of ^{3}H-serotonin release was generated using known amounts of C16:0-AGEPC (5 to 50 fmoles); the sensitivity of this assay was 3-5 fmoles. PAF activity in saliva-derived, unknown samples was then determined directly from this standard curve and expressed relative to the number of C16:0-AGEPC equivalents (fmoles). For each TLC purified saliva sample, PAF activity was corrected for losses through the determination of the amount of ^{3}H-C16:0-AGEPC tracer in the TLC fractions subjected to bioassay; ^{3}H-C16:0-AGEPC recovery was routinely between 50 to 80%.

F. HISTOLOGY

The pellets obtained during the initial lipid extraction of saliva (protein, cells, and cell debris) were stored in 10% neutral buffered formalin (NBF) and subsequently prepared for light microscopic examination. For histologic processing, the pellets were resuspended in 3.5% gelatin (Sigma Chemical Co., St. Louis, MO) at 60°C in pre-warmed polypropylene microcentrifuge tubes (Beckman Instruments Inc., Palo Alto, CA) and immediately centrifuged for 2.5 min (10,000 x g, room temperature) followed by incubation in an ice bath for 5-15 min. The plastic tubes were then bisected and the solid gelatin/pellet removed to 10% NBF prior to routine paraffin embedding, sectioning (6 μm), and staining with hematoxylin and eosin (H&E). Polymorphonuclear leukocytes or neutrophils (PMN) within each section were enumerated by light microscopy using a square reticule in a 10X eyepiece with a 40X objective. At least 3 representative fields were examined for each

specimen and utilized to calculate the mean number of PMN/mm^2. This microscopic neutrophil quantitation was performed in a blinded manner in that coded tissue specimen were used for enumeration and both sample identity and donor status were not designated.

G. STATISTICAL EVALUATION OF DATA

Data are presented as the mean $\pm$ SEM. Probability values for statistical differences between means of the routine maintenance group to that of the refractory group were determined by the Student's *t* test for unpaired data. P values of less than 0.05 were considered significant.

To evaluate possible effects of other variables on salivary PAF levels, the following data were evaluated through one and two-way analysis of variance: 1) age, 2) sex, 3) systemic medications at time of sampling, 4) time of day patient sampled, and 5) recent eating, drinking, and oral hygiene practices.

Using one way analysis of variance, the possible effects of time of sampling and patient medications on PAF levels were determined. The sampling time of 8:00 AM to 5:00 PM was broken into 9 one hour time periods and PAF values of samples taken within these time periods were evaluated. Medications being taken by the patients at the time of sampling were classified as to the type of medication. The medication categories evaluated included antibiotics, aspirin, and non-steroidal anti-inflammatory drugs (NSAID).

Unpaired *t*-tests were used to evaluate the effects of recent eating, drinking, or oral hygiene habits. Patient PAF data were

evaluated for effects of eating, drinking, or any oral hygiene practice within one hour of sampling. Additionally, analysis of covariance was used to determine any possible significant effects of the variables of age and sex on PAF values.

III. RESULTS

A. PATIENT GROUPS

1. CHRONIC ADULT PERIODONTITIS SUBJECTS REFRACTORY TO TREATMENT (kEFRACTORY GROUP)

Refractory periodontitis status was identified in 13 subjects who exhibited a less than optimal response to conventional periodontal therapy. The clinical assessment of less than optimal response was based upon a history of recurrent abscesses, increasing probing depths, continued loss of attachment, and loss of teeth despite all treatment efforts. These 13 patients had been treated by a single periodontist in a private practice environment over a five to twenty-five year period. The patients in this group were at least 30 years of age and had no history of systemic condition known to have a contributory effect during the course of therapy. Documentation of disease progression through examination of full mouth periapical radiographs and clinical information was present for at least 5 years. Radiographic loss of periodontal attachment was determined through evaluation of radiographs taken at varying intervals (data not presented).

The conventional therapy received by these patients included education concerning the character of disease, personalized plaque control instructions, full mouth scaling and root planing, and periodontal surgery. All patients were followed by regularly scheduled periodontal maintenance appointments at 3 to 4 month intervals. Surgical therapy, when performed, consisted of pocket elimination surgery, flap curettage, modified Widman procedures, and regenerative surgery.

2. CHRONIC ADULT PERIODONTITIS PATIENTS RESPONSIVE TO TREATMENT (MAINTENANCE GROUP)

Sixty-three patients with chronic adult periodontitis and responsive to maintenance therapy were randomly selected from the patient population of the same private periodontal practice as the refractory periodontitis group. These patients received similar conventional therapy as the 13 refractory subjects (see above). These patients had been members of the patient population of this periodontist for 2 to 25 years. Recent treatment consisted of maintenance recall at 3-4 month intervals. All patients in this group were at least 30 years of age and had no history of systemic condition known to have an effect on response to therapy.

B. PREVIOUS PERIODONTAL THERAPY FOR PATIENT GROUP

1. REFRACTORY GROUP

A review of the previous periodontal therapy for this group revealed that all refractory periodontitis patients had experienced periodontal surgery; the most recent surgery date for any patient in this group was 6 months prior to saliva sampling (Table III). Eleven of the 13 subjects had received adjunctive antibiotic therapy in combination with their clinical therapy. The antibiotics used included tetracycline, doxycycline, and penicillin. None of the patients had taken antibiotics within 6 months of saliva sampling with the exception of a patient requiring subacute bacterial endocarditis (SBE) penicillin prophylaxis prior to each appointment. Penicillin was given one hour prior to appointment (2 gm, followed by 1 gm six hours later). At the time of

Table III
Comparison of the Treatment and Medication History
of the Refractory and Maintenance Periodontitis Patient Groups

	REFRACTORY GROUP	MAINTENANCE GROUP
MOST RECENT APPOINTMENT (PRIOR TO SALIVA SAMPLING)	7-12 WEEKS	2-52 WEEKS
PAST PERIODONTAL SURGERY	13 (100%)*	45 (71.4%)
SYSTEMIC ANTIBIOTICS (DURING THE PAST 6 MONTHS)	1 (7.7%)*	4 (6.3%)*
CURRENT SYSTEMIC MEDICATIONS (ANTI-SIALOGUES)	0 (0.0%)*	0 (0.0%)*

*(Number of Patients / Total patients per group) multiplied by 100.

saliva sampling, all 13 subjects were on a regular maintenance schedule with the range of time from these patients' last maintenance visit to the time of saliva sampling being 7 to 12 weeks. None of the patients were on systemic medication that affected salivary flow (Table III).

2. MAINTENANCE GROUP

Forty-five of the 63 patients in this group had experienced periodontal surgery; the most recent surgery date for any patient in this group was 3 months prior to sampling (Table III). Fourteen subjects had received adjunctive antibiotic therapy in combination with their clinical therapy. The antibiotics used included tetracycline, doxycycline, erythromycin, and penicillin. None of the patients had taken antibiotics within 6 months of sampling with the exception of 4 patients requiring SBE penicillin or erythromycin prophylaxis prior to each appointment. Prophylaxis was provided to these patients prior to saliva sampling and data obtained from these 4 patients was included in the study. Penicillin was given as described above, while erythromycin was given similarly but at half the dose. The range of time for the last maintenance visit prior to saliva sampling was from 2 to 52 weeks. None of the patients were on systemic medications that affected salivary flow at the time of saliva collection (Table III).

C. DEMOGRAPHICS

The two groups of patients were not significantly different with respect to age (Table IV). The average age of the refractory group was 62.4 $\pm$ 2.3 years (mean $\pm$ SEM) with a range of 49 to 75 years of age.

The average age of the maintenance patients was 57.4 ± 1.6 years with a range of 30 to 82. The refractory group was composed of 8 males and 5 females while the maintenance group was composed 43 females and 20 males (Table IV).

D. CLINICAL DATA

At the time of saliva sampling, the clinical parameters of number of remaining teeth, number of diseased teeth, number of bleeding sites, bleeding score, probing depths, and plaque index were measured or obtained. There were significant differences in these clinical parameters between the two groups (Table V). The refractory group had a significantly greater number of diseased teeth (7.9 ± 1.3) as compared to the maintenance group (4.5 ± 0.5). In addition, the refractory group had significantly higher values for the bleeding score, the number of bleeding sites, and the number of probing depths greater than 5 millimeters (*i.e.*, 6 mm or greater). For the number of bleeding sites, the refractory group had 15.4 ± 2.4 sites compared to 5.2 ± 0.97 sites in the maintenance group. The mean value for the bleeding score in the refractory group was 24.3 ± 3.4 compared to 5.9 ± 1.4 in the maintenance group. The refractory group had 7.6 ± 2.3 sites with probing depths greater than 5 mm as compared to 2.3 ± 0.7 sites for the maintenance group.

The maintenance group had significantly higher remaining teeth compared to the refractory group, i.e., 23.7 ± 0.8 and 18.8 ± 2.1, respectively. Other clinical parameters (*i.e.*, number of probing depths >3 mm and plaque index) were not significantly different between these groups of patients (Table V).

Table IV
Comparison of Demographics of the Refractory and Maintenance Periodontitis Patient Groups

	REFRACTORY GROUP	MAINTENANCE GROUP
AGE (years)	62.4 ± 2.3* $p<0.18$	57.2 ± 1.6
FEMALES	5/13**(38.4%)	43/63 (61.6%)
MALES	8/13 (61.5%)	20/63 (38.5%)

* Value represents the mean ± SEM. There was no significant difference in age between groups as determined by unpaired t-test. .

**Number of Patients / Total Number of Patients per Group. There was a borderline significant difference in gender distribution ($p < 0.06$) by Fisher's exact test.

Table V.
Comparison of the Clinical Presentation of the Refractory and Maintenance Periodontitis Patient Groups

	REFRACTORY GROUP	MAINTENANCE GROUP
TOTAL TEETH	18.8 ± 2.1*	23.7 ± 0.8 ** p<0.01
NUMBER OF DISEASED TEETH	7.9 ± 1.3 *** p<0.007	4.5 ± 0.5
NUMBER OF BLEEDING SITES	15.4 ± 2.4 *** p<0.001	5.2 ± 1.00
BLEEDING SCORE (%)†	24.3 ± 3.4 *** p<0.002	5.9 ± 1.4
SITES WITH PROBING DEPTH >3MM	22.4 ± 5.5	16.3 ± 2.7
SITES WITH PROBING DEPTH >5MM	7.6 ± 2.3 *** p<0.01	2.3 ± 0.7
PLAQUE INDEX (5)††	67.4 ± 4.80	76.7 ± 1.5

* All data are expressed as mean ± sem.
** Significantly higher than refractory group (unpaired t-test)
***Significantly higher than maintenance group (unpaired t-test)
† Bleeding Score = (number of bleeding sites/total number of teeth x 6) x 100
†† Plaque Index = (number of plaque-free surfaces/total number of teeth x 4) x 100

E. SALIVARY PAF AND PMN LEVELS

Four of the 63 saliva samples from the maintenance group contained no detectable PAF activity, while PAF activity was identified in all but one of the saliva samples derived from the refractory group. When recovered, salivary PAF was always found in identical TLC fractions as the synthetic internal tracer, ^{3}H-C16:O-AGEPC. Salivary PAF activity in the refractory patients was significantly greater than in the maintenance group, 7897.7 $\pm$ 1941.1 and 3414 $\pm$ 554.5 C16:0-AGEPC fmole equivalents/ml of saliva, respectively (Table VI).

When PMN within the mixed saliva samples were enumerated histologically, no significant difference was noted between the groups, *i.e.*, 122 $\pm$ 32 PMN/mm^2 for the refractory group and 82 $\pm$ 11 PMN/mm^2 for the maintenance group (Table VI).

Correlations of salivary PAF levels to the measured clinical parameters of periodontal disease were determined (Table VII). Within the refractory group, salivary PAF levels was significantly correlated to the number of diseased teeth (r=0.722) (Figure III). Additional correlation was also noted in this group between PAF levels and both the number of probing depths greater than 3mm (Figure IV) and the number of probing depths greater than 5mm (r=0.697 and r=0.768, respectively). Weaker, but significant correlations were found between PAF levels and the number of total teeth and histologic PMN counts (Table VII).

Table VI.
Comparison of Salivary PAF and PMN Levels in the Refractory and Maintenance Periodontitis Patient Group

	REFRACTORY GROUP	MAINTENANCE GROUP
PAF (C16:0-AGEPC fmole equivalents/ml of saliva)	7897.7 ± 1941.1+* $p<0.003$ (n=13)	3414.0 ± 554.5 (n=63)
Number of PMN/mm^2	122 ± 32 (n=13)	82 ± 11 (n=63)

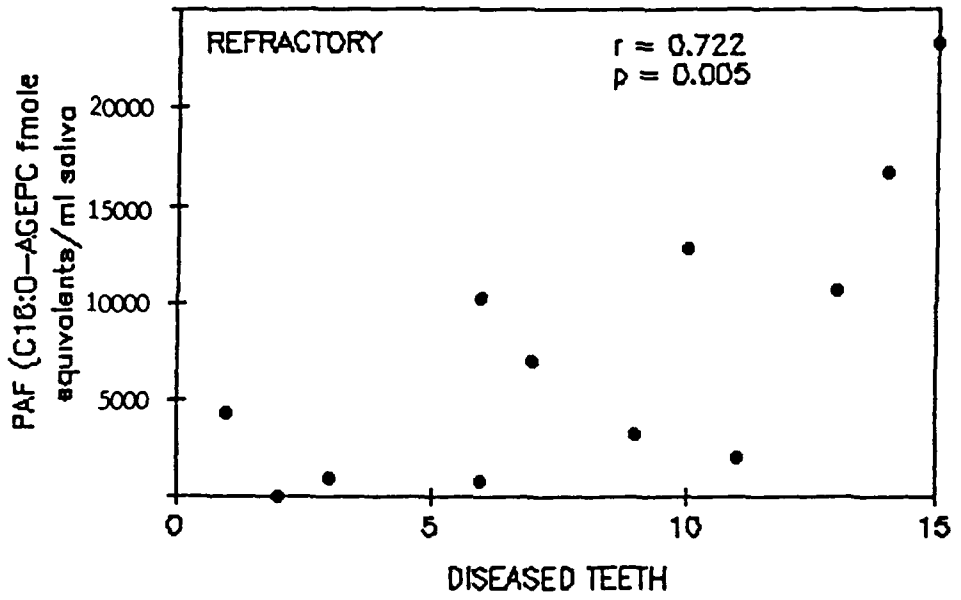
+ All data are expressed as mean ± sem.
* Significantly higher than maintenance group (unpaired t-test).

Table VII
Correlation of Salivary PAF levels with other Measured Parameters in the Refractory and Maintenance Periodontitis Patient Groups

	REFRACTORY GROUP	MAINTENANCE GROUP
NUMBER OF TOTAL TEETH	**r= 0.573*** **p<0.04**	r= 0.117 p<0.36
NUMBER OF DISEASED TEETH	**r= 0.722** **p<0.005**	r= 0.197 p<0.14
NUMBER OF SITES WITH PROBING DEPTH >3 MM	**r= 0.697** **p<0.01**	r= 0.154 p<0.23
NUMBER OF SITES WITH PROBING DEPTH >5 MM	**r= 0.768** **p<0.003**	**r= 0.253** **p<0.04**
BLEEDING SCORE	r= 0.183 p<0.50	r= 0.124 p<0.49
NUMBER OF BLEEDING SITES	r= 0.428 p<0.16	**r= 0.301** **p<0.09**
PLAQUE INDEX	r= 0.165 p<0.13	r=0.178 p<0.14
SALIVARY PMN COUNT	**r= 0.571** **p<0.04**	**r= 0.351** **p<0.006**

* r values represent Pearson's Product Moment direct correlation coefficient.

Figure 3. Comparison of salivary PAF and number of diseased teeth in the refractory and maintenance periodontitis patient


REFRACTORY
r = 0.722
p = 0.005
PAF (C16:0–AGEPC fmole equivalents/ml saliva
20000
15000
10000
5000
0
5
10
15
DISEASED TEETH

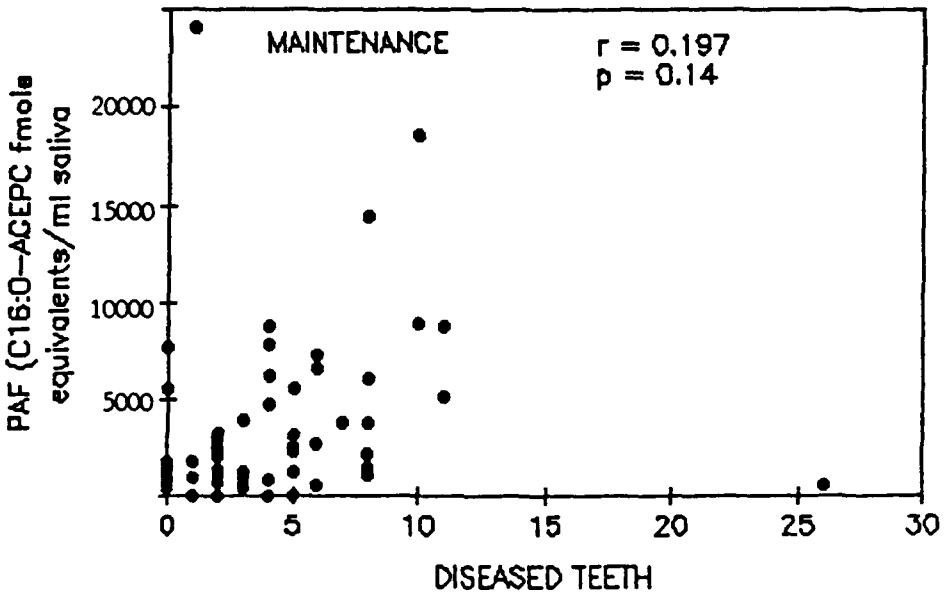
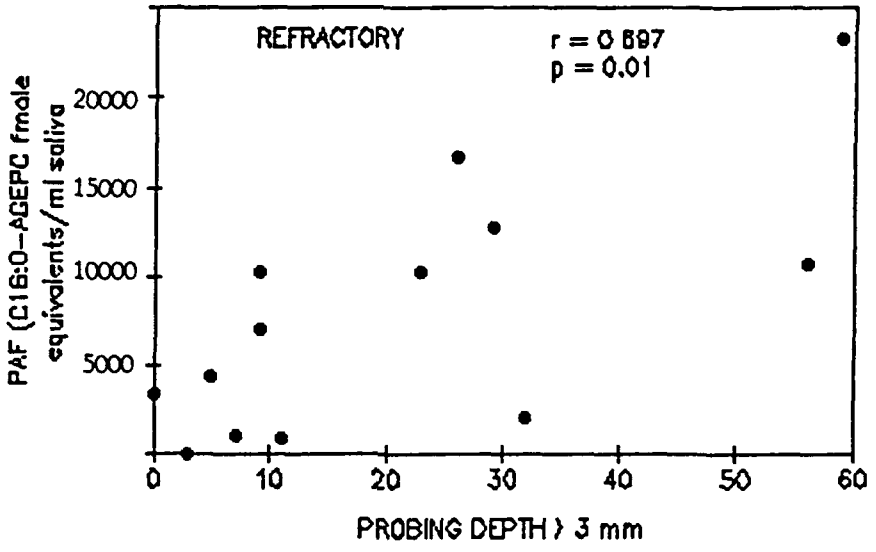

MAINTENANCE
r = 0.197
p = 0.14
PAF (C16:0–AGEPC fmole equivalents/ml saliva
20000
15000
10000
5000
0
5
10
15
20
25
30
DISEASED TEETH

Figure 4. Comparison of salivary PAF and the presentation of sites with probing depths greater than 3mm in the refractory and maintenance periodontitis patient groups.


REFRACTORY
r = 0.697
p = 0.01
PAF (C16:0–AGEPC fmole equivalents/ml saliva
20000
15000
10000
5000
0
10
20
30
40
50
60
PROBING DEPTH > 3 mm

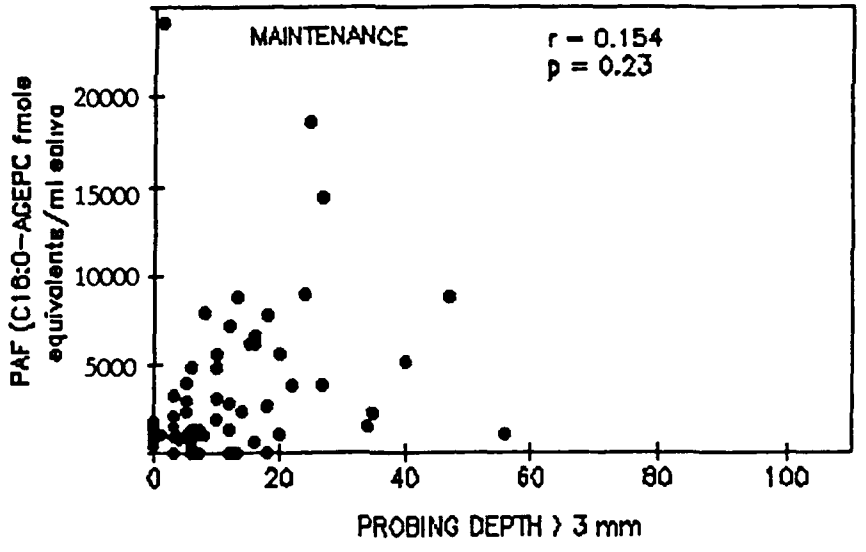

MAINTENANCE
r = 0.154
p = 0.23
PAF (C16:0–AGEPC fmole equivalents/ml saliva
20000
15000
10000
5000
0
20
40
60
80
100
PROBING DEPTH > 3 mm

Table VIII.
Effect of Hour of Sampling on Dependent Variable PAF.

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES	MEAN SQUARE ERROR	F VALUE	p VALUE
Hour	8	306412139.20	34045793.24	1.33	0.23*
-Error	67	1714584564.76	25590814.40		
-Corrected Total	76	2020996703.95			

* No significant effect on dependent variable Salivary PAF.

In contrast, PAF levels in the maintenance group correlated with only two clinical parameters. These correlations proved to be weak, but significant, for the number of probing depths greater than 5mm and histologic PMN counts (Table VII).

F. ADDITIONAL DATA ANALYSES

To evaluate possible effects of other variables on salivary PAF levels, the following data were evaluated through one and two-way analysis of variance: 1) age, 2) sex, 3) systemic medications at time of sampling, 4) time of day patient sampled, and 5) recent eating, drinking, and oral hygiene practices.

The effects of time of sampling and patient medications on salivary PAF levels were examined. The sampling time of 8:00 am to 5:00 pm was broken into 9, one hour time periods and PAF values of saliva samples taken within these time periods were evaluated. Sampling hours had no significant effect on mean PAF values (Table VIII). Medications being taken by the patients at the time of sampling were classified as to type of medication. The medication categories evaluated included antibiotics, aspirin, and other non-steroidal anti-inflammatory drugs. As previously mentioned, 4 of the maintenance patients and 1 of the refractory patients had taken antibiotics for SBE prophylaxis. Twenty-one maintenance and 3 refractory subjects had taken aspirin recently (*i.e.*, multiple doses within 6 weeks), while 3 maintenance and 1 refractory subject had taken other non-steroidal anti-inflammatory drugs. No medication was found to have an effect on salivary PAF levels (Table IX).

Table IX.
Effect of Systemic Medications on Salivary PAF
by One Way Aanalysis of Variance

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUMS OF SQUARES	MEAN SQUARE ERROR	F VALUE	p VALUE
Systemic Medication	3	38592725.42	12864241.807	0.45	0.71*
-Error	68	1937489529.333	28492493.078		
-Corrected Total	71	1976082254.753			

*No significant effect on dependent variable salivary PAF.

The effects of recent eating, drinking, or oral hygiene habits upon salivary PAF level were evaluated with the use of unpaired T-tests. Salivary PAF values were evaluated for effects of these activities which had occurred within one hour of saliva sampling. Salivary PAF levels were unaffected by these variables.

Any possible significant effects of age and sex on salivary PAF values were assessed with the use of analysis of covariance. These analyses indicated no significant effect of these variables since age $F(1,45) = 0.16$, $p = 0.68$ and sex $F(1,45) = 1.69$, $p = 0.19$. Within the refractory group, females had PAF values of 10720.9 ± 3723.4 C16:0-AGEPC fmole equivalents/ml of saliva while males had values of 7355.6 ± 1760.7 C16:0-AGEPC fmoles equivalents/ml of saliva. Within the maintenance group, females had PAF values of 3269.4 ± 618.4, while males had values of 3704.3 ± 1133.9

All patients were questioned (via written questionnaire) at the time of saliva sampling concerning their use of tobacco products. A positive or negative response with respect to smoking status was requested. Many subjects gave no answer to this question on their questionnaire. The possible effects of smoking on salivary PAF, therefore, could not be statistically determined. Of the questionnaires which were adequately answered, a positive smoking status was present in 11 subjects. Two of these 11 were present in the refractory group, while 9 were present in the maintenance group. Mean salivary PAF values were computed for these 11 subjects. When compared to group salivary PAF mean values, a trend toward lower salivary PAF levels were seen in both the maintenance (3414.0 versus 2992.9) and

refractory groups (7897.7 versus 6051.0). This trend was consistent with the results of a recent study (Garito, 1991) which showed a correlation between salivary PAF levels and smoking status in periodontally diseased subjects. Subjects who were smokers were shown to have lower salivary PAF levels than non-smokers.

IV. DISCUSSION AND SUMMARY

Periodontitis is an inflammatory disease of periodontal tissues which is characterized by loss of support of the affected teeth. Specifically, periodontal ligament fibers and the bone into which they are inserted are destroyed (AAP Glossary of Terms, 1986). It has been well established that periodontal diseases are the result of accumulation of specific bacteria in the supragingival and subgingival areas of the involved teeth (Dzink, *et al.*, 1985; Loe, *et al.*, 1965; Socransky, 1977). Although bacteria are the essential primary etiologic agents, the mere presence of bacteria alone on the tooth surface and in the subgingival area has not proven to be sufficient to explain the processes of periodontal disease (Page, 1989). The host must interact with etiologic agents if disease is to be halted. The host defense systems in periodontitis, as with other bacterial infections, attempts to neutralize, dilute, remove, or kill the bacterial agents. In periodontal disease, the host may not be able to adequately prevent disease because chronic bacterial accumulation may cause an excessive and persistent stimulus. This persistent bacterial stimulus may directly damage tissue or elicit tissue destructive host defense mechanisms.

Extensive research has elucidated certain host mechanisms that may be involved in the initiation and progression of periodontal disease. Considerable progress is being made to determine how immune and inflammatory systems mediate this disease process. Immune and inflammatory pathways hypothesized as important components of the pathologic events of periodontal disease are arachidonic acid metabolites (*i.e.*, prostaglandins, thromboxane, prostacyclin,

leukotrienes), interleukins, polyclonal B cell activation, IgG-mediated complement activation, and T cell immunoregulation (Altman, *et al.*, 1982; Asaro, *et al.*, 1983; Carpenter, *et al.*, 1983; Carpenter, *et al.*, 1984; Ebersole, *et al.*, 1985; Goodson, *et al.*, 1974; Offenbacher, *et al.*, 1984; Offenbacher, *et al.*, 1986). Inflammatory mediators, such as prostaglandins, have been found in significantly elevated levels within periodontally diseased tissues and within gingival crevicular fluid. Elevated levels of such mediators implies local cell and tissue production and suggests a role for these mediators in the periodontal disease process (ElAttar and Hsein, 1986; Offenbacher, *et al.*, 1986).

The present investigation attempted to focus upon a more recently identified family of lipid inflammatory mediators known as PAF. A myriad of recent studies suggesting that this family of molecules are indispensable to the regulation of inflammation form the background for this study (Archer, *et al.*, 1984; Fjellner and Hagermark, 1985; Humphrey, *et al.*, 1984; McManus, 1986; Pinckard, *et al.*, 1988).

Two groups of periodontally diseased patients were chosen to determine the relative salivary PAF levels and evaluate possible correlations between salivary PAF and periodontal disease status. These two groups of subjects, *i.e.*, chronic adult periodontitis patients who either responded (maintenance group) or failed to respond to conventional therapy (refractory group), expressed relatively different levels of periodontal inflammation. The refractory periodontitis group would presumably represent patients with continued periodontal inflammation based on a history of non-responsiveness to conventional periodontal therapy. This non-responsiveness was based on clinical

findings and estimates of radiographic bone loss over at least a five year period. A group of randomly selected routine periodontal maintenance patients were chosen as a group to contrast the refractory subjects with respect to overall periodontal inflammation and response to conventional periodontal therapy.

A review of the clinical presentation of these two patient groups at time of saliva sampling indicated that there were two distinct groups relative to the level of periodontal disease. The refractory periodontitis group had a significantly higher number of diseased teeth, number of bleeding sites, bleeding scores, and number of probing depths greater than 5 mm. These significant differences provide evidence to suggest an increased level of present and past periodontal inflammation or disease. The higher number of diseased teeth and number of probing depths greater than 5 mm would suggest a history of more severe periodontal disease in the refractory group as compared to the maintenance group. Since bleeding on probing has been shown to correlate well with the level and magnitude of inflammatory infiltrates in periodontal tissues (Davenport, *et al.*, 1982; Greenstein, *et al.*, 1981), the higher values for the number of bleeding sites and bleeding score for the refractory group would suggest a higher level of tissue inflammation within this group as compared to the routine periodontal maintenance group.

The difference in clinical presentation between the two groups of patients may have been affected through the different methods of probing techniques used in each group. Although a force of only 25 g was used with the Florida electronic probe in the refractory group, this

may have exceeded forces used during manual probing. Increased values for number of sites with increased probing depth and bleeding in the refractory group may have resulted. This could then have resulted in a greater interexaminer difference between the groups. It should be noted, however, that the use of the standardized electronic probe probably reduced intraexaminer error within the refractory group. Ideally, electronic standardized probing would have been desired in both groups to decrease both intra- and interexaminer variability.

Consistent with the suggested clinical difference between groups, these two groups of patients also proved to be significantly different in their salivary PAF levels. A comparison of PAF data between the groups indicated that salivary PAF was significantly greater in the refractory periodontitis group. This finding would seem to parallel the clinical difference found in these patients. Thus, salivary PAF levels were significantly higher for a group of patients with a higher and persistent level of inflammation as compared to a relatively stable group of chronic adult periodontitis patients.

The PMN within the mixed saliva of each group were enumerated histologically to provide a possible explanation for the relative difference in salivary PAF levels between the two groups. PMN were enumerated on a per unit basis to provide comparable and consistent data among subject groups. PMN were specifically enumerated since the presence of substantial numbers of PMN in affected gingival tissue and crevicular exudate that is one of the most conspicuous features of periodontal disease (Attstrom, 1975). In addition, PMN are known to actively synthesize PAF following *in vitro* activation (Ludwig, *et al.*,

1985). There proved to be no significant difference between the two groups with respect to the number of PMN in the saliva tissue pellets. Thus, although the source of salivary PAF in both groups of patients remains to be determined, it appears that salivary PMN did not reflect salivary PAF levels in these patients. Although histologic salivary PMN did not reflect the salivary PAF levels in these patients, there is a potential that a difference in quality of PMN between groups resulted in a difference in salivary PAF levels seen. Krause (Krause, *et al.*, 1990) found significant differences in *in vitro* granulocyte aggregation response between periodontally diseased and control patients. If the PMN in the refractory group were somehow more sensitive to the stimulatory effects of PAF, there may have been a greater production of PAF *from the refractory* group PMN compared to a similar number of PMN within the maintenance group.

The contribution of tissue sequestered PMN to salivary PAF remains to be established. Indeed, Noguchi *et al.* (Noguchi, *et al.*, 1989) determined that PAF levels were elevated in inflamed gingival tissues. Thus, it is conceivable that this acute inflammatory cell, *i.e.*, the PMN, may yet prove to be the source of salivary PAF in periodontal disease. It should be pointed out that if the latter is the case, the local tissue levels of PAF could be far greater than that in saliva. In this case, a role for PAF in local inflammatory response and tissue injury would be expected.

The source of PAF in saliva remains to be determined. As PMN recovery in saliva samples correlated only weakly to salivary PAF content in the refractory and maintenance groups, this cell cannot be

solely implicated as the contributor of PAF in saliva. Additional studies must be performed to estimate levels of other inflammatory cells shown to be capable of producing PAF in order to determine their possible contribution to salivary PAF. And, as outlined above, tissue cells sequestered in periodontally inflamed tissues must also be evaluated.

The strong correlation of salivary PAF to the clinical parameters of number of diseased teeth, probing depths greater than 3 mm, and probing depths greater than 5 mm in the refractory group may suggest a gingival crevicular fluid source for salivary PAF. The tooth sites with increased probing depths likely represent areas with a higher level of gingival crevicular fluid flow relative to healthier, nondiseased sites (Davenport, *et al.*, 1982; Loe, 1971). The potential for a higher level of periodontal inflammation within deeper probing sites also exists and may offer some explanation for the observed correlations (Greenstein, *et al.*, 1981).

The weaker correlation between salivary PAF and another specific clinical parameter (bleeding on probing) requires some attention. Bleeding on probing has been shown to correlate to the level of histologic tissue inflammatory infiltrate. Offenbacher (Offenbacher, *et al.*, 1984) found an association of crevicular fluid prostaglandin (PGE_2) levels to periodontal disease; however, clinical signs of inflammation could not be used to independently predict crevicular fluid levels of PGE_2 when all sites were evaluated. However, when those sites were divided into shallow (0-3 mm) and deep (4-11 mm) attachment loss, a relationship between crevicular fluid prostaglandin levels and clinical signs of inflammation was noted. Offenbacher found that tissue PGE_2

levels increased at apical aspects of the pocket; suggesting that tissue destruction is greater at these sites. Therefore, it was not surprising that clinical signs of inflammation pericoronally did not correlate to crevicular fluid prostaglandin levels. The same phenomenon may have occurred in the relationship of PAF and the clinical signs of inflammation used in this study.

The relationship of periodontal disease to prostaglandins found in the Offenbacher study (Offenbacher, *et al.*, 1984) may indicate a potential for a similar relationship between periodontal disease and PAF. Both PAF and prostaglandins are derived from a common precursor. This suggests a potential for a direct molar to molar relationship between PAF and prostaglandins within cells or tissues. The increased levels of prostaglandins seen in periodontal disease, thus, are possibly associated with increased levels of PAF and may suggest potential for a relationship between PAF and periodontal disease.

A limited number of studies have reported the direct isolation of PAF from human tissues or fluids. PAF has been obtained from both normal human, mixed saliva and inflamed human gingival tissues and found to be physiochemically similar to that of PAF isolated from stimulated inflammatory cells (Christman and Blair, 1989; Cox, *et al.*, 1981; Noguchi, *et al.*, 1989). The results of this study confirm the presence of PAF within saliva, indicating possible correlations of salivary PAF to periodontal disease parameters. The correlation of salivary PAF to numbers of deeper probing sites may suggest a crevicular fluid source for salivary PAF.

The isolation of PAF from biologic fluids and tissues has been complicated by the presence of catabolic enzymes and natural inhibitors specific for PAF. The serum enzyme acetylhydrolase rapidly degrades this phospholipid (Blank, *et al.*, 1983; Farr, *et al.*, 1980; Farr, *et al.*, 1983). Snyder *et al.* (Snyder, *et al.*, 1987) isolated similar enzymes from various cells and tissues. Natural inhibitors of PAF have been described by Miwa *et al.* (Miwa, *et al.*, 1987) and Nakayama *et al.* (Nakayama, *et al.*, 1987). Of importance in the isolation of biologic PAF is the quantity present in areas of inflammation; *in vitro* and *in vivo* studies have suggested that only small quantities of PAF may be present or necessary due its biologic potency. The isolation of PAF from biologic tissues and fluids may, therefore, be hampered by catabolic enzymes and natural inhibitors acting on potentially very small quantities of PAF within these tissues and fluids.

In this study, PAF activity was documented in 94% of all saliva samples collected. Samples were collected directly into organic solvents in order to improve salivary PAF recovery. The salivary lipid extracts were subsequently fractionated by routine TLC procedures. These procedures apparently prevented catabolic enzymes and natural inhibitors of PAF from altering salivary PAF. It is possible, however, that enzymes such as acetylhydrolase are absent in saliva. TLC purification of salivary PAF was completed prior to bioassay, since natural inhibitors of PAF are present in lipid extracts of saliva. The absence of salivary PAF within 6% of the patients may represent laboratory error either in the processing or assaying of these samples.

In summary, the results of the current study showed a significant difference in the level of salivary PAF in two groups of patients with relatively different levels of periodontal disease or inflammation. These results are consistent with the hypothesis that PAF may play a role as an inflammatory mediator in periodontal disease. The observations in this study along with the results from previous studies (McManus, *et al.*, 1990; Rasheed and McManus, 1990) and suggest a gingival crevicular fluid source for salivary PAF. Additional investigations are required to determine the level of PAF within gingival crevicular fluid and within the periodontal tissues in active sites of disease. If PAF is shown to be an inflammatory mediator of consequence in periodontal disease activity, PAF could possibly be used to detect active episodes of disease at a specific site.

Additional longitudinal studies are also required to determine if PAF may be used to detect disease susceptible individuals. Certain individuals may inherently produce greater levels of PAF. These individuals may then exhibit an overamplified inflammatory response when compared to individuals who may inherently produce lower amounts of PAF. This overamplified response may then lead to greater disease and tissue injury.

If significant relationships between PAF and periodontal disease are found in these future studies, investigations may then proceed to identify agents capable of blocking or modulating this phospholipid mediator of inflammation. PAF receptor antagonists (Terashita, *et al.*, 1987; Valone, 1985) may be agents with conceivable utility for

treatment and prevention of acute inflammatory tissue injury within periodontal tissues.

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Vita

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