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A Histologic Bioassay of the Effect of Endotoxin of Escherichia coli 0111:B4 Strain Injected Into Guinea Pig Oral Mucosa

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A HISTOLOGIC BIOASSAY OF THE EFFECT OF ENDOTOXIN OF ESCHERICHIA COLI 0111:B4 STRAIN INJECTED INTO GUINEA PIG ORAL MUCOSA

BY

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DEDICATION

To my loving parents, Juan Luis and Helga,
for their caring, understanding and who made possible
my postgraduate education.

To my fiance, Rocio, for her love and support.

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LIFE

The author, Juan Jose de Obarrio, is the son of Juan Luis and Helga de Obarrio. He was born September 27, 1953, in Panama City, Republic of Panama.

His elementary and secondary education was obtained at Colegio Javier, Panama City, Republic of Panama, where he graduated December 1969.

In January 1970 he entered The University of Panama, Panama City, Republic of Panama. He received the degree of Doctor of Dental Surgery in October 1977.

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CHAPTER I

INTRODUCTION

It has been well established that bacteria are essential in the etiology of inflammatory periodontal diseases. (Loe et al., 1965, Socransky 1970, Kelstrup and Theilade 1974, Socransky 1977, Slots 1979, van Palenstein Helderman 1981).

It has been shown that in the healthy gingival sulcus the predominant microbial flora is composed mainly of Gram-positive microorganisms and a shift toward a predominantly Gram-negative microbial flora is seen in the most destructive forms of periodontal disease (Socransky 1977, Slots 1979, van Palenstein Helderman 1981).

An important difference between Gram-negative and Gram-positive microorganisms is the presence of endotoxin in Gram-negative microorganisms.

The term endotoxin is used to denote the lipopolysaccharides (LPS) of high molecular weight which are considered to be integral structural units found on the outer membrane of the cell wall of Gram-negative microorganisms (Westphal 1957).

LPS have been shown to possess several biologic properties, which include activation of the complement system by the classical and alternate pathway (Morrison & Kline 1977, Okuda & Takazoe 1980), stimulation of macrophages to release collagenase (Doe & Henson 1978, Weinberg et al.,

1978), bone resorption (Hausmann et al., 1981), cytotoxicity for fibroblasts (Hatfield & Baumhammers 1971, Aleo et al., 1974, 1975), B-cell mitogenic properties (Andersson et al., 1972, Kunori et al., 1978) and induction of chemotaxis of polymorphonuclear leukocytes (PMNLs) in the presence of serum derived from the complement system chemotactic factors (Snyderman 1972).

The biologic properties mentioned above suggest an important role of LPS in the pathogenesis of inflammatory periodontal diseases.

It has been shown that LPS exhibits chemotactic mediator properties for PMNLs (Rizzo & Mergenhagen 1964, Jensen 1966, Snyderman 1972, Wilton & Almeida 1980). Conversely, physiologic sterile saline is not known to exhibit chemotactic properties, although tissue injury may lead to the release of chemoattractants for PMNLs (Cochrane et al., 1972, Ward 1974, Higgs & Eakins 1980).

The purpose of the present study is to determine the least concentration of E. coli 0111:B4 strain endotoxin (LPSE) which is able to produce an inflammatory response when compared to a physiologic sterile saline solution (0.85%) as a control, by a single injection into the guinea pig oral mucosa in a 24 hour period of time.

In the present study, a 0.05 ml volume of six different concentrations of LPSE (50, 25, 10, 1, 0.1 and 0.01µg/ml) and a sterile saline control were injected into the guinea pig oral mucosa. The animals were sacrificed 24 hours after the injections and a histologic examination was done to compare the presence of the inflammatory infiltrate.

CHAPTER II

LITERATURE REVIEW

A. THE ROLE OF BACTERIA IN PERIODONTAL DISEASES

There is strong direct evidence that dentogingival bacterial plaque is essential in the etiology of inflammatory periodontal diseases (Ash et al., 1964, Loe et al., 1965, Socransky 1970, Kelstrup & Theilade 1974). Conversely, in the absence of the bacterial plaque, there is essentially no detectable pathologic change (Loe et al., 1965, Theilade et al., 1966, Loe and Schiott 1970, Waerhaug 1971, Loe 1971).

Listgarten (1965) showed bacterial invasion into the gingival tissues in acute necrotizing ulcerative gingivitis (ANUG). Other reports have been shown bacterial invasion into the gingival tissues in advanced periodontitis (Frank 1980, Saglie et al., 1982) and juvenile periodontitis (Gillett & Johnson 1982). Whether bacterial penetration represents a feature of the advanced stages of periodontitis leading to exfoliation of the tooth, or occurs also in less advanced stages remains to be determined.

The periodontal literature contains abundant research material about the cytotoxic and antigenic substances from bacterial plaque which diffuse into the gingival tissues to produce inflammatory and destructive changes of the periodontium (Socransky 1970, Kelstrup & Theilade 1974, Gibbons & van Houte 1978, Fine et al., 1978).

It has been shown that there is a different microbial flora in

different periodontal diseases (Listgarten 1976, Slots 1979, van Palenstein Helderman 1981, Socransky et al., 1982), the microbial flora is different supragingivally and subgingivally (Listgarten 1976, Socransky 1977), from person to person (Loesche & Syed 1973, Bowden et al., 1975, van Palenstein Helderman 1981, Socransky et al., 1982) and in the same person from site to site (van Houte & Green 1974, Bowden et al., 1975, Newman & Socransky 1977, van Palenstein Helderman 1981, Socransky et al., 1982).

Review articles by Socransky (1977), Slots (1979) and van Palenstein Helderman (1981) summarize the microbial flora associated with different periodontal diseases.

Microbial flora associated with healthy gingival sulcus

Listgarten (1976) in a study with the electron microscope found a thin microbial layer adhered to the enamel surface. The cells were predominantly coccoid in shape with a majority exhibiting cell wall features compatible with those of Gram-positive microorganisms.

According to Socransky (1977), a scanty microbial flora is located almost entirely supragingivally on the tooth surface. Microbial cell accumulations are usually 1 to 20 cells in thickness and are comprised mainly of Gram-positive coccal forms. The microorganisms commonly encountered in such sites in adults include Streptococcus mitis, Streptococcus sanguis, Staphylococcus epidermids, Rothia dentocariosa, Actinomyces viscosus, Actinomyces naeslundii and occasionally species of Neisseria and Veillonella.

According to Slots (1979), a scant microbial flora dominated by

Gram-positive microorganisms (85%), usually Streptococcus and facultative Actinomyces species is found in the healthy gingival sulcus.

Microbial flora associated with gingivitis

According to Socransky (1977), in experimental gingivitis there is an increase in the total mass of plaque and cell layers which often extend to 100 to 300 cells in thickness. There is an increase in proportions of members of the genus Actinomyces. This group of microorganisms tends to be the dominant genus associated with supragingival plaque frequently comprising 50% or more of the isolates. In long standing gingivitis, approximately 25% of the microbial flora may be Gram-negative including species of Veillonella, Campylobacter and Fusobacterium. The Gram-negative cells appear to be located primarily on the surface of the bacterial plaque in subgingival sites.

According to Slots (1979), the development of gingivitis is accompanied by a marked increased in the total number of Gram-negative microorganisms. Fusobacterium nucleatum, Bacteroides melaninogenicus ss. intermedius., Haemophilus species, and other Gram-negative microorganisms comprised about 45% of the total gingivitis isolates. Streptococcus and facultative and anaerobic Actinomyces species constituted the majority of the Gram-positive gingivitis isolates.

Pregnancy gingivitis

Recent studies have implicated B. melaninogenicus ss. intermedius due to an increase of estrogen and progesterone in the gingival fluid which enhance the growth of the microorganism (Kornman & Loesche 1980).

Microbial flora associated with ANUG

A recent study by Loesche et al., (1982), found Treponema species 32%, B. melaninogenicus ss. intermedius 24%, Selenomonas species 6% and Fusobacterium species 3%. It is the first time that B. melaninogenicus ss. intermedius is implicated as an important pathogen of ANUG.

Microbial flora associated with periodontitis

Listgarten (1975) in a study with the electron microscope found that filamentous forms were distinctly predominant. All the cell types encountered in gingivitis could also be observed in these specimens. Among the filaments, Leptotrichia buccalis could be identified because of the characteristic appearance of its cell wall (Listgarten & Lai 1975). Spirochetes and Gram-negative bacteria with multiple flagella closely resembling the species Selenomonas sputigenum were also found in large amounts. The subgingival flora consisted of fewer cells adherent to the root surface with a concomitant increase in the population of Gram-negative and flagellated cells, as well as spirochetes.

Later, Listgarten & Hellden (1978) examined with a darkfield microscope the distribution of bacteria in periodontitis and found significant differences compared to the healthy sulcus. Coccoid cells were predominant at normal sites (74.3% vs. 22.3%), while at diseased sites, motile rods were more frequent (12.7% vs. 0.3%), as well as curved rods (1.7% vs. 0%), small spirochetes (12.6% vs. 1.1%), medium-size spirochetes (18.5% vs. 0.5%) and large spirochetes (6.7% vs. 0.2%). The ratio of motile to non-motile cells in the normal was 1:49, whereas at diseased

sites the ratio was close to 1:1.

According to Socransky (1977), rapidly destructive periodontitis revealed a predominance of Gram-negative rods. At least two patterns of subgingival colonization may be observed. One pattern appeared to be dominated by Bacteroides melaninogenicus ss. asaccharolyticus (B. gingivalis, for review see Coykendall et al., 1980), and spirochetes. A second pattern appeared to be comprised of large numbers of anaerobic vibrios, "corroding" Bacteroides and Eikenella corrodens.

According to Slots (1979), Gram-negative anaerobic rods (75%), B. melaninogenicus ss. asaccharolyticus (B. gingivalis) and F. nucleatum were the most predominant isolates in periodontitis.

Tanner et al., (1979) studied the microbial flora of eight young adult patients. Two patients presented generalized extensive bone loss, extensive clinical inflammation and suppuration, with a microbial flora being dominated by Bacteroides asaccharolyticus (B. gingivalis) and an organism with characteristics consistent with Actinobacillus actinomycetemcomitans. The other two patients presented extensive bone loss but minimal clinical inflammation and the microbial flora was predominated by Bacteroides melaninogenicus ss. intermedius and Eikenella corrodens in one patient and E. corrodens and a slow growing fusiform-shaped Bacteroides in a second patient. A third group of four patients demonstrated moderate levels of clinical inflammation and evidence of continued bone loss in the last year. Predominant microorganisms in this group were more heterogeneous and included B. asaccharolyticus (B. gingivalis), Fusobacterium

nucleatum, the fusiform Bacteroides and anaerobic vibrios. Sites with minimal disease in the patients revealed higher proportions of Gram-positive microorganisms including Rothia dentocariosa, Actinomyces naeslundii and Actinomyces viscosus. Gram-negative microorganisms were predominant in the patients which presented bone loss.

Syed et al., (1981) found that in untreated moderate-to-advanced periodontitis the microbial flora was dominated by anaerobic, Gram-negative organisms such as spirochetes, Selenomonas and Pigmented Bacteroides. Capnocytophaga and Fusobacterium were also present. Pigmented Bacteroides species were found in 84% of the sites whereas spirochetes and other motile microorganisms were found in 80% of the samples.

Kornman & Holt (1981) studied three cases of severe localized periodontitis in adults, in which a new Bacteroides which comprised more than 50% of the cultivable subgingival microbial flora was found. The isolates were Gram-negative, obligately anaerobic short rods, which required CO_2 for growth. The physiological and ultrastructural observations showed these isolates to be representative of a new Bacteroides species, Bacteroides capillus.

Mashimo et al., (1981) studied the microbial flora of juvenile diabetes patients with localized periodontitis with severe bone loss and found Capnocytophaga species 25%, "anaerobic vibrios" 18.2% and Actinomyces species 7.8%.

According to van Palenstein Helderman (1981), Gram-negative rods appear to be specifically associated with periodontitis. A growing body

of evidence implicates especially Bacteroides assacharolyticus (B. gingivalis) as one of the responsible pathogens (for review see Slots 1982).

Periodontitis have been shown to have periods of exacerbation showing an increase of Gram-negative microorganisms and periods of remission showing a decrease of Gram-negative microorganisms. Disease may progress by multiple periods of exacerbation and remission until the supporting structures of the tooth are lost (Newman 1979).

Microbial flora associated with juvenile periotontitis (periodontosis)

The microbial flora of this periodontal disease which at one time was considered to be a degenerative disease was first described by Newman et al., (1976) and Slots (1976) who found predominantly Gram-negative capnophilic and anaerobic rods. One of the microorganisms most frequently isolated in high numbers was a Gram-negative fusiform shaped rod which would glide on agar surfaces. A new genus Capnocytophaga was proposed. According to Slots (1979), the microbial flora was predominantly Gram-negative (65%), but was of a nature different from that of adult periodontitis, being predominated by isolates of Bacteroides species and other microorganisms of unknown species. According to van Palenstein Helderman (1981), the subgingival microbial flora is different from that associated with adult periodontitis and the findings implicate Actinobacillus actynomycetemcomitans and Capnocytophaga species as important pathogens.

Microbial flora associated with destructive periodontal disease in children

Destructive periodontal disease of children of ages 5 to 12, appear

to be characterized by a rather heterogeneous groups of microorganisms. Most notable has been the frequent isolation of high numbers of Bacteroides oralis, Selenomonas sputigena, Clostridium species and anaerobic actinomycetes (Sasaki et al., 1977).

Microbial flora associated with the periodontal abscess

Newman & Sims (1979) found that the healthy subgingival sites harbored a microbial flora which was predominantly Gram-positive (71%) and facultative (78.3%). In contrast the microbial flora recovered from the abscess sites were predominantly Gram-negative (66.2%) and anaerobic (65.6%). In the exudate, B. melaninogenicus subspecies, Fusobacterium species and "vibrio-corrodens" predominated. Capnocytophaga species were isolated in all exudate samples. In contrast to the healthy subgingival sites the exudate samples contained Peptococcus species and Peptostreptococcus species. The microorganisms isolated from the apical sites included Capnocytophaga, B. melaninogenicus subspecies and Fusobacterium species. This study demonstrated that the microorganisms which colonize the periodontal abscess are primarily Gram-negative anaerobic rods.

Experimental periodontal diseases in animals

Initial studies of the pathogenic potential of human isolates in gnotobiotic rat systems revealed that certain Gram-positive microorganisms are able to produce periodontal disease. Such microorganisms included Streptococcus mutans, Streptococcus salivarius, Actinomyces naeslundii, Actinomyces viscosus, Bacillus and Nocardia species (Gibbons et al., 1966, Kelstrup & Gibbons 1970). Studies with Gram-negative isolates have been

shown to accelerate alveolar bone loss when implanted as monocontaminants in gnotobiotic rats. These included a Bacteroides strain isolated from juvenile periodontitis, Capnocytophaga strains isolated from juvenile periodontitis and periodontitis, Eikenella corrodens, Bacteroides melaninogenicus ss. asaccharolyticus (B. gingivalis), Fusobacterium nucleatum and Selenomonas sputigena strains from periodontitis (Irving et al., 1975 Crawford et al., 1977). The Gram-positive and Gram-negative experimental infection, differed on a clinical and histopathologic basis. Disease induced by Gram-positive microorganisms tended to exhibit root caries, more plaque, and less abundant osteoclastic response than disease induced by Gram-negative microorganisms (Irving et al., 1974, Irving et al., 1975, Irving et al., 1976, Garant 1976).

All these studies have shown an increase of the Gram-negative microbial flora from healthy sites to different types of gingivitis and finally to the most destructive forms of periodontal disease. Gibbons & van Houte (1978), suggested that it may be reasonable to hypothesize that, if infection with Gram-negative microorganisms is superimposed on infection with Gram-positive microorganisms, the rate of tissue destruction would accelerate. The finding of higher proportions of Gram-negative anaerobic microorganisms in the depths of rapid periodontitis pockets substantiates this suggestion. These microorganisms may increase the rate of destruction because of their elaboration of endotoxin.

It is important to point out that the cell wall of Gram-negative bacteria is different (contains endotoxin) from that of Gram-positive

bacteria, which does not contain endotoxin (Nikaido 1973, Vogel & Mergenhagen 1982).

B. THE ROLE OF ENDOTOXIN IN PERIODONTAL DISEASES

The term endotoxin is used to denote the lipopolysaccharides (LPS) of high molecular weight which are considered to be integral structural units found on the outer membrane of the cell wall of Gram-negative bacteria (Westphal 1957). The term originates from the observations of early workers, such as Pfeiffer (1892), who noted that apart from the toxin released into the culture medium by living Gram-negative microorganisms (exotoxin), toxic substances were also released coincident with the bacteria undergoing lysis. Those who used the term "endotoxin" assumed that the active substance was released into the culture fluid upon autolysis of the cells; therefore, they tended to employ long incubation periods before harvest. Ecker (1917) certainly was working with endotoxin, but he rejected the term because he demonstrated heat-stable toxin in filtrates of young growing cultures where no sign of autolysis could be found. It has been shown that "free endotoxin" can be released from intact Gram-negative microorganisms as a result of excess production of cellular material during vigorous growth of the bacteria rather than as a result of cell lysis (Crutchley et al., 1967).

Chemical composition

LPS are heat-stable, high molecular weight, macromolecular structures which composed of three main regions: Lipid A, R-polysaccharides (core), and the O-polysaccharides (Fig. 1).

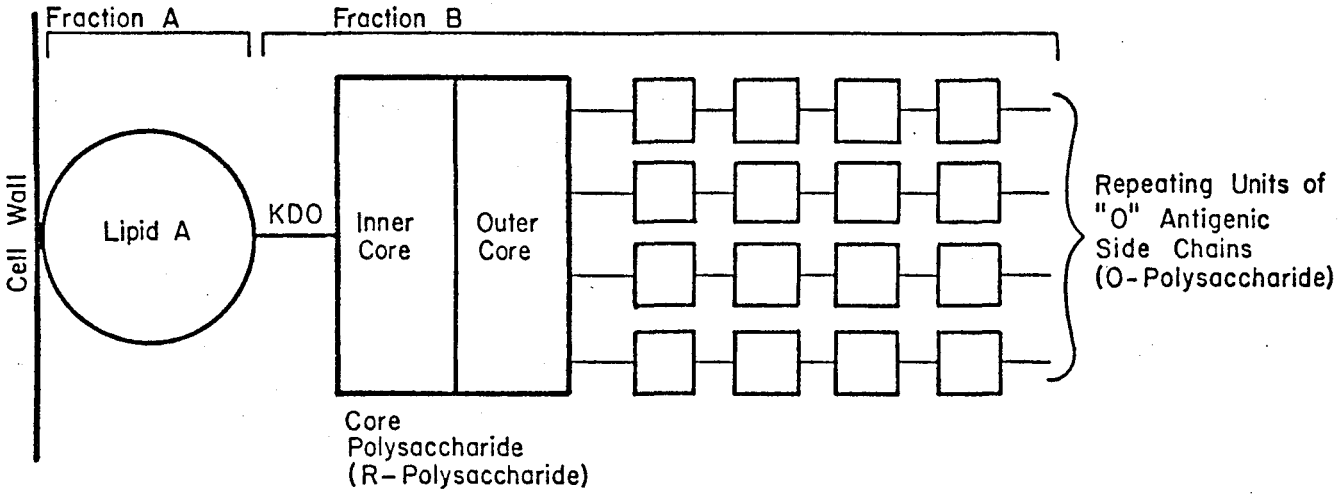

Figure 1. Diagram of lipopolysaccharide showing three main regions: lipid A, Core polysaccharide (R-polysaccharide) and "O" antigens. (O-polysaccharide). Fraction A includes the lipid portion of LPS and Fraction B the polysaccharide portion. Note key position of KDO as a link between lipid A and polysaccharides. KDO is attached via a ketoacidic linkage to glucosamine residues of lipid A.

Lipid moiety: acid hydrolysis of LPS produces a phosphorus-containing lipid precipitate, designated as Fraction A (later called Lipid A), and a soluble fraction B, which contains polysaccharides. The basic structural component of the lipid is a diglucosamine unit that contains fatty acids attached to amino and hydroxyl groups (Elin & Wolff 1976). A protein-rich component linked to the lipid moiety called Lipid A associated protein (LAP) has been described (Sultzer & Goodman 1976). The LPS molecule which is the outermost part of the cell wall of the Gram-negative bacteria is attached via the lipid moiety (Elin & Wolff 1976).

A unique sugar with the formula 2-keto-3-deoxyoctonate (KDO), links Lipid A to the core region (Heath & Ghalambor 1963).

R-polysaccharide (core): is the middle portion of the LPS molecule, linking the lipid moiety to the O-polysaccharide moiety. Chemical analyses of the R-core have shown five basal sugars, phosphate, and O-phosphorylethanolamine (Hellerqvist & Lindberg 1971).

O-polysaccharide: this structure determines the O-antigenic specificity of the bacteria. Chemical analyses of the O-polysaccharide have shown a regular sequence of the sugars (Hellerqvist et al., 1969).

Extraction procedures for LPS

Available information suggests that the LPS is bound to the cell wall primarily by physical forces, i.e., hydrophobic, ionic, or both (Wheat 1964). Several extraction procedures exist (for review see Luderitz et al., 1971), the most commonly used being the hot phenol-water procedure (Westphal & Jann 1965). LPS is usually collected from the

acqueous phase, although the isolation of a phenol-soluble LPS has been reported (Knox & Parker 1973).

Assay methods for LPS

There are in vivo and in vitro types of procedures for the assay of LPS (for review see Daly et al., 1980). In the in vivo procedures, LPS can be assayed by the localized and generalized Shwartzman reactions; pryogenicity tests; the production of primary skin inflammation in rabbits; and chick embryo and mouse lethality tests.

The localized Shwartzman reaction is produced by injecting a few micrograms of LPS into rabbit subcutaneously. An intravenous injection of the same amount of LPS given 24 hours later results in the injected skin site becoming hemorrhagic within a few hours (Davis et al., 1969).

The generalized Shwartzman reaction is a pathologic entity produced by giving two intravenous injections of LPS 24 hours apart (Davis et al., 1969).

The in vitro assay is based on the ability of LPS to induce gelation of a lysate obtained from amebocytes, the only cell in the blood of Limulus polyphemus, the horshoe crab (Levin & Bang 1964). The Limulus lysate assay (LLA) is generally considered the most sensitive test for the detection of bacterial LPS, and Yin et al., (1972) reported that picogram quantities of LPS per ml can be detected. A positive LLA can, however, also be induced by a number of other substances including thrombin, thromboplastin, ribonucleic acids and ribonuclease (Elin & Wolff 1973).

Biological properties of LPS

LPS is known to be a potent toxin which in a large dose is lethal (Drutz & Graybill 1978). Several biological properties of LPS when injected into animals include fever, transient leukopenia followed by marked granulocytosis, thrombocytopenia, disseminated intravascular coagulation (due to activation of factor XII), endotoxic shock effects on the endocrine system, (release of cortisone, ACTH and growth hormone), effects on metabolism which include hypoglycemia, hyperlipidemia, increase in several serum enzymes such as lactic dehydrogense, isocitric dehydrogenase, transaminases and creatine phosphokinase, hypoterremia and decrease in serum zinc (for review see Elin & Wolf 1976).

Galanos et al., (1972) showed the Lipid A component to be the toxic factor by removing it from LPS and testing its biological activity separately. Nowotny et al., (1975) found that isolated polysaccharides may, in certain circumstances, express LPS-like activity in the absence of Lipid A.

Other biological properties of LPS include activation of the complement system (Gewurz et al., 1968). The Lipid A moiety of LPS activates the classical complement pathway (Morrison & Kline 1977, Okuda & Takazoe 1980). The polysaccharide component has been shown to activate the alternative complement pathway (Morrison & Kline 1977, Okuda & Takazoe 1980), and carries the molecule's major antigenic determinants (Westphal 1975).

LPS has been shown to induce stimulation of macrophages to release collagenase (Wahl et al., 1974), induce bone resorption in vitro (Hausmann

1974, Raisz et al., 1981), elicit cytotoxicity for cell cultures (Aleo et al., 1974), be a B cell mitogen (Andersson et al., 1972), and a potent polyclonal antibody activator (Kunori et al., 1978). Only Lipid A (Andersson et al., 1973) and LAP (Sultzer & Goodman 1976) have been shown to possess mitogenic activity. The Lipid A and LAP also have the capacity of induce differentiation of the macrophages into killer cells (Doe et al., 1978).

It has been shown that interaction of LPS with serum generates chemotactic factors for polymorphonuclear leucocytes (PMNLs) derived from the complement system (Snyderman 1972).

LPS and periodontal diseases

Early studies: Boe (1941) produced the Shwartzman reaction in rabbits with cell-free filtrates of autolysed cultures of oral Fusobacterium and Leptotrichia establishing a suspicion of LPS as a pathogenic factor in periodontal disease. The growing suspicion of a pathogenic role for LPS was enhanced by the study of Rizzo & Mergenhagen (1964) who injected LPS from Veillonella into the oral mucosa of rabbits. They found an inflammatory infiltrate characterized by PMNLs and monocytes, and also localized areas of bone resorption. Jensen et al., (1966) found that LPS from Veillonella applied to "skin windows" in humans produced an increase in PMNL migration and phagocytosis. Mergenhagen (1967) found raised serum antibody titres in patients with periodontal disease to LPS from Veillonella and Fusobacterium.

LPS and bacterial plaque: electron microscopic examination has revealed

small rod and disc-like structures bounded by trilaminar membranes within the matrix of dental plaque resembling the electron microscopic appearance of LPS (for review see Shands et al., 1967), Selvig et al., (1971) identified them as representing free bacterial LPS in plaque. Fine et al., (1978) examined the LPS content of superficial loosely-adherent plaque as compared to the deeper, adherent plaque and they concluded that the highest LPS concentrations were found in the subgingival loosely-adherent plaque.

LPS from specific microorganisms: different destructive periodontal diseases have been associated with different microbial floras (Socransky et al., 1982). The most destructive forms of periodontal disease have been associated with the Gram-negative microorganisms (LPS Producers).

LPS from Bacteroides melaninogenicus was found to be less toxic than LPS from Salmonella when the Shwartzman reaction test was used (Hofstad 1970). Veillonella LPS has been shown to have quite potent toxic properties (Rizzo & Mergenhagen 1964, Sveen 1977) Fusobacterium LPS produced necrosis at rabbit skin sites suggesting its pathogenic importance (Sveen 1977).

The low amounts of LPS found in plaque by Johnson et al., (1976) have led them to speculate that the LPS responsible for the major tissue damage in periodontal disease may originate from microorganisms which grow in the depths of the periodontal pocket and liberate LPS with "unusual biologic properties". A gram-negative rod isolated from juvenile periodontitis, has been shown to cause rapid, extensive bone loss in

monoinfected rats in the absence of plaque formation (Irving et al., 1975). Listgarten et al., (1978) reported similar findings in gnotobiotic rats monoinfected with Eikenella corrodens.

A recent study showed that LPS from two strains of Actinobacillus actinomycetemcomitans (Y4 and N27) are potent bone destructive agents in vitro, are cytotoxic for mouse peritoneal macrophages and produced the localized Shwartzman reaction (Kiley & Holt 1980).

The concept that tissue destruction may be mediated primarily by LPS from specific bacteria rather than from the plaque as a whole has been further supported by Fine et al., (1978).

Tissue response and LPS

LPS in the gingival sulcus: LPS has been detected in the gingival fluid and reported to have a direct relationship to the degree of inflammation as assesed both clinically and histologically (Simon et al., 1970, 1971, 1972). The presence of LPS in the gingival sulcus has led to its being implicated in the complement activation in chronically inflamed gingiva (Schenkein & Genco 1977) and to PMNL migration which occurs at all stages of chronic inflammatory periodontal disease (Page & Schroeder 1976). The presence of PMNLs and macrophages in the inflamed periodontium may be attributable in part to the chemotactic factors released from the interaction of complement with LPS.

LPS penetration of gingival tissue: Rizzo (1968) observed a positive Shwartzman reaction when LPS was applied to mechanically ulcerated epithelium and suggested that LPS may gain access to gingival tissue only after

the pocket lining epithelium has become ulcerated due to the disease process.

Schwartz et al., (1972) demonstrated that topically applied, tritiated E. coli LPS (86µg over a 2 hour period) penetrated clinically healthy, microscopically intact crevicular epithelium in beagle dogs. Their finding that LPS penetrates gingival tissue has been generally accepted.

LPS and gingival inflammation: Vascular changes (Ranney & Montgomery 1973, Kahnberg et al., 1976), PMNL migration (Mergenhagen 1970, Baboolal & Powell 1971), macrophage migration, fibroblast toxicity (Allison et al., 1976) have all been attributed to LPS-activated complement products.

LPS and bone: It has been shown that LPS stimulates the release of prelabelled calcium (^{45} Ca) from fetal rat bone tissue culture (Hausmann et al., 1970, Kiley & Holt 1980, Raisz et al., 1981). The component of LPS primarily responsible for the observed resorptive activity is thought to be Lipid A (Hausmann 1972, 1975).

LPS and cementum: The possibility that LPS accounts for the cytotoxicity of periodontally involved cementum was first proposed by Hatfield & Baumhammers (1971). Later, Aleo et al., (1974) observed cytotoxic effects in cell cultures which had been incubated with phenol-water extracts of autoclaved involved cementum, tested with the LLA assay concluding that LPS was present. Aleo et al., (1975) found that cultured human gingival fibroblasts would attach only to a periodontally involved tooth surface following either hot aqueous phenol treatment, or when the entire cementum

had been removed by root planing. A recent study showed LPS penetration into the cementum (10μm) of periodontally involved roots and microbial deposits down to the level of the cemento-dentinal junction suggesting that all periodontally involved cementum should be removed during root planing, in order to achieve a root surface free of bacterial contamination (Dayle et al., 1982).

All these studies strongly suggest an important role of LPS in the pathogenesis of periodontal diseases.

CHAPTER III

MATERIALS AND METHODS

PRELIMINARY STUDIES

A preliminary study (I) was carried out utilizing six young adult female guinea pigs. Repeated injections of 100µg/ml of E. coli endotoxin 0111:B4 strain (LPSE)* in 0.85% sterile saline (0.2 ml volume) at most apical extent of the labial frenum of the mandible, and as a control, 0.85% sterile saline at the similar site in the maxilla were performed. The LPSE in sterile saline was placed in an ultrasonic bath** for five minutes to make the preparations homogeneous.

The animals were anesthetized with ethyl ether,*** ear markers were placed to identify each animal. The repeated injections were performed at 0, 24, 48 hours and 5, 6 and 7 days. The animals were sacrificed by ethyl ether asfixiation 24 hours after each injection. In all cases, a greater neutrophilic and monocytic inflammatory response was seen in the experimental injection site compared with the control. Additionally, in the 7 day specimen, a chronic inflammatory response also was seen, characterized by tissue macrophages, lymphocytes and plasma cells. This preliminary study demonstrated a dramatic inflammatory response in all the experimental sites compared with the control sites which showed a minimal inflammatory response. This fact demonstrated that at 24 hours after LPSE

* DIFCO LABORATORIES - Detroit, Michigan
** METTLER ELECTRONICS CORP. - Anaheim, California
*** MALLINCKROOT INC. - Paris, Kentucky

injection, the inflammatory response was induced.

A second preliminary study (II) was carried out utilizing five young adult female guinea pigs, using the same procedure as in the first study. However, introduced a modification by using a single injection of five different concentrations of LPSE: 10, 1, 0.1, 0.01 and 0.001µg/ml of LPSE. The animals were sacrificed by ethyl ether asfixiation 24 hours after the injections. In all cases, a greater neutrophilic and monocytic inflammatory response was seen in the experimental site compared to the control site. However, the inflammatory response with the smallest concentrations (0.001µg/ml) appeared morphologically similar to that of the control. This second study demonstrated that a very small concentration of LPSE was able to produce a greater inflammatory response compared with the sterile saline control.

ENDOTOXIN PREPARATION

One preparation was sonicated and the other one was not sonicated. Six concentrations of E. coli endotoxin 0111: B4 strain (LPSE) in a 0.85% sterile saline vehicle were made by several dilutions in the following manner:

Stock dilution of 50µg/ml was prepared by dissolving 5 mg of LPSE in 100 ml of sterile 0.85% Na Cl. Serial dilutions were prepared as follows:

GROUP I: Stock concentration of 50µg/ml

GROUP II: Stock diluted 1:1 with saline for concentration of 25µg/ml

Group III: One part of stock diluted with 4 parts of saline to obtain 10µg/ml concentration

GROUP IV: One part of 10µg/ml solution diluted with 9 parts of saline to yield 1µg/ml

GROUP V: One part of 1µg/ml solution diluted with 9 parts of saline for a concentration of 0.1µg/ml

GROUP VI: One part of 0.1µg/ml solution diluted with 9 parts of saline for 0.01µg/ml final concentration

SONICATION PROCEDURE

Sonication of the concentrations was performed with a Blackstone Ultrasonic Probe System, Probe Model BP2,* which has a mechanical resonance of 20 kc, at maximum power for eight periods of 30 seconds (modified from Shands et al., 1967). The concentrations were placed in a plastic beaker to prevent fracture of a glass beaker, and in a cold bath to prevent extreme heat (Table I).

The concentrations that were not sonicated were placed for five minutes in an ultrasonic bath** to make the preparations homogeneous (Table II).

ANIMALS

Fourteen young adult female guinea pigs, weighing 500-550 g were utilized in this experiment. The experimental animals were divided into six groups. The animals were anesthetized with ethyl ether, ear markers were placed to identify each animal. Two animals from each group were injected with a syringe mounted with a 25 gauge needle. A 0.05 ml volume

* BLACKSTONE ULTRASONICS, INC. - Sheffield, Pennsylvania
** METTLER ELECTRONICS CORP. - Anaheim, California

of sonicated LPSE was injected at the most apical extent of the labial frenum in the maxilla; and the same volume of unsonicated LPSE was injected into the most apical site of the labial frenum of the mandible. Two animals were utilized as controls and were injected with 0.85% sterile saline solution at similar sites as in the experimental animals (Table III). The animals were sacrificed 24 hours after the injections, being anesthetized with ethyl ether and then injected intraperiotoneally with 0.5 ml of Socumb - 6GR (sodium pentobarbital)*. The mucosa of each injection site was resected utilizing sharp disection with a #15 Bard Parker blade, a 0.5 cm^2 tissue sample was removed and placed immediately in buffered formalin, 10% for 24 hours. The tissue was washed, dehydrated, cleared, embedded in paraffin and sectioned at 5µm utilizing an A.O. 820 microtome**. The sections were stained with haematoxylin and eosin.

The density and morphology of inflammatory cells per unit area was measured using an A.O. 10 microscope mounted on the occular with a reticule grid calibrated in $100^2\mu\ m^2$ (A.O.). A blood cell counter was utilized to record the cell population. Five randomly selected fields where the inflammatory response was present were counted in each specimen, and the cell population density per $100^2\mu\ m^2$ averaged.

A one tail t test was done to compare: a) the mean of the inflammatory cell population density of the sonicated LPSE group with the mean of the cell population density of the control group; b) the mean of the cell

* THE BUTLER COMPANY - Columbus, Ohio
** AMERICAN OPTICAL CO. - Buffalo, New York

population density of the unsonicated LPSE group with the mean of the cell population density of the control group; c) the mean of the cell population density of the unsonicated group with the mean of the cell population density of the sonicated group; d) the mean of the cell population density of each subgroup in the sonicated LPSE group with the mean of the cell population density of the control group; e) the mean of the cell population density of each subgroup in the unsonicated LPSE group with the mean of the cell population density of the control group.

TABLE I

SONICATED GROUP - MAXILLA SITE

ANIMAL (12)	LPSE CONCENTRATION	VOLUME
Group I (2 animals)	50 µg/ml LPSE + 0.85% Sterile Saline	0.05 ml
Group II (2 animals)	25 µg/ml LPSE + 0.85% Sterile Saline	0.05 ml
Group III (2 animals)	10 µg/ml LPSE + 0.85% Sterile Saline	0.05 ml
Group IV (2 animals)	1 µg/ml LPSE +).85% Sterile Saline	0.05 ml
Group V (2 animals)	0.1 µg/ml LPSE + 0.85% Sterile Saline	0.05 ml
Group VI (2 animals)	0.01 µg/ml LPSE + 0.85% Sterile Saline	0.05 ml

Table I: Experimental LPSE sonicated group. Maxilla site.

TABLE II

UNSONICATED GROUP - MANDIBLE SITE - SAME ANIMAL OF TABLE I

ANIMAL (12)	LPSE CONCENTRATION	VOLUME
Group I (2 animals)	50 µg/ml LPSE + 0.85% Sterile Saline	0.05 ml
Group II (2 animals)	25 µg/ml LPSE + 0.85% Sterile Saline	0.05 ml
Group III (2 animals)	10 µg/ml LPSE + 0.85% Sterile Saline	0.05 ml
Group IV (2 animals)	1 µg/ml LPSE + 0.85% Sterile Saline	0.05 ml
Group V (2 animals)	0.1 µg/ml LPSE + 0.85% Sterile Saline	0.05 ml
Group VI (2 animals)	0.01 µg/ml LPSE + 0.85% Sterile Saline	0.05 ml

Table II: Experimental LPSE unsonicated group. Mandible site. Same animal as table I.

TABLE III

ANIMAL (2)	CONTROL SOLUTION	VOLUME	SITE OF INJECTION
Animal #1	0.85% Sterile Saline	0.05 ml	Maxilla
Animal #1	0.85% Sterile Saline	0.05 ml	Mandible
Animal #2	0.85% Sterile Saline	0.05 ml	Maxilla
Animal #2	0.85% Sterile Saline	0.05 ml	Mandible

Table III: Sterile saline control group.

RESULTS

Histologically the oral mucosal labial frenae sites injected with sterile saline, and unsonicated and sonicated LPSE showed a keratinized stratified squamous epithelium with a varying inflammatory cell infiltrate in the connective tissue characterized predominantly by polymorphonuclear leukocytes (PMNLs) with some monocytes. Dilation and congestion of the blood vessels and loss of collagen fibers in the supporting tissues infiltrated by PMNLs, were also observed.

STERILE SALINE CONTROL SITES

Histologically, the four sites injected with sterile saline showed only a scanty inflammatory cell infiltrate in the connective tissue characterized predominantly by PMNLs with few monocytes, dilation and congestion of blood vessles were observed (Fig. 2 and 3). The mean of the PMNLs population density was 13.5 cells/$100^2\mu m^2$ (Table IV).

SONICATED LPSE SITES

Histologically, all twelve sites showed an inflammatory cell infiltrate in the connective tissue characterized predominantly by PMNLs and some monocytes. The supporting connective tissue showed a moderate disorganization characterized by cell necrosis, edema, and loss of collagen fibers, in the sites infiltrated by PMNLs. A moderate dilation and congestion of the blood vessels were also observed (Fig. 4 and 5). This inflammatory response was more extensive than observed in the

sterile saline control sites but less than that observed in the unsonicated group. The mean population density of PMNLs were not dose dependent (Table IV), and appeared to be similar at all doses of LPSE.

UNSONICATED LPSE SITES

Histologically, all twelve sites showed a diffuse, dense inflammatory cell infiltrate in the connective tissue, characterized predominantly by PMNLs and few monocytes. The dense inflammatory cell infiltrate was more extensive as compared to the sterile saline control sites. Furthermore, the sites injected with unsonicated LPSE had a greater inflammatory cell infiltrate than sites injected with sonicated LPSE. The connective tissue showed a severe disorganization characterized by cell necrosis, edema and loss of collagen fibers in the supporting tissue infiltrated by PMNLs. A severe dilation and congestion of the blood vessels were also observed (Fig. 6 and 7). The mean population density of PMNLs were dependent on the dose of the LPSE injected, as the greatest number of PMNLs were observed in sites injected with the greatest concentration of LPSE.

STATISTICAL ANALYSIS OF INFLAMMATORY CELL DENSITIES

The results of the t test were as follows: a) the LPSE sonicated group showed a statistically significant greater population density for PMNLs than the sterile saline control at the $P< 0.01$ level; b) the LPSE unsonicated group showed a statistically significant greater population density for PMNLs than the sterile saline control group at the $P< 0.01$ level; c) the LPSE unsonicated group showed a statistically significant

density for PMNLs that the LPSE sonicate group at the $P< 0.01$ level; d) all the subgroups in the LPSE sonicated group showed a statistically significant greater population density for PMNLs than the sterile saline control group at the $P< 0.05$ level; e) all the subgroups in the LPSE unsonicated group showed a statistically significant greater population density for PMNL, than the sterile saline control group at the $P< 0.01$ level (see Appendix.)

A graph showing the PMNLs population density related to the sterile saline control, sonicated LPSE group and unsonicated LPSE group illustrates the quantitative results of the study.

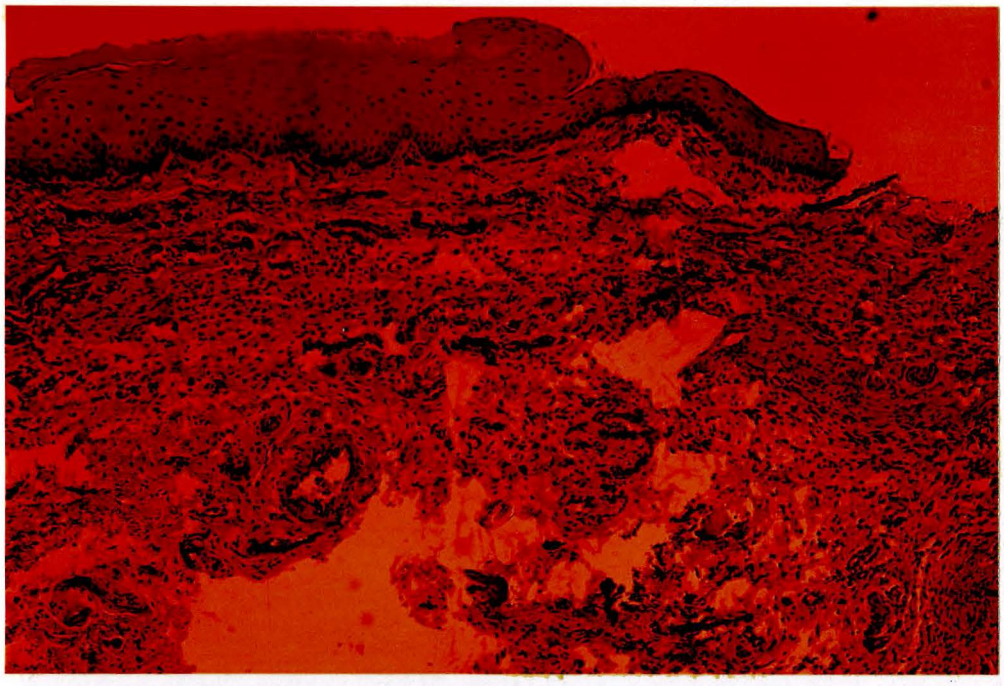

Figure 2: Histologic appearance of one of the specimens of the sterile saline control showing a scanty inflammatory infiltrate in the connective tissue (Haematoxylin and eosin stain - magnification X 100).

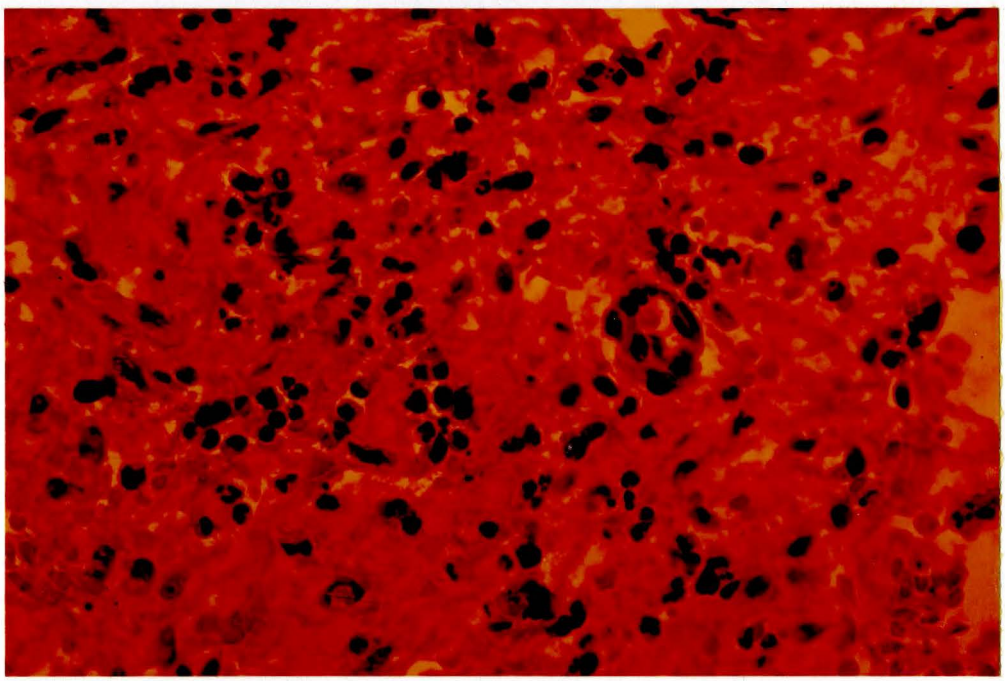

Figure 3: High power view of specimen in Figure 2, showing a scanty PMNL infiltrate and a mild blood vessel dilation and congestion (Haematoxylin and eosin stain - magnification X 400).

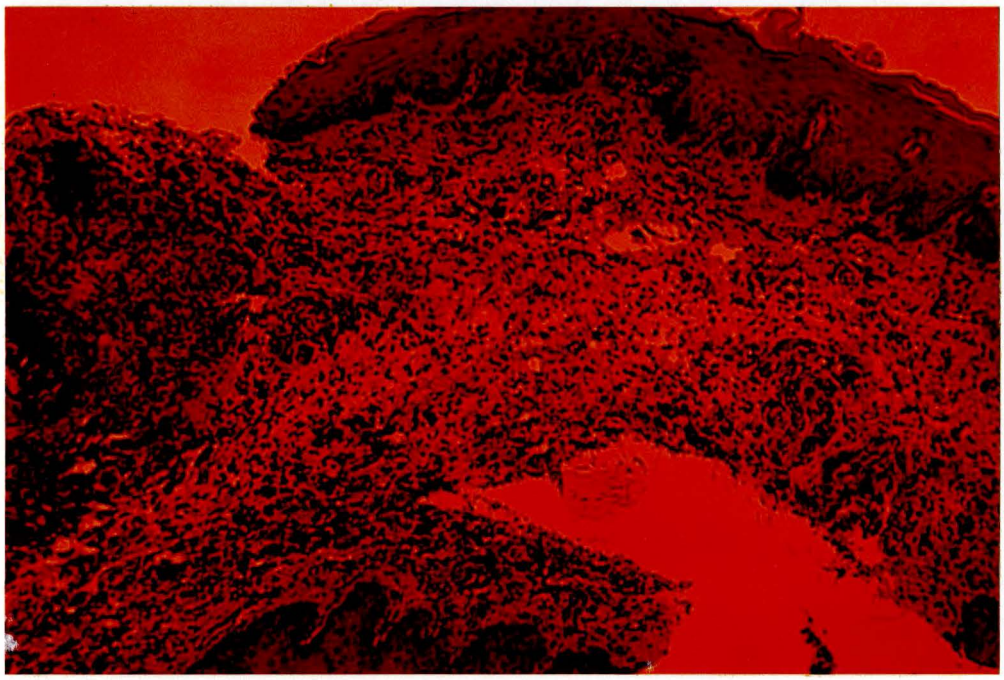

Figure 4: Histologic appearance of sonicated LPSE Group III (10µg/ml) showing a moderate inflammatory infiltrate in the connective tissue. Blood vessel dilation and congestion can be observed. (Haematoxylin and eosin stain - magnification x 100).

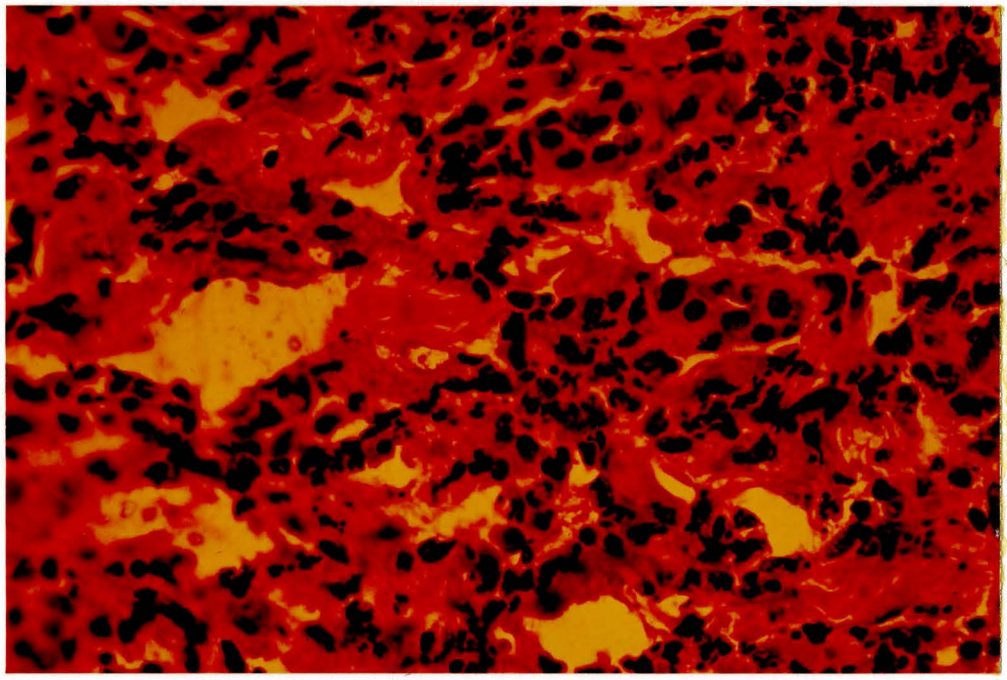

Figure 5: High power view of specimen in Figure 4 showing a moderate PMNL infiltrate, dilation and congestion of blood vessels, loss of collagen fibers can be observed. (Haematoxylin and eosin stain - magnification X 400.)

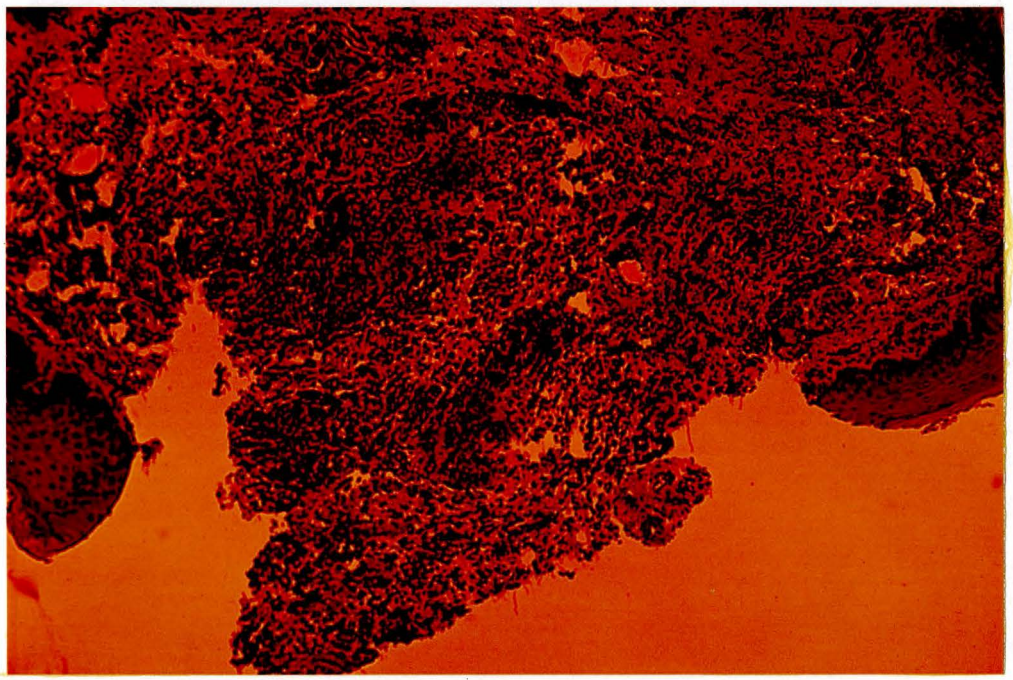

Figure 6: Histologic appearance of unsonicated LPSE Group III (10µg/ml) showing a dense inflammatory infiltrate in the connective tissue. Blood vessel dilation and congestion can be observed. (Haematoxylin and eosin stain - magnification X 100.)

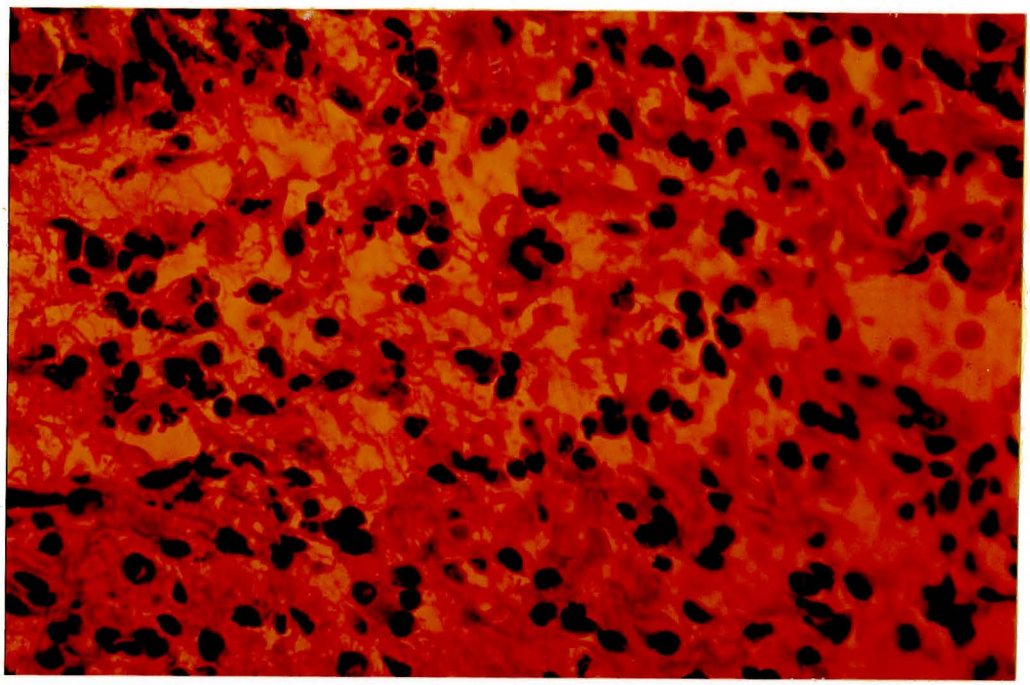

Figure 7: High power view of specimen in Figure 6, showing a dense PMNL infiltrate, dilation and congestion of blood vessels. Loss of collagen is evident surrounding the PMNLs. (Haematoxylin and eosin stain - magnification X 400.)

TABLE IV

MEANS OF THE PMNLs POPULATION DENSITY

(cells/$100^2\mu m^2$)

CONTROL

PMNLs 13.5

UNSONICATED LPSE

Group I (25µg/ml)
PMNLs 69.3

Group II (10µg/ml)
PMNLs 68.4

Group III (50µg/ml)
PMNLs 60.7

Group IV (0.1µg/ml)
PMNLs 52

Group V (1µg/ml)
PMNLs 47.6

Group VI (0.01µg/ml)
PMNLs 44

Unsonicated LPSE
PMNLs 57.0

SONICATED LPSE

Group I (50µg/ml)
PMNLs 33.3

Group II (25µg/ml)
PMNLs 34.1

Group III (10µg/ml)
PMNLs 32.5

Group IV (1µg/ml)
PMNLs 34.3

Group V (0.01µg/ml)
PMNLs 30.1

Group VI (0.01µg/ml)
PMNLs 33.9

Sonicated LPSE
PMNLs 33.03

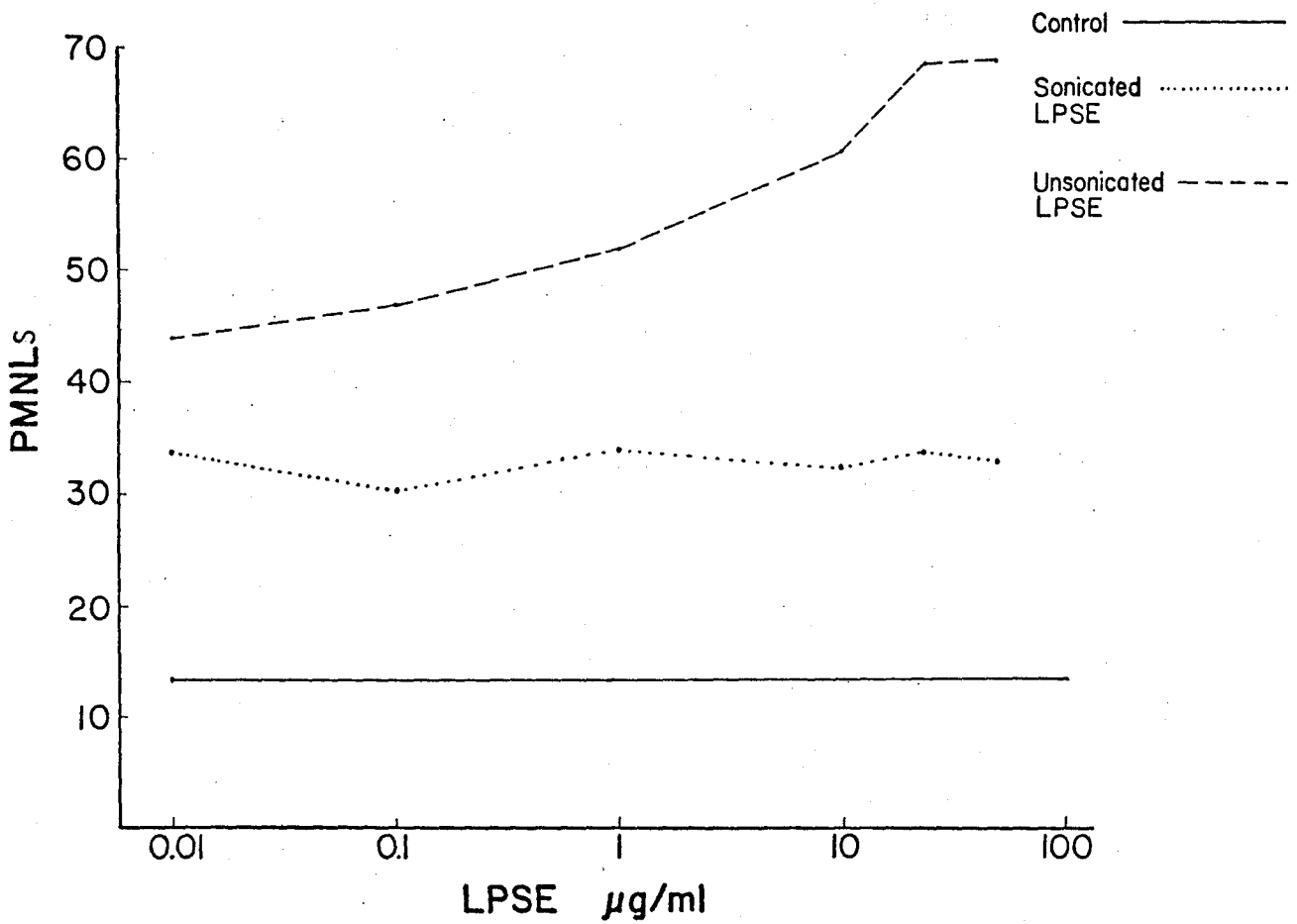

Figure 8: PMNLs population density related to control and LPSE concentrations. (cells/$100^2\mu m^2$)

CHAPTER V

DISCUSSION

An inflammatory cell infiltrate characterized predominantly by PMNLs resulted in a response to all concentrations of LPSE ranging from 50µg/ml to 0.01 µg/ml (0.05 ml volume) injected into the oral mucosa of guinea pigs, after a 24 hour period of time.

The density of PMNLs was greater in the LPSE groups than in the sterile saline control injection sites, which showed a localized and scanty inflammatory infiltrate. Monocytes were present in response both to the LPSE and sterile saline injection, but were more evident at the LPSE injection sites.

Clearly, LPSE induced an inflammatory process of significantly greater cellularity than did sterile saline, however, the unsonicated LPSE resulted in a greater response than either saline and sonicated LPSE. This finding could be related to the hypothesis that a macromolecular complex of critical size is required in order for LPSE to elicit its characteristic effect in the host (Tarmina et al., 1968). Sonication reduces the macromolecular size of LPSE (Shands, 1967) and may be responsible for its reduction in the induced inflammatory response as compared to unsonicated LPSE observed in this study. However, in another study, Sveen (1977) found that sonicated LPSE produced a greater inflammatory response, as measured by the diameter of the erythema in the skin of the rabbit

than unsonicated LPSE and it was suggested that the LPSE should be in a well suspended state. In the present study, the unsonicated LPSE was placed in an ultrasonic bath for five minutes and a good suspended state of LPSE was assumed since a greater inflammatory response was obtained as determined by the results of this study.

The question of the effect of sonication of LPSE and the inflammatory response may be related to a reduction not only in the molecular size, but also to a cleavage of the Lipid A portion from the polysaccharide moiety such as occurs following the use of sodium deoxycholate (Ribi et al., 1966). Possible, sonicated LPSE fragments of Lipid A and polysaccharide, if permitted time for reassembly, could regain the macromolecular size to induce an inflammatory response similar to unsonicated LPSE. Such a study should be done to assess this possibility.

The presence of a dramatic infiltrate of PMNLs with all the LPSE concentrations and in a lesser degree in the sterile saline injection sites, suggests the presence of chemoattractants for PMNLs in both the LPSE and sterile saline sites of injection.

There are many substances which act as chemoattractants for PMNLs (for review, see Zigmond, 1978) however, the possible chemoattractants related to this experiment would include those produced only by the injection of sterile saline and LPSE.

The physical injuries produced by a needle injection are most likely related to disturbances of the damaged or killed cells and the hemorrhage produced at the injection sites. Perturbation of the plasma

membrane results in phospholipidase A2 activation and deesterification of phospholidylcholine arachidonate, liberating arachidonic acid. Free arachidonic acid is the substrate for both the cyclo-oxygenase enzyme system resulting in prostaglandin production and the lipoxygenase system and the production of hydroperoxidation of arachidonic acid to the leukotrienes (Higgs and Eakins, 1980). According to Goetzl et al., (1977), Leukotriene B has been shown to be a potent chemoattractant for PMNLs (Fig. 9).

In hemorrhages, resulting from damage of blood vessel walls, clotting factor XII (Hageman factor) which upon contact with the collagen and sulfated glycoproteins in the vessel wall is activated to factor XIIa and XIIb (Fig. 10). This results in both a blood clotting cascade and the activation of Kallikrein and plasmin both of which are chemotactic for PMNLs (Lepow and Ward, 1972). Moreover, the fibrin clot produced by activation of factor XIa also is chemotactic as are the split products of fibrin resulting from fibrinolysis by plasmin (Stetcher, 1974). Injured cells release lysosomal enzymes, one of these, collagenase which breaks down collagen. Products of collagen breakdown have been shown to have chemotactic properties (Chang and Houch, 1970). Another type of tissue-derived chemotactic product is related to the fact that most tissues contain C3 and/or C5 cleaving enzymes (Ward, 1974). Some fragments of the complement system, C3a, C5a, $\overline{C567}$ are PMNLs chemoattractants (Lepow, 1971, Ruddy et al., 1972, Ward, 1974, Nisengard, 1977). There are certain enzymes responsible for generation of these fragments within the complement system which are chemoattractants. These include for the C3

INJURY OF BLOOD VESSELS

HAGEMAN FACTOR → COLLAGEN → KALLIKREIN → CHEMOTAXIS
(FACTOR XII) & SULPHATED GLYCOPROTEINS

Figure 9: Injury of blood vessels and chemotaxis.

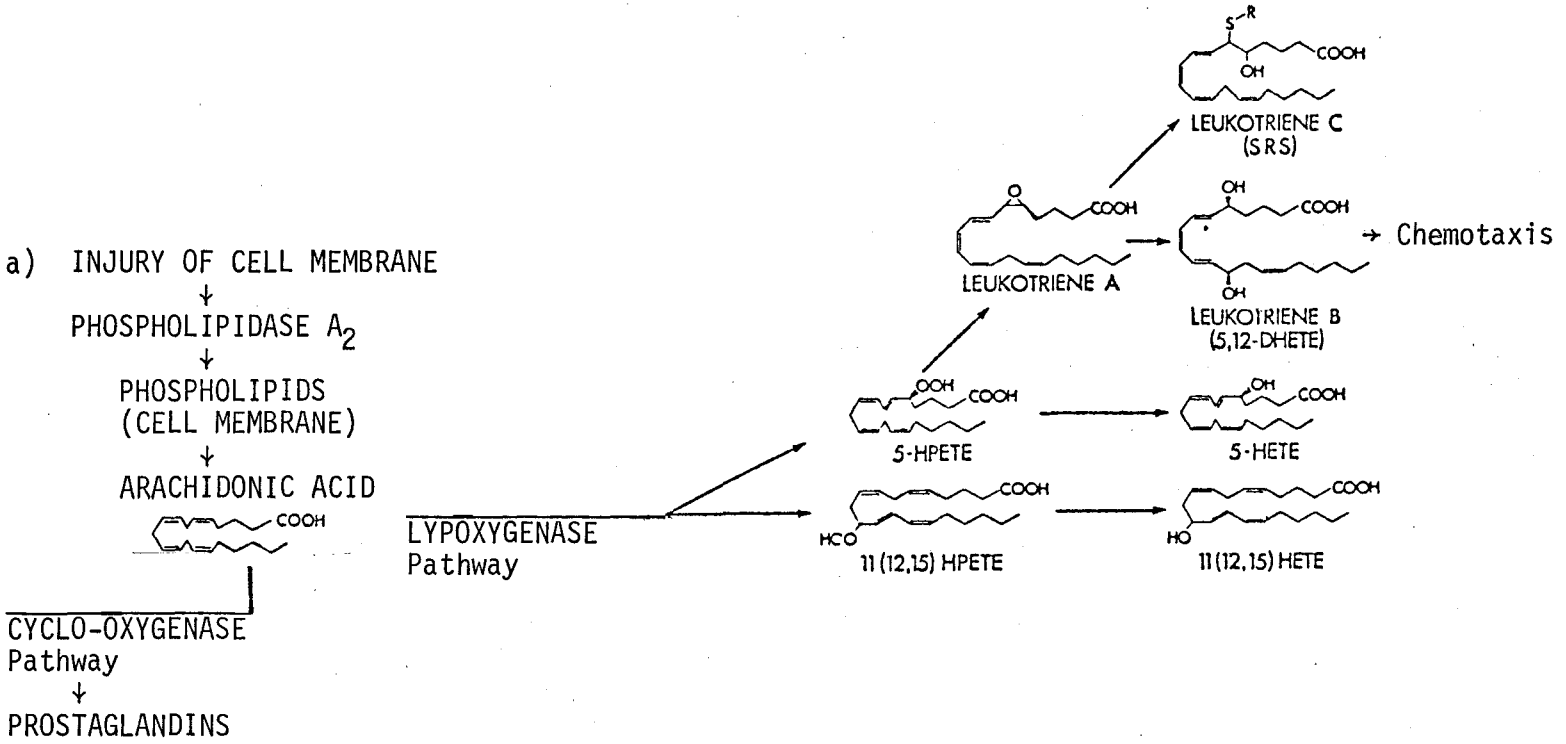

Figure 10. Injury of cells and chemotaxis

fragment: $C\overline{42}$, C3 activator, trypsin, thrombin, neutral tissue protease; for the C5 fragment: $C\overline{423}$, protease of alternate complement pathway, neutral protease of lysosomal granules; for the $C\overline{567}$ fragment: $C\overline{423}$ and trypsin (Ward, 1974).

Sterile physiologic saline does not have chemoattractant properties for PMNLs and by itself could not explain the presence of PMNLs at the site of injection. However, the needle penetration and the pressure of the volume of injected sterile saline can cause injury to the mucosa. The injury would occur to the epithelium, basement membrane, and the vascular and fibrous tissues of the lamina propria in the oral mucosa. The injury results in necrosis of epithelial cells, fibroblasts, endothelial cells, Schwann cells and the perivascular adventitia including pericytes, smooth muscle cells, monocytes, lymphocytes and PMNLs randomly present. Accordingly, in the sterile saline injected site, the physical changes can result in cellular perturbation which may result in the liberation of altered cellular products leading to the production of mediators of inflammation, including the chemoattractants discussed above. In addition to the source of chemoattractants induced by physical injury, LPSE also has been reported to mediate PMNL chemotaxis. LPSE has been shown to activate the complement system by either the classical or alternate pathway (Morrison and Kline, 1977, Okuda and Takazoe, 1980), generating C3a, C5a, and $C\overline{567}$ fragments which are PMNL chemoattractants as was discussed above. In 1964, Rizzo and Mergenhagen found in an *in vivo* study that after injection of 50µg LPSE from *Veillonella* into rabbit

oral mucosa, a PMNL infiltrate reached a peak at 24 hours after injection; monocytes and cell remnants were also present.

It has been shown in an experiment with human subjects that the capacity of only 0.19µg of LPSE from a strain of human oral Veillonella (V-1) can induce PMNL migration and phagocytosis (Jensen et al., 1966). A recent in vivo study, in mice, showed that 5.0µg LPSE from oral Veillonella is capable to attract PMNLs (Wilton and Almeida, 1980). In vitro studies of the reaction of LPSE from Veillonella alcalescens with human, mouse, rabbit, or guinea pig serum resulted in the generation of chemotactic activity for PMNLs, suggesting the activation of the complement system and generation of chemotactic factors from some of its components (Snyderman et al., 1968. In a related study, 10% autologous plasma and LPSE from Eikenella corrodens using a modified Boyden chamber technique also found to be chemotactic for PMNLs (Manoucher - Pour et al., 1981).

These studies suggest that when LPSE is released by Gram-negative bacteria into the tissues, there is an interaction with the complement system and chemotactic activity for PMNLs to occur.

In the present study, chemoattractants derived both from the needle injury and the chemoattractant mediator activity of LPSE may result in an amplification of PMNLs chemotaxis.

The extremely important protective role of PMNLs in periodontal disease is well known (for review, see Newman, 1980, Van Dyke et al., 1982, Wilton, 1982, Page and Schroeder, 1982) but also could act as a

double edge sword and cause destruction when there is an imbalance in their function.

Accumulation of PMNLs in the connective tissue and junctional epithelium of the periodontium is a characteristic feature of chronic periodontal disease (Page and Schroeder, 1976). PMNL lysosomal enzymes could have a part in the pathogenesis of periodontal disease (Taichman, 1970 , Newman, 1980). Hyaluronidase, vascular permeability promoting proteases, collagenase and other lysosomal enzymes capable of producing tissue destruction (Taichman, 1970) suggest a destructive role of these enzymes in periodontal disease. Tsai et al., in 1978 studied the interaction between PMNLs and Gram-negative microorganisms and found the release of lysosomal enzymes in the absence of serum components. Their electron microscopic observations indicated that degranulation and release of PMNL lysosomes were associated with phagocytosis of Gram-negative bacteria. They also found that serum has a modulating effect on PMNL release, generally enhancing release reaction to all bacteria.

Baehni et al., in 1978 showed that PMNLs may release lysosomes in response to bacterial plaque in the abscence of phagocytosis. Their data suggests that PMNL interaction with Gram-negative bacteria may be an important mechanism in the pathogenesis of periodontal diseases.

The findings of the present study show that LPSE exhibits potent chemotactic mediator properties even in very small amounts (0.01µg/ml). This is consistent with the studies of Rizzo and Mergenhagen (1964), who reported that after injection of 50µg LPS from Veillonella, a PMNL

infiltrate reached a peak at 24 hours post-injection and that monocytes were also present; Wilton and Almeida (1980), also reported that LPS from Veillonella when injected into the peritoneal cavity of mice will exhibit chemotactic properties for PMNLs 24 hours after injection. The present study is also closely related to the studies of LPS and its chemotactic mediator properties performed by Jensen et al., 1966, Snyderman et al., 1968 and Manoucher - Pour et al., in 1981.

The protective role of PMNLs in periodontal diseases has been studied by many investigators and its destructive role when there is an imbalance in its function has also been suggested. However, the precise relationship between PMNL chemotaxis and the development of the periodontal lesion is not well known. Further studies testing the capacity of lysosomal enzymes to induce destruction in the periodontal tissues are necessary to elucidate its destructive role in periodontal diseases.

There is abundant evidence concerning the biological activity of LPS and its destructive role in periodontal diseases. However, future studies testing the biological activities of LPS from specific oral bacteria are necessary to define the precise role of LPS in the pathogenesis of periodontal diseases.

CHAPTER VI

SUMMARY AND CONCLUSIONS

This study demonstrated that 24 hours after the injection of physiologic sterile saline into the guinea pig oral mucosa, a scanty inflammatory infiltrate characterized predominantly by PMNLs is present at the site of the injection.

Physiologic sterile saline is not known to exhibit chemoattractant properties for PMNLs, however, there is evidence that mechanical injury of the tissues, the needle injury in this case, lead to the release of tissue derived chemoattractants.

When LPSE is injected with sterile saline, there is an amplification of the inflammatory response probably due to the activation of the complement system by LPS and the release of potent chemoattractant fractions of the complement system (C3a, C5a, and $\overline{C567}$).

Both, the sonicated and unsonicated LPSE induced a greater inflammatory response than the sterile saline, however, the inflammatory response was greater in the unsonicated LPSE probably due to the reduction in molecular size and alteration of the LPS chemical structure in the sonicated LPSE.

It is difficult to determine the least concentration of LPS which is able to produce an inflammatory response 24 hours after injection, due to the fact that mechanical injury produced by the needle induces an

inflammatory response, however, it was shown that very small amounts of LPS such as 0.01µg/ml (0.05 ml volume) exhibit potent chemotactic mediator properties for PMNLs.

It is known that when an imbalance of PMNLs occurs, the release of lysosomal enzymes has the potential to produce tissue destruction. LPS has been shown to exhibit cytotoxic properties for fibroblasts. These biological properties of LPS suggest a role in tissue destruction in inflammatory periodontal diseases.

Future research testing the biological activities of LPS from specific oral bacteria are necessary to define the precise role of LPS in the pathogenesis of inflammatory periodontal diseases.

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APPENDIX

COUNTS OF PMNLs

STERILE SALINE CONTROL GROUP

Animal #1 (Maxilla)

Field *	PMNLs
#1	12
#2	14
#3	18
#4	10
#5	+ 18

72÷5=14.4

Animal #1 (Mandible)

Field	PMNLs
#1	9
#2	14
#3	6
#4	9
#5	+ 6

44÷5=8.8

Animal #2 (Maxilla)

Field	PMNLs
#1	28
#2	31
#3	23
#4	18
#5	+ 21

121÷5=24.2

Animal #2 (Mandible)

Field	PMNLs
#1	8
#2	4
#3	6
#4	6
#5	+ 9

33÷5=6.6

15.5
8.8
24.2
+ 6.6

54.0÷4=13.5 (mean of the sterile saline group)

* Fields per $100^2 \mu m^2$

SONICATED LPSE (MAXILLA SITE)

GROUP I

Animal #1

Field	PMNLs
#1	72
#2	27
#3	26
#4	24
#5	+ 51

200÷5=40

GROUP I

Animal #2

Field	PMNLs
#1	22
#2	24
#3	19
#4	36
#5	+ 32

133÷5=26.6

40
+ 26.6

66.6-2=33.3 (mean of Group I)

GROUP II

Animal #1

Field	PMNLs
#1	43
#2	54
#3	26
#4	22
#5	+ 48

193÷5=38.6

GROUP II

Animal #2

Field	PMNLs
#1	29
#2	31
#3	28
#4	26
#5	+ 34

148÷5=29.6

38.6
+ 29.6

68.2÷2=34.1 (mean of Group II)

GROUP III

Animal #1

Field	PMNLs
#1	60
#2	43
#3	28
#4	32
#5	+ 36

199÷5=39.8

GROUP III

Animal #2

Field	PMNLs
#1	16
#2	28
#3	32
#4	26
#5	+ 24

126÷5=25.2

39.8
+ 25.2

65.0÷2=32.5 (mean of Group III)

GROUP IV

Animal #1

Field	PMNLs
#1	28
#2	20
#3	40
#4	39
#5	+ 42

169÷5=33.8

GROUP IV

Animal #2

Field	PMNLs
#1	31
#2	37
#3	26
#4	42
#5	+ 38

174÷5=34.8

34.8
+ 33.8

68.6÷2=34.4 (mean of Group IV)

GROUP V

Animal #1

Field	PMNLs
#1	28
#2	33
#3	18
#4	22
#5	+ 26

127÷5=25.4

GROUP V

Animal #2

Field	PMNLs
#1	44
#2	36
#3	28
#4	32
#5	+ 34

174÷5=34.8

34.8
+ 25.4

60.2÷2=30.1 (mean of Group V)

GROUP VI

Animal #1

Field	PMNLs
#1	45
#2	20
#3	28
#4	32
#5	+ 24

149÷5=29.8

GROUP VI

Animal #2

Field	PMNLs
#1	45
#2	41
#3	46
#4	26
#5	+ 32

190÷5=38

29.8
+ 38.0

67.8÷2=33.9 (mean of Group VI)

UNSONICATED LPSE (MANDIBLE SITE)

GROUP I

Animal #1

Field	PMNLs
#1	84
#2	80
#3	49
#4	62
#5	+ 51

326÷5=65.2

GROUP I

Animal #2

Field	PMNLs
#1	107
#2	65
#3	71
#4	61
#5	+ 63

367÷5=73.4

73.4
+ 65.2

138.6÷2=69.3 (mean of Group I)

GROUP II

Animal #1

Field	PMNLs
#1	72
#2	94
#3	89
#4	76
#5	+ 81

412÷5=82.4

GROUP II

Animal #2

Field	PMNLs
#1	55
#2	47
#3	56
#4	61
#5	+ 53

272÷5=54.4

82.4
+ 54.4

136.8÷2=68.4 (mean of group II)

GROUP III

Animal #1

Field	PMNLs
#1	54
#2	52
#3	64
#4	72
#5	+ 39

281÷5=56.2

GROUP III

Animal #2

Field	PMNLs
#1	45
#2	69
#3	72
#4	62
#5	+ 78

326÷5=65.2

56.2
+ 65.2

12.4÷2=60.7 (mean of group III)

GROUP IV

Animal #1

Field	PMNLs
#1	61
#2	69
#3	52
#4	49
#5	+ 58

289÷5=57.8

GROUP IV

Animal #2

Field	PMNLs
#1	40
#2	45
#3	36
#4	52
#5	+ 58

231÷5=46.2

57.8
+ 46.2

104.0÷2=52 (mean of group IV)

GROUP V

Animal #1

Field	PMNLs
#1	51
#2	89
#3	28
#4	50
#5	+ 34

252÷5=50.4

GROUP V

Animal #2

Field	PMNLs
#1	56
#2	48
#3	34
#4	48
#5	+ 37

224÷5=44.8

50.4
+ 44.8

95.2÷2=47.6 (mean of group V)

GROUP VI

Animal #1

Field	PMNLs
#1	48
#2	36
#3	42
#4	41
#5	+ 38

205÷5=41

GROUP VI

Animal #2

Field	PMNLs
#1	37
#2	53
#3	64
#4	37
#5	+ 44

235÷5=47

41
+ 47

88÷2=44 (mean of group VI)

t TEST CALCULATIONS

t test (one tail) to compare the mean of the unsonicated LPSE group (see Table IV) with the mean of the control group.

Unsonicated LPSE group	Control group
$\overline{X}_1 = 57$	$\overline{X}_c = 13.5$
$\overline{S}_1 = 12.51$	$\overline{S}_c = 6.80$
$N_1 = 12$	$N_c = 4$

$H_o: \overline{X}_1 = \overline{X}_c$

$H_1: \overline{X}_1 > \overline{X}_c$

Formulas for the t test and σ (standard deviation)

$$\sigma = \sqrt{\frac{N_1 S_1^2 + N_2 S_2^2}{N_1 + N_2 - 2}} \qquad t = \frac{\overline{X}_1 - \overline{X}_2}{\sigma \sqrt{1/N_1 + 1/N_2}}$$

$$\sigma = \sqrt{\frac{12(12.51)^2 + 4(6.80)^2}{12 + 4 - 2}} \qquad t = \frac{57 - 13.5}{\sqrt{1/12 + 1/4}}$$

$$\sigma = \sqrt{\frac{1878.0012 + 184.96}{14}} \qquad t = \frac{43.5}{(12.138957)\ (0.57735027)}$$

$\sigma = 12.138957$ $\qquad t = \dfrac{43.5}{7.0084301}$

$t > 2.98$ (1%) $\qquad t = 6.2068$

$p < 0.01$ $\qquad df = 14$

$H_1 : \overline{X}_1 > \overline{X}_c$

t test (one tail) to compare the mean of the sonicated LPSE group (see Table IV) with the mean of the control group.

Sonicated LPSE group	Control group
$\overline{X}_1 = 33.03$	$\overline{X}_2 = 13.5$
$\overline{S}_1 = 5.56$	$S_2 = 6.80$

$H_o : \overline{X}_1 = \overline{X}_2$

$H_1 : \overline{X}_1 > \overline{X}_2$

$t = 5.36$

$df = 22$

$t > 2.82$ (1%)

$p < 0.01$

$H_1 : \overline{X}_1 > \overline{X}_2$

t test (one tail) to compare the mean of the unsonicated group with the mean of the sonicated group.

Unsonicated LPSE group	Sonicated LPSE group
$\overline{X}_1 = 57$	$X_2 = 33.03$
$S_1 = 12.51$	$S_2 = 5.56$
$N_2 = 12$	$N_2 = 12$

$H_o : \overline{X}_1 = \overline{X}_2$

$H_1 : \overline{X}_1 > \overline{X}_2$

$t = 5.80$

$df = 22$

$t > 2.82$ (1%)

$p < 0.01$

$H_1 : \overline{X}_1 > \overline{X}_2$

t test (one tail) to compare the mean of the sonicates LPSE group I with the mean of the sterile saline control group.

Sonicated LPSE group I	Control group
$\overline{X}_1 = 33.3$	$\overline{X}_c = 13.5$
$S_1 = 6.7$	$S_c = 6.8$
$N_1 = 2$	$N_c = 4$

$H_o : \overline{X}_1 = \overline{X}_c$

$H_1 : \overline{X}_1 > \overline{X}_c$

$t = 2.75$

$df = 4$

$t > 2.13$ (5%)

$P < 0.05$

$H_1 : \overline{X}_1 > \overline{X}_c$

t test (one tail) to compare the mean of the sonicated LPSE group II with the mean of the sterile saline control group.

Sonicated LPSE group II	Control group
$\overline{X}_2 = 34.1$	$\overline{X}_c = 13.5$
$S_2 = 4.5$	$S_c = 6.8$
$N_2 = 2$	$N_c = 4$

$H_0 : \overline{X}_2 = \overline{X}_c$

$H_1 : \overline{X}_2 > \overline{X}_c$

$t = 3.16$

$df = 4$

$t > 2.13$ (5%)

$P < 0.05$

$H_1 : \overline{X}_2 > \overline{X}_c$

t test (one tail) to compare the mean of the sonicated LPSE group III with the mean of the sterile saline control group.

Sonicated LPSE group III	Control group
$\overline{X}_3 = 32.5$	$\overline{X}_c = 13.5$
$S_3 = 7.3$	$S_c = 6.8$
$N_3 = 2$	$N_c = 4$

$H_o : \overline{X}_3 = \overline{X}_c$

$H_1 : \overline{X}_3 > \overline{X}_c$

$t = 2.56$

$df = 4$

$t > 2.13$ (5%)

$P < 0.05$

$H_1 : \overline{X}_3 > \overline{X}_c$

t test (one tail) to compare the mean of the sonicated LPSE group IV with the mean of the sterile saline control group.

Sonicated LPSE group IV	Control group
$\overline{X}_4 = 34.3$	$\overline{X}_c = 13.5$
$S_4 = 0.5$	$S_c = 6.8$
$N_4 = 2$	$N_c = 4$

$H_o : \overline{X}_4 = \overline{X}_c$

$H_1 : \overline{X}_4 > \overline{X}_c$

$t = 3.52$

$df = 4$

$t > 2.13$ (5%)

$P < 0.05$

$H_1 : \overline{X}_4 > \overline{X}_c$

t test (one tail) to compare the mean of the sonicated LPSE group V with the mean of the sterile saline control group.

Sonicated LPSE group V	Control group
$\overline{X}_5 = 30.1$	$\overline{X}_c = 13.5$
$S_5 = 4.7$	$S_c + 6.8$
$N_5 = 2$	$N_c = 4$

$H_o : \overline{X}_5 = \overline{X}_c$

$H_1 : \overline{X}_5 > \overline{X}_c$

$t = 2.53$

$df = 4$

$t > 2.13$ (5%)

$P < 0.05$

$H_1 : \overline{X}_5 > \overline{X}_c$

t test (one tail) to compare the mean of the sonicated LPSE group VI (the smallest concentration) with the mean of the sterile saline control group.

Sonicated LPSE group VI	Control group
$\overline{X}_6 = 33.9$	$\overline{X}_c = 13.5$
$S_6 = 4.1$	$S_c = 6.80$
$N_6 = 2$	$N_c = 4$

$H_o : \overline{X}_6 = \overline{X}_c$

$H_1 : \overline{X}_6 > \overline{X}_6$

$t = 3.187$

$df = 4$

$t = > 2.13$ (5%)

$P < 0.05$

$H_1 = \overline{X}_6 > \overline{X}_c$

t test (one tail) to compare the mean of the unsonicated LPSE group I with the mean of the setrile saline control group.

Unsonicated LPSE group I	Control group
$\overline{X}_1 = 69.3$	$\overline{X}_c = 13.5$
$S_1 = 4.1$	$S_c = 6.8$
$N_1 = 2$	$N_c = 4$

$H_o : \overline{X}_1 = \overline{X}_c$

$H_1 : \overline{X}_1 > \overline{X}_c$

$t = 8.71$

$df = 4$

$t > 3.75$ (1%)

$P < 0.01$

$H_1 : \overline{X}_1 > \overline{X}_c$

t test (one tail) to compare the mean of the unsonicated LPSE group II with the mean of the sterile saline control group.

Unsonicated LPSE group II	Control group
$\overline{X}_2 = 68.4$	$\overline{X}_c = 13.5$
$S_2 = 14$	$S_c = 6.8$
$N_2 = 2$	$N_c = 4$

$H_o : \overline{X_2} = \overline{X_c}$

$H_1 : \overline{X_2} > \overline{X_c}$

$t = 5.27$

$df = 4$

$t > 3.75$ (1%)

$P < 0.01$

$H_1 : \overline{X_2} > \overline{X_c}$

t test (one tail) to compare the mean of the unsonicated LPSE group III with the mean of the sterile control group.

Unsonicated LPSE group III	Control group
$\overline{X}_3 = 60.7$	$\overline{X}_c = 13.5$
$S_3 = 4.5$	$S_c = 6.8$
$N_3 = 2$	$N_c = 4$

$H_o : \overline{X}_3 = \overline{X}_c$

$H_1 : \overline{X}_3 > \overline{X}_c$

$t = 7.25$

$df = 4$

$t > 3.75$ (1%)

$P < 0.01$

$H_1 : \overline{X}_3 > \overline{X}_c$

t test (one tail) to compare the mean of the unsonicated LPSE group IV with the mean of the sterile saline control group.

Unsonicated LPSE group IV	Control group
$\overline{X}_4 = 52$	$\overline{X}_c = 13.5$
$S_4 = 5.8$	$S_c = 6.8$
$N_4 = 2$	$N_c = 4$

$H_o : \overline{X}_4 = \overline{X}_c$

$H_1 : \overline{X}_4 > \overline{X}_c$

$t = 5.598$

$df = 4$

$t > 3.75$ (1%)

$P < 0.01$

$H_1 : \overline{X}_4 > \overline{X}_c$

t test (one tail) to compare the mean of the unsonicated LPSE group V with the mean of the sterile saline control group.

Unsonicated LPSE group V	Control group
$\overline{X}_5 = 47.6$	$\overline{X}_c = 13.5$
$S_5 = 2.8$	$S_c = 6.8$
$N_5 = 2$	$N_c = 4$

$H_o : \overline{X}_5 = \overline{X}_c$

$H_1 : \overline{X}_5 > \overline{X}_c$

$t = 5.55$

$df = 4$

$t > 3.75$ (1%)

$P < 0.01$

$H_1 : \overline{X}_5 > \overline{X}_c$

t test (one tail) to compare the mean of the unsonicated LPSE group VI (the smallest concentration) with the mean of the sterile saline control group.

Unsonicated LPSE group VI	Control group
$\overline{X}_6 = 44$	$\overline{X}_c = 13.5$
$S_6 = 3$	$S_c = 6.8$
$N_6 = 2$	$N_c = 4$

$H_o : \overline{X}_6 = \overline{X}_c$

$H_1 : \overline{X}_6 > \overline{X}_c$

$t = 4.944$

$df = 4$

$t > 3.75$ (1%)

$P < 0.01$

$H_1 = \overline{X}_6 > \overline{X}_c$

APPROVAL SHEET

The thesis submitted by Juan J. de Obarrio, D.D.S. has been read and approved by the following committee:

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact thay any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

10-4-1982
Date


Director's Signature