June 1987

Effect of Fibrinogen Derived Factors on Polymorphonuclear Leukocyte Function In Vitro

Reed Ference

Follow this and additional works at: http://digitalcommons.uconn.edu/sodm_masters

Recommended Citation
http://digitalcommons.uconn.edu/sodm_masters/38
THE EFFECT OF FIBRINOGEN DERIVED FACTORS ON POLYMORPHONUCLEAR LEUKOCYTE FUNCTION IN VITRO

Reed Ference
B.A., State University of New York at Binghamton, 1980
D.D.S., State University of New York at Stony Brook, 1984

A Thesis
Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Dental Science
The University of Connecticut
1987
APPROVAL PAGE

Master of Dental Science Thesis

THE EFFECT OF FIBRINOGEN DERIVED FACTORS ON
POLYMORPHONUCLEAR LEUKOCYTE FUNCTION IN VITRO

Presented by

Reed Ference,
B.A., D.D.S.

Major Advisor
Donald L. Kreutzer

Associate Advisor
Mark Patters

Associate Advisor
Frank Nichols

The University of Connecticut
1987
ACKNOWLEDGEMENTS

This research effort would not have been possible with the support and guidance of Dr. Donald L. Kreutzer. His extensive knowledge of inflammation, as well as his dedication to research and teaching have made my work in his laboratory a particularly rewarding experience.

The many hours spent working in the laboratory would not have been enjoyable or productive without support from the graduate students and technical staff in the laboratory. I am grateful for their friendship and assistance.

I would like to thank my thesis advisors, Dr. Frank Nichols and Dr. Mark R. Patters, each of whom made invaluable contributions to this thesis.

I am indebted to the faculty and staff of the departments of Periodontology and Pathology. I thank the faculty for sharing their knowledge with me and the staff for their support. I am particularly grateful to Rinette Pelletier and Gayle D'Abate for their secretarial assistance and the typing of this manuscript.

Finally, I want to thank my wife, Jody, for her help, understanding and support during the preparation of this thesis.
TABLE OF CONTENTS

OVERVIEW 1

REVIEW OF THE LITERATURE AND RATIONALE 5

HYPOTHESIS AND SPECIFIC AIMS 17

METHODS 20

RESULTS 39

DISCUSSION 75

APPENDICES 90

REFERENCES 97
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Tables</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Effect of 3 FDF Preparations on PMN Viability</td>
<td>44</td>
</tr>
<tr>
<td>2. Effect of FDF on Resting and Stimulated Chemiluminescence</td>
<td>57</td>
</tr>
<tr>
<td>3. Pronase E Peptide Bond Specificity</td>
<td>70</td>
</tr>
<tr>
<td>4. Summary Table</td>
<td>78</td>
</tr>
<tr>
<td>Figures</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1. Regulatory Model</td>
<td>4</td>
</tr>
<tr>
<td>2. Fibrinogen Metabolism</td>
<td>7</td>
</tr>
<tr>
<td>3. Protocol for Fibrinogen Purification</td>
<td>22</td>
</tr>
<tr>
<td>4. Protocol for FDF Production</td>
<td>24</td>
</tr>
<tr>
<td>5. SDS-PAGE 18-hour Plasmin Digestion of Fibrinogen</td>
<td>41</td>
</tr>
<tr>
<td>6. Effect of FDF on Human PMN Adherence to Plastic</td>
<td>45</td>
</tr>
<tr>
<td>7. Scanning Electron Microscopy Evaluation of the Effects of FDF on Human PMN Morphology</td>
<td>47</td>
</tr>
<tr>
<td>8. Ultrastructural Analysis of Buffer Treated (Control) Human PMN by TEM</td>
<td>49</td>
</tr>
<tr>
<td>9. Ultrastructural Analysis of the Effects of FDF on Human PMN by TEM</td>
<td>50</td>
</tr>
<tr>
<td>10. Effect of FDF on PMN Chemotaxis</td>
<td>52</td>
</tr>
<tr>
<td>11. Effect of FDF on PMN-SRBC Adherence</td>
<td>54</td>
</tr>
<tr>
<td>12. Phagocytosis of Opsonized RBC by PMN</td>
<td>55</td>
</tr>
<tr>
<td>Figures</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>13. H$_2$O$_2$ Assay Standard Curve</td>
<td>58</td>
</tr>
<tr>
<td>14. Effect of FDF on Stimulated H$_2$O$_2$ Release</td>
<td>60</td>
</tr>
<tr>
<td>15. Effect of FDF on PMN Lactoferrin Release</td>
<td>62</td>
</tr>
<tr>
<td>16. Effect of FDF on PMN Glucuronidase Release</td>
<td>63</td>
</tr>
<tr>
<td>17. Effect of FDF on PMN Lysozyme Release</td>
<td>64</td>
</tr>
<tr>
<td>18. Killing of <em>S. aureus</em> by Human PMN 10:1 PMN/bacteria</td>
<td>65</td>
</tr>
<tr>
<td>19. Killing of <em>S. aureus</em> by Human PMN 1:1 PMN/bacteria</td>
<td>66</td>
</tr>
<tr>
<td>20. Antibody Blocking of FDF Activity</td>
<td>68</td>
</tr>
<tr>
<td>21. Separation of FDF Activity by Molecular Weight</td>
<td>69</td>
</tr>
<tr>
<td>22. Protease Sensitivity of FDF Activity</td>
<td>71</td>
</tr>
<tr>
<td>23. Effect of FDF on PMN LFA-1 Antigen</td>
<td>73</td>
</tr>
<tr>
<td>24. Effect of FDF on PMN MO-1 Antigen</td>
<td>74</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>FDF</td>
<td>fibrinogen derived factors</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamine gel electrophoresis</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salts solution</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte or neutrophil</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>SRBC</td>
<td>sheep red blood cell</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>FMLP</td>
<td>formylmethionylleucylphenylalanine</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenases</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>O.D.</td>
<td>optical density</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>Fibrin(ogen)</td>
<td>fibrin and/or fibrinogen</td>
</tr>
<tr>
<td>A.212</td>
<td>absorbance at 212 nm</td>
</tr>
</tbody>
</table>
OVERVIEW

Fibrin is ubiquitous in inflammatory disease processes and is thought to be rapidly metabolized by a variety of fibrinolytic enzymes (e.g. plasmin) producing, at the site of inflammation, a wide range of degradation products. These products of fibrinogen metabolism (designated Fibrinogen Derived Factors, or FDFs) have been shown to possess wide ranging biological activities in vitro and in vivo.\(^{63}\) For example, FDFs have been shown to affect coagulation,\(^{5}\) vasopermeability,\(^{22}\) endothelial cell integrity,\(^{64,65}\) inflammatory cell chemotaxis,\(^{21}\) as well as lymphocyte proliferation and antibody production.\(^{20}\) In light of the immunomodulatory effect that FDF's have on other inflammatory cells (e.g. lymphocytes), our laboratory has hypothesized that FDFs also modulate neutrophil function in vitro and in vivo (see Figure 1). It was hypothesized that host and/or bacterial derived factors present at a disease site generate specific FDFs which are capable of altering the PMN (neutrophil) causing down regulation or suppression of neutrophil function. For the purpose of this thesis, "down regulation" will refer to suppressing neutrophil function and "up regulation" will refer to augmentation of neutrophil function. The resulting "suppressed" neutrophils may be deficient in phagocytosis and killing of bacteria which may result in the proliferation of bacteria at the diseased site. Fibrin deposition increases from the resultant tissue damage, and the cycle repeats itself. With this hypothesis in mind, the general objective of this study was to determine if FDFs known to be produced locally in an inflammatory lesion can modulate neutrophil function and host defense in vivo.

Since no systematic investigations to date have been undertaken to study the effects of FDFs on neutrophil function, short term goals of the
present studies were developed to evaluate the in vitro effects of FDFs on key neutrophil functions involved in host defenses. Although there are a wide number of neutrophil functions that contribute to host defense, the direct microbicidal activity of the neutrophil is central to host defense. Thus, initial experiments focused on determining the direct effect of FDF on microbicidal activity. Preliminary data generated at the outset of this study demonstrated the ability of FDF to significantly inhibit PMN microbicidal activity.

PMN microbicidal activity may be divided into adherence dependent phenomena, oxygen dependent microbicidal phenomena, and oxygen independent microbicidal phenomena, and oxygen independent microbicidal phenomena. Therefore in order to gain insight into the mechanism of FDF inhibition of microbicidal activity, the effect of FDF on adherence, phagocytosis (adherence dependent), $H_2O_2$, chemiluminescence (oxygen dependent), and degranulation (oxygen independent) were studied.

The long term goal of this project is to determine the role of FDFs in inflammation and host defense in general and in human periodontitis specifically.

Periodontal disease is an excellent model for studying host-parasite interactions in a chronic inflammatory lesion. Organisms capable of producing fibrinolytic enzymes have been found in increased numbers in periodontal disease. Fibrin has been found in diseased periodontal disease tissues and in gingival fluid obtained from periodontitis lesions. Moreover, FDF have not been detected in clinically normal tissues. Since neutrophils have been shown to be essential in preventing the progression of inflammatory periodontal disease, it is hypothesized that the production of
FDF's by bacteria inhibit neutrophil function in periodontal disease allowing for disease progression. The identification of specific FDF's will not only provide insight into the mechanism involved in inhibition of neutrophil function, but may provide a useful diagnostic tool in identifying local inflammatory lesions in which connective tissue destruction is occurring. Finally, it is hoped that *in vitro* findings can be extended to a relevant *in vivo* disease model.
FIGURE: 1

Hypothetical Model for the Role of FDF in Regulating Leukocyte Function During Inflammation and Host Defense

Microbes Present At A Diseased Site

Proliferation of Bacteria

Increased:
1. tissue damage
2. fibrin deposition

Bacterial Enzymes

FDF

Binding to PMN Membrane

Down-regulation

Altered Neutrophil
REVIEW OF THE LITERATURE

AND RATIONALE

Fibrin is a ubiquitous protein found in virtually all inflammatory processes, e.g., renal disease, transplant rejection phenomena, disseminated intravascular coagulation, neoplasia, and inflammatory periodontal disease to name but a few. Previously, fibrin was thought to function primarily as a hemostatic plug, with past investigations dealing primarily with the physical manifestations of fibrin deposition, e.g., fibrin's role in hemostasis, tissue repair and limiting the spread of microorganisms from the disease site. Interestingly, relatively little work has been done concerning the biological role of peptides derived from fibrinogen metabolism. This is surprising in light of: 1) interrelationships that have been shown to exist between the coagulation system and inflammatory processes, 2) the high concentration of these peptides found in many inflammatory lesions and 3) evidence establishing that these peptides do have effects in vivo and in vitro. For example, during acute inflammatory reactions in models of acute respiratory disease, a reduction of pulmonary edema has been seen following fibrinogen depletion. In chronic inflammation, a role for fibrin has been suggested through the use of anticoagulants, e.g. heparin has been shown to inhibit intraocular tuberculin hypersensitivity. This observation has been supported by other studies that demonstrate that the anticoagulants ameliorate the effects of delayed type hypersensitivity reactions. In addition, the typical induration present in delayed type hypersensitivity reactions is not seen in individuals with a rare afibrinogenemia. Anticoagulants and genetic defects in fibrinogen production were used in the studies cited above to limit fibrin accumulation
and FDF production, however, in an inflammatory reaction \textit{in vivo} FDF production and their concentration is dependent upon the balance of protease and anti-protease systems.

Thus a variety of FDFs may be obtained from thrombin, plasmin, or cellular derived protease metabolism of fibrinogen. A brief review of the general pathways of fibrinogen metabolism and the biological activities associated with their metabolites is provided below.

\textbf{Fibrin Metabolism}

The formation of fibrin from its precursor fibrinogen is the result of a complex system of regulatory zymogen enzymes which function as serine proteases. These zymogen enzymes, or coagulation factors, have been assigned Roman numerals I-XIV, and are divided functionally into two pathways; the intrinsic pathway and the extrinsic pathway (see figure 2). The intrinsic pathway and the extrinsic pathways are linked through their conversion of Factor X which is the first key reaction leading to the formation of a clot. The other two key reactions are: 1) the formation of thrombin and 2) the formation of fibrin. Clotting involves an interplay between the two pathways and each is needed for proper clotting. The intrinsic pathway is activated by exposure of intrinsic blood components (Factor XII) to a negatively charged surface (subendothelial collagen, phospholipids). The extrinsic pathway is activated when trauma to a blood vessel releases a lipoprotein called Tissue Factor. A complex of Tissue Factor and Factor VII then catalyze the activation of Factor X, which along with Factor V and Platelet Factor 3 cleaves prothrombin to produce thrombin. The enzyme thrombin is responsible for the generation of fibrin from its precursor fibrinogen. Fibrinogen is comprised of 3 pairs of
Figure 2: Fibrinogen Metabolism Which Produces Fibrinogen Derived Factors
polypeptide chains designated Aα, Bβ and γ. Thrombin cleaves four arginine-glycine bonds in fibrinogen releasing four peptides: an "A" peptide from each alpha chain, and a B peptide from each of the two β chains. These peptides are referred to as fibrinopeptides, while fibrinogen devoid of these peptides is referred to as fibrin monomer. Fibrin monomers aggregate end-to-end and side-to-side and polymerize, being held together by hydrophobic bonds, and this fibrin is referred to as soluble fibrin. At the time of fibrinopeptide release, thrombin activates Factor XIII (i.e., fibrinoligase) which replaces the hydrophobic bonds on the polymerized fibrin with covalent bonds forming an insoluble fibrin polymer. The coagulation cascade, if unregulated, could lead to excessive fibrin formation, pathologic thrombosis, and tissue damage. Fibrinogen metabolism is regulated by a variety of proteases and antiprotease which controls fibrin deposition and the fibrinolysis.

Control of fibrin deposition is achieved by protease inhibitors such as anti-thrombin III which blocks most of the serine proteases in the coagulation cascade. Other minor inhibitors of the coagulation cascade include: anti-trypsin, α2 macroglobulin, and C1 esterase inhibitor. If these inhibitors fail to prevent excessive fibrin formation, then the fibrinolytic system plays a major role in removing potentially harmful fibrin deposits from the vasculature and/or tissues. Fibrinolysis can be considered to be the physiologic counterpart to the process of fibrin deposition. Its function, along with the inhibitors mentioned above, is to limit the accumulation of fibrin. The major enzyme responsible for fibrinolysis is plasmin and it exists in plasma as its precursor plasminogen. Plasminogen is present in serum as a single polypeptide chain with a molecular weight of 90,000. The principal
site of plasminogen synthesis is the liver although some controversy exists concerning other sources\textsuperscript{18}. Plasminogen is converted to plasmin through the enzymatic cleavage of two peptide bonds liberating two peptide chains; a light chain and a heavy chain; the active site of plasmin is located on the light chain. Numerous activators of plasminogen exist and the most important physiologic pathways for plasminogen activation are the Hageman factor pathway and endothelial synthesis of plasminogen activator. The Hageman factor pathway functions through the interaction of factor XIIa with a plasminogen proactivator. This interaction leads to production of an activator that converts plasminogen to plasmin which then degrades fibrin. Tissue plasminogen activator (t-PA), secreted by a variety of cells including macrophages and vascular endothelial cells, is a single polypeptide chain with a molecular weight of 70 kDa. The rate of plasminogen activation by t-PA increases several hundred fold by the presence of fibrin suggesting the formation of a t-PA-fibrin complex. Plasmin has wide ranging enzymatic activity and has an equal affinity for fibrin and fibrinogen. Due to its wide ranging enzymatic activity, plasmin is capable of degrading coagulation factors and activating complement. The degradation of coagulation factors can lead to hemorrhage, and the activation of complement has multiple effects such as increased vascular permeability and red blood cell hemolysis.

Since plasmin has multiple biological activities and its precursor is found in most biologic fluids,\textsuperscript{6} regulation of plasmin activity must occur. Inhibitors of plasmin activity include alpha\textsubscript{2}-antiplasmin, alpha\textsubscript{2}-macroglobulin and C1 esterase inhibitor. The lysis of fibrin and fibrinogen by plasmin proceeds by cleavage of the carboxy terminal of the A\alpha chain. The process continues with destruction of the B\beta chain and finally the \gamma chain is
degraded. This process of enzymatic digestion results in the production of four clinically recognized FDFs, the X, Y, D and E fragments, in order of production as well as low molecular weight peptides. The low molecular weight peptides are not well defined as to their structure, however they have been shown to possess biologic activity.

The degradation of fibrin and fibrinogen can occur by the action of enzymes other than plasmin. For example, inflammatory cells such as neutrophils and macrophages are capable of degrading fibrin, producing FDFs. Neutrophil derived elastase cleaves fibrin, producing peptides different from those produced by plasmin. Thus fibrinogen can be metabolized by a wide variety of cell-derived proteases producing numerous large and small peptides that have been shown to possess biologic activities.

**Biological Effects of FDF**

Fibrinogen derived factors have been shown in the literature to have a variety of biological effects in vivo and in vitro. Provided below is a brief review of the effects of FDF on 1) coagulation, 2) vasopermeability and 3) leukocyte/lymphocyte function. Information pertaining to microbicidal degradation of fibrinogen and its possible relationship to periodontal disease is also provided.

This review is not only provided for the purpose of a general background, but to provide the foundation for the hypothesis of the role of FDF in regulating PMN function. For example, information pertaining to the effect of FDF on cells and factors involved in coagulation and vasopermeability was included due to the importance of these processes in inflammation and host defense. Background information is provided on the ability of bacteria to degrade fibrinogen producing FDF was cited to suggest
a link between bacterial derived FDF and modulation of host defense. Finally, evidence pertaining to FDF and periodontal disease was included to show how these factors may affect an inflammatory disease of bacterial origin.

**FDF and Coagulation**

Inhibition of coagulation is important in diseases where fibrinolysis predominates, e.g., carcinomas, and disseminated intravascular coagulation. In the case of disseminated intravascular coagulation (DIC), the presence of FDF's determines the clinical signs and symptoms of this condition. In DIC, if fibrinolysis is dominant, hemorrhage occurs. FDF's have profound effects on the coagulation system and have been shown to be anti-coagulants in vivo. These studies demonstrated that FDFs directly inhibited coagulation by 1) interfering with fibrin monomer polymerization and 2) inducing a defect in platelet function.

**FDF and Vasopermeability**

In addition to their effects on coagulation, FDF's have direct effects on the vasculature. FDF's have been shown to increase vascular permeability and induce isolated arterial contraction. A series of studies have described the isolation and characterization of peptides which have vasopermeability effects in vivo. These peptides have been identified as Aα 224-324 from the middle region of the Aα chain and Bβ 43-47. In a series of reports, Kreutzer and colleagues have shown that FDF's related to Bβ1-42 can induce vascular endothelial cell injury in vitro. Moreover, these FDF's were shown to injure vascular endothelial cells in aorta organ cultures, and to induce vasopermeability in skin. These findings are significant when one considers the role of the vasculature in
inflammatory reactions. Endothelial cells are intimately involved with the regulation of vascular permeability. An increase in vasopermeability is one of the first signs of inflammation allowing for the exchange of inflammatory cells from the vasculature to a tissue site. This increase in vasopermeability is followed by recruitment of cells to the site of inflammation. Since fibrin is present at high concentrations at many inflammatory sites, its effect on inflammation or inflammatory cells may be significant.

Effect of FDF On Leukocyte/Lymphocyte Function

As mentioned above, recruitment of inflammatory cells to the site of inflammation is necessary for the process of inflammation to occur. FDF’s have been shown to affect leukocyte/lymphocyte function. For example, FDF’s (Bβ 30-43) have been shown to induce neutrophil chemotaxis in skin. Leukocyte accumulation at two hours was significantly greater with injection of FDF’s than with injection of leukotriene B4.68 The above report suggests that peptides that are cleaved early in the formation of a fibrin clot (Bβ 30-43) enhance chemotaxis of neutrophils. Girmann et al. first suggested that FDF derived from the plasmic cleavage of fibrinogen suppress the immune response in vivo, and inhibit the response of blood mononuclear cells to phytohemaglutinin.27 This work has been extended by Saldeen and Edgington,27,20 who found that FDF’s inhibit the proliferative response of lymphocytes to mitogens and to allogeneic cell stimulation. Inhibition was dose specific and could be elicited before, at the time of, or as late as 48 hours after mitogen stimulation.

In vivo experiments have demonstrated that low molecular weight FDF can suppress cell mediated immune reactivity61 and are associated with enhancement of tumor growth in BALB/c mice62. Recently, receptor
mediated binding of large molecular weight FDFs to macrophages has been described. The binding of FDFs to microphages has also been shown to increase production of the monokine Hepatocyte Stimulating Factor. Therefore, the binding of FDF to macrophages may represent a feedback regulatory mechanism by which fibrinogen synthesis is regulated.

**Fibrinogen Metabolism and Microbes**

The effect of FDF's on leukocyte/lymphocyte function is diverse, i.e. chemotaxis and lymphocyte responses to mitogens. Clearly, the body of literature to date supports the role of FDF's as modulators of local inflammatory reactions. If FDF's function as local modulators of inflammation, it is not unreasonable to hypothesize that bacteria have developed ways of using the regulatory effects of FDF's to their advantage.

Classically fibrin has been thought to wall-off an inflammatory lesion and act as a barrier to the spread of microorganisms contained in the lesion. However, very little work has examined the identity of FDF produced by bacterial enzymes and the effects of these products on host inflammatory cells. This is surprising in light of 1) the growing body of literature demonstrating that FDF's have effects on inflammatory cells, and 2) the ability of many pathogens to synthesize extracellular factors that produce FDF's. Streptokinase, produced by β hemolytic streptococci, is the most widely known factor responsible for FDF production. Streptokinase is a single chain protein of molecular weight 47,000. Streptokinase functions by forming a complex with plasminogen and thereby inducing a conformational change. This conformational change exposes an active enzymatic site within the complex which may cleave an arginal-valine bond in other plasminogen molecules forming plasmin. Other bacteria (in addition to β hemolytic Strep)
produce fibrino(gen)olytic enzymes. Oral bacteria such as *Bacteroides oralis* produce a streptokinase-like molecule which acts indirectly by activating plasminogen.\(^{53}\) *Bacteroides gingivalis* (an organism associated with periodontitis disease sites) possesses a membrane-bound protease which causes fibrinolysis.\(^{53}\) *Treponema denticola* produces an enzyme which acts directly on fibrin to effect lysis.\(^{53}\) In a recent study, a correlation was found between possession of fibrino(gen)olytic activity and the presence of microorganisms in inflammatory disease sites. Bacteria were isolated from deep periodontal pockets and the predominating strains isolated were tested for fibrino(gen)olytic activity. Fusobacterium, Bacteroides and Actinomyces species all exhibited fibrino(gen)olytic activity.\(^{77}\) These organisms are found in increasing numbers in adult periodontitis.\(^{69}\) These findings suggest that fibrino(gen)olytic enzymes produced by bacteria may function as virulence factors. However, to discuss a possible role for bacterial factors related to fibrinogen metabolism in the progression of chronic disease processes an experimental model must be selected, periodontal disease represents such a model.

**Fibrinogen Metabolism and Periodontal Disease**

Periodontal disease is an excellent prototypical model for studying and illustrating host-parasite-fibrinogen metabolism interrelationships. It is ideally suited because the lesions and disease activity are localized (fibrin exerts its modulating effects locally), the lesions are easily accessible, and documented animal models exist.\(^{55}\) Regardless of these advantages and the almost universal presence of fibrin in inflammatory lesions, little work has been done to examine FDF's role in periodontal disease. Provided below is a
brief review of what is known about FDF's and host-parasite relationships in periodontal disease.

Bacteria, which are suspected periodontal pathogens, have been shown to possess the ability to degrade fibrin (as discussed above). Fibrin has been shown to be present in periodontal pockets, and has been seen lining the pocket wall in close proximity to emigrating neutrophils. In related studies, neutrophil enzymes have been shown to be capable of degrading fibrinogen. A limited number of studies have looked at the capability of epithelial cells lining the periodontal pockets to cause fibrinolysis. These cells lining the pocket have been shown to secrete plasminogen activators and cause fibrinolysis in humans and a number of animals. Plasminogen activator synthesis has been reported to be a function of cell keratinization and cell turnover. Epithelial cell production of plasminogen activators may be of significance in the pathogenesis of periodontal disease due to 1) plasmin's ability to activate complement, and 2) fibrinolysis produces FDF's. One study has looked at the presence of FDF's in the gingival fluid of healthy patients and those with periodontitis. FDF's were found in 12 of 14 patients with periodontitis. FDF's were not found in patients who were clinically healthy. Therefore, the necessary enzymes (bacterial, neutrophil or tissue) and substrates [fibrin(ogen)] are present in periodontal pockets for the production of FDF's.

It is possible that the association between FDF's and periodontal disease may be due to effects that FDF's have on neutrophil function. To date, no studies have investigated the effects of FDF's on neutrophil microbicidal functions. Neutrophils are present in the gingival sulcus, and existing evidence suggests that they play a protective role. Evidence to support
this role is based on periodontal disease severity in patients afflicted with neutrophil abnormalities such as Chediak-Higashi syndrome and neutropenic states.\textsuperscript{29,14} Patients with defects in neutrophil function, or possessing decreased numbers of neutrophils exhibit early severe periodontal destruction and loss of teeth.\textsuperscript{14} In addition, it has been reported that the phagocytic activity of neutrophils in severe periodontal lesions is diminished locally.\textsuperscript{49} Therefore FDFs have been shown to be associated with a disease process in which the local control of neutrophil function is of primary importance.
HYPOTHESIS AND SPECIFIC AIMS

As was presented in the previous section, FDF have been shown to be associated with a wide range of disease processes in which host defense is of primary importance. Previous investigations of the effects of FDF on immunologic cell function have focused on the lymphocyte. Since the neutrophil plays an important role in host defense, the present study was undertaken to determine the effect of FDF on PMN function in vitro.

In light of the fact that FDF have been shown to affect lymphocyte function in vitro and in vivo, the following general hypothesis was developed: that local generation of FDF by either tissue or microbe derived proteases can result in the down regulation of neutrophil function i.e. suppression. This down regulation of neutrophil function result in depressed host defense with resulting proliferation of bacteria at a disease site. This increase in bacterial proliferation results in increased tissue damage, fibrin deposition and the cycle repeats itself.

To begin to test this hypothesis, the effect of FDF on PMN microbicidal activity was studied. Preliminary data were developed at the outset of this study which demonstrated the ability of low molecular weight peptides (FDF) derived from the plasmin digestion of fibrinogen to inhibit PMN phagocytosis and killing of bacteria. Therefore to dissect the molecular/cellular basis of FDF activity, specific aims were developed to systematically study the effect of FDF on those functions involved in microbicidal activity. Functions involved in PMN microbicidal activity can be divided into adherence dependent functions (binding of a microbe to the PMN, phagocytosis) oxygen dependent functions (H$_2$O$_2$ production) and oxygen independent functions (degranulation). The effect of FDF on chemotaxis was studied because this
function is directly related to host defense and microbicidal activity. Those specific aims are as follows:

**Specific Aims**

**SPECIFIC AIM 1:** To determine the effect of FDF on neutrophil functions involved in host defense.

**Question 1:** What is the Effect of FDF on PMN chemotaxis?

**Study 1:** Effect of FDF on chemotaxis

**Study 2:** Are FDF effects reversible

**Question 2:** What is the effect of FDF on Fc receptor function and phagocytosis?

**Study 3:** Microscopic quantification of adherent opsonized sheep red blood cells

**Study 4:** Microscopic quantification of phagocytosed sheep red blood cells

**Question 3:** What are the effects of FDF on oxygen dependent microbicidal activity of leukocytes?

**Study 5:** Chemiluminescence

**Study 6:** $H_2O_2$ production

**Question 4:** What are the effects of FDF on leukocyte degranulation?

**Study 7:** Release of granule components extracellularly

**a.** Quantification of lysozyme

**Study 8:** Primary vs. secondary granules

**a.** β glucuronidase quantification as a primary granule marker
b. Lactoferrin quantification as a secondary granule marker

SPECIFIC AIM 3: To characterize the identity of FDF involved in the suppression of leukocyte function.

Study 9: Antibody blocking of FDF activity
Study 10: Protease sensitivity of FDF
Study 11: Separation of FDF according to molecular weight and subsequent testing in a screening assay.

a. Separation of FDF according to molecular weight using Amicon ultrafiltration
b. Testing of molecular weight functions using neutrophil $H_2O_2$ production as a screening assay.

SPECIFIC AIM 4: To determine the effect of FDF on cell surface adhesion molecules.

a. Effect of FDF on LFA-1 expression
b. Effect of FDF on MO-1 expression
### METHODS

Outline

<table>
<thead>
<tr>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation and Purification of Fibrinogen</td>
<td>21</td>
</tr>
<tr>
<td>Assessment of Fibrinogen Purity</td>
<td>21</td>
</tr>
<tr>
<td>Preparation of FDFs</td>
<td>23</td>
</tr>
<tr>
<td>Reconstitution and Standardization of FDF Peptides</td>
<td>23</td>
</tr>
<tr>
<td>Preparation of Control SMW Peptides</td>
<td>25</td>
</tr>
<tr>
<td>Isolation of PMN from Whole Human Blood</td>
<td>25</td>
</tr>
<tr>
<td>Determination of the Effects of FDFs on PMN Viability</td>
<td>26</td>
</tr>
<tr>
<td>Effects of FDF on PMN Morphology</td>
<td>27</td>
</tr>
<tr>
<td>Effect of FDF on PMN Chemotaxis</td>
<td>28</td>
</tr>
<tr>
<td>Adherence (Rosetting)</td>
<td>28</td>
</tr>
<tr>
<td>Erythropagocytosis</td>
<td>29</td>
</tr>
<tr>
<td>Chemiluminescence Assay</td>
<td>30</td>
</tr>
<tr>
<td>PMN H$_2$O$_2$ Assay</td>
<td>31</td>
</tr>
<tr>
<td>Phagocytosis Induced Lysozomal Enzyme Release</td>
<td>32</td>
</tr>
<tr>
<td>Effect of FDF on Degranulation</td>
<td>33</td>
</tr>
<tr>
<td>Assay of Microbicidal Activity</td>
<td>35</td>
</tr>
<tr>
<td>Separation of FDF by Molecular Weight Using Ultrafiltration</td>
<td>36</td>
</tr>
<tr>
<td>Antibody Blocking of FDF Suppressive Activity</td>
<td>36</td>
</tr>
<tr>
<td>Protease Sensitivity of FDF Inhibitory Activity</td>
<td>37</td>
</tr>
<tr>
<td>Effect of FDF on PMN LFA-1 and MO-1 Antigen Levels</td>
<td>37</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>38</td>
</tr>
</tbody>
</table>
METHODS

Isolation and Purification of Fibrinogen

The method used to isolate and purify fibrinogen from plasma was a modification of that employed by Doolittle, et al.19 (see Figure 3). Fresh plasma was obtained (Red Cross, Farmington, CT) and fraction I (corresponding to commercial preparations) was precipitated by the addition of 0.22 volumes of cold 50% ethanol in 0.55 M trisodium citrate pH 7.1 while lowering the temperature to -3°C. After centrifugation, the precipitate was washed with 0.5 original volumes of 7% ethanol pH 6.5 at -3°C. The precipitate was then dissolved in 15 mM phosphate buffer and the pH and ionic strength were adjusted to 7.8 and 6.9 m.mho respectively. The solubilized fibrinogen was then passed through a DEAE ion exchange chromatography disk (AMF, Cuno, Meriden, CT) which has been demonstrated to remove complement component C3 from plasma (Kreutzer unpublished). After ion exchange chromatography, the fibrinogen was placed on a gelatin sepharose column to remove fibronectin contamination. The void volume was collected and cooled to 0°C and "cold insoluble material" removed by the addition of cold 20% ethanol to a final concentration of 2%. Centrifugation removes a mucous like precipitate. Addition of more 20% ethanol to a final concentration of 8% results in the precipitation of fibrinogen used in this study.

Assessment of Fibrinogen Purity

Fibrinogen preparations were assayed for contaminants using: 1) ELISA to measure C3, C5 contaminations; 2) SDS-PAGE; 3) SDS-PAGE reduced fibrinogen for fibronectin contamination; and total amino acid analysis.
Plasma or Fraction I Fibrinogen

Precipitation with a .22 volumes of cold 50% ethanol -3°

Centrifuge and collect pellet

Wash with .5 original volumes 7% ethanol pH 6.5 -3°

Centrifuge and collect pellet

Dissolve in 15 mM phosphate buffer pH 7.8; ionic strength 6.9 mmho

DEAE ion exchange

Removal of C3

Gelatin Sepharose

Removal of fibronectin

Addition of cold 20% ethanol to a final concentration of 2%

Addition of 20% ethanol to a final concentration of 8%

Precipitation of fibrinogen used in experiments

Figure 3: Protocol for Fibrinogen Purification
**Preparation of FDFs**

Purified fibrinogen was dissolved in 0.02 M Tris-HCl 0.1 M NaCl pH 7.8 to a final concentration of 4 mg/ml and warmed to 37°. Plasmin (Sigma, St. Louis, MO) (0.02 u/ml) was added to the fibrinogen and the mixture was incubated for 18 hours stirring at 37°C. Aliquots were saved at 1 min, 15 min, 1 hour, 5 hours, 18 hours, and frozen at -80° for analysis using SDS-PAGE. Following the 18 hour incubation, the large molecular weight fibrinogen was separated from the plasmin generated peptides using an amicon ultrafiltration unit. The unit contained a membrane which excludes proteins >30,000 M.W. The plasmin generated peptides <30,000 M.W. were lyophylized and stored at -20°C for use in the experiments.

**Reconstitution and Standardization of FDF Peptides**

The lyophilized peptides were reconstituted by the addition of distilled water (see Figure 4). Peptide concentration was determined by absorbance at 212 nm, since standard protein assays are unable to quantify small peptides accurately (212 nm is the region where peptide bonds absorb u.v. light). A buffer of 0.02 M tris; 0.1 M NaCl, pH 7.8 was used to blank the instrument. The peptide samples were adjusted to an absorbance of 30, 15, and 7.5 absorbance units for use in experiments. Addition of 0.2 ml of 30, 15 and 7.5 absorbance units to a 0.5 ml PMN solution resulted in a final concentration of 8.6, 4.3, and 2.15 absorbance units respectively. A 0.2 ml aliquot of a 30 absorbance unit solution was found to contain 260 μg/ml of FDF upon amino acid analysis and corresponded to a starting solution of 6 mg/ml of fibrinogen. These concentrations are biologically relevant.
Figure 4: PROTOCOL FOR THE PRODUCTION OF LOW MOLECULAR WEIGHT FDF
Preparation of Control Small Molecular Weight Peptides

Small molecular weight peptides derived from BSA were prepared to rule out any non specific effect small peptides might have on neutrophil function. To prepare these peptides BSA was dissolved in 0.02 M Tris - HCl, 0.1 M NaCl, pH 7.8 to a final concentration of 4 mg/ml. Small molecular weight BSA derived peptides were produced using plasmin and methods identical to that employed for generation of fibrinogen derived peptides.

Isolation of Polymorphonuclear Leukocytes from Whole Human Blood

Human PMN were isolated from citrated venous blood of healthy donors (1 ml 3.8% sodium citrate-dihydrate in 0.85% NaCl per 9 ml blood) using Ficoll-Hypaque gradient centrifugation followed by dextran sedimentation according to a modified method of Boyum. Briefly, a Ficoll-Hypaque Solution was prepared by mixing 35.25 ml of Ficoll (9% wt/vol) with 10.00 ml of Hypaque (50%) and 4.75 ml of distilled H2O. Then 25 ml of diluted blood (1 part whole blood to 1 part 0.85% NaCl) was layered over 13 ml Ficoll-Hypaque solution in a 50 ml polypropylene test tube and centrifuged at 500g at room temperature for 30 minutes. After centrifugation, the blood cells were separated into two fractions, an upper layer corresponding to the mononuclear cell layer at the interface region between the plasma and the Ficoll-Hypaque solution, and a cell pellet which contained erythrocytes and granulocytes. After the removal of the mononuclear cell layer, plasma and the Ficoll-Hypaque Solution, PMN were separated from erythrocytes by adding 30 mls of 3% Dextran (in 0.85% NaCl), mixing and incubating at room temperature for 45 minutes. The supernatant which contained the PMN was removed and centrifuged at 500g for 10 minutes at 4°C. To eliminate the contaminating red blood cells: The resulting pellet was resuspended with red
blood cell lysing solution (0.15 M NH$_4$Cl; 0.01M KHCO$_3$; 0.001M EDTA: pH 7.2) followed by 3 washes with HBSS. The PMNs were adjusted to the appropriate concentration with HBSS for use in experimental studies. The PMN preparations contained 82 to 95% neutrophils as determined by cytocentrifugation and Wright-Giemsa staining. Greater than 98% viability of isolated cells was obtained as determined by trypan blue dye exclusion.

**Determination of Effect of FDFs on PMN Viability**

Preliminary data demonstrated that FDFs inhibited PMN phagocytosis and killing of bacteria. However it was not known whether the FDFs or contaminants in the FDF preparation were toxic to the neutrophils. Trypan blue dye exclusion is typically used to determine cellular viability, but it is not a very sensitive technique. A very sensitive technique for measuring cellular viability is the measurement of lactate dehydrogenase (LDH). LDH is a cytosolic enzyme, therefore detection of LDH in the extracellular fluid is an indication of cell death or loss of membrane integrity. The assay is based on the spectrophotometric measurement of NADH consumption during the LDH catalyzed reduction of pyruvate to lactate.

\[
\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD} + \text{H}^+ 
\]

The protocol for treatment of the PMNs with FDFs was identical to that used in all functional assays. FDFs from three different preparations were used. 0.2 ml of FDF at 30 OD, 15 OD, 7.5 OD were added to 0.5 ml of 1 x 10$^7$ PMN/ml. For the 100% LDH content (positive control) cells were lysed with 0.1% Triton X. Buffer was added to the negative (sham) controls. The cells were then incubated in a shaking waterbath for 20 minutes at 37°C. The PMNs were centrifuged down at 400xg for 10 minutes and the supernatants collected. For the LDH assay 50µl of neutrophil supernatant
substrate was added to 1.0 ml NADH substrate 0.14 mg/ml. 50 µl of Na pyruvate (Sigma 490-1) was added to start the reaction, and the absorbance at 340nm was determined.

**Effects of FDF on PMN Morphology**

a. **Adherence to Plastic**

To determine the effects of FDFs on plastic adherence eutrophils (0.5 ml of 5x10^6 PMN/ml) were incubated with 0.2 ml of FDF at 30, 15 and 7.5 OD unit concentrations (for a final concentration of 8.6, 4.3 and 2.15 OD) for 20 minutes at 37°C. Following the incubation 1 set of samples was added to a 6 well tissue culture plate (Falcon) and incubated for 30 minutes. An identical set of samples was washed 2x with 2.5 ml of HBSS followed by addition to the 6 well plate and incubated for 30 minutes. Photos of the cells were taken using light microscopy 10x.

b. **Transmission and Scanning Electron Microscopy**

To determine the effects of FDFs on neutrophil ultrastructure neutrophils (0.5 ml of 1x10^7 PMN/ml) were incubated with 0.2 ml FDF (final concentration 8.6 OD) for 20 minutes at 37°C shaking in a water bath. Following the incubation, the cells were placed on ice and submitted in suspension for scanning and transmission electron microscopy.

**Effect of FDF on PMN Chemotaxis**

The effect of FDF on PMN chemotaxis was determined using a standard multiwell Boyden chamber assay with 8 µm nitrocellulose filters (Millipore Bedford MA). For these studies PMN were isolated from peripheral blood as previously described and adjusted to 5 x 10^6/ml. The cells (0.5 ml of 5 x 10^6 PMN/ml) were added to 0.2 ml of FDF at 30, 15 and 7.5 O.D. unit concentrations for a final concentration of 8.6, 4.3 and 2.15 O.D. and
incubated for 20 minutes at 37°C shaking in a waterbath. HBSS was added to control PMN. An aliquot (0.250 ml) of each sample was added to the top chamber of the plate and 4% activated serum was used as a chemoattractant and added to the bottom compartment. All samples were assayed in triplicate. The multiwell plates were incubated for 45 minutes in a moist CO₂ chamber followed by staining of the filters. Chemotaxis was quantified using an Olympus microscope coupled to an Optomax image analyzer. Three high power fields were counted for each filter resulting in 9 fields for each sample. Chemotactic indices were determined, and % inhibition of chemotaxis expressed as

\[
\text{mean experimental chemotactic index} - 1 \times 100% \\
\text{mean control chemotactic index}
\]

**Reversibility of FDF Induced Suppression of PMN Chemotaxis**

FDF treated neutrophils and controls were washed to determine if the effect of FDF on PMN chemotaxis was reversible. FDF treated samples and controls were washed 2x with 2.5 ml of HBSS followed by centrifugation (500 x g) for 8 minutes. After the second wash, the PMN were brought up to 1 ml in 0.5% BSA/HBSS. The cells were then assayed for chemotaxis.

**Adherence (Rosetting)**

Adherence of an organism to the neutrophil membrane is an essential step for its subsequent phagocytosis. Adherence is mediated by antibody, the complement component C3b, and their receptors. The use of opsonized sheep red blood cells in quantifying binding can provide a means of measuring Fc receptor function due to the presence of specific anti-sheep red blood cell antibody on the RBC surface. Adherence or rosetting of SRBC was
quantified microscopically to assess Fc receptor function on the neutrophil. For these studies PMNs were isolated from venous blood as described and adjusted to $5 \times 10^6$ cells/ml. FDF aliquots (0.2 ml of O.D. 30, 15, and 7.5) were added to 0.5 ml of cells and incubated for 20 minutes. The volume was then brought to 1 ml by the addition of 0.3 ml HBSS. The 1 ml samples were then split into two 0.5 ml samples and 0.5 ml of sheep red blood cells opsonized with rabbit anti-sheep IgG were added ($2.5 \times 10^6$; PNM/RBC ratio 1:1) to the PMN. The samples were placed on a rotating tube rack for 30 minutes at 37°C. After incubation the samples were placed on ice for 15 minutes to arrest phagocytosis followed by centrifugation at 300g for 15 minutes at 4°C. Extracellular RBC were not lysed. At this point the samples were centrifuged and the resulting pellet washed twice with HBSS. After the final washing the pellet was resuspended with 1 ml of HBSS containing 0.1% BSA. For each reaction sample duplicate smears were prepared by using cytocentrifuge (500 r.p.m. for 5 minutes) followed by Wrights-Giemsa staining. The smears were examined using light microscopy (100x) and the number of adherent red blood cells per PMN in a total of 200 PMN were counted. PMN that appeared to be within the PMN were counted as adherent.

**Erythrophagocytosis**

Binding of a microorganism to a PMN and its subsequent phagocytosis are the first steps involved in the microbicidal process. Therefore, it might be a potentially important step where FDF regulates microbicidal activity e.g., FDF down regulation. The effect of FDF on neutrophil binding and phagocytosis was assayed using the erythrophagocytosis assay. Many phagocytosis assays have inherent problems in that they are unable to
differentiate between adherent and ingested particles. Phagocytosis assays using antibody coated SRBC eliminate this problem because extracellular RBC can be lysed and eliminated (using NH₄Cl lysis) leaving intracellular RBCs intact. For these studies PMNs were isolated from venous blood as described and adjusted to 5 x 10⁶ cells/ml. FDF aliquots (0.2 ml of O.D. 30, 15, and 7.5) were added to 0.5 ml of PMN and incubated for 20 minutes. The volume was then brought to 1 ml by the addition of 0.3 ml HBSS. The 1 ml samples were then split into two 0.5 ml samples and 0.5 ml of sheep red blood cells opsonized with rabbit anti-sheep IgG were added (2.5 x 10⁷; PMN/RBC ratio 1:10) to the PMN. The samples were placed on a rotating tube rack for 30 minutes at 37°C. After incubation the samples were placed on ice for 15 minutes to arrest phagocytosis followed by centrifugation at 300xg for 15 minutes at 4°C. The cell pellet was then resuspended in 2 mls of cold red blood cell lysing solution (0.5 M NH₄Cl, 0.01M KHCO₃, 0.001 M EDTA, pH 7.2) to eliminate adherent RBC. At this point the samples were centrifuged at 300g for 15 minutes at 4°C and the resulting pellet washed twice with HBSS. After the final washing the pellet was resuspended with 1 ml of HBSS containing 0.1% BSA. For each reaction sample duplicate smears were prepared by using a cytocentrifuge (500 r.p.m. for 5 minutes) followed by Wrights-Giemsa staining. The smears were examined using light microscopy (100x) and the number of intracellular red blood cells per PMN in a total of 200 PMN were counted.

**Chemiluminescence Assay**

Adherence of a particle to the neutrophil membrane is associated with an increase in oxidative metabolism known as the "respiratory burst." This event is associated with the production of various chemical species important
in oxygen dependent microbicidal activity such as hydrogen peroxide, superoxide anion and singlet oxygen. Singlet oxygen is formed by the reaction of the H$_2$O$_2$ and hypochlorite which involves the action of the myeloperoxidase-halide system. The formation of singlet oxygen is followed by the emission of light known as chemiluminescence. Chemiluminescence was measured to determine the effect of FDF on PMN oxidative metabolism. Sensitivity was increased by using the cyclic hydrazide luminal which acts as a substrate for singlet oxygen. The assay can be performed on resting or stimulated cells since baseline PMN metabolic activity causes chemiluminescence. Resting chemiluminescence was performed to determine the effect of FDF on PMN devoid of any challenge.

For this assay a 5.5 mM stock solution of luminol in DMSO was prepared and diluted 1:200 prior to use in HBSS. 3.9 ml of HBSS, 0.2 ml of luminol (final concentration 1.0 μM), 4 ml opsonized zymosan (1.25 mg/ml) were added to 10 ml scintillation vials and placed in a β counter shifted to 1 photomultiplier tube (3H channel). The vials were dark-adapted for 20 minutes. Prior to the experiment, 0.5 ml of PMN (5 x 10$^4$/ml) were incubated with 0.2 ml of FDF (for a final concentration of 8.6, 4.3, and 2.15 OD units) for 20 minutes. The cells were brought to a volume of 1 ml and added sequentially to the scintillation vials every minute. Values reported are those for the peak response which routinely occurred after 9 minutes of incubation.

**PMN H$_2$O$_2$ Assay**

Formation and release of the H$_2$O$_2$ is dependent upon phagocytosis and can be used to indirectly quantitate this phenomena. Stimulated PMNs produce a respiratory burst in which oxygen is reduced to superoxide which then dismutates to form H$_2$O$_2$. 
\[ \text{H}_2\text{O}_2 + \text{NADPH} \rightarrow \text{H}_2\text{O}_2 + \text{NADP}^+ + \text{H}^+ \]
\[ \text{H}_2\text{O}_2 + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]

H$_2$O$_2$ produced during the respiratory burst can be quantitated because H$_2$O$_2$ freely diffuses from the cell. In the following experiments H$_2$O$_2$ was quantitated using an assay which makes use of a color change that accompanies the oxidation of phenol red.$^{56}$ Horseradish peroxidase (HRP) uses H$_2$O$_2$ and phenol red as substrates, and in an excess of HRP, the color change of phenol red is proportional to the H$_2$O$_2$ concentration.

PMNs isolated from venous blood were adjusted to a concentration of 2 x 10$^7$ PMNs/ml. FDF aliquots (0.2 ml) at various concentrations 30 OD, 15 OD and 7.5 were added to 0.5 ml cells for a final FDF concentration of 8.6, 4.3 and 2.15 respectively. Buffer was added to controls. The FDF cell mixture was incubated for 20 minutes in a shaking water bath at 37°. The final cell volume was brought to 1.0 ml and 10 µl of phenol red 20 mM, 10 µl HRP (14 mg/ml), and various stimuli (10$^{-6}$ M. FMLP, 10$^{-7}$ M. PMA 10:1 zymosan) were added to the PMNs followed by incubation for 1 hour on a rotating tube rack. NaOH (10 µl, 10 N) was added to stop the reaction. The cells were spun down and the absorbance of the supernatant was determined at 610 nm. A standard curve was obtained using 30% H$_2$O$_2$. The concentration of H$_2$O$_2$ for the standard curve was determined using the molar extinction coefficient for H$_2$O$_2$ (81 moles/cm$^{-1}$ at 230 nm).

**Phagocytosis Induced Lysozomal Enzyme Release**

To determine the effect of FDFs on phagocytosis neutrophils were isolated from peripheral blood and adjusted to 2 x 10$^7$ cells/ml. PMN (0.5 ml) were added to FDF at 30, 15 and 7.5 O.D. units for a final concentration of 8.6, 4.3 and 2.15 O.D. units and incubated as previously described. Enzyme
release was stimulated by the addition of 0.3 ml of opsonized zymosan (3.3 x 10^8/ml) for a final PMN/zymosan concentration of 1:10. The cells were incubated for 45 minutes at 37°C on a rotating tube rack followed by centrifugation (500 g 8 minutes) and removal of the supernatant. 3 different neutrophil supernatants were analyzed for lactoferrin, β glucuronidase, and lysozyme content.

**Effect of FDF on Degranulation**

Degranulation of lysosomal granules is an important step in neutrophil function. These lysosomal granules contain a variety of antimicrobial agents important in the microbiocidal activity of the neutrophil. In addition to their antimicrobial properties, (e.g. lysozyme, lactoferrin), certain intracellular granule constituents can act as primary (azurophil) or secondary (specific) granule markers. The effect of FDF on primary granule release was determined by quantification of β glucuronidase which is a marker for that granule. Secondary granule release was quantified by measuring lactoferrin (a marker for secondary granules). Lysozyme release was quantified since it is found in both primary and secondary granules. Provided below are the methods used to quantify these enzymes.

**β Glucuronidase**

The β glucuronidase assay is dependent on the enzymatic conversion of the chromogenic substrate phenolphthalein glucuronic acid to phenolphthalein and glucuronate:

\[
\text{phenolphthalein glucuronic acid} + \text{H}_2\text{O} \xrightarrow{\beta\text{ glucuronidase}} \text{phenolphthalein} + \text{glucuronate}
\]
The pH of the sample is changed by the addition of glycine buffer, causing a shift in color due to the phenolphthalein. The change in absorbance is directly proportional to the β glucuronidase concentration.

Neutrophil supernatant (0.1 ml) from the experimental and control samples was added to 0.7 ml of sodium acetate buffer (pH 4.5) and 0.2 ml of 0.01 M phenolphthalein glucuronic acid (Sigma). A standard curve was established using phenolphthalein (Sigma) concentrations from 2 to 200 μg/ml (0.7 ml sodium acetate buffer, 0.1 ml standards, 0.2 ml 0.85% NaCl). The standards and unknowns were allowed to incubate for 18 hours at 37°C followed by the addition of 1.0 ml .4 M glycine buffer (pH 10.5). Absorbance of unknowns and standards was determined at 540nm. β glucuronidase concentrations were determined from the standard curve.

**Lactoferrin Assay**

Lactoferrin was quantified using an enzyme linked immunoabsorbant assay. A 96 well microtiter plate was coated with 0.1 ml of 10 μg/ml IgG rabbit antihuman lactoferrin (Cappel, Malvern, PA) in carbonate buffer pH 9.6. The plate was incubated overnight at 4°C followed by washing 3 times with PBS/tween. Neutrophil supernatant (0.1 ml) or lactoferrin standard (Calbiochem, San Diego, CA) 1-64 ng/ml, (dissolved in 1% BSA/PBS) was added to the plate and incubated for 90 minutes at 37°C. The plate was washed 3 times followed by the addition of 0.1 ml of peroxidase conjugated antilactoferrin (Cappel, Malvern, PA) 1:400 dissolved in 1% BSA/PBS. The plate was incubated for 90 minutes and washed 3 times. 0.1 ml substrate (25 ml citric acid buffer, 25 ml d H2O, 5 mg phenylene diamine 0.018 ml 30% H2O2) was added at timed intervals. After sufficient color change, the reaction was stopped with 8N H2SO4. Absorbance was read at 490 nm and
the lactoferrin concentration of the unknowns determined from the standard curve.

**Lysozyme Assay**

Lysozyme hydrolyzes the mucopeptide cell wall structure of the organism *Micrococcus lysodeikticus*. This process is accompanied by loss of absorbance over time which is proportional to the lysozyme concentration.

A 0.25 mg/ml suspension of *Micrococcus lysodeikticus* (Sigma, St. Louis, MO) was prepared using 0.1M phosphate buffer. 0.1 ml of standard or neutrophil supernatant sample was added to 1 ml of the *Micrococcus* suspension and the loss of O.D. at 450 nm was determined using a spectrophotometer with kinetics software (Beckman, Brea, CA).

**Assay of Microbicidal Activity**

Chemotaxis, adherence, the generation of oxygen metabolites, and degranulation all function to defend the host against invading microorganisms. FDF have been found to affect many of the above functions and one could hypothesize that FDF might affect killing as well. To test whether FDF affect killing, the *S. aureus* killing assay was chosen. This assay has an advantage in that the compound lysostaphin can be used to lyse extracellular bacteria if needed.

*S. aureus* was prepared by overnight culture (18 hours) in trypticase soy broth, washed and adjusted to $5 \times 10^7$/ml using a standard curve (CFU vs. A$_{650}$). FDF (0.2 ml) at various concentrations 30, 15, 7.5 were added to 0.5 ml of neutrophils for a final FDF concentration of 8.6, 4.3 and 2.15, respectively. The PMN-FDF mixture was placed in a waterbath for 20 minutes at 37°C. After the incubation, 0.5 ml bacteria at $5 \times 10^7$ and $5 \times 10^6$ were added to the PMN for 1:1 and 10:1 PMN/bacteria ratios, respectively.
The PMN-bacteria samples were placed on a rotating tube rack and incubated for 1 hour at 37°C. Following the incubation, 0.2 ml of each sample was added to 1.8 ml of H₂O for hypotonic lysis of the neutrophils. Serial dilutions were carried out and the bacteria were plated out on Mueller-Hinton agar plates. The plates were counted and CFU determined following a 24 hour culture.

**Separation of FDF by Molecular Weight Using Ultrafiltration**

FDF <30,000 daltons were separated according to molecular weight using Amicon ultrafiltrations (Lexington Mass). 200 ml of FDF <30,000 MW were run over an Amicon PM-10 membrane (excludes proteins >10,000 daltons). The retentate, (10,000 - 30,000 daltons) was resuspended to its original volume with 0.02 M tris buffer and lyophylized. The filtrate was then run over a YM-2 and the resulting retentate (100 - 10,000 daltons) and filtrate (<1000 daltons) were lyophylized. Before incorporation into the H₂O₂ assay, all fractions were normalized according to peptide concentration using absorbance at 212 nm.

**Antibody Blocking of FDF Suppressive Activity**

If the inhibitory activity demonstrated in the previous experiments is fibrinogen related, then if epitopes are present antibody should be able to remove all or part of the inhibitory activity. The following experiments make use of protein A linked sepharose beads (Sigma) which bind the Fc portion of IgG thereby forming an insoluble complex.

100 mg of protein A linked sepharose beads were incubated with 1 ml of rabbit anti-human fibrinogen serum for 1 hour. Normal rabbit serum was used as a control and treated in the same way. Both samples were washed exhaustively with PBS following the incubation. The sepharose-protein A-IgG
was centrifuged for 20 minutes at 500 g. The pellets were saved and reacted with 1 ml of FDF for 1 hour. Following the incubation the beads were spun down and the supernatant (FDF) was saved. These FDF samples were diluted and used in the H$_2$O$_2$ assay using opsonized zymosan as a stimulus.

**Protease Sensitivity of FDF Inhibitory Activity**

A nonspecific neutral protease was added to the FDF to determine if the FDF inhibitory activity is protease sensitive. Protease (2 units Pronase E, Sigma) from *Streptomyces griseus* made insoluble by covalent attachment to beads was added to 1 ml FDF 30 OD for 3 hours at 30°C (1 unit will hydrolyze 1.0 µmole of Nα Benzoyl-L Arginine ethyl ester per minute, pH 7.0, 30°C). Following the incubation the beads were removed by centrifugation (500 x g 8 minutes). Protease treated samples were then incorporated into the PMN H$_2$O$_2$ assay. To control for nonspecific sticking of peptides to the beads, and equal volume of Sepharose 4b was added to 1 ml FDF removed by centrifugation and the supernatant collected and incorporated into the H$_2$O$_2$ assay. The ability of FDF and protease treated FDF to inhibit stimulated (10^-6M f-met-leu-phe) H$_2$O$_2$ production by PMN was tested.

**Effect of FDF on PMN LFA-1 and MO-1 Antigen Levels**

PMN (0.5 ml, 1.0x10^7/ml) were incubated with buffer or 0.2 ml FDF for a final FDF concentration of 8.6, 4.3 and 2.15 as previously described. Following a 30 minute incubation with the FDF at 37°C, the PMN were placed on ice and brought up to 1 ml with 0.3 ml of PBS with 0.02% azide for a final PMN concentration of 5x10^6/ml. 0.2 ml of the PMN were added to 0.005 ml mouse anti-human LFA-1 (Ortho diagnostics Raritan, NJ) or 0.005 ml of mouse anti-human MO-1 (Coulter Immunology Hialeah, FL) and
incubated for 45 minutes on ice. After the incubation period 2.5 ml of PBS (0.02% azide) were added to each tube and centrifuged 400 g 8 minutes. The supernatant was decanted and the pellet washed 2 more times with PBS with azide. After the last wash, 0.005 ml of goat anti-mouse IgG-FITC (Coulter, Hialeah, FL) was added to each tube and incubated for 30 minutes. The cells were then washed 3X as above. After the last wash 0.5 ml of 2% buffered formalin was added to each tube for 30 minutes to fix the cells; followed by centrifugation. The labelled cells were resuspended in 0.5 ml PBS with azide and stored in the dark at 4°C until analysis.

Analysis was performed at the FACS service (U. Conn. Health Center Department of Pathology) on a Becton Dickinson FACS analyzer with data analyzed on a Becton Dickinson consort 30 mini-computer. Cells were gated on the granulocyte population as observed by viewing volume versus side scatter.

Statistical Analysis

For each experiment, control cells (buffer treated) were paired with experimental (FDF treated) cells. For this reason, Student's paired t test was used to determine whether differences between control and experimental cell responses were statistically significant.
## RESULTS

**Outline**

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assessment of Fibrinogen Purity</td>
<td>40</td>
</tr>
<tr>
<td>Pattern of Plasmin Digestion of Fibrinogen</td>
<td>42</td>
</tr>
<tr>
<td>Effect of FDF on PMN Viability</td>
<td>42</td>
</tr>
<tr>
<td>Effect of FDF on PMN Morphology</td>
<td>43</td>
</tr>
<tr>
<td>Effect of FDF on PMN Chemotaxis</td>
<td>51</td>
</tr>
<tr>
<td>Effect of FDF on SRBC-PMN Adherence</td>
<td>53</td>
</tr>
<tr>
<td>Effect of FDF on PMN Phagocytosis</td>
<td>53</td>
</tr>
<tr>
<td>Effect of FDF on PMN Chemiluminescence</td>
<td>56</td>
</tr>
<tr>
<td>Effect of FDF on H$_2$O$_2$ Production</td>
<td>56</td>
</tr>
<tr>
<td>Effect of FDF on Degranulation</td>
<td>59</td>
</tr>
<tr>
<td>Effect of FDF on PMN Microbicidal Activity</td>
<td>61</td>
</tr>
<tr>
<td>Antibody Blocking of FDF Suppressive Activity</td>
<td>61</td>
</tr>
<tr>
<td>Separation of FDF Activity by Molecular Weight</td>
<td>67</td>
</tr>
<tr>
<td>Effect of Pronase E on FDF Inhibitory Activity</td>
<td>67</td>
</tr>
<tr>
<td>Effect of FDF on PMN LFA-1 and MO-1 Antigen Levels</td>
<td>72</td>
</tr>
</tbody>
</table>
RESULTS

Assessment of Fibrinogen Purity

In order to attribute any bioactivity to FDFs, establishing the purity of the fibrinogen used is clearly important. The use of pure fibrinogen is of utmost importance to these studies. Previous studies in Dr. Kreutzer’s laboratory had established the commercially available fibrinogen preparations contain impurities such as complement and fibronectin which have biologic activities. Therefore SDS-PAGE (reduced and non-reduced), as well as enzyme linked immunoabsorbent assays specific for C3 and C5, were used to determine fibrinogen purity.

SDS-PAGE Analysis: The fibrinogen preparation was found to have no impurities such as albumin detectable by SDS-PAGE (see figure 5). The fibrinogen sample ran as a single band corresponding to a molecular weight of 340,000.

SDS-PAGE Analysis Reduced Samples: Reduced fibrinogen samples possessed three bands corresponding to the α, β and γ chains. No other contaminating bands were evident. Most importantly, the fibrinogen preparations were found to be free of fibronectin contamination on SDS-PAGE. Fibronectin when reduced exhibits a band with a molecular weight of 200,000. Bands of MW 200,000 were not detectable in any fibrinogen samples run.

Complement Component Analysis: C3 and C5 antigen levels were determined using an ELISA. C3 and C5 antigen levels were determined to be 572 ng/mg fibrinogen and 70 ng/mg fibrinogen respectively. Considering a 10% molecular weight conversion of C3 to C3a, or C5 to C5a; 57 ng of C3a and 7 ng of C5a would be present for every mg of fibrinogen. This low level
FIGURE 5
7.5% SDS-PAGE
18 Hour Plasmin Digestion of Fibrinogen

Figure 5: SDS-PAGE plasmin digestion of fibrinogen

Lane 1: fibrinogen; Lane 2: 1 minute plasmin digestion;
Lane 3: 15 minutes; Lane 4: 1 hour; Lane 5: 5 hours;
Lane 6: 18 hours; Lane 7: molecular weight standards.
of C3 and C5 was found not to have chemotactic activity in our assay and therefore is unlikely to have significant impact on our in vitro studies.

**Pattern of Plasmin Digestion of Fibrinogen**

The products generated with the plasmin digestion of fibrinogen are well known. Generally, the first high molecular weight fibrinogen fragment to appear is fragment X MW 240,000, followed by fragment Y 145,000, fragment D MW 92,000, and fragment E MW 50,000. The same pattern of plasmin digestion is seen in figure 5 which is a 7.5% SDS-PAGE gel of aliquots removed during FDF preparation. At 1 minute, fragments X, Y, and D have appeared. At 1 hour fragment X is beginning to disappear and the band corresponding to fragment E is starting to appear. At 5 hours fragments X and Y are gone and there is a dark band corresponding to fragment E. At 18 hours of digestion only evidence of fragments D and E are visualized (see Figure 5, lane 6). Therefore, Figure 5 demonstrates that the starting fibrinogen preparation is pure (within the resolution limits of SDS PAGE) and that the proteolysis pattern follows a pattern which has been previously described in the literature.

**Effect of FDF on PMN Viability**

To evaluate the effect of FDF on PMN viability, the LDH assay was chosen. FDF were added to neutrophil preparations at the biologically relevant concentrations used in all assays. For example 0.2 ml of a 30 OD unit FDF preparation contains 260 μg of protein and corresponds to a starting fibrinogen concentration of 6 mg/ml. The assay for LDH measures LDH leakage from PMN cytoplasm and is designed so that the concentration of LDH is the rate limiting step. Therefore, the rate of reaction for the conversion of NADH to NAD is directly proportional to the LDH
concentration in the FDF exposed PMN supernatant. As can be seen from Table 1, the background LDH release from control PMNs (buffer treated) is much less than the total amount of LDH present in the cells (Triton-treated lysed cells). Using 3 different FDF preparations the amount of LDH released from the PMN treated with FDF is not statistically different from those treated with buffer. Therefore, it does not appear that FDF affect PMN viability or cause cell injury at biologically relevant concentrations when using LDH-release as a marker of cell viability.

**Effect of FDF on PMN Morphology**

Since FDF had effect on PMN viability experiments were carried out to investigate the effect on FDF on PMN morphology. A difference between the morphology of FDF treated cells and buffer treated cells may provide insights related to effects of FDF on neutrophil function. Morphological differences were observed between FDF treated and buffer treated (control) PMN. When FDF treated PMN were placed on plastic, they appeared round and failed to spread. Control PMN (buffer treated) were uniformly spread on the plastic culture dishes (see Figure 6).

When suspensions of FDF treated and control PMN were evaluated using electron microscopy, control cells appeared to have a granular surface with short ridges (Figure 7a and 7b). FDF treated cells appeared less granular with extended ridges (see Figure 7c and 7d). The increased length of FDF treated PMN cellular ridges or processes is confirmed using transmission electron microscopy. FDF treated PMN (Figure 9) appear to have longer and more numerous cytoplasmic processes than buffer treated cells (Figure 8). Interestingly, cytopathologic changes e.g. (vacuolization) are absent from both FDF and buffer treated cells.
# TABLE 1

**EFFECT OF 3 DIFFERENT FDF PREPARATIONS ON PMN VIABILITY**

<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITIONS</th>
<th># 1</th>
<th># 2</th>
<th># 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔO.D. (%)</td>
<td>ΔO.D. (%)</td>
<td>ΔO.D. (%)</td>
</tr>
<tr>
<td>100% LDH Release</td>
<td>-1.819</td>
<td>-1.666</td>
<td>-1.789</td>
</tr>
<tr>
<td>Background LDH</td>
<td>-0.125 (6.8%)</td>
<td>-0.098 (5.8%)</td>
<td>-0.128 (7.1%)</td>
</tr>
<tr>
<td>FDF Concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.6 absorbance units</td>
<td>-0.111 (6.1%)*</td>
<td>-0.123 (7.3%)*</td>
<td>-0.130 (7.2%)*</td>
</tr>
<tr>
<td>4.3 absorbance units</td>
<td>-0.114 (6.2%)*</td>
<td>-0.125 (7.5%)*</td>
<td>-0.132 (7.3%)*</td>
</tr>
</tbody>
</table>

1. Percentages are percent of positive control (100% LDH)

2. ΔO.D. = change(decrease) in absorbance at 340nm/time x 10, which is proportional to the rate of reaction NADH→ NAD catalyzed by L.D.H.

3. FDF concentration standardized by absorbance at 212 nm. 0.2 ml of 30 absorbance units and 0.2 ml of 15 absorbance units added to 0.5 ml PMN for a final concentration of 8.6 and 4.3 respectively.

*p > .05; no significant difference between FDF treated and buffer treated PMN
FIGURE 6

Effect of FDF on Human Neutrophil Adherence to Plastic

To determine the effect of FDF on neutrophil adherence to plastic 0.05 Ml 5 X10⁶ PMN/ml were exposed to 0.2 ml of FDF (30 OD) or buffer (37° C, 20 minutes) followed by incubation on culture plastic for 30 minutes. Experimental cells appear round and have not spread (Figure 6B) Control cells have spread (Figure 6A). Magnification 400x.
FIGURE 6

A. Buffer treated (control) PMN on plastic.

B. FDF treated PMN on plastic.
FIGURE 7

Scanning Electron Microscopy Evaluation of the Effects of FDF on Human Neutrophil Morphology

To determine the effect of FDF on neutrophil morphology 0.5 ml of PMN $1 \times 10^7$ PMN/ml were exposed to FDF (30 OD) or buffer (control) for 20 minutes at 37°C. Both experimental and control cells were submitted in suspension for scanning electron microscopy. Cells were placed on micropore filters (10,000×). Control and experimental cells have different morphological appearances. Control cells appear to have granular surfaces with slight ridges (Figure 7A and 7B). FDF treated cells appear less granular with extended ridges (Figure 7C and 7D). In a double blind study using 50 random photo micrographs, 2 independent investigators were able to distinguish control cells from FDF treated cells 79% and 72% respectively.
To control for nonspecific \textit{in vitro} effects on neutrophil ultrastructure, human PMN were exposed to buffer (HBSS) for 20 minutes at 37° C. Following this incubation, the cells were submitted in suspension for evaluation by transmission electron microscopy (magnification 8000x). No significant ultrastructural changes are evident in the photomicrograph.
PMN (0.5 ml of $1 \times 10^7$ PMN/ml) were incubated with 0.2 ml FDF 30 OD (for a final concentration of 8.6 OD) and incubated for 20 minutes. Following the incubation, PMN were submitted for transmission electron microscopy. When compared to control (buffer treated) PMN, no cytopathologic changes were evident. Additionally, TEM analysis supported SEM studies (Figure 7) which suggested that surface alterations are induced by FDF.
**Effect of FDF on Chemotaxis**

Chemotaxis is the directed movement of cells along a chemotactic gradient and results in the recruitment of cells to a site of inflammation. The regulation or control of chemotaxis functions to: 1) maintain cells at the site of inflammation, 2) limit tissue injury by limiting cell movement into a tissue site where healing is occurring.

In this present study FDF were found to inhibit chemotaxis in a dose dependent manner. As can be seen from Figure 10, inhibition was significant at the 8.6 OD units (-52%), 4.3 OD units (-42%) and the 2.15 OD units (-37%) FDF concentrations. Washing of the cells resulted in removal of almost all of the inhibitory activity for the 8.6 (-5%) and 4.3 (-3%) doses, and all of the activity for the 2.15 FDF dose. Addition of FDF to the upper compartment of the Boyden chamber resulted in significant inhibition of random migration as well, for PMN not exposed to chemotactic factors. The chemotactic index for FDF treated PMN was 696 ± 113, versus a control chemotactic index of 1121 ± 272, p<0.05. Additionally, the FDF were not found to be chemotactic at the concentrations used, i.e. FDF chemotactic index at a concentration of 8.6 OD = 1121 ± 133 as compared to a control chemotactic index 1061.25 ± 89.61, p>0.05. To control for the non specific effect of small peptides on neutrophil function, an 18 hour digest of commercially available bovine serum albumin was prepared. Due to the presence of significant amounts of contaminating gamma globulin in the BSA preparation, peptides from this protein were also produced. Methods used for production of the BSA peptides were identical to those used for FDF production. BSA/gamma albumin peptides <30,000 daltons (30 OD) were incubated with the PMN for 20
To determine the effects of FDF on neutrophil chemotaxis, FDF were incubated with 2.5 x 10⁶ cells/ml for 20 minutes at 37°C and incorporated into a standard multiwell Boyden chamber assay. Buffer treated PMN were used as controls. Positive wash samples were washed twice following FDF treatment and were incorporated into the assay. Mean chemotactic indices were calculated from 4 experiments and percent inhibition of chemotaxis expressed as (experimental chemotactic index/control chemotactic index) -1 x 100%. Significant (p<.05) inhibition of chemotaxis was observed for 2.15, 4.30 and 8.60 dose levels. The FDF inhibition was significantly reversed by washing of the FDF exposed PMN prior to addition to the Boyden Chamber Assay.

Figure 10: Effect of FDF on PMN Chemotaxis.
minutes followed by incorporation into the chemotaxis assay. The addition of BSA/gamma globulin peptides to this assay did not result in inhibition of chemotaxis, but actually caused a small, but statistically insignificant, increase in chemotaxis. The mean chemotactic index for buffer treated cells was $1912 \pm 182$ as opposed to a chemotactic index for BSA treated PMN of $2147 \pm 158$.

**Effect of FDF on SRBC - PMN Adherence**

Adherence of a particle, or microbe to the neutrophil membrane often is receptor mediated and is one of the first steps involved in phagocytosis. The effect of FDF on antibody coated SRBC-PMN adherence was measured to probe for possible effects of FDF on membrane receptors.

Figure 11 shows the immune adherence index plotted versus FDF concentration. As can be seen from the graph, FDF significantly decreased the adherence of antibody coated sheep RBC to human PMN. A significant dose response was not seen in this assay. This may be due to the large standard error and the choice of index. To rule out any effect the FDF may have on the RBC, FDF (30 OD) were added to the SRBC followed by washing. Addition of FDF to the RBC had no effect on PMN-RBC adherence when compared to non FDF treated PMN-RBC reactions (data not shown).

**Effect of FDF on PMN Phagocytosis**

Phagocytosis is an important function involved with PMN microbicidal activity and wound healing. Any effect of FDF on phagocytosis would be important in regulating a variety of responses that occur as a result of this function, e.g. cell activation, degranulation.

When the FDF were added to the PMN samples, phagocytosis was significantly inhibited (Figure 12). Inhibition is significant and increased up
To determine the effects of FDF on PMN-SRBC adherence, FDF were incubated with a final concentration of $2.5 \times 10^6$ PMN/ml. for 20 minutes at $37^\circ$C. Antibody coated sheep red blood cells were added to the PMN (PMN:RBC 1:1) and adherence was quantified microscopically. Adherence Index = Number of PMN $\geq 1$ adherent RBC/200 PMN. Sample duplicate smears were prepared and 3 fields per slide counted. Values represent the means of 3 experiments. Significant inhibition of SRBC adherence was observed for the 2.15, 4.30 and 8.60 dose levels ($p<.05$).

Figure 11: Effect of FDF on PMN-SRBC Adherence.
To determine the effect of FDF on PMN phagocytosis, various concentrations of FDF were incubated with the PMNs for a final concentration of $2.5 \times 10^6$ PMN as previously described. Opsonized RBC were added to the PMN at a 1:10 PMN:RBC ratio followed by lysis of adherent extracellular RBC by ammonium chloride. PMN were cytocentrifuged and sample duplicate smears prepared. Phagocytosis was quantified microscopically counting 3 fields of 200 PMN. Phagocytic index = Number of PMN $\geq$1 intracellular RBC/200 PMN. Values represent the means of 4 experiments. FDF significantly inhibited phagocytosis at all FDF doses ($p<.05$).
to the FDF concentration of 4.3, resulting in approximately 50% inhibition of phagocytosis. At the 8.6 FDF concentration there is a slight increase in phagocytosis, however, it is still significantly less than the control samples. As with the adherence assay, FDF (30 OD) were added to the RBC first, followed by washing to rule out any direct effect of FDF on the RBC. There was no difference between RBC treated with FDF followed by washing, and controls in the phagocytosis assay.

**Effect of FDF on PMN Chemiluminescence**

PMN chemiluminescence is an excellent indicator of PMN oxidative metabolism and has been be used as a general indicator of cell activation. To determine the effect of FDF on cell activation and PMN oxidative metabolism, PMN resting and stimulated chemiluminescence was measured.

**Resting Chemiluminescence** is significantly inhibited for all concentrations of FDF used (Table 2). FDF does not activate PMN and appears to be suppressing the oxidative metabolism of the resting cell.

**Stimulated Chemiluminescence** was inhibited for all doses tested (Table 2). Statistical significance was achieved at the 8.6 and 2.15 FDF dose level.

**Effect of FDF on PMN H2O2 Production**

**Standard Curve for H2O2 production**

Previous reports by Pick et al. have shown that the change in absorbance that accompanies the oxidation of phenol-red is linear and an accurate indication of H2O2 concentrations from 0-80 μM of H2O2. To determine H2O2 concentrations in our assay, and test the direct effect of FDF on our assay system, a standard curve for H2O2 levels was established. The standard curve (Figure 13) demonstrates that a relatively linear relationship can be obtained between absorbance at 610 nM and H2O2.
### TABLE 2

**EFFECT OF FDF ON RESTING AND STIMULATED HUMAN PMN CHEMILUMINESCENCE**

**PERCENT INHIBITION**\(^1\)

<table>
<thead>
<tr>
<th>FDF CONCENTRATION</th>
<th>RESTING PMN</th>
<th>STIMULATED PMN(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.6 absorbance units(^3)</td>
<td>-35.25±2.45 p&lt;.05</td>
<td>-31.85±3.84 p&lt;.05</td>
</tr>
<tr>
<td>4.3 absorbance units</td>
<td>-38.25±1.94 p&lt;.05</td>
<td>-28.6±6.08 p&gt;.05</td>
</tr>
<tr>
<td>2.15 absorbance units</td>
<td>-28.5±4.8 p&lt;.05</td>
<td>-20.83±4.78 p&lt;.05</td>
</tr>
</tbody>
</table>

\(n=4\) \(n=6\)

---

1. percent inhibition = (Experimental CPM/Control CPM) - 1 x 100%.

2. PMN stimulated with opsonized zymosan.

3. FDF concentration expressed as absorbance units at 212 nm.
Figure 13: H$_2$O$_2$ Standard Curve Using Phenol Red Assay.

H$_2$O$_2$ concentration was determined by using H$_2$O$_2$ molar extinction coefficient of 81 moles/cm. at 230 nm. A linear dose response (Abs. vs. H$_2$O$_2$) was seen between 0 and 50 micromoles/ml.
concentration. FDFs were added to the standard curve samples and they did not interfere with the assay.

Stimulated PMN H$_2$O$_2$ Production

H$_2$O$_2$ is an important chemical species involved in PMN microbicidal activity. It is required for the functioning of the myeloperoxidase-halide system which is thought to be of primary importance in PMN microbicidal activity. For this reason the effect of FDF on PMN H$_2$O$_2$ production was tested.

Figure 14 shows the ability of FDF to affect H$_2$O$_2$ release by 3 different stimuli. FDF significantly inhibit H$_2$O$_2$ release in a dose dependent fashion for all three stimuli. FMLP stimulated H$_2$O$_2$ production is more susceptible to low concentrations of FDF than PMA or zymosan. This effect may be due to the strength of the stimulus involved. Interestingly, FDFs inhibit H$_2$O$_2$ release by FMLP and PMA. These stimuli are not dependent on Fc receptor function or phagocytosis, but are dependent upon cytoskeletal organization. Incubation of PMN with peptides derived from an 18 hour plasmin digestion of BSA/gamma globulin failed to inhibit H$_2$O$_2$ production by PMN. H$_2$O$_2$ release from buffer treated cells was 52 μm/ml as opposed to 57 μm/ml for BSA treated PMN.

Effect of FDF on Degranulation

Degranulation is dependent upon stimulus-response coupling at the PMN membrane. Therefore the effect of FDF on zymosan stimulated degranulation was studied. Certain granule constituents may be used as markers: lactoferrin is a secondary granule marker, and 8 glucuronidase is a primary granule marker. However, lysozyme is found in both primary and secondary granules.
Figure 14: The Effect of FDF on Stimulated H$_2$O$_2$ Release by PMN.

The ability of FDF to affect H$_2$O$_2$ release by 3 different stimuli (10$^{-6}$M FMLP, 10$^{-7}$M PMA and 10:1 opsonized zymosan) was assayed using the phenol red assay for H$_2$O$_2$. Values represent the means of 5 experiments. FDF were incubated with a final PMN concentration of 1 x 10$^7$ PMN/ml. Significant inhibition of H$_2$O$_2$ release was seen for all 3 stimuli except for PMA and zymosan at the 2.30 dose level.
FDFs significantly inhibited lactoferrin release from PMN at the 8.6 and 2.15 dose levels (Figure 15). The values represent the means for three independent experiments. Inhibition of lactoferrin release occurred at the 4.3 dose level, however, it was not statistically significant. FDF statistically increased β glucuronidase release for all three dose levels (Figure 16). Interestingly, FDF had no statistically significant effect on lysozyme release (Figure 17).

**Effect of FDF on PMN Microbicidal Activity**

Adherence, phagocytosis, cell activation and H₂O₂ production are all important functions involved in PMN microbicidal activity. Therefore one would predict that the FDF that affect these functions would affect PMN microbicidal activity as well. Thus the ability of FDF to inhibit PMN microbicidal activity was investigated.

Figures 18 and 19 show that FDFs significantly inhibit PMN killing of *S. aureus* at both 1:1 and 10:1 PMN/Bacteria ratios. As reflected by an increase in *S. aureus* viability, statistical significance was achieved at the 8.6 FDF concentration for the 10:1 PMN/Bacteria samples (figure 18). It would appear that at lower dilutions, inhibition can be partially overcome if the PMN greatly outnumber the bacteria present. At the 1:1 PMN/Bacteria ratios (figure 19) significance is achieved at the 8.6 and 4.3 concentrations, but not at the lowest dilution. This is in keeping with the dose response relationships seen for the peptides; at lower dilutions the inhibitory effect is of smaller magnitude and statistical significance is lost.

**Antibody Blocking of FDF Suppressive Activity**

To verify the fibrinogen derived nature of the FDF activity, antibody blocking studies were undertaken. Thus, the removal of FDF inhibitory
Figure 15: The Effect of FDF on Lactoferrin Release from Human PMN Granules.

To determine the effect of FDF on secondary granule release, lactoferrin (a secondary granule marker) was quantified in phagocytic supernatants from control (buffer) and FDF treated PMNs. For these studies, various concentrations of FDF were incubated with $1 \times 10^7$ PMN as previously described followed by stimulation with opsonized zymosan (10:1 zymosan:PMN). Buffer treated (HBSS) samples served as controls. Lactoferrin release was quantified by ELISA using a monoclonal Ab. FDF significantly inhibited phagocytosis stimulated lactoferrin release by PMN at the 8.60 and 2.15 dose levels. Lactoferrin release at the 4.30 dose level was inhibited, however statistical significance was not achieved.
To determine the effect of FDF on primary granule release, β glucuronidase (a marker for primary granules) was quantified. FDF were incubated with $1 \times 10^7$ PMN/ml. as previously described in the methods section. β glucuronidase release was stimulated with opsonized zymosan (10:1 zymosan:PMN). Supernatants from 3 different neutrophil samples were analyzed for glucuronidase release. Values represent the means of 3 experiments. FDF significantly increased enzyme release at all dose levels ($p<.05$).
The effect of FDF on phagocytosis induced release of lysozyme, a marker of primary and secondary granules, was determined using the general protocol outlined in the methods section. FDF were incubated with a final concentration of 1 x 10^7 PMN and lysozyme release was stimulated with opsonized zymosan 10:1. Lysozyme release was quantified from 3 different PMN supernatants by measuring the lysis of Micrococcus lysodeikticus. FDF had no significant effect on lysozyme release (p>.05).
To determine the effect of FDF on PMN microbicidal activity, FDF were incubated with PMN at $5 \times 10^7$ PMN/ml for 20 minutes. Following this incubation $5 \times 10^6$ *S. aureus* were added to the PMN to a final $10:1$ PMN:Bacteria ratio and incubated. The PMN *S. aureus* mixture was subjected to hypotonic lysis and plated on Mueller-Hinton agar. *S. aureus* viability is expressed as CFU x $10^4$ surviving per sample. Values equal the means of 6 experiments. FDF significantly inhibited PMN killing of *S. aureus* at the 8.60 dose level ($p<.05$).

![Figure 18: Effect of FDF on Killing of *S. aureus* by PMN at a 10:1 PMN: Bacteria ratio.](image)
To determine the effect of FDF on PMN microbicidal activity, FDF were incubated with a final PMN concentration of 5 x 10^7 PMN/ml as previously described. Following the incubation 5 x 10^7 S. aureus bacteria were added to the PMN to a final PMN/Bacteria ratio of 1:1. PMN were subjected to hypotonic lysis followed by plating on Mueller-Hinton agar. S. aureus viability is expressed as CFU surviving per sample. The data presented represents the means of 7 independent experiments. FDF significantly suppressed PMN killing of S. aureus at the 4.30 and 8.60 dose levels (p<.05).
activity by fibrinogen specific antibody would lend support to the claim that the inhibitory activity seen is fibrinogen derived.

In a series of 3 experiments, antibody absorbed or NRS treated FDF were added to PMN in the \( \text{H}_2\text{O}_2 \) assay using opsonized zymosan as a stimulus. As can be seen in Figure 20, cells exposed to the antibody absorbed samples released significantly more \( \text{H}_2\text{O}_2 \) at the 8.6 and 4.3 dose levels. Controls demonstrated that resting cells were not activated by the Ab absorbed FDFs, thus they contained no inherent stimulatory activity (data not shown). Any effect of antibody or serum contamination can be ruled out in that the normal rabbit serum absorbed FDF samples are not different from those exposed to FDF alone.

**Separation of FDF Activity by Molecular Weight**

To develop a preliminary characterization of FDFs responsible for suppression of PMN production, FDF were separated according to molecular weight using Amicon ultrafiltration (see Figure 21). Two distinct molecular weight ranges of activity were seen. FDF<1000 daltons and 10,000 - 30,000 daltons significantly suppressed PMN \( \text{H}_2\text{O}_2 \) production. No significant activity was seen for the 1,000 -10,000 dalton fraction. Thus these data suggest a heterogeneous distribution of at least 1 FDF activity i.e. PMN \( \text{H}_2\text{O}_2 \) suppression.

**Effect of Pronase-E on FDF Inhibitory Activity**

To determine whether FDF activity was protease sensitive, a broad spectrum protease was used (see table 3 for peptide bond susceptibility). Figure 22 represents the effect of the neutral protease, Pronase E, on FDF inhibitory activity. Non protease treated FDF significantly inhibited FMLP stimulated \( \text{H}_2\text{O}_2 \) production by PMN. However, there was no significant
Figure 20: Effect of Anti-Fibrinogen Antibody on FDF Inhibitory Activity

The ability of specific antibody to remove FDF activity was tested using anti-fibrinogen antibody. Rabbit anti-human fibrinogen antibody was incubated with protein-A-sepharose for 1 hour. (Normal rabbit serum was used as a control.) The sepharose protein-A-anti-fibrinogen was added to FDF followed by centrifugation and removal of antibody. FDF, anti-fibrinogen treated FDF, and NRS treated FDF, were tested for their ability to inhibit PMN H$_2$O$_2$ production in the phenol red H$_2$O$_2$ assay. Anti-fibrinogen antibody significantly removed FDF inhibitory activity at the 4.30 and 8.60 dose levels. There was no significant difference between FDF and NRS treated FDF in their inhibition of PMN H$_2$O$_2$ production.
Figure 21: Fractionation of FDF Inhibitory (H$_2$O$_2$ production) by Ultrafiltration.

To further characterize FDF inhibitory activity FDF < 30,000 daltons were separated into 3 molecular weight fractions using amicon ultrafiltration. Each fraction was normalized with respect to protein concentration and assay for inhibitory effect on PMN H$_2$O$_2$ production. Values represent the means of 4 experiments. Two distinct molecular weight ranges of activity were found: <1,000 and 10,000-30,000 daltons. The fraction 1,000-10,000 daltons had no significant activity which rules out any non-specific effects of small fibrinogen peptides on PMN.
TABLE 3

PEPTIDE BOND SPECIFICITY OF PRONASE E

<table>
<thead>
<tr>
<th>ALA-X</th>
<th>LEU-X</th>
<th>GLY-X</th>
<th>OTHER</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA-GLY</td>
<td>LEU-TYR</td>
<td>GLY-LEU</td>
<td>ARG-LEU</td>
</tr>
<tr>
<td>ALA-PHE</td>
<td>LEU-ALA</td>
<td>GLY-TYR</td>
<td>PIIE-LEU</td>
</tr>
<tr>
<td>ALA-TYR</td>
<td>LEU-ARG</td>
<td>GLY-NORLEU</td>
<td>PHEN-ARG</td>
</tr>
<tr>
<td>ALA-ARG</td>
<td>LEU-ARG</td>
<td>GLY-NORLEU</td>
<td>PHEN-ARG</td>
</tr>
<tr>
<td>ALA-LEU</td>
<td>LEU-ARG</td>
<td>GLY-NORLEU</td>
<td>PHEN-ARG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VAL-GLY</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TYROSINE ETIOXY GROUP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TYROSINE AMINO GROUP</td>
</tr>
</tbody>
</table>
Figure 22: Effect of Pronase-E on the PMN-H$_2$O$_2$ Inhibitory Activity of FDF.

The protease sensitivity of FDF inhibitory activity for PMN production of H$_2$O$_2$ was tested using a non-specific protease from *Streptomyces griseus* covalently linked to sepharose beads. FDF were incubated with 2 units of bead immobilized pronase-E, or control beads for 3 hours. The protease immobilized or control beads were removed from the sample by centrifugation. The resulting supernatants were tested for PMN inhibitory activity using the phenol red H$_2$O$_2$ assay. Values represent the means of 3 experiments. There were no significant differences between protease treated FDF and control samples (beads alone) in their ability to inhibit H$_2$O$_2$ production by PMN.
difference between the samples treated with protease linked to beads, and those treated with beads alone \( p > 0.05 \) in their ability to inhibit \( H_2O_2 \) production. There were differences between the protease control samples, protease samples, and the FDF treated samples in their inhibition of \( H_2O_2 \) production at the 2.15 and 8.6 dose levels. These differences are not believed to be due to protease activity, since there are no differences between the samples treated with protease linked to beads and beads alone, but due to non-specific absorption of FDF to the beads thus non-specifically removing FDF activity.

**Effect of FDF on LFA-1 and MO-1 Antigen Levels on PMN**

Data developed by this project suggested the possibility that part of the FDF activity may involve adhesion molecules. Thus, the effect of FDF on two adhesion molecules on the surface of human PMN was evaluated, i.e. LFA-1 and MO-1. As can be seen from Figure 23, FDF significantly increased the amount LFA-1 antigen on the surface of PMN at the 2.15 dose level \( (p < 0.05) \). Doses 4.3 and 8.6 increased the level, however, the increase was not statistically significant. Interestingly, FDF had no significant effect on MO-1 antigen levels (Figure 24). Thus these data raise the possibility that the FDF induced alterations of adhesion molecules on the surface of the PMN may be responsible for the suppressive activity of the FDF on human neutrophils.
Figure 23: Effect of FDF on PMN LFA-1 Antigen Expression.

The ability of FDF to alter adhesion molecule expression on the surface of PMN was studied using a monoclonal Ab. to LFA-1. 5 $\times 10^6$ PMN/ml were incubated with FDF for 20 minutes and then placed on ice. PMN were labelled with mouse anti-human LFA-1 followed by washing and labelling with a secondary antibody conjugated to FITC. Cells were fixed and submitted for FACS analysis. Values represent the average of mean fluorescent values for 4 experiments. FDF significantly increased the mean fluorescence (and LFA-1 antigen levels) on PMN at the 2.15 FDF dose level ($p<.05$).
The ability of FDF to affect cell adhesion molecules on the surface of PMN was studied using a monoclonal antibody to MO-1. $5 \times 10^6$ PMN/ml were incubated with FDF for 20 minutes and then placed on ice. PMN were labelled with mouse anti-human MO-1 followed by washing and labelling with a secondary antibody conjugated to FITC. Cells were fixed and submitted for FACS analysis. Values represent the mean fluorescent values for 3 experiments. FDF had no significant effect on MO-1 antigen levels.
DISCUSSION

Fibrin is found in many inflammatory disease processes and is known to be rapidly metabolized by a variety of enzymes, including plasmin, which produce a wide range of degradation products locally at the site of inflammation. These degradation products have been shown to possess a variety of effects in vitro and in vivo. FDFs have been shown to affect coagulation, vasopermeability, endothelial cell integrity, inflammatory cell chemotaxis, angiogenesis, lymphocyte function and delayed-type hypersensitivity reactions in vivo. However, to date, no systematic studies have been undertaken to investigate the effect of FDFs on neutrophil function in vitro or in vivo. This is surprising in light of the fact that: 1) neutrophils are present in virtually all lesions in which fibrinogen is found, 2) the neutrophil is thought to be an important component of host defense.

In its role in host defense, the neutrophil is a highly specialized cell with its primary function being the destruction of microorganisms and the preparation of tissue for healing. The neutrophil response to microbial invasion can be divided into a number of stages including 1) Adherence to vascular endothelium followed by chemotaxis which recruits inflammatory cells to the site of microbial invasion. 2) Binding and Phagocytosis: the microorganism binds to the neutrophil membrane which invaginates internalizing the microorganism in a phagocytic vacuole, 3) Killing of the Microorganism: which is accompanied by a metabolic burst in which reactive oxygen metabolites are produced, fusing of enzyme containing granules with the phagolysosome, and release of granule components extracellularly. Thus factors which would modulate any or all of these events in host defense could have a marked impact on microbial invasion and tissue injury.
This thesis is the result of my preliminary studies (see Appendix experiments 1 and 2) which demonstrated the ability of FDF to inhibit PMN microbicidal activity. The results of the present studies have not only supported the ability of FDF to suppress microbicidal activity in vitro but have demonstrated the ability of FDF to inhibit a number of neutrophil functions important in host defense (see Table 4). Since many of the functions studied are adhesion dependent, the observed FDF inhibitions of neutrophil function may involve alterations in neutrophil adherence. Recently investigators have begun to recognize the importance of adhesion molecules on the surface of the PMN. It has been hypothesized that cell surface adhesion molecules represent a group of surface receptors such as the fibronectin receptor, platelet fibrinogen receptor, leukocyte adhesion molecules (LFA-1, MO-1), thought to belong to a family of wide ranging molecules linked with the cytoskeleton. In this capacity they function in wide ranging control of cell functions such as migration in embryos, wound healing, phagocytosis and T lymphocyte help to name just a few. Small molecular weight peptides produced by enzymatic activity (FDFs) may bind to these cell surface molecules inhibiting cell movement during embryogenesis and chemotaxis, thus providing fine tuned local regulation of these processes.

How would FDFs function in inflammation and what role do bacteria play in this process? My working hypothesis is that low molecular weight FDFs produced late in an inflammatory lesion function to down regulate (inhibit) the immune response limiting local tissue damage. However, bacterial proteases may take advantage of this process, producing FDF which allow for increased bacterial growth and tissue damage. Therefore, FDFs
may play a role in many disease processes where bacteria are present and local regulation of disease activity is occurring (e.g. inflammatory periodontal disease).

In order to study the effect of FDF on host defense, the effect of FDF on each stage involved in PMN function was examined. As mentioned earlier these steps include adherence, chemotaxis, phagocytosis, degranulation and oxygen metabolism. In the present studies, FDF were found to inhibit all of these steps as well as produce recognizable morphologic changes and up regulate cell surface adhesive molecules (see Table IV). Provided below is a detailed discussion related to those experimental findings and their implications for the regulation of neutrophil function in vitro and host defense in vivo.

**Fibrinogen Purity and the Use of Biologically Relevant Concentrations**

Previous studies which have investigated the effects of FDF on lymphocyte function have not addressed the question of fibrinogen purity prior to enzymatic digestion. Fibrinogen preparations may contain impurities capable of stimulating (e.g. complement) or inhibiting (e.g. fibronectin) cell function. In this study considerable effort was made to purify the fibrinogen prior to digestion. Complement contamination was sufficiently low so that it was unlikely to be of biological significance in these reactions. Since fibronectin was not evident on SDS PAGE, it appears that the starting protein was highly purified fibrinogen.

The concentration of fibrinogen or FDF used in in vitro experiments must be consistent with that found in vivo for the experiments to have biologic relevance. The highest concentration of peptides used in the various experiments presented (30 absorbance units) corresponded to a starting
TABLE 4
SUMMARY OF EFFECT OF FDF ON NEUTROPHIL FUNCTIONS

<table>
<thead>
<tr>
<th>FUNCTION</th>
<th>EFFECT</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIABILITY</td>
<td>NE</td>
</tr>
<tr>
<td>CHEMOTAXIS</td>
<td>↓</td>
</tr>
<tr>
<td>ADHERENCE</td>
<td>↓</td>
</tr>
<tr>
<td>PHAGOCYTOSIS</td>
<td>↓</td>
</tr>
<tr>
<td>CHEMILUMINESCENCE</td>
<td>↓</td>
</tr>
<tr>
<td>H$_2$O$_2$ RELEASE</td>
<td>↓</td>
</tr>
<tr>
<td>LACTOFERRIN RELEASE</td>
<td>↓</td>
</tr>
<tr>
<td>$\beta$ GLUCURONIDASE RELEASE</td>
<td>↑</td>
</tr>
<tr>
<td>LYSOZYME RELEASE</td>
<td>NE</td>
</tr>
<tr>
<td>MICROBICIDAL ACTIVITY</td>
<td>↓</td>
</tr>
<tr>
<td>MO-1 ANTIGEN</td>
<td>NE</td>
</tr>
<tr>
<td>LFA-1 ANTIGEN</td>
<td>↑</td>
</tr>
</tbody>
</table>
fibrinogen concentration of 6 mg/ml. Fibrinogen is present in plasma at a concentration of 3-4 mg/ml.\textsuperscript{19} Moreover, fibrinogen, as an acute phase reactant, can reach concentrations as high as 30-40 mg/ml\textsuperscript{19} during acute inflammation. The deposition of large amounts of fibrin in both acute and chronic inflammatory lesions may lead to even higher local concentrations of FDF. Therefore the concentration of FDF used in the experiments described is within the range seen in vivo and in fact much higher levels may be seen in vivo.

**Effect of FDF on Chemotaxis**

One of the first steps in the inflammatory process is the movement of PMN from the vascular bed to a site of injury or bacterial invasion. Once the cells arrive at the site of inflammation, mechanisms must exist to limit their further movement out of the area. Moreover, mechanisms must exist to reduce or prevent chemotaxis of additional cells into a lesion once healing has been initiated, preventing further tissue damage by PMN proteases. FDFs may play a role in these immunoregulatory pathways.

Experiments presented in this thesis have shown that FDFs can inhibit PMN chemotaxis as well as random migration. This inhibition was found to be reversible upon washing, and specific since peptides derived from BSA degradation had no effect on chemotaxis. The finding that the inhibitory effects of FDF are reversible supports their role as possible immunoregulators. Permanent down regulation of PMN by a factor (FDF) in an inflammatory lesion may not be desirable.

Present experiments have not found these small peptides (FDF) to be chemotactic. It must be noted, however, that other studies have found fibrinogen peptides to be chemotactic. However, these studies employed
either thrombin derived peptides\textsuperscript{36}, or found major chemotactic activity of molecular weight $\geq 30,000$ daltons.$^{47}$ In addition, no study to date has investigated the effect that preincubation of PMN with FDF have on PMN chemotaxis as this study has done.

In support of experimental findings related to chemotaxis presented here, recent experiments by other investigators in our laboratory have demonstrated the ability of FDF peptides, identical to that used in these experiments, to inhibit endothelial cell migration.$^{33}$ Therefore, there is evidence that FDFs affect chemotaxis of other cell types as well.

In light of these data it is hypothesized that that FDFs may bind to the neutrophil membrane mimicking matrix proteins such as fibronectin and fibrinogen. The FDFs may function to saturate membrane bound adhesive molecules reducing cell adhesiveness and motility.

**Effect of FDF on SRBC-PMN Adherence and Phagocytosis**

The first step in phagocytosis is the binding of a particle to the neutrophil membrane and FDFs were found in this study to significantly inhibit binding of antibody coated SRBC to PMN membranes. A clear dose response was not seen in the adherence assay. However, this may be due to the high standard error present and the selection of the index used for measurement of adherence. Counting all cells with 1 adherent RBC tended to obscure the finding that with increasing FDF concentration there were less cells with 2 or 3 adherent RBC (see Appendix). Inhibition of binding can affect all subsequent steps in killing and it cannot be said whether the inhibition of phagocytosis seen is due to a direct effect on phagocytosis or due to decreased binding. However, inhibition of phagocytosis was significant at all dose levels in the erythrophagocytosis assay.
A possible mechanism to explain the effects of FDF on SRBC adherence and phagocytosis might include binding of FDF to the Fc receptor and other cell surface adhesion receptors. For example, inhibition of platelet receptor binding to fibrinogen by small peptides has been described and this receptor has been found on PMNs. It is possible that FDF bind the Fc receptor of PMN down regulating adherence and subsequent phagocytosis of antibody coated cells. Any effect of the FDF on the RBC is less likely due to experiments that incubated the FDF with the RBC followed by washing and incorporation into the assay. FDF treated RBC had no effect on adherence or phagocytosis. Thus these studies suggest that FDF inhibition of SRBC-PMN adherence and phagocytosis is due to a direct effect on the PMN.

**Effect of FDF on Chemiluminescence and H$_2$O$_2$ Production**

It is well established that binding of an opsonized particle or a chemotactic peptide to a PMN results in a metabolic burst in which light (chemiluminescence) and several reactive oxygen metabolites (H$_2$O$_2$) are produced. Because of the role of these oxygen metabolites in host defense the effect of FDF on H$_2$O$_2$ and chemiluminescence was studied.

It is noteworthy that FDF had effects on the resting cell, inhibiting resting chemiluminescence. This is an effect that is independent of the binding of a chemotactic factor or opsonized particle to the cell. It appears the resting cell is down regulated by FDF.

FDFs have been shown to inhibit IgG mediated RBC adherence and phagocytosis to the PMN membrane. Addition of the FDF to the SRBC had no effect. Therefore, evidence exists for FDF affecting binding of opsonized particles to the PMN membrane. This fact may, in part, explain the effect of FDF on stimulated chemiluminescence. Part of the effect seen may be due
to the inhibition of binding of opsonized zymosan by the FDF resulting in decreased stimuli. However, the suppressive effects of FDF may be a result of other effects on PMN metabolism as suggested from the resting chemiluminescence data.

Chemiluminescence is an excellent indicator of PMN oxidative metabolism. Therefore the finding that FDF inhibit both stimulated chemiluminescence and H$_2$O$_2$ production is not surprising. The finding that FDF inhibit H$_2$O$_2$ production from 3 seemingly unrelated stimuli supported the general suppressive effect of the FDF. The general suppressive effect on H$_2$O$_2$ production may be due to FDF affecting a common cellular pathway, or due to a number of peptides affecting individual pathways. Another possible mechanism to account for these data might include FDF binding to cell surface receptors. This binding could prevent the interaction of serum opsonins (e.g. C3b on zymosan) and chemotactic factors with their individual receptors; inhibiting the stimulus for H$_2$O$_2$ production. The peptide sequence x-Arg-Gly-Asp-y, found to inhibit cell adhesion to fibrinogen and fibronectin, is found in the fibrinogen molecule and the complement component C3. Moreover, it has been found to bind to a number of cell surface receptors. It is possible that FDF with sequences related to x-Arg-Gly-Asp-y, or other peptides bind to the C3b or f-met-leu-phe receptor inhibiting binding and H$_2$O$_2$ production. Some degree of specificity was demonstrated in present experiments when peptides derived from BSA were shown not to inhibit H$_2$O$_2$ production. The effect of FDF with PMA activation is more complex since PMA is thought to have multiple effects in addition to activating protein kinase C. Protein kinase C is involved with the phosphorylation of proteins resulting in the control of calcium fluxes and
receptor transport. PMA, in its activation of protein kinase C, has been thought to be an agonist.\textsuperscript{52} However, protein kinase C can be involved with receptor down regulation and a report describes the PMA inhibition of neutrophil response to chemotactic peptides.\textsuperscript{50} FDF by binding to the neutrophil membrane may act to affect PMA transport into the cell or may function to decouple the activation of protein kinase C from cell activation.

**Effect of FDF on PMN Degranulation**

A critical aspect of the phagocytic process is the incorporation of agents toxic to microorganisms into the phagocytic vacuole. Following binding and phagocytosis, PMN cytoplasmic granules (separated into two distinctive populations, primary and secondary) move and fuse with the phagocytic vacuole releasing various proteolytic enzymes. Moreover, the contents of the granules can often be released extracellularly due to incomplete closure of the phagocytic vacuole, or rupture of the cell causing death of extracellular microorganisms or host tissue damage. The control of PMN degranulation is not well understood, however, different granule populations (primary and secondary) are believed to be responsive to independent stimuli and secondary granules are involved in receptor transport to the cell surface (e.g. FMLP). Secondary granule release can be elicited with stimuli such as PMA and Con A while primary granule release cannot\textsuperscript{76}. Release of primary granules requires a greater stimulus than secondary granules\textsuperscript{76}. In addition, secondary granules are thought to be more readily released into the extracellular environment; primary granules tend to fuse with the phagolysosome\textsuperscript{76}.

Since primary and secondary granules may require different stimuli for release, it is not surprising that FDF have the opposite effects on their
release. FDF suppress secondary granule release and potentiate primary granule release. The effect of FDF on neutrophil granule release may affect the character of PMN mediated connective tissue destruction. For example, an increase in primary granule release would cause an increase in the release of enzymes such as elastase which can degrade connective tissue proteoglycans. Inhibition of secondary granule release could function to prevent the release of an important anti-microbial agent (lactoferrin) as well as inhibit the transfer of f-met-leu-phe receptors to the cell surface. Whether FDF affect signal response coupling, cytoskeletal events or some other cellular process has not been elucidated. However, the effect of peptides found in inflammatory lesions could alter the course and outcome of disease processes in vivo.

Effect of FDF on PMN Microbicidal Activity

The primary function of phagocytosis degranulation and the generation of oxygen metabolites is to defend the host against invading microorganisms. Since FDF have been shown in this thesis to inhibit many of the functions involved in PMN microbicidal activity, it is not surprising that microbicidal activity was suppressed. Present studies demonstrated that FDF inhibition of killing was dose dependent, statistically significant, and became more pronounced with the 1:1 PMN/Bacteria ratio. PMN to bacteria ratios of 1:1 and 10:1 were chosen because at these ratios PMN killing has been shown to be the most efficient. Experimental conditions in which bacteria greatly outnumber PMN (e.g. bacterial plaque in the gingival sulcus) might introduce other variables such as bacterial toxins etc. which might cause neutrophil death.
Effect of FDF on LFA-1 and MO-1 Antigen Levels

Many of the functions that FDF have been shown to affect are dependent on adhesion of microorganisms to the cell surface. In keeping with the hypothesis that FDF affect cell surface adhesion molecules, experiments were performed to examine the effect of FDF on the cell adhesion molecules LFA-1 and MO-1. Evidence is accumulating that these molecules are important in neutrophil function and host defense. Present experiments have demonstrated the ability of FDF to significantly increase the amount of LFA-1 antigen on the surface of PMN. FDF had no significant effect on MO-1 antigen levels. The greatest effect on LFA-1 antigen levels occurred at the lowest FDF concentration. These data suggest that FDF may be binding to cell surface adhesion receptors stimulating an increase in cell surface receptors in a manner analogous to the binding of a chemotactic factor such as f-met-leu-phe. For example, when a PMN binds to a venuole in vivo, up regulation of adhesive receptors may occur. However, if FDF are present (the inflammatory lesion is resolving), FDF binding to adhesive receptors might block margination and diapedesis.

Identity of Active FDFs

The identity and relative homogeneity of FDF(s) which down regulate neutrophil function has not been demonstrated. In fact, it is clear that the FDF preparations contain a variety of peptides. Thus some peptides may be stimulatory and others may be inhibitory. Clearly, in vitro and in vivo, the relative concentration for the PMN of each of the peptides would determine the ultimate regulation of PMN function.

Data obtained from separation of FDF according to molecular weight shows two molecular weight ranges of activity: FDF<1000 d and
The finding that FDF <1,000 daltons have activity is in good agreement with other studies showing that peptides of molecular weight in the range of 500 daltons are able to bind to cell surface receptors and inhibit cell function. The total lack of activity seen in the 1,000-10,000 molecular weight range would argue against any non specific effects of small peptides on the cells. Present experiments performed to investigate the susceptibility of FDF to a non specific protease have not demonstrated protease sensitivity. This may be due to a lack of peptide bond susceptibility in the peptide(s) that account for FDF activity. Finally, antibody blocking experiments support the fibrinogen related identity of the FDF activity response for suppressing PMN-H₂O₂ production in vitro. Data presented here strongly suggests that the PMN inhibitory activity demonstrated is fibrinogen related and that significant activity is found MW<1,000. These data suggest that small molecular weight peptides of fibrinogen can inhibit PMN function in vitro and may regulate PMN function in vivo. The exact identity of these peptides remains to be determined.

Conclusion

The ability of FDF to inhibit functions related to host defense may have important immunoregulatory and immunopathologic consequences. FDF inhibition of chemotaxis may inhibit the host's initial response to microbial invasion. Increased fibrinolytic activity found surrounding tumors may inhibit recruitment of PMN to the tumor site, and subsequent killing of tumor cells. Moreover, work done by other investigators in Dr. Kreutzer's lab, using peptides identical to those used in present experiments, has demonstrated the ability of FDF to inhibit endothelial cell migration. Therefore, FDF may
affect other diverse cellular kinetic processes such as angiogenesis and neovascularizations and embryogenesis.

FDF inhibition of phagocytosis and killing of microorganisms may affect the course and severity of diseases of microbial etiology. Bacterial virulence factors in combination with FDF may act to favor bacterial proliferation over host defenses. Phagocytosis is important in wound healing. Therefore, FDF may inhibit the ability of the host to repair damage once it has occurred.

The finding that FDF preferentially stimulate primary granules and inhibit secondary granule release may affect PMN extracellular release of proteases into host tissues in vivo. The increase in release of primary granule constituents such as elastase may cause increased connective tissue destruction.

Currently, the mechanism(s) of action of FDF is unknown but we have developed preliminary data to suggest that one possible mechanism for FDF induced suppression of PMN function may involve adhesion dependent functions. The ability of small molecular weight peptides derived from fibrinogen degradation to up-regulate the cell adhesive protein LFA-1 is interesting in light of recent work done with fibronectin. The peptide x-Arg-Gly-Asp-y which represents the putative cell binding site of fibronectin, has been found to bind to a number of cell surface receptors inhibiting cellular function. The sequence x-Arg-Gly-Asp-y has been shown to: inhibit adhesion, spreading, and microfilament organization on fibrinogen coated surfaces, inhibit experimental metastasis of murine melanoma cells, block neural crest cell migration and bind to the platelet fibrinogen receptor IIb-IIIa inhibiting platelet interactions with that molecule.
The platelet fibrinogen receptor has been identified on PMN and is believed to function in adhesion. Moreover, the platelet fibrinogen receptor IIb-IIIa is thought to belong to a family of cell surface adhesion receptors (LFA-1, MO-1) involved in cell locomotion and phagocytosis. Therefore, an adhesive molecule has been described on PMN which binds fibrinogen and whose function is blocked by small peptides derived from a matrix protein.

Experimental evidence pertaining to the Arg-Gly-Asp peptide is particularly relevant because: 1) the sequence is found twice in the α chain of fibrinogen, 2) the sequence Arg-Gly-Asp is found in the complement component C3, 3) small peptides related to fibrinogen and fibronectin are able to bind to cell surface receptors inhibiting function in many cells. It is possible that small peptides (FDF) regulate cell function by occupying tissue matrix binding sites and opsonin receptors on PMN during the inflammatory processes. This process would provide an opportunity for local control of an inflammatory lesion and provide a mechanism by which pathogens could take advantage of local regulatory processes to promote their proliferation and tissue invasion.

Thus data obtained from this study provide a variety of interesting avenues of investigation. For example, the identification of specific peptides which inhibit cell function and the receptors involved will provide new insights into the regulation of local and systemic immune function. In addition, the identification of specific factors that inhibit cell migration etc. may provide exciting therapeutic possibilities. These agents may be used therapeutically to control the repopulation of wounds by specific cell types and affect wound healing (e.g. periodontal attachment procedures).
Moreover, development of inhibitors of cell movement may prevent and thereby limit PMN-mediated tissue damage (e.g. rheumatoid arthritis).

Finally, the characterization of the biologic significance of specific FDF will be instrumental in diagnosis and treatment of inflammatory diseases such as periodontal disease.

Therefore, in the future, investigation into the role of these peptides in biologic systems will provide the clinician as well as the basic scientist with new and exciting areas of research.
<table>
<thead>
<tr>
<th>FDF A. 212</th>
<th>RBC/PMN</th>
<th>PMN/200</th>
<th>% CHANGE</th>
<th>PMN/200</th>
<th>% CHANGE</th>
<th>PMN/200</th>
<th>% CHANGE</th>
<th>PMN/200</th>
<th>% CHANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUFFER</td>
<td>0</td>
<td>97</td>
<td>-</td>
<td>139</td>
<td>-</td>
<td>120</td>
<td>-</td>
<td>146</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>40</td>
<td>-</td>
<td>50</td>
<td>-</td>
<td>48</td>
<td>-</td>
<td>43</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>36</td>
<td>-</td>
<td>8</td>
<td>-</td>
<td>22</td>
<td>-</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>27</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>9</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>8.6 OD</td>
<td>0</td>
<td>138</td>
<td>51</td>
<td>171</td>
<td>23</td>
<td>167</td>
<td>39</td>
<td>180</td>
<td>23</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>38</td>
<td>-5</td>
<td>25</td>
<td>-50</td>
<td>30</td>
<td>-38</td>
<td>19</td>
<td>-56</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>17</td>
<td>-52</td>
<td>3</td>
<td>-75</td>
<td>2</td>
<td>-91</td>
<td>1</td>
<td>-88</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>7</td>
<td>-74</td>
<td>1</td>
<td>-75</td>
<td>1</td>
<td>-89</td>
<td>0</td>
<td>-100</td>
</tr>
<tr>
<td>4.3 OD</td>
<td>0</td>
<td>144</td>
<td>49</td>
<td>173</td>
<td>25</td>
<td>164</td>
<td>38</td>
<td>176</td>
<td>21</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>33</td>
<td>-18</td>
<td>21</td>
<td>-56</td>
<td>26</td>
<td>-46</td>
<td>22</td>
<td>-49</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>12</td>
<td>-66</td>
<td>6</td>
<td>-25</td>
<td>8</td>
<td>-64</td>
<td>2</td>
<td>-75</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>11</td>
<td>-59</td>
<td>0</td>
<td>-100</td>
<td>2</td>
<td>-78</td>
<td>0</td>
<td>-100</td>
</tr>
<tr>
<td>2.15 OD</td>
<td>0</td>
<td>156</td>
<td>61</td>
<td>153</td>
<td>10</td>
<td>152</td>
<td>27</td>
<td>162</td>
<td>11</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>26</td>
<td>-35</td>
<td>42</td>
<td>-14</td>
<td>46</td>
<td>-4</td>
<td>35</td>
<td>-19</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>10</td>
<td>-72</td>
<td>5</td>
<td>-38</td>
<td>2</td>
<td>-91</td>
<td>3</td>
<td>-63</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>8</td>
<td>-70</td>
<td>0</td>
<td>-100</td>
<td>1</td>
<td>-88</td>
<td>0</td>
<td>-100</td>
</tr>
</tbody>
</table>
## Appendix II

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Experiment 1 PMN/200</th>
<th>% Change</th>
<th>Experiment 2 PMN/200</th>
<th>% Change</th>
<th>Experiment 3 PMN/200</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC/PMN</td>
<td>0</td>
<td>11.6</td>
<td>0</td>
<td>1.15</td>
<td>11.6</td>
<td>1.15</td>
</tr>
<tr>
<td>Rosetting</td>
<td>8.6</td>
<td>25.4</td>
<td>1.0</td>
<td>23.6</td>
<td>1.0</td>
<td>23.6</td>
</tr>
<tr>
<td>FDF A. 212</td>
<td>4</td>
<td>53.6</td>
<td>1.10</td>
<td>1.98</td>
<td>1.10</td>
<td>1.98</td>
</tr>
<tr>
<td>Buffer</td>
<td>0</td>
<td>0.55</td>
<td>0.1</td>
<td>0.0</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Rosetting</td>
<td>0.43</td>
<td>0.25</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
### APPENDIX III

<table>
<thead>
<tr>
<th>EXPERIMENT (CPM)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDF A212</td>
<td>15375</td>
<td>543</td>
<td>822</td>
<td>15538</td>
<td>43</td>
<td>71</td>
<td>28</td>
<td>50</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>CONTROL</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CONTROL-EXP (D)</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>2.15</td>
<td>0.52</td>
<td>0.48</td>
<td>0.44</td>
<td>0.40</td>
<td>0.36</td>
<td>0.32</td>
<td>0.28</td>
<td>0.24</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Resting Chemiluminescence

Paired T test: P < 0.05
### Stimulated Chemiluminescence

<table>
<thead>
<tr>
<th>FDF A. 212</th>
<th>EXPERIMENT (CPM)</th>
<th></th>
<th></th>
<th></th>
<th>D</th>
<th>SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUFFER</td>
<td>293855</td>
<td>199496</td>
<td>120238</td>
<td>124661</td>
<td>23321</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.6 OD</td>
<td>156149</td>
<td>101936</td>
<td>85253</td>
<td>107548</td>
<td>13675</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL-EXP (D)</td>
<td>137706</td>
<td>97560</td>
<td>34985</td>
<td>17113</td>
<td>9646</td>
<td>59401</td>
<td>49895</td>
</tr>
<tr>
<td>4.3</td>
<td>251693</td>
<td>110639</td>
<td>115853</td>
<td>129945</td>
<td>14889</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>42162</td>
<td>88857</td>
<td>4385</td>
<td>3716</td>
<td>8432</td>
<td>29510</td>
<td>32933</td>
</tr>
<tr>
<td>2.15</td>
<td>175995</td>
<td>177589</td>
<td>75350</td>
<td>93787</td>
<td>18748</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>117860</td>
<td>21907</td>
<td>44888</td>
<td>30874</td>
<td>4573</td>
<td>44020</td>
<td>39168</td>
</tr>
</tbody>
</table>
S. aureus Killing Assay
Colony Forming Units
10:1 Neutrophil/Bacteria

<table>
<thead>
<tr>
<th>EXP Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>D</th>
<th>SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUFFER</td>
<td>5.00E+04</td>
<td>2.70E+03</td>
<td>1.70E+04</td>
<td>3.00E+03</td>
<td>9.00E+03</td>
<td>-</td>
<td>2.00E+04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 OD</td>
<td>4.00E+05</td>
<td>6.40E+04</td>
<td>3.40E+04</td>
<td>2.00E+04</td>
<td>4.60E+04</td>
<td>-</td>
<td>1.07E+05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL-EXP (D)</td>
<td>3.50E+05</td>
<td>6.13E+04</td>
<td>1.70E+04</td>
<td>1.70E+04</td>
<td>3.70E+04</td>
<td>-</td>
<td>8.70E+04</td>
<td>9.48E+04</td>
<td>1.16E+05</td>
<td>p&lt;.05</td>
</tr>
<tr>
<td>43 OD</td>
<td>1.80E+05</td>
<td>3.90E+04</td>
<td>3.60E+04</td>
<td>4.00E+03</td>
<td>2.00E+04</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>1.75E+05</td>
<td>3.53E+04</td>
<td>1.90E+04</td>
<td>1.00E+03</td>
<td>1.10E+04</td>
<td>-</td>
<td>-</td>
<td>4.80E+04</td>
<td>6.40E+04</td>
<td>.05&lt;p&lt;1</td>
</tr>
<tr>
<td>213 OD</td>
<td>7.70E+04</td>
<td>1.60E+04</td>
<td>1.80E+04</td>
<td>-</td>
<td>1.40E+04</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>2.70E+04</td>
<td>1.33E+04</td>
<td>1.00E+03</td>
<td>-</td>
<td>5.00E+03</td>
<td>-</td>
<td>-</td>
<td>2.61E+04</td>
<td>4.83E+04</td>
<td>p&gt;0.05</td>
</tr>
</tbody>
</table>
### Appendix VI

#### Staphylococcus Aureus Killing Assay

<table>
<thead>
<tr>
<th>Colony Forming Units</th>
<th>1:1 Neutrophil/Bacteria</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Buffer</th>
<th>Control</th>
<th>Exp. OD</th>
<th>8.5 OD</th>
<th>13 OD</th>
<th>23 OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.6E+05</td>
<td>1.6E+05</td>
<td>1.8E+05</td>
<td>1.9E+05</td>
<td>1.8E+05</td>
<td>1.4E+05</td>
</tr>
<tr>
<td>2</td>
<td>2.5E+05</td>
<td>2.0E+05</td>
<td>2.1E+05</td>
<td>1.9E+05</td>
<td>1.1E+05</td>
<td>1.1E+05</td>
</tr>
<tr>
<td>3</td>
<td>1.3E+05</td>
<td>7.1E+04</td>
<td>4.2E+05</td>
<td>4.0E+05</td>
<td>1.2E+05</td>
<td>9.5E+05</td>
</tr>
<tr>
<td>4</td>
<td>3.6E+05</td>
<td>2.0E+05</td>
<td>2.0E+05</td>
<td>2.0E+05</td>
<td>2.0E+05</td>
<td>2.0E+05</td>
</tr>
<tr>
<td>5</td>
<td>2.0E+05</td>
<td>1.1E+05</td>
<td>1.0E+05</td>
<td>1.0E+05</td>
<td>1.0E+05</td>
<td>1.0E+05</td>
</tr>
<tr>
<td>6</td>
<td>1.3E+05</td>
<td>7.1E+04</td>
<td>4.2E+05</td>
<td>4.0E+05</td>
<td>1.2E+05</td>
<td>9.5E+05</td>
</tr>
<tr>
<td>7</td>
<td>1.2E+05</td>
<td>6.0E+03</td>
<td>3.0E+05</td>
<td>2.0E+05</td>
<td>2.0E+05</td>
<td>2.0E+05</td>
</tr>
</tbody>
</table>

**P-values:**
- $P<0.05$
- $P<0.01$
- $P>0.05$
REFERENCES


55. Page R.C. and Schroeder H.E. Periodontitis in man and other animals. (Karger; New York, 1982).


