Endotoxic Lipopolysaccharide Activation of Complement: A Role for the Alternate Pathway

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ENDOTOXIC LIPOPOLYSACCHARIDE ACTIVATION OF COMPLEMENT:
A ROLE FOR THE ALTERNATE PATHWAY

by
Blase P. Brown

A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Master of Science

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VITA

The author, Blase Patrick Brown, is the son of Robert M. Brown and Joan (Waters) Brown. He was born January 5, 1953, in Chicago, Illinois. He attended Quigley Preparatory Seminary South and was graduated in June, 1971. In September, 1971, he entered Loyola University of Chicago, and in June, 1975, received the degree of Bachelor of Arts, with a major in history. Since that time, he has been a graduate student in Oral Biology at Loyola, where he is working towards a Master of Science degree.
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INTRODUCTION

Exposure of a host to a foreign antigen elicits an immune response in which specific immunoglobulins and effector lymphocytes are produced to identify and neutralize this foreign substance. Elimination and destruction of the identified antigen proceeds through the functional limb of the immune system, which includes immune effectors (complement, kinin, coagulation, lymphokines, and lymphotoxin). On activation of an immune response, an inflammatory reaction is a result of the release of biologically active products from the immune effector systems. Generally, these products cause increased vascular permeability, smooth muscle contraction, and chemotaxis of leukocytes. In the ensuing response, leukocytic phagocytosis functions as the major defense mechanism for the removal and degradation of antigens. In many bacterial infections, a humoral response facilitates this phagocytosis through the production of agglutinating and opsonizing antibodies, and by the amplification of the inflammatory response through complement activation. However, in many respects the biosynthesis of antibodies is too slow and ponderous a mechanism for the purposes of host defense. Therefore, initial biological response at a local site of infection does not rely on a de novo immune reaction, but on innate (nonspecific activation of effectors) mechanisms that trigger inflammation and enhance phagocytosis. Both innate and immune mechanisms in host defense have been reviewed (Humphrey and White, 1970; and Davis et al., 1973).
A variety of microbial factors and products cause potent biological changes in a host through initial and prolonged exposure. Complement (an alternate pathway) has been implicated as a humoral effector system in reactions with microbial factors such as bacterial polysaccharides, yeast cell walls, Gram-negative bacteria, and the endotoxic lipopolysaccharide (LPS) of certain Gram-negative species (Gotze and Muller-Eberhard, 1976). The activation of complement by endotoxin has been implicated as a potential pathologic factor in specialized tissues which are susceptible to damage by inflammatory products (Mergenhagen et al., 1968; Gewurz et al., 1971; and Synderman 1972b). These pathological conditions include both acute and chronic inflammation in tissues such as the glomerulus and periodontium. The presence of immunoglobulins to antigens, including endotoxin, have to be considered together with innate inflammatory mechanisms. The participation of an endotoxin-activated complement system, in the presence or absence of an immune response to this antigen in generating biological activity is undefined.

The objective of this study is to compare in vitro complement generated chemotaxis in an immune system to chemotaxis in non-immune LPS-complement activation. This comparison of chemotactic activity in two functional complement pathways (immune system) to the alternate pathway (non-immune activation), solely, will be related to in vivo LPS-complement participation in inflammation and destruction in specialized tissues.
Endotoxic lipopolysaccharides and Gram-negative bacterial cells caused potent biological reactions when injected into experimental animals. The activation of a histamine-releasing factor through a heat-labile serum enzyme has been implicated as the cause of the major physiological changes of endotoxin: shock, fever, local inflammatory skin reactions, and death (Greisman, 1960; Spink and Vick, 1961; and Weil and Spink, 1957). Complement activation was implicated in these in vivo reactions when all the major physiological changes due to the presence of endotoxin were reproduced in laboratory animals by employing immune complexes (Stetson, 1964). In addition, complement-induced anaphylactic shock was shown to simulate endotoxin shock (Weil and Spink, 1957). LPS was further demonstrated to cause a significant decline in rabbit serum complement levels following the injection of lethal doses (Gilbert and Braude, 1962; and Pearlman et al., 1963). In addition, the early phase of endotoxin shock in dogs could be prevented by a cobra venom factor (CVF) decomplementation, which implicated the complement system as the mediator (Gewurz et al., 1970).

The endotoxic lipopolysaccharides of certain Gram-negative bacteria have been demonstrated to function as activating factors of both the alternate pathway of complement fixation in immunoglobulin-independent reactions (figure 1), and the classic complement pathway (figure 2) in immunoglobulin-dependent reactions (Mergenhagen et al., 1973; and Westphal, 1975). These two distinct pathways of complement activation have been observed in
Figure 1

activating substance

$S_l$ = site of initiation
$S_a$ = site of attachment
$S_1$ = cytolytic assembly

(reproduced from Gotze & Muller-Eberhard, 1976)
FIGURE 2
Classical Complement Activation

(modified reproduction from Gewurz et al., 1969)
normal vertebrate serum, and function in both specific and nonspecific reactions in host defense.

The classical complement sequence is a multimolecular, self-assembling biological system which constitutes the primary humoral mediator of antigen-antibody reactions (Müller-Eberhard, 1975; and Davis et al., 1973). Operationally, this pathway can be divided into three functional units: Cl (Clq-Clr-Cls), the recognition unit; the activation unit, C2, C3, C4; and the membrane attack unit, C5b-C6-C7-C8-C9. The first three components function as a Ca++-dependent, trimolecular complex (Cl) which combines reversibly with immune complexes of IgG or IgM. The Clq component possesses recognition-binding sites for a receptor on the Fc portion of the immunoglobulin. Following binding, Clq undergoes conformational change that, in turn, activates Clr as an enzyme. Clr then cleaves a small fragment from Cls, which activates the remaining fragment as a serine enzyme (Cls).

The second stage of the classical sequence involves the sequential formation of two related enzymes initiated by Cls. C4 is the natural substrate of Cls, and is cleaved in large amounts into C4a and C4b. The C4b fragment can attach to the cell membrane (particle surface), and a small percentage becomes bound. The resultant Cls-C4b site has the capacity to neutralize certain viruses. In the following step, C2 is adsorbed by membrane-bound C4b (Mg++-dependent), and is cleaved by Cls into C2a and C2b. The C2a fragment fuses with C4b to form another enzyme with C3 as its substrate (C3 convertase), while C2b enters the fluid phase. C4b-C2a then cleaves C3 into two fragments, C3a and C3b, allowing nascent C3b to associate itself with the activating enzyme. C3a (anaphylatoxin) is given off
into the fluid phase, while C3b possesses a transient ability to attach and opsonize cell membranes. The latter has a binding site for lymphocytes and various phagocytic cells, and functions in immune adherence and in enhancing phagocytosis. The further association of C3b with C4b-C2a on the membrane surface gives rise to a trimolecular complex with C5-cleaving activity (C4b-C2a-C3b). C5 is split into C5a (anaphylatoxin and chemotactic factor), the fluid phase component, and C5b which possesses a transient binding site.

The third phase of the classical sequence is initiated by the cleavage of C5, which initiates the formation of the stable C5b-C9 complex in both the fluid phase and on the active site. C5b sequentially activates, without further enzymatic activation, the attachment of C6 and C7 (chemotactic activity) with C8 and six molecules of C9. The complex (C5b-C6-C7-C8-C9) has the ability to alter cell membranes and cause irreversible damage.

The rapid decay of activated binding sites restricts complement action to the immediate environment of the site. Rigid control also limits the time and space of classical complement reactions by the decay of C4b-C2a and C4b-C2a-C3b, and through the actions of serum C1 inactivator and C3b inactivator (Muller-Eberhard, 1975; and Mayer, 1970). These classical complement reactions have been studied in great detail and recently reviewed (Davis et al., 1973; Humphrey and White, 1970; and Muller-Eberhard, 1975).

Recent studies have demonstrated that the endotoxins of certain Gram-negative bacteria function as activating factors of the alternate pathway of complement activation. The ability of LPS to deplete complement com-
ponents was first observed to be a function of a newly discovered "prop-
erdin" system. This system was described as a group of normal serum pro-
teins which interacted with zymosan, a variety of polysaccharides, and
lipopolysaccharide and resulted in the preferential consumption of "C3"*
in the presence of Mg\(^{++}\) (Pillemer et al., 1954, 1955; Wedgwood et al., 1956;
Wardlaw and Pillemer, 1956; and Hinz et al., 1956). This group of factors
appeared to mediate several diverse activities of normal serum such as, the
killing of Gram-negative bacteria, neutralization of certain viruses, and
lysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinu-
ria (PNH).

A number of factors have been shown to activate the complement se-
quence without significant consumption of Cl, C2, and C4 (Sandberg et al.,
1970; and Gewurz et al., 1968b). The absence of any reaction of endotoxin
with each of the terminal components indicated the need for serum factors
other than Cl, C2 and C4 in complement activation (Gewurz et al., 1968b).
These results were corroborated by similar experiments that firmly estab-
lished the existence of a second complement pathway. By demonstrating the
complement consuming ability of guinea pig γ-1 antibodies, the ability of
C4-deficient serum to sustain complement activation, and the C3-cleaving
activity of LPS in C2-adsorbed guinea pig serum, an alternate pathway was
believed to exist (Frank et al., 1971; Sandberg et al., 1970, 1971; and

\*C3 was an early designation of components C3 to C9.
Through a second series of related investigations with cobra venom factor (CVF), the "C3 activator system" was identified as an alternate complement pathway (Muller-Eberhard & Gotze, 1971). Recent investigations have identified the properdin and activator systems as an identical pathway, and has currently led to the postulation of a plausible molecular concept of alternate pathway activation (Muller-Eberhard, 1975; and Gotze & Muller-Eberhard, 1976). Activation of this pathway has been demonstrated to occur through a number of substances including LPS, plant polysaccharides, a number of polymeric substances, T cell-independent antigens, B cell mitogens, levan-polymerized flagellin, dextran sulfate, and pokeweed mitogen (Mergenhagen et al., 1969, 1973; Burger et al., 1975; Hadding et al., 1973; and Bitter-Suermann et al., 1975). Immunoglobulin activation of the alternate pathway is not required, but does occur frequently. Guinea pig \( \gamma-1 \) and \( \gamma-2 \), as well as rabbit IgG, have the ability to activate the alternate pathway through a site in their F(\( \text{ab}' \))\(_2\) fragment (Burger et al., 1975; Osler et al., 1969; Reid, 1971; Sandberg et al., 1972, 1970, 1971; and Schur & Becker, 1963). Aggregates of human IgA\(_1\) and IgA\(_2\) are endowed with the same capacity, while human IgG acquires the function only under exceptional circumstances (Gotze & Muller-Eberhard, 1976).

In a yet unknown manner, an alternate pathway activator causes the conversion of the properdin precursor to active properdin (\( \text{P} \)). However, recent evidence indicates that there are several factors that precede properdin utilization (table 1) (Muller-Eberhard, 1975). Following the contact
### Table 1

Nomenclature and Proteins of the Properdin System

<table>
<thead>
<tr>
<th>Protein Component</th>
<th>Symbol</th>
<th>Molecular Weight</th>
<th>Serum Conc. (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiating Factor</td>
<td>IF</td>
<td>160-180,000</td>
<td>20-50</td>
</tr>
<tr>
<td>Properdin</td>
<td>P</td>
<td>212,000</td>
<td>25</td>
</tr>
<tr>
<td>3rd Complement Component</td>
<td>C₃</td>
<td>180,000</td>
<td>1,104-1,373</td>
</tr>
<tr>
<td>C₃ Proactivator</td>
<td>C₃Pa,Pa,orB</td>
<td>93,000</td>
<td>143-226</td>
</tr>
<tr>
<td>C₃ Proactivator Convertase</td>
<td>Pase,orD</td>
<td>24,000</td>
<td>1-5</td>
</tr>
<tr>
<td>C₃ Inactivator</td>
<td>C₃b INA</td>
<td>100,000</td>
<td>30-50</td>
</tr>
<tr>
<td>C₃ Inactivator Accelerator</td>
<td>B₁H</td>
<td>150,000</td>
<td>133</td>
</tr>
</tbody>
</table>

(reproduced with modifications from Muller-Eberhard, 1975)
with essential structures of the activating particle, an activated initiating factor (IF) interacts with Pa (factor B), Pase (factor D), and native C3 (in the presence of Mg++) to generate a C3 convertase (figure 1). Pa and C3 interact without enzymatic cleavage and can be recovered intact after activation (Fearon & Austen 1975; Nicholson et al., 1975; and Vogt et al., 1975). This soluble enzyme acts only on C3, and can deposit C3b onto the surface of the activating substance. The latter fragment subsequently acquires a transient site for various acceptors (Muller-Eberhard, 1966), a stable binding site for immune adherence reactions (Eden et al., 1973; Lay & Nussenzweig, 1968; and Vogt et al., 1975), a site which interacts with Pa and Mg++, and a binding site for activated properdin (Fearon & Austen, 1975).

In this initial cleavage of C3, C3b is also deposited in the fluid phase (with C3a), which leads to the further formation of a soluble, labile C3 convertase and a bound, labile C3–C5 convertase (Gotze & Muller-Eberhard, 1976). Analogous to classical C4b–C2a, bound and soluble C3b display a Mg++-dependent affinity for PA in the formation of a reversible inactive complex (Medicus et al., 1976; Nicholson et al., 1975, and Vogt et al., 1975, 1976). This complex is then subject to the hydrolytic action of Pase, which cleaves a fragment from Pa, producing a complex (C3b–Bb) possessing C3-cleaving activity (active site on Bb). This convertase has been shown to operate in a positive feedback mechanism through the generation of C3b fragments (Muller-Eberhard & Gotze, 1972; Gotze & Muller-Eberhard, 1976; and Brade et al., 1974). Evidence suggests that at least two spacially arranged molecules
are necessary for $\text{C3b-Bb}_n$ expression of C5 convertase activity (C3b-Bb, where $n>1$). C5 cleavage, as in the classical pathway, activates terminal components involved in the membrane attack unit. Both C3 and C5 convertase of the alternate pathway are analogous to the classical pathway enzymes, and function in producing large amounts of biologically active products (C3a and C5a) in the fluid phase.

It is now clear that properdin is utilized in the final steps of this pathway. It occurs in serum in a precursor form, and is able to form a $\text{P}$-dependent C3 convertase with Pa, Pase, C3, and $\text{Mg}^{++}$ (Gotze & Muller-Eberhard, 1976). Evidence suggests that surface-bound C5 convertase ($\text{C3b}_n$-Bb) is the properdin activating factor, and that $\text{P}$ also participates in a fluid phase C3 convertase. The attachment of $\text{P}$ to the bound C5 convertase increases the half-life of the enzyme by conferring stability on the association of C3b-B and by retarding spontaneous decay (Fearon & Austen, 1975; and Vogt et al., 1974, 1976). The stabilized enzyme ($\text{C3b}_n$-Bb-P) acts on both C3 and C5, and can undergo reverse assembly by loss of factor B. This $\text{P}$-bound site can attain enzymatic activity by the reacquisition of B in the presence of $\text{Mg}^{++}$ and Pase (factor D).

This complex pathway has, at present, three known modes of regulation. C3-C5 convertase decays spontaneously due to the loss of Bb. Secondly, soluble C3b can actively disassemble $\text{P}$-stabilized C5 convertase by releasing Bb. The third is by the action of a serum enzyme, C3b inactivator (C3b INA), which cleaves bound C3b into two smaller fragments, and causes the release of bound $\text{P}$ (Medicus et al., 1976; and Gotze & Muller-Eberhard, 1976). Optimal inhibitory regulation of C3b inactivator requires the simultaneous
presence of yet another serum protein. The effector is a 10s, $\beta_{1H}$-globulin (C3b INA accelerator), which operates in trace amounts (Whaley and Ruddy, 1976; Gitlin et al., 1975; Lachmann and Muller-Eberhard, 1968; and Nilsson & Muller-Eberhard, 1965).

Thus, through the activation of either complement pathway, C3 and C5 convertase, as well as the membrane attack unit, can be constructed and activated. The major differences of the two pathways reside in the mode and specificity of initial activation, and in the use or bypassing of components C1, C2, and C4.

Investigations utilizing reactions of Gram-negative bacteria or LPS with complement have involved bactericidal action, significant complement consumption profiles, reactivity in complement-deficient sera, and the generation of biologically active products (Wardlaw, 1964; Bladen et al., 1967; Gewurz et al., 1968a,c, 1969b, Mergenhagen et al., 1969; and Fine, 1972 and 1974).

The endotoxin from a variety of organisms (Veillonella alcalescens, Serratia marcescens, Salmonella typhosa, and Escherichia coli) has demonstrated the ability to activate complement when incubated with guinea pig, human, and primitive vertebrate sera (Pillemer et al., 1955; Gilbert and Braude, 1962; Miller et al., 1966; Gewurz et al., 1968; and Day et al., 1970). Microgram amounts of LPS caused significant depletion of complement levels in all species that exhibited antibody-induced hemolytic activity (Day et al., 1970). Complement activation was evident in these reactions by the presence of multiple lesions on the surface of LPS particles.
Complement fixation by LPS has been examined in view of the initiation of biological reactions, and in comparison to classical complement fixation. It was reported that factors exist (alternate pathway proteins) in normal guinea pig and human sera to initiate complement fixation upon incubation with LPS in the absence of exogenous antibody (Bladen et al., 1967; Day et al., 1970; Gewurz et al., 1968a and 1971). Endotoxin depleted the six terminal complement components (C3-C9) with minimal consumption of C1, C2, and C4 (Gewurz et al., 1968b). In addition, Gewurz et al., (1968b and 1971) have reported that the LPS of V. alcalescens consumed significantly greater amounts of terminal components than either the zymosan activated alternate pathway, or immune complex activated classic pathway.

These reactions were further examined utilizing sera from man, pig, and cow with developmental agammaglobulinemia (Gewurz et al., 1969a and 1970). Depletion of complement ensued in each of the vertebrate sera, except human, when challenged with LPS, and the percent fixation was comparable to immunoglobulin-normal control animals. However, the lower level of complement consumption in human agammaglobulinemic, C1-deficient, and C2-deficient sera implied the requirement of an efficient use of anti-LPS immunoglobulins with C1, C2, and C4 in the formation of an intermediate C3 convertase (Gewurz et al., 1971a,b; Synderman et al., 1971c).

Further examination of LPS-vertebrate complement reactions demonstrated complement activation and efficient consumption of terminal components in the absence of C4b-C2a (Marcus et al., 1971; and Frank et al., 1970). This supported the concept of alternate pathway activation in the absence of im-
mune complexes. The minimal consumption of C1, C2, and C4 in LPS-guinea pig serum incubations was determined to be the result of a natural antibody. In addition, chelation of human serum with EGTA (binds Ca\(^{++}\) more avidly than Mg\(^{++}\)) inhibited classical complement activation, but allowed alternate pathway activation when challenged with erythrocyte-labeled LPS (E-LPS) (Fine, 1972 and 1974; Forsgren et al., 1975). Thus, LPS activates the alternate pathway of complement fixation, and is capable of terminating in a membrane attack unit independent of antibody mediation.

Ample evidence exists to demonstrate the role of complement (C5b-6-7-8-9) in bactericidal reactions initiated by immune complexes (Muller-Eberhard, 1966 and 1975; Rapp and Boros, 1966; and Nelson, 1965). LPS is also capable of acting as a substrate for this reaction, which consumes significant amounts of the six terminal components (Boros, 1964; Hadding et al., 1966; Bladen et al., 1966; Gewurz et al., 1970 and 1971; and Mergenhagen et al., 1968). The incubation of guinea pig sera with LPS or Gram-negative bacteria regularly induces lesions on the particle surface, which are characteristic of complement activation (Bladen et al., 1967; and Mergenhagen et al., 1969). When guinea pig sera was treated with a number of complement inhibitors, the loss of the ability to lyse sensitized red blood cells was associated with the absence of lesions on the LPS surface of V. alcalescens (Bladen et al., 1967; and Mergenhagen et al., 1968). Lesions on endotoxin are consequent to the activation of complement and the interaction of multiple complement components. In the formation of such lesions, given amounts of LPS consumed greater quantities of C3 to C9 than either immune complexes
or aggregated human gammaglobulin (Gewurz et al., 1969b).

Activation of the clotting system by LPS-complement activation is another factor in the pathological effects of LPS (Thomas, 1954; Mergenhagen et al., 1969). The injection of LPS into a suitable host causes a profound thrombocytopenia, which is characterized by the deposition of agglutinated platelets in the liver, spleen, and lung (McKay and Shapiro, 1958; Stetson, 1951). In vivo reactions of LPS cause platelet degranulation and release of factors (histamine and platelet factor three, P.F.3) through a heat-labile, cation-dependent, plasma system (Horowitz et al., 1962; Des Perez and Bryant, 1966). Complement was implicated as the mediator of the LPS-platelet reaction, and a Mg\(^{++}\) requirement implicated the alternate pathway as the apparent in vitro mechanism (Spievogel, 1967; Nelson, 1965; Sequieria and Nelson, 1961; Gocke and Osler, 1965; Sandberg et al., 1971; Zucker and Grimm, 1974; and Pfueller and Luscher, 1974).

Endotoxin activation of the clotting mechanism appeared to require C3, C3 proactivator (Pa), and possibly properdin as necessary factors (Zucher et al., 1974; and Kane et al., 1973). However, a fault in the clotting mechanism of C6-deficient rabbits implicated the membrane attack unit from either pathway as the mediator of platelet degranulation (Zimmerman, 1971). The reaction appears to be a noncytolytic event (lack of C8 and C9) in which C3, C5, C6, and C7 are required (Kane et al., 1973; and Graff et al., 1976). Thus, the aggregation of platelets, degranulation, and activation of P.F.3 by endotoxin-complement interaction may represent a possible mechanism of the pathological intravascular coagulation seen in Schwartzman-
type reactions and Gram-negative sepsis (Zimmerman, 1973; and Brown and Lachmann, 1973).

Endotoxic lipopolysaccharides of Gram-negative bacteria are macromolecular complexes which are composed of two chemical moieties: a complex heteropolysaccharide and a lipid portion, lipid A (Shands, 1971; and Gewurz et al., 1971). LPS is rich in antigenic determinants with which immunoglobulins frequently react (Neter, 1969).

A number of morphological studies on LPS of various bacterial genera have shown superficial structural similarities in a trilaminar arrangement, which could be common to all gram negative lipopolysaccharides (Shands, 1971; Rothfield et al., 1966; Bladen et al., 1967; De Petris, 1967; and Knoz et al., 1967). This structure consists of linear repeating oligosaccharides ("O" antigen) linked to a core polysaccharide, which is covalently linked to the lipid A moiety (Luderitz et al., 1966b). Lipid A is a highly substituted, long chain fatty acid derivative of a phosphorylated hexosamine-disaccharide backbone: 4-phosphoglucomaminyl-β-1, 6-glucosamine-1-phosphate (Westphal, 1975). The core polysaccharide forms an ester bond to the hexosamine unit commonly with KDO (2-keto-3-deoxyoctanate). The polysaccharide contains five sugar constituents in most endotoxins, and commonly consists of a heptose, glucose, galactose, and glucosamine (besides KDO) (Westphal, 1975). Three hydroxyl groups on the disaccharide backbone are esterified with lauric (C\textsubscript{12}), myristic (C\textsubscript{14}), and palmitic (C\textsubscript{16}) acids. An ester linkage to the KDO unit is formed at a fourth hydroxyl group (Luderitz, et al., 1973).
It appears that the polysaccharide components of the LPS molecule do not play any specific role in endotoxicity (Galanos et al., 1971). However, the polysaccharides attached to lipid A may render it more soluble by influencing the conformation, which indirectly effects endotoxic reactions (Galanos et al., 1972; Morrison, 1976; Loos et al., 1974; and Lachman and Nicol, 1974. Purified suspensions of "O" antigens and core polysaccharide have been found to be ineffective in alternate complement pathway activation (Mergenhagen et al., 1961).

Complement activation is not a property common to all lipopolysaccharides, though, the isolated lipid A from both active and non-active endotoxin is strongly toxic and anticomplementary when solublized by a suitable carrier (Galanos, 1971). The reaction with complement does not result in any chemical or compositional changes, but does result in the loss of solubility in water and loss of biological activity.

The participation of complement in inflammation and tissue destruction has been partially attributed to the production of anaphylactic and chemotactic factors through both pathways (Mergenhagen, 1970,1972a; Muller-Eberhard, 1972a; Budzko et al., 1971; and Hugli, 1975b,a). The developing sequellae of reactions, following the injection of immune complex-adsorbed serum in guinea pigs, was first proposed to be the effect of a toxic factor (anaphylatoxin) generated from the serum (Freidberger, 1910). Generally, anaphylatoxin is a pharmacologically active substance that causes contraction of the guinea pig ileum, is blocked by anti-histamines, increases vascular permeability, and degranulates mast cells (Mergenhagen et al., 1969).
The function of complement in anaphylatoxin generation was first recognized by Osler et al., (1959a,b). Hemolytic complement fixation was necessary for rat skin-bluing activity when immune complexes, agar, inulin, dextran, liquoid, and zymosan were incubated with rat serum. Through the inhibition of complement activity, the immune aggregates were unable to produce anaphylatoxins upon incubation. Cl esterase activation of guinea pig sera also generated factor(s) that fulfilled the established criterion for anaphylatoxin (Ratnoff and Lepow, 1963; and Dias Da Silva and Lepow, 1966a). The reactions were dependent on the participation of complement factors beyond Cl, C2, and C4.

In separate reactions, purified complement components were incubated with a number of factors (LPS, CVF, immune aggregates, and polysaccharides) which stimulated the release of anaphylatoxin. This factor could be produced by tryptic cleavage of C5, but was also generated in solutions containing only C1, C2, C4, and C3 (Jensen, 1966, 1967; Dias Da Silva and Lepow, 1966a, 1967; and Cochrane and Muller-Eberhard, 1968). The latter stimulated the in vitro degranulation of mast cells, and was unaffected by the addition of C5. Further examination of these reactions revealed that the fluid phase anaphylatoxin was generated from a reaction of Cl, C2, C4, and C3. This factor was cleaved from C3 (C3a), and had an approximate molecular weight of 6800 (Shin et al., 1969a; Dias Da Silva et al., 1967; and Cochrane and Muller-Eberhard, 1968). Utilizing sheep red blood cell-bound complement intermediates (C2a-C4b-C3), anaphylatoxin was generated from C5 (C5a), and caused contraction of the guinea pig ileum. The
cleavage of C5 appeared to be dependent upon the formation of a cell-bound C5 convertase, and generated a factor (15,000 daltons) with both anaphylactic and chemotactic abilities. Thus, C3a and C5a serve as anaphylatoxins when activated by known initiators of the classical complement sequence (Mergenhagen et al., 1969).

The experimental evidence that endotoxins induce anaphylatoxins from whole serum strongly indicates complement factors identical to those mentioned previously, and have become apparent with the current molecular model of alternate pathway activation. The incubation of LPS (V. alcalescens and E. coli) in rat plasma and guinea pig sera produced the capacity to contract the guinea pig ileum in a manner analogous to anaphylatoxin (Greisman, 1960; and Lichtenstein et al., 1969). A positive correlation was made between LPS-activation of terminal complement components and the appearance of anaphylactic activity. Furthermore, these reactions were inhibited by anti-histamines and complement inactivation.

Several investigators have determined that LPS-complement activation cleaves small molecular weight fragments from guinea pig C3 and C5 (C3a and C5a), which retain anaphylactic and chemotactic activity (Jensen et al., 1969; and Synderman et al., 1969). Incubation of LPS, lipid A, or zymosan in human, guinea pig, or hamster sera generates C3a and C5a via the alternate pathway (Brade and Vogt, 1972; Hook, 1970; and Dierich et al., 1973). The anaphylatoxins released in these reactions caused the degranulation of mast cells and human basophilic leukocytes, releasing histamine and heparin (Hook, 1970; Muller-Eberhard, 1975; Gewurz et al., 1971; Johnson et al.,
1975; and Hook et al., 1975). Isolated C5a from LPS-stimulated serum has been shown to cause typical anaphylactic responses. The subcutaneous injection of this factor in rabbits and rats produced an intense inflammatory reaction that resembled the Arthus phenomenon in lesion formation and time course (Synderman et al., 1971, 1969; and Jensen et al., 1969).

In addition to C3a and C5a, LPS-complement reactions liberate or activate a histamine-heparin releasing factor with vasoactive properties. The expression of vasoactivity by this factor, a 60,000 molecular weight β-2 globulin, is dependent on complement initiation, but is inconsistent with published data on complement anaphylatoxins (Hook et al., 1972).

Both C3a and C5a have been recognized in human serum, but were considered insignificant due to negligible biological activity in experimental systems (Muller-Eberhard and Vallota, 1971; and Bokisch and Muller-Eberhard, 1970). The isolation and characterization of an anaphylatoxin inactivator (AI) revealed a control mechanism for human complement anaphylatoxins. The factor occurs in trace amounts as a carboxypeptidase that functions by the removal of the C-terminal amino acid residue on both C3a and C5a (Vallota and Muller-Eberhard, 1973; and Bokisch et al., 1975). The removal of AI allowed the generation of anaphylatoxins through both complement pathways by LPS, inulin, and immune complexes. It was determined that human anaphylatoxins are not significantly different from those of other mammalian species, and that C5a (16,500) is one thousand fold more biologically active than C3a (9,000), on a molar basis.

Polymorphonuclear leukocyte (PMN) accumulation follows the intra-
dermal injection of microgram amounts of endotoxin, and is one characteristic pathological change induced by LPS (Stetson, 1951; and Thomas, 1954). Similar i.p. injections of sub-microgram amounts caused a dramatic increase in PMN accumulation in peritoneal exudates (Fruhman, 1959). The mechanism mediating these responses was examined, in vitro, following the development of a suitable assay by Boyden (1962). The assay measured leukocyte migration through a Millipore filter within an acrylic diffusion chamber.

Immune complexes incubated in guinea pig serum caused the chemotaxis of PMN leukocytes through the activation of a heat-labile serum enzyme system (Boyden, 1962). Similarly, aggregated human gamma globulins alone could not generate chemotaxis, but produced this activity when incubated in rabbit serum (Keller and Sorkin, 1965). Further studies determined that a chemotactic component was derived from the complement system, and is composed of a trimolecular complex of C5b-6-7, with a molecular weight of 300,000 (Ward et al., 1965, 1966). A second factor derived from C3 (C3a), with a molecular weight of 8,000, also exhibited chemotactic attraction to rabbit PMN leukocytes (Ward, 1967).

The incubation of microgram amounts of LPS from V. alcalescens and S. marcescens in human, guinea pig, and rabbit serum induced chemotactic activity for PMNs (Synderman et al., 1968). Various inhibitors of complement also halted chemotaxis in LPS-stimulated serum. The active component was identified as a peptide with a molecular weight of 15,000 which was antigenically identical to the complement anaphylatoxin, C5a (Synderman et al., 1968, 1969; and Shin et al., 1968). C5-6-7 and C3a chemotactic factors were found to possess weak biological activity in the chemotactic
attraction of PMN leukocytes (Shin et al., 1969b).

The intradermal injection of C5a in rabbits caused increased vascular permeability and neutrophil accumulation (Jensen et al., 1969; and Shin et al., 1969b). This led to the hypothesis that complement activation, as a biological effector of inflammation through anaphylactic and chemotactic activities, mediates certain pathologic effects of bacterial LPS (Ward and Newman, 1969).

Synderman et al., (1971) investigated the role of C5a in the early accumulation of PMNs in inflammatory exudates. A 0.5% glycogen solution was injected i.p. in guinea pigs, and exudates were extracted at 30 minutes, 1, 2, 3, 4, 5, 6, 27, and 48 hours. PMN levels increased to a maximum from 2 to 6 hours (260 x 10^6/ml), and then decreased to 48 hours. The chemotactic factor was present after 30 minutes, and increased to a maximum at 2 hours. C5-deficient mice, highly susceptible to microbial infection, were unable to duplicate this experiment.

Guinea pig, mouse, and human mononuclear leukocytes also respond chemotactically to C5a, in vitro. This factor may be responsible for monocyte accumulation in wound healing, and could mediate tissue damage in sensitive tissues (Synderman et al., 1971b, 1972a, and 1975; and Lett-Brown, 1976).

Human basophilic leukocytes have been found to respond chemotactically to a number of factors, including C5a. In mixed populations of leukocytes, a 5-10% basophil population responded to zymosan, LPS, and trypsin-generated C5a, together with neutrophils and monocytes (Kay and Austen, 1970;
and Ward et al., 1975).

Eosinophils have also been demonstrated to respond chemotactically to a trypsin-derived C5a in mixed population of leukocytes. When the eosinophil population exceeded 10% in the Boyden test chamber, C5a selectively attracted these cells with minimal migration of neutrophils (Kay et al., 1973).

Thus, C5a generated from both complement pathways mediates the directed migration of blood leukocytes, especially PMNs and monocytes. This has been proposed as the second major biological activity of complement as a mediator of the inflammatory response (Muller-Eberhard, 1972a; and Mergenhagen, 1972).
Veillonella alcalescens Cl, a Gram-negative anaerobe (diplococcus) isolate from sheep rumen, was supplied by Dr. E.A. Delwiche of the College of Food Sciences, Cornell University in Ithaca, New York. Initial cultures (1.8 liters) were grown in a pre-reduced lactate medium in 2000 ml flasks for seventy-two hours at 37°C. This liquid medium was overlayed with sterile paraffin oil (degased) to insure reduced conditions under incubation. This medium was prepared according to Virginia Polytechnic Institute specification (VPI Anaerobe Lab Manual, 1975), and was used within one day of preparation. The medium was a peptone-yeast-lactate (PY-L) with the following formula: 10 gm peptone (Difco), 10 gm yeast extract (BBL), 4 ml resazurin solution (0.25 mg per ml H₂O), 40 ml salts solution (0.2 gm CaCl₂, 0.2 gm MgSO₄ 7H₂O, 1.0 gm K₂HPO₄, 1.0 gm KH₂PO₄, 10 gm NaHCO₃, and 2.0 gm NaCl per 1000 ml distilled H₂O), and 9 ml 85% lactic acid added to 1000 ml distilled water. The pH was adjusted to 7.0 with 8N NaOH and boiled until the resazurin indicator became colorless. Then 0.5 gm cysteine hydrochloride (Eastman) was added as a reducing agent, the pH readjusted to 7.0, and the medium autoclaved. Cells were harvested, washed in cold saline (0.15M NaCl), resuspended in 40 ml sterile skim milk, and lyophilized.

Viable cultures were maintained on pre-reduced brain-heart infusion agar plates containing 3.7% B.H.I., 0.5% yeast extract, 0.4% resazurin solution, and 0.05% cysteine hydrochloride. The plates were incubated in vented Gas
Pack anaerobic jars (BBL) at 37°C, and were transferred once every three days. Anaerobic conditions in the jar were established by flushing twice with anaerobic grade CO₂, and filling the jar with gas composed of 10.34% CO₂, 10.0% H₂, and the balance N₂. Disposable anaerobic indicators (BBL) with methylene blue and the resazurin in the media were used to monitor anaerobic conditions in the jar.

**Extraction of bacterial endotoxin**

For the routine extraction of endotoxin from *Veillonella alcalescens*, three flasks, each containing 250 ml of PY-L, were inoculated from single colonies of *V. alcalescens* and incubated at 37°C for forty-eight hours. These cultures were used to inoculate six (1 starter culture per 2 liters) liters of PY-L, which were subsequently incubated for seventy-two hours at 37°C. The cells were grown to 10⁹ per ml, harvested with a Beckman J-21 centrifuge at 15,380 x g for thirty minutes, washed three times in cold saline (0.15M NaCl), and resuspended in 40 ml of saline. Cell walls were disrupted by sonication (Blackstone) at two minute intervals, followed by two minutes cooling at 4°C, for a total of twenty-two minutes of actual sonication. This procedure was performed with extreme care to generate a minimum amount of heat to the cell walls by using ice baths. Cell lysis was measured by phase contrast microscopy and viable counts (pour-plate technique). Cell wall debris was harvested by centrifugation (Beckman J-21) at 27,300 x g for two hours. The cell wall pellet was resuspended in sterile distilled water and centrifuged. This washing procedure was repeated six times, and the pellet of cell wall material was finally resuspended in 50 ml of distilled water.
Endotoxin was extracted by the phenol-water method of Westphal (1952, 1971), as diagramed in figure 3. The yield of purified LPS from whole bacterial cells was approximately 1.72% (9.8 mg of endotoxin) of the dry weight.

**Experimental animals**

Young adult female guinea pigs (Scientific Small Animal), from 500 to 700 grams, were housed in a thermostatically controlled and humidified room, with a diurnal variation of light, at Loyola University Dental School Animal Care Facility. They were contained in large metal cages with mesh bottoms, and were fed Purina guinea pig chow and water (supplemented with ascorbic acid) ad libitum. Adult New Zealand rabbits, from 2 to 3 kilograms, were also housed under similar conditions. They were watered and fed Purina rabbit chow and lettuce ad libitum. Only clinically healthy rabbits were considered for use in experimental studies.

**Source of complement**

Complement was collected from healthy, female guinea pigs by cardiac puncture. An intravenous infusion set (19 gauge, 7/8 inch needle) was connected to a #5 1/2 rubber stopper with a glass-tube lead for attachment to a small vacuum pump. The complete sterile apparatus was aseptically attached to a 50 ml conical centrifuge tube (I.E.C.), and blood was collected from the guinea pigs under vacuum (2 psi). The blood was allowed to clot at room temperature for fifteen minutes, the tubes rimmed with sterile applicator sticks, and centrifuged at 160 x g for thirty minutes in an International Clinical Centrifuge (I.E.C.). The separated serum was col-
FIGURE 3

Phenol-water extraction of endotoxin

50 ml of an 89% phenol solution, heated to 56°C, was added to the 50 ml cell wall suspension, and heated for thirty minutes at 56°C with continuous stirring.

The suspension was cooled to 4°C and centrifuged at 27,300 x g for one hour, resulting in a distinct phase separation of phenol and water.

Aqueous phase I

Phenol phase

50 ml distilled water added to phenol phase, heated for 30 min. at 56°C with stirring, and cooled to 4°C.

Centrifugation was repeated at 27,300 x g for 1 hour.

Aqueous phase II
Phenol phase was discarded.

The aqueous phases (upper phase) were dialyzed against distilled water to remove phenol, as indicated by the lack of detectable odor.

Endotoxin was precipitated from the water by the addition of granular sodium acetate (Sigma) to a final concentration of 0.15M and absolute ethanol dropwise to a final volume concentration of 68% while stirring at 4°C.

The solution was kept at 4°C over night to allow precipitation.

The precipitate was separated by centrifugation at 1000 x g for thirty min.

The pellet from centrifugation was resuspended in 25 ml of distilled water and dialyzed against distilled water to remove traces of ethanol.

Lyophilization yielded the purified endotoxin.
lected by sterile Pasteur pipets, placed in sterile tubes, and immediately frozen at -58°C (Revco). When approximately 30 ml of serum had been collected, it was pooled, divided into 1 ml aliquots, and frozen at -58°C. Whole guinea pig serum was used as the complement source in all assays performed.

Source of antiserum

Anti-V. alcalescens serum was prepared by injecting six female guinea pigs with heat-killed whole cells of V. alcalescens Cl. The saline cell suspensions (0.15M NaCl) were emulsified in complete Freund's adjuvant (Difco), and injected at a rate of three injections per week for five weeks. The injections consisted of increasing doses from 0.1 mg to 1.0 mg of dry whole cells, and were administered by intramuscular and subcutaneous routes, alternately. At the end of thirty-five days the animals were bled (36th day). Six days later they were boosted with 1.0 ml of a 1 mg per ml suspension of V. alcalescens subcutaneously. The following day they were again boosted intraperitoneally with 1.0 ml of the same suspension. Six days later they were again bled. Following this schedule, the guinea pigs were boosted with the cell suspension and bled on alternate weeks until approximately 40 ml of antisera had been collected (Hagen, 1972). Serum was separated and collected as already described, and was frozen in large tubes at -58°C. All injections were performed with disposable 3cc syringes with 22 gauge, 1 1/4 inch needles.

Pre-immunization bleedings were performed with a guinea pig bleeding apparatus, which used a 15 psi vacuum to collect blood from the tarsus vein. Antisera was collected by cardiac puncture. All samples with a passive hemagglutination titer of 5120 or above, were pooled, heat-inactivated at 56°C for
thirty minutes, decanted into vials, and frozen at -58°C until used (Weir, 1967).

Rabbit polymorphonuclear leukocytes

Polymorphonuclear leukocytes were collected from peritoneal exudates by a modified method of Sbarra and Karnovsky (1959). A 110 ml solution of 8% casein (Matheson, Coleman, and Bell) in saline (0.15M NaCl), supplemented with 50 µg neomycin per ml, was injected intraperitoneally with a 50cc syringe and 18 gauge needle. Approximately eighteen hours later, the cells were collected by injecting 150 ml of heparinized saline (10 units per ml) into the peritoneal cavity. The exudate was immediately aspirated from the lowest point in the cavity. An 18 gauge, 1 1/2 inch needle mounted in heparinized rubber tubing was connected to a glass tube lead into a #5 1/2 rubber stopper as previously described. This vacuum device was aseptically inserted into an I.E.C. conical centrifuge tube, and was used to aspirate fluid from the rabbit peritoneum. The leukocyte suspension was harvested by centrifugation for ten minutes at 160 x g, and resuspended in Gey's balanced salt solution (Difco) containing 2% BSA (Difco), 50 µg streptomycin per ml, 50 units penicillin per ml, and 10 units heparin per ml (Synderman et al., 1968). Leukocytes were counted by standard clinical procedures utilizing a Neubauer hemacytometer (American Optical Co.), and adjusted to 3 x 10^6 cells per ml with Gey's medium. Differential counts of two hundred Wright stained cells determined the number of PMN leukocytes in suspension (Brown, 1975). The counts ranged from 86 to 95.5% PMNs, with minimal numbers of monocytes, lymphocytes, and eosinophils.
Chelation of complement and antisera

Pooled guinea pig complement and anti-V. alcalescens sera were chelated with ethyleneglycol-bis(β-amino-ethyl ether) N,N'-tetra acetic acid (EGTA, Sigma) according to the procedure of Fine (1972, 1974; and Forsgren et al., 1975). Chelation with 10mM MgEGTA selectively removed Ca^{++} and classical complement activity, leaving the alternate pathway intact. Sterile 0.1M MgEGTA was used to chelate reaction mixtures by adding 0.015 ml of this solution to each 0.15 ml of antiserum and complement prior to incubation.

Chelator stock solutions of 0.1 M MgEGTA were prepared by dissolving EGTA in normal saline (0.15M NaCl). The mixture was heated to 60°C, and 5N NaOH was added dropwise until EGTA went into solution. This mixture was adjusted to a final pH of 7.45 with 1N HCl. Granular MgCl\textsubscript{2} was then added to the solution, which was brought to a final concentration of 0.1M (MgCl\textsubscript{2} & EGTA) with saline, and autoclaved.

Chemotaxis reaction mixtures

In the preliminary studies, a measured quantity of lyophilized cell wall sonicate or LPS was diluted in 0.15M veronal buffered saline (VBS), pH 7.3, to 0.15 ml and incubated with 0.15 ml guinea pig complement for thirty minutes at 37°C. The mixture was then removed and heat-inactivated at 56°C for thirty minutes to prevent any further complement activity. The mixture was then diluted to 1.5 ml in Gey's medium (final serum concentration 10%), and utilized in a chemotaxis assay (Synderman et al., 1968, 1969; and Keller et al., 1976).

In the normal immune serum reactions, 50 µg of LPS in 0.15 ml VBS was
incubated with 0.15 ml guinea pig complement and 0.15 ml anti-*V. alcalescens* sera (diluted to 0.48 ml with VBS) for thirty minutes at 37°C. This was followed by heat-inactivation at 56°C for thirty minutes, dilution to 1.5 ml in Gey's medium, and measurement of chemotactic activity. In reactions of chelated serum, 50 µg LPS diluted in 0.15 ml VBS was incubated with 0.165 ml of chelated guinea pig complement and 0.165 ml of chelated anti-*V. alcalescens* sera for thirty minutes at 37°C. These mixtures were inactivated and measured for chemotactic activity. All reaction mixtures in this series of experiments had a final serum concentration of 20% (Keller et al., 1976). Controls consisted of normal and chelated serum samples mixed with 0.15 ml VBS in place of endotoxin. They were incubated, heat-inactivated, and assayed for chemotactic activity.

**Quantitation of chemotaxis**

Measurement of complement activation was quantitated using a modified Boyden diffusion chamber (Neuroprobe, Inc., Bethesda). Permeable 8 micron and impermeable 0.45 micron Millipore filters were used to separate the two chambers of this chemotaxis assay system. Prior to assembly of the diffusion chamber (figure 4), the filters were thoroughly soaked in Gey's medium to prevent the entrapment of air between them. They were immediately placed in the filter retainer groove, and the filter retainer was carefully screwed into place. Approximately 1 ml of chemotactic mixture was placed, with a Pasteur pipet, in the lower compartment of the chamber, which was tilted to remove any air bubbles collecting at the lower (0.45 micron) filter surface. The prevention of leakage into the upper compartment assured proper positioning
Chemotactic solution  PMN leukocyte solution

Teflon plug

Filter retainer plug

13 mm diameter groove for filters. 8 micron filter placed above .45 micron filter.

Chemotactic mixture

Leukocyte suspension

FIGURE 4
Modified Boyden Diffusion Chamber
of filters. Immediately following, 0.56 ml of the leukocyte suspension was placed in the upper chamber compartment (teflon filter retainer) simultaneous with the placement of the remaining chemotactic mixture in the lower compartment. The solutions in both compartments allowed a very slight overflow, which resolved fluid levels and reduced variability in leukocyte migration due to hydrostatic pressure. The charged Boyden chambers were incubated for sixty minutes at 37°C with precautionary measures taken to prevent vibrations and movement (Keller et al., 1972, 1976).

Assessment of chemotaxis was measured by the direct quantitation of PMNs which migrated across the Millipore filters. The filters were removed together from the chamber, gently rinsed in Gey's medium, placed on a standard microscope slide, and stained by the following procedure: 5 minutes fixation in 85% ethanol, rinse in distilled H$_2$O; separate filters with the lower surface of the 9 micron filter facing up, stain with hematoxylin 5 minutes, rinse in distilled H$_2$O; dehydration with 70% ethanol (2 minutes), 95% ethanol (2 minutes), and absolute ethanol (3 minutes). The filters were then cleared in xylene and mounted with permount (Fischer). The number of PMNs that completely migrated through the filters were counted in ten random fields on the extreme lower surface of the 8 micron filter and on the upper surface of the 0.45 micron filter. The cells were counted under high power (43x) with the aid of a microgrid. The mean and probable error of measurement (P.E.M.) was calculated for each test and control. A between-within analysis of variance was computed between each test and control in both the chelated and normal serum samples. In addition, the same statistical analysis was computed between the normal reaction mixtures and the chelated mixtures to determine the
difference, if any, in chemotaxis generated from the alternate complement pathway, with and without classic pathway activation.

The Boyden chambers were completely disassembled and scrupulously cleaned following each chemotaxis assay. They were rinsed in tap, de-ionized, and distilled water, and allowed to dry in a relatively dust free environment under an ultra-violet light hood. Prior to each day's use, they were submitted to ultra-violet radiation for three hours.

**Measurement of residual hemolytic complement activity**

Residual levels of hemolytic complement in chelated and normal serum were measured and reported in $C'H_{50}$ units according to the method of Mayer (Kabat and Mayer, 1961). These experiments were performed by allowing the desired amount of antigen (LPS) and antibody react with a large quantity of complement. At the end of the fixation period, accurately measured aliquots of the reaction mixtures were measured for hemolytic complement levels by a photometric procedure. Sheep red blood cells were sensitized with rabbit anti-sheep hemolysin (Difco). This was accomplished by harvesting fresh red blood cells from sheep blood, which were suspended in VBS at $5 \times 10^8$ per ml with the use of a hemacytometer. These cells were subsequently concentrated to $1 \times 10^9$ per ml. The cells were optimally sensitized by the addition of 12.5 ml of a 1:400 dilution of anti-sheep hemolysin to 12.5 ml of red cells, which brought the final concentration to $5 \times 10^8$ per ml. This mixture was gently agitated for 5 minutes, and then incubated for sixty minutes at 37°C.

A 2.5 ml dilution of 50 µg LPS in VBS was mixed with 5.0 ml of a 1:10 dilution of complement and 2.5 ml of a 1:10 dilution of anti-*V. alcalescens*...
sera. Two identical 10 ml reaction mixtures were prepared with 50 µg of LPS in similar 1:10 dilutions of chelated complement and chelated antisera. Controls consisted of VBS substituted for LPS in all three reaction mixtures. All components were accurately measured and combined at 4°C (to retard activity), and were incubated for ninety minutes at 37°C in a water bath. At the end of the incubation period the tubes were removed, placed in an ice bath, and prepared for measurement of hemolytic activity. Both 4.0 and 5.0 ml quantities of a 1:10 dilution of each reaction mixture and control were placed in sterile tubes, diluted to 6.5 ml with VBS, mixed with 1.0 ml of sensitized sheep red blood cells, and incubated for ninety minutes at 37°C. Dilution blanks with 1.0 ml red blood cells and 6.5 ml VBS were also incubated for ninety minutes. One set of chelated reaction mixtures and controls were recalcified with sterile CaCl₂ solution at a final concentration of 10mM, prior to mixture with the amboceptor.

At the conclusion of the incubation period, all the tubes were removed and centrifuged at 160 x g. The supernatant of each tube was removed, and the optical density (O.D.) of oxyhemoglobin was read at a wavelength of 550 mµ in a Coleman Jr. spectrophotometer. The supernatant from the red cell VBS blanks was used as the blank in the readings of O.D. A reading was made of a 1:10 dilution of complement and antisera against a saline blank to determine a correction factor for serum absorbance. The per cent lysis of 5 x 10⁸ cells/ml was determined by dividing the corrected O.D. of reaction mixtures by the O.D. of 100% lysis. The latter was produced by freezing and thawing 1.0 ml of sensitized cells in 6.5 ml of VBS three times. The per cent lysis was transformed into C'H₅₀ units from published values (Kabat and
Mayer, 1961) based on the von Krogh equation, \( x = K(y/l-y)^{1/n} \) (mathematical expression of the sigmoidal response curve of hemolytic complement activity). These residual complement levels represent a control for the chelation of serum by EGTA in the chemotaxis assay.
Results

Activation of complement chemotactic factors in the absence of immune sera

Chemotaxis of leukocytes in a Boyden diffusion chamber is the most sensitive assay of biological activity generated from complement activation (Synderman et al., 1968, 1969, 1971, 1975). It quantitatively measures reaction products and activity, where immune hemolysis and the C3 to C9 assay qualitatively measure the small percentage of complement capable of effecting the lysis of sensitized erythrocytes.

To insure accurate measurement of chemotaxis, only those cells which completely migrated through the 8 micron filter were counted. The cells on the lower surface of the filter were observed to have distinct segmented and lobulated nuclei, with the entire cell outline visible (figure 5). Cells in lower planes (focusing down from the lower surface) were undergoing diapedesis within the pore of the filter, and only varied shapes of the nucleus could be discerned. In addition, cells that completely migrated through the filter and became detached, were collected on the 0.45 micron filter. These varied from 5 to 30% of the total leukocytes counts in text mixtures, but accounted for only 0-5% in controls. All readings were reported as the mean of ten random high power fields ± the probable error of measurement (P.E.M.). The latter represents possible error in the readings due to judgement of the operators decision of whether a cell was within the plane of measurement or slightly below (within a pore). These are random errors associated with measurement, and are systematic.
Figure 5. PMN leukocytes on the lower surface of an 8 micron pore Millipore filter following incubation in the Boyden chamber (43 x).
Thus, if further measurements of 10 random fields were made, 50% of the readings should fall within the stated measurement ± P.E.M. (Parratt, 1961).

Varying quantities of _V. alcalescens_ Cl cell wall material ranging from 5 µg to 5 mg were incubated with guinea pig complement in chemotaxis assays. Although these were somewhat arbitrary units, their usage was loosely based on previous findings of _E. coli_ activation of complement and pilot studies using _E. coli_ LPS and Veillonella cell walls at concentrations between 0.5 µg and 500 µg (Fine, 1974). Activation of complement by cell wall material was evident in the increases of directed migration of PMNs (figure 6). The number of migrating cells almost doubled from 5 µg to 500 µg, compared to minimal increases of 90.3 ± 4.5 to 103.4 ± 7.5, for cell concentrations between 500 µg and 5 mg. This is consistent with published data on complement activation in which LPS-generated chemotaxis reaches a plateau with increasing amounts of activator (Synderman et al., 1968). Controls for these experiments were consistent with readings between 28.9 ± 1.6 and 35.1 ± 1.5. These numbers express the random migration of leukocytes occurring throughout the incubation period. In statistical analysis, all test mixtures differed from positive controls with P < .01. Thus _V. alcalescens_ Cl, the rumen strain, can generate complement chemotaxis similar to the oral strains. Strain Cl has a typical cell wall structure and chemical composition for Veillonella species (Winter & Delwiche, 1975), and also possesses biologically active lipopolysaccharide in that structure.

The endotoxin extracted from Veillonella appeared to compose a very small quantity of the whole cell dry weight (1.7%). This does not necessarily reflect weak endotoxic activity in _V. alcalescens_ (Bladen et al.,
Figure 6

Graphic expression of V. alcalescens Cl cell wall and endotoxin chemotactic activity in serum reactions.

Mean PMNs/10 fields ± P.E.A.

ug amount of complement activator

0.05 0.5 5.0 50 500 5 mg
The purified material was readily solubilized in distilled water, and adhered tenaciously to sheep red blood cells (for hemagglutination) at concentration of 50 µg per ml. An insoluble preparation of LPS would have indicated complexing with divalent cations (Ca$^{++}$ and Mg$^{++}$) and an inability to generate significant biological activity (Shands, 1971).

The purified endotoxin effected complement-mediated chemotaxis in concentrations from 0.05 µg to 50 µg. Both 0.05 µg and 0.5 µg produced similar migrations of PMNs (62.9 ± 3.9 and 60.3 ± 5.1), as did 5.0 µg and 50 µg (88.8 ± 5.6 and 102.5 ± 5.1). Although there was not as dramatic a difference within this series of experiments as with those from cell wall studies, they demonstrated a consistent ability to generate complement-mediated chemotaxis in a Boyden system, with results comparable to or greater than those observed for cell wall material at hundred-fold higher concentrations. The controls varied slightly, from 16.9 ± 2.1 to 41.2 ± 1.4, but were all significantly less than the test reading with $P < .01$.

Based on this quantitation of complement chemotaxis for varying amounts of endotoxin, 50 µg was chosen as the quantity to activate immune and chelated test mixtures. This corroborates previous findings on the ability of the LPS of oral *Veillonella* strains, *Serratia marcesens*, and *Salmonella* species to generate maximum chemotaxis from a fixed quantity of guinea pig complement (Synderman et al., 1968, 1969; Mergenhagen et al., 1969; and Gewurz et al., 1971).

LPS-chemotaxis in immune and chelated immune sera
Eight in vitro immune reaction systems containing 50 µg LPS, anti-*V. alcalescens* sera, and complement, were tested and compared to eight identical reactions mixtures with MgEGTA chelated anti-sera and complement for stimulation of chemotaxis (table 2). The normal serum reaction varied from 85.2 ± 4.5 to 227.4 ± 16.2 but not significantly enough to effect its relation to data on the chelated reactions (variance within the sample). The chelated reactions mixtures also had a varied sampling, from 60.1 ± 12.2 to 204.7 ± 18.6. There was very little variation in the normal controls (from 22.2 ± 2.5 to 38.8 ± 3.1), which were significantly less than the chemotaxis in the reaction mixtures (P ≤ .001). Variation in the chelated controls was greater than that in samples previously examined (11.7 ± 1.4 to 81.3 ± 8.8). The controls were also significantly less (P ≤ .001 for 1, 2, 3, 4, 5, 8, and P ≤ .05 for 7 and 6) than reaction mixtures. Chelation was performed with MgEGTA because of its ability to remove Ca++, causing the disassociation of the Cl complexes, and the negation of classic complement activity with alternate pathway intact (Fine, 1972, 1974; and Forsgren et al., 1975).

The chelated immune system generated chemotaxis in eight separate tests (mean = 94.6 ± 7.1) to levels which were not significantly different from the experiments with normal complement and anti-sera (108.7 ± 8.9). The latter is in a system in which a significant titer of immunoglobulin was reacting with an antigen and forming complexes capable of initiating classic complement fixation, while the alternate complement pathway was being effected. Thus, the alternate pathway in chelated serum can, in fact, generate biologically active products (C3a, C5a, C5b-6-7) for chemotaxis as
Table 2

Quantitation of Chemotaxis in Normal & Chelated Serum

<table>
<thead>
<tr>
<th>Test</th>
<th>Mean PMNs/10 fields±P.E.M.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50µg LPS normal</td>
<td>50µg LPS chelated</td>
</tr>
<tr>
<td>1</td>
<td>143.9±8.3</td>
<td>119.2±4.1</td>
</tr>
<tr>
<td>2</td>
<td>90.0±6.4</td>
<td>121.3±6.6</td>
</tr>
<tr>
<td>3</td>
<td>85.2±4.5</td>
<td>204.7±18.6</td>
</tr>
<tr>
<td>4</td>
<td>139.3±8.8</td>
<td>184.2±5.2</td>
</tr>
<tr>
<td>5</td>
<td>227.4±16.2</td>
<td>108.6±13</td>
</tr>
<tr>
<td>6</td>
<td>132.3±13.2</td>
<td>59.3±3.3</td>
</tr>
<tr>
<td>7</td>
<td>147.3±21.1</td>
<td>79.6±7.6</td>
</tr>
<tr>
<td>8</td>
<td>121.2±10.5</td>
<td>69.1±12.2</td>
</tr>
</tbody>
</table>

Quantitation of leukocytes in normal serum assayed 50 µg LPS as an activator of 0.15 ml anti-*V. alcalescens* and 0.15 ml complement. The chelator measurements were assays of 50 µg LPS with chelated antisera and chelated complement. Controls substituted 0.15 ml VBS for the LPS.
quickly and efficiently as the classic and alternate systems together (in fixed amounts of LPS, antibody, and complement).

**Measurement of residual hemolytic activity**

Hemolytic titers of complement were measured by an assay to determine the effect of chelation with MgEGTA on classic complement fixation. These experiments measured the number of C'H\textsubscript{50} units present in a dilution of reaction mixture after incubation. In reaction mixtures with 50 µg LPS, normal antisera, and complement, residual hemolysis was negligible (table 3), whereas complement controls with antisera and VBS had a mean 37.5 C'H\textsubscript{50} units. Thus in normal serum reaction mixtures with 50 µg of endotoxin, hemolytic levels were reduced to a negligible level. The control levels represent the complement hemolytic unit in all reaction mixtures prior to incubation.

Chelated reaction mixtures and controls effected no hemolysis, due to the removal of Ca\textsuperscript{++} ions and negation of classical complement activity. However, the recalcification of chelated reaction mixtures and controls, prior to incubation with amboceptor, retained classical activity. The reaction mixture with LPS had a mean 6.4% hemolysis (negligible C'H\textsubscript{50} units), while the recalcified control had a measurement of 44.4 units (48.1%). The activated recalcified mixture had a significantly low titer of hemolytic complement activity, though slightly above the normal serum reaction mixture. Hence, chelation with MgEGTA removed classic complement activity from serum reaction mixtures, which could be reactivated by saturation with Ca\textsuperscript{++}. The small, almost insignificant, hemolytic levels of recalcified LPS-acti-
<table>
<thead>
<tr>
<th>Reaction Mixture</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mixture for</td>
<td>Hemolytic</td>
<td>Hemolysis %</td>
<td>Residual C'H₅₀</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemolytic</td>
<td>Estimation Diln</td>
<td></td>
<td>units</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vol.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C', VBS, &amp;</td>
<td>4.0</td>
<td>25.7%</td>
<td>32.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>antiserum</td>
<td>1:10</td>
<td>30.7%</td>
<td>42.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS, C', &amp;</td>
<td>4.0</td>
<td>1.9%</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>antiserum</td>
<td>5.0</td>
<td>1.4%</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chelated C', &amp;</td>
<td>4.0</td>
<td>0</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>antiserum</td>
<td>1:10</td>
<td>0</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS, chelated C',</td>
<td>4.0</td>
<td>0</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&amp; antiserum</td>
<td>5.0</td>
<td>0</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recalcified C',</td>
<td>4.0</td>
<td>47.6%</td>
<td>39.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&amp; antiserum</td>
<td>1:10</td>
<td>48.6%</td>
<td>49.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recalcified C',</td>
<td>4.0</td>
<td>8.5%</td>
<td>Neg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS &amp; antiserum</td>
<td>5.0</td>
<td>4.3%</td>
<td>Neg.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Neg = negligible
* As explained in text
vated reaction was due to LPS consumption of the six terminal components. This corroborates previous studies in which LPS of *V. alcalescens* and *E. coli* reacted with large amounts of the six terminal complement components in normal and chelated serum (Synderman et al., 1968; Gewurz et al., 1968; and Fine, 1974). This measurement of residual hemolytic complement was made as a control of the effect of chelation with MgEGTA.
DISCUSSION

The objective of this study was to compare the ability of two LPS-activated complement pathways to effect chemotaxis of PMN leukocytes. This was done by activating the classic and alternate pathways in one series of reactions, and comparing them to chelated reactions which exhibited the activity of the alternate pathway. This direct measurement of complement chemotactic factors has been reported to be the most significant assay of the use of terminal complement components. Previous studies reported the ability of LPS to consume greater amounts of the six terminal complement components than zymosan, inulin, or immune complexes (Synderman et al., 1968, 1969; and Gewurz et al., 1968). However, classic complement activity generated by immune complexes could not be compared to alternate pathway activity (LPS, inulin, and zymosan) in the production of C3a, C5a, and C5b-6-7, due to the inefficiency of measuring C'H50 units in these experiments. In the kinetics of complement hemolysis, only 10 to 20% of C3b and C5b are retained on the red cell membrane for construction of the attack unit, and can not be used to determine active cleavage products in the fluid phase (Rapp & Boros, 1970; Kabat & Mayer, 1961; and Mayer, 1970). Thus, the data of this study has particular importance for the function of the alternate pathway in the production of biologically active products.
The chemotaxis and hemolysis experiments do not directly measure or describe the molecular consequences of classical or alternate complement activation, but they do provide evidence dependent on the efficiency of the two pathways to consume the six terminal complement components. There was a slight difference between the low levels of hemolysis in the normal reaction mixtures, but may have been the result of unused C1, C2, and C4 in the latter. The ability of LPS-activation of the alternate pathway to consume significantly large amounts of C3 to C9 corroborates evidence of the ability of *E. coli* and *V. alcalescens* LPS to produce the same results (Synderman et al., 1968; and Fine, 1974). Classic complement pathway activation relies on the correct spacial arrangement of IgG for contact with Clq (IgGₙ, n>1). However, the ever-increasing number of alternate pathway activators represents a more general and nonspecific complement activation that has been demonstrated in this study to equal the classic and alternate systems together. Thus, the alternate complement pathway equals the classic pathway in the efficiency of activation and consumption of the six terminal components. This could be due to the numerous repeating subunits in the lipid A portion of LPS available for contact with IF of the properdin system. This reaction is analogous to IgGₙ–Clq contact, but does not require specificity such as the Clq-receptor in the Fc fraction of immunoglobulins (Muller-Eberhard, 1975). The two pathways, thus, function as equal effector systems in producing biologically active products in nonspecific and specific reactions.

Endotoxin has been shown to cause the directed migration of blood
leukocytes through the activation of a complement peptide, C5a (Synderman et al., 1968, 1969, 1971a, and 1972; Shin et al., 1968, 1969; Gewurz et al., 1971; Kay & Austen, 1970). PMN leukocytes, monocytes, eosinophils, and basophilic leukocytes are attracted to this factor, which also possesses anaphylactic ability with C3a (Jensen et al., 1969). The inability of C5-deficient mice to halt bacterial infection following the primary lodgement of organisms is a further example of the importance of biologically active complement peptides participating in host defense mechanisms. These two minor complement peptides were also determined to possess chemotactic activity for PMNs.

Complement components C3a and C5a generated by either pathway were also identified as principle anaphylotoxins, which degranulate mast cells and basophils, cause increased vascular permeability, smooth muscle contraction, and the extravasation of fluids from vessels (Gewurz et al., 1971; Hook et al., 1970; Dierich et al., 1973; Muller-Eberhard, 1975; and Ward, 1971). Many pathological features of endotoxin-complement reactions in experimental animals are present in the Arthus phenomenon, and can be simulated by immune complexes (Jensen et al., 1969; and Stetson, 1964). Thus, C3a and C5a function in both immune and innate reactions in host defense mechanisms by mediating the inflammatory response.

The in vitro activities of LPS-complement chemotaxis have been related to the in vivo inflammatory effects caused by the experimental injection of LPS and purified C5a. The injection of endotoxin causes a number of pathological occurrences dependent on the function of a viable complement system (Thomas, 1954; Gewruz et al., 1970; Pearlman et al.,
1963; and Mergenhagen et al., 1969). These reactions include local skin inflammation with intravascular coagulation, PMN accumulation, vasculitis, and necrosis (Greisman, 1960; and Stetson, 1951). These reactions were mediated via principle biologically active peptides cleaved from LPS-activation of the alternate complement pathway (Mergenhagen et al., 1973; and Gewurz et al., 1971).

The chemotaxis experiments of this study corroborate previous data of the production of biologically active C5a in both classic and alternate pathway in vitro systems, and suggest that the alternate pathway can produce in vivo chemotactic and anaphylactic factors in reactions equal to those produced with two functional complement pathways. The alternate pathway could mediate an inflammatory response identical to classic complement amplification of an immune response, and preceed the latter in certain infections as a primary humoral defense mechanism.

The presence of C3a, C5a, and C5b-6-7 in human pathological fluids, serum, and experimental vasculitis has indicated the ability of complement chemotactic factors to mediate inflammation and the in vivo accumulation of leukocytes in causing tissue destruction (Mergenhagen et al., 1972; Muller-Eberhard, 1972a; and Ward, 1971). Phagocytosis of complement-tagged (C3b) particles by leukocytes results in degranulation of the leukocytes and release of proteolytic enzymes capable of causing connective tissue damage, extravasation of plasma, and Arthus vasculitis. Furthermore, the attraction of PMNs to alternate pathway produced factors has been shown to cause selective enzyme release in the absence of spe-
cific particles. It has also been found that if the PMN plasma membrane is perturbed sufficiently, perturbation, fusion, and extrusion of enzymes occurs through a process of reverse endocytosis (Goldstein et al., 1973). The interaction of C5a and the PMN membrane has caused the release of lysozyme, β-glucuronidase, and microtubule assembly. In vivo, this is dependent on phagocytosis or adherence to complement opsonized particles (Goldstein et al., 1975). It is probable that the classic activation of complement can function as an effector of immune tissue destruction through the accumulation and degranulation of PMN leukocytes. In addition, the alternate pathway could also function in nonspecific tissue destruction that precedes any immune involvement, and would act synergistically with the latter, upon prolonged exposure, in tissues susceptible to damage by inflammatory products.

Solid evidence for the role of the alternate pathway in disease, in the absence of classic pathway activation, is difficult to obtain due to the potential of classic generated C3b, which could react with Pa and bind P if deposited on a membrane (Gotze & Muller-Eberhard, 1976). However, in cases where the alternate pathway functions in tissue injury, it could act either as the principal complement effector, or produce biologically active products with the classic pathway (Naff, 1972). The anaphylactic and chemotactic activities of C3a and C5a in initiating and sustaining inflammation are simultaneous with the opsonization of particles or membranes with C3b to facilitate adherence to the accumulating leukocytes, and enhancing phagocytosis (Jensen, 1969). Thus, the presence of
a powerful complement activator, such as bacterial endotoxin, in either initial or prolonged exposure, could result in the continual production of inflammatory products. This tremendous inflammatory response could mediate tissue destruction, and is of particular importance in tissues susceptible to damage from inflammation (Lepow, 1972; Synderman, 1972a; and Muller-Eberhard et al., 1973). These are reactions in which immunoglobulin production would not necessarily be critical for optimum complement activation and production of phlogistic products.

The interaction of endotoxin with complement, innately or in the presence of immunoglobulins to specific "O" antigens, may be implicated, partially, in the etiology of soft and hard periodontal tissue destruction. Low, but biologically significant levels of LPS have been demonstrated in plaque as well as in Gram-negative bacteria isolated from plaque (Berglund et al., 1969; and Mergenhagen et al., 1961). Although the greater percentage of cultivable bacteria in the normal gingival crevice are Gram-positive rods and cocci (Actinomyces and Streptococcus species), Gram-negative anaerobic rods and cocci (Veillonella, Fusobacterium, and Bacteroides) compose approximately 28% of the cultivable flora (Socransky, 1970). Veillonella species have been found to occur in subgingival plaque up to 8% of the cultivable flora, while Gram-negative rods range from 6.5% to 61.2% after sixteen days of growth (Stig et al., 1976). Thus, subgingival plaque is a good source of bacterial endotoxin.

Crevicular material and plaque extracts in clinically healthy individuals harbor factors chemotactic for PMNs that are heat labile
at 56°C, and can induce experimental vasculitis (Hellden & Lindhe, 1973; Tempel et al., 1970; Egleberg, 1967; Kahnberg, 1975; Lindhe & Hellden, 1972; and Miller et al., 1975). The additional finding of C3 and C4 in clinically healthy gingival crevices, and C3pa and C5 in chronically inflamed gingivae, indicates that complement is quite active in gingival inflammation (Attstrom et al., 1975; and Shillitoe & Lehner, 1972). De-complementation of Labrador Retrievers prior to topical application of plaque extracts failed to initiate vasculitis observed in the experimental initial gingivitis in the control dogs (Kahnberg et al., 1976). The presence of endotoxin in the cell walls of Veillonella, Fusobacterium, and Bacteroides species found in subgingival plaque could account for some of the complement activation in both initial gingivitis and chronic periodontal disease.

Previous clinical studies have suggested a relationship between oral endotoxin levels and the severity of gingival inflammation (Simon et al., 1970, 1971, and 1969). The resultant tissue damage could be mediated via the complement cleavage of C5 (Porter et al., 1971; Sarknes, 1966). Increases in the levels of endotoxin in crevicular exudate, dental plaque, and gingival tissue have been correlated to the degree of clinical inflammation in periodontal patients (Shapiro et al., 1972). Increasing gingival index scores have also been shown to accompany increases in endotoxin-containing Gram-negative bacterial cells in plaque, including Veillonella species (5 to 25%) (van Palenstein & Hoogeveen, 1976; and Johnson et al., 1976). The potent ability of oral Veillonella endotoxins to activate complement in vitro and generate significant chemotaxis can be related to in
vivo inflammation in periodontal tissues (Synderman et al., 1969, 1972a). *V. alcalescens* CL is similar in the structure of its cell wall and lipopolysaccharide to the various oral strains under study (Mergenhagen et al., 1969; Synderman et al., 1968; and Mergenhagen et al., 1961). The actual form of endotoxin in the sulcular environment is probably closer to that of a lysed cell wall fragment, rather than the purified soluble product. This study has demonstrated the ability of those LPS-containing cell wall fragments to activate complement chemotaxis in various quantities. Thus, the activity of the extracted LPS can be related to the cell wall particle.

The data of this study is of special interest in acute periodontal inflammation and soft tissue destruction. Initial acute gingivitis could occur through the interaction of endotoxin with complement in the absence of a significant titer of antibody. Through the mechanical irritation of plaque, calculus, and bacterial enzymes, endotoxin-containing cell wall particles could penetrate crevicular epithelium for immediate contact with complement components and activation of the alternate pathway. The data of the chemotaxis experiments emphasize the extent of this initial complement activation as being equal to an immune response, which would occur over a longer period of time (5 to 14 days), upon primary exposure of this antigen. The biological effects in causing vascular permeability, smooth muscle contraction, mast cell degranulation, and chemotaxis of PMNs and monocytes are relevent to the onset of periodontal inflammation. The subsequent phagocytosis and metabolism of leukocytes would result in lysosomal release of enzymes, including cathepsins and collagenases (Cochrane, 1968). Thus, the products in plaque, rather than the organisms themselves, may in-
initiate a chain of molecular events resulting in the progressive destruction of supporting periodontal structures.

The accumulation and degradation of leukocytes would cause initial tissue destruction, the breakdown of intracellular substances (desmosomes and hemidesmosomes), and the further penetration into gingival epithelium and lamina propria of endotoxin and other bacterial antigens. The latter has been demonstrated with purified LPS in experimental gingivitis, and has been observed in gingival biopsies of patients with periodontal disease (Schwartz et al., 1972; Tempel et al., 1970; and Shapiro et al., 1972). The prolonged exposure of LPS and stimulation of immune mechanisms would not necessarily increase inflammatory products, according to the in vitro measurements of chemotaxis in the immune and non-immune systems. However, as the disease progresses, the environment becomes more favorable for anaerobic organisms, which includes the LPS-containing Gram-negative species. The accompanying increases in LPS lead to the further progression of periodontal inflammation, but does not necessarily depend on immunoglobulin production. Thus, the initial exposure of small amounts of endotoxin-containing particles are sufficient to activate complement and cause inflammation and tissue destruction. This cycle of LPS and antigen penetration, exposure to immune and non-immune host defenses, and inflammation would become self-sustaining, and lead to the further progression of the disease process.

In chronic periodontal inflammation, the large amounts of endotoxins found in pocket formation could be constantly activating complement through both pathways, and producing factors to act synergistically with lymphokines in the chemotactic attraction of monocytes and eosinophils (Kay et al., 1975).
The resultant bone resorption accompanying chronic inflammation could also be influenced by complement activation (Synderman, 1972). The release of heparin from connective tissue mast cells in regions of alveolar bone could cause bone resorption. Heparin had been shown to act synergistically with gingival tissue fragments in enhancing in vitro bone resorption (Goldhaber, 1965, 1971). Lyososomal enzymes, derived from inflammatory leukocytes, also have been shown to stimulate in vitro bone decalcification (Fell, et al., 1966). Lastly, endotoxin has been found to directly inhibit bone growth and stimulate chondroclast resorption in tissue culture (Hauseman, et al., 1970). Thus, endotoxin-complement generation of biologically active products are probably responsible, in part, for periodontal tissue destruction in acute and chronic inflammation through innate or immune mechanisms.

The objectives of this study have been fulfilled: the chemotaxis in two complement pathways was studied with the alternate pathway solely, and was supported by a subline of evidence in residual hemolytic complement measurements; and this in vitro function has been related to possible in vivo effects of the production of phlogistic peptides.
Summary

The cell wall and endotoxin extracted from *Veillonella alcalescens* activated the alternate complement pathway and produced chemotactic factors for polymorphonuclear leukocytes. The purified endotoxin generated chemotactic activity from whole guinea pig serum in reactions equal to chemotaxis with one hundred fold greater amounts of cell wall material. Therefore, the major complement activating ability of *V. alcalescens* cell walls is due to the biologically active endotoxin.

Lipopolysaccharide-activated complement measured in immune and non-immune reaction mixtures showed no significant differences in the chemotaxis of PMNs between the alternate pathway, solely, and both active complement pathways together. This establishes non-immune alternate pathway activation as an equal biological effector to immune activation of the classic pathway in the production of factors which mediate the inflammatory response.

Endotoxin-complement activation is significant in tissues susceptible to damage by the inflammatory response. The continual presence and infiltration of Gram-negative bacteria and endotoxin in the healthy human gingival sulcus is a factor in the initiation of periodontal disease through alternate complement pathway activation, subsequent accumulation and degradation of PMNs and monocytes, epithelial tissue destruction, and further antigen penetration to underlying lamina propria with immunocompetent cells.
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The thesis submitted by Blase P. Brown has been read and approved by three members of the Graduate School faculty.

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

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

August 19, 1977

Date

Signature of Advisor