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Longitudinal Assessment of Complement Components C3, C4, and B and Their Cleavage Products in Gingival Fluid Before and After Treatment of Chronic periodontitis in Humans

Christine Elizabeth Niekrash

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LONGITUDINAL ASSESSMENT OF COMPLEMENT COMPONENTS C3, C4, AND B AND THEIR CLEAVAGE PRODUCTS IN GINGIVAL FLUID BEFORE AND AFTER TREATMENT OF CHRONIC PERIODONTITIS IN HUMANS

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Sc.B., Brown University, 1975
D.M.D., University of Connecticut, 1981

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LONGITUDINAL ASSESSMENT OF COMPLEMENT COMPONENTS C3, C4, AND B AND THEIR CLEAVAGE PRODUCTS IN GINGIVAL FLUID BEFORE AND AFTER TREATMENT OF CHRONIC PERIODONTITIS IN HUMANS

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The University of Connecticut

1983
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THE COMPLEMENT SYSTEM PLAYS AN IMPORTANT ROLE IN THE INITIATION OF ACUTE AND CHRONIC INFLAMMATION. ACTIVATION OF THE COMPLEMENT CASCADE MAY LEAD TO TISSUE DAMAGE BY CHEMOTACTIC ATTRACTION AND CHANGES IN THE ACTIVITY OF POLYMORPHONUCLEAR LEUKOCYTES WHICH MAY RELEASE DAMAGING PROTEOLYTIC ENZYMES, DEGRANULATION OF MAST CELLS AND COMPLEMENT-MEDIATED LYSIS OF TISSUE CELLS. IN ADDITION, COMPLEMENT ACTIVATION MAY STIMULATE OSTEOCLASTIC BONE RESORPTION, THE PRODUCTION OF LYMPHOKINES AND INCREASED DIFFERENTIATION OF B-LYMPHOCYTES. DESTRUCTION OF CONNECTIVE TISSUE AND BONE ARE PROMINENT FEATURES IN PERIODONTAL DISEASE AND EMPHASIZE THE POTENTIAL IMPORTANCE OF COMPLEMENT ACTIVATION IN THIS DISEASE PROCESS.

EVIDENCE EXISTS THAT COMPLEMENT ACTIVATION OCCURS IN PERIODONTAL DISEASE. NATIVE C3 AND C4 ARE REDUCED IN GINGIVAL FLUID COMPARED TO SERUM IN PATIENTS WITH SEVERE PERIODONTAL DISEASE (SCHENKEIN AND GENC0 1977A, ATTSSTROM ET AL. 1975). COMPLEMENT ACTIVATION MAY OCCUR CONSEQUENT TO ANTIGEN-ANTIBODY REACTION OR BY SEVERAL ALTERNATIVE MEANS. CLEAVAGE OF C3 IN GINGIVAL FLUID BY THE ALTERNATIVE AND/OR CLASSICAL PATHWAYS OR BY DIRECT ENZYMATIC CONVERSION IS SUGGESTED BY THE PRESENCE OF THE CONVERSION PRODUCT C3C (ATTSTROM ET AL. 1975, SCHENKEIN AND GENC0 1977A) AND C3D (SCHENKEIN AND GENC0 1977B) IN GINGIVAL FLUID.

THESE PREVIOUS STUDIES INDICATE THAT COMPLEMENT CLEAVAGE OCCURS IN VERY SEVERE PERIODONTAL DISEASE. THESE INVESTIGATIONS,
however, were conducted only in patients with severe periodontitis. Due to the small amounts of gingival fluid obtainable from mildly inflamed tissues, no longitudinal studies of complement activation have been reported. The new measurement technique utilizing filter paper strips presented in this manuscript allows measurement of complement cleavage by crossed-immunoelectrophoresis from gingival fluid samples taken from areas of minimal inflammation. In addition, modification of this technique by the Master's candidate allows the simultaneous measurement of C3, C4, B and their cleavage products from a gingival fluid sample as small as 0.2 ul. This technique allows longitudinal studies of complement cleavage in periodontal disease.
BACKGROUND

The human complement system is a principal defense mechanism which acts to protect the host from foreign substances. However, activation of the complement system may also lead to damage of host tissue and exacerbation of the inflammatory response. The extensive biologic activities of complement result primarily from the cleavage products resulting from activation of the complement cascade. The complement system consists of a series of proteins which can be divided into three major pathways: the classical, the alternative activating, and the terminal pathways.

Classical Pathway

The classical pathway, activated primarily by antigen-antibody complexes (Hyslop et al. 1970), consists of a cascade of alternating proteolytic cleavage and protein-binding reactions with important host defense consequences. Thus, its major function is to provide an amplification mechanism consequent to the interaction of antigen with specific antibody. Other substances, such as C-reactive protein, an acute phase serum protein, also cause activation of the classical pathway (Siegel et al. 1976, Volanakis and Kaplan 1974).

The recognition unit of the classical pathway, Cl, is a trimolecular complex consisting of Clq,Clr, and Cls (Lepow et al. 1963). Clq, which consists of six peripheral subunits connected by fibrillar strands to a central core (Knobel et al.
1975, Shelton et al. 1972), recognizes the Fc portion of immunologically complexed IgG (IgG₁, IgG₂, IgG₃) or IgM antibody (Augener et al. 1971). At least two IgG molecules in close proximity or one IgM pentamer are essential for the binding of Clq (Colten et al. 1968). In the presence of calcium ions, this binding is thought to induce a change in the conformation of Clr (Müller-Eberhard 1977), causing a self-cleavage reaction that activates Clr (Naff and Ratnoff 1968). The activated Clr then cleaves a peptide bond in Cls to reveal its enzymatically active serine esteratic site.

The natural substrates of Cls in serum are C4 and C2, the next two components in the classical cascade (Müller-Eberhard and Lepow 1965). Cleavage of C4 and C2 by Cls leads to the formation of the short-lived activated proteins, C4b and C2a. Cl first cleaves C4 into C4b and C4a with C4b attaching to the immune complex (Law et al. 1980). Cls then cleaves C2 into the larger C2a and the smaller C2b fragment (Kerr 1980) with C2a binding to the attached C4b. This complex, C4bC2a, formed in the presence of Mg²⁺, functions enzymatically as the classical pathway C3 convertase which cleaves C3 into two fragments, C3a and the larger C3b.

The classical complement system is controlled by a variety of naturally occurring biochemical regulators present in serum. Cl activity is balanced by the glycoprotein Cl inhibitor (Cl INH) (Ratnoff and Lepow 1957), which forms a stoichiometric complex with the active sites of both Clr and Cls. This abrogates their
enzymatic activity and causes the dissociation of Clr and Cls from antigen-antibody-C1 aggregates (Sim and Reboul 1981). Clq inhibitor has recently been described as physically binding to Clq and blocking its activity (Ghebrehiwet 1981).

C4 is inactivated by two plasma proteins acting together. C4 Binding Protein (C4BP) acts as a cofactor for the degradation of C4b by the enzyme I (C3b inactivator, Fujita et al. 1978). When bound or soluble C4b is complexed with C4BP, it is subject to cleavage by I (Gigli et al. 1979, Nagasawa et al. 1980, Press and Gagnon 1981). Serum proteases act to release C4c, inactivating C4b. In addition, the C4b,C2a complex is inherently unstable and dissociates rapidly.

**Alternative Activating Pathway**

The alternative activating pathway of complement was postulated in 1954 by Louis Pillemer based on observations of serum proteins active in early host defenses against bacteria and viruses in the absence of antibodies (Pillemer et al. 1954). Specific immunity is therefore not required for the activity of the alternative activating pathway (Götte and Müller-Eberhard 1976). The initiation of the enzyme cascade by certain microorganisms and mammalian cells is dependent on the chemical composition of their cell surface rather than on the formation of immune complexes. The activators of the alternative activating
pathway of human complement include a variety of microbial polysaccharides such as lipopolysaccharides from Gram negative bacteria (Marcus et al. 1971), teichoic acid from pneumococci (Winkelstein and Tomasz 1978), and zymosan from yeast (Pillemer et al. 1954). In addition, this complement pathway has been shown to inactivate many viruses (Hirsch et al. 1980, Mills et al. 1979, Welsh 1977) and to kill a number of helminthic and protozoan parasites (Santoro et al. 1979) in the absence of antibody. However, antibody-dependent activation of the alternative activating pathway has been described for immune aggregates of certain IgG and IgA subclasses, and with aggregates containing IgD and IgE (Reid 1983). Also, certain immune complexes have been shown to initiate the alternative activating pathway in the absence of classical pathway components C1 and C4 (Edwards et al. 1980).

The initiating event in the alternative activating pathway of complement involves the formation of a priming C3 convertase, C3,Bb formed by the interaction of C3 (which has undergone a spontaneous hydrolysis of a thioester bond) with B to form C3,B which is then cleaved by the enzyme D in the presence of Mg$^{++}$ to yield C3,Bb (Fearon and Austen 1975). This occurs continually at a slow rate in plasma and generates small amounts of C3b by the enzymatic action of C3,Bb on C3. Initiation of the amplification loop of the alternative activating pathway is determined by the attachment of C3b to an activating surface.

In the presence of an activating surface, C3b covalently
attaches to it and is therefore less susceptible to the regulatory proteins. B then binds to C3b in the presence of Mg\(^{++}\). D (proactivator convertase), a serine esterase present in serum only in its active form with no known specific inhibitor, then cleaves the bound B into Ba, which is released, and the larger Bb (Lesavre and Müller-Eberhard 1978). Bb forms a labile bimolecular complex with C3b, C3b,Bb. P (properdin) is adsorbed to the C3b,Bb complex from the serum and results in stabilization of that complex (Medicus et al. 1980), with a five-fold increase in its in vitro half-life (Fearon and Austen 1975). The formation of C3b,Bb, the C3 or "amplification" convertase, is the major step in the activation of the alternative activating pathway. It acts to cleave C3 into C3a and C3b. Bound C3b may then recruit the alternative activating pathway components, B, D, and P to assemble more C3b,Bb complexes, creating many more molecules of C3 convertase. The C3b-dependent C3 convertase is also capable of amplifying C3 cleavage initiated by the classical pathway.

The protein B contains the proteolytic site for C3 cleavage. This activity is partially expressed in the C3,Bb complex (initiation loop) and is fully exposed in the C3b,Bb complex (activation loop, Fearon et al. 1973, Götze and Müller-Eberhard 1976, Medicus et al. 1976). This accounts for the differences in the activity of the two alternative activating pathway C3 convertases: one resulting in continuous, low-grade production of C3b, and the other incorporating the priming C3b with subsequent amplified cleavage of C3.
In the absence of an activating surface, the regulatory proteins dominate and no amplification is achieved. The C3b,B and C3b,Bb complexes are dissociated by H (beta-1H), a serum glycoprotein that competes for the B binding site on bound or free C3b (Fearon and Austen 1977). I (C3b inactivator), an endopeptidase, then cleaves the C3b complexed with H to form inactive iC3b (Law et al. 1979, Müller-Eberhard and Schreiber 1980, Pangburn et al. 1977), destroying the hemolytic and immune adherence reactivity of C3b. iC3b is susceptible to degradation by other proteases (trypsin, plasmin and leukocyte elastase (Johnson et al. 1976, Taylor et al. 1977)), generating C3c (in fluid phase) and C3d (which remains bound). C3c may then be cleaved to form C3e.

The competition between B and H for C3b determines whether or not the alternative activating pathway is triggered (Fearon and Austen 1977, Kazatchkine et al. 1979). This is decided by the presence or absence of certain cell membrane constituents (activators of the alternative pathway) which protect C3b from inactivation and block the active dissociation of P,C3b,Bb by the regulatory proteins. For example, C3b with no activator surface binds H with almost one hundred-fold greater affinity than it binds B (Kazatchkine et al. 1979). Competition between B and H for bound C3b is influenced by the amount of membrane sialic acid, heparin, and C3 receptor protein.

In addition, the appropriate activating surface must be close enough to the C3b molecule for attachment to occur before
the metastable binding site on C3b decays. The C3b receptor of human erythrocytes, polymorphonuclear leukocytes (PMNs), monocytes, and B-lymphocytes strongly binds to C3b and exhibits H-like activity. These receptors, therefore, regulate the alternative activating pathway by inhibiting formation of the C3 amplification convertase (Fearon 1979).

Terminal Pathway

The C3 convertases of both the classical and alternative activating pathways of complement become C5 convertases by binding C3b. This binding exposes active sites on C2a and Bb, respectively, allowing C4b,C2a,C3b (Cooper and Müller-Eberhard 1975) and activator-bound C3b,Bb,C3b stabilized by P to act identically to split one bond in the alpha-chain of C5. This last proteolytic step of the complement pathways yields the smaller C5a (Hugli 1975) which is released and C5b which binds to the alternative pathway activator or to the immune complex. The membrane attack complex which follows is a rapid non-enzymatic process common to both pathways. C5b combines with C6 (Yamamoto and Gewurz 1978) and C7 (Podack et al. 1978) to form the trimolecular complex C5b,C6,C7 which intimately attaches to target-cell lipid membrane via the generation of hydrophobic forces (Monahan and Sodetz 1981). C8 then binds to the attached complex and forms a structural pore through the lipid bilayer of the membrane permitting bidirectional ion and water passage. C9
combines with the C5b,C6,C7,C8 complex and acts as an accelerating factor leading to osmotic lysis and death of the target cell. Cell lysis is characterized by distinct 100Å circular lesions. This final membrane attack complex is stable and has a molecular weight of 1,000,000 daltons (Kolb and Müller-Eberhard 1975).

The regulators of this terminal pathway act by inserting into the membrane attack complex as it forms, preventing the attachment of the complex to the target cell membrane. These regulators include low density lipoproteins (Lint et al. 1977), Anti-thrombin III, and the S protein of normal serum (Podack et al. 1978b).

Sensitivity of bacteria to the lytic action of the membrane attack complex appears to be related to the thickness of the cell wall. Most gram-negative organisms can be killed by antibody and complement, but most gram-positive organisms are resistant.

**Biologic Activities of Complement**

The activation of the complement cascade induces many of the phenomena characteristic of inflammation. This response contributes to host defenses by protecting against foreign substances, but may also be responsible for damage to host tissues. Most of the biologic activities result from the release of limited proteolytic cleavage products formed by the complement cascade. Others are caused by the binding of complement cleavage products
to other cells, such as macrophages, PMNs, lymphocytes, erythrocytes, and platelets.

**Effect on the vasculature.** Increased vascular permeability results from two potent stimulators of inflammation, C3a (Hugli 1975, Hugli and Müller-Eberhard 1978) and C5a (Gerard and Hugli 1979, Gerard et al. 1981). These low molecular weight anaphylatoxins (generated by both the classical and alternative activating pathways of complement) cause degranulation and release of histamine from human mast cells and basophils by non-lytic mechanisms, resulting ultimately in smooth muscle contraction (Gerard et al. 1980) and increased vascular permeability. In fact, C3a and C5a elicit an immediate edema and erythema reaction when injected into human skin even at extremely low concentrations. C4a has also been shown to exhibit anaphylatoxic activity (Gorski et al. 1979). The vascular permeability effects of C3a, C4a, and C5a are controlled by anaphylatoxin inactivator, a carboxypeptidase present in human serum that removes the carboxy-terminal arginine residue (Bokish and Müller-Eberhard 1970). Changes in vascular permeability (a kinin-like activity) also result from a vasoactive peptide cleavage fragment of C2 (Klemperer et al. 1969, Becker et al. 1974).

**Effect on granulocytes.** The accumulation of leukocytes is characteristic of inflammation and is a prime defensive mechanism of the host. C5a, a chemotactic factor produced by proteolytic cleavage of C5, stimulates directed movement of neutrophils, basophils, eosinophils, and monocytes (Ward and Newman 1969).
C3b, Bb and Ba (Hadding et al. 1978), formed by the alternative activating pathway, have been shown to be chemotactic for neutrophils (Takahashi et al. 1980). In addition, the decay product of C5b, C6, C7 exhibits chemotactic activity in vitro (Ward et al. 1966). Complement-derived chemotactic factors have been shown to have potent in vivo activity. They have been isolated from inflamed tissues and local injection of these factors causes an accumulation of leukocytes (Ward and Becker 1977). A factor present in serum is able to destroy the complement-derived chemotactic activity (Kreutzer et al. 1979). The chemotactic activity of C3a is controversial (Damerau 1978, Rother 1972).

A systemic leukocyte mobilizing effect has been shown for C3e, leading to leukocytosis by stimulating PMN release from bone marrow (Ghebrehiwet and Müller-Eberhard 1979). C3e, therefore, increases the pool of responding cells in inflammation.

In addition to the chemoattraction of leukocytes to sites of complement activation, several complement cleavage products lead to changes in the activity and responsiveness of leukocytes. C3b has been shown to increase neutrophil oxidative metabolism (Goldstein et al. 1976) and also to permit subthreshold amounts of immunoglobulin to cause lysosomal enzyme release. A cleavage fragment of C5 causes aggregation of granulocytes (O'Flaherty et al. 1977a), reflected by an increase in their adhesiveness (Craddock et al. 1977), which may serve a role in margination, an early inflammatory response (O'Flaherty et al. 1977b). C5a has been shown in vitro to stimulate the secretion of lysosomal
enzymes from neutrophils (Becker et al. 1974, Goldstein and Weissman 1974).

Several complement components are also active in promoting immune adherence and opsonization. C3b-coated target cells or immune complexes have been shown to bind to certain cells which possess specialized membrane receptors for C3b. PMNs, macrophages, monocytes, eosinophils, and lymphocytes all possess stable surface receptors for C3b. This immune adherence through the C3b receptors facilitates the involvement of these cells in the inflammatory response (Gigli and Nelson 1968). When opsonized by C3b, one hundred times more bacteria bind to a neutrophil membrane than when bacteria are opsonized by IgG alone (Menzel et al. 1978). Although binding appears to be strongly promoted, C3b alone does not seem to induce phagocytosis. However, a synergy exists between IgG and C3b in IgG-mediated phagocytosis (Fearon 1979). C4b also promotes the immune adherence phenomenon (Cooper 1969) and C5b may act to increase the phagocytosis of C3b-opsonized bacteria.

Effect on mononuclear cells. Bb activates macrophages, causing spreading of peripheral monocytes (Götze et al. 1979) and migration inhibition. In addition, C3b has been shown to cause IgG-independent lysosomal enzyme secretion by cultured guinea pig and murine peritoneal macrophages (Schorlemmer and Allison 1976). Evidence exists that activated mouse macrophages bind and ingest C3b-coated particles in the absence of IgG (Bianco et al. 1976).
Macrophages also have iC3b receptors, thought to promote attachment but not phagocytosis. The macrophage C3b receptors may serve a critical role in presentation of antigen to antibody-producing cells. B-lymphocytes (Ross et al. 1973), peripheral blood monocytes (Ehlenberger and Nussenweig 1975), and macrophages (Reynolds et al. 1975) bear receptors for C3d which may be involved in immune adherence.

Complement cleavage products from both complement pathways influence lymphocyte activity. The adherence reaction of C3b-bearing particles stimulates the secretion of chemotactic lymphokines by B-lymphocytes (Sandberg et al. 1975), and promotes the development of B-cells into memory cells (Klaus and Humphrey 1977) and antibody-secreting cells (Lewis et al. 1977). C3c and C3d have been recently shown to inhibit T-lymphocyte proliferation by antigen (Schenkein and Genco 1979).

Indirect evidence exists to suggest that the differentiation of lymphocytes is affected by C3b. In vivo, the depletion of C3 prevents the development of the primary response to T-dependent antigens, thought to be mediated through C3d receptors present on some B-lymphocytes (Pepys 1974).

Other effects. Complement activation also leads to irreversible damage to biological membranes through the common terminal pathway. The membrane attack complex (C5b,C6,C7,C8,C9) does not involve specific receptor binding and leads to uptake of salt and water by the target cell with ultimate lysis. This occurs in target bacterial or tumor cells, but can also cause damage to
host tissues by the innocent bystander phenomenon or through autoimmune mechanisms.

Complement-dependent prostaglandin-mediated bone resorption has been shown when the bone tissue has been incubated with antiserum or heterologous serum (Raisz et al. 1974). In addition, it has recently been shown that C3b, iC3b and C3c induce human mononuclear phagocytes to synthesize and secrete prostaglandin (Rutherford and Schenkein 1983).

C3b may inhibit cross-linking and lattice formation of immune complexes, resulting in the solubilization of large immune complexes. These solubilized complexes can neither bind cells nor activate complement (Takahashi et al. 1980).

Other complement products have a variety of effects including: C5 split products may trigger the extrinsic clotting pathway (Muhlfelder et al. 1979), C4b may be important in virus neutralization (Daniels et al. 1969).

Complement and Inflammatory Disease

Activation of the complement cascade results in a variety of biologic effects. These include: 1) irreversible damage to biologic membranes, 2) deposition of molecules on the surface of particles to opsonize and to promote clearance, and 3) formation of potent mediators of the inflammatory response. These activities, although beneficial to the host, also possess an inherent potential for coincidental damage. The complement system has, therefore, been implicated in a variety of inflammatory diseases.
Direct cytotoxic injury involves antibody reacting directly with a cell and activating the complement enzyme cascade. Inflammatory mediators are released and the cells are damaged. Autoimmune hemolytic anemia results from this process with the development of antibodies to erythrocyte antigens. Nephrotoxic nephritis is also thought to result from complement-mediated cytotoxic reactions.

Circulating immune complexes induce tissue injury through activation of the complement system. These antigen-antibody complexes deposit in tissues that have no antigenic relationship to the antibody, such as renal glomeruli or vascular endothelium and initiate complement cleavage and the consequent inflammatory sequelae. In instances of antibody excess, Arthus-type vasculitis reactions result. Farmer's lung (hypersensitivity pneumonitis) is an example of this phenomenon, with inhaled antigens combining with antibodies in the alveoli, activating complement, and inducing an inflammatory response. Antigen excess results in "serum sickness" type reactions with immune complex deposition and complement activation culminating in glomerulonephritis, arthritis, and vasculitis. This occurs following exposure to sensitizing antigens in drugs or foreign serum. The localization of the immune complexes and inflammatory sequelae depend on the chemical and physical nature of the antigen-antibody complexes in addition to the properties of the target tissue (Cochrane 1971). Immune complexes of IgG₁, IgG₂, IgG₃, or IgM are known to activate the classical pathway of complement. However, in immune
complex disease, the alternative pathway of complement is thought to participate at least through its amplification loop. C3b production by the classical pathway leads to alternative pathway C3 convertase generation. In vivo, C3, P and H deposits are found in addition to immune complexes in the glomeruli (Wyatt et al. 1979).

Autoantibodies formed against soluble antigens to which they are continually exposed, give rise to a constant supply of immune complexes and to lesions similar to serum sickness. In Systemic Lupus Erythematosus (SLE) with renal dysfunction, complexes of DNA and other nuclear antigens with IgG and complement have been visualized in the glomeruli of patients by using immunofluorescent techniques (Koffler et al. 1971). Electron microscopic studies have confirmed the presence of "lumpy-bumpy" immune complex deposits on the epithelial side of the glomerular basement membrane (Koffler et al. 1971). Further evidence exists to solidly link complement cleavage with SLE. The active phase of SLE (increased clinical severity of the disease) has been associated with a drop in total serum complement levels attributed to cascade activation by immune complexes in the circulation and in the kidneys (Williams and Law 1958, Ellis and Felix-Davies 1959). In longitudinal observations, Schur and Sandson (1968) observed decreased complement levels in advance of clinical exacerbation. In fact, clinical symptoms, immune complex concentrations, and complement levels appear to be strongly associated. Patients with persistently depressed C3 had more symptoms of nephritis
(Stastny and Ziff 1968), associated with cold-insoluble immune complexes (Stastny and Ziff 1969) and circulating antibodies to nuclear antigens (Tojo and Friou 1968, Townes et al. 1963) which activate complement. Therefore, it is hypothesized that circulating immune complexes deposit beneath the glomerular basement membrane, activate complement and incite an inflammatory response. This process may be compounded by the phenomenon of defective immune complex solubilizing capacity observed in sera from patients with active SLE (Sakurai et al. 1982).

The most consistently depressed complement component in SLE serum is C4 (Perrin et al. 1973). However, elevated serum levels of Ba and reduced levels of B have been observed during periods of intense classical pathway activation (Hunsicker et al. 1972, Perrin et al. 1975). These alternative pathway cleavage products have been seen chiefly in cases where the classical pathway has been activated (Perrin et al. 1973, Hunsicker et al. 1972); serum B depressions have been observed in sera with already depressed C4 (Perrin et al. 1973), suggesting the primary role of the classical pathway and an amplification role for the alternative pathway. Similar mechanisms have been implicated in a variety of diseases such as polyarteritis nodosa with inflammatory lesions in the blood vessel walls.

Complement serum depressions have been associated with other systemic rheumatic diseases including mixed IgG-IgM cryoglobulinemia (Riethmuller et al. 1966, Wilson et al. 1977), Waldenstrom's macroglobulinemia (Glovsky and Fudenberg 1970), arthritis
with cutaneous vasculitis (McDuffie et al. 1973), and polyarteritis (Gocke et al. 1970). Changes in the complement catabolic rate have also been associated with disease processes, such as Ankylosing Spondylitis (Brinch 1982).

Local formation of immune complexes with consequent complement cleavage leads to the development of inflammatory disease within a specific organ or extravascular site. For example, analysis of synovial fluid, cerebral spinal fluid, or pleural effusions may reveal immunopathologic processes not necessarily detectable in peripheral blood. Rheumatoid arthritis (RA) is though to have an immune complex-mediated inflammation centered primarily in the intra-articular region. The immune complexes present in RA consist of Rheumatoid Factor (IgG, IgA, and IgM immunoglobulins) in combination with IgG antigen or self-associated aggregates of IgG rheumatoid factors (Winchester et al. 1970). These complexes (especially IgM) activate complement (Townes and Marcus 1972, Tanimoto et al. 1975) through the classical pathway and then are phagocytized by the infiltrating polymorphonuclear leukocytes with consequent tissue damage. Evidence to support this concept of RA etiology is strong. Immunoglobulins, Clq, C4, and C3 have been found within the intra-articular phagocytic cells (Hollander et al. 1965) and deposited in the synovial lining tissue when examined with immunofluorescence (Britton and Schur, 1971). In addition, the RA synovium is heavily infiltrated with mononuclear cells, including macrophages (Ishikawa and Ziff 1976) and many plasma cells with a high rate
of synthesis of immunoglobulins, including rheumatoid factor. Rheumatoid synovial tissue has been shown to synthesize many complement components: C2, C3, C4, and C5 (Ruddy and Colten 1974, Whaley 1980) and C2, B, D, P, I and H (DeCeulaer 1980). Therefore, within the synovium all the participants necessary for complement-mediated immune complex-induced inflammation are present.

Considerable additional evidence adds support to the implication of complement-fixing immune complexes in the pathogenesis of rheumatoid arthritis. Synovial fluid in RA patients has been shown by several investigators to have depressed whole complement levels (Pekin and Zvaifler 1964, Hunder et al. 1977). Individual complement components also are reduced in the synovial fluid of these patients; depressed C4 levels are most consistent (Hunder and McDuffie 1973). Serum from RA patients may also have low levels of C4, but most have normal or even elevated levels in serum when rheumatoid vasculitis is not present (Franco and Schur 1971, Ruddy and Austen 1970, Versey et al. 1973). In addition, the few with decreased complement serum levels have more severe disease and high rheumatoid factor titers (Ruddy and Austen 1970, Vaughan et al. 1968). Perrin et al. (1977) observed significantly increased levels of breakdown products of C3, C4, and B in the joint fluid from RA patients when compared with osteoarthritis patients. The parallel increase of C3d (Nydegger et al. 1977), C4d and Ba levels in the RA patients suggest true activation of
the complement system rather than a non-specific enzymatic breakdown in the synovial fluid. C3 conversion was found in all synovial fluids of sero-positive RA patients tested by Hunder et al. (1977), with an accelerated functional catabolic and synthesis rate of C3 measured using radio-labelled C3 (Weinstein et al. 1972, Ruddy et al. 1975). C3 levels in sero-positive RA were shown to be decreased when measured by radial immunodiffusion (Ruddy et al. 1969), crossed immunoelectrophoresis (Hedberg 1964) or hemolytic titration (Ruddy and Austen 1970) methods. Low levels of both C2 and C4 suggest that the lowered synovial fluid complement levels are due to classical pathway activation (Ruddy and Austen 1970, Gabay et al. 1975).

Winchester et al. (1969) established a correlation between the amount of immunoglobulin-containing complexes and the diminution of total hemolytic complement activity in synovial fluid from patients with sero-positive RA. This was also confirmed by Townes and Marcus (1972). In addition, it has been demonstrated that complement proceeds to cytolytic completion in the RA joint space. Ruddy et al. (1971) found decreased C9 levels in joint fluid and Dourmaskin and Patterson (1976) observed membrane lesions in synovial cells characteristic of the membrane attack complex using electron microscopy.

Complement activation by the classical pathway thus appears to be a factor in RA with intra-articular cleavage of C3, its deposition in tissues with PMN phagocytosis and appearance of cleavage fragments. However, the alternative pathway components,
B and P, have diminished levels in RA joint fluid and their cleavage products also appear in these fluids (Zvaifler 1974, Ruddy et al. 1975b, Lambert et al. 1975). Increased catabolism of B has been demonstrated in the serum of patients with RA (Krick et al. 1978). Kaplan et al. (1980), using radio-iodide labelled C4 and B, concluded that the C4 fractional catabolic rate was predominant over the B fractional catabolic rate. He found that the catabolism correlated significantly with rheumatoid factor, supporting the concept of RA as a systemic, extravascular immune complex disease with rheumatoid factor immune complexes playing a significant pathogenic role through the activation of the classical complement pathway, with the alternative activating pathway acting in an amplifying role. However, Hunder et al. (1979) showed that direct activation of the alternative pathway in RA is also possible. Using C4-deficient serum, he observed complement cleavage induced by unidentified substances in the sera and synovial fluid of RA patients. The amount of complement cleavage was approximately the same whether the classical pathway was operational or not. Other investigators have demonstrated that Type I and Type II collagen are able to induce C3 and B conversion in both normal and C4-deficient serum, suggesting a mechanism for the direct alternative pathway activation in RA synovial fluid (Takahashi et al. 1975).

The alternative pathway has been shown to be active in a number of other pathologic conditions. Complement activation as evidenced by hypercatabolism of serum C3 has suggested a role for
complement in Ulcerative Colitis and Crohn's Disease (Hodgson et al. 1977), involving the activation of complement within the inflamed gastrointestinal mucosa and sequestration of C3 in the extravascular space. Lake et al. (1979) observed abnormalities of the alternative pathway (decreased serum P) in the serum of patients at the time of diagnosis of inflammatory bowel disease, especially regional enteritis and Crohn's disease. The classical pathway appeared intact in this study.

Other diseases are characterized by aberrations in the alternative pathway. Membranoproliferative glomerulonephritis and partial lipodystrophy (Peters et al. 1972) have been associated with a prolonged reduction in total serum hemolytic complement (low serum C3 with normal C1, C4, and C2 (West et al. 1965)). Investigators have described C3 Nephritic Factor present in the serum of some of these patients (Spitzer et al. 1969). C3 Nephritic Factor is an IgG autoantibody capable of activating the alternative pathway of complement by combining with and stabilizing C3b, B convertase (Fearon et al. 1975), therefore causing a rapid consumption of C3. Peters et al. (1972) examined 15 patients with this disease and found reduced levels of C3 and C7 in the serum, with C3 breakdown products and C3 Nephritic Factor also present. In addition, C3 and immune complex deposits were present in the kidneys.

Another disease, Paroxysmal Nocturnal Hemoglobinuria, has also been linked to the activation of the alternative pathway. It is an acquired disorder of erythrocyte membranes which
heightens their susceptibility to complement-mediated lysis. The erythrocytes therefore have a shortened life span (Hinz et al. 1956).

In gram negative bacteremic individuals, decreased serum C3 levels have been associated with shock (McCabe 1973). P, C3, C5, C6, and C9 levels are lower in bacteremic individuals with shock than in those not in shock. Palestine and Klemperer (1976) observed circulating B cleavage products in normotensive bacteremic patients, despite the presence of normal or even elevated B levels, suggesting involvement of the alternative pathway.

**Complement and Periodontal Disease**

Chronic inflammatory periodontal disease, the major cause of tooth loss in adults, is characterized by infiltration of the gingival tissues by inflammatory cells and loss of connective tissue substance. Page and Schroeder (1976) have described the various stages of the progression of periodontal disease. The initial lesion, which occurs after a few days accumulation of microbial plaque, is characterized by acute exudative vasculitis, edema, loss of perivascular collagen, and the presence of PMNs intercellularly migrating through the gingiva into the sulcus. PMNs are present in the progressing lesions, also. The early lesion, which develops within four to ten days of plaque accumulation, consists of a dense infiltrate of small lymphocytes and other mononuclear cells, accompanied by degenerative change in fibroblasts and continued loss of connective tissue substance.
The established lesion, present in two to three weeks, reveals a predominance of plasma cells, and may remain stable for several years, or progress to the advanced lesion. The advanced lesion is characterized by a loss of alveolar bone and periodontal ligament, many plasma cells, fibrosis, bone resorption and destruction of the normal periodontal architecture.

Since the histopathologic features of periodontal disease are similar to other chronic inflammatory disease processes which involve the complement system, the potential role of the complement system in the initiation and progression of acute and chronic periodontal inflammation has been the subject of considerable study. Analyses of the exudate of periodontal lesions, gingival fluid, have provided an avenue for examination of the complement system. Several investigators have confirmed that gingival fluid is an altered inflammatory exudate. This has been accomplished by examining the proteins (Brill and Bronnestrom 1960, Mann and Stoffer 1964, Weinstein and Mandel 1964, Schenkein and Genco 1977a), carbohydrates (Hara and Løe 1969), ions (Krasse and Egelberg 1962), and enzymes (Gustafson and Nilssen 1961, Frank and Cimasoni 1972) present in gingival fluid and comparing them with serum content. However, studies of gingival fluid are hindered by the small amounts which can be collected and by the vast assortment of microbial and host-derived substances which are present.

Complement components have been identified in gingival fluid. Shillitoe and Lehner (1972) observed concentrations of C3
in gingival fluid which were somewhat less than those found in serum. Courts et al. (1977) noted the existence of a functional complement system in the gingival fluid of patients with periodontitis. The authors reported that the addition of dental plaque to gingival fluid led to Cl fixation and they determined both Cl hemolytic activity and whole complement hemolytic activity.

Complement utilization appears to be associated with periodontal disease. Reduced levels of native C3 and C4 (Attstrom et al. 1975, Schenkein and Genco 1977a) relative to other serum proteins have been found in the gingival fluid obtained from severe periodontal lesions. Schenkein and Genco (1977a) observed that native C3 in gingival fluid from diseased sites was approximately 25% of the serum level while other serum components of similar molecular weight existed at 80% of serum levels. Activation of C3 in gingival fluid obtained from periodontal lesions has been suggested by the presence of its cleavage products, C3c (Attstrom et al. 1975, Schenkein et al. 1976, Schenkein and Genco 1977b) and C3d (Schenkein and Genco 1977b). Patters et al. (1979) reported a larger percent of C3 cleavage in gingival fluid from inflamed sites when compared with healthy sites. C4c, a conversion product of C4 (Shiraishi and Stroud 1975) has been observed in the gingival fluids of patients with juvenile periodontitis (Schenkein et al. 1976) and in some patients with severe rapidly progressive periodontitis (Schenkein and Genco 1977b). The cleavage product of B, Bb, has been detected in the gingival
fluid from severely diseased sites (Attstrom et al. 1975, Schenkein et al. 1976, Schenkein and Genco 1977b). In all patients examined in these studies, no complement cleavage products were observed in serum.

Additional indirect evidence exists to support the concept of complement cleavage in periodontal disease. Extracts of gingival tissues from patients with periodontitis were observed to be chemotactic for monocytes (Okada and Silverman 1979). This was not true for tissue from healthy subjects. Antibodies to C5 substantially inhibited this chemotactic activity. In addition, these gingival extracts induced chemotactic activity from fresh serum. Complement-related compounds may, therefore, be important in the chemotaxis of mononuclear cells in periodontal disease and in the change of predominant cell type that occurs.

The above studies do not address the mechanism or the location of the complement conversion in the periodontal diseases, but many of the initiating factors of both the classical and the alternative pathways are present in the periodontium. Bacteria and their products, immunoglobulins, and complement components are all located in the area of the periodontal pocket, supporting the concept of a potential role for complement in periodontal disease.

Although immune complexes have not been identified in tissues taken from either dogs or humans with chronic periodontitis (Clagett and Page 1978), other evidence suggests a role for the classical pathway of complement activation in periodontal
disease following the interaction of antigens and antibodies. Immunoglobulins were first described in gingival fluid by Brandtzaeg (1965). Several laboratories since have identified predominantly IgG, but also IgA without a secretory piece (Holmberg and Killander 1971), and IgM in gingival fluid (Shillitoe and Lehner 1972, Schenkein and Genco 1977a). Antibodies also have been demonstrated in the serum of individuals with periodontal disease that are reactive with many species of periodontopathic bacteria, including Actinomyces, Actinobacillus, Bacteroides, Fusobacteria and Leptotrichia (Mergenhagen et al. 1965, Genco et al. 1974, Evans et al. 1966, Mansheim et al. 1980). In juvenile periodontitis patients, high serum and gingival fluid antibody titers to Actinobacillus actinomycetemcomitans have been observed. Additionally, local synthesis within the gingival tissues of antibodies and complement have been reported (Schlossberg 1971, Payne 1975). Gingival plasma cells have been shown to synthesize immunoglobulins specific for oral bacterial antigens. Berglund (1971) demonstrated antibody activity in extracts of inflamed gingival tissue. Results have been conflicting in determining the type of immunoglobulins produced within the periodontium; Brandtzaeg (1965) noted primarily IgG production, Platt et al. (1970): IgM, and Mackler et al. (1978): IgG1, IgG3, IgG4. Lally and coworkers (1980), using 14C-labelled amino acids with immunoelectrophoresis and autoradiography, detected IgG and IgA synthesis locally in chronically inflamed gingival tissues. They observed no immunoglobulin synthesis in
healthy gingival tissue. Using a similar method, Lally et al. (1982) demonstrated local biosynthesis of C3 and C5 in the majority of individuals with chronically inflamed gingiva, but not in healthy tissue. Immunofluorescent studies have been reported which describe IgG, IgM, and C3 within the gingival tissues of gingivitis (Toto et al. 1978a) and periodontitis (Toto et al. 1978b).

Investigators have used immunofluorescent techniques to determine the presence of immunoglobulins and complement bound to subgingival bacteria. Only a fraction of the total bacteria in each smear were positive for the immunoglobulins or complement (Platt et al. 1970, Genco et al. 1974). Nisengard and Jarrett (1976), however, reported that all bacterial samples obtained from periodontitis and most from gingivitis revealed in vivo coating of some bacteria with IgG, IgA, IgM, IgE, and/or complement. Recently, bacteria coated with Clq and C4 have been demonstrated in dental plaque from chronic periodontitis pockets, visualized by light and electron microscopy with specific antibodies to human Clq and C4 and reacted with peroxidase-antiperoxidase (Chisikovsky et al. 1982). Many constituents of dental plaque have been shown to consume complement by the alternative activating pathway or the classical pathway, or both. Okuda and Takazoe (1980) observed complement cleavage in all sera following exposure to dental plaque. A. viscosus, B. melaninogenicus, B. oralis, polysaccharide from S. mutans, S. sanguis, F. nucleatum cell wall and other oral bacteria cause complement activation
(Nilsson et al. 1975, Allison et al. 1976, Shivers et al. 1974, Hawley and Falker 1977). The incubation of dental plaque and serum results in the production of a factor which causes histamine release from basophils. This factor is inhibited by antibody to C5 and it is thought to be C5a (Olsson-Wennstrom et al. 1978). This abundance of oral bacteria, both Gram-positive and Gram-negative, capable of activating complement through the alternative pathway, and the presence of specific antibodies strongly suggests that many of the inflammatory events associated with periodontal inflammation are complement-related.

Studies in decomplemented animals provide further support to the importance of complement in periodontal disease. Dogs, immunized with plaque extract and decomplemented with carragheenan (prevents complement activation by blocking the reaction between C1 and the immune complexes (Borsos et al. 1965, Ward and Cochrane 1965) showed markedly diminished PMN migration and vascular exudation when challenged with plaque extract (Kahnberg et al. 1977). However, when Cobra Venom Factor (a C3b analog and alternative pathway activator) decomplemented dogs which had not been previously immunized were challenged with dental plaque, only a minor change was observed in the number of leukocytes in the challenged junctional epithelium (Kahnberg et al. 1976). The vascular response to the applied plaque extract was decreased in these decomplemented dogs, however. Wennstrom and coworkers (1980) applied extracts from pure cultures of Actinomyces viscosus and Capnocytophaga ochracea to the gingival margin of Cobra
Venom Factor decomplemented dogs and observed a markedly diminished leukocyte accumulation in the junctional epithelium, suggesting that complement plays an \textit{in vivo} role in leukocyte chemotaxis and gingival inflammation.

Other consequences of complement activation correspond with events in periodontal disease. Complement cleavage generates mediators of vascular and cellular inflammatory phenomena through chemotaxis, anaphylatoxin production and host and bacterial cytotoxicity. In addition, the opsonizing effect of C3b, important in host defense in the gingival sulcus, contributes to tissue damage by release of histolytic enzymes by phagocytizing cells. C3b also induces the release of lymphokines from lymphocytes, and has been implicated in stimulating the release of hydrolytic enzymes from macrophages (including collagenase and elastase), resulting ultimately in connective tissue destruction, a hallmark of periodontal disease. Complement has also been linked to bone resorption, an important phenomenon in periodontitis. Fetal rat bone resorption was observed when bone cultures were incubated in fresh rabbit serum. The resorption did not occur when the bones were cultured in heated rabbit serum or C6-deficient serum but recurred when purified C6 was added (Raisz et al. 1974). The mechanism of complement cleavage in gingival fluid in periodontal disease is unknown, but it is clear that C3 is degraded to its final products. This can be achieved by 1) activation of the classical pathway, 2) initiation of the alternative activating pathway, or 3) non-complement proteolytic cleavage of complement.
components. The presence of constant antigenic challenge in the periodontal pocket, plaque-specific serum antibodies and the observation that plasma cells are found in tissues adjacent to the pocket, suggest the potential for antigen-antibody reactions with consequent classical pathway complement activation. Experimentally, Arthus reactions induced in the gingiva of immunized monkeys resulted in an inflammatory infiltrate and osteoclastic resorption similar to that found in periodontitis (Nisengard et al. 1977). IgG and C4 deposits were observed in perivascular areas in these animals. It is therefore possible that specific antibodies which reach the periodontal tissues via serum and locally synthesized immunoglobulins with specificity for bacterial antigens may interact with bacteria and their products to initiate classical pathway activation.

The diseased gingival tissues contain endotoxin and other bacterial products capable of fluid-phase alternative pathway activation. In addition, cleavage of complement components by non-complement proteolytic enzymes may also occur.
GENERAL OBJECTIVES

It is clear that the activation of complement plays an important role in the mediation of inflammation in many chronic inflammatory diseases. Conversion products of C3, C4, and B have been reported to be present in gingival fluid from severe periodontitis lesions. Because of the limited amounts of gingival fluid available from lesions of minimal inflammation, no longitudinal studies of complement activation in periodontal disease have been reported. Using an assay developed by the Master's candidate, sufficient sensitivity is now available to assess longitudinal changes in complement conversion in periodontal disease. Therefore, the purpose of this study was to determine the extent of conversion of complement components of both the classical and alternative pathways in human gingival fluid obtained from lesions of chronic periodontitis, prior and subsequent to initial periodontal treatment. The differences in the measured extent of conversion of the complement components C3, C4, and B from the same site were statistically compared before and after therapy. The information gained may provide a better understanding of the role of complement activation in the initiation and progression of periodontal disease.
SPECIFIC OBJECTIVES

The specific objectives of this study were to:

1) develop a sensitive method to simultaneously assay C3, C4, and B conversion from a single gingival fluid sample

2) measure the extent of conversion of C3 in gingival fluid from patients with chronic periodontitis

3) measure the extent of conversion of C4 in gingival fluid from patients with chronic periodontitis

4) measure the extent of conversion of B in gingival fluid from patients with chronic periodontitis

5) determine the differences in the measured extent of conversion of C3, C4, and B in gingival fluid from the same periodontal sites after initial periodontal therapy.
MATERIALS and METHODS

Selection of subjects Participants were selected from patients presenting for treatment at the out-patient clinics of the University of Connecticut School of Dental Medicine. The nature and risks of the study were explained to all participants and informed consent was obtained. Subjects received a complete oral examination including Gingival Index (Løe and Silness 1963), Plaque Index (Silness and Løe 1964), measurement of pocket depth and loss of periodontal attachment in millimeters, presence of suppuration and bleeding on probing (recorded as 1 if present, 0 if absent). These indices were recorded at a visit prior to the initial sampling, and confirmed immediately subsequent to sampling.

Subjects chosen for study had clinical and radiographic evidence of 20 to 50% bone loss on many teeth. The severity of periodontal disease in this group was consistent with that which would be expected due to age and local factors. Subjects ranged in age from 40 to 60 years, and had a mean Plaque Index greater than 1, with pocket depths of 4-9 mm, and loss of attachment ranging from 1-9 mm.

Gingival fluid from nine individuals with chronic periodontitis was obtained at selected times before and after treatment from representative sites. In some patients, samples were taken twice prior to treatment (baseline). The subjects were then instructed in oral hygiene (brushing and flossing) and received thorough mechanical debridement (root planing and scaling). Sites
were sampled again at 2-4 weeks after therapy. All clinical indices were repeated after each sampling. Some sites were sampled twice (2 and 4 weeks) after therapy. Blood samples were also taken at the initial visit and serum separated.

Collection of gingival fluid: The sites selected for study were interproximal areas which best reflected the disease status of the patient. Standardized filter paper strips (PER-601, Harco Electronics, Winnipeg, Manitoba), wetted with 0.01M Na₂EDTA and completely dried before use to prevent C4 conversion upon electrophoresis (Sjoholm and Laurell 1973), were inserted a pre-marked length (3 mm) into the pocket and allowed to remain undisturbed for 3 min. Prior to fluid collection, the tooth was gently washed with water, dried with air and isolated with cotton rolls. Samples contaminated with blood or plaque were discarded. The samples were then placed in individual plastic containers coded by a person other than the investigator and subjected to crossed-immunoelectrophoresis within 1 hr.

Antisera: Solid-phase absorbed antisera specific for C3 (beta-1-C, beta-1-A), C4, transferrin, and B, respectively, were obtained from Atlantic Antibodies (Scarborough, Maine) and tested for specificity by immunoelectrophoresis vs. human serum. Fresh serum and zymosan-activated serum served as negative and positive controls, respectively, for C3 conversion to C3c and B conversion to Bb. EDTA-anticoagulated plasma and immune-complex treated serum (Sjoholm and Laurell 1973) were used as negative and positive controls, respectively, for C4 conversion to C4c.
Assay of Complement Components in Gingival Fluid: Complement conversion was assessed by crossed immunoelectrophoresis. The coded filter paper strips were removed from their containers, trimmed to 5 mm in length, positioned over a 1.6 mm hole cut in agarose, and folded into the hole with a sharp instrument (Patters et al. 1979). Fluid components migrated out of the strip into the agarose when electrophoresis was begun.

First dimension electrophoresis was carried out in 1.0% agarose (SeaKem) in barbital buffer (pH 8.6, ionic strength=0.02) containing 0.01M Na₂EDTA. An 8 volts/cm current was applied to the 1.5 mm thick gel containing the filter paper strips for 90 min at 15°C.

Second dimension electrophoresis was carried out using a technique developed by the Master's candidate, in which the conversion of C3, C4, and B could be simultaneously assayed from a single gingival fluid sample. The first dimension gel was cut with a razor blade into a 1 cm slab containing the filter strip and its migrated components and transferred to a 50x50x1 mm glass plate. Agarose (3.1 ml) containing a predetermined optimal concentration of specific antisera to B and to C3 was poured onto the remaining 4 cm of the plate. After this gel had solidified, 2.0 cm of this agarose was removed, leaving the 1 cm wide first dimension gel and 2.0 cm of anti-B and anti-C3. Finally, 1.5 ml of agarose containing specific antisera to C4 and to human transferrin (reference protein) was poured onto the remaining part of
the plate. In this manner, a 50x50x1 mm gel had been formed containing the first dimension gel, a 2.0 cm wide slab of agarose with anti-B and anti-C3, and a 2.0 cm wide gel containing anti-C4 and anti-transferrin.

The solidified gel was placed on the electrophoresis apparatus at 90° to the direction of the first dimension with the first dimension gel toward the cathode. The gel was connected with the buffer by means of filter paper wicks. Current (2volts/cm) was then applied at 150°C for 16 hours. The gels were rinsed with distilled water, dried at 60°C, and stained with Coomassie Brilliant Blue R-250, and the precipitates measured as described below.

The areas under the curve formed by each precipitate were estimated on a 7X magnified projection by measuring the rocket height and multiplying by the rocket width measured at one-half the rocket height (Weeke 1973). The percentage of C3 converted to C3c was then determined by the following formula:

\[
\% \text{C3 conversion} = \frac{\text{Area of the C3c rocket}}{\text{Area of C3c rocket} + \text{Area of C3 rocket}} \times 100
\]

Conversion of C4 to C4c was assessed by appearance of a less anodally migrating rocket when compared with C4 in EDTA-containing serum. Transferrin, which produced a single rocket which migrated reproducibly between the C4 and C4c rockets, served as a marker to aid in the identification of a single rocket as either
C4 or C4c (Scharfstein et al. 1978). The percentage of C4 conversion was calculated as described for C3c/C3. Because of the heterogeneity of human C4, serum samples were used as controls to indicate the migration pattern of C4 for that individual.

B produced a single rocket which migrated anodally, while Bb, under the conditions of pH and buffer strength used, produced a rocket with minimal gamma mobility. Since the Bb rocket was always small, quantitative measurements were not possible.

Codes were broken after the gels were analyzed.

Statistical analyses. Statistical significance of longitudinal changes in clinical indices and complement conversion following therapy was assessed by both parametric and non-parametric methods. Interval data (pocket depth, loss of attachment, area of precipitates formed by complement components, and percentage conversion) were analyzed using the correlated (paired) t-test. Using this test, t is determined by dividing the mean of the differences between pairs by the standard error of the mean difference. The probability value, p, is found from t at the degrees of freedom equal to the number of pairs minus one. Non-interval data (plaque index, gingival index, bleeding on probing, and suppuration) were analyzed using the Wilcoxon matched-pairs signed rank test. In this test, the differences between pairs are ranked and the smaller of the sums of the positive or negative ranks is T. Although means and standard deviations are presented for descriptive purposes, they were not used in the statistical analyses of non-interval data.
RESULTS

The Method: To establish the crossed-immunoelectrophoresis method introduced in this study, fresh serum was used as the negative control. Zymosan-activated serum served as the positive control for C3 and B conversion and immune-complex activated serum was utilized as the positive control for C4 conversion. The concentrations of antisera in the gels were adjusted to achieve C3, C4, and transferrin rockets with an area of approximately 1000 units on the 7X projection when 0.5 ul of serum was used. The optimal concentrations for these antisera were 2.0 ul/ml agarose for anti-C4, 1.4 ul/ml agarose for anti-C3, and 10 ul/ml agarose for anti-transferrin. Due to the low concentration of B in serum, rockets of this magnitude were never possible. Therefore, the Anti-B antiserum was adjusted to a concentration of 4.0 ul/ml agarose, which produced a rocket of about 250 units on the 7X projection from 0.5 ul of serum. Bb appeared only as a flat unmeasurable precipitate, and thus was recorded only as present or not present. Throughout the course of this study, a single lot of each antiserum was used.

Precipitates formed by multi-dimension crossed-immunoelectrophoresis were compared with those from standard crossed-immunoelectrophoresis using a single antiserum. The precipitates of all proteins examined: C3, C3c, C4, C4c, B, Bb and transferrin were of similar area and mobility when compared to the multi-layer gels. Therefore, the multi-dimension technique did not cause significant distortion of the precipitates.
Reproducibility of Method: To establish the reproducibility of the method, 26 sites were examined twice prior to treatment. Additionally, 16 sites were sampled two times post-treatment. Analysis of the two pretreatment sample plaque index values by Wilcoxon (for non-interval data) revealed a significant variation between Time 1 and Time 2 ($p \leq 0.005$, Table 1). During this time, the plaque index declined from a mean of 1.88 (Time 1) to 1.35 (Time 2). Comparison of the two post-treatment plaque index values showed no significant difference between Time 3 (mean=0.63) and Time 4 (mean=0.75).

Wilcoxon analysis of the two pretreatment gingival index values (Table 2) disclosed no significant variation between Time 1 (mean GI=1.54) and Time 2 (mean GI=1.35). Also, no significant difference existed between the post-treatment values, (Time 3 mean GI=1.12) and Time 4 (mean GI=1.25).

The two pretreatment values for bleeding on probing (Time 1 mean bleeding on probing=0.81, Time 2=0.73) were not significantly different (Table 3). Comparison of the two post-treatment mean bleeding on probing values showed no significant variation (Time 3=0.50 Time 4=0.63). Analysis of the two pretreatment and the two post-treatment suppuration values by Wilcoxon revealed no significant changes in either comparison (Table 4).

The correlated t-test was used for analysis of the interval data. Table 5 contains the values for the reproducibility of pocket depth measurements. No significant change in pocket depth occurred between the two pretreatment values (Time 1 mean pocket
depth=6.38 mm and Time 2 mean pocket depth=6.46 mm). However, a slight but significant difference was apparent between the two post-treatment readings (p=0.015). The Time 3 mean pocket depth was 5.81 mm and the Time 4 mean pocket depth was 5.31 mm.

Loss of attachment reproducibility analysis followed a similar pattern (Table 6). Using the correlated t-test, no significant variation was found between the two pretreatment values (Time 1 mean loss of attachment=5.38 mm and Time 2 mean loss of attachment=5.19 mm). There was a small but significant difference between the two post-treatment measurements (p<0.0034). The Time 3 mean loss of attachment was 6.00 mm and the Time 4 mean loss of attachment equalled 5.38 mm.

Table 7 contains the reproducibility analysis of the percentage C3 conversion by correlated t-test. The mean percentage of C3 conversion was 78.2% and 62.2% for Time 1 and Time 2, respectively. No significant differences were apparent between the two pretreatment or between the two sets of post-treatment percentage C3 conversion values (Time 3=51.4% and Time 4=39.4%).

Therefore, for all pretreatment parameters measured on two occasions only the plaque index differed significantly. In comparing the two sets of post-treatment values, a slight but significant decrease in both pocket depth and loss of attachment occurred between Time 3 and Time 4. All other variables examined did not significantly differ.

Clinical Longitudinal Results: For analysis of the changes in the clinical variables over the course of treatment, 37
chronic periodontitis sites were examined. Time 1 values were compared with Time 4 values. Wilcoxon analysis was employed for non-interval data and correlated t-test for interval data.

Comparison of plaque index values (Table 8) revealed a significant decrease (p<=0.005) following periodontal treatment. The pretreatment plaque index mean was 1.76 and the post-treatment plaque index mean equalled 0.95. The Gingival Index followed a parallel pattern (Table 9), and declined significantly (p<=0.005) between pretreatment and post-treatment measurements. The mean pretreatment GI was 1.54 and the mean post-treatment GI was 1.11. Bleeding on probing (Table 10) also showed a significant decrease after periodontal treatment (p<=0.005), decreasing from a pretreatment mean of 0.86 to a post-treatment mean of 0.41. Suppuration values (Table 11) declined significantly (p<=0.025) over the course of treatment. The mean pretreatment suppuration value was 0.22, but the post-treatment mean was 0.05.

From the correlated t-test analysis of pocket depth measurements (Table 12), it was apparent that a highly significant decrease (p<=0.00005) occurred between the pretreatment measurements (mean pocket depth=6.32 mm) and the post-treatment values (mean pocket depth=5.05 mm). A reduction in loss of attachment (Table 13) was also highly statistically significant (p<=0.00005), changing from a pretreatment mean of 5.65 mm to a post-treatment mean of 4.84 mm.

**Complement and Transferrin Longitudinal Results:** For analyses of the changes in complement cleavage during periodontal
treatment, the pretreatment crossed-immunoelectrophoresis rocket area measurement was compared with the same site post-treatment value by correlated t-test. Thirty-seven chronic periodontitis sites were examined and Time 1 was compared with Time 4.

Table 14 contains the comparison of pretreatment C3 (mean area=985) with post-treatment C3 (mean area=1675). This increase was statistically significant at p=0.040.

The C3c rocket area (Table 15) decreased significantly (p=0.0019) between pretreatment (mean=1580) and post-treatment (mean=904) measurements.

Table 16 illustrates the measurements of C3 percentage conversion before and after periodontal treatment. A highly significant (p<=0.00005) decrease occurred between the pre-treatment value (mean=70.0%) and the post-treatment value (mean=37.5%).

The C4 rocket area (Table 17) did not change significantly during periodontal treatment. The mean value was 382 pre-treatment and 347 post-treatment. C4c precipitates were never seen in the gingival fluid of the 37 chronic periodontitis sites selected.

Table 18 depicts the comparison of the pretreatment B (mean=125) and the post-treatment B (mean=138). This difference was not statistically significant. The Bb precipitates were too small to measure, so no statistical analysis was possible.

The area under the transferrin rocket (Table 19) showed no significant change between the pretreatment (mean=1626) and the post-treatment values (mean=1246).
Therefore, several significant changes in complement components occurred following periodontal treatment. The area of the C3 precipitate increased; the area of the C3c precipitate decreased, and the percentage of C3 conversion declined sharply. C4, C4c, B, Bb and transferrin did not show any statistically significant change.

Figure 1 is a photograph of a gel from a gingival fluid sample taken prior to periodontal treatment. Precipitates representing transferrin (T), C4, C3, C3c, B, and Bb are visible. Approximately 60% of the available C3 has been converted to C3c.

Figure 2 is a photograph of a gel obtained after crossed-immunoelectrophoresis of gingival fluid taken from the same site as in Figure 1, but sampled following periodontal therapy. Again precipitates representing transferrin (T), C4, C3, C3c, B and Bb can be observed. The C3c rocket is diminished in area relative to the C3 rocket in this post-treatment sample and represents 27% C3 conversion.
DISCUSSION

The Method. The sampling and crossed immunoelectrophoresis technique introduced in this paper was highly sensitive and allowed the assessment of the complement components from a single gingival fluid sample obtained from a specific site. No pooling of samples was necessary as the proteins could be measured in gingival fluid samples as small as 0.2μl. Since no elution of the samples was required, the dilution of C3 beyond detectable levels as occurred in the report of Attstrom et al. (1975) was also avoided. The sensitivity of the new procedure and the sampling method, therefore, allowed the analysis of complement components in areas of minimal periodontal inflammation, making possible a longitudinal study of complement activation in chronic periodontitis over the course of treatment. No previous longitudinal studies of complement activation in gingival fluid have been reported. Additionally, it was possible to ascertain not only whether the complement terminal pathway was activated by the presence of C3 conversion products, but also which initiation pathway might be utilized. If C4 cleavage products are present, then the classical pathway is likely activated. If B cleavage products can be observed, alternative pathway initiation is suggested.

Reproducibility of the method. The major objective of this study was to determine longitudinal changes in complement cleavage in gingival fluid that occurred with treatment of the periodontal lesions. Prior to making these observations, it was
necessary to determine the stability of the clinical and complement measures. Therefore, a subset of periodontal lesions was assessed clinically and for complement cleavage on two occasions prior to and two occasions after therapy. A comprehensive clinical examination was performed at both pretreatment and both post-treatment visits and included the plaque index (Silness and Løe 1964), gingival index (Løe and Silness 1963), bleeding on probing, suppuration, pocket depth and loss of attachment in millimeters, all recorded after the gingival fluid samples were taken.

In comparing Time 1 with Time 2 samples (Table 1), only the plaque index decreased significantly (p<.005). This improvement in the plaque index can be attributed to the fact that the patient had started a series of periodontal treatment visits and had received some hygiene instruction. All other clinical parameters and complement component measurements were not statistically different between Time 1 and Time 2 (Tables 1-7). In addition, reproducibility of complement component measures between the two pretreatment samples was excellent. Therefore, Time 1 values were selected for further statistical comparisons.

Identical periodontal sites were also examined and sampled at two and four weeks post-treatment. Comparison of the two posttreatment samples revealed a small decrease in pocket depth (0.5 mm) and loss of attachment (0.6 mm) with the longer healing time (Table 5 and 6). This decrease in loss of attachment may be caused by a decrease in inflammation of the periodontal tissues with a consequent tighter adaptation of the tissue to the tooth.
and a greater resistance to periodontal probing. All other clinical parameters and complement measurements did not vary significantly between the two post-treatment samples. Results from these experiments indicated that both clinical and complement parameters appeared reproducible before and after therapy. Therefore, changes in these measurements after therapy likely resulted from treatment rather than random fluctuations. For analysis of the changes occurring longitudinally with treatment, the first pretreatment sample (Time 1) was compared with the last post-treatment sample (Time 4).

Complement cleavage in periodontitis. It is clear from the results of this study that C3 is cleaved to C3c in the gingival fluid of chronic, untreated periodontal lesions. This observation is supported by the cross-sectional findings of Attstrom et al. (1975), Schenkein and Genco (1977a), and Patters et al. (1979). The cleavage of C3 may be caused by three primary mechanisms: classical pathway, alternative activating pathway, or proteolysis due to enzymes present in the periodontal pocket.

With the continual presence of antigenic challenge and the observation of IgG antibodies reactive with oral bacterial antigens in the gingival fluid, it is feasible that a local antigen-antibody interaction is possible with consequent classical complement pathway activation. However, few immune complexes have been observed in the periodontal tissues of chronic periodontitis (Clagett and Page 1978, Platt et al. 1970, Genco et al. 1974). In all 37 chronic periodontitis sites examined, C4 cleavage
products were not observed in the gingival fluid. It therefore, seems unlikely that complement activation occurred primarily by this pathway.

There is evidence that soluble bacterial products capable of fluid-phase alternative pathway activation (such as endotoxins) can enter the periodontal tissue through the damaged crevicular epithelium (Schwartz et al. 1972). Many agents are capable of activating the alternative activating pathway of complement, and several of these are present both in the periodontal pocket and the surrounding tissue. T-independent antigens and B-cell mitogens can activate this pathway. In addition, several bacteria have been shown to initiate this alternative cascade, including gram-negative organisms, Actinomyces viscosus and a polysaccharide from Streptococcus mutans. Microorganisms are certainly present in the periodontal pocket and have been observed in the tissue of severe periodontitis and juvenile periodontitis (Sagli 1982, Frank 1980). Gram negative organisms, including Fusobacterium nucleatum and Bacteroides gingivalis found in chronic periodontitis have been shown to be capable of initiating the alternative activating pathway (Nilsson et al. 1975, Allison et al. 1976, Shivers et al. 1974, Hawley and Falker 1977). This evidence, coupled with the fact that B and its cleavage product Bb were consistently present in untreated periodontitis, while C4 cleavage products were not, suggests that in the periodontal pocket, nonspecific direct activation of C3 may occur via the alternative activating pathway.
Proteolytic Enzymes. C3 and C5 have been split in vitro by complement-unrelated enzymes such as purified neutral proteases from PMNs (Johnson et al. 1976, Bronzna et al. 1977, Taylor et al. 1977). In the above studies, it was observed that elastase, collagenase and cathepsin G were capable of splitting C3 and C5 into smaller fragments electrophoretically similar to C3a and C5a. These and similar enzymes may be present in the periodontal pocket and the possibility does exist that complement cleavage in periodontal disease occurs by this means. No attempt was made in the current study to evaluate the contributions of complement-unrelated enzymes in complement cleavage in the gingival fluid.

Longitudinal changes following periodontal therapy. Over the treatment period, plaque index, gingival index, bleeding on probing, suppuration, pocket depth, and loss of attachment all decreased significantly (Tables 8-13). These substantial changes in the clinical parameters demonstrated the effectiveness of the initial periodontal therapy. Measurements of transferrin showed no variation between the pretreatment and post-treatment samples (Table 19). Transferrin, a protein unrelated to changes in inflammation, served as a marker and, therefore, should not have changed with periodontal treatment. Other factors which might affect the results were minimized as follows: C4 conversion due to electrophoresis was suppressed by the treatment of the filter paper strips used for sampling with Na$_2$EDTA (Sjoholm and Laurell 1973). Samples were processed within one hour to minimize in vitro exposure of the gingival fluid to bacterial plaque on the
filter strip. Patters et al. (1979) demonstrated that plaque present on the sampling strip did not activate the complement present in fluid absorbed into EDTA-containing filter paper. In addition, serum samples from individuals in the study were handled in an identical manner to gingival fluid. No C3, B, or C4 cleavage products were observed in the serum tested and they, therefore, served as the negative controls. This was especially critical in the evaluation of C4 which is heterogeneous in humans and thus has slightly variable mobilities. The transferrin marker also served to reproducibly distinguish C4 from C4c mobility (Scharfstein et al. 1978). Immune complex-activated serum served as the positive control to visualize C4c mobility. Additionally, clinical measurements and filter strip samples were taken by the Master's candidate to eliminate inter-operator inconsistency. All samples were coded by a person other than the Master's candidate and these codes were not broken until the gels had been measured. The evidence of clinically successful therapy, the stability of the reference protein, transferrin, and the above precautions and controls suggested that any changes in C3, C4 or B would be the result of therapy rather than random changes.

For all paired samples (pretreatment vs. post-treatment), however, C3 levels increased (Table 14) and C3c amounts decreased (Table 15) significantly. Therefore, the percent of C3 conversion also decreased significantly (Table 16, p<0.00005), decreasing from 70.0% to 37.5% overall. Less complement cleavage was therefore present in the gingival fluid after initial therapy
than in the untreated chronic periodontitis lesions. B (Table 18) and Bb levels did not change significantly between pretreatment and post-treatment levels, but a trend did exist for decreased Bb after treatment. This could not be statistically confirmed due to the difficulty of quantitation of Bb. C4 conversion products were never observed in the pretreatment samples, and it remained uncleaved throughout treatment. Native C4 levels did not statistically change with treatment (Table 17).

This demonstration of a reduction in the C3/C3c ratio following initial periodontal therapy suggests a correlation between conversion of C3 in gingival fluid and the state of periodontal health. The observation of complement cleavage products in gingival fluid from diseased sites and an understanding of their biological implications makes complement-mediated tissue injury a distinct possibility in periodontal disease. This is underscored by the fact that the conversion products decrease with a concomitant decline in clinical signs of disease.

**Protective and destructive aspects of complement activation.** The activation of complement may play a protective role in the periodontal tissues. The opsonization of bacteria and their products, chemotaxis, bacterial cell cytotoxicity, and modulation of cell-mediated immunity are all first-line defense mechanisms involving complement. Since the periodontal tissues are constantly challenged by pathogenic bacteria and their toxic products, greater tissue damage than is observed might be expected
to occur. Chronic periodontitis, however, is a slowly progressive pathologic process. Complement likely figures prominently in this protective function, both through enhanced phagocytosis and bacteriolysis.

However, activation of the complement cascade induces many phenomena associated with inflammation and concomitant tissue damage. This is accomplished in many ways, primarily through the release of limited proteolytic cleavage products formed by the complement cascade. The results of this study indicate that the percentage of C3 cleaved to C3c correlate with clinical measures of inflammation suggesting an ongoing pathologic process. The balance between host protection and tissue damage remains to be determined.

Complement in other chronic inflammatory diseases. Chronic periodontitis and rheumatoid arthritis are similar in several ways. They both involve the development of inflammatory disease within a specific extravascular site. The majority of inflammatory cells present locally in both disease entities are mononuclear cells. Despite the fact that the involved tissues in both cases have been shown to contain cells which synthesize many complement components, complement components in both gingival fluid (Schenkein and Genco 1977a) and synovial fluid (Pekin and Zvaifler 1964, Hunder et al. 1977) are at reduced levels. This suggests that complement may be utilized in both of these disease processes. In fact, C3 cleavage products are found in both

However, it is suggested from this study that the mechanism of complement utilization may differ between the two disease entities. Immune complexes are commonly found in RA patients (Winchester et al. 1970, Townes and Marcus 1972) and are thought to initiate the complement cascade and contribute to the progression of the disease. However, immune complexes have not been regularly observed in chronic periodontitis. Results of this study indicate that in chronic periodontitis, complement cleavage occurs primarily via the alternative activating pathway or by non-complement enzymes. No C4 cleavage (a classical pathway component) was observed in the gingival fluid of these patients. In RA synovial fluid, C4 is the most consistently depressed complement component (Perrin et al. 1973) and complement cleavage is believed to occur primarily through the classical pathway with the alternative activating pathway in an amplifying role (Kaplan et al. 1980). It is apparent, therefore, that chronic periodontitis is not principally an immune-complex disease, like RA or SLE, and that complement cleavage does not seem to occur by the classical pathway. A multitude of factors which initiate the alternative activating pathway, including bacteria and their products, are present in the periodontal pocket and may explain the cleavage of complement.
CONCLUSIONS

The following conclusions can be made from the studies presented in this thesis:

1) The technique introduced in this paper was suitable for the assessment of complement components C3, C4, and B and their conversion products. The method was adequately quantitative for the complement components studied with the exception of Bb.

2) C3 is converted to the cleavage product C3c in the gingival fluid of chronic periodontitis lesions.

3) C4 cleavage products were never identified in the gingival fluid of chronic periodontitis lesions.

4) B cleavage product, Bb, was present consistently in the gingival fluid of chronic periodontitis lesions.

5) The percentage of C3 converted to C3c in gingival fluid from chronic periodontitis lesions decreased significantly following periodontal therapy. This change paralleled the decline in clinical measurements of inflammatory disease.
FURTHER INVESTIGATIONS

The development of the new sampling method and crossed immunoelectrophoresis technique introduces the possibility for numerous additional studies. The longitudinal evaluation of complement changes during treatment should be continued throughout the surgical phase of treatment. In addition, complement cleavage in the gingival fluid of patients in the maintenance phase of periodontal care could be monitored periodically and the complement changes examined for correlation with clinical measurements and disease recurrence. Complement cleavage may occur prior to changes in measurable clinical parameters and, thus, complement cleavage measurements could be the first sign of recurring inflammation.

Other periodontal diseases such as juvenile periodontitis and rapidly progressive periodontitis could be thoroughly examined both cross-sectionally and longitudinally to evaluate the mechanism of complement initiation and complement changes with treatment. In a preliminary report, evidence for C4 cleavage in juvenile periodontitis was presented by Schenkein and coworkers (1976), but further studies are indicated. In addition, the technique presented here is ideally suited for longitudinal analysis of complement cleavage in experimentally induced gingivitis because the small amounts of gingival fluid available from healthy sites can be analyzed.

To provide further information as to the mechanism of complement cleavage in periodontal disease, the role of complement-
unrelated proteolytic enzymes could be explored. Radio-labelled C3 would be incubated with gingival fluid in the presence of an inhibitor of the complement system such as EDTA or heat inactivation, electrophoresis conducted, and the amount of labelled C3 converted could be evaluated by autoradiography to establish the presence of proteolytic enzymes in gingival fluid.
CITATIONS


DeCeulaer, C. 1980. Increased biosynthesis of complement components by cultured monocytes, synovial fluid macrophages and synovial membrane cells from patients with rheumatoid arthritis. Immunology 41:37-43.


PLAQUE INDEX
(SILNESS and LOE)

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UNTIED N = 10  
p \leq 0.005

POST-TREATMENT

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WILCOXON T = 10.5  
UNTIED N = 7  
p = \text{NS}\textsuperscript{b}

\text{a)} Means, standard deviations, and standard errors are shown for descriptive purposes only and were not used in the statistical analyses.

\text{b)} \text{NS} = not significant (p > 0.05)

TABLE 1
GINGIVAL INDEX
(LOE and SILLNESS)

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UNITED N = 11

p = NS<sup>b</sup>

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UNITED N = 6

p = NS<sup>b</sup>

a) Means, standard deviations, and standard errors are shown for descriptive purposes only and were not used in the statistical analyses.

b) NS = not significant (p > 0.05)
BLEEDING ON PROBING
(0=NEGATIVE, 1=POSITIVE)

PRETREATMENT

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p = NS

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Wilcoxon T = 7
Untied N = 6
p = NS

a) Means, standard deviations, and standard errors are shown for descriptive purposes only and were not used in the statistical analyses.

b) NS = not significant (p > 0.05)
SUPPURATION  
(0=NEGATIVE, 1=POSITIVE)  

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p = NS^b

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UNITED N = 2  
p = NS^b

a) Means, standard deviations, and standard errors are shown for descriptive purposes only and were not used in the statistical analyses.

b) NS = not significant (p > 0.05)
POCKET DEPTH (MM)

PRE-TREATMENT

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\[ t = 0.53 \quad df = 25 \quad p = 0.60 \text{ (NS)} \]

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\[ t = 2.74 \quad df = 15 \quad p = 0.015 \]

b) \text{NS = not significant (p > 0.05)}

TABLE 5
LOSS OF ATTACHMENT (MM)

PRETREATMENT

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\[ t = 1.15 \quad \text{df} = 25 \quad p = 0.26 \quad (\text{NS}^b) \]

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<tbody>
<tr>
<td>TIME 3</td>
<td>16</td>
<td>6.00</td>
<td>4</td>
<td>9</td>
<td>0.62</td>
<td>0.18</td>
</tr>
<tr>
<td>TIME 4</td>
<td>16</td>
<td>5.38</td>
<td>3</td>
<td>8</td>
<td>0.62</td>
<td>0.18</td>
</tr>
</tbody>
</table>

\[ t = 3.49 \quad \text{df} = 15 \quad p = 0.0034 \]

b) NS = not significant \( (p > 0.05) \)

**TABLE 6**
PERCENTAGE OF C3 CONVERSION
(C3c/[C3 + C3c])

Pretreatment

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean Difference</th>
<th>Standard Error of Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time 1</td>
<td>26</td>
<td>78.2</td>
<td>20.8</td>
<td>100.0</td>
<td>16.0</td>
<td>8.4</td>
</tr>
<tr>
<td>Time 2</td>
<td>26</td>
<td>62.2</td>
<td>0</td>
<td>100.0</td>
<td>16.0</td>
<td>8.4</td>
</tr>
</tbody>
</table>

\[ t = 1.90 \] \hspace{1cm} \[ df = 25 \] \hspace{1cm} \[ p = 0.07 \text{ (NS)}^b \]

Post-treatment

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean Difference</th>
<th>Standard Error of Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time 3</td>
<td>16</td>
<td>51.4</td>
<td>0</td>
<td>100.0</td>
<td>12.0</td>
<td>11.7</td>
</tr>
<tr>
<td>Time 4</td>
<td>16</td>
<td>39.4</td>
<td>0</td>
<td>87.3</td>
<td>12.0</td>
<td>11.7</td>
</tr>
</tbody>
</table>

\[ t = 1.03 \] \hspace{1cm} \[ df = 15 \] \hspace{1cm} \[ p = 0.32 \text{ (NS)}^b \]

b) NS = not significant \( (p > 0.05) \)

**Table 7**
PLAQUE INDEX
(SILNESS and LOE)

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>MEANᵃ</th>
<th>STANDARDᵃ DEVIATION</th>
<th>STANDARDᵃ ERROR</th>
<th>MINIMUM VALUE</th>
<th>MAXIMUM VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIME 1 (PRE)</td>
<td>37</td>
<td>1.76</td>
<td>0.60</td>
<td>0.10</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>TIME 4 (POST)</td>
<td>37</td>
<td>0.95</td>
<td>0.74</td>
<td>0.12</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

WILCOXON T = 6.5
UNTIED N = 22
p <= 0.005

a) Means, standard deviations, and standard errors are shown for descriptive purposes only and were not used in the statistical analyses.
GINGIVAL INDEX  
(LOE and SULNESS)

<table>
<thead>
<tr>
<th>N</th>
<th>MEAN(^a)</th>
<th>STANDARD(^a) DEVIATION</th>
<th>STANDARD(^a) ERROR</th>
<th>MINIMUM VALUE</th>
<th>MAXIMUM VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIME 1 (PRE)</td>
<td>37</td>
<td>1.54</td>
<td>0.51</td>
<td>0.08</td>
<td>1</td>
</tr>
<tr>
<td>TIME 4 (POST)</td>
<td>37</td>
<td>1.11</td>
<td>0.31</td>
<td>0.05</td>
<td>1</td>
</tr>
</tbody>
</table>

WILCOXON T = 9.5  
UNTIED N = 18  
p <= 0.005

a) Means, standard deviations, and standard errors are shown for descriptive purposes only and were not used in the statistical analyses.

TABLE 9
**BLEEDING on PROBING**
(0=NEGATIVE, 1=POSITIVE)

<table>
<thead>
<tr>
<th>N</th>
<th>MEAN(^a)</th>
<th>STANDARD(^a) DEVIATION</th>
<th>STANDARD(^a) ERROR</th>
<th>MINIMUM VALUE</th>
<th>MAXIMUM VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIME 1 (PRE)</td>
<td>37</td>
<td>0.86</td>
<td>0.35</td>
<td>0.06</td>
<td>0</td>
</tr>
<tr>
<td>TIME 4 (POST)</td>
<td>37</td>
<td>0.41</td>
<td>0.50</td>
<td>0.08</td>
<td>0</td>
</tr>
</tbody>
</table>

**WILCOXON T = 22**
UNTIED N = 21
p <= 0.005

---
a) Means, standard deviations, and standard errors are shown for descriptive purposes only and were not used in the statistical analyses.

**TABLE 10**
**SUPPURATION**
(0=NEGATIVE, 1=POSITIVE)

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>MEAN</th>
<th>STANDARD</th>
<th>STANDARD</th>
<th>MINIMUM</th>
<th>MAXIMUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIME 1 (PRE)</td>
<td>37</td>
<td>0.22</td>
<td>0.42</td>
<td>0.07</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>TIME 4 (POST)</td>
<td>37</td>
<td>0.05</td>
<td>0.23</td>
<td>0.04</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

WILCOXON T = 0
UNTIED N = 6
p <= 0.025

a) Means, standard deviations, and standard errors are shown for descriptive purposes only and were not used in the statistical analyses.

**TABLE 11**
<table>
<thead>
<tr>
<th>N</th>
<th>MEAN</th>
<th>MINIMUM</th>
<th>MAXIMUM</th>
<th>MEAN DIFFERENCE</th>
<th>STANDARD ERROR OF DIFFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIME 1 (PRE) 37</td>
<td>6.32</td>
<td>4</td>
<td>9</td>
<td>1.27</td>
<td>0.25</td>
</tr>
<tr>
<td>TIME 4 (POST) 37</td>
<td>5.05</td>
<td>3</td>
<td>9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ t = 5.02 \]
\[ df = 36 \]
\[ p \leq 0.00005 \]

**TABLE 12**
LOSS of ATTACHMENT
(MM)

<table>
<thead>
<tr>
<th>N</th>
<th>MEAN</th>
<th>MINIMUM</th>
<th>MAXIMUM</th>
<th>MEAN VALUE DIFFERENCE</th>
<th>STANDARD ERROR OF DIFFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIME 1 (PRE) 37</td>
<td>5.65</td>
<td>1</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIME 4 (POST) 37</td>
<td>4.84</td>
<td>1</td>
<td>9</td>
<td>0.81</td>
<td>0.17</td>
</tr>
</tbody>
</table>

\[ t = 4.82 \]
\[ df = 36 \]
\[ p \leq 0.00005 \]

TABLE 13
<table>
<thead>
<tr>
<th>N</th>
<th>MEAN</th>
<th>MINIMUM</th>
<th>MAXIMUM</th>
<th>MEAN DIFFERENCE</th>
<th>STANDARD ERROR OF DIFFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIME 1 (PRE)</td>
<td>37</td>
<td>985</td>
<td>0</td>
<td>4536</td>
<td>690</td>
</tr>
<tr>
<td>TIME 4 (POST)</td>
<td>37</td>
<td>1675</td>
<td>80</td>
<td>6525</td>
<td></td>
</tr>
</tbody>
</table>

\[ t = 2.13 \quad \text{df} = 36 \quad p = 0.040 \]

**TABLE 14**
### C3c

*(Area Under Curve)*

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean Difference</th>
<th>Standard Error of Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIME 1 (PRE)</td>
<td>37</td>
<td>1580</td>
<td>186</td>
<td>5350</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIME 4 (POST)</td>
<td>37</td>
<td>904</td>
<td>0</td>
<td>2806</td>
<td>676</td>
<td>202</td>
</tr>
</tbody>
</table>

\[ t = 3.35 \]
\[ df = 36 \]
\[ p = 0.0019 \]

**TABLE 15**
PERCENTAGE C3 CONVERSION  
(C3c/[C3 + C3c])

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>MEAN</th>
<th>MINIMUM</th>
<th>MAXIMUM</th>
<th>MEAN DIFFERENCE</th>
<th>STANDARD ERROR OF DIFFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIME 1 (PRE)</td>
<td>37</td>
<td>70.0</td>
<td>12.5</td>
<td>100.0</td>
<td>32.5</td>
<td>6.3</td>
</tr>
<tr>
<td>TIME 4 (POST)</td>
<td>37</td>
<td>37.5</td>
<td>0</td>
<td>87.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ t = 5.19 \]
\[ df = 36 \]
\[ p \leq 0.00005 \]

**TABLE 16**
### Table 17

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>MEAN</th>
<th>MINIMUM VALUE</th>
<th>MAXIMUM VALUE</th>
<th>MEAN DIFFERENCE</th>
<th>STANDARD ERROR OF DIFFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TIME 1 (PRE)</strong></td>
<td>37</td>
<td>382</td>
<td>0</td>
<td>1680</td>
<td>35</td>
<td>109</td>
</tr>
<tr>
<td><strong>TIME 4 (POST)</strong></td>
<td>37</td>
<td>347</td>
<td>0</td>
<td>1755</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ t = 0.32 \quad \text{df} = 36 \quad p = 0.75 \ (\text{NS}^b) \]

\( b \) \text{ NS = not significant (p > 0.05) }
<table>
<thead>
<tr>
<th>N</th>
<th>MEAN</th>
<th>MINIMUM</th>
<th>MAXIMUM</th>
<th>MEAN DIFFERENCE</th>
<th>STANDARD ERROR OF DIFFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIME 1 (PRE)</td>
<td>37</td>
<td>125</td>
<td>0</td>
<td>777</td>
<td>13</td>
</tr>
<tr>
<td>TIME 4 (POST)</td>
<td>37</td>
<td>138</td>
<td>0</td>
<td>1287</td>
<td></td>
</tr>
</tbody>
</table>

\[ t = 0.25 \quad \text{df} = 36 \quad p = 0.81 \text{ (NS\textsuperscript{b})} \]

\textbf{b) } NS = not significant \quad (p > 0.05)

\textbf{TABLE 18}
### TRANSFERRIN
(AREA UNDER CURVE)

<table>
<thead>
<tr>
<th>N</th>
<th>MEAN VALUE</th>
<th>MINIMUM VALUE</th>
<th>MAXIMUM VALUE</th>
<th>MEAN DIFFERENCE</th>
<th>STANDARD ERROR OF DIFFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TIME 1 (PRE)</strong></td>
<td>37</td>
<td>1626</td>
<td>0</td>
<td>10545</td>
<td>380</td>
</tr>
<tr>
<td><strong>TIME 4 (POST)</strong></td>
<td>37</td>
<td>1246</td>
<td>0</td>
<td>5760</td>
<td>t = 1.09</td>
</tr>
</tbody>
</table>

b) NS = not significant (p > 0.05)

**TABLE 19**
FIGURE 1. A photograph of a stained crossed-immunoelectrophoresis gel of gingival fluid from an untreated site of periodontitis. GF=gingival fluid. The precipitates are labelled.
FIGURE 2. A photograph of a stained crossed-immunoelectrophoresis gel of gingival fluid from the same site as figure 1 after periodontal therapy. A reduction in C3c can be observed. GF=gingival fluid. The precipitates are labelled.