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IDENTIFICATION OF IgG, IgM,

C1q AND C4 ON

BACTERIAL PLAQUE ORGANISMS IN PERIODONTITIS

By

JACOBO CHISIKOVSKY

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL

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DEDICATION

To my wonderful parents:

Dr. Abraham Chisikovsky
Esther Chisikovsky

Especially for their years of
love, support, encouragement and inspiration.

To my loving wife Susy

Whose love, patience and understanding
have been a never-ending source of inspiration.

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VITA

The author, Jacobo Chisikovsky Nazari, is the son of Dr. Abraham Chisikovsky Perkis and Esther Nazari de Chisikovsky. He was born January 13, 1957, in México City, México.

His elementary, secondary and high school education was obtained in "Colegio Hebreo Tarbut" where he graduated in June 1974.

In September 1974, he enrolled at the Universidad Tecnológica de México, School of Dentistry. He graduated in June 1978 with the degree of Cirujano Dentista, and the award of Mención Honorífica.

In October 1978, he took for three months a Post Graduate course in Fix Partial Prosthodontics at the Centro de Investigación y Especialización en Rehabilitación Oral in México City.

From Septmeber 1979 through June 1981 he took Graduate Education towards a Certificate of Specialty in Periodontics under Dr. Anthony W. Gargiulo, and a Master in Science in the Department of Oral Biology at Loyola University School of Dentistry, Chicago, Illinois.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The literature has indicated that patients with periodontal in-
flammatory disease and pockets contain a variety of bacterial organisms
within plaque. The cell membrane of these bacterial organisms contain
antigens on their surfaces.

Immunoperoxidase staining has been shown to be an effective method
to localized antigens in tissue and immunoglobulins components on bacter-
ial plaque organisms.

Since the immunoperoxidase reaction has been utilized for the iden-
tification of IgG and IgM within bacterial plaque, the author presents
evidence that IgG, IgM, Clq and C4 are present on the surface of bacteria
implying an antigen-antibody reaction activating complement by the classic
pathway.

CHAPTER II

LITERATURE REVIEW

BACTERIAL DENTAL PLAQUE IN PERIODONTITIS

Ash, Gitlin, and Smith (1964) reported that there is a direct correlation between the amount of bacterial debris on teeth and the severity of gingivitis. The debris, as measured by oral hygiene index scores, is almost entirely bacterial in nature. This would suggest that the amount of bacterial debris on teeth is related to the severity of gingivitis.

Löe, Theilade, and Jensen (1965) showed that a group of 12 dental students who abstained from oral hygiene accummulated bacterial debris (plaque) on their teeth, and, clinically, developed gingivitis. When oral hygiene procedures were reinstituted, the gingivitis subsided. The investigators repeated the experiment and achieved essentially the same results (1966). The implication of dental plaque in gingivitis was confirmed in a later, short-term, study on humans (1967), as antibiotics were effective in reducing gingival exudate and leucocyte emigration, signs of subclinical inflammation.

Saxe, Greene, Bohannon, and Vermillion (1967) found that the mechanical removal of bacteria plaque in dogs also led to a decrease in periodontal disease. Thus, according to Socransky (1970), the severity of

experimental gingivitis in man or animals can be controlled by reducing the accumulation of bacterial plaque in the gingival crevice.

Waerhaug (1956, 1957, 1960) showed that bacterial plaque was more important in the initiation of severe inflammation in the gingival tissues than mechanical irritation. In a series of experiments he used a variety of mechanical irritants alone to traumatize the gingiva. In all instances, the inflammatory response was mild or absent. However, bacterial plaque and trauma with an offending mechanical device resulted in a greater inflammatory process.

Cross-sectional radiographic studies of 737 individuals conducted by Schei, Waerhaug, Lovdal, and Arno (1959) indicated that toothbrushing efficiency, measured by a visual estimation of bacterial debris, correlated with the severity of bone loss in Periodontitis. The most severe bone loss occurred in individuals who were judged to have poor oral hygiene. It seems clear that the bone loss was related to the mass of bacteria accumulating in the gingival crevice region.

The bacteria resident in the human gingival crevice have pathogenic potential. They caused infection in bite wounds within a variety of labortory animals as found by McMaster (1939), and Fritzell (1940). Thus, studies by Foley and Rosebury (1942), Shpuntoff and Rosebury (1949), Rosebury, MacDonald, and Clark (1950), indicate that human gingival organisms and their products can lead to inflammation and destruction of oral tissues.

Socransky (1970) found that the mixed microbiota or pure cultures of human gingival crevice bacteria, produced a variety of enzymes that were capable of destroying constituents present in human gingiva. These included collagenase, a variety of proteases, hyaluronidase, chondroitin sulfatase, B-glucuronidase, fibrinolysin, deoxyribonuclease, and ribonuclease.

Pure cultures of human oral bacteria, including streptococci and Gram-positive rods, can initiate a periodontal syndrome in gnotobiotic rats and hamsters were reported by Gibbons, Bernan, and Knoettner (1966); Gibbons and Banghart (1968) Socransky (1970). Although these data did not conclusively prove the etiologic role of these organisms in human periodontal disease, it did demonstrate the pathogenic relation of such organisms to periodontal destruction.

Collectively, the evidence indicates a significant role for oral bacteria in the etiology of human periodontal disease.

Studies of the composition of the microbiota in the human gingival crevice present problems in sampling. The plaque microbiota are extremely complex; no single species has yet been identified as the agent responsible for gingival inflammation according to Socransky (1970). In considering the technical problems of enumerating microorganisms, it should be pointed out that not all of the organisms resident in the gingival crevice can be successfully cultivated, and those organisms which can be cultivated do not always fit current classification schemes.

In spite of these difficulties, a reasonable amount of data concerning the microbiota of the gingival crevice regions is available. Several of the organisms present produce various irritants, enzymes, cytotoxic metabolites, lipopolysaccharide endotoxins, mucopeptides, chemotactic factors and antigens, all which may contribute to the inflammatory response. Thus, Kelstrup and Theilade (1974) reported that there may be more than one combination of bacteria with the ability to induce clinical responses of gingivitis and periodontitis.

ORGANISMS OF THE HUMAN GINGIVAL
CREVICE REGION. SOCRANSKY (1970)

Group	Approximate Percentage of Cultivable Microbiota	Genera and/or Species Commonly Found in this Site
Gram-positive facultative cocci	28.8	staphylococci enterococci Streptococcus mutans Streptococcus sanguis Streptococcus mitis
Gram-positive anaerobic cocci	7.4	Peptostreptococcus
Gram-positive facultative rods	15.3	Corynebacterium Lactobacillus Nocardia Odontomyces viscosus Bacillus matruchotii
Gram-positive anaerobic rods	20.2	Actinomyces bifidus Actinomyces israelii Actinomyces naeslundii Actinomyces odontolyticus Propionibacterium P acnes Leptotrichia buccalis Corynebacterium
Gram-negative facultative cocci	0.4	Neisseria
Gram-negative anaerobic cocci	10.7	Veillonella alcalescens Veillonella parvula
Gram-negative facultative rods	1.2	
Gram-negative anaerobic rods	16.1	Bacteroides melaninogenicus Bacteroides oralis Fusobacterium nucleatum Selenomonas sputigenum
Spiral organisms	1 to 3	Treponema denticola Treponema oralis Treponema macrodentium Borrelia vincentii

IMMUNOLOGIC FACTORS IN PERIODONTITIS

The thymus produces small lymphocytes, (T cells), which are necessary for cellular hypersensitivity and cooperative effects in the induction of circulating antibody. B cells are plasma cells precursors necessary for antibody production. Five major classes of immunoglobulins are recognized in man: IgG, IgA, IgM, IgD, and IgE.

The various immune components which appear to participate in periodontal disease include humoral factors such as immunoglobulins and complement, effector molecules such as prostaglandins, as well as the immunologically competent macrophages and lymphocytes and their products.

While a specific infectious microorganism has not yet been established in the etiology of Periodontitis, one type of response of the human host to bacterial antigens in dental plaque is the production of antibodies by B-lymphocytes and their progeny, the plasma cells. It has been shown experimentally by Ranney (1970); Ranney and Montgomery (1973); Rizzo and Mitchell (1966), that the topical application of a single antigen to animal gingiva causes a serum antibody response as well as gingival accumulations of plasma cells containing specific antibody. Thus, Genco, Mashimo, Krygier, and Ellison (1974) have suggested that the presence of antibodies reactive to oral bacterial antigens in the sera of numerous individuals, and the predominance of plasma cells in inflamed gingiva strongly suggest that immunoglobulins are involved in the maintenance and progression of periodontal disease.

In inflammatory disease, the plasma cells secrete these immunoglobulins into the surrounding connective tissue. These specialized proteins, especially IgG, are found in greater abundance in areas of pronounced inflammation than in noninflammed areas. Rizzo and Mergenhagen (1977) noted that the presence of IgG, IgM, and IgA in the sulcular washings of clinically normal individuals indicates these immunoglobulins may gain access to the gingival sulcus. They are even easily detected in the sulcular fluid of individuals afflicted with inflammatory periodontal disease.

The demonstration of the local synthesis of antimicrobial antibodies and of immunoglobulins-coated bacteria in subgingival debris indicates that at least some of these immunoglobulins may be specific for oral microorganisms as shown by Nisengard and Jarrett, (1976). Although these interactions may be protective, the immune complexes may contribute to inflammation, as they do in other pathological conditions. Presumably, bacterial products penetrate into the underlying gingival tissue where they interact with specific antibody to form immune complexes. As a result of their phagocytosis by polymorphonuclear leukocytes and macrophages, antigens are destroyed, but tissue-destructive enzymes are released locally. Additional tissue damage by cytolysis may be incurred through activation of the complement sequence by such complexes as shown by Rizzo and Mergenhagen (1977).

Two mechanisms may participate in complement activation within periodontal disease. They consist of the classic and alternate pathways.

1) Classic Pathway:

The classical mechanism of complement activation involves the interaction of C1 with immune complexes and immunoglobulin aggregates resulting in cytolysis. Not all antibodies are able to bind C1. IgA appears to lack the property to interact with C1, while IgM and IgG do so, effectively. Only one molecule of pentameric IgM is required to bind C1 while two of monomeric IgG are necessary to form a potentially cytolytic site.

The following is the schematic diagram of classic complement activation which occurs on the membrane:

$$AgAb + C1q \xrightarrow{Ca^{++}} AgAb\overline{C1q}$$

$$\overline{C1q} \longrightarrow \overline{C1r} \longrightarrow \overline{C1s} \text{ (C4, 2 convertase)}$$

$$\overline{C1s} + C4 \longrightarrow \overline{C4a} + \overline{C4b}$$

$$\overline{C1s} + C2 \longrightarrow \overline{C2a} + \overline{C2b}$$

$$\overline{C4b2a} \text{ (C3 convertase)} + C3 \longrightarrow \overline{C3a} + \overline{C3b}$$

$$\overline{C4b2a3b} \text{ (C5 convertase)} + C5 \longrightarrow \overline{C5a} + \overline{C5b}$$

$$\overline{C5b} + C6, C7 \longrightarrow \overline{C567}$$

$$\overline{C567} + C8, C9 \longrightarrow \overline{C5b - 9}$$
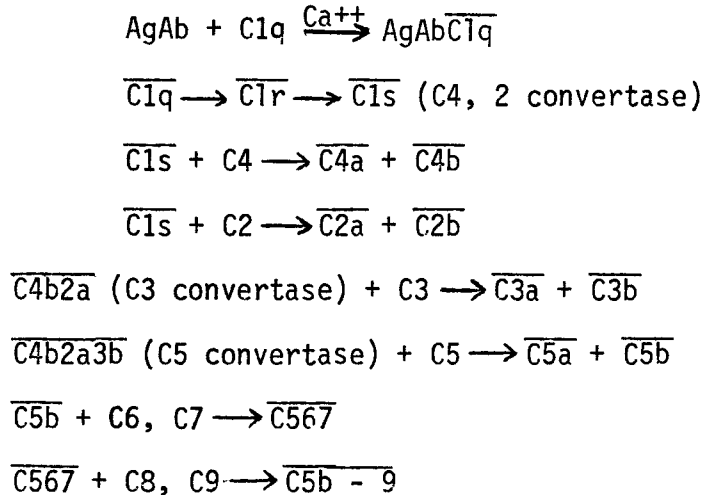
Final assembly:

$$\overline{C142356789}$$

——— : active form

2) Alternate pathway

The alternate pathway of complement activation bypasses the need
for antigen-antibody complex activation. C3 is cleaved by a serum pro-
tein or a system of proteins known as properidin. Properidin may be
activated by endotoxins and lipopolysaccharides, sulfate dextran, Ag-
gregated IgA. The properidin system is a nonimmune alternative mechanism
of defense Anderson and Kissane,(1977).

The properidin system is activated as follows:

Lipopolysaccharide, zymosan, aggregated IgG, IgA, IgE,

Plasmin, Tripsin + C3 $\longrightarrow$ $\overline{C3a}$, $\overline{C3b}$

$\overline{C3b}$ + C5 (Lysosmal enzyme Tripsin) $\longrightarrow$ $\overline{C5a}$ + $\overline{C5b}$

$\overline{C5b}$ + C6, C7 $\longrightarrow$ $\overline{C567}$

$\overline{C567}$ + C8, C9 $\longrightarrow$ $\overline{C5b - 9}$

Final assembly:

$\overline{C3b,56789}$

$\overline{}$: active form

According to Schenkein and Genco (1977) the alternate pathway of
complement activation occurs in the periodontal pocket as demonstrated
by conversion of C3 proactivator (factor B) to C3 activator Bb. C3b
can also be provided by the classic pathway and from there the alter-
nate pathway could be activated.

Ward (1972) reviewed the biologic activities of complement. They are listed as follows:

1. Inflammatory mediators

    A. Vascular permeability factors

        C3a   the anaphylatoxins: contract smooth muscle

        C5a   direct effect on blood vessels

                                         and/or releae of histamine

                                       from mast cells

    B. Leukoattractants

        C3a   attract polymorphonuclear

        C5a   leukocytes, eosinophils,

        C567   monocytes

2. Promotion of Phagocytosis - C3b, C5

3. Coagulation Modifying Activity

    A. Promotion of blood coagulation - C6

    B. Promotion of clot lysis - C3, C4 by plasmogen proactivation   plasmin

4. Viral Neutralization - C1, C4, and C1, 4, 2, 3

5. Cytotoxic Activity - C1-9

6. Inactivation of Bacterial Lipopolysaccharide - C5, C6

A variety of antigens have been shown to induce inflammation upon repeated application to the periodontium of several animal species.

Thonard and Dalbow (1965) challenged gingival tissues of conventionally reared rats and guinea pigs with whole sheep erythrocytes. The animals received biweekly injections for 3 weeks in the papilla between

the maxillary incisors. Antibody forming cells were found in approxi-
mately 50% of the inoculated gingivae. Circulating hemolysins were
found in the sera of all rats and over 80% of the guinea pigs. They
concluded antibody production associated with indigenous cells can take
place in suitably stimulated oral mucosa.

Rizzo and Mitchell (1966) exposed rabbits to protein antigen by
triweekly insertion of egg albumin pellets into labial gingival pockets of
mandibular incisors. Egg albumin treated rabbits developed serum anti-
body titers against egg albumin while saline treated control rabbits did
not. The gingival lamina propria exhibited a moderately severe chronic
inflammatory reaction consisting mainly of plasma cells and lymphocytes.
Their findings supported the concept that gingival plasmacytosis of
chronic periodontal disease was brought about by local absorption of
bacterial antigens. The results indicated antigen was absorbed into the
gingiva and caused both a local and systemic immunologic response.

Antibody was localized by electron microscopy within differentiat-
ing and mature plasma cells in the spleens of hyperimmunized rabbits
according to Leduc, Avrameas and Bouteille (1968). Horseradish peroxi-
dase was used as the antigen. Intracellular antibody to peroxidase was
revealed in glutaraldehyde-fixed tissue by coupling it with its antigen
and then revealing the sites of peroxidase activity cytochemically.

Antibody first appeared in the perinuclear space of hemocytoblasts
where it persisted through differentiation into immature plasma cells,
but disappeared from this site in mature plasma cells. However, anti-

body was present in the lamellar portion of the Golgi apparatus in all
phases of plasmacytic differentiations. Mature plasma cells exhibited
two types of antibody distribution: a concentration into large spherical
intracisternal granules or an overflowing into all parts of the cytoplasm.

Many investigators have used immunohistochemical techniques in
studying human inflammatory periodontal disease.

Brandtzaeg and Krause (1965) exposed sections of alcohol-fixed
fresh frozen gingival biopsies to specific labeled antisera. The speci-
mens were obtained from 8 subjects with a clinically healthy periodont-
ium and 21 subjects with varying degrees of periodontal inflammation.
Using the direct immunofluorescence technique, an intense fluorescence
was noted in severely inflamed specimens. The most striking difference
demonstrated between clinically healthy and inflamed gingiva was the
frequent marked increase in the number of plasma cells containing IgA
in diseased tissues. Fibrous tissue, which fluoresced the weakest of all
tissue components, was reduced. The number of blood vessels, which stain-
ed brightly, was increased. However, in the squamous epithelium, inter-
cellular staining was more common in the prickle cell layer. The stain-
ing increased towards the epithelial surface and appeared to be associat-
ed with an increase in cellular separation. A band of staining occurred
beneath the parakeratotic layer indicating a decrease of permeability.

Immunoflorescence tracings by Brandtzaeg (1966) indicate that IgG
and IgA permeate the connective tissue ground substance and further dif-
fuse through both the oral epithelum and sulcular or crevicular epithelium

of the gingiva. They pass the epithelium by an intracellular and inter-
cellular routes, and, especially, intercellular when inflammation is
marked. The extravascular distribution of serum proteins is highly de-
pendent upon concentration and molecular size: the majority of IgM
(70%) is located in the intravascular space while the majority of IgG
and IgA (52%-58%) is distributed extravascularly. Immunofluorescence
revealed large concentrations of IgG, much less IgA and very little IgM
contained within the gingival connective tissue.

Schneider, Toto, and Gargiulo (1966) demonstrated a local defense
mechanism of the gingival tissue to bacteria present in the adjacent
sulcus or pocket by identifying antigen-antibody reactions with fluore-
scent antibodies. Gingival biopsies and bacterial samples were taken
from 18 adult males whose periodontal conditions ranged from marginal
gingivitis to periodontitis. Frozen sections were reacted with specific
fluorescein conjugated antibody to show the presence of globulins in
the tissue. Perivascular connective tissue, collagenous fiber bundles,
basement membrane, intercellular spaces in the epithelium, and within
the cytoplasm of plasma cells. Bacteria, stained with a contrasting
fluorescent dye, Rhodamine B, were incubated with serial sections and
the sections were reacted with fluorescein labeled antibody. An attrac-
tion of bacteria to the gingival tissue was always shown in areas of glob-
ulin concentration.

Using immunoelectrophoresis, Brandtzaeg (1965) showed that IgG,
IgA, IgM, albumin and fibrinogen were present within gingival pocket

fluid in concentrations comparable to plasma. IgG, IgA, and IgM were pre-
sent in a ratio of 12:4:1. In this study, gingival pocket fluid, serum,
and saliva were collected from 4 subjects exhibiting periodontal condi-
tions. Only IgG, IgA and albumin were detected in whole saliva, and
their concentrations were lower than in serum. The ratio of IgG: IgA
was 1:1 in saliva as compared to 8:1 in serum and gingival fluid. He sug-
gested plasma was the principal source of the five proteins detected in
gingival tissue and pocket fluid. However, IgA and IgG may originate in
part from plasma cells in the gingiva. As the IgA is elevated in a
higher ratio to IgG in sulcus fluid as compared to that in serum.

Results of a study by Nisengard and Beutner (1970) showed that the
sera of 9 humans had IgG directed against bacteria from the gingival sul-
cus. The sera of 70 humans had antibodies to Actinomyces. Many individ-
uals from this group were allergic or hypersensitive to actinomyces.
Moreover, the levels of antibody to Actinomycetes and percentage of aller-
gic individuals increased with the severity of gingival inflammation.

Organisms are present in periodontal plaque in extremely high con-
centrations, approximately 10^{11} organisms per mg in direct contact with
the tissues. In spite of this, they do not seem to invade the tissues
to any appreciable degree. Rizzo (1970) found topically applied anti-
gens did not seem to be capable of penetrating an intact gingival sulcus
barrier.

Simon, Goldman, Ruben and Baker (1970) showed the quantity of endo-
toxin found in the gingival exudate of 39 patients was correlated at the

1% level with the degree of clinical inflammation. Clinical signs of gingival inflammation became manifested just after a shift from a predominance of Gram-positive flora to a Gram-negative one in bacterial plaque. Gram-negative organisms are capable of liberating endotoxin upon their destruction. The authors related their results to those of Loe, Theilade, and Jensen (1965) and Theilade, Wright, Jensen and Loe (1966).

Toto, Gargiulo and Kwan (1970) conducted an immunofluorescence study of 25 biopsy specimens of chronic gingivitis. The intact epithelium contained intercellular and intracytoplasmic immunoglobulin. They suggested that the epithelium could serve in defense against antigenic substances found in the gingiva.

Platt, Crosby, and Dalbow (1970) investigated gingival tissues from patients with varying stages of periodontal disease. An immunofluorescence study revealed all immunoglobulin components were present. Their relative concentrations appeared to be related to the stage of pathology. IgM producing plasma cells were predominant in acute gingivitis, though cells with IgG were abundant. Sections from severe periodontitis showed numerous IgM, many IgA and few IgG containing plasma cells and fluorescing endothelial cells of blood vessels. Also, the intense fluorescence of residual plaque adhering to gingiva indicated immunoglobulins combined with bacteria in the gingival region.

Berglund (1971) demonstrated immune complexes were formed by the reaction of gingival immunoglobulins obtained from thin slices of gingi-

val tissues and from spent media of gingival organ cultures with antigens from microorganisms of dental plaque containing Fusobacterium, Veilonella, and Escherichia coli. Immunoglobulins from diseased human gingiva apparently originated also from cells in the inflamed tissue as well as from serum, since larger amounts of antibody activity were observed over some gingival specimens than was expected from the magnitude of serum titers.

Indirect immunofluorescence staining by Nisengard, Beutner and Gauto (1971) revealed cells containing IgE in inflamed human gingival connective tissue. Morphologically the mononuclear cells resembled plasma cells. The number of IgE containing cells appeared to be related to the severity of inflammation. Microorganism from subgingival debris but not supragingival debris were coated with IgE.

Mayron and Loiselle (1973) treated tissue sections obtained from patients with periodontal disease with fluorescein conjugated Streptococcus mitis, Streptococcus salivarius, and Neisseria catarrhalis. Microscopic examination revealed the presence of fluorescence primarily in the epithelium; theory suggesting the presence of antibodies to these microorganisms. Antigen was located in the subkeratin layers of the epithelium.

Brandtzaeg (1973) considered the number of activated B cells (plasma cells) in inflammatory periodontal lesions to be impressive in view of the great dominance of T cells (90%) in the pool of peripheral blood lymphocytes. He noted that infiltrates within established periodontitis lesions in man are dominated by IgG contains plasma cells. As IgG has

great potency for complement activation, the consequences of the immune
response may be determined by a balance in the local synthesis of IgG
and IgA antibodies. Brandtzaeg (1973) states that a shift in favor of
complement fixing IgG over low complement fixing IgA may disturb immune
homeostasis with aggravation and perpetuation of inflammatory process.

Using gel diffusion techniques, Genco, Mashimo, Krygier and Elli-
son (1974) found that a large percentage of human sera contain precipita-
ting antibodies directed to antigens found in extracts of sonicated Lep-
totrichia buccalis.

They treated flame-fixed L. buccalis smears with a series of di-
lutions from subjects' sera and then reacted such smears with fluorescein
labeled goat antihuman IgG, IgM, IgA or IgE. Antileptotrichial antibod-
ies of either complement fixing IgG and/or IgM were detected in all of
the adult sera tested. This suggests that the host is sensitized to
dental plaque bacteria. The levels of IgG and IgM antibodies to L.
buccalis in 5 edentulous adults were comparable to those found in 14
dentulous adults, suggesting that the persistence of L. buccalis in the
gingival sulcus is not necessary for the maintenance of adult serum anti-
body levels.

Immunofluorescent localization studies carried out on human gingiva
showed the presence of IgA, IgG, IgM, C3, albumin and transferrin in the
connective tissues and in the sulcular epithelium.

Marttala, Toto, and Gargiulo (1974) demonstrated that antibodies
to specific bacteria were extractable locally in the gingiva. Three pedi-

gree strains of lyophilized Actinomyces (israelii, naeslundii, and viscosus) and one of Streptococcus mutans were prepared as smears on slides and reacted with gingival tissue extracts obtained from 12 patients with chronic periodontitis. The treated smears were reacted with goat anti-human antiserum to IgG, IgA, and IgM, conjugated to fluorescein isothiocyanate. Fluorescence was predominantly positive to all test organisms in all subjects.

Byers, Toto, and Gargiulo (1975) using low-level diffusion plates, assayed resected inflamed gingival tissue obtained from 16 periodontal patients and a pooled sample of noninflamed gingiva from 5 patients. Marked increases in levels of IgG, IgA, and IgM were seen in inflamed tissues. These were attributed to the greater antigenic stimulus present in gingival pockets. The IgA/IgG and IgM/IgG ratios in inflamed gingiva were substantially different than those in normal serum. This study supported the concept of local antibody production in inflamed gingiva.

The ratio of IgA/IgG was similar in inflamed and normal gingivae. This may be the result of the body's homeostatic mechanism regulating levels of blood and tissue immunoglobulins. IgM was present in 6 of 16 inflamed specimens. IgM may not be a consistent feature of chronically inflamed gingiva.

Attstrom, Laurel, Lahsson, and Sjoholm (1975) conducted an electro-immunoassay of supernatants from gingival crevicular material. Higher concentrations of C3 and C4 were demonstrated in samples from chronically inflamed gingiva when compared to those from healthy gingiva. The

concentration of C4 in samples from inflamed gingiva was significantly lower when related to plasma levels. This finding might indicate this factor had been consumed or was present in an altered form. C5 could be demonstrated in material only from inflamed gingiva. C3 proactivator was present in material from inflamed gingiva in the converted form. Analysis of C3 in samples from inflamed gingiva using crossed immuno-electrophoresis showed C3 was converted to degraded fragments in the samples. This would appear to indicate that the complement system may be activated in gingival crevice material from inflamed gingiva.

Courts, Boackle, Fudenberg and Silverman (1977) collected crevicular fluid from patients with periodontitis and tested it for the presence of a functional complement system. Functional C1 and whole complement activities were rapidly inactivated by dental plaque. Results suggested complement was responsible for the hemolytic activity of gingival crevicular fluid.

Taichman, Tsai, Baehni, Toller and McArthur (1977) demonstrated that human peripheral blood polymorphonuclear leukocytes actively released lysosomal constituents upon in vitro exposure to either viable or irradiated, supragingival or subgingival dental plaque. Fresh sera amplified the release reactions. Modulation of polymorphonuclear leukocyte lysosomal release may be modified by complement components and/or antibodies to plaque bacteria. Complement factor C5a can trigger lysosome release by neutorphils while antibodies facilitate phagocytosis by opsonization.

Schenkein and Genco (1977) determined the concentrations of select-
ed proteins from gingival crevicular fluid and serum by the single radial
immunodiffusion method. Gingival crevicular fluid from severely inflamed
periodontal tissue represented only 15 to 30 percent dilution of serum
IgG, IgA, IgM. However, IgG showed a tendency towards higher gingival
crevicular fluid concentration than IgA and IgM. They felt this was due,
in part, to the production of the predominant IgG containing plasma cells
(70%-80%) located in the chronically inflamed gingiva.

A marked decrease in C3 levels was found in most gingival crevicu-
lar fluid samples; whereas, a marked decrease of C4 levels was found in
several crevicular fluid samples. Therefore, it was suggested that comple-
ment may be activated during periodontal inflammation. The finding of
the C3 activation products C3c and C3d indicated C3 had been degraded.
Possible mechanisms suggested for C3 degradation included an antigen-
antibody reaction promoting complement activation by the classical path-
way, a nonspecific direct activation by the alternate pathway, and pro-
teolysis due to enzymes in the gingival sulcus. Quantitatively, C3d
levels were comparable to levels attained consequent to immune complex
and tryptic activation of human serum.

Altman, Chassy and Mackler (1975) found that lymphocytes, which
were activated by immune complex binding through membrane complement
(C3) receptors or Fc receptors and with an affinity for the Fc part of
IgG, produced pharmacologically active lymphokines equivalent to those
induced by antigenic stimulation.

Mackler, Frostad, Robertson and Levy (1977) studied gingival specimens from patients with varying degrees of periodontal disease with hematoxylin-eosin and immunofluorescence staining. They found that the normal gingiva contained few lymphocytes and plasma cells. Ninety-four percent of the cellular infiltrate present within the lamina propria at the epithelium junction in mild gingivitis were lymphocytes lacking membrane-associated immunoglobulins. Tissues associated with periodontitis contained significant numbers of immunoglobulin bearing lymphocytes and plasma cells. In lymphocytes, their immunoglobulin distribution was 78% IgG, 9% IgM and 4% IgA. The plasma cells seen in these tissues had the following distribution: 67% IgG, 24% IgM and 8% IgA. Distinctly differnet cellular infiltrates were seen associated with the two stages of inflammatory periodontal disease.

Toto, Lin and Gargiulo (1978) demonstrated the presence of immunoglobulin and C3 by means of the direct immunofluorescence technique in sections of frozen gingival specimens obtained from 20 patients with gingivitis and chronic periodontitis. The presence of IgG was seen in 20 cases, IgM in 10 cases and C3 in 7 cases. Fluorescence was localized mainly in the lamina propria. Examination of the basement membrane at high magnification showed an irregular and diffuse fluorescence in all positive reacting sections. They concluded that the presence of IgG, IgM and C3 suggested an antigen-antibody response binding and activating complement. Similar results were obtained by Suzuki, Gargiulo and Toto (1979).

The immunoglobulins and complement components in crevicular fluid are associated with bacterial plaque as shown by Gilmour and Nisengard (1974), Gerenscer and Slack (1976), Marttala, Toto and Gargiulo (1974), Nisengard and Jarrett (1976) and Loe, Thielade and Jensen (1965).

The comparison of normal gingiva with inflamed gingiva can be performed by utilizing specific antisera to the human immunoglobulins IgG, IgM, IgA and C3 conjugated to horseradish peroxidase. This procedure provides for immunoglobulins and complement localization in plasma cells and extracellular tissues similar to the immunofluorescent method as shown by Toto, Ruiz and Gargiulo (1981).

The peroxidase antiperoxidase technique (PAP) as shown by Sternberger (1979), is a three stage method and does not involve amplification at each step as is employed in the immunofluorescent method reported by Nakane (1966), Davey (1970), and Taylor (1974). A primary antibody of goat antihuman immunoglobulin is applied to each section. This is followed by a reaction with a second antibody such as rabbit antigoat IgG. This second antibody serves as a link between the primary antibody and goat antiperoxidase antibody conjugated to horseradish peroxidase; 3,3' diaminobenzidine (DAB) then is added as a substrate, which by an oxidative polymerization is localized as a brown granular precipitate, in the tissue antigen.

Evidence for the binding of antibody to plaque bacteria was shown in a recent study by Newman (1980) who used 51 different gingival margin plaques obtained from children's premolar teeth extracted for orthodon-

tic reasons. The plaque bacteria were subjected to transmission electron and immunoelectron microscopy using the peroxidase antiperoxidase technique (PAP) to determine how subcontact area plaque survives the host response and enters the gingival crevice. Newman found that both IgG and polymorphonuclear leukocytes were found preferentially at the actual apical border of plaque especially in embrasures. Some polymorphonuclear leukocytes showed evidence of phagocytosis. Polymorphonuclear leukocytes in contact with plaque generally showed lysosome and glycogen loss and occasional phagocytosis. The presence of IgG was not coincidental with bacteriolysis. Labelling was limited to a zone at the apical border of the plaque corresponding approximately to that area occupied by polymorphonuclear leukocytes when present. There were instances, especially in relation to subcontact area plaque, where IgG-coated organisms were observed in the absence of polymorphonuclear leukocytes. Positive PAP labelling usually coated the organisms. Occasionally, bacterial microcolonies were enveloped in an IgG positive matrix. The principal bacteria at the plaque border were cocci. The cocci organism frequently IgG-positive, whether located inside or outside polymorphonuclear leukocytes.

Immunofluorescent studies conducted by Nisengard and Jarrett (1976), revealed that 2 of 3 patients with gingivitis and all patients with periodontitis (20) had crevicular bacteria that were coated in vivo with immunoglobulins IgG, IgA, IgM, and C3. However, the observation both of IgG, IgM and C3 fragments on the surface of microorganisms may represent

either immune comlex formation between antibody and the antigen on the bacteria or it may only represent nonspecific adsorption of the immunoglobulins and C3 on the cell surface.

In order to determine whether or not the presence of immunoglobulins and C3 on bacteria represent an antigen-antibody reaction or an immune complex it is essential to demonstrate the presence of C1q and C4 which are involved in the classic pathway of complement activation.

Currently the development of a variety of new procedures utilizing C1q have presented for the evidence of immune complex formation. Since C1q has been successfully used by Nydegger, Lambert, Gerber and Miescher (1974) to assay immune complex formation, it seems probable that C1q activity may represent the classic pathway of complement activation. The reaction of C1q with 1g aggregants can be demonstrated with the following techniques.

1) Gel diffusion:

Specimens to be tested are diffused against a solution C1q at 200 ug/ml. Plates are incubated at 22 C for 48 h. Precipitin lines obtained can then be further intensified by incubation at 0 C for up to 72 h. Pathological sera are initially screened undiluted. Positive specimens are then tested in two fold dilution to 1:16. A normal serum and positive control are routinely run with wach batch of unknowns.

2) Radioimmunoassay methods

a) Radiolabeled C1q binding test utilizing polyethylene glycol: This method is based on two phenomena, the binding of C1q by anti-

gen-antibody complexes and precipitation of antigen-antibody complexes
by polyethylene glycol. Creighton, Lambert and Miescher (1973). The
test specimens are heated at 56 C for 30 min, and 200 uliters is incubat-
ed with Clq labeled with I^{125}. Polyethylene glycol is then added to a
final concentration of 2.5% to precipitate gamma globulin complexes. Trace
amounts of Clq bound by complexes can then be quantitated by measuring the
radioactivity in the precipitate.

b) Clq deviation test - Sobel, Bokish and Müllen-Eberhard
(1975).

This method is based on the inhibition by immune complexes of ra-
diolabeled Clq uptake by sheep erythrocytes coated with antierythrocyte
IgG. A 50-uliter amount of serum is diluted fivefold so that the con-
ductance of the final solution is 7 mmho/cm. The solution is then heat-
ed at 56 C for 30 min, (^{125}I) Clq is added, and the mixture is incubated
for 15 min. at 20 C. Sensitized erythrocytes are then added, and another
15-min incubation at 20 C is performed. Cells are then separated from
the fluid phase in 40% sucrose. The uptake of (^{125}I) Clq by cells with
and without the test specimen is determined, and the percent inhibition
is calculated.

As shown by Torabinejad and Kettering (1979), immune complexes
have also been demonstrated within periapical lesions by the anticomple-
ment fluorescence technique (ACIF).

Since diseased periodontal tissue contains immunoglobulins and
antigens from oral bacteria, it seems likely that immune complexes do

form in these tissues. Nevertheless, neither specific immune complexes, nor specific antigens, nor even specific bacteria antibodies have been unequivocally identified in spontaneously diseased human tissues according to Rizzo and Mergenhagen (1977).

The purpose of this study is to microscopically examine human plaque bacteria for the presence of complement C1q and C4 using specific anti-human C1q and C4 antisera bound to immunoperoxidase labeled reagent and to determine whether or not C1q and C4 are present. Should the evidence show that C1q and C4 are present it will be supportive evidence that an immune complex does form between an antibody and bacterial antigens in plaque contained within the gingival sulcus.

CHAPTER III

MATERIALS AND METHODS

Seventeen patients with moderate periodontitis, Type III, were se-
lected for this study. They were chosen according to the ADA classifi-
cation (1976) which consists of moderate to severe bone loss, abnormal
bone crest topography and slight mobility. Dental plaque was removed
from the gingival sulcus of 5-6 mm pockets employing a sterile curette.
The plaque substance was used to prepare seven separate smears on glass
slides approximately 25 mm x 75 mm in size. The slides of each patient
were separately treated as follows:

Gram stain slide: The Gram stain method, Bartholomew's Modifica-
tion (1962), was used to identify the morphologic characteristics of bac-
teria collected in the smear taken from the dental plaque.

Technique: The plaque sample was collected with a periodontal
curette, smeared into the center of a glass microscope slide and fixed
by three passes through a flame. The following staining procedure was
performed on one slide per patient:

    1) flood slide with crystal viatel for 1 minute

    2) wash 5 seconds in running water

    3) Weigert's iodine, 2 changes, 5 seconds and 1 minute

    4) wash 5 seconds in running water

5) decalorize in 3 changes N-propanol 1 minute each

6) wash 5 seconds in running water

7) 2 changes of sefranin, 5 seconds and 1 minute

8) wash 5 seconds, dry and examine

The results were interpreted as follows: Blue Black indicated gram positive organisms; red indicated gram negative organisms.

Immunoperoxidase stain for Clq, C4, IgG, IgM modification of Ludwig L. Sternberger technique, (1979): Glass slides were cleaned with H_2SO_4 - dichromate solution then washed in distilled water, followed by an alcohol rinse and then dried. The cleaned slides were dipped in a solution composed of 0.5% gelatin + .01% chrome alum and dried. This treatment facilitated adherence of the plaque smear to the slide.

The bacterial plaque smear was fixed with acetone 100% at 4°C overnight. Each smear was treated with 0.3% Hydrogen peroxide in 100% methanol for 30 minutes; this reacts and consumes any soluble endogenous peroxidase in the smear. Each smear was then rinsed in 70% ethanol and washed 3 times in distilled water.

In order to disclose antigens that may have been masked by the fixation, each smear was immersed in 25% sodium bisulfite for five minutes.

Between each of the following stages there were five minute washes or rinses in .01M P.B.S. (Phosphate buffer saline). The slide was flooded with 0.1 ml of 3% normal rabbit serum[1] in P.B.S. for 30 minutes at

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[1]Normal Rabbit Serum; Cappel Laboratories, Cochranville, PA 19330.

room temperature (23°C). Normal rabbit serum was used to react with non-specific immunoglobulin binding substances in the smear.

The slides were drained and blotted dry. One slide each was flooded for 30 minutes at room temperature (23°C) with 0.1 ml of one of the following monospecific antisera to human IgG,[2] IgM,[3] Clq,[4] and C4,[5] prepared in the goat. Antisera to IgG, Clq, C4 were used in a dilution of 1:1000 of the original concentrations (IgG 10.0 mg/ml), and the original IgM concentration of 3.5 mg/ml was diluted in a 1:500 solution. All original concentrations were diluted with P.B.S. containing 1% normal rabbit serum.

The smears next were flooded with 0.1 ml of rabbit anti-goat IgG[6] at dilutions of 1:50 in P.B.S., for 30 minutes at room temperature (23°C). This reaction links rabbit antibody to goat antibody.

The smears were then flooded with a labeled antibody consisting of 0.1 ml goat anti-peroxidase conjugated to horseradish peroxidase (PAP),[7] containing 1% normal rabbit serum and incubated at room temperature (23°C).

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[2]Goat Anti-human IgG; Cappel Laboratories, Cochranville, PA 19330.

[3]Goat Anti-human IgM; Cappel Laboratories, Cochranville, PA 19330.

[4]Anti-human Clq (goat), Biowane products, Whippany, New Jersey 07981.

[5]Anti-human C4 (goat), Biowane products, Whippany, New Jersey 07981.

[6]Rabbit Anti-goat IgG; Cappel Laboratories, Cochranville, PA 19330.

[7]Goat Peroxidase - anti-peroxidase (PAP); Cappel Laboratories, Cochranville, PA 19330.

The antibody complex formed in the smear was fixed by a graded series of acetones for one minute each at room temperature (23°C), in order to reduce the solubility of the immunoglobulins. The smears then were treated with the substrate 3,3' - Diaminobenzidine Hydrochloride (DAB) powder[8]. The preparation of the DAB solution is as follows: 44 mg% of DAB was added to amonium acetate buffer (.05 M) at ph 5.0; and dissolved by shaking in a dark bottle, and filtered just before using; 0.0075% H_2O_2 was then added.

The slides were placed into the DAB solution contained in a dark coplin jar for 2½ minutes at room temperature with continuous agitation; they were then rinsed in distilled water. Diamino-benzidine hydrochloride in the presence of H_2O_2 was oxidized by the peroxidase to a brown precipitant, oxidized polymerization, as described by Sternberger (1979).

The slides were counter stained by placing them in a solution of 1% methyl green in phosphate buffer ph 4.0 for 2 minutes, blotted, dehydrated in alcohol, cleaned in saline and mounted in permount.

Control slides of plaque smears (Clq, C4):
These slides containing plaque smears were treated with 1:100 Goat anti-human IgG[9] for 30 minutes at room temperature (23°C) before applying PAP, in order to block the linking antibody rabbit anti-goat IgG. The subsequent steps were the same as the experimental slides.

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[8]3,3' Diaminobenzidine Hydrochloride, powder, V.T. Baker Chemical

[9]Goat Anti-human IgG; Cappel Laboratories, Cochranville, PA 19330

Interpretation:

The slides were examined under the AO 10 microstar light microscope in order to detect deposits of a brown oxidative precipitate DAB, located on the surface of the microorganisms. A brown precipitate localized the antigens Clq, C4, IgG, or IgM.

CHAPTER IV

RESULTS

Morphologic Examination: Gram stain

The histomorphologic examination of bacteria within periodontal pockets demonstrated both gram positive and gram negative organisms.

The standard smear showed the characteristics of bacterial plaque specifically gram positive thread forms, actinomyces, bacilli and coccal forms, and gram negative rods. Polymorphonuclear leukocytes were commonly present. (See Figure 1)

Immunoperoxidase PAP reaction:

The plaque samples taken from seventeen patients with periodontal disease showed a positive immunoperoxidase staining reaction for IgG, IgM, Clq, and C4 (Table 1).

This positive reaction was characterized by a brown precipitate formed into aggregates of bacteria which outlined the bacterial cell wall. (See Figure 2)

Control reaction: Clq and C4

No reactions occurred in any specimen that was inhibited or blocked with goat anti-human IgG. The positive brown precipitate was absent in the seventeen control sections. (See Figure 3)

Light microscopy sometimes showed epithelial cells with attached microorganisms. These epithelial cells were easily recognized because of the PAP positive brown aggregates which surrounded the attached microorganisms. (See Figure 4)

TABLE I

IMMUNOPEROXIDASE STAINING
FOR IMMUNOGLOBULINS AND COMPLEMENT ON DENTAL PLAQUE

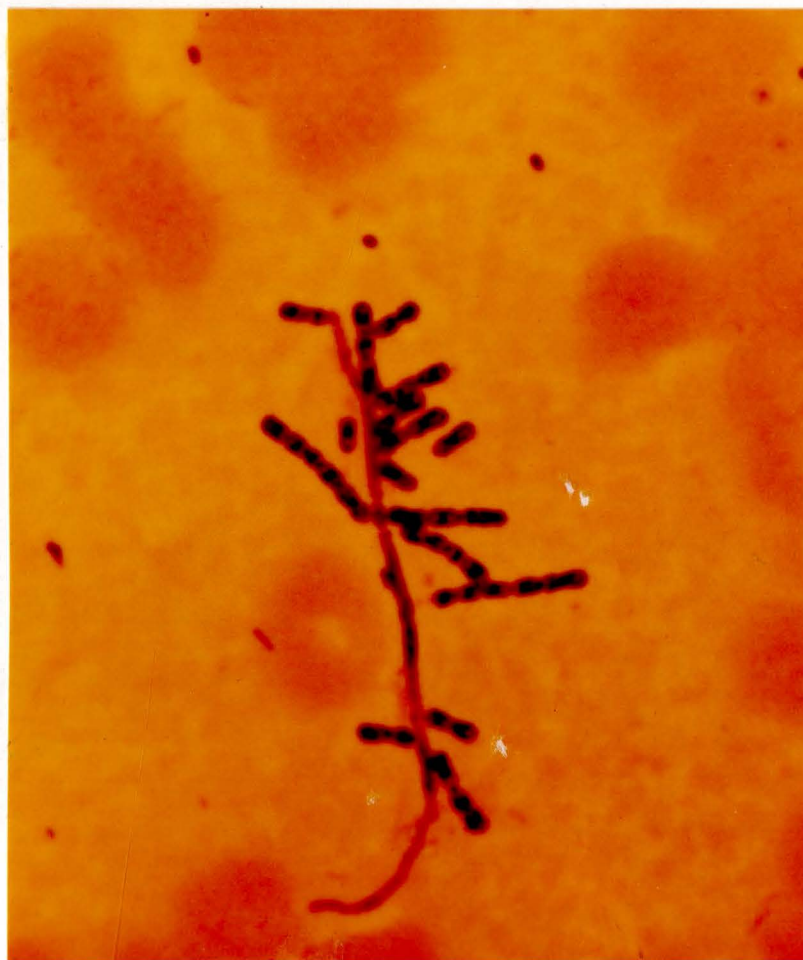|	IgG	IgM	C1q	C4	C1q Control	C4 Control
PATIENTS						
1	+	+	+	+	-	-
2	+	+	+	+	-	-
3	+	+	+	+	-	-
4	+	+	+	+	-	-
5	+	+	+	+	-	-
6	+	+	+	+	-	-
7	+	+	+	+	-	-
8	+	+	+	+	-	-
9	+	+	+	+	-	-
10	+	+	+	+	-	-
11	+	+	+	+	-	-
12	+	+	+	+	-	-
13	+	+	+	+	-	-
14	+	+	+	+	-	-
15	+	+	+	+	-	-
16	+	+	+	+	-	-
17	+	+	+	+	-	-

FIG. 1. Gram positive Cocobacillus apparently
complexed to Actinomycetes form.
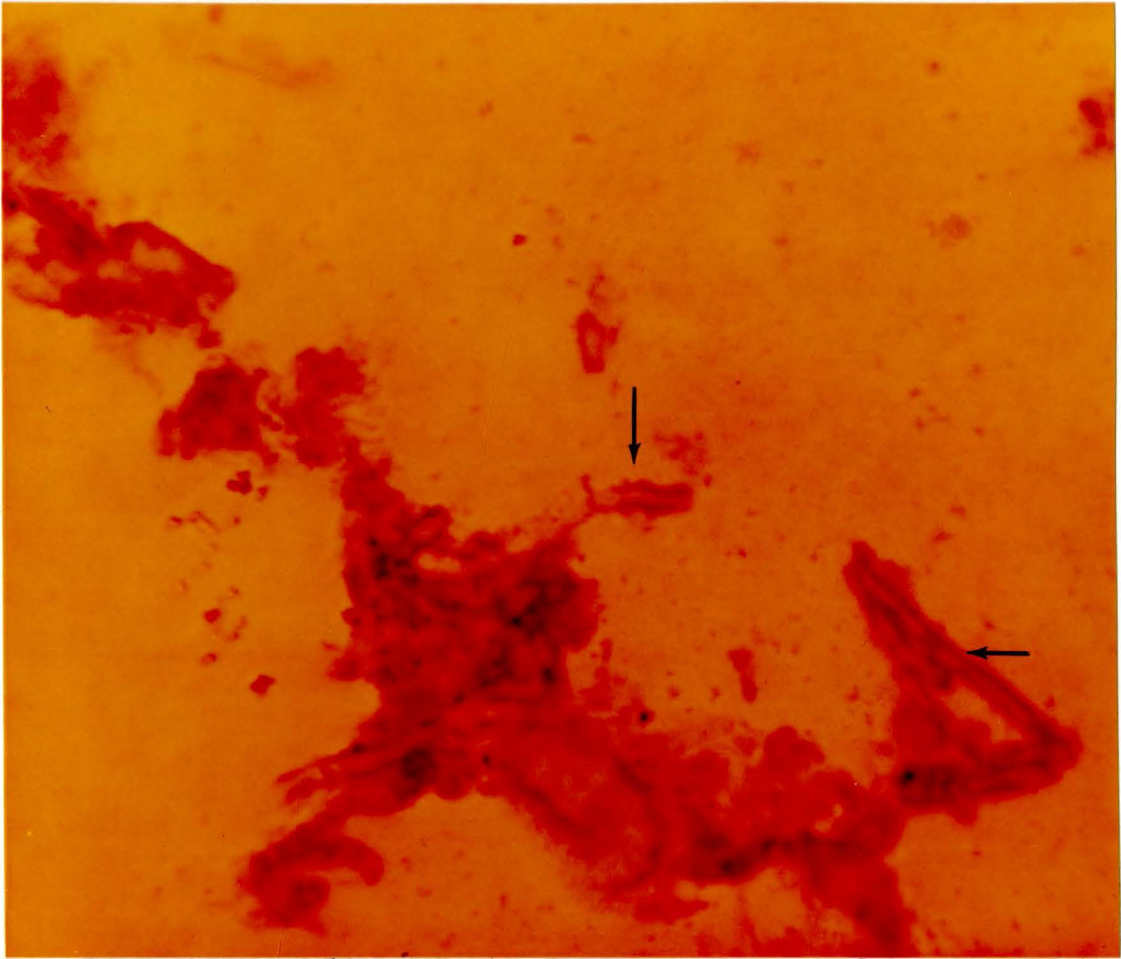Gram stain. 1000X magnification.

FIG. 2. C1q deposited on the surfaces of plaque
bacteria appear as adherent granules in
this Actinomyces like microorganism.
Goat Peroxidase-antiperoxidase stain.
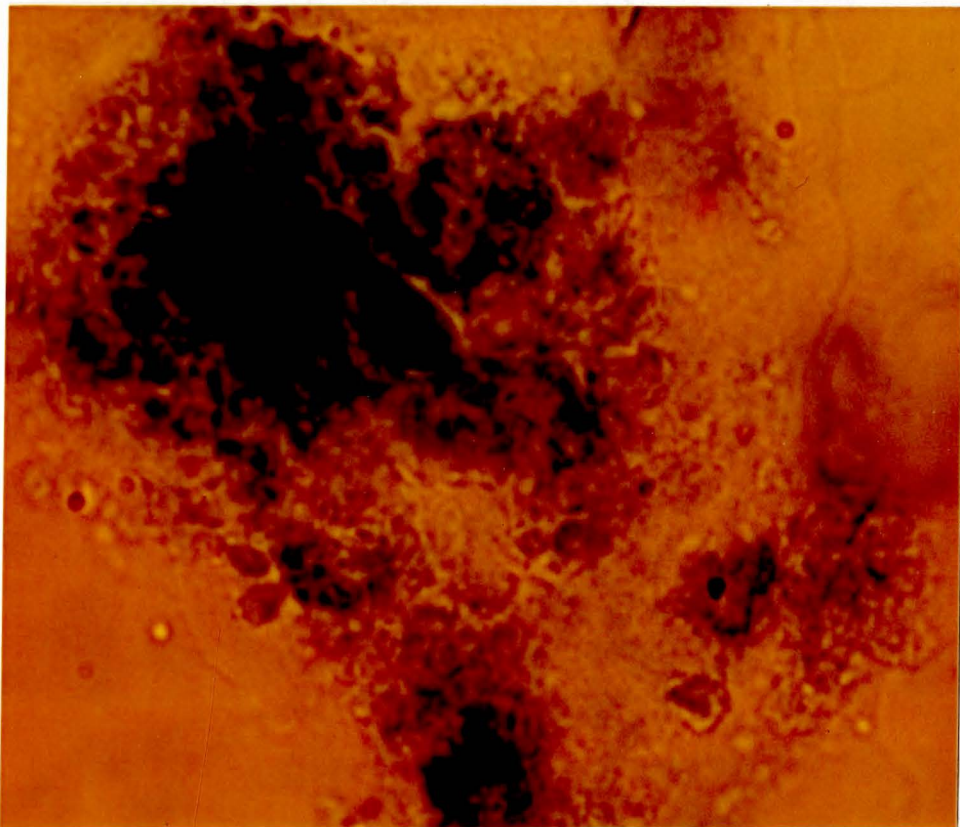1000X magnification

FIG. 3.   Negative control (blocking).
The microorganisms lack brown
staining and appear refractle.
Goat Peroxidase-antiperoxidase
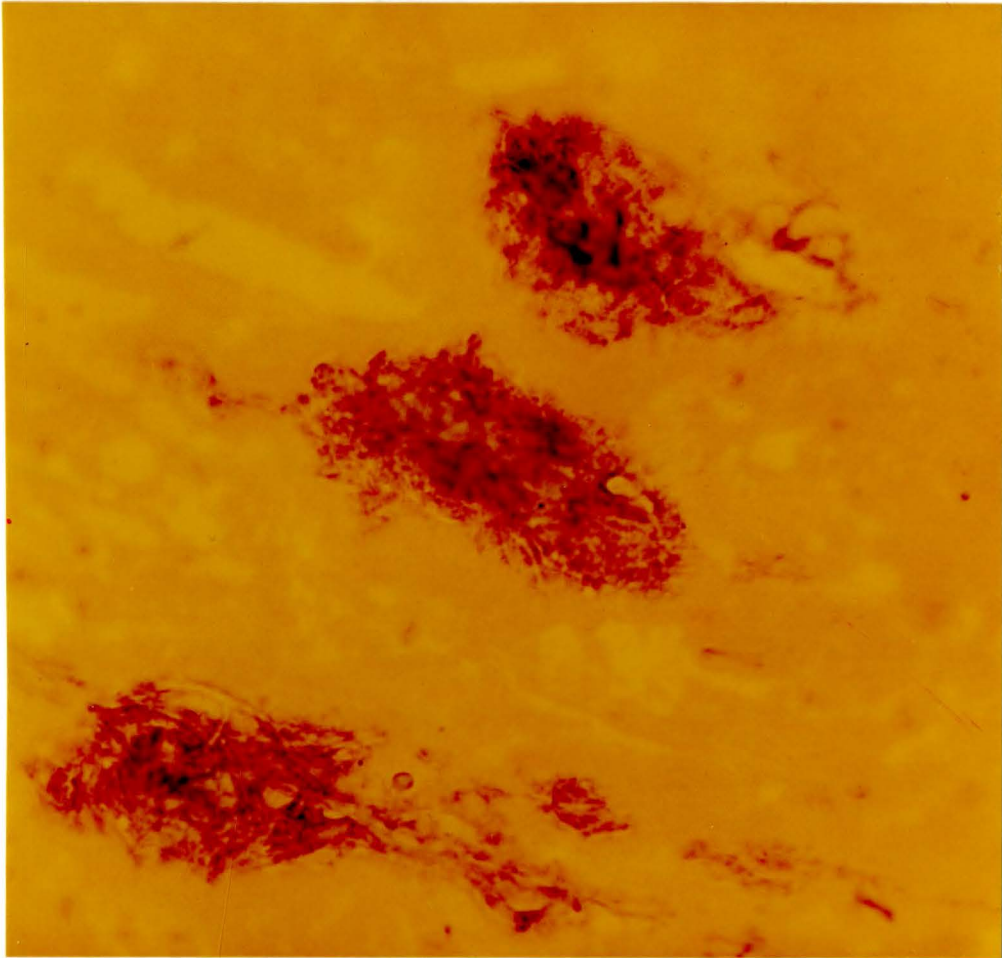stain, and methyl green.
1000X magnification.

FIG. 4.   C4 deposited on the surface of
plaque bacteria appear as fine
adherent brown granules. The
organisms are adherent to the
epithelial cells.
Goat Peroxidase-antiperoxidase
stain, and methyl green.
450X magnification.

CHAPTER V

DISCUSSION

The finding that Gram-positive and Gram-negative bacteria are present within the plaque of patients with periodontal disease substantiates the studies by Socransky (1970), Kelstrup and Theilade (1974).

This study concerns with the findings of Platt, Crosby, and Dalbow (1970), Mayron and Loiselle (1973) that the immunoglobulins IgG and IgM, are associated with plaque bacteria thereby giving evidence for the immune complex formation mentioned by Berglund (1971). Unlike the previous studies which were performed within gingival tissues, this study substantiates the presence of immunoglobulins on plaque bacteria.

Genco, Mashimo, Krygier and Ellison (1974) reported specific human sera antibody with the plaque antigen Leptotrichia bucalis and they suggested that the host was sensitized against the plaque bacteria. Marttala, Toto and Gargiulo (1974) showed specific immunoglobulins to Actinomyces (israelii, naeslundii, and viscosus), and Streptococcus to be contained within bacterial plaque. These studies support the finding contained within this paper in that antibody reacts with bacterial plaque antigens and can be dictated on Actinomyces, rods and cocci forms.

The literature has substantiated the use of the immunofluorescence technique for the demonstration of immunoglobulins and complement distribution in periodontitis.

40

This literature includes studies by Brandtzaeg and Krause (1965), Toto, Lin and Gargiulo (1978), Nisengard, Beutner and Gauto (1971), Nisengard and Jarrett (1976), Mackler, Frostad, Robertson and Levy (1977).

This study demonstrates that the immunoperoxidase technique is an effective procedure to show the presence and distribution of IgG, IgM, C1q and C4 on bacterial plaque organisms. Dorling, Johnson, Webb and Smith (1971) noted that the immunoperoxidase technique compares favorably with immunofluorescence. Horseradish peroxidase procedures have the following advantages over immunofluorescence:

1. They are applicable to routinely processed tissues;

2. A simple light microscope is required;

3. Preparations are permanently mounted;

4. Good morphological results are obtained;

5. The reaction product is not subject to change during examination.

Newman (1980) applied the PAP technique to electron microscopy and found IgG and IgM associated with dental plaque. Similar to Newman (1980), this light microscopy study shows plaque bacteria associated with IgG and IgM. It further evidences the presence of C1q and C4 with bacterial plaque, which to the authors knowledge has not been previously reported utilizing the peroxidases anti-peroxidase (PAP) technique under light microscopy.

The association of IgG, IgM, C1q and C4 with bacterial plaque is dictated by the positive oxidative polymerization of 3,3' Diaminobenzi-

dine which results in formation of a brown precipitate as described by Sternberger (1979).

The employing of a blocking step such as goat anti-human IgG preciding the PAP reaction results in a negative response. This negative PAP response indicates that the staining of Clq and C4 is not a technique artifact and therefore further verifies that the immunoperoxidase technique is as useful as the immunofluorescein technique.

Under light microscopy, brown PAP positive aggregants were seen surrounding microorganisms which were sometimes attached to epithelial cells. A possible explanation for aggregation of epithelial cells and bacteria is:

1. The presence of antibody intercellulary or on epithelium as demonstrated by Brandtzaeg (1966) with IgG.

2. The carbohydrites on the surface of bacteria have either a specific or nonspecific affinity to the carbohydrite on the surface of epithelial cell.

3. Immunoglobulins reacting with bacterial antigens and epithelial cells.

As previously stated in this paper, the presence of IgG, IgM, Clq and C4 are indicated with the immunoperoxidase technique. This presence of IgG, IgM, Clq and C4 in dental plaque suggests that antibody complexes, which have the potential to bind and activate complement, may form with bacterial antigens in periodontal disease.

It is known that IgG and IgM have complement receptor sites and can bind complement. Nydegger, Lambert, Gerber and Miescher (1974) used C1q to assay immune complex formation. It seems probable that C1q activity plus the presence of IgG, IgM, and C4 may indicate that complement is activated via the classic pathway in dental plaque.

Furthermore, the findings by Attstrom, Laurel, Lahsson and Sjoholm (1975) indicated that C4 is decreased in inflamed gingiva as compared to plasma levels. The results of this study would seem to indicate that this reduction is due to the consumption of C4, meaning that C4 is being activated during periodontal disease. Likewise, Courts, Boackle, Fudenberg and Silverman (1977) demonstrated that C1q was consumed within dental plaque.

The existance of immune complex formation between antibody and bacterial antigen is substantiated by the study of Nisengard and Jarrett (1976) who showed that IgG, IgM and C3 are present on the surface of bacterial plaque. The findings in this study of C1q and C4 on bacterial plaque, which are involved in the early steps of C3 activation, appears to support the occurance of C3 on bacteria as previously reported in the literature. C3 can be activated by C1q and C4 by the classic pathway, or by endotoxins, C3bB within the alternate pathway.

This study appears to reinforce the association of C3 as being activated within the classic pathway. It further suggests the occurance of an antigen-antibody reaction in periodontal disease.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Dental plaque samples were obtained from the gingival sulcus of seventeen patients with type III periodontal disease.

The plaque samples were fixed, prepared, and treated with the immunoperoxidase technique utilizing goat anti human IgG, IgM, Clq, and C4 bound to horseradish peroxidase (PAP-technique). A brown precipitate indicated a positive reaction. The Gram stain technique was used to idnetify and differentiate Gram positive and Gram negative organisms.

The conclusions are as follows:

1. Gram positive and Gram negative bacteria were associated with dental plaque.

2. The immunoperoxidase technique did react specifically with bacterial cell surfaces as evidenced by a brown precipitate associated with the cell membrane.

3. The positive immunoperoxidase reaction would appear to indicate the presence of IgG and IgM on the bacterial cell surface.

4. The presence of Clq and C4 was demonstrated on the cell membrane.

5. The finding of IgG, IgM, Clq, and C4 in bacterial plaque would seem to give supportive evidence for the classic pathway of complement activation.

6. This classic pathway of complement activation suggest the occurence of an immune or antigen-antibody reaction in periodontal disease.

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