Doctorate of Philosophy
Department of Operative Dentistry
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Pathogenic mechanisms in human carious pulpitis

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Contents

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This work is dedicated to the memory of my grandmother, Sarah Mabel Kibby, whose support of the varied talents of her family, and in particular this author, has been an inspiration.

Publications and presentations of work described in this thesis

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Dentinal microflora and pulp histopathology in human carious pulpitis.

J Dent Res. 67, 263.

Massey WL, Romberg D, Hunter N, Hume WR (1992)

The association of dentine microflora with tissue changes in human carious pulpitis.

Oral Microbiology and Immunology (in press).

Massey WL, Hunter N (1992)

A study of cell death in mitogenically-stimulated spleen cell populations.

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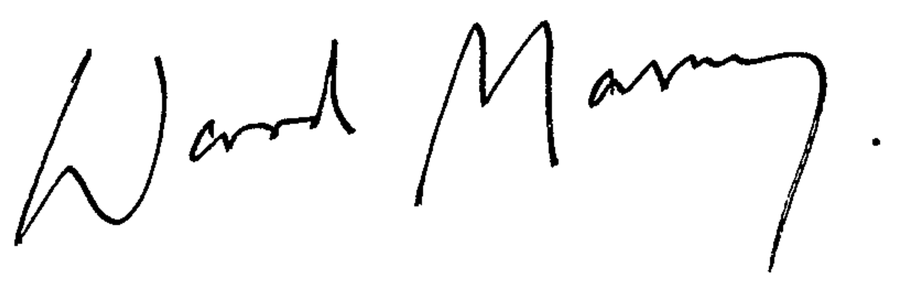
Massey W, Hunter N (1992)

Cytofluorographic analysis of phenotypic changes in mitogenically stimulated spleen populations.

J Dent Res. 71, 497.

Statement of authorship

The work presented in this thesis is wholly the work of Ward Massey undertaken at Westmead Dental Clinical School and the Institute of Dental Research between January 1988 and December 1991, towards the degree of Doctorate of Philosophy at the University of Sydney. To the knowledge of the author, this work is original and has not yet been published or presented elsewhere by other workers.



Thesis abstract

The oral cavity is host to a rich and complex microbial environment. Few, if any, infectious diseases exemplify as well as caries and periodontitis the diverse and complex range of interactions between host, microorganism and environment. Details of both the microbial "driving" force and the exact nature of tissue destruction remain unclear. A review of the literature indicates the great diversity of the microbial flora within the carious lesion both from a qualitative and a quantitative viewpoint. The various components of a humoral or cell-mediated immune response have been identified in inflamed pulpal tissues but the immunopathogenic mechanisms are not well understood. A review of the available literature is presented in Chapter one.

Correlations were therefore sought between microbial type and number in sampled carious dentine and cellular responses in dental pulp from vital, carious human teeth. A significant association between the number of *Prevotella* subspecies in carious dentine and extensive, principally mononuclear, cell infiltration was demonstrated. The presence of a significant B lymphocyte and plasma cell infiltrate was noted in those specimens associated with a high incidence of *Prevotella* species. There was evidence that polyclonal B cell activation played a significant role in the progressive and destructive lesion of carious pulpitis. This data is presented and discussed in Chapter two and is followed by a review of B lymphocyte development, and the regulation of B lymphocyte activation and differentiation in Chapter three.

Although many studies have investigated the possible immunoregulatory roles of T cells and accessory cells in chronic oral disease, very few have been concerned with B cell phenotype and function. B cell hyper responsiveness in oral and systemic disease and the potential contribution of the $CD5^+$ B cell subset, and its murine homologue Ly-1, in this process is discussed in Chapter four.

In Chapter five a murine splenic cell culture model is used to study lymphocyte blastogenesis and mitogenesis. A preferential role for the Ly-1 murine homologue of the $CD5^+$ B cell subset was postulated and investigated using this *in vitro* model. Mitogens, including lipopolysaccharide, a gram negative cell wall component, were used to stimulate whole and depleted lymphocyte cultures *in vitro*. The relative responsiveness of the T lymphocyte population and the Ly-1^+ and Ly-1^- B lymphocyte subsets was then examined over a 72 hour period by cytofluorographic analysis, colorimetric assay and viability staining. Dose responsiveness to mitogen, peak effects and the relative independence of the B lymphocyte mitogenic response were also studied.

Details of the fate of splenic B cells are not well described but the majority of mitogen-reactive splenic B cells appear to possess an accelerated turnover rate. The mechanism of this high turnover is not understood but it relates to death within the splenic B cell population. The nature of this cell death, be it apoptotic or necrotic, is investigated in Chapters six and seven. A review of the roles of apoptosis and necrosis in the development and function of the immune system is presented and followed by a light and electron microscopic study of morphological changes within control and mitogen-stimulated populations. This morphological data is supplemented by DNA electrophoresis.

Cytokine levels were also investigated on the basis of background evidence for a role of cytokines in mediating B lymphocyte growth and differentiation and potentially, in controlling B lymphocyte death. Cytokine levels were studied by bioassay and ELISA techniques. The cytokines interleukin 6 (IL-6) and tumour necrosis factor alpha (TNFa) were assayed from "whole" and depleted culture supernatants. This data is detailed in Chapter eight.

The overall significance of the data presented in experimental and review chapters is discussed in Chapter 9. Future lines of investigation are also documented in a general discussion of the implications of the work.

Part One

Chapter 1

The pathogenesis of carious pulpitis- specific bacterial effects and a potentially destructive host response

1.1.Introduction:

Human beings are colonised by a variety of microorganisms that comprise collectively the "indigenous microflora" or "normal flora" of skin, mucous membranes, and the gastrointestinal tract. All are potentially disease-producing, depending on the state of host defence mechanisms.

The term caries (derived from the Latin "caries" meaning decay; rottenness; dry rot) refers to the localised, progressive destruction of the teeth by organic acids produced locally by bacteria fermenting dietary carbohydrate (Figure 1.1). Progressive loss of hard tissue by continued bacterial action is often followed by infection of the dental pulp and surrounding tissues. Few, if any, infectious diseases exemplify as well as caries the diverse, and invariably complex, range of interactions between host, parasite and environment.

Understanding of many aspects of the pathogenesis of caries has advanced substantially in recent years. Nevertheless, knowledge is incomplete and caries continues to be a major public health problem.

Many descriptions of caries of the teeth are found in ancient literature, however most are too imprecise to allow any interpretation as to concepts of its cause. G.V. Black (Classics Of Dentistry Library 1980) has quoted an anonymous German author as the earliest writer giving a rational description of the cause of caries in relatively modern terms.

The statement given in the year 1550 reads as follows: "Caries is a disease and evil of the teeth in which they become full of holes and hollow...when they are not cleaned of clinging particles of food which decompose producing an acid moisture which eats them away and destroys them so that finally with much pain they rot away little by little". This concept, of acid derived from adherent food particles dissolving tooth structure, was shared by Black and formed the basis of recommendations for oral hygiene procedures as a caries preventive measure during the first half of this century.

Figure 1.1 : Gross clinical caries in a young adult patient. Extensive loss of enamel and underlying dentine with pulpal involvement is evident in a deciduous molar (DM). Caries involving enamel and dentine is also evident in the adjacent first permanent molar (FM).

FM

DM

Figure 1.1.

The French writings in dentistry of the 18th century also acknowledge the problem of caries but do not contain clear statements as to the nature of the pathology. Fauchard (Classics Of Dentistry Library 1979) in 1746 wrote, "Caries is one of the most baleful afflictions which can attack the teeth...Its progress destroys and consumes them..Once the cavity in the middle of the tooth is exposed by caries or otherwise, no cure is possible except by divers operations during which specific remedies are employed." Fauchard described methods of tooth restoration to effect such a cure.

Until the 19th century, caries was perceived as a purely chemical disease i.e. chemical substances developed in the mouth or produced by food dissolved out the calcium salts of which the teeth were composed. In 1881, Miles and Underwood determined, by using aniline dyes developed by Koch, that the enlarged tubules seen in dental caries contained micro-organisms. W.D. Miller, working in Koch's laboratory, was able to cultivate these micro-organisms and tentatively classify them on the basis of acid fermentation and other forms of decomposition. The demonstration, using sugar or starch substrates, that organic acids were produced by these microorganisms and that these acids were capable of dissolving calcium salts in tooth tissue, added to the explanation of the local changes in dental caries. It was not, however, until the work of Orland and colleagues (Orland *et al.* 1959) with gnotobiotic animals, that it was realised that the presence of micro-organisms was essential for both the initiation and the development of the carious lesion.

1.2.The enamel-dentine-pulp complex:

Enamel is a material consisting of two phases, mineral and organic (Orban 1980; Ten Cate 1989). The mineral phase, an apatite of calcium phosphate (hydroxyapatite) is the major component, comprising from 96 to 98% of the weight of the tissue. This mineral phase is composed of microscopic fibrillar crystals arranged into long bundles or prisms averaging 5 μm in diameter. The crystals are cemented together by the organic phase, a matrix of a number of proteins with molecular weight range 25-55 kilodaltons, collagen in its type 1 form being dominant (Figure 1.2 a &b).

Dentine is an avascular mineralised connective tissue characterised by the presence of tubules which radiate outwards from the pulp and containing the processes of the dentine-forming odontoblasts (Orban 1980; TenCate 1989). The mineral component of dentine comprises 70% of the dry weight in the form of apatite. The apatite crystals are similar in size and shape to those of bone and cementum and may form long tubular coats to the collagen microfibrils of the organic matrix.

Figure 1.2 (a): Scanning electron micrograph of enamel structure. Note characteristic morphology of enamel prisms (EP) and angulation of enamel rods (R) within prism structures. Scale bar indicates 10μm. Magnification= X 5000.

Figure 1.2 (b): Scanning electron micrograph of enamel structure. Note central zone (CZ) showing rapid action of dilute acid (Coca-Cola). Scale bar indicates 10μm. Magnification= x 2100.

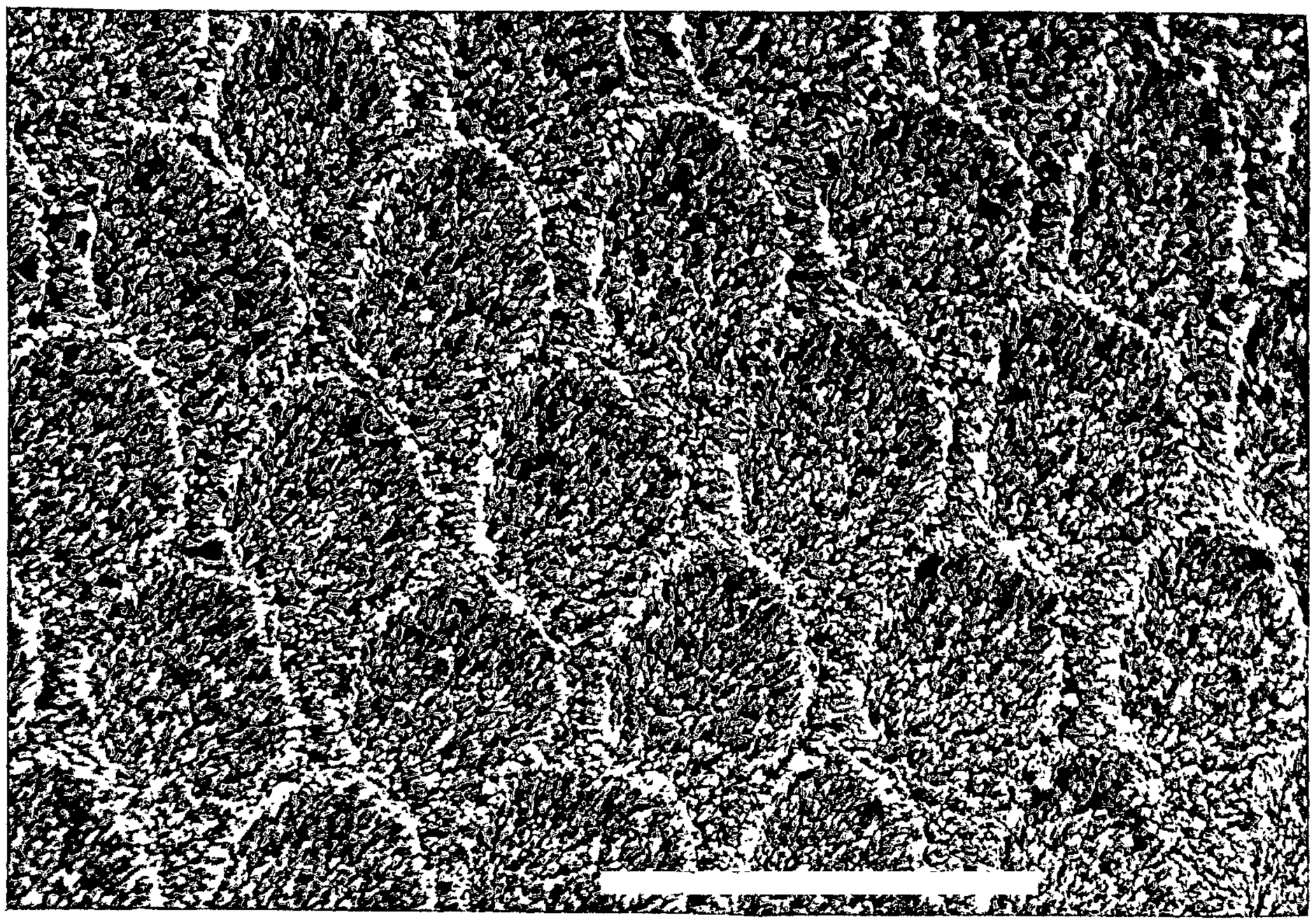
EP

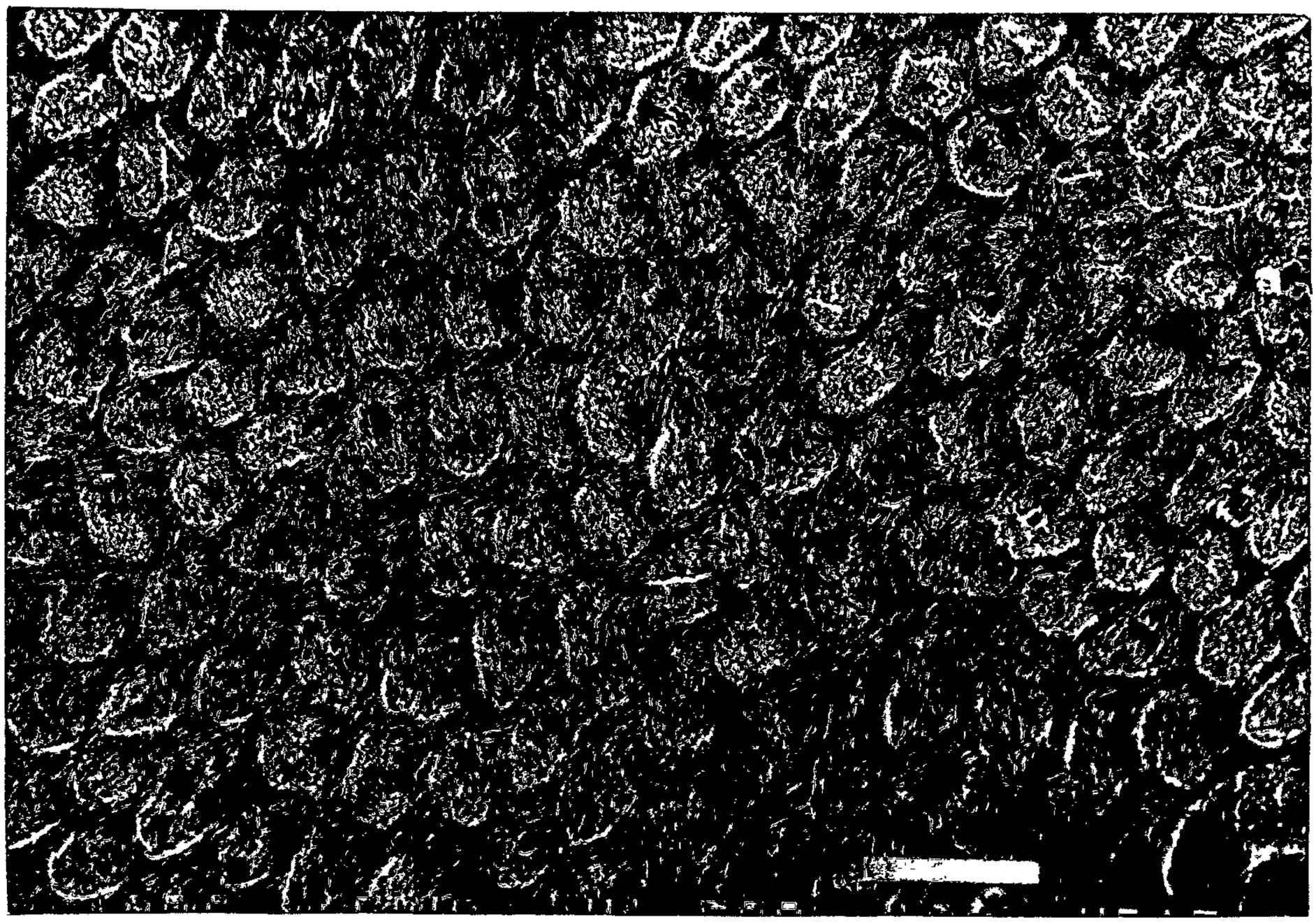
R

Figure 1.2 (a)

Figure 1.2 (b)

CZ





This arrangement allows for a large crystalline surface area and increases the reactivity of the dentine mineral.

The organic phase (18% of the overall weight of dentine) is almost entirely (90%) made up of the protein collagen in its type 1 form. The remaining protein, carbohydrate and lipid components of the organic matrix are believed to play a role in the extracellular aggregation of collagen and the nucleation and growth of apatite crystals. The organic phase (together with H_2O) makes up 50% of the total volume of dentine.

Dentinal tubules are responsible for the permeability of dentine. They are approximately 2µm in diameter in mid-dentine, but expand closer to the pulp and narrow at the outer limit of dentine. The cross-sectional areas of adjacent tubules can vary markedly due to variations in the amount of peritubular dentine infilling. There are approximately 30,000 tubules / mm^2 in mid-dentine but approximately half and double that number in the peripheral and pulpal regions respectively. The tubules branch finely close to the dentino-enamel junction (DEJ) forming side branches. These side branches are more numerous in root dentine than in the crown. Each dentinal tubule houses an odontoblast process surrounded by a potential space which may contain nerve fibres. It is generally accepted that the odontoblast process extends right into the peripheral dentine and is not withdrawn as the odontoblast migrates.

1.3.Pulpal histology:

The dental pulp is a loose connective tissue, different from that elsewhere in the body only in that it is located within a hard tissue shell. It is composed of cells, extracellular matrix, blood vessels and nerves and is gelatinous in consistency (Figure 1.3). The major extracellular matrix component is water. Collagen, again predominantly in its type 1 form, comprises approximately 40% of total pulpal protein. Elastic fibres are virtually absent being seen only in the walls of blood vessels, however two other fibre types have been identified in pulp tissue: oxytalan and argyrophilic reticular fibres. Silver staining reticular fibres are composed of an association of fine fibrils of type III collagen with glycoprotein moieties, while oxytalan fibres are believed to represent an immature form of elastin. The principal non-collagenous proteins of dental pulp are fibronectin and the protein component of proteoglycans. The extracellular matrix is also rich in glycosaminoglycans.

The most numerous cell type in the periphery of the healthy, mature pulp is the dentine-forming odontoblast (Pulver *et al.* 1977; Ten Cate 1989). The stroma of the pulp also contains typical connective tissue cells which, unlike the odontoblasts, are not unique to that

Figure 1.3 : Normal pulpal histology. A predentine layer (PD) is seen with subjacent odontoblastic (OL) and cell-free pulpal zones (CF). The inderlying cell-rich layer is seen to merge with a pulpal stroma of typical loose connective tissue (PP). Scale bar indicates 50µm.

PD

OL

CF

PS

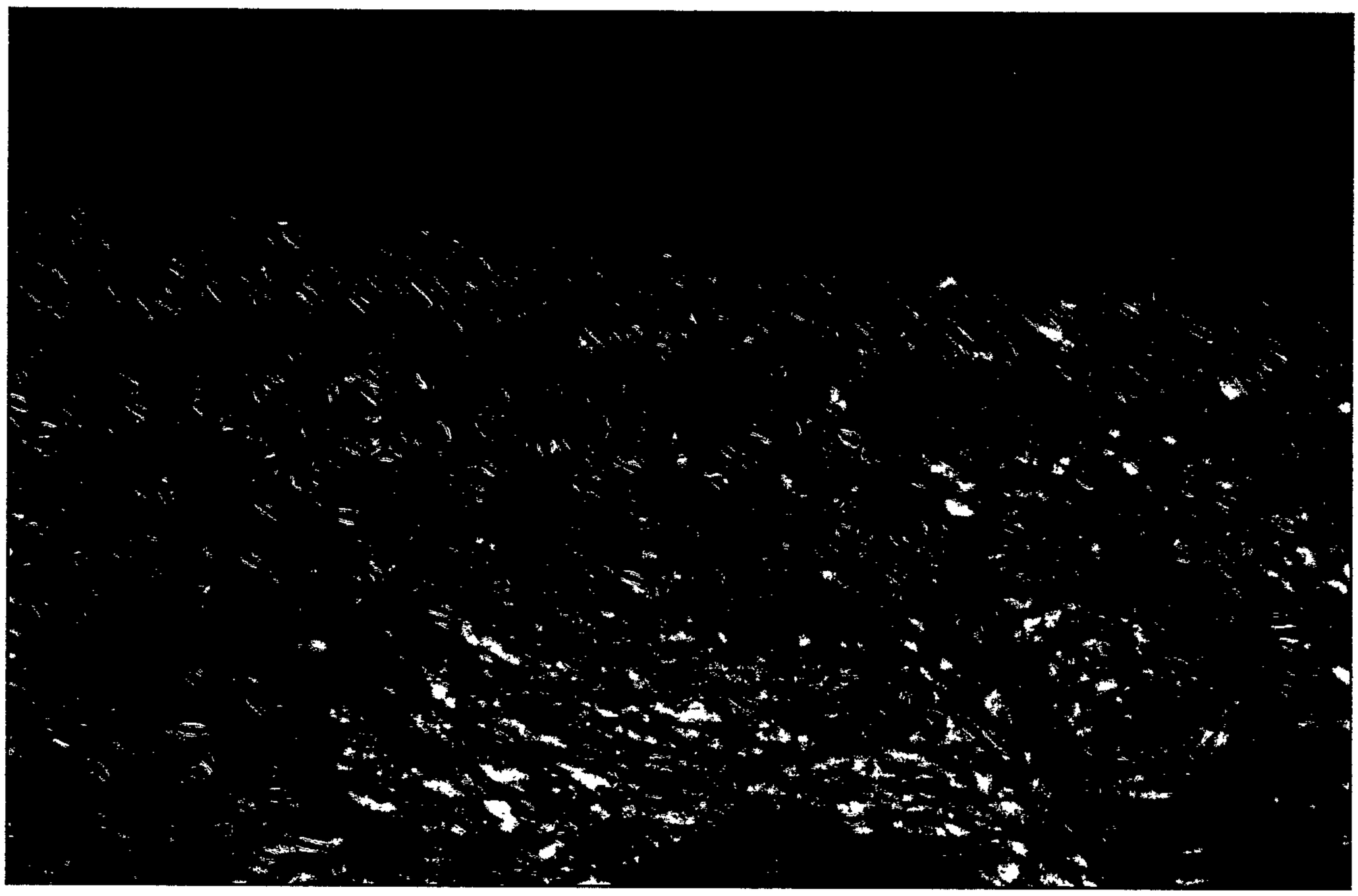


Figure 1.3

tissue. Fibroblasts, for example, are the second most common cell type and are found throughout the pulp at all ages. Little is known about the immunocompetence of the dental pulp. Preliminary experimental work (Pulver *et al.* 1977; Jontell *et al.* 1987) has demonstrated the presence of small numbers of immunocompetent cells in the normal dental pulp.

Pekovic and Fillery (1984) and Jontell *et al.* (1987) have demonstrated the presence of T lymphocytes (both $CD8^+$and $CD4^+$) and macrophages in normal pulp tissue. The absence of B lymphocytes in normal pulp tissue in both studies was taken to suggest that B lymphocytes are not permanent residents of healthy dental pulp connective tissue. The role of B lymphocytes in the early phases of pulpal responses to injury is therefore unclear.

The distribution of cells in the pulp is not noteworthy except in the region adjacent to the odontoblasts. Here, in the mature pulp, a cell-free zone (called the cell-free Zone of Wiel) is often described separating a cell-rich layer from the odontoblasts. This layer cannot always be demonstrated and may represent an artefact of histological preparation. Compared with most other loose connective tissues, the fibrous component is sparse even in the mature pulp.

Silver-stained reticular fibres predominate throughout the young pulp, whereas collagen fibres are mainly found close to the dentine and near neurovascular bundles. Large fibres are not a normal feature of even the older pulp. Fibronectin is present throughout the dental pulp, but is concentrated in the walls of blood vessels and in the apical region of the pulp. Oxytalan fibres have also been shown to be present throughout the mature pulp.

The pulp contains sensory nerve fibres, both myelinated A-fibres and unmyelinated C fibres, with the reported ratio being approximately 1:3. The mixed fibres enter the pulp through the apical foramen and travel to the coronal pulp with little branching. It has been estimated that less than 10% of the nerve fibres entering the tooth branch within the apical pulp. Fine unmyelinated fibres have been identified, however, in the outer regions of the radicular pulp. These either run singly or are accompanied by blood vessels. These fibres transmit only pain impulses. A delta fibres are fast conductors with low threshold, while C fibres have a slow conduction rate and a high stimulation threshold. In the coronal pulp, the nerve bundles branch and rebranch as they radiate out towards the periphery. They finally form a plexus subjacent to the odontoblasts. Axons, mainly as unmyelinated fibres, then extend from this plexus below and between the odontoblasts. There is no good evidence, however, that the odontoblast functions as a sensory receptor.

Nerve fibres are found in dentinal tubules but not until the majority of the primary dentine has been formed. The total number of pulpal sensory nerve fibres appears to decrease with age, as does the sensitivity of the pulp, although the innervation of tubules is reported to remain unchanged.

Sympathetic nerve fibres enter the pulp with the blood vessels and form a plexus around them supplying the smooth muscle in arterial walls. These nerves play a significant role in the regulation of pulpal blood flow.

Pulpal blood vessels vary in number, size and distribution both during development and with ageing. The vascular arrangement also differs in the coronal and radicular regions of the pulp and from tooth to tooth. The main trunk vessels are usually thin-walled arterioles and venules which run longitudinally through the root canals branching as they go. Small arterioles supply a network of fenestrated and continuous capillaries at the pulpal periphery from which a terminal network develops to supply the coronal pulp immediately subjacent to the odontoblast layer. Some of these capillaries loop terminally through the odontoblast layer. Anastomoses occur between venules, as well as between arterioles and venules.

The presence of lymphatic vessels in the pulp has been the subject of controversy largely because of their close morphologic resemblance to veins and capillaries. Observations (Seltzer & Bender 1984) at both the light and electron microscopic level point to the probability that the pulp contains lymphatics and this is supported by some animal studies. Investigations of the pulpal lymphatic system in dogs have shown lymph capillaries as blind openings near the odontoblast layer. These drain into small thin-walled collecting vessels. The collecting vessels have frequent interconnections and pass apically accompanying blood vessels and nerve fibres to exit through the apical foramen and drain into large lymph vessels in the periodontal ligament (Seltzer & Bender 1984). However, Takada and his co-workers (Rev. in Seltzer & Bender 1984), using an intra-arterial dye injection method, were unable to detect lymphatic vessels in the pulp tissues of a variety of animal species including the dog.

1.4. Caries initiation:

Although bacteria were isolated from a human carious lesion in 1924, proof for the causative role of bacteria came only in the 1950's and 1960's following experiments with germ-free animals (Orland *et al.* 1959).

The normal oral flora are responsible for the two most prevalent infections known to man, caries and periodontal disease. Mixed cultures are a characteristic feature of these oral infections and anaerobic techniques increase the number of populations recovered from most sites. Anaerobic organisms are of significance in many oral infections. Caries is a result of an outcome of the interaction involving diet, the host, and microbial activity. Diet can influence the colonisation and activity of the microbial flora. Host factors include the anatomy of the site, and the composition and properties of saliva and crevicular fluid.

The oral cavity is not a uniform habitat for microbial growth and colonisation. Different oral structures provide different habitats due to their physical nature and biological properties. The teeth, for example, allow the accumulation of large masses of bacteria by virtue of their hard, non-shedding surfaces. The microbial community associated with the teeth is referred to as dental plaque and its composition varies at each tooth surface due to the local environmental conditions. Dental plaque is an essential precursor of the dental diseases, caries and periodontitis.

1.4.1 Dental Plaque

Morphologically, plaque is a dense, organised deposit of bacteria within a salivary protein-derived framework (the pellicle) which usually contains, in addition, extracellular polysaccharides of bacterial origin (Newman & Nisengard 1988). Plaque is normally found on a pellicle-covered tooth surface but neither pellicle nor teeth are absolute requirements. Plaque may vary in thickness from a few bacterial cells up to a millimetre thick in stagnation areas.

Pellicle formation is a purely physicochemical process in which salivary proteins rapidly and selectively adsorb to the enamel surface.

Plaque formation includes the processes of:

(a) bacterial attachment and proliferation

(b) salivary protein incorporation

(c) polysaccharide accumulation

Later inclusion of inorganic components like calcium, phosphate and fluoride results in calculus formation.

1.4.2 Bacterial attachment and proliferation

Bacterial attachment to the pellicle covered surface occurs via several mechanisms: electrostatic (Van der Waals) forces, hydrophobic interactions, and selective binding involving the production of extracellular polysaccharides or surface features such as fimbriae or capsules.

Increases in plaque thickness are the result of growth of the initial colonising organisms as well as the attachment of new organisms. The same forces that favour attachment of bacteria to the pellicle promote stability within the plaque matrix.

The overall quantity of plaque in the mouth contributes to its pathogenicity at specific sites, since plaque thickness determines to some degree the diffusion and therefore the local concentration of substrates, acids and other bacterial products.

1.4.3 Microbial species in plaque

The bacterial morphology of plaque is highly variable. Discrete bacterial colonies or heterogeneous aggregates may develop.The principle factors determining the microbial composition of plaque are the age of the plaque and the anatomical site involved (Rev. in Burnett & Schuster 1978). An aetiological role for a particular microbial species in pulpal inflammation has not been demonstrated.

The majority of studies have indicated that early plaque (up to 24 hrs) is composed mainly of coccal forms, particularly *Streptococcus* spp. and *Neisseria* spp. Few rod-shaped organisms or anaerobic species are present. In older plaque (over 7 days old), filamentous organisms become dominant by 14 days, at which time the proportion of streptococci has fallen but that of the facultatively anaerobic organisms, for example, *Lactobacillus* spp. and *Veillonellae* spp. has risen.

Bacterial interactions can have important effects on the pathogenic potential of plaque. The synthesis of an extracellular polysaccharide by one species which can be utilised for energy by another is one example. The utilisation of lactate (produced from glucose by numerous acidogenic species) by *Veillonellae* species is another.

1.4.4 Plaque Metabolism

Organisms within plaque utilise a variety of selective transport mechanisms to take up dietary sugars. The central metabolic activity of dental plaque organisms is the breakdown of glucose to form acids with the release of energy via the glycolytic pathway. Pyruvate, at the

end of the common pathway can be metabolised in various ways. When glucose levels are high, many bacteria convert the majority of this glucose to lactate; but at low glucose levels a greater proportion is converted to acids such as acetic, formic, propionic, butyric and succinic. This shift in acid end-product reflects both changes in bacterial type and changes in enzyme activity within a particular species.

Many sugars are, however, converted to intracellular or extracellular polysaccharides. Extracellular (matrix) protein and polysaccharides comprise 10% of the dry weight of plaque. These proteins are taken up by the plaque via adsorption to bacterial surfaces or precipitation from saliva.

Intracellular polysaccharides are formed from glucose by many oral streptococci as a carbohydrate store for energy production when dietary carbohydrate is low. This allows maintenance of growth and is related to an organism's cariogenicity. Also extracellular polysaccharides are formed by many plaque microorganisms, mostly from sucrose.

The polysaccharides are polymers of glucose (glucans) or fructose (fructans) or heteropolysacccharides with mixed monosaccharide units including hexosamines and other more complex sugars. These polysaccharides are involved in bacterial adhesion, and in the retardation of the diffusion of acids, and perhaps other charged moieties in plaque as well as providing a store of carbohydrate for later energy production (Rev. in Burnett & Schuster 1978).

1.5.The carious lesion in enamel and dentine:

The hard tissue barrier of enamel, dentine and cementum provides strong mechanical support and protection to the loose connective tissue of the pulp. The oral environment's numerous harmful influences are kept at bay as long as this hard tissue barrier is intact. Bacteria and their by-products are associated with most pulpal disease processes whereas dietary items and components from restorative materials play relatively minor roles (Kakehashi *et al.* 1965).

Dental caries can be defined as a condition in which hard tissue is demineralised as a result of bacterial action. Loss of mineralised tissue leads to a collapse of the organic matrix and a cavity is formed. The acidogenic or chemicoparasitic theory of caries aetiology is the most widely accepted concept of the mechanism of caries initiation. In this concept, the fermentation of dietary carbohydrate by organisms within dental plaque on the tooth surface

Figure 1.4 (a): Scanning electron micrograph showing carious enamel. Note intact enamel (dark area at top of micrograph) (IE) and decalcified enamel and microorganisms (DE) in lower portion of section. Scale bar indicates 10μm. Magnification= x 3200.

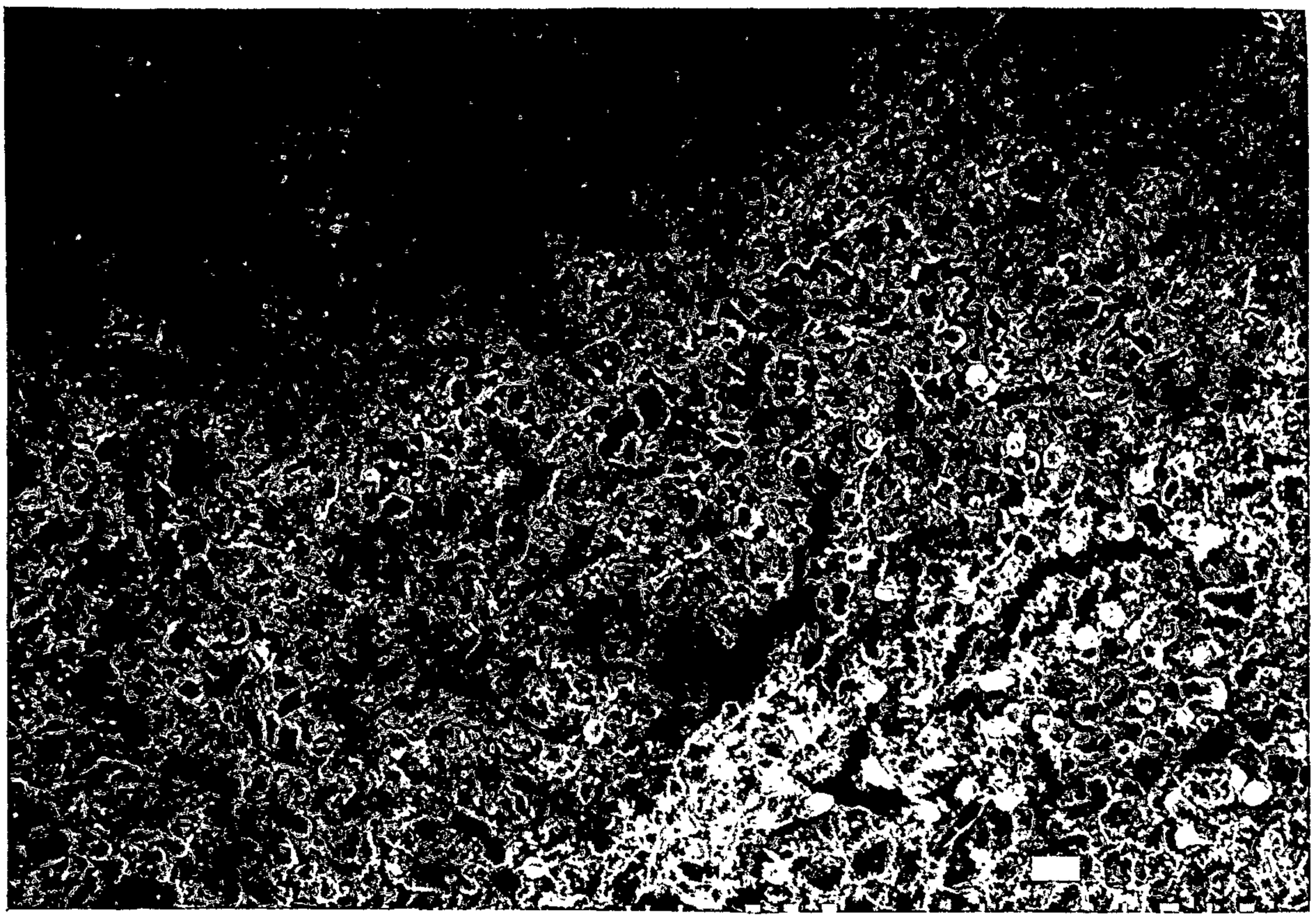
Figure 1.4 (b): Scanning electron micrograph showing variable morphology of dental plaque. Note the presence of rod, filamentous and coccal organisms. Scale bar indicates 1μm. Magnification= x 6500.

IE

DE

Figure 1.4 (a)

Figure 1.4 (b)



results in the production of acid which dissolves the apatite crystal structure of the dental hard tissues (Figure 1.4 a & b).

The organic acids (principally lactic acid) are produced as end products or intermediaries of glycolysis and the Kreb's tricarboxylic acid cycle and through other pathways which bacteria use to breakdown carbohydrate.The formation of dental plaque, which through limitation of diffusion holds these acids adjacent to the enamel surface and which also limits their buffering and dilution by saliva, is essential to the process of enamel dissolution.

Early enamel caries involves the formation of subsurface holes or spaces in the enamel. These "pores" are not of uniform size or distribution within the enamel layer. The organic matrix ensheathing the enamel prisms may play a significant role in both caries initiation and progression via the control of ion movement into or out of enamel. There appear to be two major patterns of destruction of enamel crystals. In the first the surface of the prisms is repeatedly etched; in the second, a central core or defect forms. Controversy still exists over whether the primary site at which demineralisation occurs is the interprismatic area or whether the prism is the more susceptible structure, although there is agreement that the two sites are identical apart from crystal orientation.

1.5.1 The progress of the lesion in dentine:

On reaching the relatively lightly mineralised dentino-enamel junction (DEJ), the process of demineralization spreads laterally along a plane of least resistance thereby undermining sound enamel. Spread in this fashion results in a broad base to the dentine lesion. The lesion then follows the primary orientation of the dentinal tubules so it develops a conical shape with its base on the DEJ and its truncated apex pointing towards the pulp.With fissure caries, lateral spread at the DEJ results in a larger area of involved dentine compared with smooth surface lesions and because the tubules are relatively straight there is less taper of the lesion towards the pulp.

The small dentinal lesion consists of two zones, as follows.

(a)A Translucent zone - a hypermineralised sclerotic region which walls off the lesion from surrounding dentine and which is formed as a result of mild stimulation. Mineral in the form of apatite is laid down within the dentinal tubules to form a mineralised barrier against the diffusion of acids (and later proteolytic enzymes and bacteria) towards the pulp.This zone probably represents an acceleration of the normal physiological process of peritubular dentine formation and therefore does not occur in situations where the stimulus is severe and the odontoblast is damaged or killed.

Both the peri-tubular and inter-tubular matrix appear normal at this stage.

(b)The Body of the lesion - this is contained within the translucent zone and, until a cavity forms in the overlying enamel, is uninfected. In the superficial part of the body of the lesion, both peri-tubular and inter-tubular dentine are partially demineralised.

In the case of a small dentine lesion deep to intact enamel, bacteria have not yet invaded the tissue. However, because carious enamel is extremely porous, acids, metabolites and breakdown products are able to diffuse into the underlying dentine and provoke a response in the dentine-pulp complex.

At this stage there is a layer of normal dentine deep to the body of the lesion. At the surface of the pulp chamber a layer of reactionary (secondary) dentine is also formed deep to the lesion. The structure of this tissue may resemble regular reactionary dentine or approximate severely dysplastic tissue and is dependent on the severity of the stimulus and resultant damage to dentine-forming odontoblasts.

Once cavitation has occurred, bacteria are able to penetrate the tissue. The pathway of invasion of the dentine by the pioneer organisms is along the dentinal tubules (Figure 1.5 a & b). The first wave of bacteria appears to be primarily acidogenic, and the acid they produce diffuses deep to, and in advance of, the organisms causing demineralisation. The organisms of this primary wave are believed to be principally lactobacilli which occupy tubules towards the periphery of the lesion, but which do not produce tubule enlargement (McKay 1976). The second invasion appears to comprise a greater mixture of microbes and it produces widening of the occupied tubules and considerable damage to the surrounding dentine. Toward the DEJ, the diversity of the bacterial population may increase. Proteolytic enzymes and other hydolases produced at this site may add to the effect of the acid and destroy the collagenous matrix.

At this stage also, the dentinal lesion can be described as consisting of a translucent zone and a body. The pulp shows mild inflammatory change and more reactionary dentine is deposited.

The body of the lesion is further subdivided at this stage into

(a) a zone of demineralisation - this is the deepest part of the body of the lesion and is usually free of bacteria although partially demineralised. Large plate-like crystals are seen to form within the tubules.

Figure 1.5 (a): Scanning electron micrograph showing dentinal structures in cross section. Note dentinal tubules (DT) and cut odontoblastic processes (OP). Scale bar indicates 10μm. Magnification= x 3050.

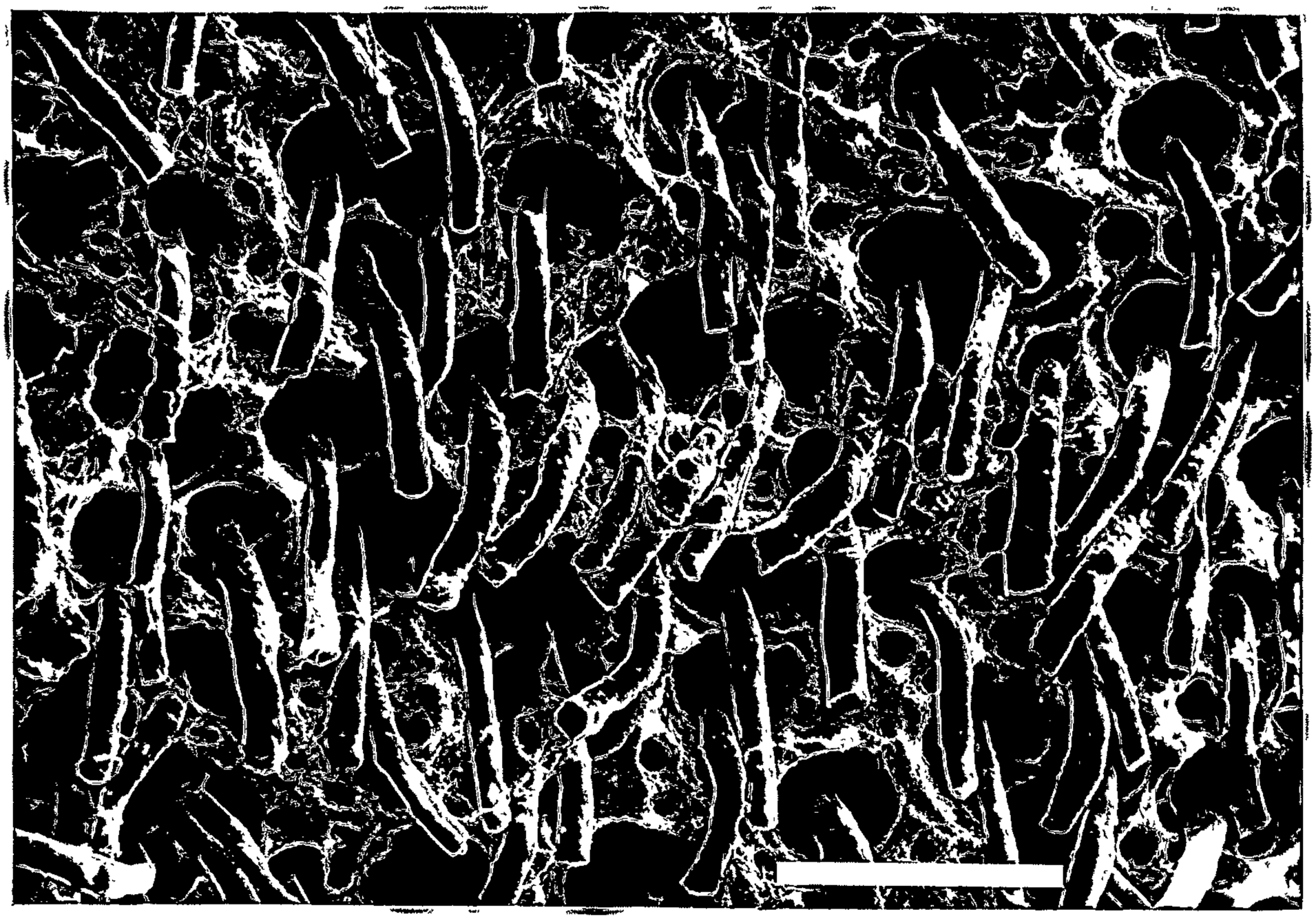
Figure 1.5 (b): Scanning electron micrograph showing microorganisms penetrating dentinal tubules. Note the presence of coccal and rod-like microorganisms. Scale bar indicates 1μm. Magnification= x 10300.

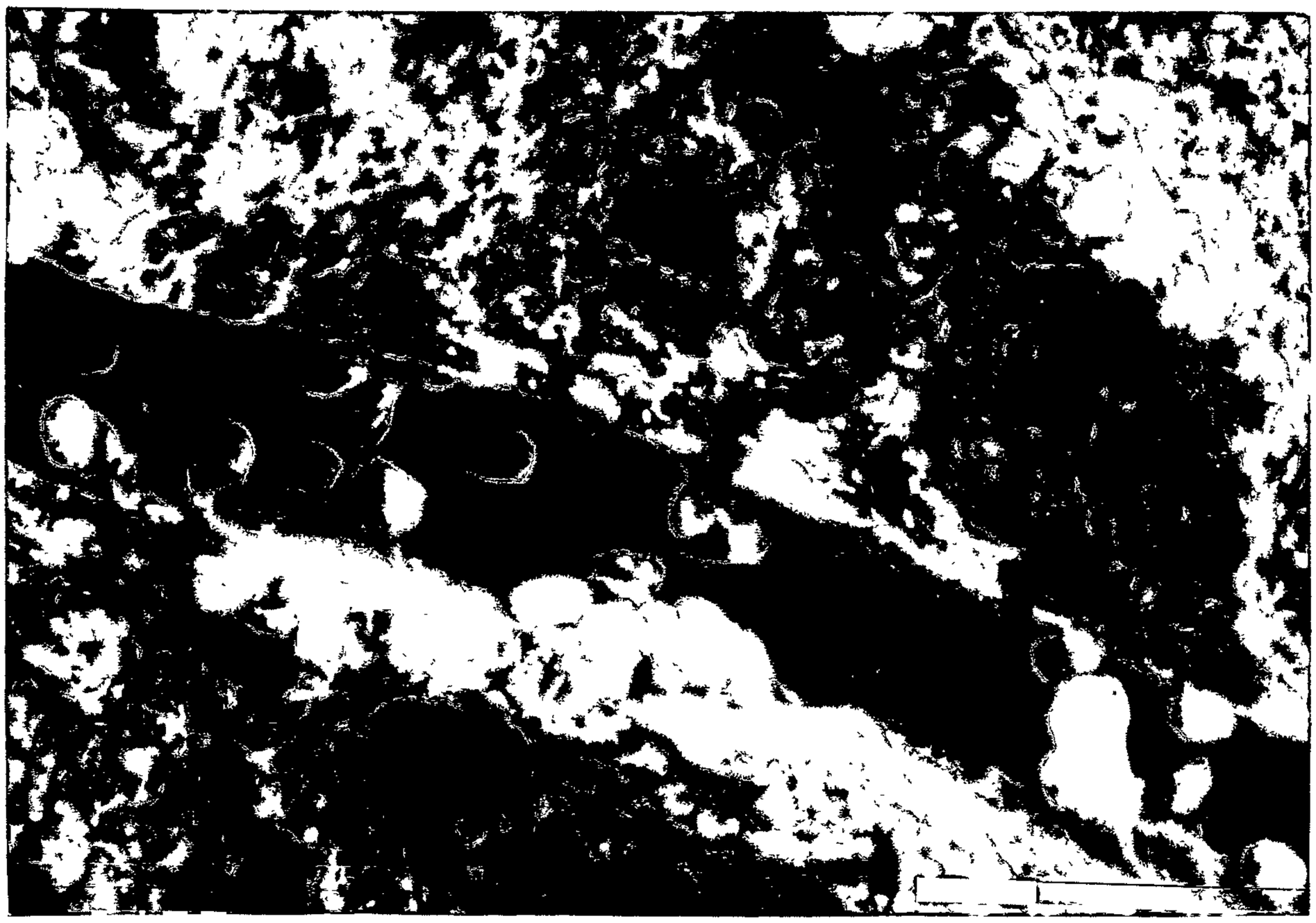
DT

OP

Figure 1.5 (a)

Figure 1.5 (b)





(b) a zone of penetration - this refers to the region of the body of the lesion which has been penetrated by bacteria. Throughout most of the zone bacteria are confined to the tubules which are distorted by their presence. Remnants of densely mineralised tissue are usually present in the bacteria-filled tubules. The intertubular dentine in this zone of penetration is extensively demineralised.

(c) a zone of destruction - this zone is frequently fragmented and little of the normal architecture of the dentine remains due to the presence of microbial cavities. Microorganisms are now able to invade peritubular and intertubular dentine. Liquefaction foci are formed by the coalescence of necrotic tissue and bacteria.

1.5.2 Protective mechanisms:

Whether dentine can provide protection to the pulp from bacterial influences when it is exposed to the oral environment through caries is a critical determinant of pulpal survival or death.

Bacterial substances from pooled dental plaque (Bergenholtz and Lindhe 1975), lyophilised sonicates of pure cultures of plaque bacteria (Bergenholtz 1977, Warfvinge and Bergenholtz 1986), and high molecular weight complexes of bacterial cell walls (Warfvinge *et al.* 1985) all can initiate acute inflammatory lesions in the pulp within 8-32 hours after application to cut dentine. Similar observations have been reported after provocation with carious dentine (Langeland 1987). These experiments demonstrate the rapidity with which the dentine/pulp complex can respond to bacterial influences. Furthermore, oral bacteria do not have to invade the pulp tissue or the dentinal tubules to initiate pulpal injury. The diffusion of bacterial substances through dentinal tubules precedes the passage of organisms.

The earliest evidence of a pulpal reaction to caries appears to occur in the odontoblastic layer (Langeland 1987). These changes, which include reductions in odontoblast size and number and changes in odontoblast shape, precede obvious inflammatory changes in the subodontoblastic layer even in acute caries. The formation of reparative dentine at this and subsequent stages represents a defensive reaction of the pulp to caries. The amount and quality of reparative dentine formed is variable and dependent on factors such as the rate of carious attack and the amount of primary dentine lost.

Defensive reactions within dentine are overcome as the carious lesion of dentine progresses. The translucent zone is lost via the action of acids and enzymes released by bacteria in the zone of penetration. The body of the lesion then consists only of zones of penetration and destruction. Pulpal inflammatory change increases progressively and reactionary dentine, if

formed, is extremely dysplastic. Eventually dentinogenesis is arrested, organisms invade reactionary dentine and finally, the pulpal tissue. Lateral spread of the lesion along the DEJ, will, by this stage, have undermined much of the enamel. Eventually this unsupported enamel fractures off leaving a large open cavity.

Although it has been demonstrated experimentally that bacteria can invade dentinal tubules after bacterial plaque has accumulated, penetration of microorganisms through non carious dentine to the pulp is rare even if the period of exposure to the oral environment is extensive (Lundy & Stanley 1969).

Experimental work has, however, shown that freshly cut dentine is highly permeable to bacterial components and/or products (Bergenholtz & Lindhe 1975; Bergenholtz 1977; Bergenholtz & Warfvinge 1982; Warfvinge *et al.* 1985) although it has not yet been proven that bacterial components are actually transported through dentine to the pulp where the inflammatory responses are induced. The initial reactions of host defence may be initiated via signal substances present within the dentinal fluids.

It has been reported (Warfvinge & Bergenholtz 1986) that dentine which has been exposed to microbial activity for extended periods, loses its permeability to bacterial products. Although reparative dentine can provide pulpal protection, in several studies the disappearance of inflammatory cell infiltrates was noted before the appearance of reparative dentine (El-Kafrawy & Mitchell 1963).

Based on the results of *in vitro* and *in vivo* experiments, Pashley and co-workers (Pashley *et al.* 1983; Pashley *et al.* 1984a; Pashley *et al.* 1984b) noted that dentine permeability, measured by fluid filtration and isotope clearance, decreased rapidly within hours of dentine exposure and suggested that the precipitation of high molecular weight proteins, such as fibrinogen, was the reason for this. Complement components, plasma proteins and immunoglobulins have all been isolated from dentinal fluid (Okamura *et al.* 1980; Ackerman *et al.* 1981).

The complex changes occurring in dentine after bacterial and/or chemical challenge mean that pulpal responses to routine clinical procedures and insults such as caries and trauma are unpredictable. However, one may conclude on the basis of both clinical and experimental data, that bacterial irritation of an area of exposed dentine generally causes pulpal lesions of limited duration and often leaves little permanent damage.

In teeth with functional and vital pulp tissue, dentine appears to provide considerable resistance to bacterial invasion of the dental pulp.

1.6. Microflora of carious dentine:

Carious dentine is considered to be anaerobic (Edwardsson 1974) and it is therefore reasonable to predict that anaerobes, either facultative or obligate, would predominate in lesions of dentine. Indeed, Hoshino (1985), found that the overwhelming majority of organisms isolated from both deep and shallow layers of carious dentine were strictly anaerobic.

In open-dentine ecosystems, such as smooth intact exposed dentine or cemental surfaces, bacterial types appear comparable with those found on smooth enamel surfaces i.e. 1/3 gram negative, 2/3 gram positive (Burnett & Schuster 1978 ; Edwardsson 1982). The gram positive organisms are primarily *Streptococcus* and *Actinomyces* species. *Lactobacillus* spp. are usually present in low numbers and *Streptococcus mutans* is not consistently present. The gram negative organisms appear to be primarily *Veillonella, Neisseria* and *Bacteroides* (now termed *Porphyromonas / Prevotella* species) (Burnett & Schuster 1978 ; Edwardsson 1982).

In closed dentinal systems, both in soft necrotic dentine and in deeper areas, the gram positive organisms dominate (Rev. in Edwardsson 1974; Edwardsson 1982). As the deeper areas are compared with the more superficial soft necrotic dentine, a decrease in the numbers of gram positive cocci and gram negative organisms has been noted by some observers. The numbers of lactobacilli appear to be high in both areas, as are the numbers of gram positive facultative and pleiomorphic organisms eg eubacteria, bifidobacteria, arachnia, propionibacteria and actinomyces. Gram negative rods such as prevotella, porphyromonas and fusobacteria and anaerobic cocci such as peptococci and peptostreptococci have also been isolated from deeper areas but they constitute a minor part of the flora. Carious dentine may therefore contain a mix of microaerophilic, aerobic and anaerobic bacteria, varying considerably from tooth to tooth and site to site within the carious lesion.

1.7. Inflammatory and immunological aspects of dental caries :

In contrast to the detailed understanding of the principles of caries initiation, details of the nature of pulpal responses to the carious process remain elusive.

Related to this, the pulp and dentine form a single biological unit due to the presence of odontoblastic processes in dentine.

Therefore infection in the dentine (caries), surgery in the dentine (cavity preparation) and the placement of foreign material on the dentine (tooth restoration) all involve interactions with a living tissue. As with any living tissue, there is the potential for inflammation, cell damage and death.

The common response of pulpal tissue to a short-term insult, be it physical, chemical or bacterial, is inflammation. If the insult is mild, healing will occur, but the capacity to respond to subsequent insults could be compromised.

If the insult is of greater degree, then part of the pulp tissue may become necrotic. It is possible that calcific repair can then occur and that the adjacent tissue is again compromised. Partial death of pulpal tissue may also progress to total necrosis.

Because caries is an intermittent and chronic disease process, with periods of rapid activity alternating with periods of rest, the histopathological response of pulpal tissue may vary depending on whether the lesion is in an active or arrested phase (Langeland 1987). This has resulted in some uncertainty over the response of the pulp to the carious attack and to confusion over appropriate therapy. Attempts to relate clinical features with pulpal histopathology have not yielded the expected close correlation. Some studies have shown pulpal inflammation in response to incipient caries, while others have shown no apparent pulpal change even when the carious process has extended to within 0.2 mm of the pulp (Massler 1967).

The complexity of pulpal response patterns is illustrated by the finding of inflammatory cells in the pulps of impacted teeth and even in clinically intact erupted teeth. No correlation has been demonstrated between any single clinical symptom and the inflammatory process within the pulp. In some cases there is partial necrosis and severe inflammation with no history of pain. Nor is there a relation between extensive caries and a history of pain (Massler 1967; Langeland 1987).

Despite this clinical and histopathological variability, it is valid to propose that bacteria and their products play a major role in long-term pulpal insult through both dental caries and restoration microleakage (Brannstrom 1981). Although this concept is widely held, there have been surprisingly few detailed studies attempting to relate the microbiology of carious

dentine (Edwardsson 1974) with the histopathology of carious pulpitis (Torneck 1974). This is in sharp contrast to the extensive and detailed studies on the microbiology of caries initiation i.e. enamel dissolution.

Further to this, there is no clear basis for assigning an aetiological role in pulpitis to any particular microbial species. Our understanding of the microflora of the carious lesion is limited. Studies of the carious microflora have, to date, indicated a mix of species dominated, numerically, by gram-positive rods and cocci; however, the microbial flora isolated from carious dentine also shows extreme variability.

Activation of any host defence, which may involve acute inflammation, the complement cascade, the immune system, phagocytic leukocytes or various combinations of these, has great potential for causing further tissue damage. Pulpitis and periodontitis appear to represent examples of infectious diseases in which the host response may well be the cause of more damage than the microorganisms produce. Compared with other tissues in the body, cell damage may be less readily repaired in these tissues because of limited access for repair-competent tissues or cells. In pulpitis all the components of acute and chronic inflammation are present and may each play a role, but the consequences may be very different from one time to another.

It is only through an understanding of the nature of both the microbial "driving" force and the tissue response that clinicians will be able to maintain pulpal vitality with greater certainty.

Inflammatory reactions in the dental pulp have been described in response to a variety of clinical insults such as caries, periodontal disease, operative procedures and dental trauma (Ingle & Langeland 1985).Vascular reactions, as well as migration and accumulation of a variety of inflammatory cell types, have been seen during both the initial and reparative phases of the inflammatory response. The critical factor in any tissue repair process is to attract the appropriate inflammatory cells to combat infection and to debride the wound.

In the sub-odontoblastic layer a diffuse infiltration of lymphocytes, plasma cells and macrophages has been described as the earliest evidence of inflammation (Langeland 1987). In earlier stages subtle increases in vascularity with proliferation of small vessels have been documented. As the dentinal lesion nears the pulp, enlarged arterioles as well as capillaries and dilated venules become evident.

An acute inflammatory response has been demonstrated only in the late stages of development of caries and as the lesion approaches the pulp. This intensifies as bacteria begin to penetrate deep into primary or reparative dentine. Increased numbers of neutrophil polymorphs (NPM's) have been shown to emerge from adjacent venules presumably in response to chemotactic activity associated with the carious lesion. Neutrophils have also been demonstrated migrating into dentinal tubules (Langeland 1987). As this continues, discharge of lysosomal enzymes probably results in destruction of pulpal tissue and in suppuration, either localised or diffuse.

Experimental animal data has shown that antigens from oral microorganisms can induce inflammatory changes in pulpal tissue when exposed to freshly cut dentine (Bergenholtz & Lindhe 1975; Bergenholtz & Warfvinge 1982; Warfvinge *et al.* 1985). The ability of the pulp to mount an immune response has been inferred from the development of periapical lesions after the introduction of known organisms into the root canal systems of experimental animals. A change in serum protein fractions following prolonged or repeated sensitisation of the animals to antigens introduced into root canal areas has also been demonstrated (Welsh *et al.* 1936; Kennedy *et al.* 1957; Okada *et al.* 1967). Barnes & Langeland (1966) have also demonstrated antibody production as a response to non-microbial antigens placed in the pulps of experimental animals. Experimental data (Lundy and Stanley 1969) suggests both that the dentine-pulp complex has a significant capacity to defend itself against bacterial insults, and that oral microorganisms and their products are not easily able to sustain a long-term inflammatory lesion in the pulp unless the intervening dentine barrier is weakened. Host defence factors passing outwards in dentinal fluids from the dental pulp appear to significantly inhibit bacterial pathogenicity.

When the antigenic challenge is transient in nature, as it is in most cases, the inflammatory process appears short lived and self limiting. However, an examination of chronic pulpal lesions, and of situations in which the intervening dentine barrier has been destroyed, has revealed the presence of granulation tissue infiltrated by lymphocytes, plasma cells, macrophages, polymorphonuclear leukocytes, giant cells and mast cells (Torneck 1974; Seltzer & Bender 1984). Investigators have examined the relation of caries penetration to pulp pathology. Reeves and Stanley (1966) correlated the depth of bacterial penetration into dentinal tubules and the degree of pulpal pathology, and found no significant pathology until the bacteria came within 0.75 to 0.8 mm of the pulp tissue. Abscess formation and chronically inflamed granulation tissue occurred only when the organisms were located in the last 0.30-0.50mm of dentine.

These pathological changes are probably the result of activation of non-specific inflammatory reactions as well as specific immunological responses. The non-specific inflammatory mediators that may play a role in the pathogenesis of the pulpal lesions include vasoactive amines, kinins, complement components and arachidonic acid metabolites.

The presence of various classes of immunoglobulins and of immunologically-competent cells suggests a role for specific immune responses also.

1.7.1 Non specific inflammatory reactions:

The two major vasoactive amines involved in inflammatory reactions are histamine and serotonin. Both exist preformed in a variety of cells but principally mast cells, basophils and platelets. In humans, histamine is by far the most important of the vasoactive amines (Owen & Woodward 1980). Preformed granules containing histamine are released from mast cells in response to tissue injury (Wilhelm 1962), complement activation (Willoughby & Dieppe 1976), interaction with activated T lymphocytes (Van Lovern 1984) and bridging of membrane bound IgE by allergens (Ishikaza & Ishikaza 1963).

Controversy still exists over the significance of mast cells in the inflamed human dental pulp. Several authors (Dockrill 1961; Anneroth & Brannstrom 1964) have reported their absence. However, Zachrisson (1971) showed that pulps containing few or no inflammatory cells were devoid of typical mast cells, while mast cells were noted in all inflamed pulps in regions where the plasma cell populations, both mature and immature were relatively high. It is now generally agreed that mast cells exist in inflamed pulps in variable but low numbers. Although controversy exists with regard to the significance of mast cells, their major product, histamine, has been shown to be present in inflamed pulpal tissue (Del Balso *et al.* 1976).

There is also controversy concerning the role of complement in inflammatory change in the dental pulp. Speer & Heueur (1977) used immunodiffusion techniques and pooled pulpal tissue samples to demonstrated an increase in immunoglobulins IgG and IgA and a decrease in the C3 fraction of complement in inflamed pulp tissue when compared to non-inflamed pulp tissue. A role for C3 was indicated by a decrease in the mean level of this component in inflamed pulps. Okamura *et al.* (1980) have also shown the presence of immunoglobulins IgG, IgM, IgA and complement components C3 and C4 in the cytoplasm of odontoblasts, in adjacent pulp cells and in dentine. These substances were most likely present due to changes in membrane permeability associated with the pulpal response and cellular damage. In addition, most of the activators of the classical and alternate pathways of the complement

system have been found in pathologically involved pulp tissue or within periapical lesions (Malmstrom 1975; Kuntz *et al.* 1977; Pulver *et al.* 1978).

Although complement components can be found in inflamed pulpal tissues, they are not present in forms indicative of a role in immune-complex disease, that is, as indicated by C3 conversion. In all studies except one (Pulver *et al.* 1978) the components have been easily washed out indicating the presence of soluble protein rather than fixed immune-complexes. No attempts have been made to extract insoluble complexes from the tissues.

Complement activation therefore remains a potential contributory factor in pulpitis, as activation *in-vivo* is amply seen by the detection of split-products in tissue fluid. Complement activation by bacterial substances or bacterial- or host-derived proteinases rather than immune-complex disease may, however, occur.

Products of the arachidonic acid cascade are not stored, but are synthesised from cell membranes under the influence of phospholipase A_2 (Torabinejad & Bakland 1980). The two major metabolic products of this pathway are the prostaglandins and the leukotrienes. The prostaglandins, particularly PGE_2 and PGI_2 are associated with vascular permeability and pain via the action of the kinin system and histamine. The biological activities of leukotrienes include chemotaxis, lysosomal enzyme release and alterations in vascular permeability. Prostaglandins are thought to play an important role in the pathological changes associated with human pulpal disease and elevated levels have been demonstrated in inflamed and symptomatic pulps (Cohen *et al.* 1985).

1.7.2 Acquired Immunity:

Acquired immune reactions involve a specific interaction with invading antigens, are the result of lymphoid cell memory, and are supported by studies which demonstrate dense aggregations of lymphocytes, macrophages and plasma cells in inflamed pulps (Torneck 1977, Torneck 1978). Although roles for cell-mediated and humoral immunological responses in pulpal tissue have been inferred from the presence of the various components of the response, or studies of periodontal and periapical tissues, the immunopathological mechanisms involved have received scant attention. It is not known, for example, to what extent specific antibodies to invading organisms are produced locally, nor is it known if past caries experience and the possibility of a memory response, influence prognosis.

The uninflamed human dental pulp has been found to contain immunocompetent cells capable of immunological responses. Jontell (1987), used indirect immunohistochemistry to

demonstrate that the normal dental pulp is equipped with small numbers of cells (<18 mm^3) capable of initiating immune responses. Dendritic cells expressing class II antigens (ie HLA DR/DQ positive) were seen in the odontoblastic layer as well as in the central pulp tissue.T lymphocytes, both helper/inducer ($CD4^+$) and cytotoxic/suppressor ($CD8^+$), were observed in all specimens.

B lymphocytes were not detected in any of the pulp specimens examined suggesting that B cells may not participate in the initial phase of immune response of the dental pulp.

A local infiltration of lymphoid cells including lymphocytes, plasma cells, monocytes/macrophages and neutrophils has been shown to occur in chronically inflamed pulpal tissue (Torneck 1977). Pulver *et al.* (1977) used fluorescent antisera to demonstrate the presence of IgG (dominant) IgA, IgE and IgM- immunoglobulin containing cells in inflamed dental pulps.

Pekovic and Fillery (1984) claim to have first demonstrated the presence of T lymphocytes in inflamed pulp tissue using monoclonal antibodies and an indirect immunofluorescence technique. Using pulpal tissue sections stained with anti-T lymphocyte antibody and anti human Ig serum, these workers demonstrated plasma cells and T lymphocytes in pulps from teeth with advanced caries. Hahn *et al.* (1989) used monoclonal antibodies to study T & B lymphocytes in inflamed pulp tissue and confirmed the presence of T suppressor phenotype and T helper phenotype cells.

1.7.3 Potential immunopathological mechanisms:

Discussions of immunological mechanisms of tissue injury in chronic pulpitis have largely centred on the four types of hypersensitivity rections as classified by Coombs and Gell (Coombs & Gell 1975). Their characteristics do not, however, entirely account for the usual clinical and histopathological signs of pulpitis.

Type I (reaginic, atopic, anaphylactic) hypersensitivity reactions depend on IgE antibody bound to the surface of mast cells which, upon reaction of specific antibody with antigen, release histamine and other local inflammatory mediators into the local environment. The finding of IgE immunoglobulin-containing cells in inflamed pulp tissue and the detection of mast cells in inflamed pulps suggests that the components necessary for an anaphylactic type of hypersensitivity reaction are present. However, mast cells are present in variable numbers and IgE can be localised to relatively few cells.

Because of the relative paucity of IgE in inflamed pulpal tissue, type I reactions seem unlikely to be major contributors to pathogenesis.

Type II (cytotoxic) reactions are dependent on IgG or IgM antibody which fixes complement, resulting in the lysis of antibody-coated cells or of cells with surface antigens to which complement fixing antibody binds. The presence in inflamed pulp tissue of IgG molecules, neutrophil polymorphs and macrophages, as well as complement fragments, and the persistence of IgG staining of connective tissues and endothelium even after prewashing (indicative of some degree of tissue binding or complex formation) (Pulver *et al.* 1978) supports the possibility of cytotoxic hypersensitivity reactions occurring in pulp tissue. While damaged and lysed cells are clearly present in pulpitis, cell lysis is arguably not the major event in pathogenesis. Thus the coincidence of complement and antibody on the surface of cells undergoing lysis in the pulp tissue has not been demonstrated. This would be necessary to establish conclusive evidence for type II reactions.

TypeIII (Arthus) reactions also involve complement-fixing antibody. They typically occur in vessel walls and are also dependent on large precipitates of antigen-antibody complexes which activate complement. Through the release of chemotactic split complement components, phagocytic cells are attracted; these phagocytose the complexes and release tissue-degrading hydrolytic enzymes. These complexes not only bind to neutrophil polymorphs and macrophages but to platelets and endothelial cells as well. Damage to endothelial cells and surrounding tissues as a result of complement fixation intensifies the inflammatory reaction. As previously mentioned, complement components can be found in the tissue in pulpitis, but not in forms indicative of immune-complex disease. In all but one study (Pulver *et al.* 1978) , the components have been easily washed out, indicating the presence of soluble protein rather than fixed immune complexes. Complement activation therefore remains only a potential contributory factor in pulpitis.

Type IV reactions (delayed type hypersensitivity) are not considered to be antibody-mediated. Rather, they depend on the production by stimulated T cells of other mediators. Cell mediated immune reactions are mediated either by direct cytotoxicity, release of lymphokines, or both. Available data from studies of the cellular composition of periapical lesions indicates that the majority of lymphocytes present are of the T lineage (Torabinejad & Kettering 1985). In addition,numerous macrophages have been demonstrated in periapical lesions. Natural killer (NK) cells have also been demonstrated in periapical and periodontal lesions (Kettering & Torabinejad 1985; Cobb *et al.* 1989). This data and the localization by antibody techniques of significant amounts of osteoclast-activating factor in studies of

inflamed gingival tissues and periapical lesions are taken to imply that cell-mediated immune responses play a role in both the progression of periapical lesions and chronic periodontitis.

It is unlikely that pulpitis (and in fact periodontitis) are pure type IV reactions, because a large number of immature and mature B cells are present in these diseases. In addition, if periodontal and pulpal lesions are compared with "classical" delayed hypersensitivity, lymphoid cells and macrophages are present in far fewer numbers in the oral diseases. Quite clearly, also, pulpitis is not a simple B cell lesion as T helper phenotype and T suppressor phenotype cells are present.

There exists the possibility that, as in periodontitis, there is a stable T cell / cell-mediated lesion and a progressive B cell lesion in pulpitis. By using panels of monoclonal antibodies (pan-B, pan-T, T_4 and T_8), both Hahn (1989) and Falkler *et al.* (1987)have been able to demonstrate an alteration in the ratios of T suppressor (T_8) to T helper cells (T_4) and T to B lymphocytes when normal, uninflamed pulpal tissue is compared with reversibly inflamed and irreversibly inflamed (clinical diagnosis) pulpal tissue. In pulpal tissues of the reversibly inflamed group, more than 90% of the identified lymphocytes were T lymphocytes with a T_4/T_8 ratio of 0.56:1. Higher numbers of all T, T_4, T_8 and B lymphocytes were observed in the pulps from the severely, and irreversibly, inflamed group. A ratio of 1.14:1 of T_4/T_8 was observed in the irreversible group. B lymphocyte numbers increased markedly so that the ratio B:T increased from 0.04:1 (clinically normal group) to 0.05:1 (reversibly inflamed group) to 1.6:1 in the irreversibly inflamed group (Hahn 1989). When compared with the pulps of teeth from the normal and reversible groups, the irreversible group in this study contained not only higher numbers of T (T_4 and T_8) and B lymphocytes, but also plasma cells and neutrophils.

These changes, and those observed in other studies (Torneck 1981; Lin & Langeland 1981; Trowbridge 1981) suggest that immunocompetent cells are important in the pathogenesis of pulpal diseases. A high T_4/T_8 ratio and a predominance of B lymphocytes in irreversibly inflamed pulpal tissue could allow excessive T_4 (ie T helper cell) regulation of B cell function. The reduction in T suppressor cell numbers and the continuing antigen influx from the carious lesion has also been predicted to lead to an uncontrolled activation of T helper cells and B lymphocytes. The resultant cytokine and antibody release could then lead to immunopathological changes. The pro-inflammatory mediators released in response to an interaction between antigen or polyclonal activators and immunologically competent and associated cells, are believed to be integral to the disease process.

Another potentially destructive cellular mechanism is autoimmunity, wherein destruction might be produced by an immune reponse specifically directed at the pulpal tissues. Direct evidence for such a possibility in chronic pulpal lesions is limited. However, indirect evidence exists in several forms. In periodontitis, a similar oral disease with respect to aetiology and histopathology, serum antibodies against type I collagen have been reported and found at higher levels in subjects with advanced destructive periodontal lesions than in control subjects (Ftis *et al.* 1986). Advanced periodontal lesions are also characterised by dense aggregates of mature and immature cells of the B lymphocyte lineage and cells producing rheumatoid factor (IgM anti-IgG) in the gingiva of adult periodonitis patients have also been identified (Hirsch *et al.* 1989). In both diseases, destructive lesions are characterised by changes considered the hallmarks of polyclonal B cell activation. Polyclonal B cell activation is also the common feature of a number of autoimmune diseases and autoantibodies are frequently detected following polyclonal B cell activation (Klinman & Steinberg 1987)

Despite this knowledge, a number of key questions remain to be answered in relation to cellular immunity in pulpitis.

1.What is the importance of cell-mediated versus humoral responses?
2.Which is the more important mediator of the pathological responses, B or T lymphocytes?
3.Is the B cell activation evident in the disease, antigen-specific, polyclonal or both?
4.What is the role of the immune system in tissue destruction?
5.Overall, is the activation of the immune system protective or detrimental?

The immune response in pulpitis is most likely to be, on balance, protective against extensive local tissue destruction from widespread infection by oral bacteria, but the net slow local tissue destruction may well be the result of the pro-inflammatory effects of cytokines and the induction of other inflammatory mediators and tissue destructive materials from cells.

1.8. Microbiological aspects of the pathogenesis of pulpal lesions:

Two types of microbial virulence factors are important in the pathogenesis of disease. First are those that facilitate colonisation and assist evasion of host defences, and second are those factors causing tissue damage. Examples of the former include the elaboration of adhesins for saliva coated and mineral coated surfaces by *Actinomyces* and *Fusobacteria* and adhesins for basement membrane collagen type IV possesed by *Bacteroides* species. Coaggregation between gram-positve and gram-negative organisms is also widely seen.

In addition, organisms such as bacteroides are able to evade host defences by the secretion of proteinases which cleave immunoglobulins G & A and the possession of a capsule. Inhibition of phagocytosis, of superoxide production and of the formation of phagolysosomes are examples of mechanisms used by other organisms to evade host defences.

Some, or all, of the tissue destruction in pulpitis may result from an immunopathological reaction, triggered by a bacterial species, which is sustained until that species is eliminated or suppressed. Alternatively , tissue damage may be the result of specific toxins. Tissue damage can therefore be the result of invasion or a "long-range" attack by fragments of organisms and other "virulence factors" while the organisms remain outside the pulpal tissue. These factors cause pathology by direct damage or immune-mediated reactions.

Many of the microorganisms that are found in carious dentine produce products which cause inflammation via cytotoxic or direct effects. Such compounds may either be identical to host inflammatory mediators or may mimic the host's natural mediators. Greenman (1988) was able to demonstrate the production of vasoactive amines (histamine and tryptamine) and short chain fatty acids by dental plaque microorganisms. These compounds either acting alone or synergistically could contribute to pulpal inflammation.

Enzymes and metabolic by-products released from microorganisms, may play an important role in the early transient acute inflammatory phase of pulpitis, as well as the late acute inflammatory changes seen when organisms are deep in dentine or within pulpal tissue.

It is evident from the available data on the microbiology of carious dentine, that the carious process is not only episodic in nature, but complex in terms of its flora and architecture (Edwardsson 1982). There appears to be a primary bacterial attack (McKay 1976) involving lactobacilli and aciduric streptococci, followed by a secondary infection zone of mixed flora. The defence reactions of the pulpal dentine may slow the rate of invasion of the primary bacterial attack and the secondary infection zone may then catch the primary invasion and the two become confluent. Lactobacilli are easily overgrown by the other organisms found in carious dentine under these non-selective conditions . Both in soft necrotic dentine and in deeper areas of the lesion, gram positive organisms appear to predominate (Edwardsson 1982 ; Hoshino 1985). Organic acids and microbial enzymes are produced by micro-organisms both in the more superficial soft necrotic dentine and the deep areas of the lesion (McKay 1976; Edwardsson 1982). Such end-products not only damage pulpal cells but may also contribute to the induction of defensive responses.

Surface structures such as capsules and fimbriae may also be of importance for the pathogenicity of many of these organisms. Capsules and fimbriae have been demonstrated on several strains of *Prevotella* and *Porphyromonas* spp.(Okuda *et al.* 1981; Sundqvist *et al.* 1982; Brook *et al.* 1983; Handley & Tipler 1986) and results suggest that the presence of a capsule increases the capacity of an organism to generate an abscess (Brook 1986).

Lipopolysaccharide (LPS / endotoxin) from gram negative organisms may be a pathogenic factor in pulpal infections. It has been demonstrated that endotoxin is present in amounts which correlate well with the number of gram negative bacteria present (Dahlen & Bergenholtz 1980). *Veillonellae* and *Fusobacterium* spp, both commonly isolated from dentinal carious tissue, appear to have a high endotoxic activity (Nygren *et al.* 1979; Dahlen & Bergenholtz 1980;). Gram negative species may also produce extracellular toxins. The leukotoxin producer *Fusobacterium necrophorum* has been found in infected root canals of monkeys (Fabricius *et al.* 1982) but has not been recovered from human root canals. *Prevotella* and *Porphyromonas* species, organisms identified in carious dentine and infected root canals, produce several enzymes which may contribute to their pathogenicity through cytotoxic or direct effects (Rev. in Slots & Genco 1984). DNAse, RNAse, gelatinase, lipase, lecithinase, neuramidase, hyaluronidase, fibrinolysin, chondroitin sulphatase, aminopeptidases, glycosidases, phospholipase A_2 and alkaline and acid phosphatases have been described in several species of *Porphyromonas* and *Prevotella* (Rudek & Hague 1976; Bulkacz *et al.* 1979; Steffen & Hentges 1981).

The production of the enzyme collagenase by *Porphyromonas* species has generated much interest. Recent results suggest, however, that *Porphyromonas gingivalis* is the only oral bacterium possessing specific collagenolytic activity (Killian 1981; Kato *et al.* 1984; Mayrand & Grenier 1985; Sundqvist *et al.* 1987).This organism has not , however, been identified in carious dentine or infected root canals.

It has also been shown that enzymes produced by several species of *Prevotella*, *Porphyromonas* and *Fusobacterium*, as well as gram positive pleiomorphic organisms (Carlsson *et al.* 1984) are capable of inactivating or degrading plasma proteins involved in host defence.

Immunoglobulins and complement factors, as well as plasma proteinase inhibitors and plasma proteins of the fibrinolytic, coagulation and kinin systems have all been shown to be degraded or inactivated (Killian 1981; Carlsson *et al.* 1984; Kato *et al.* 1984; Nilsson *et al.* 1985).

The capacity of pathogenic microorganisms to adhere to host tissues is a prerequisite for colonisation and initiation of disease. Studies by Lantz *et al.* (1991) have demonstrated that strains of *P.gingivalis* bind human fibrinogen with high affinity and specificity. These organisms are also able to degrade human fibrinogen. Since fibrin forms the matrix of blood clots and the fibrin network is the scaffold upon which tissue repair in inflammatory lesions occurs, the ability of *P.gingivalis* (and possibly other *Prevotella* and *Porphyromonas* species which have been isolated from carious dentine and infected root canals) to interact with fibrinogen and/or fibrin may allow these bacteria to attach and detach from inflamed tissues, to persist in inflammatory lesions, and to interfere with tissue repair.

Metabolic by-products which are harmful to the host are produced by many of the bacteria in carious dentine. *Prevotella/Porphyromonas* species produce ammonia which may be toxic (Van Steenbergen *et al.* 1986). Butyrate, succinate and propionate have a toxic effect on fibroblasts (Singer & Buckner 1981), and succinic acid inhibits the function of neutrophil polymorphs (Rotstein *et al.* 1985).

The composition of the dentinal flora influences the relative toxicity and concentration of any product because some metabolites are further degraded following consumption by other species. Volatile sulphur compounds are very toxic due to their reactivity with metal-containing enzymes of the organisms encountered within carious dentine. *Fusobacterium, Prevotella/Porphyromonas, Actinomyces, Eubacterium* and *Veillonella* species have all been shown to produce volatile sulphur compounds *in- vitro* (Mink *et al.* 1983).

It is extremely unlikely that a single virulence factor is responsible for all of the tissue damage. A given factor may be essential, but it is unlikely to be sufficient to account for the pathology. Studies of general microbiological systems have shown that a series of virulence factors may well be produced under co-ordinated regulation ie a common regulator is present which co-ordinates the simultaneous production of multiple virulence factors and which is in turn regulated by the environment (eg temperature, osmolarity, Fe and Mg levels).

As an example of this type of co-ordinated regulation, studies of the organism *P.gingivalis* have indicated that iron levels (as haemin) in the immediate environment affect the expression of certain surface proteins involved in adhesion and in virulence in animal models. Local environmental control of virulence factors could explain the presence of presumed pathogens in sites which are healthy or which no longer show disease progression (Gmur *et al.* 1989; Higgins and Hunter 1990 [personal communication])

Overall, there are a number of problems to be overcome in attempting to establish a role for specific organisms in pulpitis. Firstly, the microbial populations in caries in humans have not been fully identified, although certain characteristic organisms are found in carious dentine. These communities of bacteria are presumably determined by multiple environmental factors and are stabilised by interspecies interactions.

Particular difficulties in obtaining further knowledge in this area include:
i) the problem of sampling deep carious dentine without contamination by plaque from unaffected areas where the microbial population may not be relevant;
ii) the problem, in a chronic, episodic disease of determining when active tissue destruction is taking place (ie sampling during an inactive phase may not reveal the presence of the actual bacterial pathogens);
iii) the organisms in dental caries include highly fastidious anaerobes whose successful culture requires special techniques.

In summary, the production of pulpal disease is not always the rule. The first step in the pathogenesis of wound infection is bacterial contamination. Whether this contamination produces infection of the pulp depends on the number and virulence of the organisms and the nature of the host's defences. Studies on germ free animals have shown that exposed pulps will heal completely in the presence of saliva even though 1ml (30 drops) of saliva in the average patient contains 10^9 anaerobes and 10^8 aerobes (Thomas 1972; Glasgow 1972).

Quantitative factors alone may not be responsible for the necrosis of pulpal tissue following the carious process. It is possible that loss of pulp vitality results from the combined effect of specific bacterial actions (ie the type of infection) and the occurrence of a destructive host response.

In an attempt to address the role of specific microorganisms in the aetiology and pathogenesis of pulpitis, and as a first step in the present work, correlations were sought between microbial type and number in sampled carious dentine and cellular responses in dental pulp tissue from vital, carious human teeth. As a result of this work, a significant association between the number of *Prevotella intermedia* and *Prevotella melaninogenica* in carious dentine and extensive, principally mononuclear, inflammatory infiltration was demonstrated, despite a high degree of complexity and inter-subject variability in the microbial flora. There was no apparent association between other microorganisms or total microbial load and histopathological category. Details of this study form the basis of the next chapter of this thesis.

Chapter 2

The association of carious dentine microflora with tissue changes in human pulpitis

2.1. Introduction:

It is known that bacteria and their products play a major role in long term pulpal insult through dental caries and microleakage (Brannstrom 1981). However, there have been surprisingly few detailed studies of either the microbiology of carious dentine (Edwardsson 1974) or of the relation between dentinal microflora and the histopathology of chronic pulpitis (Torneck 1974). Because of this there has been no clear basis for assigning an aetiological role in pulpitis to any particular microbial species.

The present work describes a study of the microbiology and histopathology of carious pulpitis in human teeth. Correlations were sought between microbial type and number and cellular responses in order to elucidate pathogenic mechanisms which may contribute to the destruction of the dental pulp.

2.2. Materials and methods:

2.2.1 Tissues:

Vital carious teeth were obtained with informed consent at the United Dental Hospital of Sydney, Australia, from separate, randomly selected patients of either sex. Patient age ranged from 16 to 52 years with an average age of 34.6 years. Patients from whom teeth to be included in the study were obtained did not report a history of significant medical disease or antimicrobial therapy. Patients had requested extraction to relieve the symptoms of pulpitis and had declined the option of tooth restoration.

Premolar and molar teeth were selected for the study on the basis of clinical diagnostic tests which indicated that they were vital and with clinical symptoms of reversible pulpitis (stimulated pain and heightened sensitivity to hot and cold stimuli) and although all possessed a single large and open untreated primary carious lesion (occlusal or approximal), were without gross (i.e. clinically apparent) exposure of the pulp tissue to the oral environment and were free from all but minimal inflammation of their supporting tissues (with less than 4mm pocket depth).

2.2.2 Tissue processing for microbial analysis:

Teeth were subjected to both microbiological and histopathological analysis. Immediately (less than 30 sec) after extraction under local anaesthesia each tooth was placed in a container of pre-reduced transport fluid (RTF) (Syed and Loesche 1972) and then transferred (in less than 5 mins after extraction) to an anaerobic (85% N_2, 5% CO_2, 10% H_2) glove chamber (Coe Laboratories)and rinsed several times in RTF. Using sterile sharp curettes and excavators, all softened and necrotic dentine was then removed as small fragments from the carious lesion of each tooth. In addition, shavings of dentine were taken from the stained but minimally decalcified deep layers of the lesion using sterile sharp curettes and added to the total dentine tissue sample for each tooth. Carious pulpal exposure at this stage was noted. Dentinal sampling was completed less than 20 min after tooth extraction.

The total sampled dentine from each tooth was transferred separately from the anaerobic chamber in an airtight container, weighed, and a standard solution of 10 mg wet weight dentine/ml of RTF was prepared for each tooth. The fragments of carious dentine were dispersed by vortexing for 20 seconds before being homogenized in a 2ml glass homogenizer using a standardized technique of vertical and rotational movements.

Serial dilutions in the range 10^{-3} to 10^{-6} in RTF were then prepared for plating. Plates were duplicated for each dilution and each medium and incubated in either the glove chamber or for microaerophilic conditions in a small anaerobic jar with a CO_2 gas pack (Oxoid Ltd, Hampshire, England) at 37°C for 48 hours as described below.

Isolates representing six microbial genera: *Lactobacillus, Streptococcus, Fusobacterium, Actinomyces, Prevotella* and *Porphyromonas* were chosen for more detailed study. The following media and incubation conditions were used to study general and selective growth:

Cadmium fluoride acriflavine tellurite (CFAT) agar and gelatin metronidazole cadmium (GMC) agar were used for the selective isolation of *Actinomyces* spp. and related gram-positive filamentous organisms under anaerobic conditions (Zylber and Jordan 1982). These media will also support the growth of *Bifidobacteria, Propionibacterium* and *Arachnia* species (Lennette: Manual of Clinical Microbiology 1985).Because of the limitations of the two recommended media, both were utilised to allow a comparison of their relative selectivity for anaerobic gram positive filamentous organisms.

A kanamycin-vancomycin containing anaerobic blood agar (KVA) was used for the identification of the obligately anaerobic *Prevotella* species. This medium will also support the growth of *Fusobacterium* spp. (Dowell 1975). *Porphyromonas* species were identified on an anaerobic blood agar containing kanamycin alone (KA) (Van Winkelhoff and De Graaf 1983). This medium has supported the growth of *Porphyromonas gingivalis* and *Porphyromonas endodontalis* (Van Winkelhoff *et al.* 1985).

Crystal violet erythromycin (CVE) agar was used as a selective medium for the isolation of *Fusobacterium nucleatum* (Walker *et al.* 1979; Vincent *et al.* 1985).

The selective media detailed above were prepared as described in the Manual of Clinical Microbiology (Lennette, 1985) and the handbook "Media for Isolation, Characterization and Identification of Obligately Anaerobic Bacteria" (US Dept Health & Human Services, 1982).

Rogosa agar (Bacto Laboratories Detroit, Michigan) and Mitis-Salivarius agar (Oxoid Ltd. Hampshire, England) were used to study the growth of lactobacilli and streptococci respectively under microaerophilic conditions and anaerobic conditions (Difco manual (10th edition), Difco Labs, 1984)

An initial group of 20 specimens was examined with the aim of identifying the main colonial forms on selective media. One colony forming unit (CFU) of every CFU-form observed on the plate was identified, then subcultured, gram-stained and tested biochemically using diagnostic kits of the RAPID ANA II, RAPID STR (Innovative Diagnostic Systems, Atlanta, GA.) and ROSCO systems (ROSCO A/S Denmark). Subsequent to this identification, plates were examined and the numbers of each main colony type noted. The presence of *Streptococcus salivarius* colonies was used as an index of salivary contamination of the sample. Results confirmed minimal contamination by this organism.

To confirm the accuracy of this testing, strains of known *Actinomyces*, *Prevotella* and *Porphyromonas* species were used to calibrate the system (NCTC 9931 *A.odontolyticus*, NCTC 9336 *Prevotella intermedia* , ATCC 33277 *P.gingivalis*, respectively). Colonies previously identified as the main morphological types were selected at random and retested in the remaining samples.

Selective microbial load per mg wet weight of dentine was determined by the number of colony forming units (cfu) on the selective media described above. Total microbial load was similarly determined by growth on Trypticase Soy agar enriched with menadione, haemin,

L-cystine and blood (ETSA) under anaerobic and microaerophilic conditions (US Dept Health & Human Services 1982).

2.2.3 Light microscopic examination of pulpal tissue:

Immediately following dentine sampling for microbial analysis, the teeth were removed from the chamber and sectioned incompletely using a high speed dental handpiece with a tungsten carbide bur and water spray. The remaining thin layer of intact dentine over the pulp was split using hand instruments and the whole coronal dental pulp removed. Pulpal tissues which were torn or incompletely recovered, or were obviously necrotic, were rejected from the study along with their associated carious dentine samples. After this step, paired tissues (carious dentine and pulp) from 65 teeth remained in the study.

Pulpal tissue samples were processed by fixation for 4 hours at 4^{o}C with paraformaldehyde-lysine-periodate in cacodylate buffer at pH 7.2 (Senoo 1978). Specimens were dehydrated in graded acetones and infiltrated and embedded at 4^{o}C in JB4 glycolmethacrylate resin embedding material (Polysciences Inc. Warrington, PA.). Three serial sequences of ten sections of 2µm thickness and 100µm apart were cut from each processed tissue block using glass knives and mounted on glass slides, which were then stored with dessicant at 4^{o}C. Representative sections were stained for assessment using haematoxylin or toluidine blue.

Tissue sections were coded prior to examination so that the examiner was unaware of the donor identity. The pulpal tissue area for each section was divided into a series of fields using a graticule eyepiece. All fields were examined initially at low magnification (x 50). Within these fields, areas were selected at random and studied at higher magnification (x 312).

With frequent use of a reference slide for each category, fields were assigned to one only of the following 4 categories in which a particular tissue appearance was dominant.

Category. A. Minimal inflammatory change; an essentially normal tissue pattern with minimal inflammatory infiltrate and disturbance of soft tissue.

Category. B. Soft tissue degeneration; some abnormality of connective tissue architecture but without widespread and significant calcific or inflammatory involvement.

Category. C. Hard tissue degenerative change; evidence of dystrophic calcification as a widespread and dominant pathology.

Category. D. Inflammatory degenerative change; widespread and significant infiltration with chronic inflammatory cells separated by less than one cell diameter.

Each section comprised a number of fields between 4 and 45 depending on cross-sectional area available; 30 sections were examined for each pulp specimen. In this way two indices were derived for each specimen:

(a) the relative incidence of each pathological category and therefore
(b) the dominant pathological category.

2.3. Statistical procedures:

Since a normal distribution of the data could not be assumed, non-parametric methods of statistical analysis were applied. Chi-squared tests, correlation matrices, an analysis of variance, Kruskal-Wallis rank sum and Friedman rank-tests were used to assess interspecies relations and the relations between pathological category and microbial load. All *p*-values given were derived from these tests. For all tests see Dixon and Massey (1983).

2.4. Results:

2.4.1. Qualitative and quantitative assessment of microbial data:

A high degree of complexity and inter-subject variability in both type and number of the flora of the carious lesion was apparent (Table 2.1). Thousandfold differences were noted in total microbial loads between samples. The data for selective microbial groups were very skewed with mean values much greater than median values in all cases. Selective microbial counts varied between teeth by up to 5 orders of magnitude per milligram wet weight of dentine.

The relative numbers of organisms grown on non-selective media under microaerophilic conditions compared with anaerobic conditions also varied considerably. On average, however, the number of organisms cultivable anaerobically was one order of magnitude greater than that cultivable microaerophilically.

Gram-negative organisms were seen to comprise a significant percentage of the total flora in some specimens (with *Prevotella / Porphyromonas* species comprising at peak incidence up to 20% and *Fusobacterium* species up to 9%.) In contrast, *Lactobacillus* spp. were isolated in a lower frequency than in previous studies of carious dentine.

Five predominant colony forms were identified using CFAT and GMC media on the basis of morphological criteria of which two were *Actinomyces* species and three were related gram-positive filamentous organisms. Similar colony forms were identified on each selective medium. After gram staining these were subcultured and biochemically tested for tentative identification at species level. Biochemically these colonies resembled *Actinomyces israelii*, *Actinomyces odontolyticus*, *Bifidobacteria* species, *Propionibacterium propionicus* and other *Propionibacteria* species. The two *Actinomyces* species were numerically dominant.

Four main colony types were consistently identifiable morphologically on KVA and KA of which three were *Prevotella* species on the basis of gram stain and biochemical testing. Biochemically these resembled *Prevotella intermedia, Prevotella melaninogenica* and *Prevotella buccae* groups. *P. melaninogenica* was numerically dominant. Neither *P. gingivalis* nor *P. endodontalis* species were identified on either medium. Two colony types were evident morphologically using CVE and biochemically both resembled *F. nucleatum.*

Streptococci were studied via the selective medium Mitis-Salivarius agar. Four main colony types could be identified morphologically. Biochemically two of these resembled *Streptococcus sanguis* and two *Streptococcus anginosus.*

Lactobacilli were grown on Rogosa agar and two main colony forms were identifiable. When tested biochemically these resembled *Lactobacillus acidophilus* and *Lactobacillus casei.*

2.4.2. Quantitative assessment of pulpal histopathology:

Of the specimens studied, 47% presented an essentially normal histology on the basis of minimal inflammatory infiltrate and disturbance of soft tissue architecture in all fields examined (Category A). The remainder of the specimens showed pathological changes within fields in one or more of the following categories. The categories were not mutually exclusive, i.e. a single specimen may have included more than one type of pathology; however, for purposes of description they are considered by dominant category.

In 21% of specimens the dominant category was a degenerative change within the connective tissue but without significant inflammatory cell infiltration or dystrophic calcification (Category B, Figure 2.1 a&b). These degenerative changes included pulpal fibrosis with corresponding reductions in cellularity similar to changes seen within connective tissue as the individual ages.

In addition, changes to the microvasculature were noted when fields were examined under high power. Many of the small vessels showed marked thickening of their walls with corresponding decreases in lumen diameter. This finding was noted in pulp specimens from patients of widely varying ages.

The dominant pathological change was dystrophic calcification (Category C) in 11% of samples. Calcific deposits were invariably associated with amorphous partially mineralized matrix. The remaining soft tissue showed evidence of fibrosis and small vessel change as noted previously (Figure 2.2).

In sections with widespread and significant inflammatory change (Category D) the infiltrate was principally mononuclear with the plasma cell dominant (Figures 2.3 & 2.4 a&b). This type of pathology predominated in 21% of specimens. Lesser numbers of lymphoid cells and macrophages were also present. Both mature and degenerate plasma cells were noted lying in a degraded connective tissue matrix. In some sections, necrotic foci surrounded by neutrophil polymorphs and large numbers of "foamy" macrophages separated the chronic inflammatory infiltrate (Figure 2.5). In many of these foci, bacteria could be detected under high power. Many of the small vessels in these areas of inflammatory activity also displayed features resembling high endothelial venules.

In some Category D specimens, large numbers of plasma cells were clustered and concentrated in the vicinity of small blood vessels. Whether in clusters or singly, the plasma cells were often seen in close association with normal or degenerate fibroblasts. Lymphoid cells and macrophages were also seen with this arrangement. Lymphoid cells or immature plasma cells comprised the second most common cell type in inflamed areas. Degenerative changes appeared more often in association with the plasma cells than other cell types. The clusters of plasma cells did not necessarily represent groupings of cells of the same level of maturity.

Disruption of phagocytic cells led to the presence of extracellular lysosomes in several areas. The extracellular areas of the pulp showed evidence of generalised oedema. In some such specimens vacuoles were noted. Microorganisms were seen extracellularly in areas where necrotic debris was present. Compared with less severely inflamed pulpal tissue, where the lymphocyte and plasma cell were the dominant cell types, there were large numbers of neutrophil polymorphs and macrophages. Macrophages were particularly numerous and frequently seen with groups of lymphocytes and plasma cells. Multinucleate giant cells were also occasionally seen.

In more inflamed portions of these pulps, there was evidence of fibroblast degeneration and degradation of intercellular collagen. Degenerative changes in blood vessels ranged from subtle changes in cell morphology to total cell necrosis. Endothelial cell lysis, disruption of vessel walls, thrombosis of capillaries and venules, as well as extravascular haemorrhage was noted. In 11% of those specimens showing significant inflammatory change there was evidence of haemorrhage and necrosis in relation to leukocyte infiltration (Figure 2.6 a-d).

2.5. Correlation:

Multivariant analyses were performed on the histopathological and microbiological data (data is presented in Tables 2.2-2.6) Examination of the association between microbial load and histopathological category revealed:

i) no significant relation between total (anaerobic plus microaerophilic) microbial load per mg wet weight of dentine and histopathological category.

p values 0.817 and 0.274 respectively (Kruskal-Wallis).

ii) no significant relation between histopathological category and selective microbial load for the microbial genera of *Streptococcus, Actinomyces, Lactobacillus* and *Fusobacterium.*

p values 0.167, 0.239, 0.259 and 0.778 respectively (Kruskal-Wallis).

iii) that although *Prevotella* spp. counts comprised at peak only 21% of total isolates regardless of pathological category, there was a significant ($p < 0.05$) relation between the number of *P. intermedia* and *P. melaninogenica* and those specimens in which the dominant pathological picture was one of extensive inflammatory infiltration (Category D).

p value 0.038 Kruskal-Wallis.

Using correlation matrices, relations between pairs of microbial species were examined. Correlation coefficients were calculated on all of the data and for individual pathological categories (Tables 2.7-2.11).

Overall, there was a direct relationship, i.e., a correlation significant at the 5% level amongst the numbers of the anaerobic microbial species (Table 2.7). There was also a correlation of significance between counts of the *Prevotella* species in all categories of pathological change (Tables 2.7-2.11). In contrast, there was poor correlation between *Lactobacillus* species counts and all pathological categories (Table 2.7).

Figure 2.1 (a &b) : Soft tissue degenerative changes (Category B) included fibrosis and basement membrane thickening in associated small vessels.Note basement membrane duplication (BM), sparse lymphocytic infiltrate (L), and dystrophic calcification (C). Scale bar indicates 50μm.

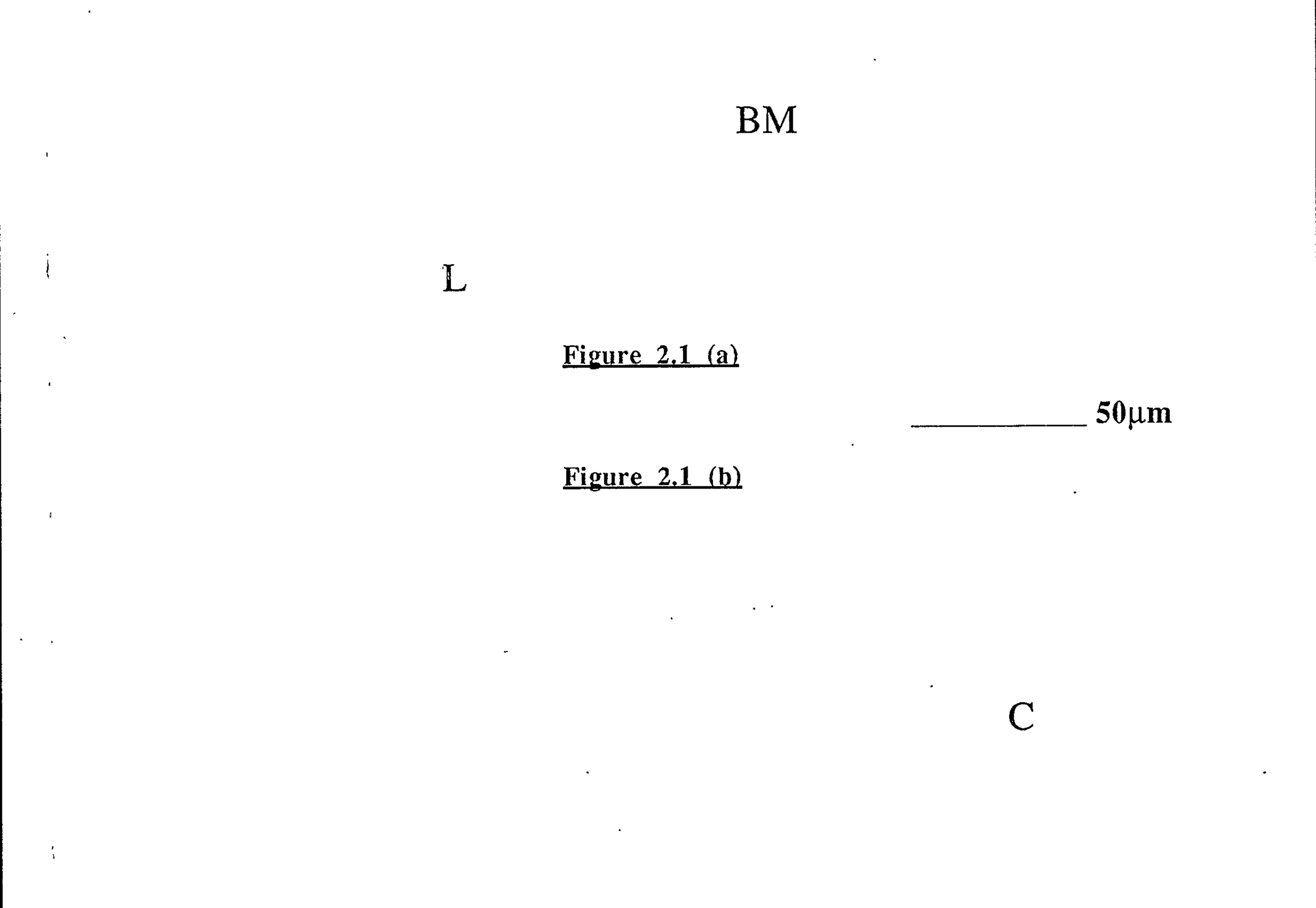
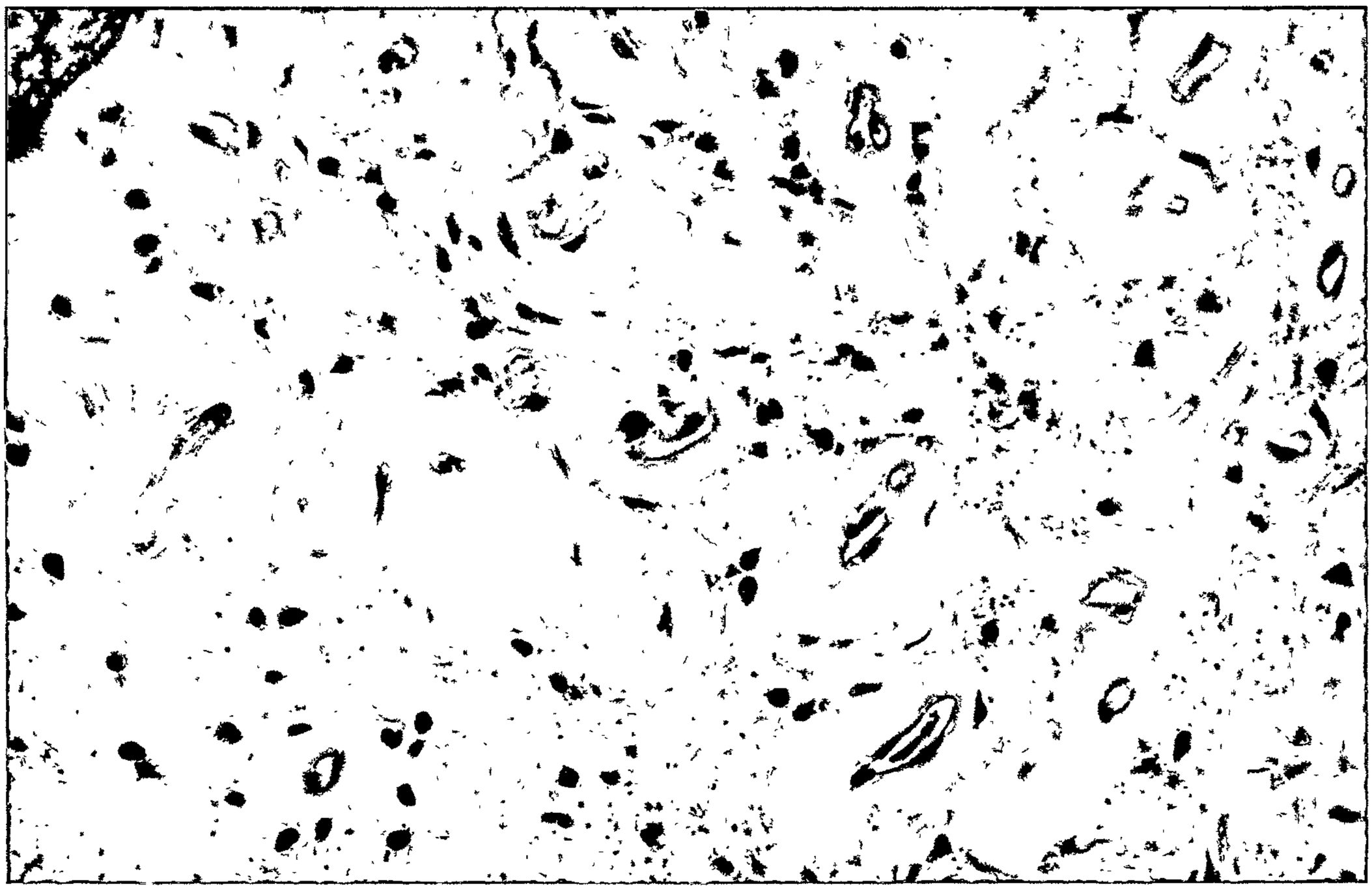


Figure 2.1 (a)

Figure 2.1 (b)



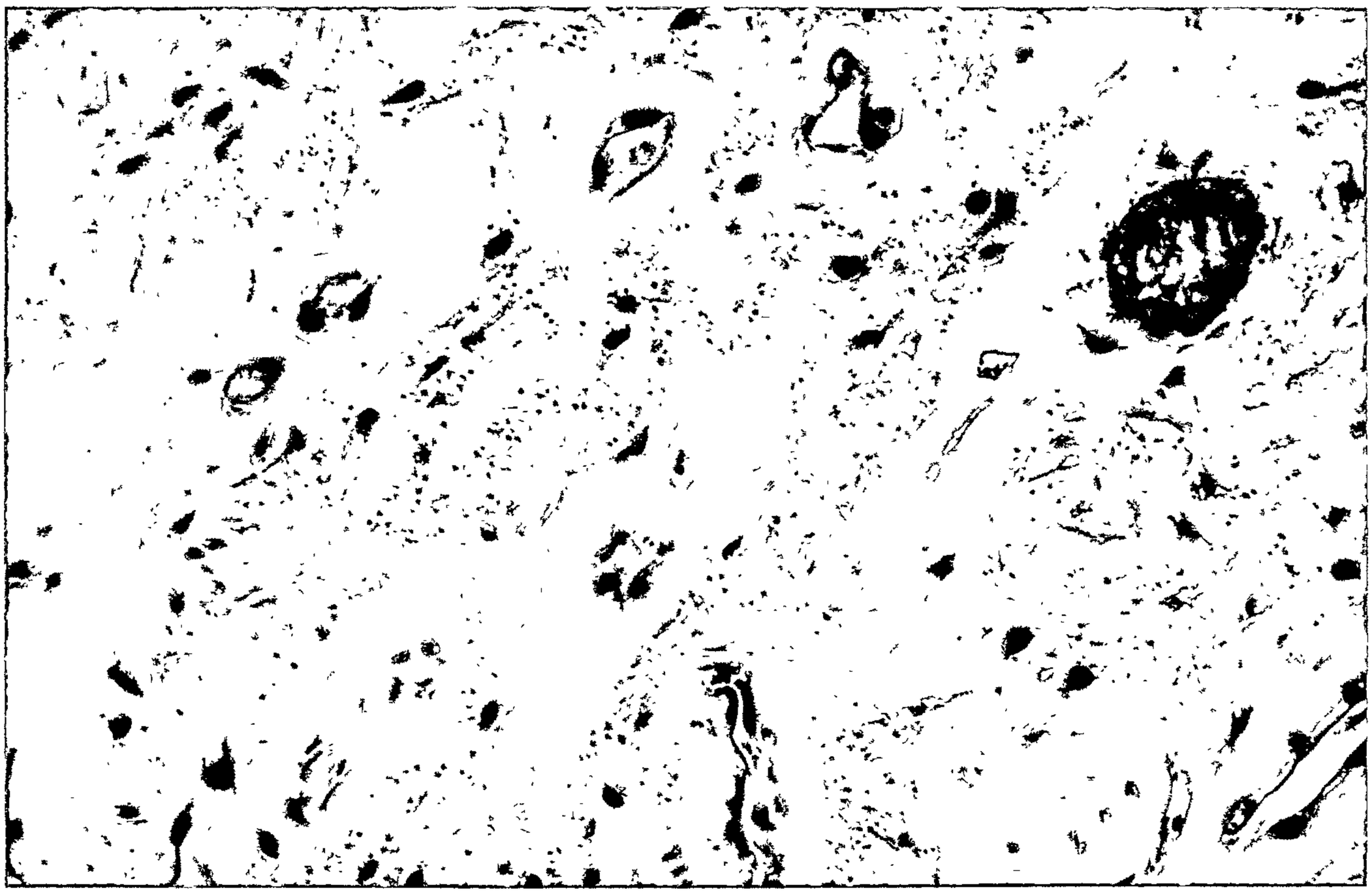


Figure 2. 2: (Category C) Calcific foci (C) are associated with amorphous, partially mineralized connective tissue matrix (M) and degenerate cells and tissue. Scale bar indicates 50μm.

C

M

50μm

Figure 2.2

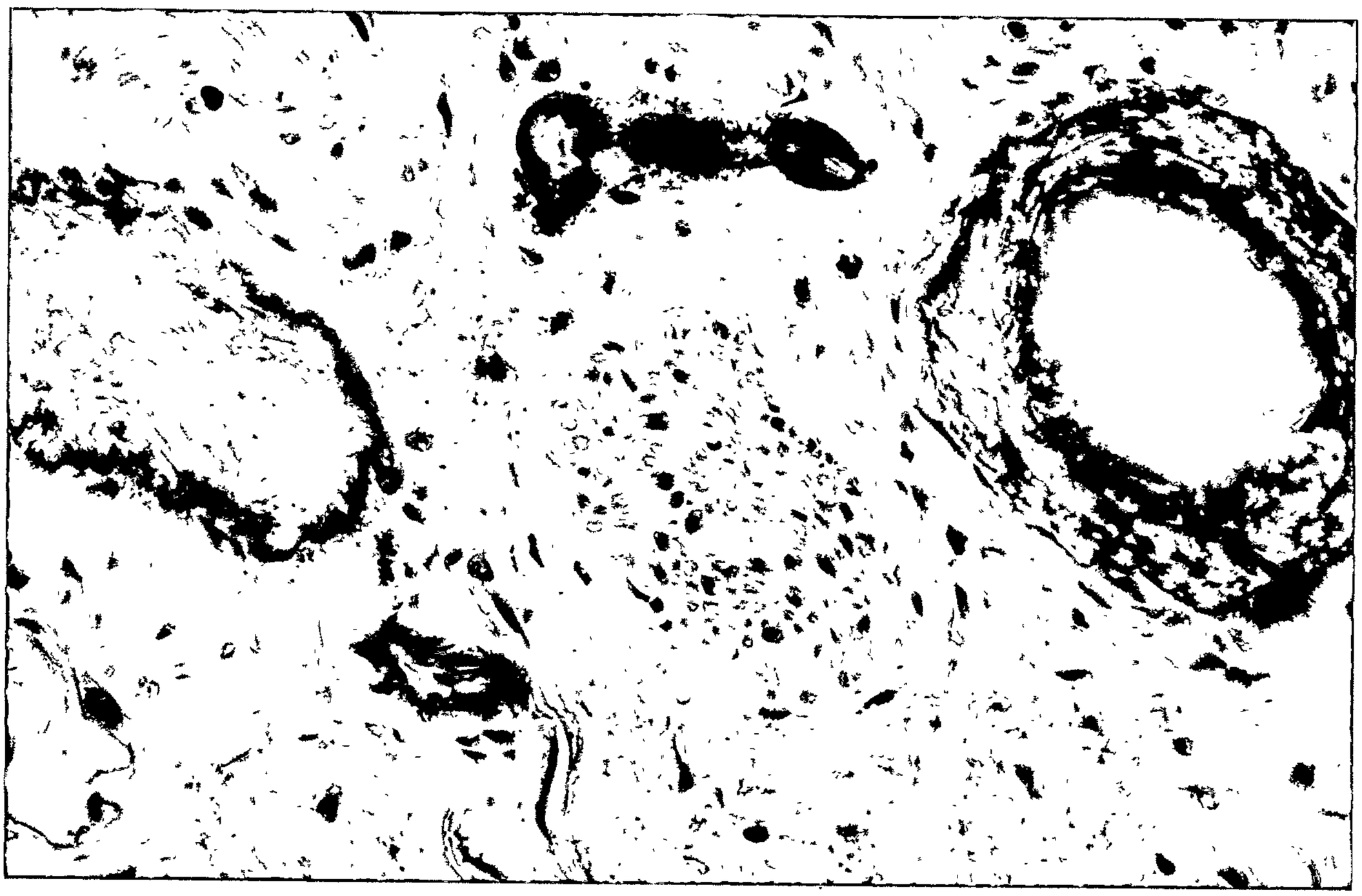


Figure 2.3: (Category D) An infiltrate of varying numbers of lymphocytes, monocytes/macrophages and plasma cells forms within the pulpal connective tissue. Capillaries may be engorged and increased in number and many small vessels in the area of infiltration display features resembling high endothelial venules (HEV).Scale bar indicates 50μm.

HEV

50μm

Figure 2.3

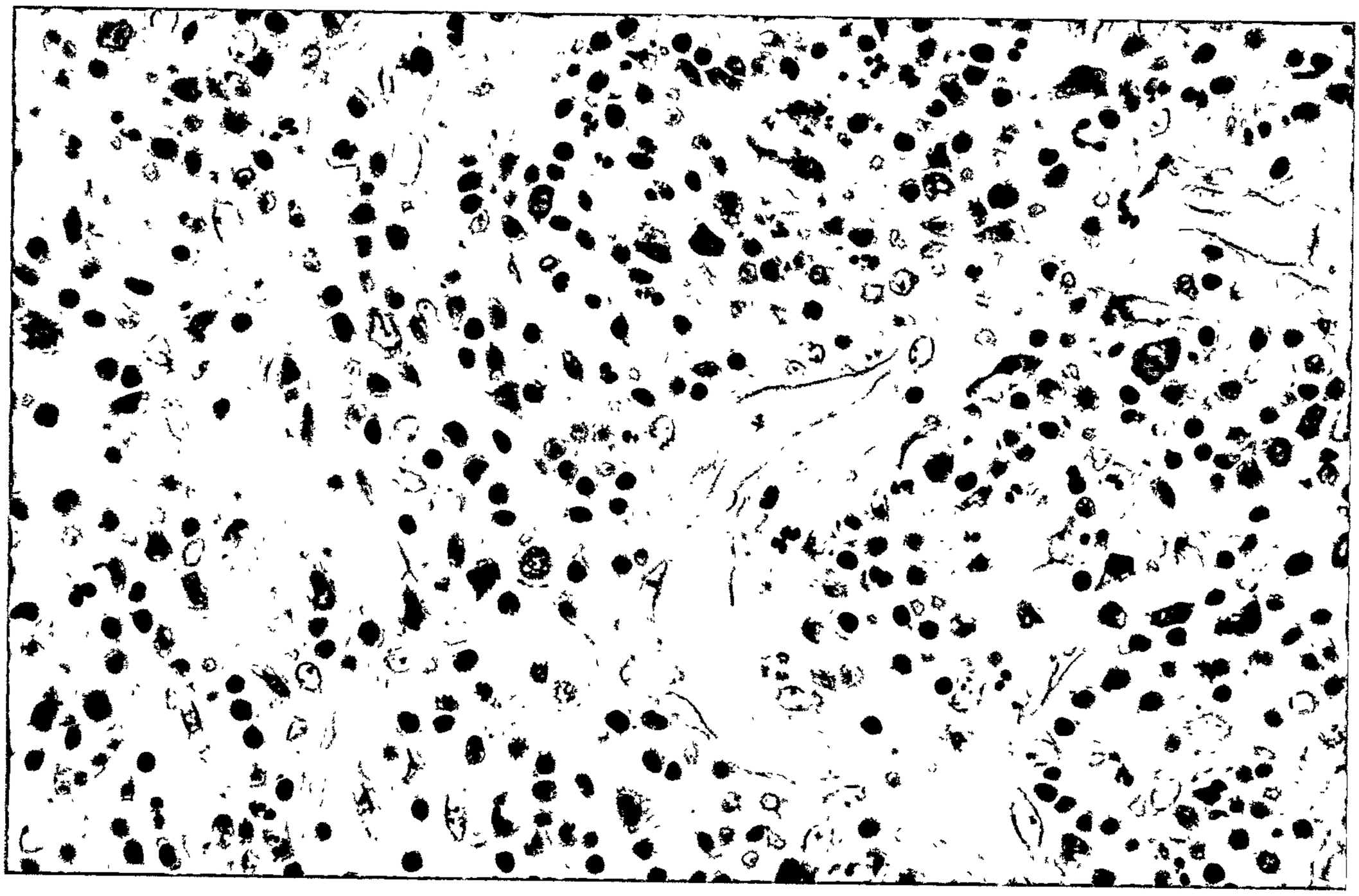


Figure 2.4 (a & b): (Category D) Plasma cell aggregates become established within the degraded connective tissue framework (PL). Many of these plasma cells are degenerate (D). Lymphocytes and macrophages are present but in lesser numbers.Scale bar indicates 50μm.

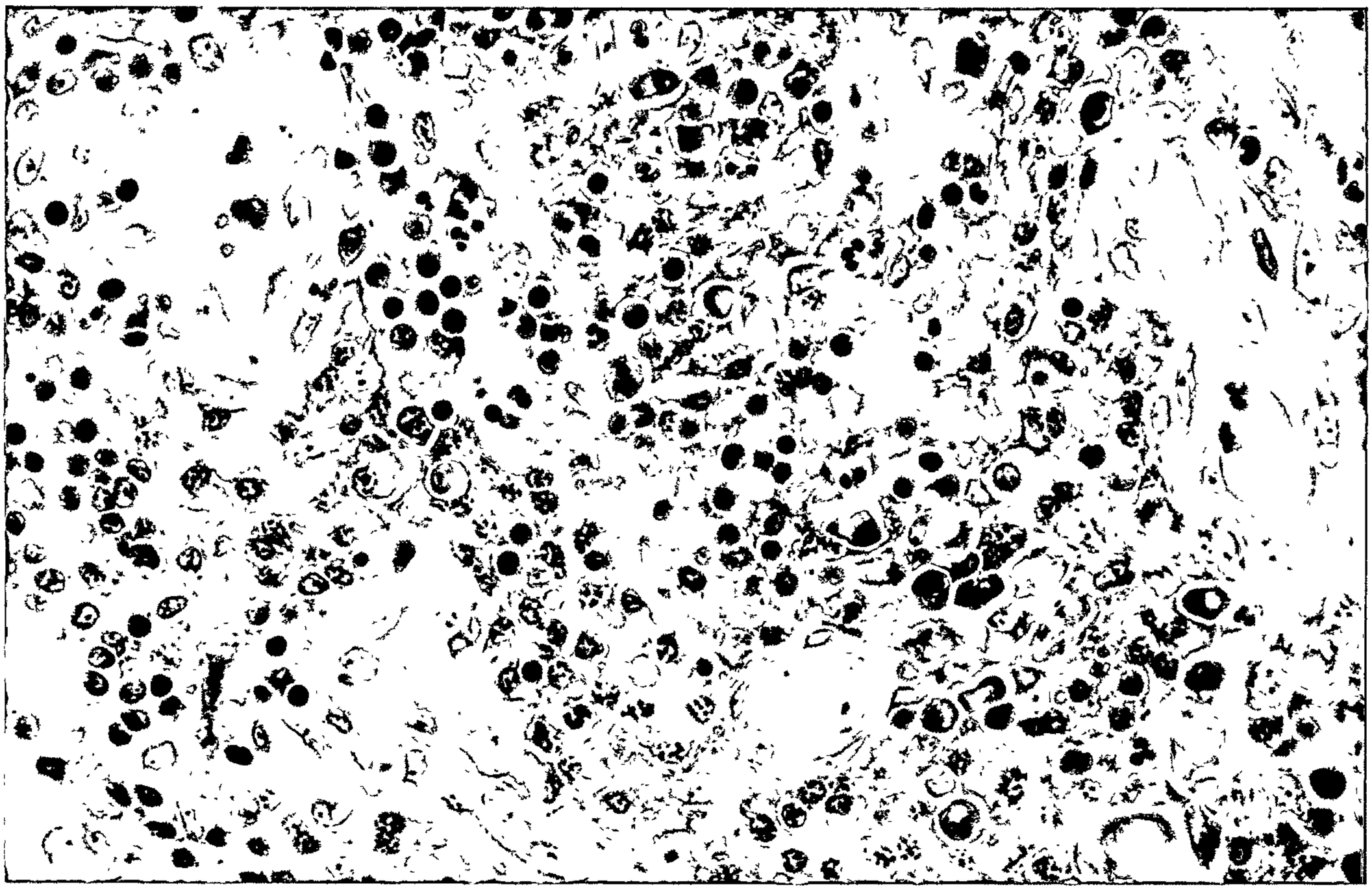
D

Figure 2.4 (a)

50μm

Figure 2.4 (b)

PL



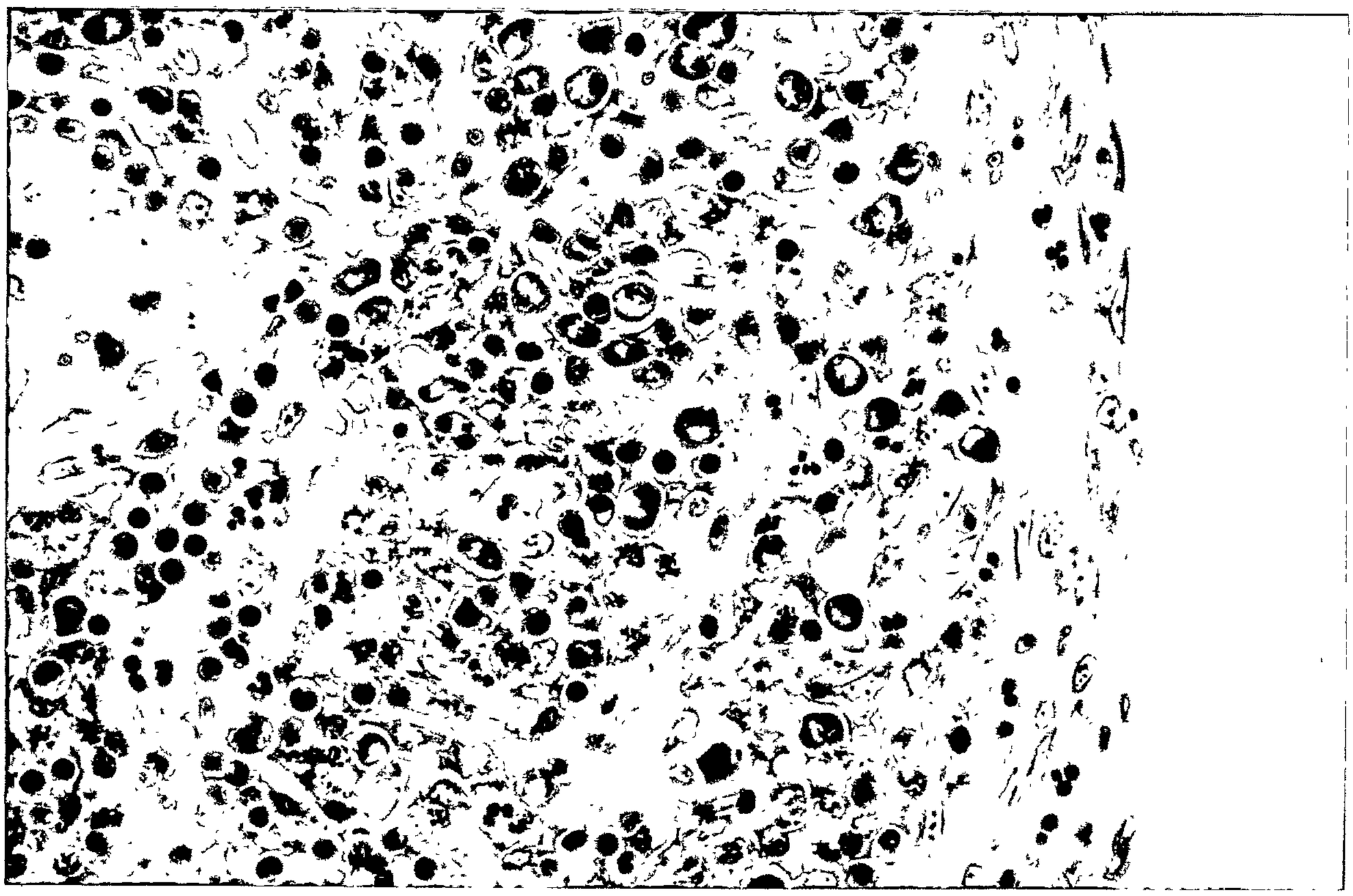


Figure 2.5: (Category D) Areas of acute inflammation and necrosis are also noted within the tissue. "Foamy" macrophages (F) can be seen surrounding neutrophil polymorphs (N), bacterial clumps (B) and necrotic cells and tissue. Scale bar indicates 50μm.

F

N

B

50μm

Figure 2.5

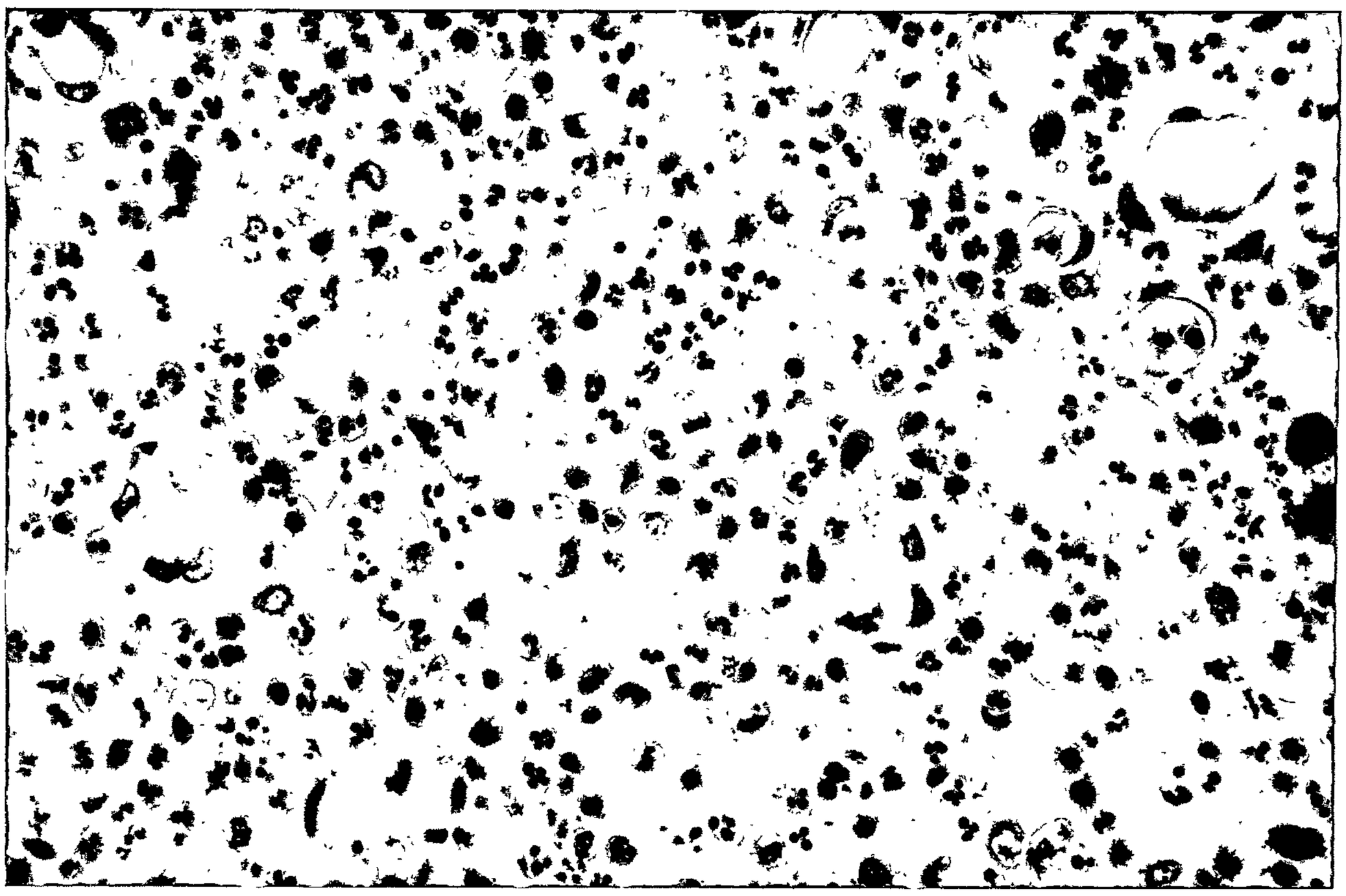


Figure 2.6 (a-d): (Category D) Haemorrhagic changes were seen in 11% of sections showing leukocyte infiltration. Note extravascular haemorrhage (EH) and chronic inflammatory infiltrate (L). Scale bar indicates 50μm.

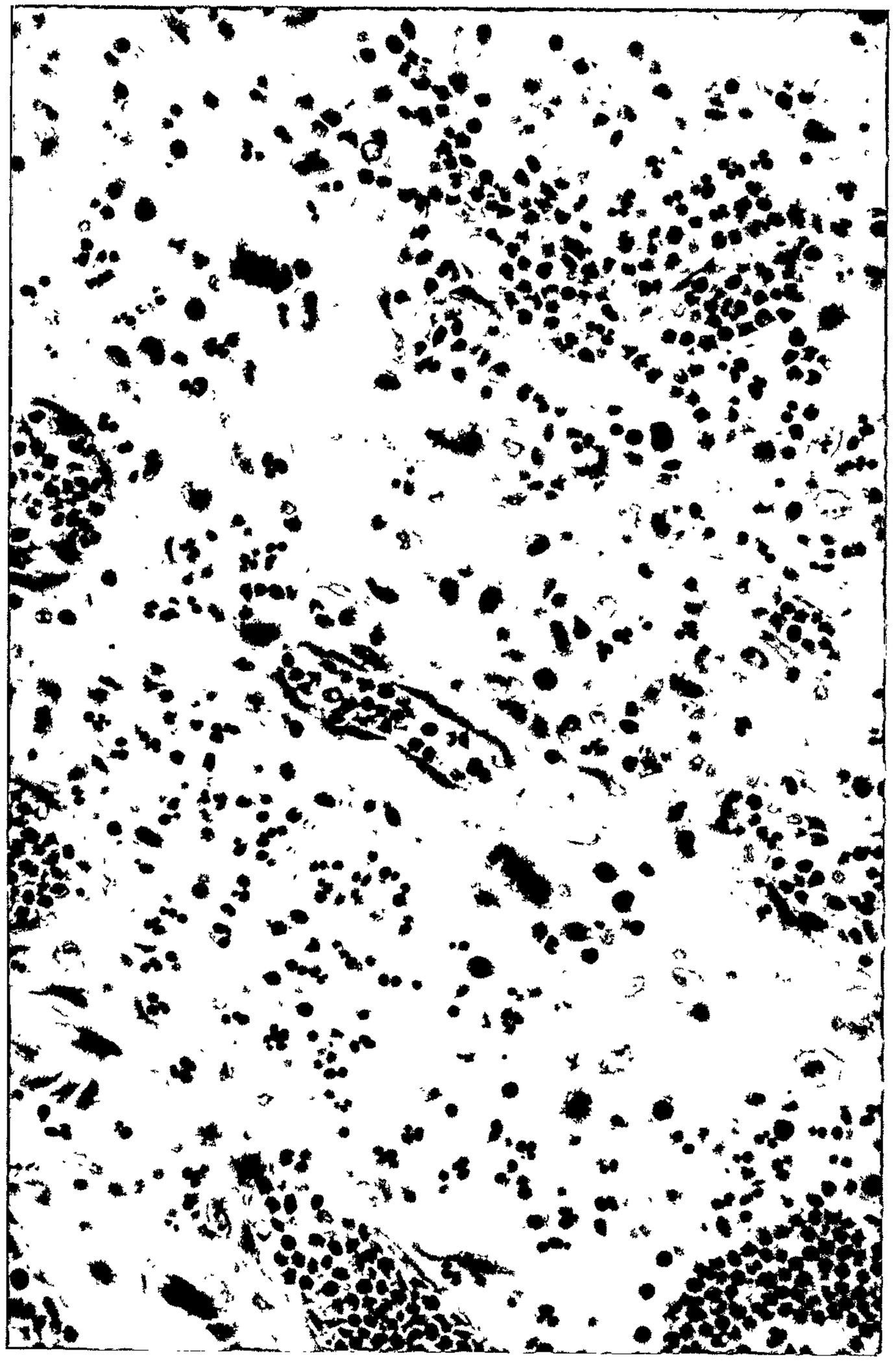
EH

Figure 2.6 (a)

50μm

Figure 2.6 (b)

L



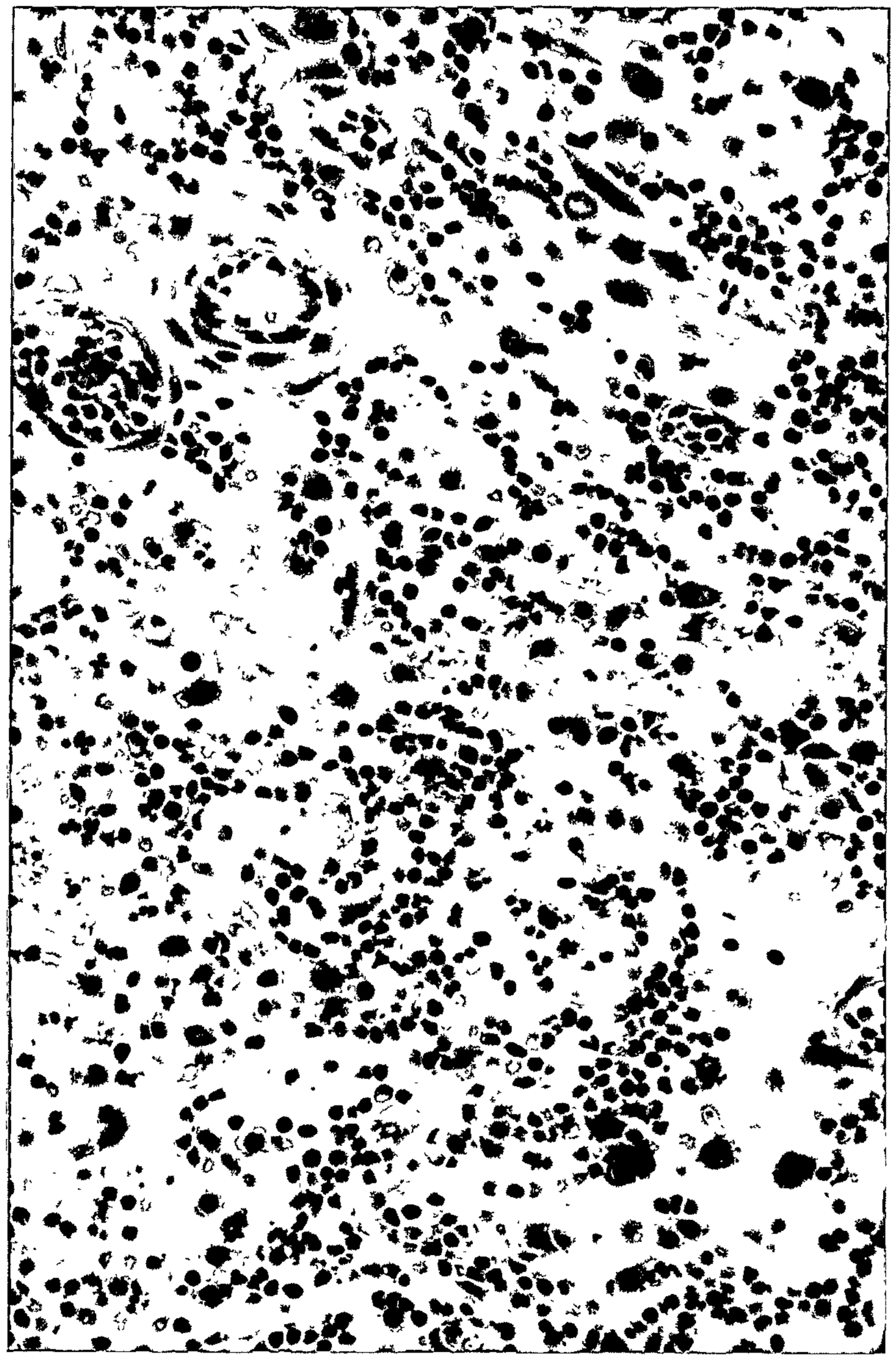
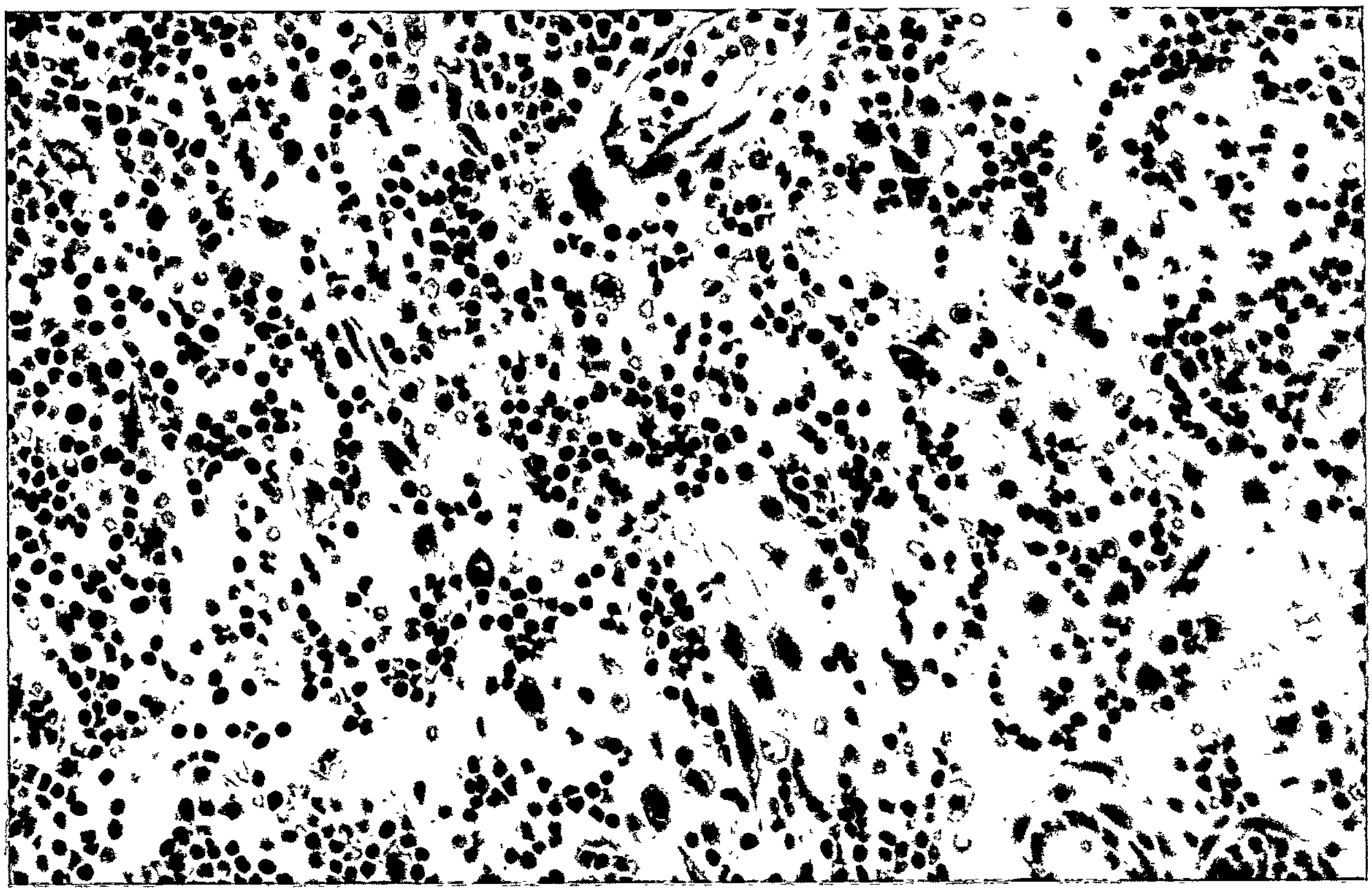
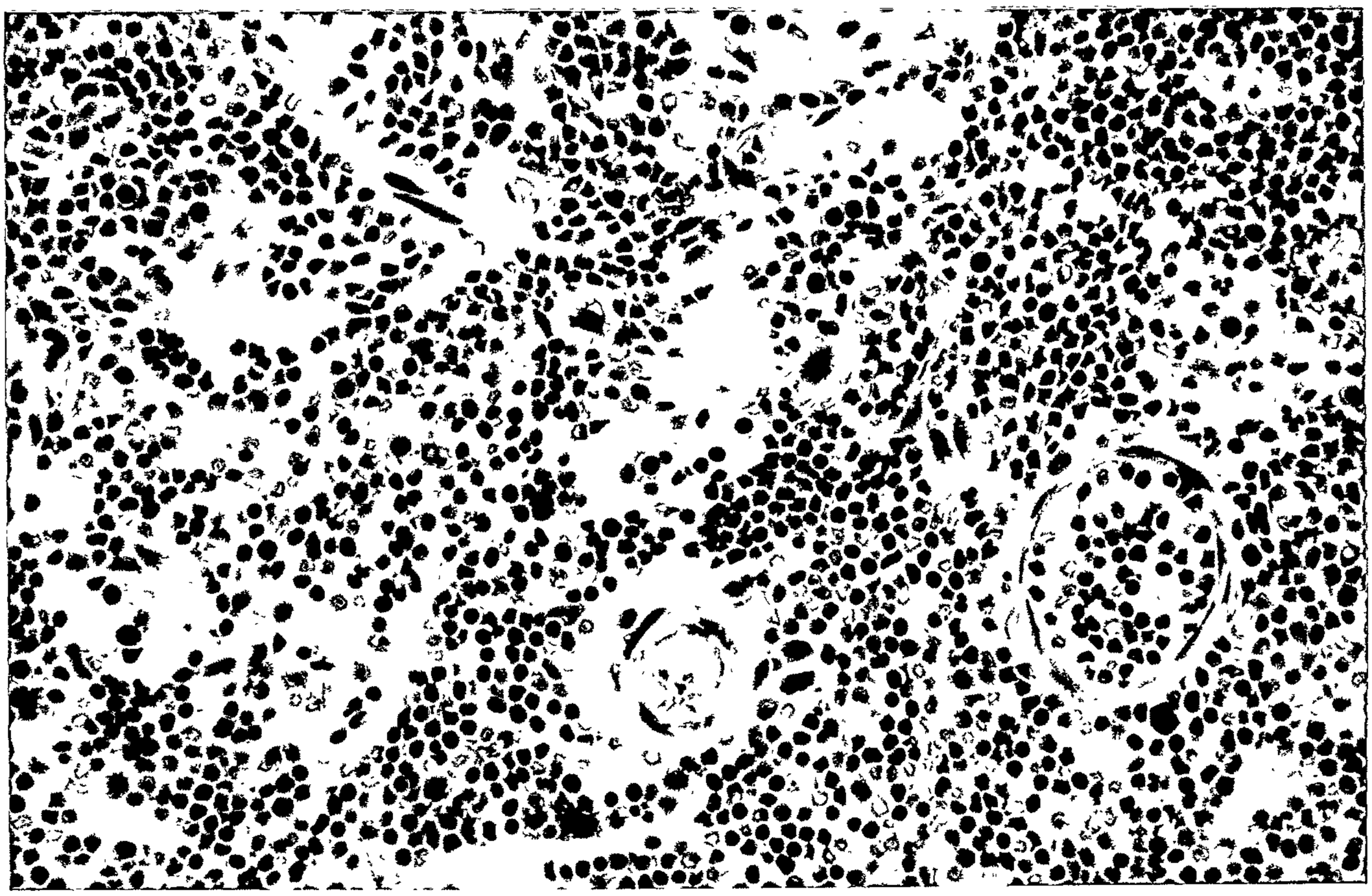


Figure 2.6 (c)

50μm

Figure 2.6 (d)





The correlation between *Prevotella* and *Actinomyces* species counts was statistically significant in all categories except Category A ($p = 0.005$, 0.012 and 0.00015 respectively). Statistical significance was highest when inflammatory degenerative change was noted as the dominant pathology ($p = 0.00015$) (Table 2.12).

2.6.Discussion:

2.6.1. Microbiological results:

The variability in microbial counts between specimens has been described in previous studies of carious dentine (Loesche and Syed 1973; Edwardsson 1974; Hoshino 1985). However, results from the present study differed in that gram-negative organisms were seen to comprise a significant percentage of the total flora in some specimens with *Prevotella / Porphyromonas* species comprising at peak incidence up to 21% and *Fusobacterium* species up to 9%. In contrast, *Lactobacillus* spp. were isolated in a lower frequency than in previous studies of carious dentine. This may reflect the selection process for teeth in our study; teeth were chosen because of extraction for relief of symptoms of pulpitis, rather than for the presence of carious lesions alone.

The interdependence of bacterial species was highlighted in the present study by the strong association between *Prevotella* and *Actinomyces* species, particularly in those specimens with pronounced inflammatory cell infiltrates. As none of the *Actinomyces* species studied was significantly associated with pathological change, the role of such organisms may be to provide factors such as vitamin K and succinate for the growth of prevotella strains (MacDonald *et al.* 1963; Mayrand and McBride 1980).

It is also known that intercellular adhesion between bacterial species is a mechanism through which pathogenic bacteria can be retained *in-situ*. Oral commensals, such as streptococci and actinomyces, are known to utilise a network of specific cell-to-cell interactions to maintain heterogeneous aggregates *in vitro* (Cisar *et al.* 1979).

In a recent study, (Kolenbrander *et al.* 1985) it was demonstrated that the *Prevotella* species *intermedia* and *melaninogenica,* and *Porphyromonas gingivalis* were able to coaggregate with a variety of gram-positive organisms including *Actinomyces israelii, Actinomyces viscosus, Actinomyces naeslundii, S.sanguis* and *Streptococcus mitis*.

Of the species studied only the *Prevotella* spp. were significantly associated with pathological change, namely the plasma cell infiltrate. This finding was related to the grouping of *P.intermedia* and *P.melaninogenica.* Neither species alone , or *P.buccae* in any combination, was implicated in an aetiological role.

On the basis of cultural characteristics and biochemical properties it appeared likely that for *P.intermedia* and *P.melaninogenica* ,the same organisms were isolated from multiple samples.

It is possible that the association between *Prevotella* species in dentine and extensive cellular damage in pulp was a function of time only, that is, that both occurred late in the sequence of advance of the carious lesion and that there was no causal relation between the two. However, given the association between the presence of these bacterial species and the advanced and destructive lesion of periodontitis (Van Winkelhoff *et al.* 1988), the present observation is worthy of note, since it may well explain the clinically observed variability of pulpal responses to remote carious lesions.

In this study *P.endodontalis* and *P.gingivalis* were not isolated from carious dentine. The ecological niche of *P. gingivalis* seems to be the dental plaque, although it has been isolated from mucosal surfaces and saliva (Zambon *et al.* 1981). *P.endodontalis* has been isolated from alveolar abscesses of endodontic origin and occassionally from dental plaque and oral mucosal surfaces (Sundqvist 1976).

The pathogenicity of *Prevotella* and *Porphyromonas* species has been extensively studied as synergistic anaerobic infections in animal models (MacDonald *et al.* 1963). Ten species are recognised and the majority of these can be isolated from the oral cavity where the group are associated with gingivitis, periodontitis, endodontic infections and alveolar abscesses.Three of the species have been implicated in oral infections: *P.gingivalis* in active periodontitis (Slots 1977; Van Winkelhoff 1986); *P.endodontalis* in endodontic infections and alveolar abscesses (Van Winkelhoff *et al.* 1985; Van Winkelhoff 1986); and *P.intermedia* in gingivitis, periodontitis, endodontic infections and alveolar abscesses (Slots 1982; Van Winkelhoff *et al.* 1985).

The large number of virulence factors demonstrated by *Prevotella* and *Porphyromonas* species explains their pathogenic potential and virulence. Proteolytic activity for a number of biologically important substances including inflammation-associated proteins such as C3,C5, immunoglobulin and α2-macroglobulin appears to be most important (Carlsson *et al.* 1984;

Sundqvist *et al.* 1985). *Prevotella* and *Porphyromonas* species also possess a number of structural features that aid colonisation and promote resistance to phagocytosis (Slots and Gibbons 1978; Van Steenbergen 1981; Sundqvist *et al.* 1982).

In addition,these organisms can produce a range of leukotoxic substances which directly inhibit chemotaxis (Rotstein *et al.* 1985). *Prevotella* and *Porphyromonas* species can also produce a number of other substances such as phospholipase, alkaline and acid phosphatases and volatile sulphur compounds that are deleterious to the host (Bulkacz *et al.* 1979; Tonzetich and McBride 1981).

Previous investigators (Edwardsson 1974; Van Winkellhoff *et al.* 1985) have also noted that the composition of the microflora of the carious lesion alters as microorganisms advance towards the pulpal tissues. There is an alteration in the nature of the microflora from gram positive facultative rods in the shallow carious lesion to facultative cocci and anaerobic rods in infected and symptomatic pulp tissue to anaerobic gram negative organisms in the necrotic pulp and periapical abscess.

The significance of anaerobic gram negative rods in deep carious dentine is not yet understood. However, the recent demonstration of a positive correlation between the presence of *Bacteroides* (a term which embraces *Prevotella* species) in sampled carious dentine and reported thermal sensitivity (Hahn *et al.* 1991a) is of relevance to the present work .

Additional support for the present study derives from another recent investigation of the microbiology of carious dentine obtained from human teeth with irreversible pulpitis (Hahn *et al.* 1991b). Two main categories of carious lesion could be identified on the basis of the type and number of the predominant flora: a *Lactobacillus* spp. high lesion and a low *Lactobacillus* spp. lesion. Over 70% of lesions studied (a total of 29 tooth samples were obtained from consenting adults) were composed of predominantly gram-positive rods, and the majority of these tested biochemically and enzymatically as lactobacilli. Gram negative rods were identified in only low numbers except in three specimens where they comprised a significant proportion of the total cultivable flora (12-20%). These specimens also contained only low numbers of lactobacilli, but increased numbers of gram positive filamentous organisms such as propionibacteria, eubacteria, bifidobacteria, arachnia and actinomyces as well as gram negative rods such as black pigmented *Bacteroides* (now *Prevotella* species), *F.nucleatum* and *Eikenella corrodens*. Propionibacteria, eubacteria, arachnia, bifidobacteria and actinomyces were also recovered from carious dentine in the present study and have

been isolated from necrotic dental pulps (Sundqvist *et al.* 1979) and infected root canal dentine (Ando and Hoshino 1990).

Hahn *et al.* (1991b) postulated that the transition from a predominantly gram positive facultative microflora to a far more diverse and increasingly anaerobic flora may be associated with the development of pulpal necrosis.

On the basis of the results presented in this chapter, it appears worthwhile to examine further the hypothesis that *Prevotella/Porphyromonas* species may play an important aetiological role in pulpitis.

2.6.2. Histopathological results:

In the present study data obtained by high resolution and semi-quantitative techniques emphasised the variability of the tissue response in chronic pulpitis. Of particular note, however, is the frequency of extensive plasma cell infiltration in 21% of specimens studied. Cells in varying degrees of degeneration were evident in some portions of all of these specimens. The cells of the inflammatory infiltrate represented the majority of cells affected in this way. Resident cells of the pulp, primarily fibroblasts, also displayed degenerative changes. The incidence and extent of these changes was far greater in those specimens associated with a high incidence of *Prevotella* spp. than in those showing early inflammatory change.

The plasma cell infiltrates were also associated with a degraded connective tissue matrix in a similar way to changes seen in chronic periodontitis where they are the hallmark of the destructive lesion (Page and Schroeder 1976). There were other similarities to the histopathology of chronic periodontitis including the vascular reponse where hyaline-thickening of microvascular vessels (Zoellner and Hunter 1989a) and the presence of high endothelial-like venules were noted (Zoellner and Hunter 1989b).

Lymphocytes have been postulated to play a role in periodontitis and pulpitis since their detection as a major component of the inflammatory cell infiltrate. In both pulpitis and periodontitis, histology and immunochemistry indicate that B lymphocytes and plasma cells are the predominant inflammatory cells in established lesions (Torneck 1974). In both diseases there is also evidence that lesions progress from an initial acute inflammatory and polymorph dominated lesion to a T cell dominated lesion and finally to a B cell dominated and progressive lesion (Pulver *et al.* 1977; Seymour *et al.* 1979). The B cell lesion is characteristic of advanced tissue destruction.

A recent study of B and T lymphocytes in inflamed pulpal tissue has demonstrated an increased proportion of T helper cells and B lymphocytes in irreversible lesions (Hahn *et al.* 1989). On the basis of this, the authors hypothesised that the irreversibly inflamed pulpal lesion was the result of an uncontrolled activation of T helper cells and B lymphocytes in response to intense and continued antigen influx. Details of possible immunopathogenic mechanisms are discussed and investigated in the following chapters of this thesis.

Table 2.1

MEAN BACTERIAL SPECIES* COUNT PER MG DENTINE

	MEAN	ST.DEV	MIN	MEDIAN	MAX	PEAK INCIDENCE
ANAEROBIC	$5.0x10^8$	$7.5x10^8$	$5.0x10^4$	$9.2x10^8$	$3.0x10^9$	
MICROAEROPHILIC	$6.3x10^7$	$8.6x10^7$	10^5	$3.0x10^7$	$3.7x10^9$	
PREVOTELLA	$1.9x10^7$	$5.0x10^7$	0	$1.3x10^6$	$3.1x10^8$	21
P. INTERMEDIA	$5.2x10^6$	$1.2x10^6$	$10^{3\#}$	$2.5x10^5$	$6.3x10^7$	
P. MELANINOGENICA	$1.8x10^7$	$4.5x10^7$	$5x10^{2\#}$	$1.4x10^6$	$2.4x10^8$	
P. BUCCAE	$2.0x10^6$	$3.9x10^6$	$5x10^{2\#}$	$3.8x10^5$	$1.8x10^7$	
FUSOBACTERIA	$1.2x10^7$	$2.2x10^7$	0	$2.6x10^6$	$1.2x10^8$	8.2
ACTINOMYCES	$2.8x10^7$	$5.1x10^7$	0	$7.9x10^6$	$3.2x10^8$	31
STREPTOCOCCI	$3.1x10^7$	$5.6x10^7$	$3x10^4$	$7.2x10^6$	$2.7x10^8$	61
LACTOBACILLI	$1.3x10^7$	$2.8x10^7$	0	$1.3x10^6$	$1.4x10^8$	71

Data collected from 65 samples. Mean bacterial count for the species listed for all teeth sampled per mg wet weight of dentine; standard deviation for each mean; minimum, median and maximum count values; peak incidence as a percentage of cultivable flora. Number of teeth positive for isolates: *Prevotella* spp 57/65 (88%); *Fusobacterium* spp 63/65 (96%); *Actinomyces* spp 64/65 (98%); *Streptococcus* spp 65/65 (100%); *Lactobacillus* spp 62/65 (95%)

*for *Prevotella* species data for the three subspecies *intermedia*, *melaninogenica* and *buccae* are shown separately.

#when present.

Table 2.2

Microbiological statistics for all pathological groups

All data	mean	standard deviation	min	median	max
Microaerophilic	$6.3x10^7$	$8.6x10^7$	10^5	$3.0x10^7$	$3.7x10^9$
Anaerobic	$5.0x10^8$	$7.5x10^8$	$5.0x10^4$	$9.2x10^8$	$3.0x10^9$
Prevotella	$1.9x10^7$	$5.0x10^7$	0	$1.3x10^6$	$3.1x10^8$
Fusobacterium	$1.2x10^7$	$2.2x10^7$	0	$2.6x10^6$	$1.2x10^8$
Actinomyces	$2.8x10^7$	$5.1x10^7$	0	$7.9x10^6$	$3.2x10^8$
Streptococcus	$3.1x10^7$	$5.6x10^7$	$3x10^4$	$7.2x10^6$	$2.7x10^8$
Lactobacillus	$1.3x10^7$	$2.8x10^7$	0	$1.3x10^6$	$1.4x10^8$
P.intermedia	$5.2x10^6$	$1.2x10^6$	10^3 *	$2.5x10^5$	$6.3x10^7$
P.melaninogenica	$1.8x10^7$	$4.5x10^7$	$5.0x10^2$ *	$1.4x10^6$	$2.4x10^8$
P.buccae	$2.0x10^6$	$3.9x10^6$	$5.0x10^2$ *	$3.8x10^5$	$1.8x10^7$

* when present

Mean bacterial count for the species listed for each pathological category ; standard deviation for each mean; minimum, maximum count values.

Table 2.3

Microbiological statistics for group # 1 (Minimal degenerative change)

Group #1	mean	standard deviation	min	median	max
Microaerophilic	$1.9x10^7$	$2.7x10^7$	10^5	$8.0x10^6$	$9.2x10^7$
Anaerobic	$2.1x10^8$	$3.2x10^8$	$5x10^4$	$7.7x10^7$	$1.2x10^9$
Prevotella	$1.6x10^7$	$3.5x10^7$	0	$2.6x10^6$	$1.3x10^8$
Fusobacterium	$1.0x10^7$	$1.5x10^7$	10^3	$6.6x10^6$	$5.7x10^7$
Actinomyces	$4.1x10^7$	$7.2x10^7$	$2.0x10^3$	$1.3x10^7$	$3.2x10^8$
Streptococcus	$3.7x10^7$	$6.7x10^7$	$2.0x10^5$	10^7	$2.7x10^8$
Lactobacillus	$8.6x10^6$	$2.0x10^7$	0	$9.4x10^5$	$9.8x10^7$
P.intermedia	$3.2x10^6$	$7.5x10^6$	10^3 *	$3.5x10^5$	$3.0x10^7$
P.melaninogenica	$1.3x10^7$	$3.0x10^7$	$5.0x10^2$ *	$1.6x10^6$	$1.0x10^8$
P.Buccae	$2.7x10^6$	$5.3x10^6$	$6.0x10^3$ *	$3.9x10^5$	$1.8x10^7$

Table 2.4

Microbiological statistics for group #2 (soft tissue degenerative change)

Group #2	mean	standard deviation	min	median	max
Microaerophilic	$3.6x10^7$	$5.4x10^7$	$1.0x10^5$	$5.0x10^6$	$1.4x10^8$
Anaerobic	$3.8x10^8$	$5.8x10^8$	$7.0x10^5$	$1.0x10^8$	$2.0x10^9$
Prevotella	$1.2x10^6$	$1.8x10^6$	0	$6.5x10^5$	$6.0x10^6$
Fusobacterium	$5.7x10^6$	$1.0x10^7$	$2.1x10^6$	$1.1x10^6$	$3.7x10^7$
Actinomyces	$1.2x10^7$	$1.7x10^7$	0	$3.3x10^6$	$6.0x10^7$
Streptococcus	$2.9x10^7$	$4.3x10^7$	$3.0x10^4$	$8.6x10^6$	$2.1x10^8$
Lactobacillus	$1.7x10^7$	$3.8x10^7$	0	$2.3x10^6$	$1.4x10^8$
P.intermedia	$6.4x10^4$	$8.2x10^4$	$2.5x10^3$ *	$3.2x10^4$	$2.5x10^5$
P.melaninogenica	$1.1x10^6$	$1.8x10^6$	10^3 *	$3.4x10^5$	$5.7x10^6$
P.buccae	$4.8x10^5$	$5.6x10^5$	$5.0x10^2$ *	$2.0x10^5$	$1.4x10^6$

* when present

Mean bacterial count for the species listed for each pathological category ; standard deviation for each mean; minimum, maximum count values.

Table 2.5

Microbiological statistics for group #3 (hard tissue degenerative change)

Group #3	mean	standard deviation	min	median	max
Microaerophilic	$1.1x10^8$	$1.3x10^8$	$2.5x10^6$	$8.8x10^7$	$3.2x10^8$
Anaerobic	$1.0x10^9$	$1.4x10^9$	$3.0x10^6$	$1.5x10^8$	$3.0x10^9$
Prevotella	$1.8x10^7$	$2.4x10^7$	$1.8x10^4$	$5.7x10^6$	$5.7x10^7$
Fusobacterium	$1.9x10^7$	$3.2x10^7$	$2.8x10^4$	$8.3x10^6$	$8.4x10^7$
Actinomyces	$2.8x10^7$	$2.7x10^7$	$1.8x10^6$	$2.2x10^7$	$7.1x10^7$
Streptococcus	$5.4x10^7$	$8.1x10^7$	$5.4x10^5$	$2.3x10^7$	$2.1x10^8$
Lactobacillus	$2.9x10^7$	$3.7x10^7$	$5.3x10^5$	$9.7x10^6$	$7.9x10^7$
P.intermedia	$1.1x10^7$	$1.3x10^7$	10^3	$7.4x10^6$	$3.1x10^7$
P.melaninogenica	$6.6x10^6$	$7.9x10^6$	10^3	$2.4x10^6$	$1.6x10^7$
P.buccae	$3.2x10^6$	$4.6x10^6$	$1.4x10^4$	$1.5x10^6$	$1.1x10^7$

Table 2.6

Microbiological statistics for group # 4 (Inflammatory degenerative change)

Group #4	mean	standard deviation	min	median	max
Microaerophilic	$9.0x10^7$	$1.4x10^8$	10^6	$1.7x10^7$	$3.7x10^8$
Anaerobic	$4.0x10^8$	$6.4x10^8$	$1.1x10^6$	$4.0x10^7$	$1.8x10^9$
Prevotella	$4.8x10^7$	$9.2x10^7$	0	$4.7x10^6$	$3.1x10^8$
Fusobacterium	$1.9x10^7$	$3.5x10^7$	$5.0x10^3$	$3.5x10^6$	$1.2x10^8$
Actinomyces	$2.5x10^7$	$3.2x10^7$	$8.0x10^3$	$2.0x10^6$	$8.5x10^7$
Streptococcus	$1.3x10^7$	$2.1x10^7$	$2.1x10^5$	$4.5x10^6$	$7.0x10^7$
Lactobacillus	$1.3x10^7$	$2.8x10^7$	0	$3.6x10^5$	$7.8x10^7$
P.intermedia	$1.2x10^7$	$2.2x10^7$	$1.3x10^4$ *	$1.5x10^6$	$6.3x10^7$
P.melaninogenica	$5.6x10^7$	$8.7x10^7$	$1.2x10^5$ *	$5.4x10^6$	$2.4x10^8$
P.buccae	$2.3x10^6$	$2.8x10^6$	10^3 *	$1.6x10^6$	$8.5x10^6$

*** when present**

Mean bacterial count for the species listed for each pathological category ; standard deviation for each mean; minimum, maximum count values.

Table 2.7

Correlations: all data

	Fusobacterium	Actinomyces	Streptococcus	Lactobacillus	P.intermedia	P.melaninogenica	P.buccae
Fusobacterium	1	0.368*	0.477*	0.008	0.596*	0.579*	0.45*
Actinomyces	0.368*	1	0.535*	0.206	0.533*	0.451*	0.476*
Streptococcus	0.477*	0.535*	1	0.29*	0.513*	0.499*	0.441*
Lactobacillus	0.008	0.206	0.29*	1	0.018	0.042	0.201
P.intermedia	0.596*	0.533*	0.513*	0.018	1	0.838*	0.693*
P.melaninogenica	0.579*	0.451*	0.499*	0.042	0.838*	1	0.631*
P.Buccae	0.45*	0.476*	0.441*	0.201	0.693*	0.631*	1

***= p< 0.05.**

Table 2.8

Correlations: group#1 (minimal inflammatory change)

	Fusobacterium	Actinomyces	Streptococcus	Lactobacillus	P.intermedia	P.melaninogenica	P.buccae
Fusobacterium	1	0.604**	0.144	-0.173	0.646**	0.563**	0.508**
Actinomyces	0.604**	1	0.378	-0.109	0.59**	0.452**	0.202
Streptococcus	0.144	0.378	1	0.078	0.575**	0.491**	-0.011
Lactobacillus	-0.173	-0.109	0.078	1	-0.103	-0.095	0.287
P.intermedia	0.646**	0.59**	0.575**	-0.103	1	0.914**	0.464**
P.melaninogenica	0.563**	0.452**	0.491**	-0.095	0.914**	1	0.493**
P.Buccae	0.508**	0.202	-0.011	0.287	0.464**	0.493**	1

***=p< 0.05.**
****=p< 0.01.**

Table 2.9

Correlations: group#2 (soft tissue degeneration)

	Fusobacterium	Actinomyces	Streptococcus	Lactobacillus	P.intermedia	P.melaninogenica	P.buccae
Fusobacterium	1	0.165	0.675**	0.072	0.273	0.245	0.234
Actinomyces	0.165	1	0.507	0.47	-0.029	0.275	0.603*
Streptococcus	0.675**	0.507	1	0.562*	0.303	0.466	0.55*
Lactobacillus	0.072	0.47	0.562*	1	-0.187	0.116	0.359
P.intermedia	0.273	-0.029	0.303	-0.187	1	0.838**	0.839**
P.melaninogenica	0.245	0.275	0.466	0.116	0.838**	1	0.661**
P.Buccae	0.234	0.603*	0.55*	0.359	0.839**	0.661**	1

***=p< 0.05.**
****=p< 0.01.**

Table 2.10

Correlations: group#3 (hard tissue degeneration)

	Fusobacterium	Actinomyces	Streptococcus	Lactobacillus	P.intermedia	P.melaninogenica	P.buccae
Fusobacterium	1	0.582	0.633	0.626	0.905*	0.765	0.617
Actinomyces	0.582	1	0.718	0.876*	0.885*	0.977**	0.894*
Streptococcus	0.633	0.718	1	0.799	0.961**	0.773	0.941**
Lactobacillus	0.626	0.876*	0.799	1	0.889*	0.894*	0.865*
P.intermedia	0.905*	0.885*	0.961**	0.889*	1	0.9*	0.988**
P.melaninogenica	0.765	0.977**	0.773	0.894*	0.9*	1	0.986**
P.Buccae	0.617	0.894*	0.941**	0.865*	0.988**	0.986**	1

***=p< 0.05.**
****=p< 0.01.**

Table 2.11

Correlations: group#4 (inflammatory degenerative change)

	Fusobacterium	Actinomyces	Streptococcus	Lactobacillus	P.intermedia	P.melaninogenica	P.buccae
Fusobacterium	1	0.73**	0.809**	0.265	0.482	0.624*	0.577*
Actinomyces	0.73**	1	0.865**	-0.123	0.74**	0.468	0.628*
Streptococcus	0.809**	0.865**	1	0.19	0.576*	0.698**	0.609*
Lactobacillus	0.265	-0.123	0.19	1	0.134	0.379	-0.07
P.intermedia	0.482	0.74**	0.576*	0.134	1	0.624*	0.726**
P.melaninogenica	0.624*	0.468	0.698**	0.379	0.624*	1	0.406
P.Buccae	0.577*	0.628*	0.609*	-0.07	0.726**	0.406	1

***=p< 0.05.**
****=p< 0.01.**

Table 2.12

The relation between Prevotella and Actinomyces species in the four pathological categories

	Minimal inflammatory change	**Soft tissue degeneration**	**Hard tissue degeneration**	**Inflammatory degeneration**
Correlation Coefficient	0.19	0.71	0.91	0.86
p-value	*not significant*	0.005	0.012	0.00015

Part Two

Introduction to Part 2

Immunohistochemical studies have indicated a predominance of B cells and plasma cells in advanced lesions of adult periodontitis. A number of workers have also cited polyclonal B cell activation as being important in the pathogenesis of this disease (Seymour & Greenspan 1979; Reinhardt *et al.* 1988).

In chronic pulpitis also, immunohistochemistry indicates that B lymphocytes and plasma cells are the predominant inflammatory cells in established lesions (Torneck 1974; Chapter 2 of this thesis). As the B lymphocyte is the dominant cell type in destructive pulpal and periodontal lesions it is reasonable to propose that those individuals whose lymphocytes are most responsive to mitogens should be the most susceptible to tissue destruction. Studies of early-onset severe generalised periodontitis support this. The basis for this hyperproliferation is not at this stage understood, however, studies to date indicate that expansion of the lymphocyte pool and altered lymphocyte/macrophage ratios, rather than altered T helper/Tsuppressor ratios, may be significant. (Engel *et al.* 1984, Mc Anulty *et al.* 1985).

The production of antibodies reactive with non-oral antigen in periodontal tissues is interpreted as an indicator of polyclonal B cell activation (PBA). Mallison *et al.* (1988) immunised rabbits suffering from experimental periodontitis with the antigens horseradish peroxidase (HRP) and glucose oxidase (GO) and nine days after secondary immunisation, HRP and GO-binding plasma cells were found in the inflamed gingival tissues as were large amounts of HRP and GO-specific antibody in the gingival crevicular fluid. The presence of these antigen specific cells localising to an area where no antigen is present confirms the work of Bice *et al.* (1982) in that inflammation alone can be an adequate stimulus to recruit recently primed B cell populations. It also appears that recently stimulated B cells are hyper-responsive to polyclonal B cell-activating (PBA) factors. This would explain the high numbers of non-oral antigen specific plasma cells and the high levels of immunoglobulin (Ig) in the gingival crevicular fluid. B lymphocytes specific for non-oral antigens would be expected to accumulate in inflamed gingival and pulpal tissue as a result of inflammatory stimuli. Similar work in humans has produced parallel data (Mallison *et al.* 1989).

Support for polyclonal B cell activation in pulpitis and periodontitis also comes from studies of T-cell deficient animals. Periodontal destruction has been studied in congenitally athymic rats and in these animals there was an increase in periodontal bone loss when compared to immunologically-competent animals (Yoshie *et al.* 1985). Thymic reconstitution subsequently resulted in decreased bone destruction.

The pulpal response to caries in a patient with thymic dysplasia has also been shown to be unusual (Trowbridge and Daniels 1977). It appears therefore that T lymphocytes have a regulatory and perhaps protective role in both disease processes.

Overall, polyclonal activation has been postulated to represent a primordial response to foreign antigenic challenge. Such a response to bacterial invaders, involving the rapid activation of up to 40% of all B cell clones, would be likely to generate antibodies at least partially reactive with the foreign components and these can block adhesion and colonisation or enhance complement-mediated destruction of microorganisms.

In addition, the specific immune response to plaque antigens is enhanced by the adjuvant effects of polyclonal B cell activators (PBA's). Antigens from oral organisms could drain to local lymph nodes and activate B lymphocytes. These activated cells could then seed from the blood into pulpal and periodontal tissue.

However, the combination of antigen-specific activation and polyclonal activation in an environment of acute and chronic inflammation is a many-fold more powerful stimulus than either alone and as such, the potential for a destructive tissue response is extreme.

In human systems, unlike the murine, optimal responses of B lymphocytes to polyclonal B cell activators require the presence of T lymphocyte and macrophage help (Kishimoto & Hirano 1988). Cytokines such as interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 5 (IL-5) and interleukin 6 (IL-6) produced by macrophages and T lymphocytes are important regulators of B lymphocyte growth and differentiation and should all be available from populations of these cells in pulpal and periodontal lesions (Arai *et al.* 1990).

Circumstantial evidence for tissue damage through polyclonal B cell activation is provided by the detection of auto-reactive antibodies, as well as the activation of previously suppressed B cell clones, following stimulation with polyclonal B cell activators. The actual significance of these auto-reactive antibodies is not known. In periodontitis an increase in the number of cells producing rheumatoid factor and antibody to types I and III collagen has been noted as the disease progresses (Hirsch *et al.* 1988). Related to this, the immunoglobulin class distribution of antibody to human collagen type I has been examined in the sera and gingival extracts of patients with chronic adult periodontitis (Anusaksathien *et al.* 1992). Tissue extracts demonstrated higher levels of IgG and IgA antibodies to collagen than in autologous serum, whereas no significant differences were found for IgM antibodies.

Antibodies to human type1 collagen are usually "natural" antibodies of the IgM class and therefore these results suggest that the periodontium is a major site of collagen antibodies in disease and that a class switch to IgG occurs in inflamed gingiva, presumably in response to prolonged antigenic stimulation. These antibodies of the IgG class may be of importance in contributing to the progression of periodontal disease and lend support to the concept of potential auto-immune damage.

A tissue-destructive reaction resulting from the pro-inflammatory activities of a T or B cell derived cytokine is also a possibility. Polyclonal activation and B cell recruitment could contribute to the pathogenesis of pulpal and periodontal disease through the production of cytokines such as IL-1, IL-6 and tumour necrosis factor alpha (TNFα) which have potent and widespread inflammatory effects. IL-1, IL-2, gamma interferon (γIFN), TNF α and IL-6 are of particular interest because of their autocrine and paracrine effects on B lymphocytes (O'Garra *et al.* 1990). Monocytes /macrophages and B lymphocytes have been shown to release TNF α when exposed to mitogenic stimuli such as LPS. Haemorrhagic necrosis is a typical consequence of the action of this cytokine in sensitised animals (Garrett *et al.* 1987). A similar haemorrhagic response noted in inflamed pulpal tissues (Torneck 1977, Chapter 2 of this thesis) may therefore be the result of inappropriate cytokine levels generated by a polyclonal B cell response.

Although the time course of tissue damage through cytokine action, hypersensitivity and auto-reactive antibodies is unknown, a defective disposal of immune complexes and polyclonal activation persist long after exposure to polyclonal activators ceases (Granholm & Cavallo 1989).

To understand both the regulation of polyclonal B cell activation and the contribution of this response to the tissue pathology seen in advanced periodontal and pulpal lesions, an *in vitro* system widely used for the study of B cell growth and differentiation, that is the murine splenic cell culture system, was employed.

By way of introduction, a general discussion of B lymphocyte growth and differentiation is presented. This is followed by a series of experimental chapters detailing work in which the murine splenic culture model was utilised to examine the nature of the B lymphocyte proliferative response to mitogens, the decline phase of the mitogenic profile, and of cytokine release in mitogenesis.

Chapter 3

B cell growth and differentiation

3.1. Introduction

B cells respond to three different types of antigen. Type 1 thymus-independent antigens such as bacterial lipopolysaccharide (LPS) and peptidoglycan (PGN) have the capacity to activate a substantial proportion of the B cell pool polyclonally, that is without reference to the surface antigen receptor. Peptidoglycan is a major cell wall constituent of all bacteria while lipopolysaccharide (LPS) is the major constituent of the outer membrane layer of the cell wall of gram-negative bacteria (see Figures 3.1 a &b) Certain antigens which are not readily degraded by phagocytes and which have appropriately spaced, highly repeating determinants (eg pneumococcal polysaccharide) are also thymus-independent in their ability to stimulate B cells directly without the need for T cell involvement. In general these molecules, classified as type 2 thymus-independent antigens, give rise to a predominantly IgM response and relatively poor, if any, memory. The majority of antigens are classified as thymus-dependent because they provoke little or no antibody response in animals which have been thymectomised at birth and have few T cells. Antigen processing and T cell help are required for B cell growth stimulation and differentiation in these cases.

The antigen-independent non-specific interaction of bacterial LPS with B cells includes blastogenic and mitogenic stimulation resulting in the synthesis and secretion of immunoglobulins. B cell blastogens initiate the transformation of B cells to blast cells. B cell mitogens always cause DNA synthesis and mitosis whereas polyclonal B cell activators can induce B cells to differentiate into plasma cells without dividing, and may or may not also induce DNA synthesis and mitosis (Tew *et al.* 1989). LPS of various *Enterobacteriaceae* genera share a common structure which consists of 3 polysaccharide structures (O-specific chain, outer core and inner core) and a lipid component, termed lipid A (see Figure 3.1b). Lipid A is the least variable component of biologically active LPS although variations in the amount and structure of almost all lipid A constituents have been observed (Sveen and Skaug 1992). It is well recognised that the lipid A portion of LPS is essential for LPS-induced B cell responses. Lipopolysaccharides of the oral organisms *Porphyromonas gingivalis, Fusobacterium nucleatum* and *Prevotella intermedia* have been reported to be potent mitogens and polyclonal B cell activators for murine responder spleen cells (Sveen and Skaug 1992). As non-specific triggering of B cells may be involved in antibody-mediated disease processes, it is conceivable that organisms such as *F.nucleatum, P.intermedia* and *P.gingivalis* may play a non-specific pathogenic role in pulpal and periodontal disease.

Figure 3.1a

Schematic representation of lipids and proteins of the gram-positive cell wall. Circles represent the polar headgroups of phospholipids. MDO are membrane-derived oligosaccharides. Both gram-positive and gram-negative organisms have an inner membrane and peptidoglycan wall. Gram-negative organisms also have an outer lipid bilayer in which lipopolysaccharide is found (see figure 3.1b).

Adapted from Raetz *et al.* 1991

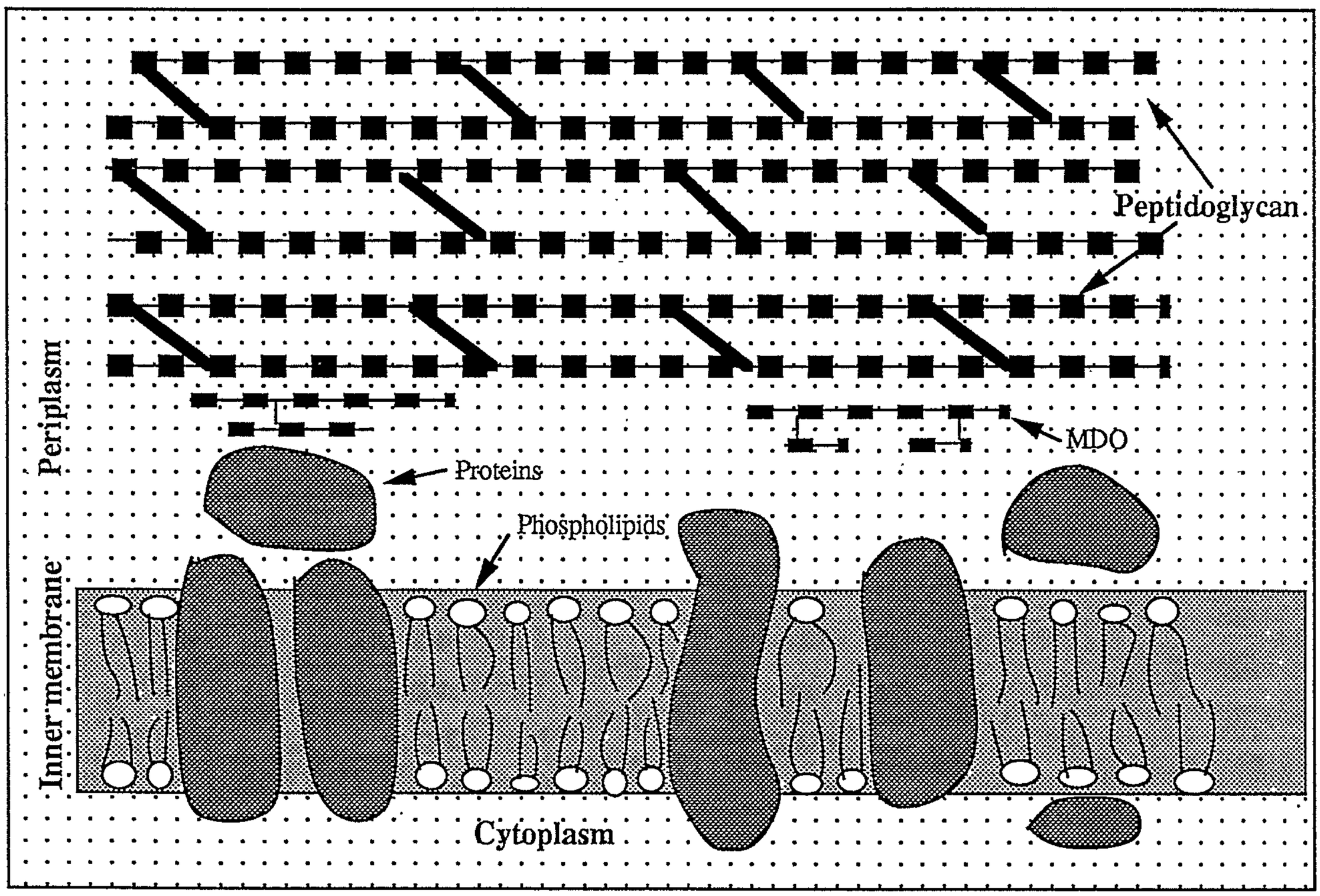
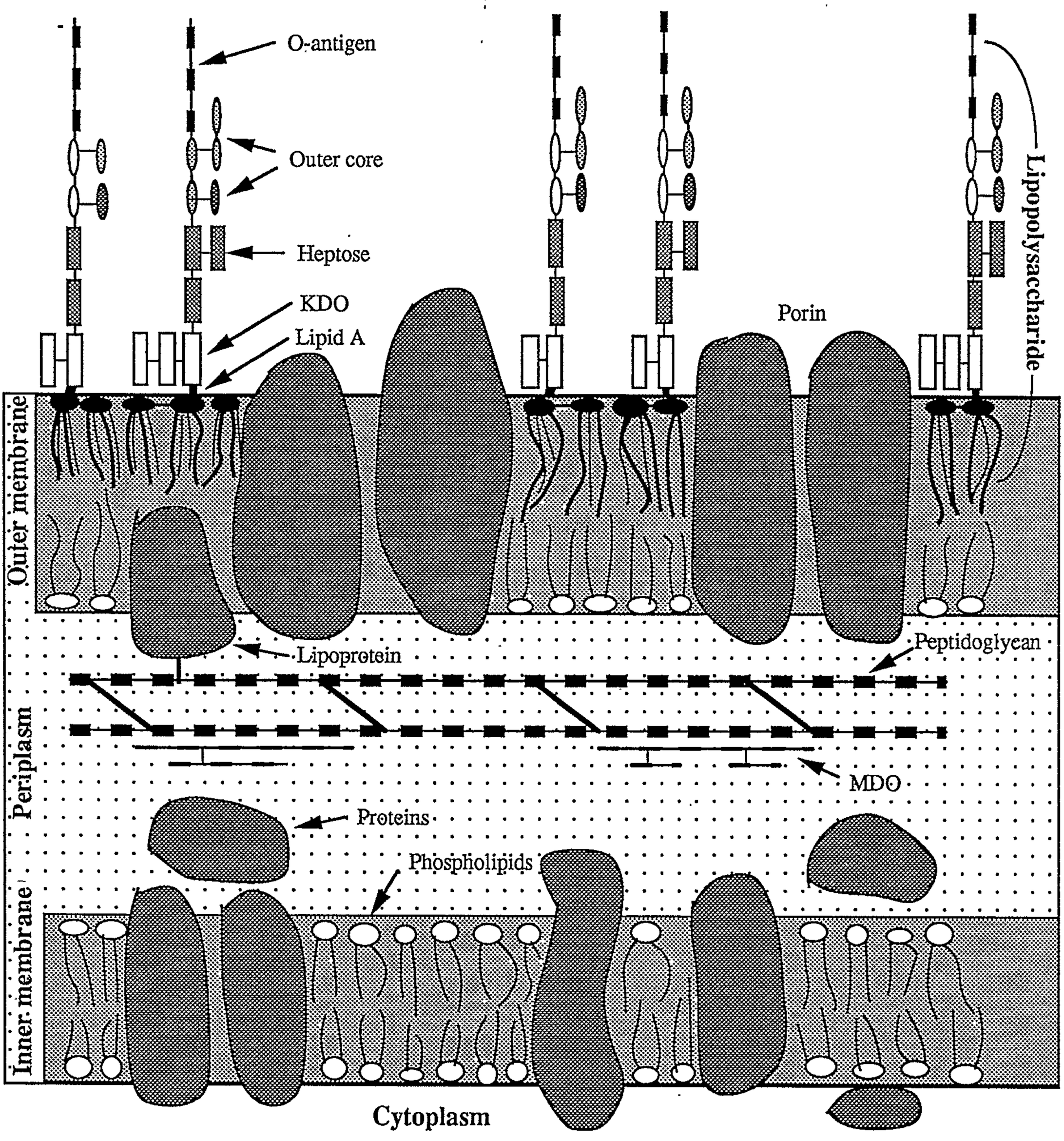


Figure 3.1b



Schematic representation of lipids and proteins of the gram-negative cell wall. Ovals and rectangles represent sugar residues. Circles represent the polar headgroups of phospholipids. MDO are membrane-derived oligosaccharides and KDO is 3-deoxy-D-*manno*-octulosonic acid. KDO and heptose make up the inner core of LPS.

Adapted from Raetz *et al.* 1991

Several *in-vivo* effects of the type 1 thymus-independent antigens PGN and LPS are strikingly similar. Both PGN and LPS reproduce most of the major signs and symptoms associated with bacterial infections, and they both act as immunoadjuvants and complement activators (Beutler & Cerami 1988). *In vitro,* both PGN and LPS are strong macrophage and polyclonal B cell activators (Sveen & Skaug 1992).

Little is known about the biochemical mechanisms of PGN and LPS action on host cells; however, available evidence indicates that these mechanisms may also be very similar (Dziarski 1988; Dziarski 1989). Some of the biological effects may be due to PGN and LPS-induced release of cytokines from macrophages and other cells, whereas other effects may be due to the direct action of PGN and LPS on various target cells.

There is now strong experimental evidence to support the concept that specific receptors exist for the lipid A component of LPS. Recently a 70kDa glycoprotein was described as a functional LPS receptor on murine and human B cell membranes (Dziarski 1991a, 1991b). Recent results also suggest that PGN and LPS bind to this same 70-kDa protein on the surface of B lymphocytes (Dziarski 1991b). This 70-kDa binding protein has also been identified on T lymphocytes and macrophages, but not erythrocytes (Dziarski 1991a). The presence of PGN and LPS-binding sites on T lymphocytes suggests that while PGN and LPS, like several other T-cell activators, in some species may not be a mitogen itself, they may still function as an activator or co-mitogen in conjunction with other stimuli. Not all of the biological effects of PGN and LPS are identical. For example, PGN is an effective B and T cell mitogen and inducer of polyclonal antibodies in cultures of human peripheral blood lymphocytes and LPS is not. It remains to be determined if any of these biological effects of PGN and LPS are triggered by the binding of PGN or LPS to cellular receptors and if all of the effects of PGN and LPS are triggered by binding to the same receptor.

3.2. Controls of B cell growth and differentiation:

B cells form a major functional component of the lymphoid system. Although their role in humoral immunity is well defined, the processes whereby functional B cell populations are generated and the exact properties of these populations are less well-defined and of great current interest.

B cell growth and differentiation can be considered as occurring in two stages (Hardy 1990). The first occurs in haemopoietic tissues (foetal liver and bone marrow), is antigen independent and involves the differentiation of pluripotent haemopoietic stem cells into

"virgin" B lymphocytes. These cells then synthesise immunoglobulin and express these molecules on the cell surface as receptors for antigen (Figure 3.2).

The second stage of B cell differentiation occurs outside the haemopoietic tissues and is triggered by contact with antigen. The end result is expansion of antigen-specific clones and the production of terminally differentiated plasma cells which synthesise and secrete large amounts of antibody. Long-lived memory cells which retain the ability to respond to the same antigen are also generated.

A further recently described B cell population known as Ly-1 B (or $CD5^+$ B cells) also appears to be a long-lived self-regenerating and selected population, but apparently arises by a different differentiation pathway from conventional memory B cells (Hayakawa & Hardy 1988).

Membership of a cell to the B lymphocyte lineage is indicated by the synthesis of immunoglobulin and this requires rearrangement of immunoglobulin gene segments. The mechanisms controlling this gene rearrangement are not yet fully understood. The environmental factors which influence the early differentiation of B lymphocytes are also poorly understood.

In mammals, in contrast to birds, B lymphocytes are produced in large numbers throughout life. B lymphocytes produced early in development do, however, show some differences in antibody repertoire and in requirements for activation compared with those found in the adult. The expression of the CD5 (Leu-1, Pan-T) antigen by B lymphocytes in young animals is an example of this. The possession of this marker is associated with the development of B cell lines which spontaneously secrete immunoglobulin capable of self-reactivity and which are of long-lifespan (Hayakawa & Hardy 1988).

Both T cell-dependent and T cell-independent B cell responses require, or are at least amplified by soluble factors (Kishimoto & Hirano 1988). At present there is a great deal of controversy concerning the number and type of molecules acting on B cells. Although it is clear that soluble factors can influence B cell growth and differentiation, there are a number of uncertainties both in relation to the sequence of events and the target cell for each of the factors.

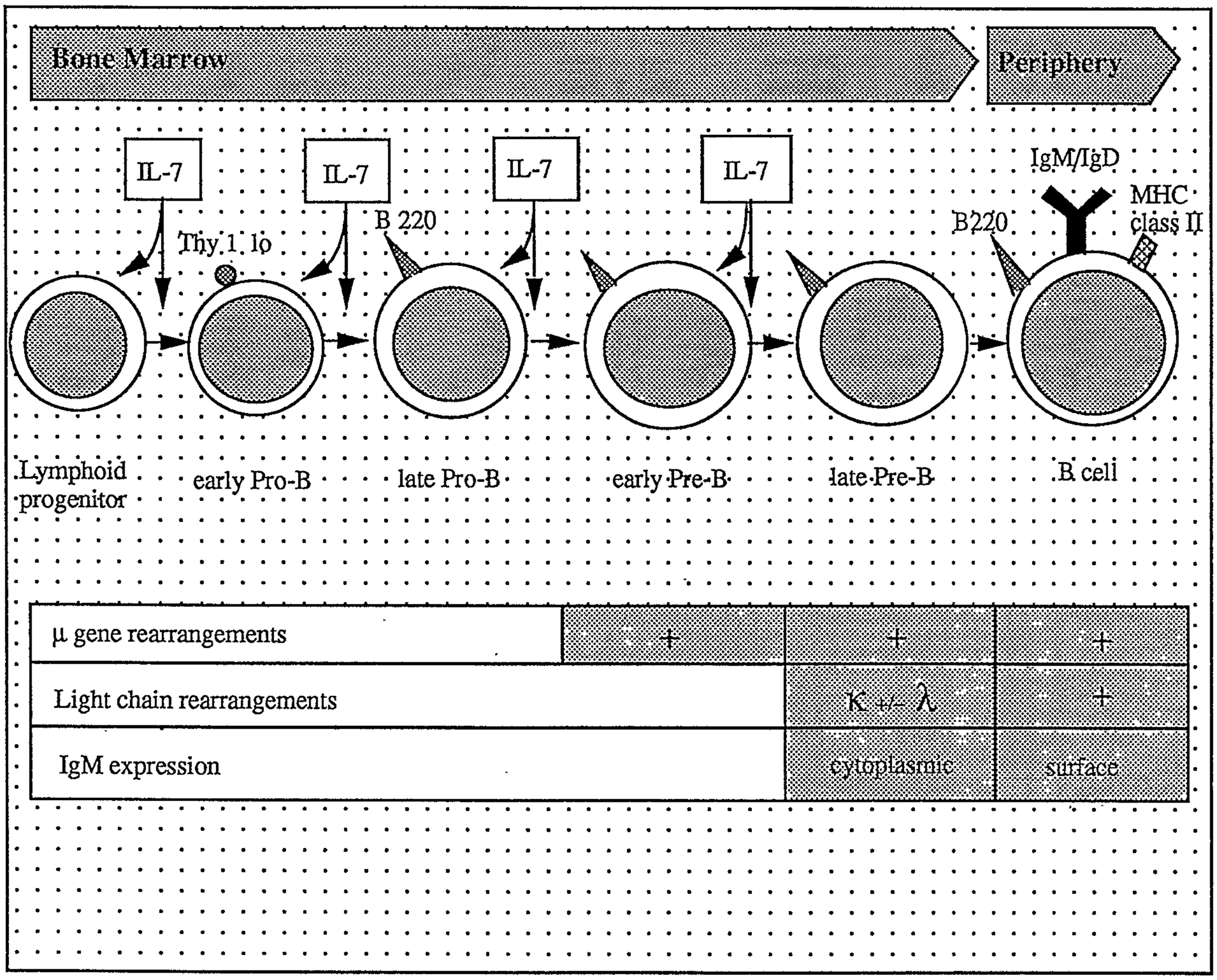


Figure 3.2 : B cell differentiation:

The earliest identifiable B lineage cell, the pro-B cell, is generated from a lymphoid progenitor capable of limited self-renewal. Ig-H genes rearrange initially, followed by K genes. Non-productive K rearrangement results in λ rearrangement.

At the late pre-B cell stage, cytoplasmic IgM is synthesised. The virgin B cell expresses surface IgM and IgD and class II MHC. The CD45 (B220) antigen is expressed throughout B cell development. Thy-1 is expressed at low levels only on pro-B cells. Early pro-B cells are smaller than late pro-B cells. IL-7 plays a role in pro- and pre-B cell proliferation although this capacity decreases at each subsequent stage.

*Adapted from Roitt, I.M., Brostoff, J., Male, D. "Immunology", second edition (1989). Gower Medical Publishing. London, England.

The activation of resting B cells *in vivo* and *in vitro* appears limited by cognate T cell help, therefore the biological role of B cell growth and differentiation factors must be more limited than in situations in which the target B cell population is driven by non-limiting soluble factors (Melchers & Andersson 1986).

3.3. B cell development:

B cells are produced continuously in the bone marrow. After leaving this tissue they migrate to the peripheral lymphoid tissues where most of them die. The mechanism by which a small part of the B cell population is selected for survival is thought to depend at least partially on encounter with antigen at extrafollicular sites (MacLennan & Gray 1986). The established recirculating population of small B lymphocytes probably consists mostly of memory cells.

Proliferating B cells within the germinal centres are derived from the progeny of activated "virgin" B cells and memory cells and are responding to antigens attached to follicular dendritic cells. In the absence of antigenic stimulation, primary B cells are thought to survive for only a very short period (MacLennan & Gray 1986).

B lymphocytes and their progeny commence immunoglobulin synthesis soon after activation and the level of secretion increases as differentiation proceeds (Swain 1989). Surface immunoglobulin and other B cell receptors are lost as terminal differentiation is reached; most pan-B and B cell-restricted antigens are lost for example. High level immunoglobulin secreting cells are frequently, but not always, of plasma cell-like morphology. They may, however, also be blast-cell or lymphocyte-like in morphology (Liu *et al.* 1989).

3.4. Regulation of Human B cell activation:

Mature B lymphocytes, if successfully incorporated in the body pool, reside in a quiescent state unless and until they engage the antigen recognised by their surface immunoglobulin. Despite debate in the 1970's as to the role of the B cell antigen receptor, there now exists overwhelming evidence in favour of a direct signalling function following binding of suitably processed antigen. Transduction of the antigen-derived signal from the surface immunoglobulin receptor involves rapid hydrolysis of cell-membrane lipids, generating a series of products among which are inositol 1,4,5-tris phosphate and diacylglycerol (Gordon & Cairns 1991). The significance of these two products is that they each elicit a key event in the so-called "dual-pathway" of signalling, the former by releasing intracellular stores of

calcium ions into the cytoplasm, and the latter by binding to, and activation of, protein kinase C.

Integration into this bifurcating pathway at some, as yet unknown, later point, is believed to be critical to the initiation of the mitogenic trigger delivered through, or independently of, surface Ig. If the signals are applied persistently, as might be expected with repeating multiepitopic thymus-independent antigens, the B cell proceeds independently to replication. If it is applied transiently, the quiescent B cell moves into an excited state where it requires additional information to progress further into the mitotic cycle. This latter situation would be seen to reflect the response of B cells to thymus-dependent antigens which, to be fully realised requires, by definition, interaction with T cells and their products .

Human B lymphocyte differentiation is coordinated by signals transmitted after the binding of specific cytokines and/or cross-linking of cell to cell adhesion receptors. A number of B cell-associated surface molecules have been identified, in addition to surface immunoglobulin, which may regulate activation and adhesion of B cells (Rev. in Clarke and Lane1991). These include members of the immunoglobulin (Ig) supergene family such as CD19, CD22, cell surface enzymes such as CD10, CD73 and CDw75, and proteins with multiple transmembrane domains such as CD20 and CD37. These accessory molecules may control signalling via antigen receptors and influence primary and secondary immune responses.

Ligation of CD21 or CD22 can, for example, augment responses to anti-Ig and the receptor CD40 may transmit a protective signal to germinal centre B lymphocytes thereby preventing apoptosis of these cells (Clarke and Lane 1991).

The signals transmitted to B lymphocytes via cytokines and cell surface receptors at one stage of differentiation lead to the expression of new or additional receptors which allow the cells to move to appropriate new microenvironments and recieve another signal via antigen, cytokine or cell-cell adhesion (Rev. in Clarke and Lane 1991).

3.5. B cell regions in lymphoid tissues:

The peripheral B lymphocyte pool is in constant flux. Some B lymphocytes recirculate through the tissues but most do not. Non-circulating B lymphocytes include those in the marginal zone of the spleen and in similar structures in other secondary lymphoid tissues (MacLennan & Gray 1986; Liu *et al.* 1989).

Most peripheral B lymphocytes have a relatively long intermitotic span of weeks rather than days. In addition, large numbers of short-lived B cells are produced continuously, both in the bone marrow and by antigen-driven stimulation of peripheral memory B cells (MacLennan & Gray 1986). Within secondary lymphoid tissue, B cells are located in follicles and marginal zone-related areas (MacLennan *et al.* 1989). A few B cells are also seen in T cell areas (Lortan *et al.* 1987) but most of these are in transit from blood or lymph to B cell areas. B cell responses are regulated in these tissues by three major types of accessory cell (Clarke & Lane 1991): interdigitating dendritic cells (bone marrow derived cells of short lifespan); follicular dendritic cells (long lived cells of uncertain origin which are able to retain antigen in its immunogenic form for long periods); and marginal zone macrophages / dendritic cells (derived from bone marrow). In primary T cell dependent responses, B cells proliferate initially in the T cell dependent areas of the spleen and lymph node. The second stage of B cell proliferation in the T cell dependent response involves migration of activated B cells into follicles where marked proliferation occurs and germinal centres are formed. Within a few days, antigen specific B cells that are not in the cell cycle, colonise the marginal zone compartment of the spleen (Liu *et al.* 1988). These cells, by virtue of their intimate association with the afferent blood supply, are ideally situated to re-encounter antigens. Marginal zone B cells and follicular B cells have distinctive surface marker phenotypes.

During secondary immune responses, memory B cells in the splenic marginal zones are selectively activated by antigen and proliferation occurs extensively in the T cell areas immediately adjacent to the marginal sinus. Large numbers of proliferating and differentiating plasmablasts migrate from these T cell areas to the splenic red pulp and bone marrow where they differentiate into plasma cells (MacLennan *et al.* 1989). A fresh wave of memory B cells also colonises the marginal zone at this time. Antibody responses to T-dependent antigens and T-independent (type1) antigens both elicit the appearance of memory B cells in the marginal zone. This is not the case for T-independent (type2) antigens (Lane *et al.* 1986; Zhang *et al.* 1988). Established responses to T-independent type 2 antigens appear to be maintained by the continuous recruitment of marginal zone B cells by polysaccharide antigens retained by marginal zone macrophages. Splenic marginal zone memory B cells turnover slowly in the absence of antigen. There is, however, continuous turnover of B cells adjacent to follicular dendritic cells (FDC's) and it appears that long-term antibody responses are maintained by chronic stimulation of B cells by antigen trapped by FDC's (Szakal *et al.* 1989).