ANALYSES OF MICROBIAL POPULATIONS ASSOCIATED WITH CARIOUS PULPITIS

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MDS

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STATEMENT OF AUTHORSHIP

The experimental work presented in this thesis was undertaken at the Institute of Dental Research. All work presented in this thesis was performed by the candidate except where stated otherwise. This work has not been submitted in whole or in part for any other degree, or at any other University.

Fjelda Elizabeth Martin
June 2002
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PUBLICATIONS

The following papers contain results described in this thesis.


Martin FE, Hunter N. Histopathological changes in chronic human pulpitis; *in preparation*.

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Paper describing studies peripheral to the thesis.

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LIST OF ABBREVIATIONS

%  percentage
±  plus or minus
≥  greater than or equal to
≤  less than or equal to
°C  degrees Celsius
Rn  fluorescence signal
μm  micrometre
16S rDNA  16 small subunit ribosomal deoxyribonucleic acid
16S rRNA  16 small subunit ribosomal ribonucleic acid
A_{260}  absorbance at 260nm
ALP  alkaline phosphatase
ANOVA  analysis of variance
bp  base pair
CFAT  cadmium fluoride acriflavine tellurite
CFU  colony forming units
C_T  threshold cycle
d  day
dNTP  mixture of four types of deoxynucleotides: deoxyadenosine 5'-triphosphate (dATP); deoxyguanosine 5'-triphosphate (dGTP); deoxycytidine 5'-triphosphate (dCTP) and deoxythymidine 5'-triphosphate (dTTP)
DEPC  diethylpyrocarbonate
DNA  deoxyribonucleic acid
DNase  deoxyribonuclease
EDTA  ethylenediamine tetra-acetic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>EGTA</td>
<td>ethylene glycol-bis (β-aminoethyl ether)-N, N', N'-tetra-acetic acid</td>
</tr>
<tr>
<td>ETSA</td>
<td>enriched trypticase soy agar</td>
</tr>
<tr>
<td>FAM</td>
<td>6-carboxyfluorescein</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IPC-BT</td>
<td>internal positive control-<em>Bactrocera tryoni</em></td>
</tr>
<tr>
<td>KVA</td>
<td>kanamycin vancomycin agar</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MES</td>
<td>2-[N-morpholino] ethane sulphonic acid</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PMNL</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RTF</td>
<td>reduced transport fluid</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TAMRA</td>
<td>6-carboxy-tetramethylrhodamine</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus aquaticus</td>
</tr>
<tr>
<td>t_d</td>
<td>bacterial doubling time</td>
</tr>
<tr>
<td>T_m</td>
<td>melting temperature of DNA</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>UNG</td>
<td>uracil-N-glycosylase</td>
</tr>
<tr>
<td>VIC</td>
<td>proprietary buffer supplied by Qiagen</td>
</tr>
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These abbreviations were used for frequently mentioned terms; otherwise normal chemical nomenclature was used throughout this thesis.
ABSTRACT

Dental caries continues to be a significant public health problem affecting mankind in many parts of the world. Microbial activities induce the progressive localised destruction of teeth that without treatment, would eventually result in infection of the dental pulp and surrounding periapical tissues. Although the bacteria responsible for caries initiation and early caries progression have been extensively studied, the microbiology of dentine caries is reported to show considerable diversity and has not yet been fully identified. Few studies have analysed the microbiology of deep caries or examined the relationship between the microflora and the histopathology of chronic pulpitis in symptomatic teeth.

Matched carious dentine samples and dental pulps were obtained from teeth without evidence of periodontal disease but with coronal caries and symptoms of pulpitis. Bacteria were cultured from the carious dentine samples under both anaerobic and microaerophilic conditions. Real-time polymerase chain reaction (PCR) technology was also used to identify and enumerate the bacteria. Development of techniques for the efficient extraction of bacterial DNA from both Gram-negative and Gram-positive bacteria found in carious dentine was an essential prerequisite for molecular analysis. In addition, the dental pulps were processed and categorised into one of four groups on the basis of the dominant pathology of the tissue (minimal inflammation, soft tissue degeneration, hard tissue degeneration and inflammatory degeneration).

Analysis of the culture data indicated a predominance of Gram-positive bacteria, particularly lactobacilli, while Gram-negative bacteria were also present in significant numbers with Prevotella species the most numerous anaerobic group cultured. Real-time PCR indicated a greater anaerobic microbial load than that determined by colony counting. The total number of anaerobes detected by PCR was 41-fold greater, while Prevotella spp. and Fusobacterium spp. were 82-fold and 2.4-fold greater respectively. PCR also identified the presence of Micromonas
micros, Porphyromonas endodontalis and Porphyromonas gingivalis in 71%, 60% and 52% of carious dentine samples, respectively. Correlation matrices from the real-time PCR data revealed significant multiple associations involving Fusobacterium spp. in combination with P. endodontalis, M. micros and/or Prevotella in the tissue response categories of minimal inflammation, soft and hard tissue degeneration. A positive correlation was also observed between M. micros and P. endodontalis for the category of inflammatory degeneration of the dental pulp. These anaerobes have been strongly implicated in endodontic infections that occur as sequelae to carious pulpitis. Accordingly, the data suggest that the presence of threshold levels of these bacteria in carious dentine may be indicative of irreversible pulpitis. Knowledge of the microbial predictors associated with irreversible pulpitis creates potential for the development of a diagnostic tool, and for restorative materials with antimicrobial properties.
INTRODUCTION

The problem

Despite the recognised advantages of fluoride in reducing tooth decay in the community, dental caries and associated pulpal symptoms continue to be a major public health problem. The development of dental caries requires certain prerequisites including the presence of cariogenic microorganisms, fermentable carbohydrates, susceptible tooth surfaces and time. The microorganisms involved in caries initiation and early caries development have been well documented and those most frequently implicated include streptococci, particularly the mutans group, and lactobacilli. Through the production of lactic acid, these species promote an acidic environment capable of demineralising enamel and exposing the dentine tubules. This phase is followed by the invasion of more opportunistic microbes such as veillonellae, neisseria and eubacteria that utilise lactic acid to produce less acidic products. The altered environment then favours the obligate anaerobes including fusobacteria, prevotellae and the porphyromonads. These organisms produce enzymes and toxic by-products with potential to degrade the pulpal matrix, attack immune components and interfere with repair.

Despite this knowledge, it is not known which microorganisms drive the carious process culminating in pulpal infection or what key interactions exist between microbes to assist this process. Additionally, the available literature provides no understanding of the relationship between the microbial profile of caries, pulpal histopathology and patient symptoms. Although the microbial populations of dental caries are known to be highly complex and variable, they have not been fully identified and it is possible that the organisms primarily responsible for pulpal infection have not yet been found. This problem is further exacerbated by the small number of studies that have investigated the microbiology of carious dentine and the single report on the relationship between the dentinal microflora and the histopathology of chronic pulpitis. As a result there is no reliable evidence to assign an aetiological role for caries pulpitis to a particular microbial species.
Aim of the study

This study will extend the results of previous work (Massey et al., 1993) by examining the microbiology of carious dentine using traditional culture techniques. In addition, molecular techniques (polymerase chain reaction) will be used to enumerate the more fastidious anaerobic species. Data from both sources will then be correlated with the histopathology of chronic pulpitis from human teeth in an attempt to identify microbial predictors associated with pulpal infection.

Research approach

To achieve this aim, an overview of the early theories of dental caries as well as a description of the dental structures, components and processes involved in the development of dental caries is presented in Chapter 1. A review of the significance of anaerobic infections including pathogenic attributes associated with dental caries and associated periapical disease is covered in Chapter 2. In Chapter 3, the protective and destructive aspects of the inflammatory and immunological reactions of the dental pulp to the carious process are examined. Chapters 4 to 7 encompass the experimental work. In Chapter 4, sample collection of carious dentine from extracted teeth, microbiological identification and enumeration using traditional culture methods are described. The detection of bacterial DNA by polymerase chain reaction (PCR) is presented in Chapter 5. The first part of the chapter includes a review of PCR technology as a method of identifying and enumerating bacteria. The remainder of the chapter contains experimental work divided into two stages; the first stage describes the development and testing of the universal amplicon, designed to detect the total bacterial load from samples. The second stage uses the universal amplicon to examine different methods of bacterial DNA extraction and protection in preparation for PCR. In Chapter 6, PCR technology is used to identify and enumerate anaerobic bacteria from the carious dentine samples. In Chapter 7, the histopathology of the dental pulps is reported and the results correlated with the microbial quantitation from both the culture data (Chapter 4) and PCR data (Chapter 6). The final chapter evaluates the achievements of the studies, identifies limitations and includes suggestions for the direction of future research resulting from this work.
CHAPTER 1

THE DEVELOPMENT OF DENTAL CARIES

1.1 Introduction

Dental caries is one of the most prevalent diseases affecting mankind. It is a unique disease in that humans have life-long susceptibility as the presence of carious teeth does not confer protective immunity (Bowen, 1999). Although caries rarely endangers life, it accounts for substantial pain and loss of masticatory function. Despite an increase in recent years in understanding of the pathogenesis of caries, knowledge is incomplete and dental caries continues to be a major public health problem.

Dental caries is described as a "dieto-bacterial" disease related to the pathogenic activities of bacteria in dental plaque. It involves the localised, progressive destruction of teeth by organic acids produced as a result of the fermentation of carbohydrates by oral bacteria. It is a chronic disease, which progresses very slowly, is seldom self-limiting and in the absence of treatment usually results in destruction of the tooth (Figure 1.1a), with subsequent infection of the dental pulp and surrounding periapical tissues (Figure 1.1b).

Dental caries is an ancient disease as interpreted from skeletal remains that provide a permanent record of caries through the ages. A pattern of sporadic caries has been documented from the beginning of the Iron Age (500BC) to the end of the medieval period (1500AD). In the seventeenth century the pattern of caries changed to one of increasing prevalence. These changes were associated with the establishment of the New World sugar industry and improvements in flour milling. Sugar consumption in Britain then remained stable until the mid 1800's when the removal of sugar import duties and repeal of the Corn Laws resulted in a rapid increase in consumption, especially by the lower socio-economic groups in industrial areas. This was accompanied by an increased incidence of gross damage to tooth structure
Figure 1.1a. Cavitation of the occlusal surface of the first permanent molar indicating gross clinical caries involving destruction of enamel and dentine.

Figure 1.1b. Diagram illustrating the spread of infection from the necrotic dental pulp to the surrounding periapical tissues.
resulting from caries (Corbett and Moore, 1976). By the 1950’s dental caries had reached epidemic proportions with 90-95% of the population in the developed world affected (Clarkson, 1999).

Historical evidence indicates the importance of sugar in caries aetiology. Sucrose is probably the most cariogenic sugar because it more readily facilitates the generation of organic acids and as a result of its low cost and ubiquitous availability in the diet of people in developed countries. In addition, sucrose is unique as a substrate for the production of extracellular storage of polysaccharides and insoluble matrix polysaccharides. Thus favouring the colonisation of teeth by microorganisms, increasing the retentiveness of plaque to the tooth surface and providing a reservoir of energy for continued cariogenic metabolism (Thylstrup and Fejerskov, 1994).

Descriptions of the carious process were recorded as early as the 1500’s. There have been many theories advanced over the years to explain the aetiology of dental caries. Some of these have included worms, imbalance of body humours, the vital theory, chemical, parasitic, proteolytic and chelation theories, and autoimmunity. More recently, G.V. Black and P. Fauchard noted the association between caries and the acid derived from food particles. The basic concept which remains unchanged today was that proposed by WD Miller in 1890, the chemico-parasitic theory. According to observations by other researchers and work carried out by Miller in the 19th century, dental caries is caused by acid attack on the enamel resulting from the fermentation of dietary carbohydrates by oral bacteria (Hardie, 1982).

These early workers reported the presence of masses of microorganisms on the tooth surface, now known as dental plaque, and studied the effects of acids produced by different oral bacteria on enamel. In addition, they demonstrated the presence of bacteria within the dentinal tubules of more advanced carious lesions. Results from studies on experimental enamel lesions indicated that dental plaque acted as a diffusion limiting membrane, influencing the passage of ions into and out of the enamel surface and limiting the access of salivary buffers to the site of acid attack, thereby producing sub-surface demineralisation.
Direct evidence for the aetiological role of bacteria came from experimental studies with animals carried out by Orland and colleagues in the 1950's. They found that germ-free rats failed to develop caries even when fed a sucrose-rich diet. Inoculation of oral bacteria into these animals resulted in the development of caries. Many researchers have now confirmed the finding that bacteria are essential to both the initiation and development of caries. It has also been shown that caries can be transmitted from infected to non-infected individuals. Young children without teeth usually do not carry Streptococcus mutans, a species implicated in the development of dental caries, however, following tooth eruption the ecological conditions for colonisation are established and the child acquires the bacteria from the primary care-giver, usually the mother (Caufield, 1997).

Dental caries has been described as a multifactorial disease. As with any other infectious process, the severity and occurrence of the disease depend on a balance between the microbial challenge, the host defences and environmental conditions (Hardie, 1982). The host defence mechanisms that operate in the mouth are complex. These include the chemical and structural composition of the teeth, their morphology and position in the mouth; factors that are critical in determining caries susceptibility. Other considerations include the buffering capacity and antibacterial components of saliva, the behavioural factors of the host, and the presence of restorations and appliances. One of the major environmental factors known to affect dental caries is the frequency of consumption of fermentable sugars. Another important consideration is the amount of fluoride that has been incorporated into tooth structure and the amount available locally. Dental caries can be prevented by adequate oral hygiene, reduced frequency of sugar consumption and the use of fluoride supplements.

The caries preventive measures aimed at controlling bacterial build-up include mechanical removal of plaque with a tooth brush or floss, chemical methods using topically applied solutions and gels, such as fluoride and chlorhexidine and development of a vaccine to protect against caries using S. mutans as the immunising agent. Although these measures have been shown to be effective in controlling the disease, dental caries persists and the core of
traditional dental practice involves the removal of carious dentine and its replacement with a restorative material.

1.2 Enamel-dentine-pulp complex

The hard tissue barrier of enamel, dentine and cementum provides strong mechanical support and protection to the loose connective tissue of the dental pulp. The harmful influences of the oral environment will not affect the pulp while the hard tissue barrier remains intact.

1.2.1 Enamel

Tooth enamel is a highly mineralised tissue containing, by weight, 96% mineral (hydroxyapatite) and 4% organic material and water. The mineral phase is made up of densely packed hydroxyapatite crystals arranged to form cylindrical rods with an average width of 5µm. The crystal orientation within the rod varies and those crystals distant from the centre flare laterally as they approach the periphery; this forms the inter-rod enamel. It is the susceptibility of these crystals to dissolution by acid that provides the chemical basis for dental caries (Figures 1.2a and 1.2b). The organic material forms a fine, lacy network between the enamel crystals. The bulk of this organic material consists of tyrosine-rich amelogenin polypeptide tightly bound to the hydroxyapatite crystals. Microscopic spaces filled with water separate the crystals (Ten Cate, 1998).

1.2.2 Dentine

Dentine is an avascular, mineralised connective tissue forming the bulk of the tooth. It is characterised by the presence of multiple closely packed dentinal tubules that radiate from the pulp and contain the cytoplasmic extensions of the dentine-forming odontoblasts. Mature dentine consists of approximately 70% inorganic material, 20% organic material and 10% water, by weight. The inorganic component consists mainly of hydroxyapatite and the organic phase is type I collagen with minor amounts of glycoproteins, proteoglycans and phosphoproteins (Ten Cate, 1998).

Dentine tubules are responsible for the permeability of dentine. They are tapered structures approximately 2.5µm in diameter near the pulp, 1.2µm mid-dentine and 0.9µm at the dentino-
enamel junction (DEJ) (Figures 1.3a and 1.3b). However, the cross-sectional areas of the tubules differ markedly due to variations in the amount of peritubular (intratubular) dentine. There are approximately 35,000 dentine tubules/mm² at the DEJ with approximately double that number/mm² at the pulp surface. This increase per unit area is a result of crowding of the odontoblasts at the pulp surface compared with the greater surface area available at the DEJ. Dentine tubules branch to form a profuse anastomosing canicular system of side branches noted to be more numerous in root dentine than in the coronal portion. It is speculated that dentinal tubules contain the process of an odontoblast bathed in tissue fluid, nerve fibres and types I and V collagen fibrils. The tissue fluid has been described as a gel containing proteoglycans, glycoproteins including fibronectin and the plasma proteins albumin and transferrin (Ten Cate, 1998).

1.2.3 Dental pulp

The dental pulp is a loose connective tissue encased in a hard tissue shell. Histologically, four distinct zones can be distinguished: the odontoblastic zone at the pulp periphery, the cell-free zone of Weil beneath the odontoblasts, the cell-rich zone and the pulp core (Figure 1.4). The pulp is composed of cells, extracellular matrix, blood vessels and nerves. The principal cells of the pulp include the odontoblasts, fibroblasts, putative undifferentiated mesenchymal cells, macrophages and other immunocompetent cells (Ten Cate, 1998).

The odontoblasts are responsible for the development of dentine, and form a distinctive lining of columnar cells around the periphery of the pulp with processes extending into dentine (Figure 1.5). Fibroblasts are the most numerous cells in the pulp and particularly in the coronal portion where they form the cell-rich zone. The function of the fibroblasts is to establish and maintain the pulp matrix consisting of collagen and ground substance. Undifferentiated mesenchymal cells represent the pool of cells from which connective tissue cells of the pulp are derived. Little is known about the immunocompetence of the dental pulp, however, small numbers of macrophages, T lymphocytes and antigen-expressing dendritic cells have been demonstrated in healthy pulp tissue (Pulver et al., 1977; Jontell et al., 1987).
Figure 1.2a and 1.2b. Scanning electron micrographs of enamel structure following acid etching. Note the characteristic keyhole pattern of enamel prisms (EP) with preferential dissolution of the enamel crystallites in the central region (CR) of the prisms. Scale bars indicate 10μm.

Figure 1.3a and 1.3b. Scanning electron micrographs of dentine. The structure, following acid etching, shows the dentine tubules (DT) surrounded by peritubular dentine (PD). Figure 1.3a has been prepared from dentine near the dental pulp. Figure 1.3b has been prepared from dentine near the dentino-enamel junction. Scale bars indicate 5μm.
Figure 1.4. Dentine-pulp interface showing dentine (D) and pre-dentine (PD) with subjacent odontoblastic layer (OD), cell-free zone of Weil (CFZ) and the underlying cell-rich zone (CRZ). Scale bar indicates 100μm.

Figure 1.5. Scanning electron micrograph showing the structure of dentine in cross-section. Note the dentinal tubules (DT) and cut odontoblastic processes (OP). Scale bar indicates 5μm.
The matrix of the pulp consists of ground substance and collagen fibres. The fibres are principally type I and type III collagen, present in a ratio of 55:45 which remains constant throughout life. The amount of collagen increases with the age of the pulp, but the ratio remains stable. The ground substance is similar to that of any other loose connective tissues and is composed of glycosaminoglycans, glycoproteins and water. It functions to support the cells and provides nutrient and metabolite transfer between the vasculature and the cells. With increasing age the composition of the matrix alters producing metabolic changes, reductions in cellular function and irregular mineral deposition (Ten Cate, 1998).

The blood vessels of the pulp enter and exit through the apical and accessory foramina accompanied by sympathetic nerve fibres that form a plexus around the blood vessels and innervate the smooth muscle in the arterial walls. These nerves play a significant role in regulating pulpal blood flow. The main vessels are thin-walled arterioles and venules that branch as they move coronally through the pulp. Further branching extends into the subodontoblastic area creating an extensive vascular capillary network with terminal loops and anastomoses between venules, as well as arterio-venous anastomoses. Lymphatic vessels are also present in the pulp and originate as small, blind, thin-walled vessels in the coronal pulp and between the odontoblasts. These drain into collecting vessels with numerous interconnections and exit apically as larger vessels accompanying blood vessels and nerve fibres, finally draining into lymph vessels in the periodontal ligament (Bernick, 1977).

Nerves enter the pulp through the apical and accessory foramina and are generally accompanied by blood vessels forming the neurovascular bundle. The nerves travel coronally, branching and rebranching to ultimately form an extensive plexus of nerves in the cell-free zone (subodontoblastic plexus of Raschikow) just beneath the odontoblasts in the crown of the tooth. The nerve bundles contain both myelinated and unmyelinated sensory nerve fibres. The myelinated axons are primarily A delta fibres associated with sharp, localised pain; these are described as fast conducting with a low stimulation threshold. The unmyelinated C fibres are
associated with dull more diffuse pain; these fibres are slow conducting with a high stimulation threshold. As the nerve bundles move coronally, the myelinated axons gradually lose their myelin sheath resulting in an increase in the numbers of unmyelinated axons in the coronal part of the tooth. While most of the unmyelinated nerve bundles terminate in the subodontoblastic plexus, a small number of axons pass between the odontoblast cells and enter the dentine tubules in close proximity to the odontoblastic processes. The presence of intratubular nerves has been shown primarily in coronal dentine extending for only for a limited distance within dentine (Ten Cate, 1998).

1.3 Saliva

Saliva is made up of fluid produced by six major salivary glands and 200-400 minor glands with different loci in the mucosa of the mouth (Thylstrup and Fejerskov, 1994). The functions of saliva include lubrication for swallowing and speech, solvent action for those substances with taste properties, defence mechanisms against microorganisms and protection of the oral cavity against damaging pH changes by salivary buffering. Saliva is a highly complex fluid containing a variety of both organic and inorganic components. The organic components comprise proteins and carbohydrates. The inorganic components include calcium, phosphate, fluoride, sodium, potassium, chloride, hydrogen ion and bicarbonate. Saliva is normally supersaturated with regard to hydroxyapatite, but contains proteins such as statherin and proline-rich proteins that prevent spontaneous precipitation of the calcium phosphate salts, thus encouraging a protective and reparative environment important to maintain the integrity of the tooth surface. Numerous cellular elements are also present which include desquamated epithelial cells from mucous membranes, neutrophils and microorganisms.

Saliva plays a significant role in maintaining an appropriate balance within the oral environment. While saliva provides proteins for development of the pellicle, which subsequently allows the formation of plaque and glycoproteins that provide essential bacterial nutrients, other features assist the elimination of microbes. These include an adequate saliva flow, which allows for debris and food to be cleared from the mouth along with the removal of
microorganisms by protein agglutination from tooth surfaces and mucous membranes. Saliva and crevicular fluids both contain neutrophils, polymorphonuclear leukocytes and immunoglobulins (IgA, IgG and IgM) which assist bacterial removal. In addition, components such as salivary peroxidase, lactoferrin, lysozyme and histidine-rich and proline-rich peptides may reduce the numbers of bacteria in plaque or diminish colonisation within the mouth (Thylstrup and Fejerskov, 1994).

Once plaque has formed on the tooth surface, the buffering effect of the bicarbonate and phosphate components of saliva control the pH of superficial plaque. Other alkaline salivary components contribute to this effect; for example salivin stimulates base production by oral bacteria and encourages the early cessation of glycolysis. Another component is urea, which is transformed by urease into carbon dioxide and ammonia. These components limit the demineralisation of tooth structure and by raising the pH of plaque reduce the advantage of the aciduric bacteria. Other positive effects of saliva include the potential of the inorganic components to increase the resistance of hydroxyapatite to acidic dissolution and the ability to remineralise early carious lesions (Thylstrup and Fejerskov, 1994).

The importance of saliva in controlling dental caries is demonstrated most effectively by the aggressive progression of disease when salivary flow is reduced or absent (xerostomia). Salivary dysfunction may be caused by lifestyle factors, disease or the side effects of medications. Lifestyle factors include stress, fluid balance problems such as strenuous exercise or excessive caffeine intake, smoking and the use of addictive drugs. Medical factors include diabetes mellitus, autoimmune disease such as Sjögren’s syndrome, chronic renal failure, alcoholism and pathology of the salivary glands including infection, neoplasm or prior radiation treatment. With regard to medication, there are several hundred medications known to induce xerostomia; some of these include psychotropic agents, diuretics, antihistamines, anticonvulsants, anti-emetics and anti-Parkinson’s disease drugs (Walsh, 2000).

1.4 Dental plaque

The oral cavity is the only site in the body where hard non-shedding surfaces are colonised
by microorganisms. This unique feature allows the accumulation of large masses of bacteria, known as dental plaque, on the tooth surfaces. This plaque is a highly organised structure of bacteria that adheres to the pellicle of the tooth surface (Figures 1.6a and b). Plaque forms on all exposed tooth and restoration surfaces especially where it is not disturbed. The plaque may vary in thickness from a few bacterial cells up to a millimetre thick in areas of stagnation and has been described as an open structure with fluid filled voids and channels extending through its entire thickness (Wood et al., 2000). Microorganisms do not adhere directly to the apatite crystals of tooth enamel. Following cleaning of the tooth surface, salivary proteins almost instantaneously adsorb onto the enamel forming a protein coating described as the pellicle. This film varies in thickness from 1-10μm and plays an important role in microbial colonisation by providing receptors for the adherence of specific bacteria. The major constituents of the pellicle are salivary glycoproteins, phosphoproteins and lipids. Development of the plaque biofilm occurs with the attachment of bacteria to the pellicle covered enamel surface via a number of mechanisms. Initially there is a loose association of bacteria to the tooth surface resulting from the action of van der Waal's forces which counteracts the negative electrostatic charges exerted by the bacteria and tooth surfaces. Bacterial attachment then occurs through selective inter-molecular interactions via lectin-like or hydrophobic ligands of the bacteria called "adhesins"; these bridge the space and form bonds with the carbohydrate groups in the pellicle glycoprotein (Gibbons, 1984). Examples include Streptococcus sanguinis (formerly Streptococcus sanguis) and Streptococcus oralis which bind specifically to terminal sialic acid residues in the glycoprotein, and Actinomyces viscosus and Actinomyces naeslundii which adhere to proteins such as proline-rich protein and statherin (Thylstrup and Fejerskov, 1994). Amylase, lysozyme, albumin and immunoglobulins have also been identified in the acquired pellicle, as well as types I and IV collagen which can act as ligands for a range of oral bacteria (Socransky and Haffajee, 1991; Marsh and Martin, 1992). Most bacteria exhibit multiple binding sites allowing attachment to a range of surfaces (Gibbons, 1984; Whittaker et al., 1996).

The microbial flora of the oral environment is complex and may contain up to 500 different
Figure 1.6a. Scanning electron micrograph showing the structure of dental plaque depicting a mixture of bacteria including long and short rods, bacilli and cocci. Scale bar represents 1μm.

Figure 1.6b. "Corn-cob" arrangement of bacteria found in plaque illustrating the coaggregation of smaller cocco-bacilli organisms with long rods. Scale bar represents 1μm.
bacterial species, with any individual harbouring between 150-200 different species (Socransky and Haffajee, 1994; Paster et al., 2001). The bacterial content of plaque varies depending on its age and location. In newly formed plaque, the Gram-positive facultative organisms dominate; these include S. sanguinis, Streptococcus mitis, Streptococcus gordonii and Actinomyces viscosus which elaborate adhesins for saliva coated and mineralised surfaces. Both the streptococci and actinomycetes groups are facultatively anaerobic and their doubling times for microbial growth during initial plaque development are less than one hour. As plaque develops into a biofilm, metabolism of the initial bacterial species creates conditions suitable for the colonisation of species that grow more slowly and have more fastidious growth requirements (Whittaker, et al., 1996). Other bacteria then colonise plaque through attachment to existing bacteria (coaggregation) using specific partnerships mediated by complementary surface structures on the partner cells. Each newly adherent cell type then becomes a nascent surface and bridge for additional cells. Some specific interactions include the attachment of S. mutans to A. viscosus and S. sanguinis (Lamont et al., 1991), Prevotella intermedia to actinomycetes (Nesbitt et al., 1993), Porphyromonas gingivalis to A. viscosus and S. sanguinis (reviews in Hamada, 1998 and Lamont et al., 1992), Actinomyces israelii to Cytophaga spp. (Kolenbrander and Celesk, 1983) and S. sanguinis to Fusobacterium nucleatum creating a “corn-cob” configuration of bacterial coaggregation (Figure 1.6b) (Kaufman and DiRenzo, 1989).

Of particular importance are the anaerobic fusobacteria that, as a group, coaggregate with some strains of all oral bacteria and act as a bridge to connect the early with the late colonisers (Kolenbrander and London, 1993). Each strain of fusobacteria coaggregates with only a certain set of partners and does not exhibit intrageneric coaggregation. Fusobacteria coaggregate with strains from a number of species of early colonisers including streptococci, actinomycetes, veillonellae and capnocytophaga. The late colonisers that favour coaggregation with fusobacteria include eubacteria, Haemophilus actinomycetemcomitans (formerly Actinobacillus actinomycetemcomitans), treponemes and strains of Prevotella and Porphyromonas spp. (Kolenbrander et al., 1993; Kolenbrander and London, 1993).
Further plaque development relies on the multiplication of existing organisms and coaggregation of additional bacteria to produce confluent growth and biofilm formation (Marsh and Martin, 1992). Thus, as plaque matures and becomes thicker there is a lowering of the oxygen concentration causing a shift in the diversity of the microflora towards increasing numbers of filamentous, Gram-negative and anaerobic bacteria (Thylstrup and Fejerskov, 1994).

The structure and development of plaque differs depending on its location. The plaque found in fissures is generally less complex, being composed mainly of Gram-positive cocci, particularly Streptococcus spp.; it also contains some rods and less inter-bacterial matrix. The fissure site tends to provide a more severe environment with a limited range of nutrients. Plaque that forms in the gingival crevice contains a greater diversity of bacterial species with higher levels of the obligately anaerobic bacteria, many of which are Gram-negative. This reflects the anatomy of the area and the flow and properties of gingival crevicular fluid. Many of the organisms found in this location are proteolytic and derive their energy sources from the degradation of host proteins and the catabolism of amino acids. Microorganisms commonly found in this region include representatives of the genera Actinomyces, Capnocytophaga, Fusobacteria, Prevotella and Porphyromonas (Marsh and Martin, 1992).

To cope with the fluctuating nutritional conditions and environmental variations within the mouth, the microflora must be biochemically flexible. The primary source of nutrients comes from the proteins and glycoproteins in saliva and gingival crevicular fluid. In addition, the diet provides carbohydrates and proteins. Carbohydrates such as glucose, sucrose and lactose easily diffuse into plaque and are readily metabolised. Carbohydrates can be transported directly into the cell and used to synthesise intracellular polysaccharides as carbohydrate storage for subsequent energy production when dietary supplies are low. Many species of oral bacteria are also able to synthesise extracellular polysaccharides from carbohydrates. These polysaccharides are polymers of glucose or fructose and form a considerable bulk of the acellular matrix of plaque. This binds the plaque together, aids its adherence to the tooth.
surface and provides a ready store of energy for bacterial metabolism, thus enhancing the pathogenicity of plaque (Thylstrup and Fejerskov, 1994).

The production of acid by acidogenic and aciduric bacteria in dental plaque is an absolute prerequisite for the development of dental caries. The bacterial species within plaque capable of fermenting carbohydrates to produce acid include *Streptococcus* (particularly *S. mutans*), *Lactobacillus* spp. and *Actinomyces* species. Diet is the primary source of carbohydrate available to plaque bacteria and excessive amounts, of particularly glucose and sucrose, result in sustained acid production, low plaque pH and an increased production of extracellular polysaccharides.

1.4.1 Plaque metabolism

Most saccharolytic oral bacteria catabolise sugars via the Embden-Meyerhof glycolytic pathway to generate energy and the precursors for cellular material. In addition, many organisms are also able to utilise the pentose phosphate shunt pathway. The main end-product of glycolysis is pyruvate, which can then be processed into a range of organic acids depending on the particular microorganism and the availability of oxygen. When available glucose levels are high, many bacteria convert the glucose to lactate. When glucose levels are low a greater proportion is converted to a variety of acids including formic, acetic, butyric, propionic and succinic acids, and ethanol. The acid end-products may be further degraded by other oral microorganisms, for example, *Veillonella* spp. consume lactic acid as an energy source and produce propionic and acetic acids, and *Eubacterium alactolyticum* converts acetic acid into butyric and caproic acids. The consumption of lactic and acetic acids with the subsequent production of butyric, propionic and caproic acids results in a significant decrease in the acidity of plaque (Thylstrup and Fejerskov, 1994).

Following the ingestion of carbohydrates, the pH of plaque decreases rapidly within the first few minutes which is followed by a slow increase to approach baseline levels after 30-60 minutes (Stephan, 1944). The extent and duration of the pH drop depend on the type of bacteria and the age of the plaque. The pH drop becomes more pronounced with increasing age
of the plaque. The resting pH of plaque has been estimated to be approximately 5.6-7.0, however, following a sucrose challenge Stephan curves show a pH drop to 5.5 in 2 day old plaque and to pH 4 in 6 day old plaque (Imfeld and Lutz, 1980).

In contrast to the large amount of information available regarding the fermentation of carbohydrates, far less is understood about the metabolism of nitrogenous components by oral bacteria. Amino acids may be required not only as building blocks in cellular macromolecules, but also as an energy source for bacterial metabolism. Some oral species demonstrate peptidase or urease activities, for example S. sanguinis has been shown to degrade casein and A. viscosus and Streptococcus salivarius can metabolise urea into carbon dioxide and ammonia; the latter can then be converted into a number of amino acids. Many of the periodontopathogens are assacharolytic and hence proteolytic, and their growth depends on an ability to utilise nutrients from gingival crevicular fluid. These bacteria also produce enzymes that are capable of degrading host molecules associated with the pocket epithelium, thus providing peptides and amino acids. Further, amino acid utilisation might also be an important mechanism whereby oral microorganisms counter the extreme pH changes caused by the metabolism of carbohydrates and urea (Marsh and Martin, 1992).

van der Steeg and van der Hoeven (1989) studied the succession of bacterial species during batch growth of subgingival plaque organisms in human serum and distinguished three phases of growth. The first phase involved consumption of carbohydrates by rapidly growing saccharolytic organisms such as the Streptococcus spp. with the subsequent production of lactic and formic acids. In the second growth phase proteins were hydrolysed with some amino acid fermentation taking place, the remaining carbohydrates were metabolised and the lactate and formate consumed. This stage of growth was dominated by Prevotella, Porphyromonas, Veillonella and Fusobacterium species. The final phase was characterised by extensive amino acid fermentation and progressive protein degradation predominantly by Peptostreptococcus and Eubacterium species. At this stage numerous toxic products including sulphide, ammonia, butyric acid and other fatty acids had accumulated.
1.5 Dental caries

Caries is a condition in which the dental hard tissues are demineralised by organic acids produced by bacteria following the metabolism of carbohydrates. The formation of dental plaque is essential to the process of enamel dissolution as it controls the diffusion of acids away from the enamel surface and limits the buffering action of saliva. This tissue loss leads to the collapse of the inorganic matrix and gradually a cavity is formed.

Early enamel caries involves the formation of subsurface holes or spaces within the enamel. This occurs with acid dissolution of individual hydroxyapatite crystals resulting in an enlargement of the intercrystalline spaces. The change in structure causes an increase in tissue porosity observed clinically as a whitish, opaque area of enamel due to alterations in the optical properties. In the initial stages the outer 20-50μm of enamel remains relatively unaffected. Although understanding of the demineralisation process is incomplete, there is speculation that the protective role of the salivary proline-rich proteins and statherin may initially inhibit surface demineralisation. Also, the surface enamel in close proximity to the plaque could be protected by the dynamics at the solid/solution interface (Ten Cate, 1998).

Enamel caries is a slowly progressing, phasic disease that is partially reversible with appropriate plaque and dietary control. However, with progress of the lesion, increasing porosity occurs along the direction of the rods towards the dentino-enamel junction, forming a conical shaped lesion with its base at the enamel surface. The previously intact surface enamel develops defects, cracks and micro-cavities before finally collapsing upon cavitation.

On reaching the relatively lightly mineralised dentino-enamel junction (DEJ) the process of demineralisation spreads laterally. The lesion follows the direction of the dentinal tubules towards the pulp and develops a conical shape with its base at the DEJ. At this stage the enamel surface is still usually intact. The enamel is avascular and acellular and cannot respond to injury, whereas the dentine and odontoblasts are integral components of the pulpo-dentinal organ and as such possess specific defence reactions that respond to external insults. Thus, when the surface dentine becomes demineralised, the odontoblasts react to the diffusion of
acids, metabolites and breakdown products from the microorganisms with two main responses, tubular sclerosis and formation of reactionary dentine.

Tubular sclerosis is the most common defence reaction of the pulp and involves the deposition of mineral within the dentinal tubules. Mineral in the form of apatite is laid down to form a mineralised barrier to protect the underlying odontoblasts from the advancing front of the carious lesion. It has been suggested that tubular sclerosis is probably an accelerated form of peritubular dentine formation, which occurs in response to mild stimulation, but progresses until the tubules become completely obliterated (Pitt Ford, 1992). In addition to the presence of these intratubular hydroxyapatite crystals, large rhombohedral crystals have been observed and identified as whitlockite crystals (Frank and Vogel, 1980). When examined under transmitted light this sclerotic zone is seen as translucent dentine because the mineral content within the tubules reduces the scattering of light causing the tissue to appear homogeneous. The second type of response is the formation of reactionary (reparative) dentine laid down at the surface of the pulp chamber by those odontoblasts under the caries-affected tubules. The structure of this tissue may resemble regular reactionary dentine or severely dysplastic tissue, depending on the severity of the stimulus (Thylstrup and Fejerskov, 1994).

Once cavitation occurs, bacteria are able to penetrate the demineralised dentine via the dentinal tubules. It is inevitable that bacterial succession will occur during development of the carious lesion, with the microflora responsible for the initiation of the lesion differing from those causing progression. Positive associations between microflora and those lesions that progress into dentine include lactobacilli and actinomycetes, and veillonellae and mutans streptococci (Marsh and Martin, 1992). This phase is followed by the invasion of a more diverse group of microorganisms including the Gram-negative anaerobes. Examination of the acid profiles of carious dentine by Hojo et al. (1991, 1994) found that lactic, propionic and acetic acids accounted for approximately 90 per cent of the total acid found in dentine caries. They reported that active carious lesions that had a low pH (4.9) and demonstrated a high lactate production were associated with the presence of Lactobacillus, Streptococcus and
Actinomyces species. Where there was restricted diffusion of both carbohydrates and acids in deep caries, arrested caries or under restorations, there was a shift in the bacterial profile. In these locations lactate was subsequently converted to acetate and propionate by a group of bacteria including Eubacterium, Propionibacterium, Arachnia and Veillonella species. Other acids, including butyrate, valerate and caproate were also found, indicating an environment with a higher pH (5.7) dominated by collagen-decomposing and amino acid fermentative bacteria. This is in agreement with earlier studies indicating an alkaline or neutral pH of carious dentine that the authors attributed to the presence of ammonia (Mäkinen et al., 1969).

Progression of the carious lesion requires bacterial invasion via the dentinal tubules (Figure 1.7). Studies in vitro have reported the bacterial invasion of root dentine by Gram-positive bacteria including Enterococcus faecalis, S. gordonii, S. sanguinis and Actinomyces spp. (Akpata and Blechman, 1982; Perez et al., 1993; Siqueira et al., 1996). However, the penetration of Gram-negative rods in monoculture in vitro has not been recorded. Akpata and Blechman (1982) noted that Bacteroides melaninogenicus ssp. melaninogenicus (Prevotella melaninogenica) did not invade root canal dentine after three weeks, while Perez et al. (1993) could not detect any dentine penetration by Prevotella intermedia after 28 days using either light microscopy or scanning electron microscopy. A scanning electron microscope study by Siqueira et al. (1996) reported that Porphyromonas endodontalis and Porphyromonas gingivalis had penetrated only a small number of dentine tubules after 21 days incubation. In contrast, studies in vitro investigating the ability of mixed cultures to invade dentine have shown that the penetration of Lactobacillus casei was improved when co-cultured with Streptococcus sobrinus or A. viscosus (Nagaoka et al., 1995), and the invasion of P. gingivalis was enhanced in mixed culture with S. gordonii (Love et al., 2000). It has been proposed that the invasion of dentinal tubules by P. gingivalis was facilitated by its ability to bind to the SspA or SspB polypeptides expressed on the surface of S. gordonii which is capable of dentine penetration through collagen binding (Love, et al., 2000).
Figure 1.7. Scanning electron micrograph illustrating the attachment of cocci-shaped bacteria to the walls of the dentinal tubules. Scale bar represents 1μm.
Once formed, the dentinal lesion can be divided into three zones.

i) The zone of destruction which is an area of softened, discoloured dentine where the basic architecture has been destroyed by the action of acids and proteolytic enzymes from bacteria. Bacterial invasion is extensive and microorganisms are found occupying areas where dentine has been destroyed. The soft dentine represents a combination of necrotic tissue and the bacterial biomass.

ii) The zone of penetration which is the region within the lesion that has been penetrated by bacteria. Lactobacillus spp. predominate because of their ability to survive the acidic conditions and are found occupying the dentine tubules. The basic structure of dentine is intact although many tubules become confluent and distended as a result of demineralisation. The intertubular dentine in this zone is extensively demineralised, with evidence of collagen breakdown interspersed with remnants of densely mineralised tissue.

iii) The zone of demineralisation results from acids produced by the biomass of anaerobic and aciduric microorganisms. The tubules in this zone are essentially normal, although there is some loss of peritubular dentine and crystals can be found within the tubules (Trowbridge, 1981). This is the deepest part of the lesion and is reportedly free of bacteria although the dentine may be partially demineralised (Pitt Ford, 1992).

The pH of the tooth environment is therefore not only influenced by the number and type of microorganisms present, but also by the buffering capacity of saliva, its flow rate and viscosity, the diffusion characteristics of plaque, the presence of fluoride, the type of diet and frequency of sugar ingested. Microbial deposits on tooth surfaces are constantly metabolically active, producing a variety of acidic and alkaline components even in the absence of a continuous supply of dietary substrate. It is the net result of the balance between these different processes that will determine the rate of progression of the carious lesion (Thylstrup and Fejerskov, 1994).
1.6 Pulp defence mechanisms

The pulp tissue reacts to caries before microorganisms penetrate the pulp chamber. When demineralisation of the dentine is 0.5 to 1 millimetre from the pulp, inflammatory reactions appear in the subodontoblastic layer. It has been proposed that this inflammation results from the diffusion of biologically active substances including bacterial enzymes and peptides, endotoxins, polysaccharides, somatic antigens, antibodies, immune complexes, chemotaxins, chemotaxigens, complement proteins, organic acids, products of tissue destruction and ammonia (Trowbridge, 1981). Numerous studies have shown that bacteria and their products produce severe pulpal reactions. Bacterial substances from dental plaque (Bergenholtz and Lindhe, 1975), lyophilised sonicates of plaque bacteria (Bergenholtz, 1977; Warfvinge and Bergenholtz, 1986), material from carious dentine (Mjör and Tronstad, 1972) and high molecular weight complexes of bacterial cell walls (Warfvinge et al., 1985) are capable of initiating acute inflammatory lesions in the pulp within 8-32 hours after their application to cut dentine surfaces.

The earliest reaction of a pulp to dental caries can be found in the odontoblastic layer underlying the lesion. Observations include a reduction in the size and number of odontoblasts, and a change in their shape from tall/columnar to flat or cuboidal. These changes precede obvious inflammation of the pulp. As the zone of demineralisation advances, reparative dentine is produced as a defensive reaction to the injury. The formation of reparative dentine is restricted to that portion of the pulp adjacent to the tubules communicating with the lesion and its elaboration encroaches on the cell-free zone. Reparative dentine is poorly calcified, contains irregular dentinal tubules and is unpredictable in the amount formed. Therefore, it cannot provide an impervious barrier but does afford some degree of protection to the pulp. The success of this defence, and therefore survival of the pulp, will depend on the rate of progression of the carious lesion, as more reparative dentine is formed in response to a chronic rather than acute lesion (Trowbridge, 1981).
The formation of sclerotic or reparative dentine is not the only factor that determines the defence capabilities of the pulp. Clinically the dental pulp has been shown to demonstrate healing and repair despite being continuously exposed to the oral environment and studies have shown the disappearance of inflammatory cell infiltrates before the appearance of reparative dentine (El-Kafrawy and Mitchell, 1963; Bergenholtz, 1990). Results of experiments by Pashley et al. (1983) indicated that dentine permeability decreased significantly within hours after the dentine was exposed. It has been proposed that dentinal fluid contains high molecular weight proteins, such as fibrinogen, which could reduce the functional radius of the tubules (Pashley et al., 1984). Other possible contributions to tubular occlusion could include the retention of immunoglobulin molecules (Hahn and Overton, 1997) and binding of penetrating bacterial antigens forming immune complexes (Okamura et al., 1980; Ackerman et al., 1981), the deposition of crystalline precipitates or the presence of large collagen fibrils within the tubules (Pashley, 1996). In addition, the outward flow of plasma fluid into the dentinal tubules in response to injury would provide a further line of defence against the inward diffusion of noxious substances.

As caries progresses, the defence reactions of the pulp become less effective. Sclerotic dentine is lost by the action of acids and enzymes from the invading bacteria. The body of the lesion then consists of zones of penetration and destruction, containing a large biomass of bacteria. Lateral spread of the lesion along the dentino-enamel junction undermines much of the adjacent enamel, which is prone to fracture with the potential to create a large open lesion. The pulp does not become inflamed until caries has involved the reparative dentine and wide areas of dentine have become demineralised. When microorganisms enter the pulp tissue acute inflammation supervenes with an influx of polymorphonuclear leukocytes followed by the formation of micro-abscesses. Infection of the pulp is a relatively late event in the carious process and its spread through the radicular portion of the pulp can lead to subsequent infection of the periapical tissues (Pitt Ford, 1992).
1.7 Carious dentine microflora

The microbial populations of dental caries are known to be highly complex and variable and have not yet been fully identified, although characteristic organisms are found. The microbes involved in caries initiation and early caries development have been well studied and are primarily Gram-positive. The organisms most frequently implicated include *Streptococcus* spp., especially the mutants streptococci group, and lactobacilli, in addition to the Gram-positive pleomorphic rods, including *Actinomyces* spp. (van Houte and Kent, 1994). The main Gram-negative bacteria identified are *Veillonella* spp., *Bacteroides* (*Prevotella* and *Porphyromonas* spp.) and *Fusobacterium* spp. (Brown *et al.*, 1986; van Houte and Kent, 1994). While it is commonly accepted that early carious lesions contain a high proportion of aerobic microorganisms, progression of the lesion changes the physiological environment including the pH and oxygen tension causing a shift in the bacterial population from aerobic to anaerobic species. A number of studies have shown the predominance of anaerobic microorganisms within caries (Loesche and Syed, 1973; Edwardsson, 1974; Hoshino, 1985; Torii *et al.*, 1986; Hahn *et al.*, 1991; Massey *et al.*, 1993).

In deep caries the Gram-positive microorganisms again dominate, with rods, especially *Lactobacillus* spp. being numerically dominant followed by the Gram-positive cocci (Hahn, *et al.*, 1991). As the lesion progresses in depth the number of Gram-positive cocci, particularly mutants streptococci decreases while the anaerobic cocci such as *Peptostreptococcus* spp. and *Peptococcus* spp. increase. The numbers of lactobacilli are reportedly high in both superficial and deep lesions with the Gram-positive facultative and pleomorphic species actinomycetes, propionibacteria, eubacteria, arachnia and bifidobacteria also present (Hahn, *et al.*, 1991; Massey, *et al.*, 1993). The Gram-negative anaerobic rods prevotellae, porphyromonas and fusobacteria have been isolated from deep carious dentine in greater numbers than from shallow caries, although they constituted only a minor component of the flora in some studies (Ando and Hoshino, 1990; Hahn, *et al.*, 1991). In contrast, Massey, *et al.* (1993) reported that the Gram-negative microbes comprised a significant percentage of the total flora. Carious
dentine, therefore, presents as a mixed infection with representative bacteria from aerobic, facultative and anaerobic groups which display a high degree of complexity and variability. However, as few studies have investigated the presence of anaerobic bacteria in dental caries, their true profile is, as yet, undetermined and little is known of their role in the pathogenicity of this disease process. The next chapter will examine the role of anaerobes in infections, with particular reference to dental caries and periapical disease.
CHAPTER 2

ANAEROBIC INFECTIONS

2.1 Introduction

Prior to 1970, a significant proportion of samples cultured from the sites of post-operative wound infection produced no pathogens, yet Gram staining indicated the presence of microbes. This inability to identify the causative pathogens ultimately resulted in incorrect treatment choices, and thus failure of therapy (Nichols and Smith, 1994). It is now known that many of these unrecoverable microbes were endogenous anaerobic bacteria that constitute the predominant human microflora, and can be found on the skin, mucous membranes of the oral cavity and upper respiratory tract, the intestinal tract, vagina and skin near the anus. It is reported that anaerobes outnumber aerobes in those regions above the diaphragm (including the oral cavity) by 100:1, and below the diaphragm by 1000:1 (Goldstein, 1999). Although anaerobes are part of the normal flora, they have the capacity to cause serious human infections as demonstrated in early clinical studies of patients with empyema and aspiration pneumonia where anaerobes were cultured from 76% and 93% of patients, respectively, compared with aerobes from 65% and 54% of patients, respectively (Bartlett et al., 1974a and 1974b). Anaerobes have been associated with a variety of infections including brain abscess, meningitis, sinusitis, peritonitis, appendicitis, diverticulitis and bacteremia (Goldstein, 1999). Sometimes these infections are associated with significant morbidity and mortality; therefore, inadequate or improper treatment can have dire consequences (Rosenblatt, 1997).

Despite many studies citing anaerobes as the predominant microorganisms there is widespread opinion among wound care practitioners that aerobic and facultative organisms are the primary pathogens involved in delayed wound healing (Bowler et al., 2001). This opinion was formed on the basis of numerous studies over the last two decades where little or
no attempt was made to isolate anaerobes, while studies involving detailed microbiological analyses of wound samples revealed that anaerobes constituted, on average, 38% of the total number of isolates (Bowler, et al., 2001). In this context, endogenous anaerobic bacteria have been reported to be the likely cause of postoperative infection when wound specimens failed to yield bacterial growth on culture (Nichols and Smith, 1994).

Failure to recognise the importance of anaerobes in infections may be the result of a number of factors: they are overlooked because it is assumed they are not detrimental, specimen collection and transport requires care and the culture, isolation and identification of anaerobes is more laborious, time-consuming and expensive than that required for aerobic and facultative organisms. As a result of these difficulties, the cultivation of anaerobes is often regarded as too demanding for diagnostic laboratories (Bowler, et al., 2001). Of concern are results of surveys of anaerobic culturing from hospital microbiology laboratories which indicate a low interest in anaerobic microbiology, lack of standardisation of procedures involving specimen collection through to identification and limited technological resources (Goldstein et al., 1995; Botta and Arzese, 1997). Additional problems have included the pressure of cost containment with a tendency for laboratories to cut back on procedures regarded as unusual or costly. Anaerobic culture and identification could be regarded by some to fall in this category (Rosenblatt, 1997). Indeed, Goldstein et al. (1995) reported that 12% of microbiology laboratories had considered stopping or reducing anaerobic bacteriology and susceptibility testing, and suggested that educational efforts be directed towards emphasising the relevance of anaerobes in infection and increasing the performance of anaerobic bacteriology.

Even with appropriate anaerobic culturing and identification, the role of anaerobes in infections may be difficult to estimate because anaerobic infections are typically polymicrobial, also involving aerobic and facultative organisms. Therefore it is difficult to differentiate between pathogenic and non-pathogenic organisms within the infected site. Bowler and Davies (1999) reported a significantly increased number of anaerobes (particularly *Prevotella* and *Peptostreptococcus* spp.) within infected leg ulcers compared with non-infected ulcers, and
proposed that the role of microbial synergistic interactions in chronic wound infection may be more important than the presence of any specific pathogen. A common group of anaerobic organisms have been reported in chronic infections found in diverse locations. These include *Bacteroides, Prevotella, Porphyromonas, Peptostreptococcus, Fusobacterium, Veillonella, Clostridium* and *Eubacteria* spp. (Chaudhry et al., 1998; Durmaz et al., 1999; Goldstein, 1999; Bowler, et al., 2001). A similar group of organisms have also been reported to be associated with oral infections (Tanner and Stillman, 1993).

### 2.2 Isolation and identification of anaerobes

Where anaerobes are likely to be present, careful attention must be applied to several key areas involving their isolation and identification including: collection of appropriate specimens of adequate size or volume, and transport to the laboratory and processing of samples using optimal media and atmospheric conditions to ensure anaerobic growth from the specimen. The most appropriate sampling method for the culture of the anaerobic pathogens involves the aseptic collection of abundant amounts of purulent fluid or excised tissue samples not contaminated by normal flora (Bowler, et al., 2001). Generally, collection of a sample using a swab is not adequate, because the sample is either insufficient or contaminated (Rosenblatt, 1997). Once collected, tissue samples can be placed in jars previously gassed with carbon dioxide or into a pre-reduced, non-nutritive transport medium to protect the anaerobes from exposure to oxygen and from dessication. Despite the perception that anaerobes die in air, a number of studies have shown that many of the common wound colonisers will survive in air for several days and that the sampling and transport methods might be less critical than the microbiological methods required to isolate the organisms (review in Bowler et al., 2001). The ability to survive for a period in the presence of air has been attributed to the reducing environment of the necrotic tissue in which they reside.

Ideally, specimens should be stored correctly and processed as rapidly as possible after collection to maintain microbial viability and preserve the relative proportions. A variety of media are required to ensure optimal recovery for both aerobic and anaerobic organisms. These
should be carefully selected, freshly made, and reduced prior to use (Peterson, 1997). Non-selective media is used to assess total growth from the sample; however, none of the media tested in one study grew all anaerobes when inoculated at low density (Rosseel and Lauwers, 1997). Additionally, selective or differential media are considered essential to allow the rapid detection and separation of anaerobes from facultative organisms. Anaerobic culture can be achieved using an anaerobic chamber for large-scale processing or, on a smaller scale, jars with chemically generated anaerobic systems. However, manipulation of anaerobes in air prior to anaerobic incubation can compromise optimal results (Cox et al., 1997). Also, quality control procedures must ensure that the jar lids and catalysts are functioning adequately (Rosenblatt, 1997). A study by Doan et al. (1999) assessed the recovery of periodontal pathogens using two different chemically generated anaerobic systems and an anaerobic chamber, and found that the recovery of bacteria by the different systems varied considerably from sample to sample. In addition to these variations anaerobes grow at different rates; although some anaerobes will grow within 24 hours, the more fastidious species, such as Porphyromonas, will take 5-7 days to grow adequately before they can be further investigated, and require up to 14 days for pigmentation (Petit et al., 1993). Further identification involves Gram stain, the use of antimicrobials, biochemical kits to detect the presence of enzymes and gas-liquid chromatography (Rosenblatt, 1997). The value of Gram staining for anaerobes is limited unless staining can be carried out within the anaerobic chamber, as exposure to oxygen compromises the cell wall integrity of some Gram-positive anaerobes causing them to stain as Gram-negative (Johnson et al., 1995a).

2.2.1 Major groups of anaerobic bacteria

Many changes in the taxonomy of anaerobic bacteria have occurred in recent years. Following the introduction of nucleic acid analyses for the identification of bacteria, new species have been found and old species have been renamed. The nucleic acid-based methods include DNA-DNA hybridisation, determination of GC content, and 16S and 23S rDNA sequencing. The use of 16S rDNA sequencing has provided a revolutionary breakthrough in
both taxonomy and bacterial identification, especially for those species that have not yet been cultured. The 16S rDNA sequencing method classifies bacteria according to their phylogenetic relatedness. Those bacteria with a similar evolutionary history will have nucleic acid sequences that are closely related (Pace, 1997). However, the phylogenetic approach does not necessarily correlate with phenotypic characteristics such as Gram-staining, morphology and growth requirements that are accepted as established methods of bacterial identification.

Of the main anaerobic bacterial groups, most changes have occurred in the taxonomy of the Gram-negative rods, particularly the genus *Bacteroides*. The genus *Prevotella* now includes most of the saccharolytic pigmented and non-pigmented species from *Bacteroides* and the former *Mitsuokella dentalis* and *Hallela seregens*. The genus *Porphyromonas* includes the asaccharolytic species from *Bacteroides*. Organisms from the genera *Prevotella* and *Porphyromonas* are found as part of the oral flora, and also in the gastrointestinal and urogenital tracts and are recovered as components of a mixed flora from infections related to those sites. The genus *Fusobacterium* contains a number of recently added species and subspecies that are found as indigenous flora in the oral cavity, respiratory, gastrointestinal and urinary tracts. *Fusobacterium nucleatum* is the species most commonly detected in clinical infections in humans. This organism comprises a number of subspecies that share similar nucleic acid sequences. Members of the genus *Bacteroides* are found in the normal flora of the gastrointestinal and genital tracts, and the oral cavity. They are isolated from infections of the head and neck, intra-abdominal and urogenital sites, and also from soft tissue and bone infections. The genus *Campylobacter* now includes the organisms *Wolinella recta* and *Wolinella curva*. Strains of *Campylobacter* have been isolated from infections of the head and neck, lungs and appendix. (Finegold and Jousimies-Somer, 1997; Jousimies-Somer, 1997).

The most clinically important group of anaerobic Gram-positive cocci is represented by the genus *Peptostreptococcus*, members of which have been cultured from a wide variety of sites, particularly abscesses and infections of the mouth, other soft tissues, bone, upper respiratory and female genital tract. Data from surveys of anaerobic infections indicate that they account
for 25-30% of all anaerobic isolates (Murdoch, 1998). Other Gram-positive cocci found in clinical infections include species of streptococci, particularly the *S. milleri* group which are associated with purulent infections. Although not strictly anaerobic, these microaerophilic bacteria are often isolated by anaerobic culture (Jousimies-Somer, 1997).

Other anaerobic Gram-positive organisms include the genera *Clostridium*, *Actinomyces*, *Eubacterium* and two new groups, *Atopobium* and *Pseudoramibacter*. Although none of the organisms in the genus *Actinomyces* is strictly anaerobic, many of the species are associated with chronic suppurating infections with a tendency to form draining sinuses in sites including the soft tissues, urinary tract, oral cavity, prostate, chest, eyes and ears. The genus *Eubacterium* presently includes more than 40 species and is phenotypically heterogeneous. Species have been isolated from oral infections, and abscesses of the rectum, brain, pelvis and lungs. The genus *Clostridium* is currently undergoing a phylogenetic reorganisation that will subsequently result in relocations and changes to nomenclature. These organisms are often isolated from significant infections in humans and also from wounds and soft tissue infections, although not from the oral cavity (Finegold and Jousimies-Somer, 1997).

Of the new species, *Pseudoramibacter* now includes *Pseudoramibacter alactolyticus* (formerly *Eubacterium alactolyticus*) which has been isolated from patients with periodontal disease, root canal infections and oral abscesses in addition to other infections such as brain, intestinal and lung abscesses, cellulitis and post-operative wound infections. The new genus *Atopobium* was created to include a new species, *Atopobium rimae*, a renamed *Lactobacillus* species (*L. minutum*) and a renamed *Streptococcus* species (*S. parvulus*). The clinical significance of these oral organisms is at this stage poorly defined (Finegold and Jousimies-Somer, 1997; Jousimies-Somer, 1997).

2.3 Dental caries

The following review outlines the development of dental caries as an example of a mixed infection initiated by aerobic and microaerophilic bacterial colonisation of the enamel surface which evolves into a complex infection dominated by anaerobes. Of the approximately 500
bacterial species that reside in the oral cavity only a small percentage may play an important role in the pathogenesis of dental caries, although it is not known which organisms drive the carious process or cause pulpal death. Despite the fastidious nature of the organisms identified in dental caries, they possess pathogenic attributes that enable colonisation of the tooth surface, survival in plaque, invasion and destruction of dentine and pulpal tissue, and evasion of host defence mechanisms.

2.3.1 Bacterial colonisation

As described earlier, bacterial colonisation is facilitated by bonding through adhesins found on filamentous appendages and outer cell membranes or vesicles of bacteria. Initially the early colonisers, streptococci and actinomycetes, attach to pellicle covered tooth surfaces via adhesins. In the presence of dietary sucrose the mutans streptococci synthesise complex mixtures of extracellular polysaccharides which further assist in the early colonisation of the enamel surface (Gibbons, 1984). The later colonisers coaggregate primarily through fusobacteria which provide a bridge between the aerobic and anaerobic microbial constituents of plaque. This is supported by Bradshaw et al. (1998) who showed that coaggregation-mediated interactions between F. nucleatum and Prevotella spp. or Porphyromonas spp. facilitated survival of the black-pigmented anaerobes in an aerated environment.

During the development and maturation of dental plaque, densely packed mixtures of different bacterial species produce many bacterial interactions. Successful interactions are important in the promotion of virulent strains and may be found where one species aids the attachment or provides growth factors for another. Reports of the progression of caries via bacterial invasion of dentine tubules illustrate further favourable interactions between microaerophilic and anaerobic bacteria (Love, et al., 2000). Other relationships are antagonistic due to competition for binding sites or nutrients, or the production of substances toxic to another species. For example, some strains of streptococci elaborate organic acids or hydrogen peroxide which inhibit the growth of Gram-negative anaerobes (Hillman et al., 1985; Mashimo et al., 1985). The black-pigmented Gram-negative anaerobic bacteria in turn limit the growth of
streptococci and other Gram-positive bacteria including actinomycetes, by the elaboration of bacteriocins (Hammond et al., 1987; Hillman, et al., 1985) and haematin (Takazoe et al., 1984). Selective habitats, such as the deep carious lesion, that favour a shift in the bacterial population towards more anaerobic microflora are also accompanied by changes in the environmental and nutritional requirements of the predominant organisms.

2.3.2 Environmental requirements

Maturation of plaque over time has been associated with a decline in the number of aerobic organisms and an increase in the number of anaerobes with an accompanying lowering of the oxidation-reduction potential. From this observation evolved the concept, published in the 1960’s, that describes the survival of obligate anaerobes in plaque as dependent on the presence of microaerophilic or facultative organisms to maintain a low redox potential. More recent information indicates that obligate anaerobes can survive in the presence of oxygen and that it is the ability to defend against the toxic effects of reactive oxygen by-products that determines survival (review in Bowden and Hamilton, 1998). Survival of organisms against oxidative stress is dependent on an ability to metabolise oxygen to harmless components or to remove toxic oxygen by-products, such as superoxide anion, hydrogen peroxide and hydroxyl radicals which may damage cell membranes, proteins, DNA and other nucleic acids. Removal of the toxic by-products occurs through the action of defensive enzymes including superoxide dismutase, peroxidase and catalase. Aerobic and facultative microorganisms utilise oxygen to produce hydrogen peroxide and water which lowers the oxygen concentration and redox potential in plaque. They are also well equipped with defensive enzymes against oxidative stress, whereas the anaerobes lack the ability to metabolise oxygen and possess limited defensive enzymes. Bowden and Hamilton (1998) reported that a number of oral anaerobes colonising humans including the porphyromonads, prevotellae and bacteroides showed some defensive enzyme activity involving superoxide dismutase and NADH oxidase, although not catalase or peroxidase.

An additional modulating factor is the pH of the environment. Marsh (1994) proposed
that a low pH, rather than the availability of carbohydrates, was the main factor involved in the selection of potentially cariogenic bacterial species. At a pH below 5, the predominant population was reported to be the more aciduric bacteria including *S. mutans*, *L. casei* and *Veillonella dispar*. However, as the pH increased to 7 or higher, anaerobic *Porphyromonas* and *Prevotella* spp. dominated. This transition is enhanced by the metabolism of lactic and acetic acids to less acidic products (Thylstrup and Fejerskov, 1994), and the breakdown of organic material in deep carious lesions, root canals and periodontal pockets by proteolytic enzymes producing amino acids for utilisation by anaerobic organisms (ter Steeg and van der Hoeven, 1989; Sundqvist, 1994). A combination of these effects results in a less acidic environment favouring anaerobic microorganisms.

### 2.3.3 Nutritional requirements

Bacteria in dental plaque receive nutrients from saliva and dietary components, with a significant group utilising carbohydrates as the main energy source resulting in the production of powerful acids capable of demineralising enamel. As plaque matures and the carious lesion advances into dentine, the more fastidious bacteria utilise serum proteins, haemin and degraded dentine components. This transition requires the development of favourable nutritional relationships that benefit fastidious anaerobic bacteria and include the production of various components by co-habiting bacteria (Loesche, 1968). *Micromonas micros*, for example, makes amino acids and peptides available from the breakdown of serum glycoproteins and connective tissue components for use by other anaerobes as energy sources (Sundqvist, 1994). Some oral organisms have specific nutritional requirements that are provided by other bacteria; examples include *Campylobacter rectus* which is reliant on the presence of proteolytic and formate-producing organisms such as *P. melaninogenica* for its growth (Grenier and Mayrand, 1986; Sundqvist, 1994) and *Porphyromonas gingivalis* which utilises protohaem produced by *C. rectus* in situations of haemin limitation (Grenier and Mayrand, 1986).

Favourable bacterial interactions have also been reported in animal models where the pathogenicity of one bacterial species was enhanced by the presence of other species. An
example of this includes *P. melaninogenica* which in pure culture is not pathogenic, but mixed with plaque bacteria produces abscess formation (MacDonald *et al.*, 1963). Similar interactions have been reported for *F. nucleatum* with *P. intermedia* or *P. gingivalis* (Baumgartner *et al.*, 1992) and *P. endodontalis* with *P. intermedia* or *P. nigrescens* (Siqueira *et al.*, 1998).

### 2.3.4 Destructive bacterial elements

Although the mechanism of pulpal infection is not known, microorganisms from superficial and deep caries produce enzymes and organic acids capable of inducing both defensive as well as destructive responses in the pulpal tissue, effectively compromising the immune and repair mechanisms of the pulp. Those substances that have the potential to induce tissue damage can be broadly classified into three overlapping groups: chemicals that injure tissue cells, chemicals that cause cells to release biologically active factors and those that affect the intercellular matrix (Socransky and Haffajee, 1991). A wide variety of bacterial metabolic by-products produced by carious microorganisms have been shown to adversely affect the growth and metabolism of mammalian cells. Butyric, succinic and propionic acids produced by *Porphyromonas* spp. are toxic to human cells (Singer and Buckner, 1981; Rotstein *et al.*, 1985). Other toxic products include indole, ammonia, hydrogen sulphide and methyl mercaptan produced by strains of fusobacteria, actinomycetes, eubacteria, veillonellae, prevotellae and porphyromonads (MacDonald *et al.*, 1963; Mink *et al.*, 1983; Persson *et al.*, 1990).

The cell walls of Gram-negative bacteria contain lipopolysaccharide (LPS/endotoxin), a major virulence factor, which exerts a range of biological effects resulting in the amplification of inflammatory reactions. Lipopolysaccharide is capable of activating the complement cascade via the alternate pathway, degranulating mast cells, activating platelets, basophils and endothelial cells, and inducing the release from cells of collagenase, tumour necrosis factor (TNF), interferons (α, β, γ), prostaglandins and interleukins (IL-1, IL-6, IL-8). It also enhances neutrophil-mediated injury with the resultant release of oxygen radicals and proteases (review in Seltzer and Farber, 1994). Studies have demonstrated that administration of endotoxin to experimental animals (Hirafuji and Shinoda, 1994) and topical application to cut dentine
(Warfvinge, et al., 1985) provokes a pulpal inflammatory reaction. Dahlen and Bergenholz (1980) documented the presence of endotoxin in necrotic pulps and found that higher levels of endotoxin were associated with greater numbers of Gram-negative bacteria. Further, increased amounts of endotoxin were associated with symptomatic teeth (Khabbaz et al., 2000), teeth with apical radiolucencies and with exudate associated with root canal therapy (Horiba et al., 1991).

Gram-positive bacteria are also capable of influencing inflammatory reactions via cell wall components that include peptidoglycans and lipoteichoic acids. These components are known to initiate and sustain a chronic inflammatory reaction. The peptidoglycans can induce the secretion of IL-1, TNF α, lymphokines and prostaglandins, in addition to nitric acid, oxygen radicals and eicosanoids from macrophages and lymphocytes. Both cell wall components have been implicated in activating the complement cascade via the alternate pathway with the subsequent release of C3a which is chemotactic for neutrophils (reviews in Farber and Seltzer, 1988 and Seltzer and Farber, 1994).

Both Gram-positive and Gram-negative microorganisms from carious dentine and infected root canals have been shown to produce a range of enzymes that contribute to pathogenicity by either direct or cytotoxic effects (Slots and Genco, 1984). Proteinases, such as keratinase, collagenase, fibronectin degrading enzymes, trypsin-like enzymes, gelatinase and other enzymes including neuraminidase, phospholipase A, DNase, RNase, catalase, superoxide dismutase and hyaluronidase have been associated with Prevotella and Porphyromonas spp. (Socransky and Haffajee, 1991; Seow et al., 1992; Sundqvist, 1993). Of the Gram-positive species, S. gordonii and P. micros produce a number of proteinases (Rams et al., 1992; Juarez and Stinson, 1999). These enzymes have been shown to evoke a wide range of potentially harmful activities including dysregulation of the complement cascade, degradation of immunoglobulins and bactericidal proteins, alteration of neutrophil antimicrobial activity and hydrolysis of collagen (Travis et al., 1997).

Other pathogenic characteristics of Prevotella, Porphyromonas, Fusobacterium and
*Lactobacillus* spp. include the ability to bind fibronectin and type I and V collagen, and to degrade fibrinogen (Falkler *et al.*, 1982; Harty *et al.*, 1994; Haraldsson and Holbrook, 1998). Some of these microorganisms also have the ability to haemagglutinate erythrocytes, aggregate platelets and release haemolytic enzymes. Hillman *et al.* (1993) frequently recovered haemolytic bacteria from sites with active periodontal disease where haemolysin production was regulated by the availability of environmental haemin. However, it has been suggested that haemolysins may be capable of lysing a number of cell types other than erythrocytes, which may provide a means for bacterial invasion (Beem *et al.*, 1998) and interference with tissue repair (Haraldsson and Holbrook, 1998).

To establish an infection in host tissue, bacteria must also be able to survive the host defence mechanisms. This can be achieved by disturbing the inflammatory response, preventing opsonisation, evading phagocytosis or killing the phagocyte. *Prevotella* and *Porphyromonas* spp. produce several proteolytic enzymes that are capable of degrading or inactivating human plasma proteins involved in host defences, such as the immunoglobulins and complement components (Kiillian, 1981; Sundqvist *et al.*, 1985; van Winkelhoff *et al.*, 1992). Further, these organisms can resist phagocytosis through capsulation (Sundqvist *et al.*, 1991) or by altering the shape of neutrophils (Seltzer and Farber, 1994), and can avoid destruction by interfering with the killing mechanisms of polymorphonuclear leukocytes (Rotstein *et al.*, 1989; Yoneda *et al.*, 1990). Other microorganisms are able to produce leukotoxins capable of killing leukocytes (Taichman *et al.*, 1980). These effects result in paralysis of the immune system of the host, thus facilitating the penetration and spread of potentially toxic substances, enzymes and antigens released by microorganisms.

As described, pulpal tissue damage can be elicited by components of microorganisms permeating the dentine or, in more advanced caries, causing direct effects on pulpal cells. Progression of the carious lesion has been reported to be episodic in nature in relation to a changeable, diverse and complex flora. The initial bacterial attack by streptococci and lactobacilli becomes modified as the environmental conditions alter to produce a mixed
bacterial infection. The defence reactions of the pulp initially involve the formation of reparative dentine and occlusion of the dentinal tubules by plasma proteins (Bergenholtz, 1990). These reactions may slow the rate of carious attack and the inflammatory reaction dissipates if the causative microorganisms are removed with restoration of the tooth. However, if allowed to continue, the carious lesion develops more anaerobic populations with increasing depth, eventually invading reparative dentine. Continued progression of the lesion results in development of acute inflammation and micro-abscess formation as microorganisms directly attack the pulp tissue, culminating in pulpal death with possible extension to involve the periapical tissues.

2.4 Periapical disease

As the dental pulp is contiguous with the periapex, bacterial invasion of the pulp as a result of untreated caries can extend to involve the periapical tissues. Periapical inflammation develops in response to irritants from microorganisms, their by-products, pulp breakdown products and inflammatory mediators from within the root canal. The host defence reaction initiated by irritants emanating from the root canal induces numerous inflammatory mediators from a number of cell types. These processes operate in an attempt to limit invasion of pathogens, however the host response may account for much of the resultant tissue damage. The sequelae of pulpal infection and necrosis produce two types of lesions: periapical abscess or periapical granuloma. The periapical abscess is characterised histologically by an accumulation of polymorphonuclear leukocytes and clinically by the rapid development of pain, swelling and elevation of the tooth from its socket. The periapical granuloma represents a mass of chronic inflammatory cells typically including lymphocytes, macrophages, mast cells, plasma cells and fibroblasts that have developed in response to inflammatory stimuli from the root canal. The types and pathogenicity of the bacteria involved as well as the response of the host determine which type of lesion develops. It has been speculated that more virulent organisms are responsible for abscess development, whereas granulomas are formed by less virulent microorganisms (Trowbridge and Stevens, 1992).
Fortunately, serious complications arising from periapical infections are rare. However, in some cases, infection spreads to adjacent areas creating abscesses in the soft tissues of the face, the submandibular or sublingual tissues, and the parapharyngeal, submaxillary, or infratemporal spaces. Extension of the infection into the maxillary sinuses and then to the nervous system can cause serious complications. The finding of anaerobic bacteria in dentine caries and periapical infections is of importance because these organisms have been associated with serious infections arising from dental foci including bacteraemia, endocarditis, meningitis, subdural and pulmonary empyema (Brook et al., 1991).

Reports of microbiological studies from teeth with infected root canals indicate that the majority of cases involve polymicrobial and predominantly anaerobic flora. The principal isolates include fusobacteria, porphyromonads, prevotellae, peptococci, peptostreptococci and eubacteria, with facultatively anaerobic streptococci found in the coronal part of the canal. This group represents a limited selection of the total oral flora and is similar to that found in the periodontal pocket, although less complex. Development of this restricted group of predominantly anaerobic microorganisms occurs as a result of synergistic microbial interrelationships and selective environmental conditions. Environmental factors contributing to the creation of a selective habitat include the lack of oxygen and development of a low reduction-oxidation potential with increasing time. Conditions within the root canal support anaerobic bacteria capable of fermenting amino acids and peptides from tissue fluid and disintegrating connective tissue, whereas bacteria that rely on the fermentation of carbohydrates as an energy source will be restricted by a lack of nutrients. Microbial interrelationships include the production by some bacteria of a wide range of proteolytic enzymes that assist the supply of nutrients and the production of haemin and vitamin K, which are essential nutrients for the black-pigmented species (Sundqvist, 1994).

In the majority of cases a mixed infection was reported to be responsible for the development of clinical symptoms, with an increase noted in both the bacterial load and the diversity of species found in cases with symptoms compared with those that presented
symptom-free (Sundqvist, et al., 1989; Gomes et al., 1994). However, there is no documented evidence for the role of a single organism or group of microorganisms in the aetiology of periapical disease. Equally, attempts to relate the presence of certain bacteria in root canal infections with the development of clinical symptoms have been unsuccessful, as few studies have compared the flora from symptom-free with symptomatic teeth (Seltzer and Farber, 1994). In some studies the presence of particular microorganisms has been implicated in the development of acute symptoms. For example pain, swelling, tenderness and exudate have been associated with peptostreptococci, prevotellae, fusobacteria, eubacteria, P. endodontalis and P. gingivalis (Yoshida et al., 1987; Haapasalo, 1989; Hashioka et al., 1992; van Winkelhoff et al., 1992; Gomes et al., 1996). However, the presence of Prevotella and/or Porphyromonas spp. in a microbial mix provides no certainty of acute symptoms. This is illustrated in a study by Sundqvist et al. (1989) where Prevotella and Porphyromonas spp. were isolated from 22 teeth with apical periodontitis; 16 of the teeth presented with symptoms, the remaining six were asymptomatic. Difficulties in developing associations have also arisen from a lack of specificity in the terminology used to describe the clinical problem and from difficulties in earlier studies of accurately culturing and identifying clinically important organisms. In addition, organisms previously identified have now been reclassified and there is continuing description of new organisms and taxa (Sundqvist, 1994).

The infected root canal has been reported to be inhabited by a relatively low number of species comprising mainly anaerobes. Periapical lesions induced in rats following exposure of the pulp to oral fluids showed a change in the anaerobic bacterial counts infecting the root canal of 24% at 7 days to 47% after 15 days (Tani-Ishii et al., 1994). Published studies indicated that obligate anaerobes have been cultivated in 80 to 100% of cases with between one and 11 different species found in each sample (Oguntebi et al., 1982; Lewis et al., 1986; Fukushima et al., 1990; Baumgartner and Faulkler, 1991; Brook et al., 1991; Sundqvist, 1992; Lana et al., 2001). More recent reports involving molecular methods of identification have extended the number of species in individual canals to 17 (Siqueira et al., 2000). The most
frequently isolated species include the previously listed anaerobes and facultative anaerobic streptococci, in addition to clostridia, lactobacilli, eubacteria, actinomycetes, veillonellae and treponemae (Baumgartner and Falkler, 1991; Sundqvist, 1992; Xia et al., 1999; Lana et al., 2001). The strains of *Prevotella* spp. most commonly found include *P. nigrescens*, *P. intermedia*, *P. melaninogenica* and *P. tannerae* (Sundqvist et al., 1989; Bae et al., 1997; Xia, et al., 1999). Of the *Porphyromonas* group, *P. endodontalis* has been identified in infected root canals and associated with periapical pathoses more frequently than *P. gingivalis* (van Winkelhoff et al., 1985; Haapasalo, 1989; Sundqvist et al., 1989; Siqueira et al., 2001).

The anaerobic microorganisms in polymicrobial root canal infections are likely sources for the production of endotoxins and the up-regulation of cytokines and enzymes resulting in inflammation, pain and bone resorption associated with periapical infections. The peptostreptococci are of particular interest as their capacity to produce proteolytic enzymes may play an important role in the pathogenesis of periapical infections (Seltzer and Farber, 1994). *Prevotella intermedia*, *P. endodontalis* and *P. gingivalis* are also capable of degrading a number of glycoproteins from serum to yield amino acids and peptides for utilisation by these and other species such as *Fusobacterium* and *Eubacterium* spp. which possess insufficient proteolytic activity (Sundqvist, 1992).

It is unlikely that any single virulence factor is responsible for the tissue damage noted in dental caries or apical periodontitis. For these conditions a range of virulence factors have been implicated at different stages of the infection. Colonisation and inter-bacterial aggregation form the first stages of disease initiation, followed by specific bacterial successions in favour of the development of anaerobic microorganisms as the environment alters. Nutritional interactions then represent a second stage in the population shift. As most bacteria from the oral cavity are only weakly virulent on an individual basis, inter-bacterial cooperation is essential to provide the necessary components for a species to become pathogenic. Thus, various combinations of microorganisms have a synergistic pathogenic effect not possessed by any individual species. Apart from supplying nutrients and anaerobic conditions, the different bacterial species
contribute to a pool of virulence factors which include enzymes, toxic products and mechanisms that are capable of inactivating or resisting host defence mechanisms. The response of the dental pulpal and periapical tissues to the microorganisms and their pathogenic effects is reviewed in the next chapter.
CHAPTER 3

INFLAMMATORY REACTIONS IN RESPONSE TO DENTAL CARIES

3.1 Introduction

As the dentine and pulp form an integral unit through the extension of odontoblastic processes into dentine, any insults affecting the dentine will directly affect the pulp. These insults may take the form of caries, restorative procedures or trauma. Each of these interactions has the potential to cause pulpal inflammation, cell damage or even pulp death. The typical response of the pulp to any physical, chemical or bacterial insult is inflammation. Generally, if the insult is minor the pulp will heal and return to normal, with the possible exception of sclerotic dentine development. With dentine caries the inflammatory reaction will resolve when the irritation is removed, although in some instances the pulp may heal despite constant bacterial irritation, which is probably related to changes in the permeability of the dentinal tubules. However, if the insult is more severe the pulp may become necrotic (Bergenholtz, 1981).

Massler (1967) documented the nature of caries as an intermittent and chronic process with periods of activity alternating with periods of stasis. As a result, the histopathology of the pulp tissue will vary depending on when the lesion is sampled; whether it is in an active or passive phase. As a consequence, it is difficult to predict how the pulp will respond to either caries progression or restorative treatment. Studies have reported a range of responses and have demonstrated pulpal inflammation associated with minimal carious lesions in newly erupted teeth (Massler, 1967), while others have shown no apparent pulpal change although caries had progressed to within 0.8mm of the pulp (Shovelton, 1968).

There is a lack of consensus in the literature regarding how early the pulpal response to caries can be detected. This is further confused by the finding of inflammatory cells in the pulps
of unerupted or newly erupted, clinically intact teeth (Massler, 1967). Unfortunately, no correlation has been found between any single clinical symptom and the degree of inflammation in the pulp. Dummer et al. (1980) used electric pulp tests, thermal tests, percussion tests and pain history and could not find any clear association between symptoms or tests and pulpal disease. In addition, Massler (1967) noted the occurrence of superficial lesions in association with sharp pain but without evidence of pulpal inflammation. These results contrasted with a report of partial necrosis and severe inflammation of the pulp without a history of pain (Langeland, 1987).

Despite these clinical and histopathological discrepancies, it is well documented that bacteria and their products play a major role in pulpal inflammation through dental caries and microleakage around restorations (Brännström, 1981). However, there have been relatively few detailed studies reporting the microbiology of carious dentine or the relationship between dentinal microflora and the histopathology of carious pulpitis. Therefore, our understanding of the microbiology of the carious lesion is limited and incomplete.

The response of the pulp to the advancing carious lesion reportedly involves both specific and nonspecific inflammatory reactions, with resultant healing and repair as well as irreversible tissue damage (Bergenholtz, 1981). These reactions are related to the host defence mechanisms including the complement cascade, the immune system and the action of phagocytic leukocytes. Although inflammation is usually a protective mechanism, it also has destructive aspects that may be responsible for further tissue damage. In contrast to other tissues in the body, cell damage in the pulp may not be as easily repaired due to limited access of appropriate repair-competent cells and the constraints imposed by the hard tissue encasement. As a result, pulpitis is an example of an infection where the host reaction may produce more damage than that caused by the effects of the microorganisms.

Despite decades of research, little is known of the response of the pulp to dental caries. One mechanism is proposed, whereby the initial reaction is one of a mononuclear infiltration dominated by lymphocytes, plasma cells and macrophages, with acute reactions involving the
mobilisation of polymorphonuclear leukocytes only when the pulp is in danger of imminent invasion by bacteria. This is in contrast to the accepted progression of inflammation where the acute phase generally precedes the chronic condition. Carious pulpitis represents a form of chronic inflammation described as an insidious, low-grade response also seen in some common and disabling human diseases such as rheumatoid arthritis, atherosclerosis and chronic lung diseases (Cotran et al., 1999).

3.2 Pulpal inflammatory reactions

The earliest sign of pulpal inflammation involves a disturbance of the odontoblastic layer of the pulp with a reduction in the number and size of odontoblasts. Early inflammatory changes following this are subtle and difficult to detect with the light microscope. The first indication of more obvious inflammation appears as a diffuse infiltration of mononuclear chronic inflammatory cells including lymphocytes, plasma cells and macrophages. It has been suggested that this inflammatory infiltrate is a local immune response to the components or metabolites of bacteria, as the prime function of the plasma cell is to produce immunoglobulin (Torneck, 1974). Accompanying this infiltrate is an increase in vascularity with the proliferation of small vessels and tissue oedema. In addition, there is some degeneration of pulp fibroblasts and destruction of collagen. As the lesion advances, enlargement of arterioles and capillaries, as well as the dilation of venules becomes evident. The small blood vessels become congested and oedema increases. At the microscopic level, alterations to endothelial cell morphology and disruption of the basement membrane with an accompanying increase in local vascular permeability have been reported (Langeland, 1987).

As the carious lesion progresses towards the pulp, the intensity and character of the inflammatory infiltrate changes. Inflammation begins as a low-grade chronic response, and it is only in the late stages that acute inflammation has been demonstrated. As the microorganisms penetrate deeper and invade reparative dentine, the adjacent pulpal tissue becomes infiltrated by macrophages and polymorphonuclear leukocytes that have been shown to emerge from adjacent small blood vessels (Torneck, 1977). Polymorphs have also been identified entering
and migrating into dentinal tubules (Trowbridge, 1981). Other forms of pulpal damage include lytic changes affecting fibroblasts with disruption of the plasma membrane and release of cytoplasmic organelles. Diffuse oedema has been reported throughout affected pulp tissue appearing as vacuoles surrounded by electron dense material at the periphery (Torneck, 1981).

Following pulp exposure, the incidence and degree of vascular change increases with more frequent disruptions of vessel walls, thrombosis and haemorrhage. By contrast, damage to the nerve tissue occurs later than in adjacent tissues possibly due to the abundance of collagen, which is less susceptible to proteolytic activity, surrounding the nerve bundles (Torneck, 1974). Evidence of microbial penetration into the pulp tissue is found by the presence of rod and coccal-shaped microorganisms both extracellularly and intracellularly within polymorphs and macrophages. These inflammatory cells are attracted to the area of microbial penetration in response to chemotactic activity and following phagocytosis of the bacteria they disintegrate. This releases the engulfed bacteria (dead or alive) and lysosomal enzymes into the pulp producing destruction of pulp tissue and liquefaction necrosis (Page et al., 1973; Langeland, 1987). An increase in the number of phagocytic cells in the pulp in advanced inflammation may be responsible for much of the cellular damage produced and be detrimental to the survival of the pulp (Torneck, 1977).

The progressive accumulation of polymorphs inevitably results in suppuration which clinically may become localised to form a small abscess within the coronal portion of the pulp. Surrounding this are chronic inflammatory cells found at a distance from the central area of irritation. The area of acute inflammation then becomes localised by the proliferation of fibrous tissue and small blood vessels to “wall-off” the affected region. At this stage, the remainder of the pulp may show no signs of pathology. If chronic inflammation persists for a long period, dystrophic mineralisation can occur with dead and dying inflammatory cells becoming mineralised through the deposition of calcium salts accumulating to form a mineralised mass. Although calcifications occur frequently, even in intact and unerupted teeth, the number and amount increases during the carious process and, therefore, they are regarded as pathological
entities as their presence reduces the ability of the pulp to respond to injury (Langeland, 1987).

Degeneration of the pulp occurs when the number of bacteria entering the pulp exceeds the ability of the pulp to withstand the insult. Whether or not the pulp then undergoes total necrosis depends on several factors including the virulence of the bacteria, the ability to offset an increase in intrapulpal pressure from tissue oedema and the resistance of the host. Host factors include the age and health of the pulp, which in turn influence the efficiency of the circulation and lymph drainage (Walton and Torabinejad, 1996).

Animal experiments have demonstrated the development of inflammation and abscess formation of surgically exposed pulps in control, but not germ-free animals (Kakehashi et al., 1965). In other studies, bacterial plaque extracts placed on freshly cut dentine produced inflammatory changes in the pulp presumably from penetration of bacterial products through the dentinal tubules (Mjör and Tronstad, 1972; Bergenholtz, 1977; Bergenholtz and Warfvinge, 1982). It was speculated by Mjör and Tronstad (1972) that immunological reactions had also occurred. In this context, following sensitisation of experimental animals to antigens applied through the root canal system, there was a strong local immunological response with accumulation of plasma cells, accompanied by a systemic reaction (Barnes and Langeland, 1965; Okada et al., 1967).

When the antigenic challenge is temporary, the inflammatory response is generally short-lived and self-limiting. However, where the process continues chronic pulpitis develops producing a predominantly mononuclear cell infiltrate (Torneck, 1977; Torneck, 1981). The relationship between depth of caries penetration and histopathology of the pulp has revealed that no significant pathology is noted until the carious lesion is approximately 1.1mm from the pulp. Once the carious process is within 0.3-0.5mm there is an increase in pathological changes with considerable inflammation (Reeves and Stanley, 1966). These pathological changes are considered to be the result of the activation of both non-specific inflammatory reactions as well as specific immunological responses.
3.3 Non-specific inflammatory reactions

Pulp injury involving cell damage and/or cell death is followed by the release of non-specific chemical mediators of inflammation. The release of histamine, interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor (TNF) is responsible for the earliest events in inflammation. Later effects are mediated by arachidonic acid metabolites, bradykinin, plasma-derived factors, other cytokines and the neuropeptides such as calcitonin gene related peptide and substance P (Walton and Torabinejad, 1996).

Histamine and serotonin are the two major vasoactive amines involved in early inflammatory reactions. Both occur in a variety of cells including mast cells, platelets and basophils. Histamine is considered to be the most important vasoactive amine and is released by inflammatory stimuli causing dilation of arterioles and increased vascular permeability. Mast cells are the main source of histamine; in addition they also release leukotrienes and platelet activating factor. Although histamine has been shown to be present in inflamed pulp tissue, controversy exists regarding the presence of mast cells in the human dental pulp. It has been reported that normal pulps lack mast cells, while small numbers have been found in inflamed pulps (Zachrisson, 1971; Miller et al., 1978).

Cytokines are peptides produced by different cell types, such as activated lymphocytes, macrophages, endothelium, epithelium and connective tissue cells, that modulate the function of other cell types by binding to specific receptors on target cells. The major cytokines that mediate inflammation include IL-1 and TNFα and β. Secretion of these products can be induced by endotoxins, immune complexes, exotoxins, cell injury and various inflammatory stimuli. The most important inflammatory effects include endothelial changes; specifically inducing the synthesis of endothelial adhesion molecules and chemical mediators, producing molecules associated with matrix remodelling, and the priming of neutrophils (Cotran, et al., 1999). The presence of elevated levels of the cytokines IL-6 (Barkhordar et al., 1999), IL-8 (Huang et al., 1999), IFN-γ, IL-4 and IL-10 (Hahn et al., 2000) have been demonstrated in inflamed pulps from carious teeth.
The main products of arachidonic metabolism include prostaglandins and leukotrienes which are synthesised from cell membranes by the activation of phospholipases in response to inflammatory stimuli. These are described as short-range mediators that are rapidly formed by most cell types, exert their effects locally and are enzymatically destroyed or spontaneously decay. Of the various prostaglandins that are produced by different cell types in the body, some are powerful vasodilators that increase vascular permeability and potentiate the effects of other vasodilator agents. Prostaglandins have been identified in inflamed pulps and elevated concentrations of PGE$_2$ have been located in pulps from symptomatic teeth (Cohen et al., 1985). Leukotrienes are also mediators of acute inflammation and are secreted by neutrophil polymorphs in inflammatory lesions. They are capable of inducing vascular dilation and of increasing venular permeability. In addition, they are potent chemotactic agents and can activate neutrophil responses such as adhesion to venular endothelium and the release of lysosomal enzymes (Anderson, 1985).

Three interrelated plasma-derived factors including complement, kinin and the clotting systems mediate a variety of activities in inflammation. The complement system consists of approximately 20 component proteins found in plasma. This system functions to provide defence by lysing microbes and facilitating increased vascular permeability, chemotaxis and opsonisation. Of the numerous steps involved in the cascade, the activation of C3 is probably the most critical and the most important inflammatory mediators are C3a and C5a (Cotran, et al., 1999). Most of the activators of the classical and alternate pathways of the complement system have been identified in inflamed pulp tissue or in periapical lesions. Both C3 and C4 have been located in dentinal tubules, in odontoblasts and associated with bacteria isolated from human carious dentine (Pulver et al., 1978). The kinin system generates vasoactive peptides from plasma proteins with the final release of bradykinin, a potent agent that increases vascular permeability.

Platelet-activating factor is another phospholipid derived mediator known to have multiple inflammatory effects. A variety of cell types elaborate platelet-activating factor,
including platelets, basophils, neutrophils, monocytes/macrophages and endothelial cells. The functions of this mediator, in addition to platelet stimulation, include vasodilation or vasoconstriction depending on concentration, increased venous permeability, chemotaxis and degranulation of leukocytes (Cotran, et al., 1999).

Pulpal innervation, in addition to mediating pain perception, may also modulate some aspects of the inflammatory response through the secretion of neuropeptides. Neurogenic responses include an increase in blood flow, plasma extravasation and leukocyte accumulation (Stashenko et al., 1998). Sensory nerve fibres containing calcitonin gene-related peptide and substance P have been shown to sprout into areas surrounding inflamed pulp tissue following injury (Byers, 1994). It has been proposed that these responses promote tissue healing by encouraging perfusion through angiogenesis, the removal of noxious substances by phagocytosis and by the close association of sprouting nerve fibres secreting calcitonin gene-related peptide at sites of reparative dentine formation. Support for this theory comes from experimental data reported by Byers and Taylor (1993) who demonstrated that innervated teeth with pulp exposures showed far less tissue necrosis than denervated teeth. In addition, Jontell (1998) has described an intricate network involving contact between nerve fibres and pulp micro-vessels via the cell processes of dendritic cells. These findings indicate that the nervous system and the pulp vasculature interact to recruit immunocompetent cells to the sites of tissue injury.

Phagocytosis of bacteria by polymorphonuclear leukocytes and macrophages is accompanied by the generation of reactive oxygen and nitrogen intermediates as the cells undergo a respiratory burst upon encountering invading microorganisms or other appropriate stimuli (Marton and Kiss, 2000). These cells also contain lysosomal enzymes which when released may contribute to tissue damage (Sandberg et al., 1988). Contents of the primary and secondary granules of PMNL include myeloperoxidase, lysozyme, cationic proteins, neutral proteinases, lactoferrin, elastase and cathepsin G. These components contribute to both the elimination of bacteria as well as the destruction of structural components of host tissues (Trowbridge and Stevens, 1992). Confirmation of the presence of some of these enzymes in
inflamed pulps was reported by Rauschenberger et al. (1991) who noted elevated levels of elastase, cathepsin G and lactoferrin in moderate to severely inflamed pulpal tissue samples compared with normal pulps.

3.4 Immunopathological mechanisms within the pulp

Once the integrity of the hard tissue barrier of the tooth is broken, noxious substances gain entry to the pulp tissues potentially activating immune responses by acting as antigens. These immune responses involve specific interactions with invading antigens which result in dense aggregations of macrophages, lymphocytes and plasma cells in inflamed pulp tissue (Torneck, 1977; Torneck, 1981). Although the roles for cell mediated and humoral immune responses have been inferred from the presence of specific cell types within the injured pulp, it is not apparent how the immunopathological mechanisms are organised.

Contrary to the findings of Seltzer and Bender (1984), the normal dental pulp has been found to contain a variety of immunocompetent cells capable of immunological responses. Jontell et al. (1987) first reported the presence of class II major histocompatibility complex (MHC) antigen-expressing dendritic cells in healthy pulps. These cells were noted in the odontoblastic layer and the central pulp tissue, and are believed to be responsible for the initiation of immune responses by presenting foreign antigens to CD4\(^+\) T lymphocytes. Inflammatory stimuli also promote the activation and differentiation of macrophages. The presence of resident macrophages in normal pulpal tissue has been documented from light and electron microscopic observations (Seltzer and Bender, 1984). Macrophages act primarily as scavengers by virtue of their phagocytic activity and are important for the elimination of antigen-antibody complexes. However, they also produce several bioactive substances including microbicidal enzymes, cytokines and growth factors for fibroblasts and endothelial cells. Class II MHC-expressing macrophages play a critical role in activating T lymphocytes (Jontell et al., 1998) and later in the inflammatory process B cells require assistance from macrophages for optimal stimulation and differentiation (Tew et al., 1989). Bergenholtz et al. (1991) reported a rapid and intense influx of MHC class II antigen expressing cells in
inflammatory lesions of the pulp induced by challenge with lipopolysaccharide from *P. gingivalis*. It is therefore reasonable to assume that macrophages and dendritic cells play a key role in the initiation of immune activities in the pulp in response to bacterial antigens.

Using monoclonal antibodies and indirect immunofluorescence, Pekovic and Fillery (1984) were the first to demonstrate the presence of T lymphocytes in inflamed pulpal tissue from teeth with advanced caries. Confirmation of this finding was reported by Hahn *et al.* (1989) using immunohistological staining with monoclonal antibodies. These investigators noted the presence of low numbers of T lymphocytes in normal tissue with increased numbers of T and B lymphocytes in inflamed pulpal tissue indicating the presence of immune reactions as part of the pathogenesis of the carious lesion. In early inflammation T lymphocytes play a crucial role in orchestrating immune responses following recognition of antigens. They secrete several cytokines that regulate the duration and intensity of immune reactions and are further classified into Th1 and Th2 cells depending on the cytokines released. The CD4⁺ Th1 lymphocytes produce mainly IL-2 and IFN-γ and are involved in macrophage activation, whereas Th2 cells produce IL-4, IL-5 and IL-6 and stimulate the proliferation and differentiation of B cells (Jontell, *et al.*, 1998).

In contrast to T lymphocytes, B cells are rarely seen in normal dental pulp tissue. Researchers have failed to identify B lymphocytes using immunohistochemistry to detect cytoplasmic or surface immunoglobulin (Pulver, *et al.*, 1977; Pekovic and Fillery, 1984) or with monoclonal antibodies against B cell markers (Jontell, *et al.*, 1987), while others have observed only occasional B lymphocytes (Hahn *et al.*, 1989). These data suggest that B cells do not participate in the initial phase of the immune response in pulpal tissue.

Early inflammatory changes in the pulpal tissue show a response composed of predominantly T lymphocytes and monocytes (Izumi *et al.*, 1995). As the carious lesion progresses, chronic inflammatory infiltrates of macrophages, lymphocytes and plasma cells accumulate in pulpal tissues (Massler, 1967; Torneck, 1974; Torneck, 1977; Langeland, 1987). Carious exposure of the pulp produces an increase in the severity of the inflammatory reaction
with an elevation in the number of macrophages and polymorphonuclear leukocytes (Torneck, 1977; Torneck, 1981). The transition from an early cellular immune response induced by mainly T-lineage cells to an advanced phase showing a humoral response containing B cells and plasma cells, has been described by Izumi et al. (1995). Accompanying these changes is an increase in the numbers of IgG-containing cells in inflamed pulps (Pulver, et al., 1977; Nakinishi et al., 1995). Significant numbers of IgA immunocompetent cells were also observed, with few IgM immunocompetent cells noted. Both Falkler et al. (1987) and Hahn and Falkler (1992) support the finding of predominantly IgG in irreversibly inflamed pulpal tissue. In contrast to the increasing quantity of immune components found with inflammation, levels of the third component of complement (C3) reportedly become diminished in inflamed pulps compared with normal pulpal tissue, which may indicate that the inflammatory response is consuming C3 (Speer et al., 1977; Okamura, et al., 1980; Pekovic and Fillery, 1984;). One mechanism for complement removal during carious pulpitis is the cleavage of C3 and C5 into fragments by proteolytic activity from Gram-negative anaerobes such as P. gingivalis, P. endodontalis and P. intermedia (Sundqvist et al., 1985). In conjunction with these changes, an increase in soluble bacterial products, components of the complement system and arachidonic acid metabolism products accumulate causing cellular destruction. These components are also chemotactic for polymorphs and macrophages, which on release of lysosomal enzymes cause further destruction of the pulpal tissue.

Studies over the last decade have further characterised the profile of cellular infiltrates using immunohistochemistry. Hahn et al. (1989) identified and enumerated the T and B lymphocytes in pulpal tissues from carious teeth that were clinically diagnosed with reversible or irreversible pulpitis. They found that CD8+ T cells outnumbered CD4+ T cells in normal pulpal tissue, which is in agreement with the findings of others (Jontell, et al., 1987), although Mangkornkarn et al. (1991) detected greater numbers of CD4+ T cells. In inflamed pulps, Hahn et al. (1989) reported increased numbers of CD4+ helper cells, CD8+ T cells and B lymphocytes. In the group with reversible pulpitis, more than 90% of the lymphocytes were identified as T
lymphocytes, with a CD4⁺/CD8⁺ ratio of 0.56, while the irreversible group was characterised by a higher number of all T lymphocytes, B lymphocytes, plasma cells and neutrophils. The CD4⁺/CD8⁺ T cell ratio in this group was 1.14. B cell numbers increased markedly with increasing inflammation, with B/T ratios of 0.04 (normal), 0.05 (reversibly inflamed) and 1.6 (irreversibly inflamed). These findings were confirmed by Izumi et al. (1995) who found numerous T lymphocytes in shallow dentine caries with an increase in B cell and plasma cell numbers in teeth with deep caries.

These findings, in addition to the observations of other authors (Torneck, 1981; Trowbridge, 1981), suggest that immunocompetent cells are important in the pathogenesis of carious pulpitis and emphasise the central role of T lymphocytes in regulating immune functions. Whether the inflammatory lesion has destructive or protective characteristics will depend on the regulatory functions of the T lymphocytes. A high ratio of CD4⁺/CD8⁺ T cells with a predominance of B lymphocytes in the irreversibly inflamed pulpal tissue could be explained by the regulation of the CD4⁺ T cells on B cell activities. Despite the high numbers of CD8⁺ T cells noted in these studies, a continuing influx of antigens from the carious lesion as well as excessive activation of the CD4⁺ helper T cells, B cells and plasma cells, could create immunopathological changes caused by lymphokine release and immunoglobulin production.

The periapical lesion represents a local immune reaction subsequent to pulpal infection. Continuous flow of bacterial antigens and tissue breakdown products through the apex from the pulpal tissue causes either an acute or chronic response in the periapical tissues, depending on the balance between the microbial factors and the host defences. An acute response is characterised by an influx of polymorphonuclear leukocytes and monocytes into the periapical area. This is followed by more chronic cell infiltrates including T and B cells, macrophages, plasma cells, eosinophils and mast cells together with connective tissue cells. The chronic response is characterised by granulomatous tissue infiltrated by chronic inflammatory cells. Both CD4⁺ and CD8⁺ subsets of T cells have been identified in periapical lesions with CD4⁺ cells dominating in the active phase of lesion expansion, followed by higher levels of the CD8⁺
and plasma cells appearing as the lesion becomes more chronic. These changes suggest that CD8+ T cells may function to dampen excessive immunoreactivity within periapical lesions (Stashenko and Yu, 1989; Marton and Kiss, 2000).

Studies using the rat model indicate that two days after pulpal exposure and infection, pulpal necrosis occurs, followed at seven days by periapical bone destruction. A period of rapid destruction then takes place between 7-20 days followed by a chronic phase characterised by slowed resorption of bone. The rapidity of bone destruction, often preceding total pulpal necrosis, suggests that many of the pathogenic effects are the result of soluble mediators such as cytokines rather than the necrotising effects of bacterial components acting directly on the tissues (Stashenko, et al., 1998). Interleukin 1α has been identified as a pro-inflammatory cytokine associated with periapical bone destruction. It is produced by a variety of cells including macrophages, fibroblasts and endothelial cells in response to lipopolysaccharide. In addition, there is evidence that matrix metalloproteinases from host cells contribute to periapical tissue breakdown through collagen destruction and that bone resorption can also be induced by inflammatory factors and antibodies produced by B cells and plasma cells in the more chronic phase of infection (Takahashi, 1998).

The immune system has both a protective and destructive role. In a protective mode it is capable of mobilising protection of the pulp against injurious stimuli. Conversely, these mechanisms also have the capacity to cause damage during the inflammatory process by activation of complement, release of chemotactic agents and attraction of neutrophils to phagocytose immune complexes. Consequent release of lysosomal enzymes and inflammatory mediators from phagocytic cells and invading bacteria can cause considerable destruction to the surrounding tissue.

In an attempt to evaluate the role of microorganisms putatively involved in the aetiology and pathogenicity of carious pulpitis, thesis studies were designed to identify and enumerate bacterial species found in the carious dentine of vital human teeth with chronic pulpitis. In the first study traditional culture techniques were used to identify and enumerate the microbial
species retrieved. In the second study identification of microorganisms, from the same samples, involved molecular methods of identification using polymerase chain reaction technology. Finally, the histopathology of the pulps was examined and correlations were sought between the microbial type and number in the sampled carious dentine and the cellular responses of the dental pulp. Details of the first study form the basis for the next chapter.
CHAPTER 4

A CULTURE STUDY OF THE MICROBIOLOGY OF HUMAN CARIOUS DENTINE

4.1 Introduction

Dental caries has been recognised as a bacterially mediated disease since 1890 when Miller introduced the chemoparasitic theory of tooth decay. Despite many decades of research, the bacterial profile of dental caries remains incomplete and understanding of microbial effects on the dental pulp, inadequate. Therefore, dental caries continues to be a major public health problem in many parts of the world despite the recognised advantages of fluoride in reducing tooth decay at a community level.

While it is known that bacteria and their products are responsible for pulpal inflammation as a result of dental caries, few studies have investigated the microbiology of carious dentine or the relationship between dentinal microflora and the histopathology of chronic pulpitis. As a result there is no reliable evidence to assign an aetiological role for carious pulpitis to a particular microbial species. Massey et al. (1993) demonstrated a significant relationship between the number of Prevotella spp. in carious dentine and an advanced inflammatory pulpal change. This study expands on the results of previous work by examining the microbiology of carious dentine in human teeth using culture techniques.

4.2 Materials and methods

4.2.1 Sample collection

Sixty five vital carious teeth were obtained with informed consent from separate, randomly selected patients of either sex from the United Dental Hospital in Sydney, Australia. The age of the patients ranged from 17 to 75 years with an average age of 37 years. Patients were excluded from the study if they reported a history of significant medical disease or antimicrobial therapy within four months prior to tooth extraction. The patients presented with
clinical signs and symptoms of pulpitis and requested extraction to relieve symptoms. The nature of symptoms was noted at the time of sampling.

Anterior, premolar and molar unrestored teeth with coronal enamel and dentine caries were selected for the study on the basis of clinical diagnostic tests which indicated that they were vital, with clinical symptoms of reversible pulpitis (pain and heightened sensitivity to hot and cold stimuli). These teeth had no obvious exposure of the pulp tissue and periodontal pocket depths of less than 4mm. This study was approved by the Central Sydney Area Health Service Ethics Review Committee, Sydney, Australia (Reference No: 6/96).

4.2.2. Dentine processing

Immediately after extraction under local anaesthesia, each tooth was placed in a container of pre-reduced transport fluid (RTF) (Syed and Loesche, 1972) and transferred immediately to an anaerobic glove chamber containing 85%N₂, 5%CO₂, 10%H₂. Superficial plaque and debris overlying the lesion was first removed, the teeth were rinsed several times with RTF, then using sterile sharp curettes and excavators, all softened and necrotic dentine was collected 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. Evidence of carious pulpal exposure was noted at this stage. Dentinal sampling was completed less than 20 minutes 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 in RTF was prepared for each tooth. The fragments of carious dentine were dispersed in the chamber by vortexing for 20 seconds before being homogenised by hand in a 2ml glass homogeniser for 30 seconds using a standardised technique of vertical and rotational movements.

Serial dilutions in the range 10⁻³ to 10⁻⁶ in RTF were prepared for plating. Plates were duplicated for each dilution and each medium then incubated in either the glove chamber for up to 2 weeks or, for microaerophilic conditions, in an anaerobic jar with a CO₂ gas pack (Oxoid,
Basingstoke, UK) at 37°C for 48 hours. Isolates representing five microbial genera: *Streptococcus*, *Lactobacillus*, *Actinomyces*, *Fusobacterium* and *Prevotella* were chosen for more detailed study on the basis of previous studies of carious dentine (Hoshino, 1985; Massey et al., 1993). The following media and conditions were used to examine both selective and general growth as described by Massey et al. (1993). Total microbial load per mg wet weight of dentine was determined by the number of colony forming units (CFU) on Trypticase Soy agar (Oxoid) enriched with menadione, haemin, L-cysteine and horse blood (ETSA) under both anaerobic and microaerophilic conditions (US Department Health and Human Services–Centres for Disease Control). Mitis Salivarius (Oxoid) (Chapman, 1944) and Rogosa agar (Bacto Laboratories, Detroit, MI, USA) (Rogosa et al., 1951) were used to study the growth of streptococci and lactobacilli respectively under microaerophilic conditions (95%N₂ and 5%CO₂). Cadmium fluoride acriflavine tellurite (CFAT) agar was used for the selective isolation of *Actinomyces* spp. and related Gram-positive filamentous organisms under anaerobic conditions (Zyler and Jordan, 1982). A kanamycin-vancomycin containing anaerobic blood agar (KVA) was used for identification of the obligately anaerobic *Prevotella* spp. (Dowell, 1975). Crystal violet erythromycin (CVE) agar was used under anaerobic conditions for the isolation of *Fusobacterium nucleatum* (Walker et al., 1979).

An initial group of twenty specimens was examined to identify the main colony forms on selective media. One colony forming unit of each type seen was subcultured, Gram-stained and assessed with biochemical diagnostic test kits: RapID ANA II (Innovative Diagnostic Systems, Atlanta, GA, USA) for anaerobic bacteria; Rapid ID 32 Strep for streptococci and, api 50 CH and api 50 CHL medium for lactobacilli (bioMérieux, Marcy-l’Étoile France). To confirm the accuracy of testing, strains of known species were employed: *Streptococcus mutans* LT11 (Tao et al., 1993), *Lactobacillus acidophilus* ATCC 4356 (Institute of Dental Research Culture Collection, Westmead Centre for Oral Health, Westmead, NSW, Australia), *Actinomyces israelii* ATCC 12102, *Prevotella melaninogenica* ATCC 25845 and *F. nucleatum* ATCC 25586 (American Type Culture Collection, Rockville, MD, USA) were used as controls. Following this identification, plates from each of the clinical samples were examined and the numbers of each main colony
type recorded. The presence of *Streptococcus salivarius* colonies was used as an index of salivary contamination of the samples.

4.2.3. Statistical analyses

Non-parametric methods of statistical analysis were applied as preliminary scrutiny indicated that the data were markedly skewed. Basic descriptive statistics were applied to the microbial counts from the culture data including mean, standard error, median, minimum and maximum counts.

4.3 Results

The number of colony forming units per mg of carious dentine showed considerable diversity and inter-subject variability of the microflora (Table 4.1). Hundred fold differences in total microbial loads were apparent between samples and selective counts varied between teeth by up to three orders of magnitude. Data for selective microbial groups were noticeably skewed, with the mean values greater than the median values in each case. Anaerobic microorganisms were isolated from all samples and the numbers of colonies cultivated anaerobically, on non-selective plates, were approximately six times greater than those grown under microaerophilic conditions. Culture data showed a predominance of Gram-positive microorganisms, with *Lactobacillus* spp. cultivated in greatest numbers on selective media. Gram-negative organisms were also present in significant numbers with *Prevotella* spp. being the largest anaerobic group. These data showed 97% of teeth positive for *F. nucleatum* and *Streptococcus* spp. (63/65), 95% for *Lactobacillus* spp. (62/65), 91% for *Prevotella* and *Actinomyces* spp. (59/65) and 88% for *P. melaninogenica* (57/65).

On the basis of colony form, five main types were identified on CFAT medium. Biochemically, these resembled *A. israelii, Actinomyces odontolyticus, A. naeslundii, Bifidobacterium* and *Propionibacterium* species. Seven main colony types were evident on the KVA medium which were identified biochemically as *P. melaninogenica, P. intermedia, Prevotella buccae, Prevotella oris, Prevotella corporis, Capnocytophaga* spp. and *Lactobacillus* species. Of these, the black pigmented prevotellae were isolated in the greatest numbers. Three colony types were
evident using CVE media and biochemically they were identified as *F. nucleatum*. *Leptotrichia buccalis* was also noted.

Streptococci were studied on Mitis Salivarius agar. Four main colony types were distinguishable and biochemically these resembled *S. mutans*, *S. salivarius*, *Streptococcus anginosus* and *S. sanguinis*. *Lactococcus, Leuconostoc* and *Enterococcus* species were also present. Lactobacilli were grown on Rogosa agar and nine main colony forms were identifiable. These resembled *L. acidophilus, Lactobacillus paracasei, Lactobacillus rhamnosus, Lactobacillus fermentum* and *Lactobacillus plantarum* when tested biochemically. *Lactobacillus acidophilus* presented in five different colony configurations and was numerically dominant.

The use of biochemical tests to identify microorganisms from dentine caries provided variable results and indicated that microbes of different appearance could be identified as the same organism. One example is described in more detail; the appearance of *P. melaninogenica* varied from a 1mm circular mid-brown, shiny, pulvinate colony with a smooth translucent edge to a 2.5mm tan to dark brown umbonate colony with a ruffled edge. The different colony morphologies were identified by a range of codes using the RapID ANA II System (Table 4.2).
### Table 4.1. Bacteria detected in carious dentine by colony counting.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>CFU (mg dentine)</th>
<th>Range</th>
<th>Median</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobic</td>
<td>$1.4 \times 10^5$ – $3.7 \times 10^7$</td>
<td>$55 \times 10^5$</td>
<td>$73 \pm 9.0 \times 10^5$</td>
<td></td>
</tr>
<tr>
<td>Microaerophilic</td>
<td>$2.5 \times 10^4$ – $4.7 \times 10^6$</td>
<td>$8.4 \times 10^5$</td>
<td>$12 \pm 1.0 \times 10^5$</td>
<td></td>
</tr>
<tr>
<td><em>Prevotella</em></td>
<td>$0.0$ – $5.1 \times 10^6$</td>
<td>$1.1 \times 10^5$</td>
<td>$4.7 \pm 1.3 \times 10^5$</td>
<td></td>
</tr>
<tr>
<td><em>P. melaninogenica</em></td>
<td>$0.0$ – $5.1 \times 10^6$</td>
<td>$1.0 \times 10^5$</td>
<td>$4.3 \pm 1.3 \times 10^5$</td>
<td></td>
</tr>
<tr>
<td><em>F. nucleatum</em></td>
<td>$0.0$ – $1.0 \times 10^6$</td>
<td>$0.8 \times 10^5$</td>
<td>$1.8 \pm 0.3 \times 10^5$</td>
<td></td>
</tr>
<tr>
<td>Actinomycetes</td>
<td>$0.0$ – $5.3 \times 10^6$</td>
<td>$0.7 \times 10^5$</td>
<td>$2.3 \pm 0.9 \times 10^5$</td>
<td></td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>$0.0$ – $1.9 \times 10^7$</td>
<td>$5.2 \times 10^5$</td>
<td>$10 \pm 3.0 \times 10^5$</td>
<td></td>
</tr>
<tr>
<td>Streptococci</td>
<td>$0.0$ – $3.1 \times 10^7$</td>
<td>$1.7 \times 10^5$</td>
<td>$3.7 \pm 0.7 \times 10^5$</td>
<td></td>
</tr>
</tbody>
</table>

* Data collected from 65 samples.
* All *Prevotella* colonies grown on KVA
* Colonies identified biochemically as *P. melaninogenica*

### Table 4.2. Different colony morphologies and identification codes for *P. melaninogenica* using the RapID ANA II System*.

<table>
<thead>
<tr>
<th>Colony morphology</th>
<th>ANA identification code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small, mid to dark brown, smooth or rough edge, pulvinate or umbonate</td>
<td>027340</td>
</tr>
<tr>
<td>Mid-brown, smooth edge, convex</td>
<td>037340</td>
</tr>
<tr>
<td>Small, shiny brown, smooth translucent edge, pulvinate</td>
<td>427340</td>
</tr>
<tr>
<td>Light to mid-brown, smooth, translucent edge, pulvinate or umbonate</td>
<td>437300</td>
</tr>
<tr>
<td>Mid to dark brown, ruffled or clean edge, wet surface, brown or tan coloured, umbonate</td>
<td>437340</td>
</tr>
</tbody>
</table>

*While the identification codes supported classification of the colonies as *Prevotella*, it should be noted that colony morphology was highly variable.*

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4.4 Discussion

The results of the present study are in agreement with others in finding a large variation in microbial counts between samples. There was also agreement that anaerobes comprised the majority of the organisms cultured and that the largest group of isolates were the facultative Gram-positive rods, although *Lactobacillus* spp. were isolated in lower frequency than in previous studies (Loesche and Syed, 1973; Hoshino, 1985; Hahn et al., 1991; Massey, et al., 1993) which may reflect the selection of carious, symptomatic teeth used in this study.

Compared with the data reported by Massey et al. (1993), CFU counts from this study indicated approximately a one hundred-fold reduction in the mean bacterial counts from non-specific and selective media. However, these values were within the range reported in previous studies (Loesche and Syed, 1973; Hoshino, 1985; Hahn, et al., 1991) and may represent differences in the efficiency of liberating microorganisms from the carious dentine during homogenisation of the samples. There was also a considerable reduction in the peak incidence of some species noted in this study, with values of 14% for *Actinomyces* spp., 46% for *Lactobacillus* spp. and 18% for *Streptococcus* spp., which contrasted with the data from Massey et al. (1993) of 31%, 71% and 61% respectively. Low recovery of streptococci, particularly *S. mutans*, from carious lesions has been reported previously (Edwardsson, 1974; Hoshino, 1985; Hahn, et al., 1991), with none recovered from pulpal and deep caries sites (Hahn, et al., 1991). However, recovery of *S. mutans* may have been enhanced using a Mitis-Salivarius agar containing kanamycin or bacitracin (Kimmel and Tinanoff, 1991). Takazoe et al. (1973) showed minimal invasion of calcified dentine by *S. mutans in vitro* unless proteinase was added, indicating the potential for invasion only after the organic dentine matrix had been partially decomposed. In addition, only those strains of *S. mutans* expressing adhesins specific for type I collagen were found to be capable of invading dentine tubules (Love et al., 1997).

In agreement with Massey et al. (1993), Gram-negative bacteria comprised a significant percentage of the total cultivable flora in this study where, in some specimens, *Prevotella* spp. comprised at peak incidence up to 21% and *F. nucleatum* up to 12%. However, other authors noted few or no Gram-negative rods (Loesche and Syed, 1973; Edwardsson, 1974; Hoshino,
1985; Hahn, et al., 1991) or did not investigate for anaerobic microorganisms (McKay, 1976). This may relate to a lack of awareness of anaerobes or to difficulties involving their isolation, especially in early studies where samples were exposed to air (Loesche and Syed, 1973) or processed without an adequate anaerobic environment (Edwardsson, 1974). Similar concerns have also been reported in the area of wound infection where studies during the last two decades have involved minimal or no isolation of anaerobes because of a tendency to regard aerobic or facultative microorganisms as the primary pathogens (Bowler et al., 2001).

In a study of carious dentine from 29 human teeth with irreversible pulpiteis, Hahn et al. (1991) found two main types of carious lesions. The first with high numbers of lactobacilli and low counts of streptococci and bacteroides (Prevotella and Porphyromonas spp.), the second with low lactobacilli numbers, numerous black-pigmented bacteroides, increased numbers of streptococci and non-lactobacilli Gram-positive rods including actinomycetes, propionibacteria, bifidobacteria, arachnia and eubacteria. Although this Lactobacillus spp. microbial pattern was not seen in the present study, examples of non-lactobacilli Gram-positive rods were recovered from the carious dentine samples and have been previously isolated from carious dentine (Massey, et al., 1993), necrotic dental pulps (Lana, et al., 2001) and infected dentine of root canals (Ando and Hoshino, 1990).

Studies have demonstrated the development of bacterial succession in periodontal microflora (ter Steeg and van der Hoeven, 1989) and root canal microflora in rat periapical lesions (Tani-Ishii et al., 1994). Both studies showed a transition from rapidly growing, microaerophilic, saccharolytic bacteria to an increasingly diverse group of isolates dominated by a Gram-negative, anaerobic microflora. It has been postulated that this transition in carious dentine may be associated with the development of pulpal necrosis (Hahn, et al., 1991). This bacterial succession, presumably, results from changes in bacterial relationships due to alterations in the environment and nutritional interactions. Early bacterial coaggregation in dental plaque has been demonstrated between Actinomyces spp. and Streptococcus spp. (Crowley et al., 1987) and is followed by late colonisers that include fusobacteria, eubacteria, prevotellae and porphyromonads (Kolenbrander and London, 1993). The decrease in
facultative organisms with time may be due to a restriction of available nutrients with a lowering in the oxidation-reduction potential of the surrounding environment which then favours the growth of anaerobes. In addition, anaerobes metabolise amino acids and proteins from tissue fluids, produce a number of factors which kill other microbial species (Socransky and Haffajee, 1991), assist colonisation and resist phagocytosis (Slots and Genco, 1984; Sundqvist, 1993), which enhances their dominance in this environment.

Prevalence of the Gram-positive microaerophilic bacteria following culture of the carious dentine may be a result of their high numbers or due to the relative ease with which these microbes can be cultivated compared with Gram-negative anaerobes. It is well recognised that obligately anaerobic bacteria are difficult to culture and the more fastidious varieties may be suppressed by the rapid growth of more robust species creating an inherent bias. During cultivation of the carious samples a number of inconsistencies were noted: in some samples plentiful growth of black-pigmented microbes on non-specific plates was not reproduced on the specific KVA plates, although it should be noted that the *Porphyromonas* species could have been inhibited by the presence of kanamycin; in other samples shown by Gram stain to contain a large number of Gram-negative rods or cocci, the number of Gram-negative anaerobes cultured was low; and variation was noted in the colony morphology and biochemical identification codes of a number of bacteria including *P. melaninogenica*. To clarify these anomalies it became apparent that additional, molecular, methods of bacterial identification were required. In the following chapter the application of PCR technology to microbial identification and enumeration is reviewed and experiments investigating the preparation of DNA for real-time PCR are described.