DEVELOPMENT OF A MODEL TO STUDY THE INTERACTION OF *Staphylococcus epidermidis* WITH PHAGOCYTIC CELLS ON THE SURFACE OF BONE AND PROSTHETIC JOINT MATERIAL

By

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"There is at bottom only one genuine scientific treatment for all diseases, and that is to stimulate the phagocytes. Drugs are a delusion."

Act 1
The Doctor's Dilemma
George Bernard Shaw, 1909

This thesis is dedicated to my family

I would like to thank the following people for their help throughout this work:

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The genus *Staphylococcus* contains common pathogens of both humans and animals. Staphylococci are non-motile, nonspore-forming, gram-positive facultative anaerobes. In humans two species are important. Originally, *Staphylococcus aureus* was considered to be the only pathogenic species. This was based on epidemiological studies and characteristics of *S. aureus* such as: coagulase production, mannitol fermentation and the presence of protein A. However, this has changed rapidly and *Staphylococcus epidermidis* is increasingly recognised as an important human pathogen in a variety of clinical settings. This may be explained by the increase in the use of prostheses, catheters, immunosuppressive therapy or the ability of *Staphylococcus epidermidis* to become resistant to the antibiotics originally successful in treating such infections.

Biomaterials have become an essential component of current medical and surgical practice. Their functional applications range from contact lenses to joint replacement systems. Implanted biomaterials may elicit a range of local, and sometimes, general host responses. One of these is activation of the complement system and the generation of mediators of inflammatory response which involves blood and tissue cells. In certain situations a chronic inflammatory response may occur. This results in a layer of inflammatory cells between the implant and the surrounding tissue. Bacterial adhesion onto biomaterials is an essential step in the pathogenesis of biomaterial-associated infection. Factors affecting bacterial adhesion include hydrophobicity; of both the bacterium and the substrate. Hydrophobic interaction involves long and short-range forces. Long range forces come in to play first and are generally attractive in nature, but as the bacterium approaches the substratum short-range repulsive forces between the bacterium and the substrate then occur. These repulsive forces hold the bacterium close to the surface. *S. epidermidis* has certain factors that allow it to adhere to, and subsequently colonize, indwelling devices. This was first reported by Bayston and Penny when they noticed that strains of *S. epidermidis* able to cause CSF shunt infections formed adherent mucoid deposits on the shunt in vitro. SEM studies

have shown that after an initial attachment phase there is an accumulation phase, where an extracellular slime substance is produced. This slime is loosely bound to the surface of the bacterial cell and consists of: glucose, galactose, mannose, glucosamine and glucuronic acid. This slime layer provides a protective environment for the bacteria to proliferate, away from the detrimental effects of the host immune system, such as phagocytosis. Phagocytosis involves attachment of the surface membrane to the foreign material and then engulfment of the membrane around it. Like adherence phagocytosis may also be influenced by certain conditions. One is the presence of opsonins. Opsonins include the complement component C3 and specific IgG molecules. Opsonins are reported to facilitate the uptake of bacteria by neutrophils.

In this study the phagocytic ingestion of two strains of *Staphylococcus epidermidis* adherent to the surface of a biomaterial was investigated. The two strains used were M7 and RP62A, and although both can adhere, strain M7 cannot accumulate onto a substrate. The biomaterial chosen for the study was polymethyl methacrylate, a polymer commonly used in the manufacture of orthopaedic implants. Phagocytic ingestion of the bacteria from the biomaterial was then compared to phagocytosis from bone and polystyrene. Factors affecting phagocytosis such as: slime production, hydrophobicity and the differences in phagocytic capacity of the polymorphonuclear leukocytes, by comparing neutrophils from different patient populations, was investigated. The phagocytic capacities of different cells were compared. These cells were neutrophils, monocytes and J774 cells- a murine macrophage-like cell line.

This study showed that both strains of bacteria were able to adhere to the substrates tested in equal amounts. Differences arose when one strain, (RP62A) was then able to accumulate and produce slime whereas M7 could not. Accumulation was noted by the absence of any slime production by strain M7. Both strains of bacteria were found to be hydrophilic in nature. This was determined using two methods, MATH and HIC, both of which correlate well. When phagocytosis of the two strains was compared, no significant difference was found between strains. Strain M7 was slightly easier ingested by neutrophils than RP62A. When neutrophil phagocytosis was compared to phagocytosis by monocytes and J774 cells the order of increasing phagocytic capacity was monocytes > J774 cells > neutrophils.

The nature of the substrate can also affect the ingestion of adhered bacteria. Both strains were found to be hydrophilic in nature. Unfortunately the hydrophobicity of the substrates was not determined but strain to strain differences in the ingestion of adhered bacteria was observed. M7 was phagocytosed with no significant difference between any of the substrates. However, with RP62A there was no significant difference between bone and prosthetic joint material, but a highly significant difference was observed between bone and polyethylene.

The phagocytic capacity of patient groups to ingest adhered bacteria was also investigated. Two patient groups, those with Diabetes Mellitus and those with Rheumatoid Arthritis were studied. They were chosen as both were groups of patients who have known immunodeficiencies and may require joint replacement therapy due to the nature of their disease. There was no significant difference in the phagocytic capacity of the neutrophils from patients with Rheumatoid Arthritis or with Diabetes Mellitus compared to the control group. When the groups themselves were further investigated only a gender difference in patients with diabetes mellitus was found. A significant difference between male and female neutrophils to ingest strain RP62A was highlighted. No such difference was observed in patients with Rheumatoid Arthritis. Thus is it not the phagocytic capacity of the neutrophils from these patient populations that pre-disposes them to infection after joint arthroplasty.

In order to understand better the mechanisms of such bacterial infections it is important to devise a model where the clinical setting is aptly reconstructed in the laboratory. The aims of this study were as follows;

- Determine the hydrophobicity levels of two strains of coagulase-negative staphylococci that are known to differ in their ability to produce slime
- Investigate the role of bacterial cell surface hydrophobicity in mediating adherence of coagulase-negative staphylococci to substrates
- To measure the kinetics of adherence of coagulase-negative staphylococci to prosthetic joint material and bone
- To measure the effect of prosthetic joint material on the activation of neutrophils and the subsequent effect (if any) on their phagocytic capacity
- To determine the most efficient phagocytic cells at internalisation of adherent coagulase-negative staphylococci
- To determine the phagocytic capacity of neutrophils isolated from different patient populations of adherent coagulase-negative staphylococci on either prosthetic joint material or bone

AIDS	Acquired immune deficiency syndrome
ATP	Adenosine triphosphate
BATH	Bacterial adhesion to hydrocarbons
CAM	Contact angle measurement
CL	Chemiluminescence
CoNS	Coagulase-negative Staphylococci
CRP	C-reactive protein
CSC	Cell surface charge
CSH	Cell surface hydrophobicity
DM	Diabetes mellitus
DVLO	Deraguin-Landlau-Verwey-Overbeek
EDTA	Ethylene-diamine tetra-acetic acid
ELISA	Enzyme-linked immunoabsorbent assay
ESR	Erythrocyte sedimentation levels
ESS	Extracellular slime substance
FCS	Fetal calf serum
GAGS	Glycosaminoglycans
GRI	Glasgow Royal Infirmary
HBSS	Hanks balanced salt solution
HIC	Hydrophobic interaction chromatography
HIV	Hepatitis immunodeficiency virus
HLA	Human leukocyte antigens
HS	Highly significant
IBAD	Ion-beam-assisted deposition
IDDM	Insulin-dependent diabetes mellitus
LPS	Lipopolysaccharide
MAA	Methacrylic acid
MAC	Membrane attack complex
MATH	Microbial adhesion to hydrocarbons
MNs	Monocytes
NADPH	Nicotinamide adenine dinucleotide phosphate

NIDDM	Non-insulin dependent diabetes mellitus
NPS	Normal pooled serum
NS	Not significant
NSAIDs	Non-steroidal anti-inflammatory drugs
OH	Hydrogen peroxide
PBS	Phosphate buffered saline
PIA	Polysaccharide intracellular adhesin
PMA	Phorbol myristate acetate
PMMA	Polymethyl methacrylate
PMNLs	Polymorphonuclear leukocytes
PS/A	Polysaccharide adhesin
PTFE	Polytetrafluoroethylene
RA	Rheumatoid arthritis
SAA	Slime associated antigen
SAARDs	Slow-acting anti-rheumatic drugs
SAT	Salt aggregation test
SD	Standard deviation
THR	Total hip replacement
TPP	Two-phase partition system
TSB	Trypticase soy broth
UHMWPE	Ultra-high molecular weight polyethylene

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Coagulase-negative Staphylococci

Coagulase-negative Staphylococci (CNS) are commonly found among the normal bacterial inhabitants of human skin and mucous membranes (Peters, von Eiff & Hermann, 1995). They are currently the predominant cause of nosocomial infections, especially those involving prosthetic devices (Rupp & Archer, 1994). Staphylococcal infections are a major clinical problem as they are associated with a high mortality rate (Graninger, Wenisch & Hasenhundl, 1995).

In 1882 Sir William Ogston introduced the name *staphylococcus* to describe the cluster-forming, cocci-shaped bacteria he observed to be the cause of pyogenic abcesses. Two years later Rosenbach further classified staphylococci into two groups on the basis of their pigment; the virulent, golden *Staphylococcus aureus* and the avirulent, white *Staphylococcus albus*. Currently there are thirty-one members of the genus (Kloos & Bannermann, 1994) which can be classified into four groups, the *Staphylococcus epidermidis* species group, the *Staphylococcus saprophyticus* species group, the *Staphylococcus simulans* species group and the *S. sciuri* group based on DNA-DNA hybridisation studies (Brooks *et al.,* 1991).

Of the thirty-one species only twelve are mainly found in specimens of human origin (Peters, von Eiff & Herrmann, 1995). Within the *S. epidermidis* group, *S. epidermidis* is the predominant coagulase-negative staphylococcus species found followed *by S. haemolyticus* and *S. hominis*. Recognition of the importance of *Staphylococcus epidermidis* as a pathogen has evolved over the past two decades. One of the major problems of daily diagnostic work is to distinguish clinically-significant coagulase-negative staphylococci from contaminant strains (Peters, von Eiff & Herrmann, 1995). Often dismissed in the past as harmless commensals or contaminants, such bacteria must now be individually evaluated as potential true pathogens as they are frequently isolated from various clinical specimens in the laboratory (Rupp & Hamer, 1998). This evolution has paralleled advances in medical technology, and *S. epidermidis* is now recognised as a major cause of infection of orthopaedic devices (Barton, Sagers & Pitt, 1996; Moussa *et al.*, 1996; Isiklar *et al.*, 1996 and An & Freidman, 1997), intravenous catheters (Greenfeld *et al.*, 1995 and John *et al*, 1995), intravascular catheters (Christensen, Baldassarri &

Simpson, 1995; Khardori, Yassien & Wilson, 1995; Khardori & Yassein, 1995 and Ley *et al.*, 1996), urinary catheters (Brisset *et al.*, 1996; Kiremitci-Gumusderelioglu & Pesmen, 1996), prosthetic valve endocarditis (Shiro *et al.*, 1995; Hedin, 1996; VanWijngaerden *et al.*, 1997 and Perdreau-Remington *et al.*, 1998), peritoneal dialysis catheters (Findon & Miller, 1995; Harris, Tebbs & Elliott, 1995 and Hussain *et al.*, 1997), and contact lenses (Fleiszig *et al.*, 1996 and Gabriel *et al.,* 1996). CNS infections are also commonly found in immuno-compromised hosts such as those who have undergone cardiac surgery, premature neonates (Hubner & Kropec, 1995 and Karamanos *et al.*, 1997) and cancer patients (van Belkum *et al.,* 1996). The prevalence of such infections may be explained by the existence of bacterial virulence factors such as extracellular slime substance, cell surface hydrophobicity, cell wall components and proteins which are discussed in greater detail in this and chapters Two and Three.

Humans are the natural reservoir for *S. epidermidis*. The bacteria are an important part of the normal skin flora and are the commonest staphylococcal species isolated from cutaneous sites (Hesdin, 1996). *Staphylococcus epidermidis* is easily shed from these sites, contaminating the air, other persons, or inanimate environmental surfaces. *Staphylococcus epidermidis* infections result from the contamination of a surgical site by organisms from the patients' skin or nasopharynx or from exogenous sources such as hospital personnel or during skin preparation and draping. This was shown in a study of operating personnel. Theatre assistants who were unscrubbed and ungowned resulted in bacterial counts being 4.4 times higher during preparation and draping than during the operation itself. When the assistants were scrubbed and gowned the counts were reduced but were still 2.4 times greater than that observed intraoperatively (Brown, Taylor & Gregg, 1996). It was also noted during this study that instrument packs should be opened only after skin preparation and draping has been completed. Surveillance studies of operating room environments have shown that the bacteria most commonly isolated from the operative site of patients during cardiac and hip surgery account for most early postoperative infections (Tsukayama, Estrada & Gustilo, 1996). Although *S. epidermidis* has been identified as a pathogen in urinary tract, surgical wound, and haematogenous bone infections, association with infections involving prosthetic devices is unique and has the greatest morbidity (An & Freidman, 1997).

1.2 Host-pathogen interaction

For an organism to cause disease, it must be capable of fulfilling the minimum requirements of any pathogen, the first being entry to the host. Human host defence against bacterial invasion is based on a complex of both specific and non-specific defence mechanisms. Defence mechanisms can be physicochemical (skin and mucous membranes), cellular (phagocytes and lymphocytes), circulatory molecules (complement and antibodies) or soluble mediators active on other cells (macrophage- and lymphocyte-derived cytokines). Skin, the mechanical barrier to infection protects against invading organisms, but breaks in the host defence caused by surgery, catheter replacement, prosthesis insertion, or immunosuppression can result in infection. Once the organism has gained entry into the host it must be able to survive and multiply.

Two distinct pathogenic mechanisms, adhesion to polymer surfaces and the subsequent accumulation of sessile bacterial cells, are considered important pathogenic steps in foreign body infections caused by *S. epidermidis* (Hussain *et al.*, 1997 and Busscher, Bos & Vandermei, 1995). In the majority of natural situations in which bacteria are found, they are associated with and attached to surfaces (Allison & Gilbert, 1995). The molecular pathogenesis of the adherence process can be divided into an early phase of adhesion and a late phase of accumulation and slime production. The early stages of adherence occur quickly and are mediated by non-specific forces such as surface-charge, polarity, van der Waals forces and hydrophobic interactions (Costerton *et al.,* 1987) (see figure I). The surface energy theory of bacterial adhesion of Deraguin-Landlau-Verwey-Overbeek (DVLO) (Rijnaarts *et al.*, 1995) was devised by a group of scientists in an attempt to explain the mechanism of bacterial adhesion and the necessity of surface appendages for adhesion to occur in such a manner. It originally applied to particles of like size and charge and can be applied to bacteria. As two bodies of like size and charge approach each other, they are subjected to attraction and infections involving prosthetic devices is unique and has the greatest morbidity (An & Freidman, 1997).

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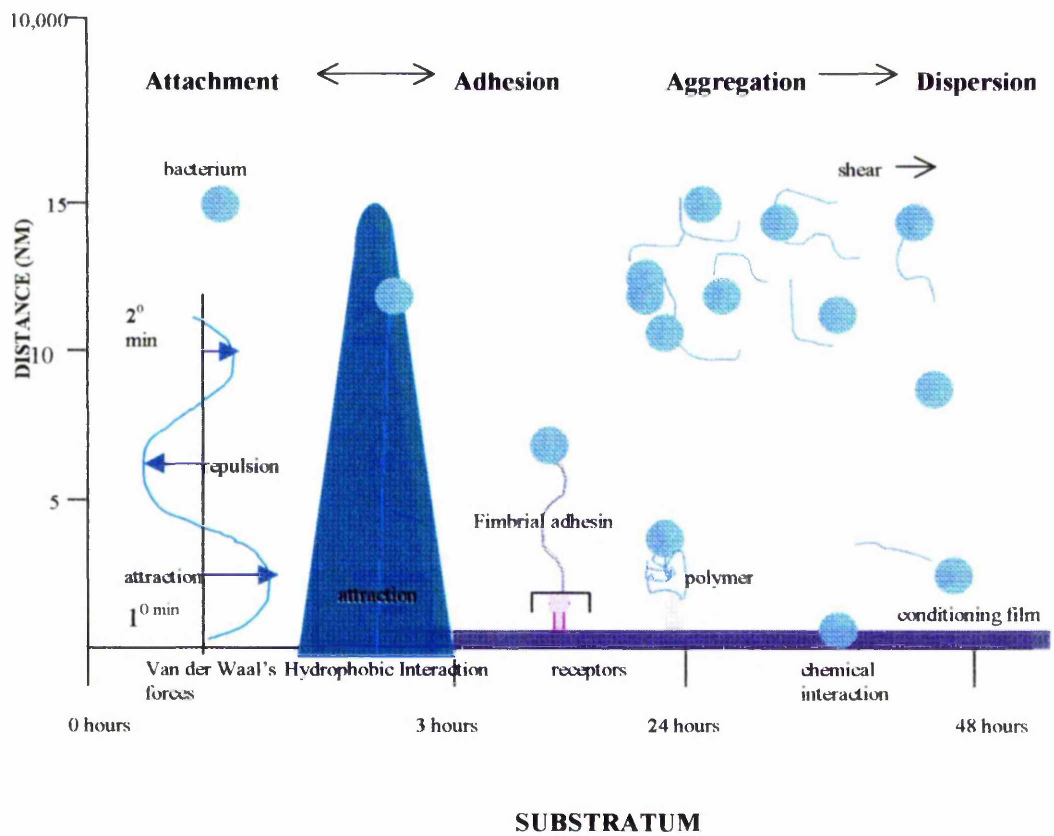
Figure 1 **The molecular sequence in bacterial attachment, adhesion, aggregation and dispersion at the substratum surface**



A number of possible interactions may occur depending on the specificities of the bacteria or the substratum (graphics, nutrients, contaminants, macromolecules, species and materials).
From, Gristina, A.G. (1987) Biomaterial centered infection: Microbial adhesion versus tissue integration. Science, **287**, 1588-1595.

repulsion forces which are additive in effect. The net negative surface charges of tissue cells and bacteria repel adhesion. This repulsion increases as the distance between the bacteria and the substrate decreases. At 15 nm there is little repulsion and the DLVO theory does not apply but, as bacteria approach 10 nm the forces of attraction are greater, there is a net free energy decrease in the system and the two bodies are held in a state of attraction by van der Waals forces and electrostatic interactions. At this stage particles can be separated by fluid shear forces. This is referred to as the secondary minimum of repulsion. At distances less than 10nm, the forces are greater in terms of repulsion rather than attraction. Repulsion between the two bodies prevents them getting closer than ~10nm, as the counter ion "clouds" on each surface repel each other. A potential energy maximum corresponds to the forces of repulsion and this is known as the primary maximum of repulsion. Organisms cannot usually overcome this primary maximum of repulsion as a whole but they use either secretory gums or fimbriae to do so. The magnitude of attractive forces as well as repulsive forces increases with the diameter of the approaching body (Roberts, 1996). Bacterial fimbriae, which are of a much smaller diameter, and therefore have a smaller radius of curvature, allow bacterial adherence that might not otherwise occur with the fimbriae reaching cell surface receptors for firm adherence to the cell surface (Jones & Isaacson, 1983). Once adherent, the normal flow of body fluids, such as mucus or urine does not wash away the bacteria and bacterial growth can then reach a critical mass for infection (Roberts, 1996).

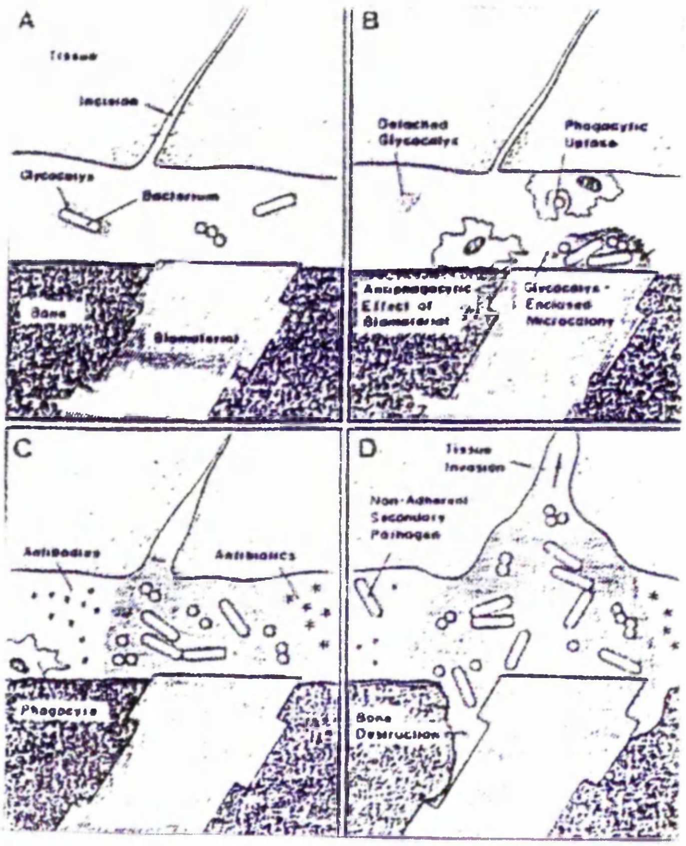
The term adhesin is a class designation of any structure leading to bacterial adhesion to a cell or tissue. Adhesion may also occur because of a non-specific characteristic of the cell such as hydrophobicity (discussed in section 1.5), but more frequently it is due to a specific receptor-lectin interaction by means of surface hair-like appendages, termed fimbriae (Duguid *et al.*, 1951) or pili (Brinton, 1965) (discussed in section 1.2.4.4). Fimbriae are not the only means of bacterial attachment since carbohydrate polymers such as glycosaminoglycans (GAGS), the structural components of the extracellular matrix which are essential for adhesion, migration and regulation of cellular growth, can also overcome the repulsive forces (Jones & Isaacson, 1983). Therefore, the adhesins include fimbriae and the afimbrial adhesins such as carbohydrate polymers, polysaccharides, lipoteichoic acid and high molecular weight proteins.

The primary adhesion phase is then followed by a secondary accumulation phase where the bacteria such as staphylococci proliferate on the substrate. This accumulation phase is characterised by the production of an extracellular slime substance or biofilm. Certain strains of bacteria are able to produce an extra-cellular substance in their pathogenicity of infection. This substance has been reported by different authors as extracellular slime substance (ESS) (Shiau & Wu, 1998), polysaccharide adhesin (PS/A) (Higashi *et al.*, 1998) slime-associated antigen (SAA) and as a capsule (McKenney *et al.*, 1998). Production of a biofilm is thought to be pivotal in the pathogenesis of infection by *S. epidermidis* (Rupp *et al.,* 1995) as it enables the staphylococci to adhere (see figure II). Two polysaccharide antigens and a protein antigen have been associated with the secondary accumulation phase (Christensen *et al.,* 1990; Mack, Siemssen & Laufs 1992 and Schumacher-Perdreau *et al.,* 1994). As the process continues, a thick matrix is established on the polymer surface containing multiple staphylococcal cell layers embedded in the biofilm. In this matrix the staphylococci are protected against host-defence mechanisms, especially those of the circulating phagocytes (Johnson *et al.,* 1980). There is some clinical and experimental evidence that staphylococci are protected from the action of antibiotics during the accumulation phase associated with slime production (Evans & Holmes, 1987; Peters *et al.,* 1987; Schumacher-Perdreau, 1988 and Sheth, Franson & Sohle 1985).

1.3 Factors affecting bacterial adhesion

A number of factors influence an organism's ability to adhere to prosthetic material. These include the surface characteristics of the bacteria and the biomaterial and also the nature of the ambient milieu. In many instances the substances involved in bacterial adhesion are extracellular, where bacteria release substances such as toxins and enzymes which facilitate adhesion and help to break down and permeate the host. In others, structural components of the bacterial cell envelope are important such as capsules in *Klebsiella pneumoniae, Salmonella*

Figure II Diagrammatic evolution of a biomaterial-associated infection



Bacteria are introduced into the wound (A) and express their natural tendency to adhere (B) to an inert surface. When the bacterial microcolony has formed and burgeoned to a greater size (C), the glycocalyx affords a measure of protection from antibiotics and appears to protect the bacteria from both bactericidal and opsonising antibodies. Later pathogens may cause destruction of bone and other tissue changes.(D) The colonies may shed secondary, less adhesive pathogens which are not necessarily representative in number type or pathogenicity of the adhesive colonies which may therefore confuse the diagnosis.

Gristina, A.G., Costerton, J.W (1984). Bacterial adherence and the glycocalyx and their role in musculoskeletal infection. *Orthop. Clin. North Am.,J* 15, 517-535.

typhi, Escherichia coli, Streptococcus pneumoniae and Staphylococcus aureus and epidermidis. Although the anatomy of the envelope of pathogens is highly variable between different organisms, the structures contributing to the ability of an organism to cause disease can be broadly grouped into four categories, namely (1) the capsular layer; (2) lipopolysaccharides; (3) protein components and (4) surface appendages.

1.3.1 The Capsular Layer

Capsules and capsular antigens are usually high-molecular weight acidic polysaccharides, which surround the bacterial cell in the form of a hydrophilic gel (Hammond, Lambert & Rycroft, 1984). Polysaccharides adhering weakly to the cell are known as slime layers. Bacterial slime can be defined as a substance produced by bacteria that may part freely from the bacteria after dispersion in a liquid medium (Duguid, 1951). The thickness of capsular gels may vary enormously. In some strains such as *E. coli* the capsule may be visualised microscopically as a zone 0.2-1.0μ, thick surrounding the cell in negatively stained preparations (Vogt, Berker & Mayer, 1995). In others a microcapsule of 10-30nm thickness is only detectable using sensitive chemical or immunological methods such as bioluminescence (Rozalska & Ljungh, 1995 and Stollenwerk *et al.*, 1998) and ELISA (Mempel *et al.*, 1996 and Thomas *et al.*, 1997). The size and form of capsule produced may also vary with the physiological state of the cell. The effects of a particular nutrient limitation or change in growth rate, in the controlled environment of the chemostat, have shown that exopolysaccharide production by different strains responds in different ways to a given environment (Perezgiraldo, 1995 and Sanford *et al.*, 1995). The growth medium affects the characteristics of individual bacterial strains and strains within one species can adhere to a very different degree. Growth of *S. epidermidis* in trypticase soy broth (TSB) supplemented with glucose resulted in a higher level of slime production (Elci *et al.*, 1997). In a study by Atmaca, Elci & Gul (1996), it was found that slime production by coagulase-negative staphylococci was less under anaerobic conditions.

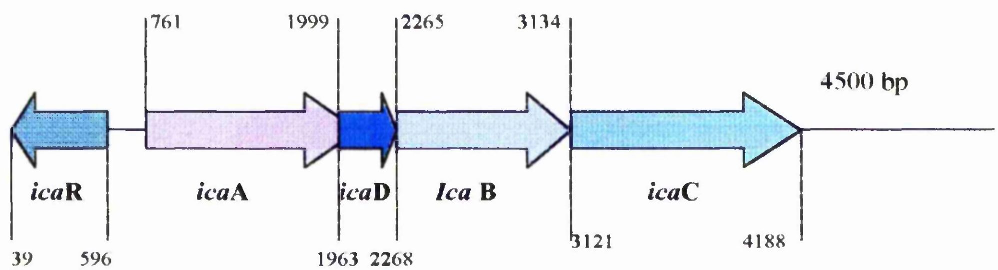
Bacteria also obtain nutrients essential for growth by sequestration. Staphylococci express a 42 kDa cell-wall protein which functions as a receptor for the mammalian iron-binding glycoprotein, transferrin during the infection process (Modun *et al.,* 1998 (b). Studies have demonstrated that removal of the extracellular exopolysaccharide slime layer by repeated washing does not diminish the ability of *S. epidermidis* to adhere to the biomaterials (Hogt *et al.,* 1986). Interference with the mechanisms involved in adherence is seen, therefore, as an attractive way of preventing or shortening infective episodes. In the same way, an organism entering host tissues encounters complex environmental conditions that may markedly influence capsular polysaccharide production.

The ability to produce large quantities of biofilm on solid surfaces is believed to distinguish the potentially pathogenic strains of *Staphylococcus epidermidis* from the commensals (Deighton, Borland & Capstick, 1996). The large number of papers addressing this property reflects the difficulties that have arisen in defining the agent or agents responsible for slime production. Recent developments often lead to the renaming of an important agent or the realisation that two agents are actually the same. This is exemplified in the discovery by Baldassarri *et al.*, in 1996 that the substance named SAA by Christensen *et al* in 1990 is chemically the same as that named PIA by Mack *et al.*, (1996). Purification of slime associated antigen (SSA) (or indeed, PIA) showed that N-Acetyl-glucosamine accounted for 70% of the dry weight of SAA (Baldassarri *et al.*, 1996). There are two stages in the biofilm formation by bacteria. Initial attachment is rapid and is followed by a more prolonged accumulation phase which involves cell proliferation and intercellular adhesion (Heilmann *et al.,* 1997). The intercellular adhesin involved in biofilm accumulation of *S. epidermidis* is a linear beta-1, 6-linked glucosaminoglycan (Mack *et al.,* 1996). This unbranched polysaccharide structure is composed of at least 130 2-deoxy-2-amino-D-glucoputanosyl residues of which 80-85% are acetylated. This favours long-range contacts and interactions between polysaccharide strands and the cell wall and/or lectin-like proteins, leading to intercellular adhesion and biofilm accumulation. The structure of the polysaccharide is considered to be unique and, according to its function, is referred to as *S. epidermidis* polysaccharide intercellular adhesin (PIA). The *S. epidermidis* genes icaADB and C (ica, *i*ntercellular *a*dhesin) are located in an operon and were identified in 1996 (Heilmann *et al.*, 1996) (see figure III). These genes are involved in cell-cell adhesion and synthesis of the polysaccharide adhesin, which has been shown to be located mainly on the cell surface by immunofluorescence studies with PIA-specific antiserum. A transposon insertion into the icaABC gene cluster leads to the loss of several traits such as the ability to form a biofilm on a polystyrene surface, cell aggregation and PIA production (Heilmann *et al.,* 1996). Complementation of the mutant by transformation with the icaABC-carrying plasmid pCN27 into the heterologous host *Staphylococcus carnosus* led to the formation of large cell aggregates, the formation of a biofilm on a glass surface, and PIA expression. It was further shown that *icaA* and *icaD* together mediate the synthesis of sugar oligomers *in vitro*, using the substrate UDP-*N*-acetylglucosamine. This UDP-*N*-acetylglucosaminyltransferase activity together with the activity of *icaC* produces a product *in vitro* that is recognised by an antibody raised against PIA (Gerke *et al.*, 1998).

The capsular polysaccharide (PS) has been reported to be a component of the cell surface and biofilm layer. It protects the cell from host defence mechanisms such as opsonophagocytosis. This lead to the PS being named capsular polysaccharide adhesin, PS/A. McKenney *et al.*, (1998) showed that *in vivo* an N-succinylated glucosamine polymer of larger molecular size with the biological properties of PS/A is also made from the *ica* locus. Thus, both PIA and PS/A share a common β-I,6-linked-polyglucosamine backbone but differ in the primary substituent on the amino groups. Antibodies to PIA also react with antibodies to PS/A. The activities of PIA and PS/A were distinguished by an assay for *in vitro* production of biofilm (McKenney *et al.,* 1998). A significant proportion of clinical CoNS isolates make a biofilm and are highly adherent to catheters *in vitro* but do not subsequently form a detectable biofilm. Thus the elaboration of PS/A does not always precede biofilm formation.

Shear forces are important in the early stages of microbial adhesion and biofilm formation as dynamic shear conditions exist in both natural and human environments such as a fast flowing river bed or the human oral cavity or urethra. A biofilm may become detached during high shear conditions, and once the bond between the initially adhering organisms and the surface is broken, it is difficult for

Figure III The *ica* operon



Overview of the *ica* operon and the adjacent *ica*R gene
From Ziebuhr, W., Krimmer, V., Rachid, S., Lossner, I., Gotz, F. & Hacker, J. (1999) A novel mechanism of phase variation of virulence in *Staphylococcus epidermidis*: evidence for control of the polysaccharide intracellular adhesin synthesis by alternating insertion and excision of the insertion element IS*256*. Molecular Microbiology, **32**, No 2, 345-356.

the biofilm to re-establish itself (Busscher, Boss & Vandermei, 1995). *In vivo*, other, more specific, mechanisms seem to be important. There is substantial evidence that several serum and tissue proteins, such as fibrinogen, fibronectin, thrombospondin and laminin, may act as mediators for adhesion (Costerton *et al.*, 1985; Benson, Burns & Mohammed, 1996 and Bos *et al.*, 1996). Such proteins rapidly coat implanted materials introduced into the human body. Irreversible adhesion produced by receptor-like interactions between epitopes of the matrix proteins and surface proteins of the staphylococci then occurs.

1.3.2 Lipopolysaccharides and other amphiphiles

Amphiphiles are a diverse range of polymers that have both hydrophobic and hydrophilic regions. A variety of amphiphiles are found in the bacterial cell envelope such as: lipopolysaccharide (Gram-negative bacteria only), lipoteichoic acid and lipoprotein. Lipopolysaccharide (LPS) is also known as endotoxin as it can induce mammalian cells to produce cytokines. It is found in the outer membrane of gram-negative cells. One end of the LPS is hydrophilic the other end is hydrophobic. It is composed of three distinct regions, lipid A, the R core region and the O side chain. Lipid A is hydrophobic and anchors the LPS to the outer membrane. The central part is the R core composed of sugars which is linked to the hydrophilic O side chain, also composed of sugars. LPS can thus attach to hydrophobic surfaces by means of this hydrophilic chain. The importance of hydrophobicity and infection is discussed later and in greater detail in Chapter Two.

1.3.3 Protein components

Surface protein layers have been observed on a number of bacterial species and those present on pathogens appear to contribute to the ability of the organism to cause disease. Studies of two classes of biofilm-negative mutants of *S. epidermidis*, created by insertion of the transposon Tn917, showed a lack of five cell surface proteins with molecular masses of 120, 60, 52, 45 and 38kDa (Heilmann *et al.*, 1997). Class A mutants were affected in initial attachment whereas class B mutants were affected in intercellular adhesion. The mutated strains were more hydrophobic and were affected in primary attachment to polystyrene but were still able to form multilayered cell clusters (Heilmann *et al.,* 1996). When the mutated strains were complemented by transformation with a 16.4kb wild-type DNA fragment the resulting complemented mutant was able to attach to a polystyrene surface, form a biofilm and produce most of the proteins missing from one of the class A mutants. Further experiments have shown that the 60kDa protein is sufficient for initial attachment to a substrate. Immunofluorescence microscopy showed that this protein is located on the cell surface. When the DNA sequence of the complementary region was analysed, a single open reading frame consisting of 1335 amino acids with a predicted molecular mass of 148kDa was discovered. The amino acid sequence was highly similar (61%) to the major autolysin of *Staphylococcus aureus* which has two bacteriologically active domains. A 60kDa amidase and a 52kDa glucosaminidase domain generated by proteolytic processing. The 120 kDa protein missing from one of the class A mutants probably represents the unprocessed amidase and glucosaminidase domains. The 45 and 38kDa proteins are probably the degradation products of the 60 and 52kDa proteins respectively (Heilmann *et al.,* 1997). The loss of several surface proteins may have led to reduced surface hydrophobicity by unmasking hydrophilic structures, thus favouring primary attachment to a glass surface and leading to subsequent biofilm formation.

In 1988, Tojo *et al.*, reported a possible involvement of extracellular polysaccharide substances as adhesins in the binding of coagulase-negative staphylococci to biomaterials. Later, in 1991,Timmermann *et al.*, reported that early adhesion of *S. epidermidis* to polystyrene was mediated by a proteinaceous adhesin. This biomaterial adhesin of *S. epidermidis* strain 354 was characterised by using a strain-specific monoclonal antibody (Mab 36.4). This monoclonal antibody was strongly and exclusively reactive with *S. epidermidis* 352 as shown by ELISA when the whole cell was used as the antigen. When cell wall preparations from *S. epidermidis* were tested, immunoblotting revealed a strong reaction with a protein between 200 and 220 kDa, and a weaker reaction with a protein band of 110kDa. When the bacteria was pre-incubated with the monoclonal antibody, adhesion to polystyrene spheres was reduced by a mean value of 74% (+/- 14%, n=10). Fab fragments from this monoclonal antibody also inhibited adhesion indicating specific blocking of an adhesion antigen as opposed to aspecific inhibition. Further studies using immuno-gold electron microscopy with Mab 36.4 showed gold particle deposits on both the cell surface and on fimbriae-like surface projections. These results all led to the conclusion that a surface-located protein antigen of *S. epidermidis* 354, recognised by monoclonal antibody 36.4, reacts as an adhesin facilitating bacterial attachment to an uncoated foreign material.

1.3.4 Surface appendages and microbial virulence

Mucosal and endothelial surfaces are constantly bathed in fluids such as blood, urine and mucus (Mims, 1991). These secretions are often kept in motion by a variety of anatomical mechanisms serving to cleanse the surfaces such as sneezing, coughing, ciliary action or peristalsis. The ability to adhere to epithelial surfaces is thought to permit colonising bacteria to resist the secretory flow of such fluids that would ultimately result in the flushing of the organism from the body.

Bacterial surface structures important in adhesion are fimbriae (pili). Fimbriae are bacterial structures 4-7nm in diameter and up to 1000nm in length. There are usually several hundred fimbriae present on the bacterial surface of species such as *E.coli*, Corynebacteria sp., and Streptococcus sp.. Bacterial adhesion pili are designed to bind specifically and maintain attachment of bacteria to target cells, such as the uropathogenic P-pili of *E. coli,* which are sufficiently mechanically resilient to resist the cleansing action of urine flow that removes most other bacteria (Bullit & Makowski, 1995 and Roberts, 1996). Flagella are involved in motility and do not mediate adherence. Neither appendage are involved in staphylococcal adhesion to natural and synthetic surfaces.

The cell wall structure differs in gram negative and gram positive bacteria as classified by their reaction to Gram stain. In gram-negative bacteria the peptidoglycan layer surrounds the plasma membrane. Gram-positive bacteria have a thick cell wall which consists mainly of peptidoglycan together with polysaccharides and various proteins such as the M protein of streptococci and protein A of staphylococci. The proteins may extend out from the outer surface of the organism interacting with the environment. Such interactions can protect the bacteria from the scavenging effects of phagocytes. Protein A does so by binding IgG, thus diverting the opsonising antibody from the cells (Hammond, Lambert & Rycroft, 1984).

1.4 Effect of antibiotics on bacterial adherence

Biofilm-embedded bacteria are widely recognised to be more resistant to antimicrobial agents than are planktonic bacteria (Qian, Sagers & Pitt, 1997). Resistance may be due to the glycocalyx secreted by the bacteria reacting with and neutralising the antimicrobial agent. Another hypothesis is that the matrix creates a diffusion barrier to the antimicrobial agent (Brown, Aldrich & Gauthier, 1995). The role of sub-inhibitory concentrations of antibiotics on the adherence of bacteria to biomaterials has been investigated (Schmitt *et al.*, 1985; Pfaller *et al.*, 1986; Root *et al.*, 1988; Taylor, Prosser & Cleland, 1988; Elliott *et al.*, 1988 and Timmermann *et al.*, 1990). From a large range of antimicrobial agents investigated including clindamycin, erythromycin, ciprofloxacin, chloramphenicol and vancomycin: vancomycin gave the best results showing that pre-treatment with vancomycin resulted in decreased adherence of *S. epidermidis* to biomaterials. Also, it was shown to inhibit adherence of strains embedded in extracellular slime substance (Widmer *et al.*, 1990). This finding was of great clinical importance as antimicrobial agents are generally reported to penetrate biofilms poorly and bacteria embedded in such biofilms appear to be resistant to a broad spectrum of such agents (Dunne, 1990).

1.5 Hydrophobicity and its affect on bacterial adherence

Microorganisms can attach to their targets both by specific adhesin-receptor mechanisms and also by non-specific mechanisms. Among the latter, hydrophobic interactions seem to be the most common. The hydrophobic interaction, that is the interaction between two non-polar groups, is of fundamental importance in both the attachment of bacteria to each other, and the bacterial adherence to tissues and foreign bodies. Bacterial attachment to human cells is modulated by a change in interfacial free energy, and this is correlated with surface hydrophobicity of bacterial cells (Bragga & Regio, 1995). Hydrophobic interaction was first reported in 1978 by Smyth *et al.,* when he investigated the differences in the hydrophobic surface characteristics of porcine *E. coli* using hydrophobic interaction chromatography (HIC) (Smyth, 1978). Whilst there are a number of methods available to characterise the cell surface hydrophobicity (CSH) and cell surface charge (CSC) of microorganisms, there is still some debate concerning the correlation of results between individual methods (Jones *et al.*, 1996). They are shown in Table 1 below.

Table 1 Methods to measure bacterial cell surface hydrophobicity

Method	Reference
Two phase partition systems	Albertson, 1958
Hydrophobic interaction chromatography (HIC)	Hjerten, 1974
Binding of ^{14}C-dodecanoic acid	Kjelleberg *et al.*, 1980
Bacterial adherence to hydrocarbons (BATH)	Rosenberg, Gutnick & Rosenberg, 1980
Microsphere adhesion to cells	Zita & Hermansson, 1997
Salting out in ammonium sulphate and sodium chloride(SAT)	Lindahl, 1981
Improved salt aggregation test (ISAT)	Rozgonyi, 1985

Bacterial cell-surface hydrophobicity is basically determined by proteins and lipids. The coagulase-negative staphylococci possess only a small amount of surface proteins (Hogt *et al.*, 1986 and Reifsteck, Wee & Wilkinson, 1987). The method by which a particular surface component is seen to be a major determinant of hydrophobic nature is based on the use of mutants lacking that particular surface component (Jonsson & Wadstrom, 1983; Jonsson & Wadstrom, 1984 and Rosenberg, Gutnick & Rosenberg, 1980).

Many factors affect cell surface hydrophobicity. One is the method of bacterial cultivation. Cell surface hydrophobicity is often best expressed after growth under nutrient-poor conditions, or "starvation" (Ljung & Wadstrom, 1995). *Staphylococcus epidermidis* exhibits both hydrophobicity and hydrophilicity when its growth conditions are varied (Rozgonyi *et al.,* 1985). This wide adaptability makes it possible for the bacteria to colonise a wide variety of substrates. The use of antibiotics also affects hydrophobicity. Pre-treatment of bacteria with antibiotics results in an increase in hydrophobicity and a decrease in the negative charge of the bacterial cell surface (Nomura, Kuroiwa & Nagayama, 1995).The substrate surface also affects hydrophobic interaction. A hydrophobic strain of *S. epidermidis* showed stronger adherence to an unpolished polymethylmethacrylate (PMMA) surface compared to a polished surface (8,000 bacteria/mm(2) compared to 5,200 bacteria/mm(2). In contrast, the hydrophilic strain adhered in greater numbers to the polished PMMA surface rather than the unpolished surface (42,090 bacteria/mm(2) compared to 2,000 bacteria/mm(2) (Schoricke *et al.*, 1997). The hydrophobic nature of *S. epidermidis* and its ability to adhere to polymethylmethacrylate is investigated further in Chapter Three.

1.6 The host response to infection

Polymorphonuclear leukocytes (PMNLs) form the first line of defence against infection, by their ability to phagocytose and kill invading microorganisms. They are the most numerous of the immune system cells. They respond rapidly to chemotactic stimuli, phagocytose and destroy foreign particles such as microbes. PMNLs can be activated by cytokines produced primarily by macrophages and endothelial cells and are the major cell population in the acute inflammatory response. The ingestion of an invading particle is termed "phagocytosis". This process was first described almost a century ago by Metchnikoff (Metchnikoff, 1905). He deduced that phagocytic cells protected higher creatures from assault by the ubiquitous microorganisms in the internal and external environment (Stossell, 1974). Phagocytosis has many stages which include, activation of the neutrophil, migration to the site of infection, attachment to the activated endothelial cell, migration through the endothelial cell, consequent opsonisation of the bacteria leading to attachment of the bacteria to the neutrophil, ingestion and killing of the bacteria (see Figure V, Chapter Two).

Phagocytosis can be made more efficient by making the material to be phagocytosed more attractive to the phagocyte by opsonisation. "The phagocytes won't eat the bacteria unless the bacteria are nicely buttered for them. Well, the patient manufactures the butter for himself all right; but my discovery is that the manufacture of that butter, which I call opsonin, goes on in the system by ups and downs" (The Doctor's Dilemma, George Bernard Shaw, 1909). Generally microorganisms are only phagocytosed after they have been properly opsonised, i.e loaded with activated C3 and IgG (Verhoef & Visser, 1993). The phagocytic cells express receptors for the Fc portion of the IgG antibody. When the IgG molecules bind to and coat antigenic particles during opsonisation, the bound IgG is recognised by the receptors on the phagocytic cell. There are two receptors for IgG on the surface of the phagocyte, FcRII and FcRIII. FcRII binds IgG1 and IgG3 equally well and does so better than IgG2 and IgG4, whereas FcRIII only binds monomeric IgG. Opsonisation also occurs when the fragment C3b, derived from the third component of complement (C3), binds to the leukocyte receptor. The complement system consists of more than 30 protein components that circulate in the blood and body tissues and act in a cascade to bring about the destruction of micro-organisms (see figure VII, Chapter Two). C3b is generated when other complement components cleave the main component C3. The neutrophil receptor that recognises C3b is CR_1, while the receptor that recognises iC3b is CR_3 (CD11b/CD18). For most opsonised particles, especially those which are encapsulated such as *Haemophilus influenzae*, the iC3b-CR_3 interaction only enhances attachment of the bacterium to the neutrophil but not it's actual ingestion.

1.7 Evasion of the immune response and the establishment of infection

Once established, the bacteria must then resist and avoid the various host defence mechanisms that are produced or activated in response to infection. The ultimate fate of an invading organism is ingestion and destruction of phagocytic cells.

Certain strains of bacteria have established methods of improving their survival within the host. Some produce substances that paralyse the neutrophils and hinder the chemotaxis of the neutrophils to the infection site. Others are equipped with cell wall structures that enable them to resist opsonisation. Others have capsules, which are not very immunogenic therefore antibodies to the capsules do not exist. These capsules can also prevent complement activation and reaction between IgG and the cell wall epitopes and may also prevent the neutrophil from actually killing the internalised bacteria. Survival rates increase when the organism is encapsulated (Khardori & Yassien, 1995). Studies with the heavily encapsulated strain *Staphylococcus aureus* M have shown methods by which bacteria use their capsule to evade phagocytosis (Hammond, Lambert & Rycroft, 1984). Peptidoglycan, one of the components of the bacterial cell wall, will fix complement, either by the classical or the alternative pathway. In encapsulated strains the complement component becomes bound beneath the capsule at a depth which is inaccessible to the phagocyte cell receptors hence avoiding uptake by the phagocytic cell. In this case the capsule is interfering with the exposure of opsonins at the external surface of the organism. For this method to be effective the capsular polysaccharide itself must not be capable of activating the phagocyte as this would result in enhanced phagocytosis.

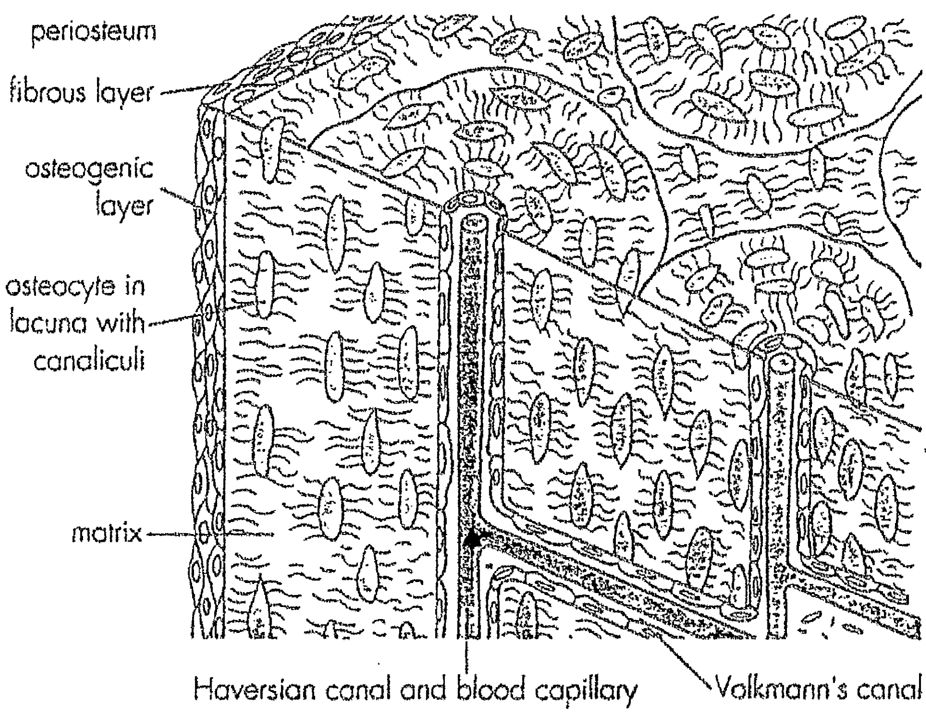
As a last protest, ingested bacteria also manage to escape toxic events in the phagolysosome which may result in secondary infections. *In vivo* phagocytosis not only occurs in a fluid milieu, but also on the surface of tissues, described by Wood as surface phagocytosis (Wood, Smith & Watson, 1946). Several more recent studies have shown that bacterium-phagocyte interaction is different when phagocytosis takes place on a surface as compared to in suspension. The process of phagocytosis is discussed in greater detail in Chapter Two

1.8 Bone

Although bone is the hardest tissue in the body it is subject to wear and in many circumstances needs to be replaced with a prosthetic device. When fully developed, bone is composed of: water (20%), organic material (30 to 40%) and inorganic material (40 to 50%). Development of bone begins before birth and is not complete until about the twenty-fifth year of life. Living bone is a mineralised connective tissue containing metabolically active cells. Inorganic (non-carbon-containing) constituents are mineral salts a large proportion of which is calcium, and phosphorus. The organic component includes the bone cells and part of the matrix. This tissue has a central role in the mineral homeostasis of the body. Both individually and in groups, bones have mechanical functions: they are acted upon by the muscles and protect the vital structures of the human body, such as the brain and the heart. Bone may be divided into four categories: tubular (e.g. the long bones in the limbs), short (e.g. carpus and tarsus) or flat (e.g. the vault of the skull) and other bones such as the vertebrae which do not fit into any of the other categories (Housden, 1995).

Bone is a fibre-reinforced calcified tissue which is continually remodelling itself. There are three cell types;- osteoblasts, osteocytes and osteoclasts. The actions of two major bone cells, the osteoclasts and the osteoblasts control this remodelling process. These two cells have opposing actions: the osteoblast forms the matrix of the bone and the osteoclast resorbs the bone matrix. Osteoblasts are single-nucleated cells, 20-50μm in diameter. The osteoblast exists in three forms: the preosteoblast, the mature osteoblast, and the osteocyte which is trapped within the bone (see figure IV). Its major functions are to produce the components of the matrix (mainly type 1 collagen with lesser amounts of proteoglycans and glycoproteins) and to control the activity of the osteoclast. When bone formation is taking place the osteoblasts may be identified as a single layer of cells covering the layer of osteoid on the surface of the bone. During active bone synthesis, they are the large cells whose cytoplasm contains an abundant rough endoplasmic recticulum and Golgi apparatus for protein synthesis and processing. These cells are also rich in alkaline phosphatase for mineralisation. After completing a cycle of activity osteoblasts become smaller resting cells. The osteoclast is believed to derive from the same myeloid precursor cells that give rise to monocytes. Osteoclasts are large, multinucleate cells. They are found on the surface of mature trabecular bone lying in shallow depressions called Howship's lacunae. On their surface adjacent to bone, the cytoplasm forms projections. Fragments of bone and

Figure IV The Microscopic Structure of Bone



Diagrammatic representation of the microscopic structure of bone. From McMinns Functional Anatomy of Bone. Chapter Two, Cells and Tissues. Eds. McMinn, R.M.H., Gaddum-Rosse, P., Hutchings, R.T. & Logan, B.M. Mosby, 1995.

collagen have been identified within this brush border using electron microscopy showing that the osteoclasts are responsible for bone resorption (Bonucci, 1981). Osteoclasts are derived from marrow precursors, are closely related to macrophages, and are best regarded as belonging to the mononuclear phagocyte system. The osteoclast and the osteoblast proliferate and differentiate into mononuclear preosteoclasts which fuse together. Bone resorption requires the osteoblast to release collagenase which removes the non-mineralised organic matrix which covers bone surfaces. The osteoclast then migrates to this revealed site, attaches itself onto the calcified matrix, and acidifies it by pumping protons outwards, thus solubilising the calcium salts. The adhered osteoclast then releases various lysosomal enzymes which remove the exposed organic matrix. As the solubilisation of the matrix occurs, this process is believed to release growth factors (produced by the osteoblasts), which will in turn stimulate mesenchymal cells to proliferate and differentiate into preosteoblasts and osteoblasts which will replace the previously removed bone matrix. Thus, there is an ongoing, balanced process of renewal and removal of the bone matrix which requires the synergistic actions of the osteoclast and the osteoblast. Bone remodelling is controlled by a wide variety of systemic factors such as prostaglandins, leukotrienes, cytokines and growth factors. The majority of these factors act upon the osteoblast which in turn transduces the action of these factors onto the osteoclast. About 5 percent of the adult skeleton is replaced annually.

Current orthopaedic practice relies on the ability of surgeons to drill, cut, ream and realign bone; to fix one piece to another with screws, plates, wires, or rods; and to obtain union between bone and plastic or metal implants. The mechanical properties of bone are readily apparent (Canalis & Lian, 1988). While the tensile strength of bone is nearly equal to that of cast iron, bone is three times lighter and nearly ten times more flexible. It is these properties of bone that manufacturers model artificial joint systems on. The effect of bioactive materials on bone formation has been investigated using *in-situ* hybridisation (Neo *et al.*, 1996).

Osteolysis and aseptic loosening are relatively common problems associated with joint replacement; therefore most research is focused on their prevention. Osteolysis was one of the first problems associated with joint replacement surgery

(Maloney & Smith, 1995). Bone loss can become a clinical problem for several reasons, including: failure of fixation of the implant, periosthetic fracture of the host bone, pain and disability. Localised bone resorption in association with loose cemented joint replacement systems, was first reported by Harris *et al.,* in 1976; later, Hasty *et al.*, 1986 and Maloney *et al.,* 1990 reported osteolysis in patients with cemented joint replacement systems. Wear debris from the replacement joint gives rise to a foreign-body reaction that leads to the formation of a characteristic soft-tissue membrane between the implant and bone. Immunohistochemical and biochemical studies have shown that this soft tissue membrane is composed of mainly macrophages and fibroblasts, similar to that found in patients with aseptic loosening. Such tissue has been shown to have the common features of a foreign-body granuloma (Goldring *et al.*, 1983 and Willert *et al.,* 1996). It has the capacity to produce soluble factors that may stimulate bone resorption, which leads to osteolysis and aseptic loosening. The soluble factors that appear within the membrane, or that are released from the membrane cells, include pro-inflammatory cytokines such as interleukin-1 alpha and beta, TNF-alpha and interleukin-6 (Jiranek *et al.*, 1993); arachidonic acid metabolites (prostaglandins and leukotrienes) and degradative enzymes. The release of such pro-inflammatory mediators occurs, both in macrophages exposed to wear debris in culture, and in retrieved specimens of implant bone interface membrane maintained in organ culture. The method by which wear particles from different joint replacement systems initiates granuloma formation and stimulates cellular metabolism remains unclear. The size of the particles and their composition is likely to be important (Maloney *et al.,* 1995).

Bone loss, in association with cemented implants, became known as 'Cement Disease' (Jones & Hungerford, 1987). Histologically, cement disease is characterised by the presence of variable amounts of cement, ultra-high molecular weight polyethylene and metal debris, tissue infiltrated with macrophages, giant cells and vascular granulation tissue (Freidman *et al.,* 1993). In order to avoid cement disease, the development of cementless joint replacement systems was encouraged. However, osteolysis was also diagnosed in total joint prostheses designed to be inserted without cement (Brown & Ring, 1985 and Maloney *et al.,* 1995). In fact the prevalence of such systems has increased with time (Maloney *et* *al.,* 1994 and Tanser *et al.*, 1992), with the size of the osteolytic lesions also increasing the longer the implant has been in place (Maloney *et al.*, 1995). Improvement in the long-term results of joint replacement systems will depend upon the ability to minimise wear debris. Despite technological improvements it is unlikely that wear debris will be eliminated, therefore the mechanisms of particle-induced bone resorption need to be fully investigated, so that this adverse reaction may be modulated or prevented using pharmacological techniques. This is particularly in the view of the non-toxic, non-immunogenic and chemically inert nature of most implantable biomaterials (Tang *et al.*, 1996).

1.9 Prosthetic joint surgery

Nosocomial infection in the hospitalised patient has become a progressively more difficult problem for both prevention and management of the infection. Hospitalised patients have a greater complexity of disease which requires a more frequent use of invasive devices both to monitor and treat their infection (Fry, Fry & Borzotta, 1994). *S. epidermidis* has been very successful in its colonisation of implanted materials and is the most frequent organism isolated from infected sites. Because of this there has been much research into the prevention of such infections. This research has focused on many different aspects of the prosthesis: from design, the choice of material and also the manufacturing and insertion process.

1.9.1 Choice of biomaterials in total joint arthroplasty

One of the most important properties of a biomaterial is its biocompatibility: the ability to perform its purpose without causing any detrimental effect to the host. According to the modern definition of biocompatibility, a biocompatible material need not be inert, but be bioactive (Peluso *et al.*, 1994). The clinical success of any implant is directly dependent upon the behaviour in the immediate vicinity of the interface established between the host tissue and the biomaterial(s) used to fabricate the device (Chesmel & Black, 1995). Little is known of the factors that determine biocompatibilty of the material used in prosthetic devices (Tang & Eaton, 1995). The use of prosthetic biomaterials has made a huge impact on modern medical care but as yet complete biocompatibility remains elusive (Kaplan *et al.,* 1994).

Of the materials available for total joint replacement, only polymethylmethacrylate cement has been reported to encourage infection although no material for fixation of an implant seems to present a better or worse risk of infection at the site of the prosthesis (Petty *et al.*, 1985). It has been shown that corrosion of multi-part metallic fracture-fixation devices may be increased in the presence of such an infection (Heirholzer *et al.*, 1984). The most commonly used material in the production of a prosthetic joint is Ultra-High-Molecular-Weight Polyethylene (UHMWPE). This material was chosen because polyethylene has little biological and chemical reactivity and its high molecular weight makes it resistant to wear. However there is an increasing body of evidence showing that debris from UHMWPE may limit its long-term use. Particles accumulated by the wear process may cause an inflammatory reaction and lead to bone lysis. Implant loosening, made worse by severe bone loss presents the surgeon with a difficult task of revision, and this is becoming a major problem in limiting the life of joint replacements. The first hip prosthesis made of this material was implanted in 1962. In the 1970's and 80's efforts were made to design a material with better wear properties than UHMWPE. Polyacetal was used in the Christensen hip prosthesis and Poly Two (carbon-fiber-reinforced ultra-high molecular weight polyethylene) (Zimmer, Warsaw, Indiana) in acetabular cups, in total hip replacements, tibial components in total knee replacements, and patellar replacements. Polyacetal has the advantages that it has a higher yield strength, higher crystallinity, and ease of manufacturing compared with UHMWPE. Unlike its counterpart, Polyacetal can be formed into parts through injection molding processes, which are both faster and cheaper than the conventional machining process. During the 70's and 80's at least four types of polyacetal were implanted: Delrin 150, Delrin 100, Delrin 500, and Celcon. Early performance data was encouraging, but a report by Dumbelton showed significantly higher rates of failure of this prosthesis compared to the Charnley prosthesis, so their use was discontinued shortly thereafter. Other than in rare clinical trials, Delrin is no longer used as a bearing surface in total joint replacement. Wear debris may also predispose to local infection through ionic suppression of leukocyte chemotactic ability and suppression of the respiratory burst of leukocytes (Pascual *et al.*, 1992; Rae, 1981 and Shanbaag *et al.*, 1992).

1.9.2 Design of the prosthesis

Variables important in the design and choice of a material for replacement are flexibility, fatigue strength and surface hardness. There is no acknowledged 'best' material for the fabrication of orthopaedic implants (Black, 1992; Ducheyne & Cohn, 1991 and Galante *et al.*, 1991). Many biomaterials in clinical use were not originally designed as such, but were off-the-shelf materials that clinicians found useful in solving a problem. An example of this is dialysis tubing, which was originally made of cellulose acetate, a commodity plastic which was found in some instances to activate platelets and the complement system (Peppas & Langer, 1994). Advances in the design of total joint replacements have suggested that certain biomaterials are more appropriate for certain applications which can be grouped according to anatomical location. Each material has a particular combination of properties determined by composition and processing, and each set of properties produces both benefits and limitations within the bounds of any device design. Therefore flexible plastics are used for urinary catheters and Hickman lines, whereas joint prostheses require a more rigid weight-bearing substance. Some materials used in joint replacement systems are better suited for load-bearing applications whilst others are more suitable for articulating surfaces. For a femoral component which will be cemented in place during a total hip or knee arthroplasty, two materials are available commercially: cobalt-chromium alloys and titanium alloys. Of the two, cobalt-chromium alloys are preferred due to their strength and bearing surfaces, whereas titanium alloys have been found to generate large amounts of particulate metallic debris (Agins *et al.*, 1988; Black *et al.,* 1990 and McKellop *et al.*, 1990). Ceramic materials may be an alternative in knee surgery due to their surface bearing properties. The flexibility of a prosthesis depends on both the material and geometric properties (Sarmiento & Gruen, 1985). The absolute flexibility of a joint prosthesis is not important, its flexibility in relation to the flexibility of the bone is the primary concern. Flexibility of a cemented prosthesis is low due to the poor mechanical properties of cement. The lowest stress in the cement prolongs the life of the prosthesis. Flexibility has been inversely related to pain in the thigh, after hip revision (Franks *et al.*, 1992 and Skinner & Curlin, 1990).

The number of total hip replacements (THR) implants available in the UK is rapidly increasing, but there is little or no scientific evidence that the newer, more expensive implants are better than established designs. THR is a very successful operation, about 40,000 are carried out annually (Morris, 1996). The reports published suggest that the revision rate is currently about 10 % at ten years (Schulte *et al.,* 1993 and Alsema, 1994). Some THR systems have good results at five years but once ten years have elapsed they have developed high failure rates (Owen, 1994). The cost of the primary implant represents only a fraction of the total cost of the operation. Revision arthroplasties are a lot more costly and do not last as long or give as good a functional result as the primary operation. About 5000 revisions are performed annually in the UK and this number is rising (Williams *et al.,* 1992). Long-term outcome is usually measured as time to revision using survival analysis, but this method is subject to distortion, making comparison of outcome difficult (Murray, Carr & Bulstrode, 1993). The THR with the longest follow up is the Charnley design. It is widely considered to be the best standard for comparison (Bulstrode, 1987 and Wroblewski & Siney, 1993) with a published twenty-year survival rate of between 85% and 90% (Schulte *et al.*, 1993). The result of a THR depends on many factors, only one of which is the implant. The technique performed, the skill of the surgeon and experience with the type of THR are all important to the overall success of the operation.

1.9.3 Manufacturing the prosthesis

The manufacturing process of polyethylene for use in prosthetics is a complex one. Three methods are currently used to make orthopaedic devices with ultra-high molecular weight polyethylene powder. The first method is direct moulding, in which the powder is placed in a mould that compresses it into the final shape of the device. The powder is then heated under pressure to consolidate it and form the part. Devices manufactured in this fashion have no external machining lines and often exhibit a highly glossy surface finish. The second method is ram-extrusion of the powder into a cylindrical bar stock ranging from two to six inches in diameter. The implant is then machined from this bar stock. The third method is the moulding of large sheets of ultra-high molecular weight polyethylene from which the implant is then moulded.

During standard processes such as injection moulding, the long-chain molecules of the UHMWPE are broken down thus rendering the material more susceptible to the wear process. Due to the costs involved in moulding the polymer from a powder state into an implant most manufacturers acquire their polymer in the form of sheets or bars, which are then manufactured into the prosthesis. As the production of prosthetics only accounts for less than 1% of the total world production of UHMWPE, the manufacturers do not view the problems incurred during this process with great concern. Recent concern about litigation is causing many polymer manufacturers to consider withdrawing this application of their product. Li and Wright (Li & Wright, 1995) have recently offered to provide well-characterised UHMWPE stock to researchers world-wide, so that valid comparisons can be made.

A major problem which occurs with the use of UHMWPE are the changes which occur during sterilisation of the finished product. Gamma irradiation accelerates oxidative degradation both 'on the shelf' before operation and after implantation (Amis, 1996). Studies by Li & Burnstein (1994) and Fisher *et al.,* (1995) have shown that this may lead to an increase in wear. Gamma irradiation may also alter the material just below the bearing surface of the material. This renders the polymer more brittle. There is little understanding as to why these changes occur below the surface rather than at the surface. It is known that the gamma irradiation process breaks down some of the polymer chains and creates reactive sites in the polymer structure, which renders it more susceptible to oxidative attack. An alternative to the use of gamma irradiation is the use of ethylene oxide. This has however been associated with inflammatory reactions due to traces of the ethylene oxide gas leaching into the tissues from microcavities in the polymer. Vacuum - extraction processes have been developed to obviate this problem (Plester, 1970).

The actual shape of the implant is very important as it can create dead space. The blood flow may be compromised in this area thus the defence mechanisms of the body have limited access. Detritus and necrotic tissue provide favourable conditions for bacterial growth.

1.9.4 Wear and tear problems with prosthetic joints

Biomaterials are used in many forms of orthopaedic surgery from remodelling to joint replacement (Rae, 1981 and Shanbaag *et al.*, 1992). Materials such as iron, cobalt and titanium are designed to be permanent. However, because such materials are subject to corrosion and wear because of the difference in mechanical properties between metal and bone, the bone surrounding the implant may weaken (Wu *et al.*, 1990). This problem lead to the introduction of polymers as joint replacement systems. Most polymers are too weak to be used in load-bearing implants and the materials used should be sufficiently strong enough to withstand the stresses to which bones are normally subjected. Sixty-two different replacement hip joints manufactured by 19 companies are currently available in Britain, half of which have been introduced since 1991. A minimum follow up of ten years is normally required to judge a joint replacement successful, but only eight of these prostheses have published follow ups of over five years (Sochart, Long & Porter, 1996). Only the Charnley low friction arthroplasty, widely regarded as the gold standard, has 20 year follow up results (Wroblewski & Siney, 1993). The principal problem is the quantity of wear debris that is produced from the bearing surfaces of the joint replacement system. This wear debris can be voluminous, stimulating cellular osteolysis in the vicinity of the joint resulting in loosening of the prosthesis (Harris *et al.*, 1976; Howie *et al.*, 1988; Skinner & Maybe, 1987 and Willert & Semlitsch, 1977 and 1974). Wear debris may also predispose to local infection through ionic suppression of leukocyte chemotactic ability and suppression of the respiratory burst of leukocytes (Pascual *et al.*, 1992; Rae, 1981 and Shanbaag *et al.*, 1992).

All metallic elements in materials that are used in implant applications with the possible exception of titanium are recognised as playing either an essential or a toxic role in human metabolism (Merzt, 1986). Current dietary recommendations include a maximum intake of metals such as magnesium, zinc, copper, manganese, molybdenum and chromium, all recognised as safe. At least one of manganese, molybdenum or chromium is found in all stainless steel and cobalt-based super alloys (National Research Council Subcommittee on the Tenth Edition of the RDA's, 1989).

1.9.5 Complications of total joint replacement

As the life span of joint replacement systems *in vivo* now exceeds ten years, attention has turned to the role of the host immune response in its ability to elicit a response either systemically or at a remote site from the implant (Freidman *et al.*, 1993). A greater understanding of such an immune response will result in better applications, especially in the coupling of the biomaterial and the host. Most biomaterials, especially metals when properly manufactured, processed and handled both before implantation and whilst being placed *in situ* in the operating room, are stable and do not generally present biological response problems in the short term (Friedman *et al*, 1993). It is difficult to observe remote and systemic responses to biomaterials (Black, 1991). Most of the responses observed can be expected to occur normally in a population of orthopaedic patients (Black, 1984), or in any patient population. Thus, the identification of an infection at a remote site from the implant as being caused by that implant is difficult, unless tests can be performed both before and after the removal of the implant. This may result in unnecessary and costly removal of the implant if it is not the culprit, so prosthesis removal is often a last resort if all other options, especially antimicrobial therapy has failed. In order to prevent this happening, Black (1991) proposed four necessary criteria:

1. The basic mechanism of the biological response must be demonstrated in at least one biological model, either *in vitro* or in an animal model.
2. After the causative implant-related species (the particular element, molecule or ion) has been identified, it's release by a functional implant and it's systemic distribution either in an animal model or in a patient (preferably both) must be shown.
3. The putative biological response must be identified either in an animal model or in patients (preferably both) with functional implants.
4. If demonstrated in patients, the biological response must be recognised on the basis of a statistically sound epidemiological study with suitable non-exposed controls, and unless the response is of a threshold type, a dose-response or exposure-incidence relationship must be demonstrated.

In most cases, evidence to satisfy the last condition is most lacking. Therefore research continues on the reality and clinical importance of systemic and remote-site effects of implants. The physico-chemical properties of a biomaterial and its surface texture greatly influence the type of tissue reaction (Meyle *et al.,* 1994). Such materials, though generally inert and non-toxic, can mediate a variety of adverse reactions, including inflammation, fibrosis, coagulation, and infection (Tang & Eaton, 1995). In some instances material-mediated inflammatory responses may cause degradation of the material itself via oxidative products released by implant-associated inflammatory cells.

Prosthetic joint infections have been arbitrarily classified into three groups on the basis of the time that elapses from the date of the surgery until they become apparent.

1. Group 1 infections present within the first 2-3 months are usually wound infections that do not involve the joint and can be treated with antibiotics and debridement (acute infections).
2. Group 2 infections present within 2 years and are thought to result from bacterial seeding of the joint at the time of surgery (subactute infections).

3. Group 3 infections present > 2 years postoperatively and are thought to represent haematogenous seeding of the joint (late infection) (Based on Gristina and Kolkin, 1983).

The infection rate for any surgical prosthesis insertion should be less than 1% in the first postoperative year (Strachan, 1995). Charnley's early experience with total hip replacement yielded a rate of deep infection, in association with his first 190 operations of almost 9 per cent. With the introduction of air-flow systems within the operating theatre, prophylactic antibiotics and body-exhaust suits the infection rate was reduced to 1.3 per cent (Charnley, 1972). Further developments have reduced the rate to below 1 per cent (Marotte *et al.*, 1987; Salvati *et al.*, 1982; Schultzer & Harris, 1988 and Wymenga *et al.*, 1992). If infection occurs, then patients will lose their newfound mobility, lose their independence, be hospitalised with sepsis, both local and systemic and at the worst outcome, may die.

Susceptibility to infection may be affected by a number of factors relating to the implant including its size and shape, the nature of its surface, the material of which it is made, the stability which it provides, and the method of insertion. Direct toxicity, corrosion and foreign-body reaction are all possible. The probability of bacterial adhesion increases with the size of the implant and may interfere with surface and tissue integration (Gristina & Costerton, 1985 and Gristina, 1987). Most nosocomially-acquired isolates are often resistant to a number of antimicrobial agents, including methicillin and other β-lactam antibiotics. Data has shown that the skin flora of patients entering the hospital contains predominantly antibiotic-susceptible CNS. However, once the patients are hospitalised, their skin flora becomes altered by the acquisition of antibiotic resistant isolates from hospital personnel or by the selection of antibiotic-resistant bacteria from the patients' endogenous flora on account of systemic antibiotic administration. In either case, colonised skin serves as a huge reservoir for dissemination of multiresistant CNS throughout the hospital. It has been demonstrated that vigorous preoperative scrubbing with a variety of topical antiseptics will not sterilise the sanctuary sites for CNS within the sebaceous glands and aprocine pits.

Preoperative antibiotics only serve to select an antibiotic resistant population of organisms.

Cephalosporins continue to be the antibiotic of choice for most surgeons because of their broad spectrum of activity against the common pathogens associated with hip infections (Hill *et al.,* 1981), their low toxicity to patients (Davies *et al.*, 1986; Leigh, 1986 and Wiggins *et al.*, 1978) and the high concentrations that are achieved in the bone and soft tissues. One drawback of systemic antibiotics is the relative impermeability of bone due to poor vascularisation. This can be overcome when the antibiotics are impregnated into the cement. A number of antibiotics are employed, including erythromycin and gentamicin. The antibiotic must be heat-stable if it is to survive the polymerisation of the cement, and water-soluble if it is to elute. Cements can be inserted either manually, or by the use of a wide-bore syringe or cement gun. Use of a pressurised syringe or gun increases the subsequent strength of the cement (Lee, 1979), and improves the quality of the mechanical airlock between cement and bone by forcing the cement into irregularities in the bone's surface. A commercially available example of this is Palacos R manufactured by 3M Ltd which contains gentamicin. This antibiotic leaches out of the cement after setting, providing protection against deep infection within the bone. Protection is provided for months or even years without the patient suffering any side effects. The use of antibiotic impregnated cement has a high success rate in revision arthroplasty (Carlsson, Josefsson & Lindberg, 1987).

Bone cements represent a significant advance over the cementless prosthesis. Recovery time is considerably reduced as there is no need to wait until the bone grows round the prosthesis. In over thirty years of use, very few adverse reactions have been recorded with the use of bone cement. Bone cement implantation syndrome is characterised by an abrupt decrease in arterial blood pressure (or, less frequently, an increase) minutes after implantation. Very occasionally this is followed by irreversible cardiac arrest and hypoxaemia, resulting in an intraoperative mortality (Charnley, 1972).

1.9.6 Loosening of the joint

Differentiating an infected joint from aseptic loosening of the prosthesis is a difficult task as treatment of the two conditions differs. Pain is an almost universal symptom in both conditions. Traditional manifestations of infection such as fever, erythema, increased warmth or purulent discharge are rarely present. Therapy for an infected joint prosthesis generally involves removal of the prosthesis in order to eradicate the infection. This is both time consuming and costly. Four to six weeks of parenteral antibiotic therapy is usually prescribed following surgical debridement. Charnley recorded a decrease in the incidence of prosthetic hip infections over a seven-year period after altering various details of the hip replacement technique. Reduction of implant infection has been achieved by meticulous asepsis around the time of surgical implantation (Charnley, 1972). Further difficulty arises in determining whether an isolate is a true pathogen or simply a contaminant. Isolates with similar patterns of biochemical and antibiotic agent susceptibility, obtained from several cultures of the same site, are readily recognised as pathogens. However with a prosthetic device, even a single isolate must be carefully evaluated. Diagnosis is even more difficult in suspected prosthetic hip infections, because even carefully obtained joint aspirates may yield *S.epidermidis* in the absence of other supporting signs of infection. Nevertheless, semiquantitative culture techniques may be useful in differentiating contamination from infection in certain well-studied situations, such as intravascular catheters.

An additional factor that may affect the pathogenicity of disease is alterations to the prosthetic device that can occur after interaction with *S. epidermidis*. Implants are finished to a highly polished state, leaving the surface smooth and shiny with no molding lines. Small scratches naked to the human eye can provide bacteria with a comfortable "niche", from which to establish themselves and manifest infection. The characterisation of the biomaterial surface is considered of ultimate importance for the development of improved biomedical device (Andrade & Gregonis, 1983 and Ratner, 1983). Surgeons must therefore exert great care when inserting the prosthesis. Erosive changes in the inert surface of polyethylene catheters have been demonstrated after adherence of coagulase-negative staphylococci *in vitro.* Anti-adhesive polymers would prevent the adhesion of microorganisms to the prosthetic device ("zero adherence"). However, unfortunately no such material has been discovered (Kohnen & Jansen, 1995). Materials with antimicrobial properties contain antimicrobial substances that are incorporated into the biomaterial or bound to the polymer surface. Such devices seem to be effective in the prevention of "early onset infections". One of the mechanisms to evade phagocytosis is the production of a capsule. Bacterial strains which possess capsules are almost always more resistant to phagocytosis than unencapsulated strains of the same species. These studies may in part explain both the unique affinity of *S. epidermidis* for prosthetic devices and the great difficulty in eradicating infections without first removing the foreign body.

1.9.7 Adverse tissue reactions to the prosthesis

The release of metallic ions such as cobalt, chromium and nickel from implants can serve as haptens, eliciting type-IV delayed hypersensitivity reactions in previously sensitised individuals (Hildebrand & Champy, 1988). A very loose implant with a higher release rate of metallic material may also sensitise previously unsensitised individuals (Elves, 1981). Immune responses have also been reported against titanium-alloy devices (Lalor *et al.*, 1991), polymethylmethacrylate bone cement (Gil-Albarova *et al.*, 1992) and silicone elastomers (Goldblum *et al.*, 1992) giving rise to concern about a possible generality of response. There are also increasing reports of relating dermatitis, urticaria, eczema and other more subtle immunological reactions to implants and their wear products, although no clear picture of either the magnitude of the immune response or its causative clinical development has emerged (Black, 1992). Another problem that occurs is with adverse tissue reactions due to the monomer. Residual monomer remaining in the cement after polymerisation is toxic to tissue but is normally present in too small a quantity to present a serious problem. However, in patients who have required re-operation for other reasons, tissue in contact with the cement has been found to be stained varying hues of yellow, orange, brown, purple and black. The cause of this tissue discoloration has not been determined, but further cement contact, such as recementing of a loose prosthesis in the face of this reaction does not seem to provoke an untoward reaction.

On a more serious note, increasing numbers of tumours are reported in individuals who have undergone joint replacement therapy (Jacobs *et al.*, 1992). Although the incidence of tumours remains low there is little question of the carcinogenic potential of metallic species, especially chromium, cobalt and nickel on their release from implants (Black, 1986). Surveys of the incidence of remote-site and systemic tumours have revealed a trend towards greater risk of lymphoma and leukaemia in patients after total hip arthroplasty (Gillespie *et al.,* 1988 and Visuri & Koskenvuo, 1991). Metallic ions produced with wear of the implant constitute a chronic stress on the immune system (Rae, 1981), which although initially may be beneficial could possibly result in immune system depletion in the longer term. A study by Rock in 1983 failed to show an increase in tumour incidence (leukaemia and lymphomas were not studied) but did show the tumours in the same limb as the joint replacement progressed more rapidly. As patients live longer and undergo joint replacements at an earlier age these concerns may well become more important (Friedman *et al.,* 1993).

CHAPTER TWO

CONDITIONS FOR OPTIMUM INGESTION OF *S. epidermidis* STRAINS M7 AND RP62A BY ISOLATED HUMAN POLYMORPHONUCLEAR LEUKOCYTES.

2.1 INTRODUCTION

The immune system is a collection of tissues, cells and molecules whose prime physiological function is to maintain the internal environment of the body by destroying invading infectious organisms (Staines, Brostoff & James, 1993). The immune system does not act in isolation. It works in a synergistic manner with the other physiological systems such as the neuroendocrine, gastrointestinal, respiratory, urogenital and musculoskeletal systems. An invading microorganism is exposed to three important host defence systems. These are (i) the tissue fluids, (ii) the lymphatic system leading to the lymph nodes and (iii) phagocytic cells (Mims, 1995). Specialised phagocytic cells are divided into two main types; the macrophages scattered throughout the major compartments of the body such as the liver, spleen, kidney and lymph nodes and the circulating neutrophil polymorphonuclear granulocytes (neutrophils).

2.1.1 The polymorphonuclear leukocyte

The leukocyte constituents of the immune system include granulocytes, specialised antigen-presenting cells and lymphocytes (Dexter & Spooncer, 1987). The granulocytes include neutrophils, eosinophils, basophils and mast cells which can be distinguished by the staining reactions of their cytoplasmic granules. Neutrophils, monocytes and macrophages are important effectors for phagocytic destruction of antigens targeted by antibody and complement and predominate in acute inflammatory responses, especially against the extracellular bacteria and in immune-complex-mediated diseases (Huston, 1997). Eosinophils are specialised cells whose main concern is the destruction of helminths and other parasites and participate in late-phase allergic inflammation. Basophils and mast cells are distinct lineages that both express cell surface high affinity receptors for IgE and serve as a central role in immediate hypersensitivity immune responses.

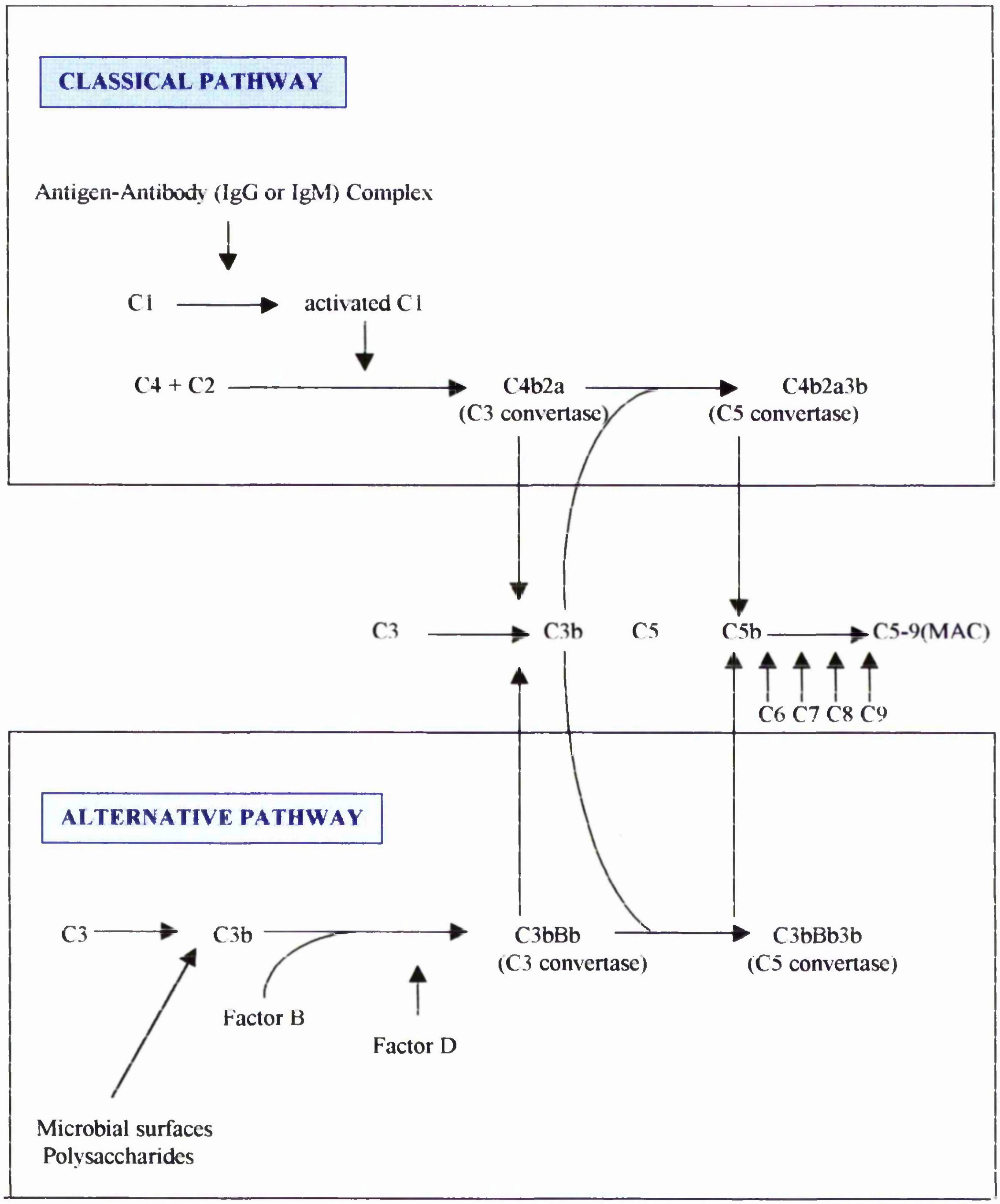
Neutrophils are the most numerous, comprising 70% of the total blood leukocyte population (eosinophils 1% and basophils 0.5%). These cells are generally referred to as "polymorphs". They contain 3 or 4 types of granules (Boxer & Smolen, 1988). These granules may be primary, secondary or tertiary granules depending on the maturation stage of the neutrophil. Primary or azurophilic granules are first seen in promyelocytes, secondary or specific granules appear in both myelocytes and metamyelocytes and tertiary granules appear during the myelocyte stage. In the mature neutrophil, only one third of the granules are primary granules (Kanwar & Cairo, 1993). The granules contain enzymes such as myeloperoxidase, alkaline phosphatase, ribonuclease, deoxyribonuclease, nucleotidases, glucuronidase, lysozyme and cathepsins. Often, myeloperoxidase is used as a marker for the presence of granules. In addition they contain cationic peptides which have a specific antibiotic-like activity by virtue of their pore-forming activity. These cationic peptides, 30 - 33 amino acids in length are called defensins and are active against a wide range of pathogens such as *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa.*

Neutrophils arise in the bone marrow and are continuously discharged in vast numbers into the blood. The 3×10^8 neutrophils that are present in normal human blood carry out their functions after leaving the circulation and entering sites of inflammation in tissues. These cells are non-dividing, live only for a few days and each day about 10^{11} disappear from the blood even in the absence of significant inflammation (Verhoef & Visser, 1993). This daily loss is balanced by entry into the blood from the bone marrow. In order to a supply a sudden demand, the bone marrow contains an enormous reserve of roughly 3×10^{12} neutrophils. Although macrophages are found throughout the body, they are not as numerous as neutrophils and are not found in great numbers in reserve in tissues.

Phagocytosis and killing of microorganisms are two major tasks which cells of the myeloid system have to fulfil (Guzik *et al.*, 1999). Strategically positioned throughout the body, the cells of the phagocytic system are equipped for immediate and lethal action against invasion of the host by bacterial and fungal microorganisms. Phagocytosis is the name given to the process by which bacteria are ingested and killed by the white blood cells. It is the major defence mechanism by which the immune system protects the body against bacterial infection. Phagocytosis involves recognition of a particle by the phagocyte followed by attachment and ingestion. Optimal ingestion conditions include the need for the particle being ingested to be opsonised. The role of opsonins in phagocytosis is well documented (Patel, 1992; Gemmell & Felmingham, 1990 and Gemmell, 1996). Opsonisation is achieved by coating the bacterium with specific IgG molecules or the complement component C3b. The complement system consists of more than 30 proteins activated along an enzymic cascade which results in a spectrum of bioactive molecules that facilitate opsonisation, osmotic lysis of targeted cells and recruitment of phagocytic cells (Frank & Fries, 1991). Complement has the unique ability to distinguish between self and non-self in that it is activated as soon as it recognises a foreign structure. Activation of the complement system normally occurs only at certain localised sites. First, immunoglobulin molecules that have bound specific antigens can activate complement and for this reason complement serves as a major effector mechanism for specific humoral immunity. The sequence of complement activation initiated by antibody-antigen complexes is called the classical pathway. Second, some complement components are directly activated by binding to the surfaces of infectious organisms. In this way complement activation also participates in natural immunity (Mollnes & Harboe, 1996). The sequence of complement activation that occurs on microbial surfaces in the absence of antibody is termed the alternative pathway, so called because it was named after the discovery of the classical pathway. These two pathways differ in their initiation as shown in figure V but share many late stages and effector functions.

The central component of the complement system is a protein called C3. Both pathways have distinct protein components that are activated to generate enzymes called C3 convertases which cleave C3 to produce C3a and C3b. In the classical pathway the C3 convertase is a combination of proteins called C1, C4 and C2 known as the C4b2a complex. In the alternative pathway, C3b generated spontaneously at low levels or by the classical pathway binds to the protein fragment Bb which has been generated by proteolytic cleavage of the protein factor B. This forms the alternative pathway convertase complex C3bBb which further breaks down C3 to produce more C3b. The next step in both pathways is the

Figure V **The classical and alternative pathways of complement activation**



Adapted from Cellular and Molecular Immunology by Abbas, Lichtman and Pober.
Second Edition (1994)

binding of C3b to the C3 convertase enzymes changing them to C5 convertases which catalyse the cleavage of the C5 protein. Cleavage of C5 is followed by the terminal steps where there is no further proteolysis but in fact sequential binding of the soluble complement proteins C6,C7, C8 and C9 to the activating surface to form the lipid-soluble pore structure called the membrane attack complex (MAC) which causes osmotic lysis of cells.

2.1.2 The process of phagocytosis.

This process can be divided into five stages (figure VI).

1. Attachment: After arrival at the site of inflammation the polymorph must attach to the microorganism and phagocytose it. This involves receptor-ligand binding between the polymorph and the microbe. In order to facilitate this, polymorphonuclear leukocytes (PMNLs) and macrophages posses a variety of receptors and enzymatic mechanisms (Hampton, Vissers & Winterbourn, 1994 and Morel, Doussiere & Vignais, 1991). This is often facilitated by neutrophil surface receptors for C3bi and the Fc portion of immunoglobulin (the opsonins coating the bacterium).

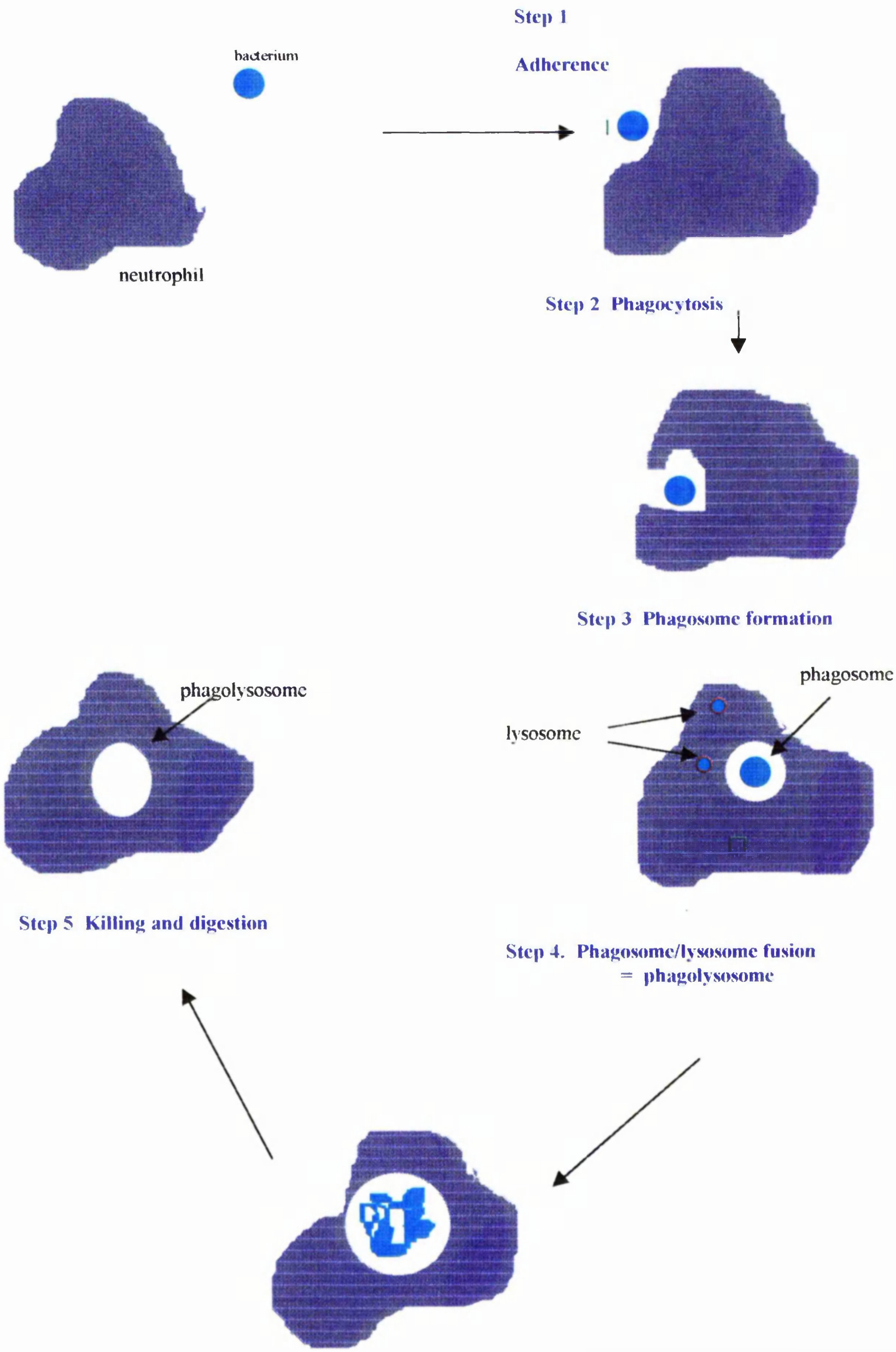
2. Engulfment: The receptor-ligand interaction activates the ingestion phase (or engulfment phase as it is also known). This involves actin, myosin, and actin-binding proteins. The actin microfilaments in the portion of the cytoplasm, which underlie the site of particle attachment undergo polymerisation. This polymerisation leads to puckering of the cytoplasm at the site of contact, because the microfilaments attach to the membrane. The membrane then envelops the particle and new particle-membrane contacts develop. Pseudopodia (finger-like extensions of the plasma membrane) are produced which surround the particle.

3. Formation of a phagosome: The particle is then engulfed by the macrophage cytoplasm, and a vacuole, called a phagosome is formed.

4. Fusion of the phagosome with a lysosome: Intracellular events involving interaction between lysosomes and phagosomes then occur. Lysosomes produced in the Golgi apparatus, are minute bodies in the cell cytoplasm which contain various hydrolytic enzymes. Initially, the phagosomes are found near the cell membrane, but in time they migrate toward the perinuclear area where they fuse with lysosomes to form phagolysosomes. The fusion process discharges lysosomal contents, including lysosomal enzymes (i.e. acid hydrolases and peptidase) into the phagolysosomes. The lysosomal enzymes are subsequently activated by the low pH, 3.5-4.0 (produced by proton pumps located in the phagosome) and can digest the engulfed substances, either storing them as 'dense bodies' or eliminating them by exocytosis.

5. Killing and digestion: PMNLs can kill microorganisms by two distinct mechanisms. One antimicrobial system is oxygen-dependent, while the other can kill bacteria in the absence of oxygen. The oxygen dependent mechanisms are set in motion when the PMNLs undergo a 'respiratory burst'. In oxygen-independent systems PMNL cytoplasmic granules contain additional antimicrobial agents that are released into phagolysosomes and do not require the production of oxidants for activity. These agents include proteases, other hydrolytic enzymes such as phospholipases, glycosidases and lysozymes, and other proteins and peptides that disrupt microbial functions or structural components. During the ingestion process, phagocytes consume oxygen from the surrounding milieu. Most, if not all of this oxygen undergoes enzyme-catalysed univalent reduction to form the superoxide anion (O_2^-), singlet oxygen and hydroxy radicals are also produced. Two O_2^- molecules, one of which may be in the form of H_2O, interact with each other in a rapid, spontaneous dismutation reaction to form hydrogen peroxide (·OH)-perhaps the most important microbiocidal oxygen species and oxygen. A flash of light is associated with the release of the oxidising species. This is known as 'native chemiluminescence' (See figure VIII).

Figure VI The process of phagocytosis.



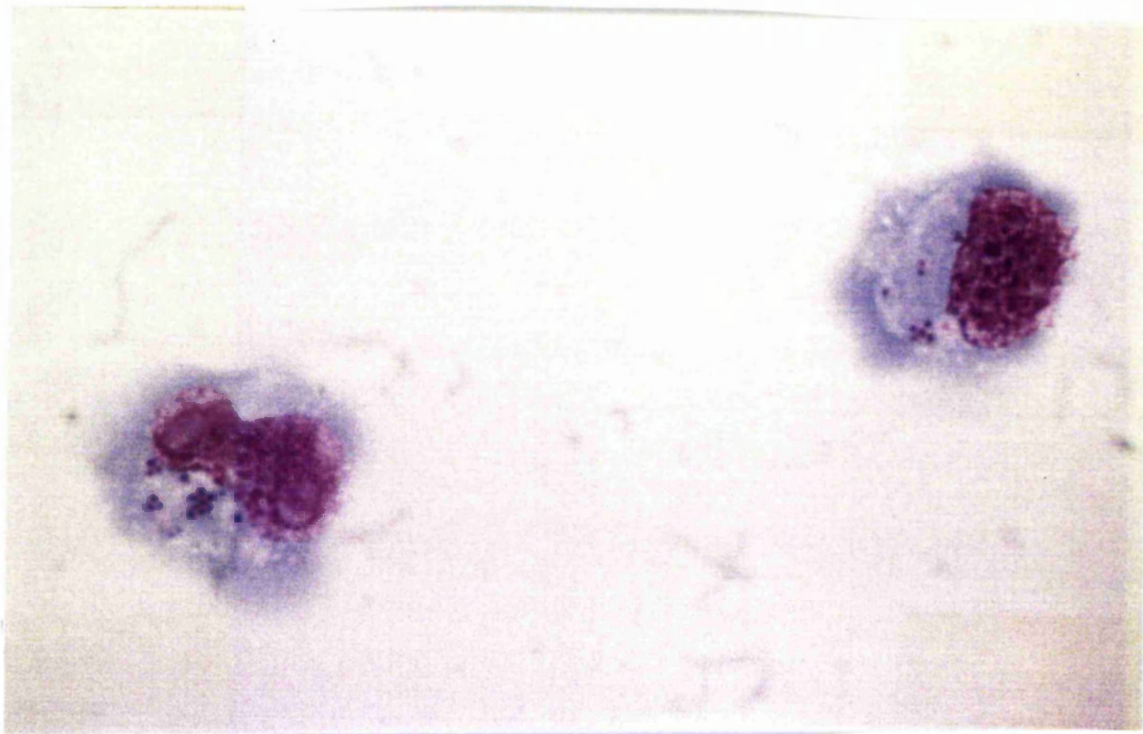
2.1.3 Measurement of phagocytosis.

In an effort to understand better this process, investigators have developed numerous *in vitro* assay systems, which can be divided into categories-

- microscopy, where samples are taken at various times and the number of ingested particles counted. Electron microscopy overcomes the difficulties encountered when counting small bacteria (Rozenberg-Arska *et al.,* 1985)
- ingestion of labelled particles monitored by either fluorescence, radioactivity or visible absorbance (Absolom, 1986)
- microbiological assays where viable bacteria are measured by their ability to form colonies after plating (Leijh *et al.,* 1979)
- and those which assess the metabolic consequences of phagocytosis such as oxygen uptake and chemiluminescence (Easmon *et al.*, 1980).

Each of the above methods has their advantages and disadvantages. A major problem in the measurement of phagocytosis is that it is difficult to distinguish particle ingestion from particle attachment, especially in the microscopic evaluation of phagocytosis (Hed, 1986). A bacterium may appear to be inside the phagocytic cell whereas it is actually sitting on the surface of the phagocyte. Also, when assaying bacterial phagocytosis by viable bacterial counts it is important to remember that microbes attached to phagocytes may be killed but not ingested and that ingested organisms may not always be killed. Examples include Legionella, Chlamydia and Mycoplasma - pathogens renowned for their ability to survive intracellularly (Arrunda *et al.*, 1993). An advantage of the viable count method is that extracellular i.e. non-ingested bacteria can be selectively killed with an antibiotic that does not enter the cell. Ingested *Staphylococcus epidermidis* is shown in figure VII.

Figure VII Ingested *S. epidermidis*



Photograph shows neutrophils containing ingested *S. epidermidis* M7. The gram-positive cocci can be seen in the phagosome/phagolysosome of the neutrophil. Slides were prepared by a cytospin preparation of the well contents at the final stage of the procedure

Magnification x1000.

2.1.4 Measurement of chemiluminescence by luminometry.

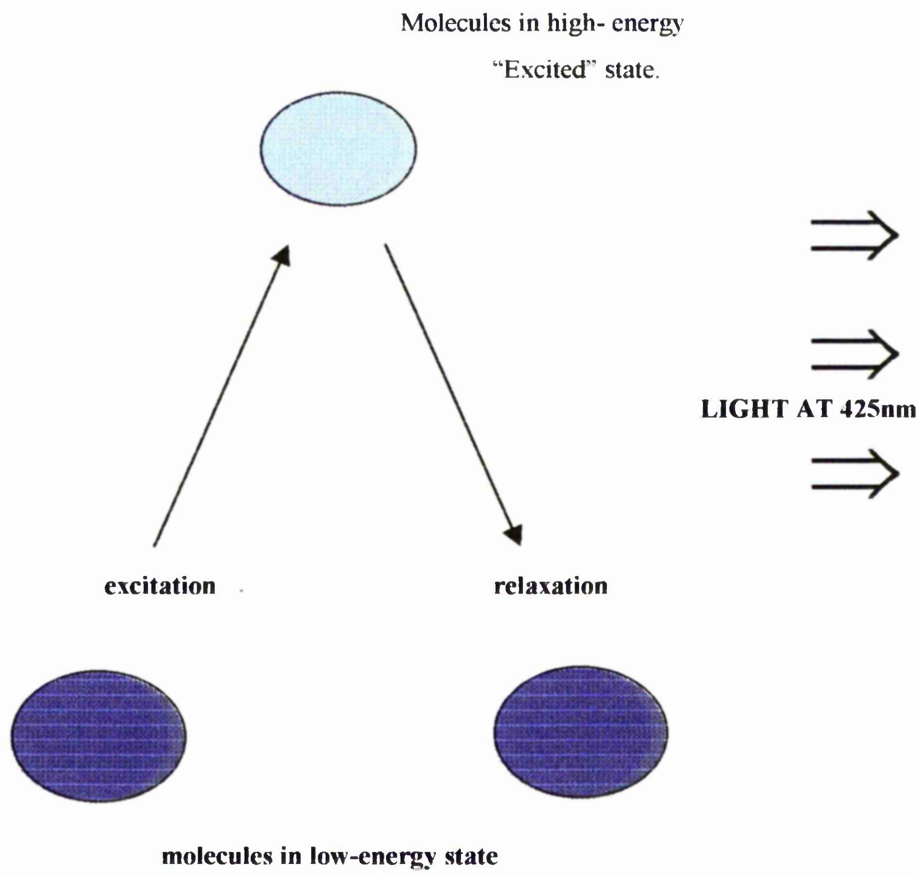
It is possible to follow the progress of phagocytosis by monitoring light emission using a luminometer. Luminescence is the emission of light by a non-thermal process. In the case of luminescence analysis, the light is produced by a chemical reaction. Molecules responsible for emitting the light absorb the free energy released by the chemical reaction and become excited. In this excited state, the peripheral electrons of the molecule are raised to a higher energy level. When they return to the lower energy level, they lose energy. This lost energy is emitted as photons of light. When the electrons have lost all their absorbed energy, the molecule is once more back in its stable ground state (see figure VIII).

Even though the application of luminescence has grown considerably it is still based primarily on three light-producing systems-

1. Firefly bioluminescence for the measurement of ATP
2. Bacterial bioluminescence for the measurement of NAD(P)H
3. Luminol chemiluminescence for the measurement of H_2O_2

Allen, Stjernholm & Steele (1972) first reported emission of light by phagocytes during the process of phagocytosis of bacteria. He studied luminol-dependent chemiluminescence in peritoneal and alveolar macrophages using a liquid scintillation counter. This method required a relatively large number of cells and the chemiluminescence generated was dependent on the interaction of some reactive species with the phagocytosed material or other component in the medium (Nelson *et al.,* 1977). The use of a stimulus such as phorbol myristate acetate (PMA) failed to generate measurable chemiluminescence unless protein or some other secondary emitter was present in the reaction mixture (Westrick *et al.*, 1980). It was for this reason the researchers used light enhancing molecules such as luminol or lucigenin as a method of amplifying the chemiluminescence response (Allen & Loose, 1976; Stevens, Winston & van Dyke, 1978 and Prendergast & Proctor, 1981). These chemicals react to different reactive oxygen metabolites thus permitting a distinction to be made between the nature and the response of the cells.

Figure VIII The emission of light during luminescence.



There are two key enzymes involved in oxygen-dependent microbiocidal activity of polymorphs; myeloperoxidase and NADPH oxidase. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) reacts with the $H_2 O_2$ in the presence of a peroxidase (i.e. myeloperoxidase) in neutrophils whereas lucigenin, (N, N`-Dimethyl-9,9`-biacridinium dinitrate) has been widely used to assess the generation of superoxide by the NADPH oxidase. NADPH oxidase, responsible for the respiratory burst is inactive until the neutrophil is stimulated by engagement of receptors for chemoattractants (chemotaxins or chemokines), receptors for opsonising phagocytosis or receptors for various cytokines e.g. IL-1R (types I and II), IL-2R, IL-4R, IL-6R, INF-γR and TNF-R. The signalling processes induced result in the rapid appearance of NADPH oxidase activity. The active enzyme catalyses the following reaction

$$\text{NADPH} + O_2 \rightarrow \text{NADP}^+ + 2\,O_2^- + H^+$$

The activation of NADPH oxidase thus accounts for the burst of O_2 consumption by neutrophils during cell stimulation.

The advantages luminol-enhanced chemiluminescence include permitting the use of far lower numbers of cells, obviating the need for working under dark-adapted conditions (Prendergast & Proctor, 1981 and DeChatelet & Shirley, 1981) and can in fact be used to measure whole-blood chemiluminescence (Faden & Maciejewski, 1981 and Selvaraj *et al.*,1980). Whole-blood chemiluminescence is important where the volume of blood sample available is very small e.g. when working with animal models.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains

Staphylococcus epidermidis strains M7 and RP62A were clinical isolates kindly provided by Dr G. Christensen (University of Tennessee, Nashville). They were maintained on Columbia Blood Agar (CBA see appendix A) at 4^0C. (Gibco). Strain RP62A is a known slime producer (Christensen *et al.*, 1982). Strain M7, which is a slime-negative mutant, was obtained after chemical mutagenesis of strain RP62A (Schumacher-Perdreau *et al.,* 1994). Before each experiment a fresh overnight culture in Mueller-Hinton broth (Oxoid, Unipath Ltd, Basingstoke, Hampshire) at 37°C was prepared. The cultures were then washed three times with sterile saline and diluted to a concentration of 5×10^8 bacteria/ml (OD=0.45 at 620nm).

2.2.2 Isolation of polymorphonuclear leukocytes.

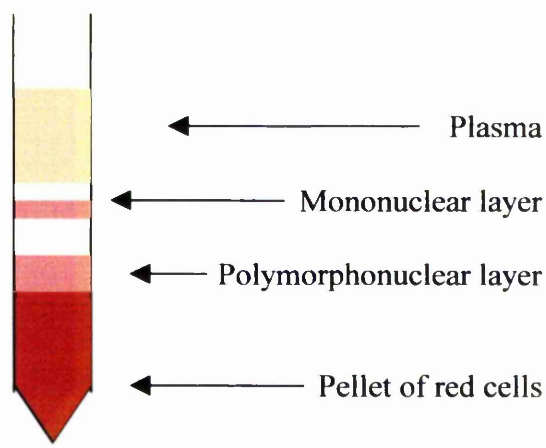
This was performed according to the method of Bøyum *et al.,* (1968). 5 ml of heparinised blood was carefully layered over 5ml Polymorphprep (Nycomed Pharma AS, Oslo, Norway) in a 10ml centrifuge tube. The sample was then centrifuged at 1800rpm for 30 minutes in a swing-out rotor (IEC Centra-4X, International Equipment Company, Dunstable, England).

After centrifugation, two leukocyte bands were visible. The top band at the sample-medium interface consisted of mononuclear cells and the lower band consisted of polymorphonuclear cells (See figure IX). The polymorphonuclear band was removed using a pasteur-pippette and residual isolation media removed by washing the cells with a solution of Hanks Balanced Salt Solution (HBSS, ICN Biomedicals Inc. USA) containing 1% Gelatin (BDH Chemicals Ltd, Poole, England) [Gel-Hanks] at 1800rpm for 10 minutes. Cells were counted in a haemocytometer and adjusted to a concentration of 5×10^5 PMNLs/ml by the addition of Gel-Hanks solution.

Figure IX **Procedure used for the isolation of polymorphonuclear and mononuclear leukocytes from whole blood**

1. Carefully layer 5 ml whole blood containing anti-coagulant over 5ml polymorphprep

2. Spin at 1800rpm for 30 minutes



3. Polymorphonuclear band is removed using a fine-tipped pasteur pipette and transferred to another test-tube. Cells are then washed in Gel-hanks solution. This results in a pellet of polymorphonuclear cells.

2.2.3 Isolation of mononuclear leukocytes.

The method is for that of isolation of polymorphonuclear leukocytes. The top band consisting of mononuclear cells was then removed and placed in a tissue culture flask containing RPMI 1640 medium (Gibco BRL, Life Technologies Ltd, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS)(Sigma Chemical Company, UK). The flasks were then incubated overnight at 37^{o}C under 5% CO2. After incubation the residual medium was removed and replaced with fresh RPMI 1640 medium. The flask was then placed on ice for 5 minutes and the cells recovered using a cell scraper. Cells were counted using a haemocytometer and adjusted to a concentration of $5x10^{5}$ mn/ml with RPMI. Cell viability was determined by exclusion of Trypan Blue Solution (0.4%) (Sigma-Aldrich Company Ltd., Irvine, UK). Cells with a viability < 95% were discarded.

2.2.4 Serum Isolation

Blood was obtained from healthy adult volunteers and was allowed to clot in sterile universal containers (Sterilin, England) for 30 minutes at room temperature. After centrifugation at 1500rpm for 15 minutes, the serum was pooled, aliquoted and stored at -70^{0}C.

2.2.5 Measurement of chemiluminescence.

Luminol-enhanced chemiluminescence of the isolated PMNLs was measured after exposure of the PMNLs to opsonised or non-opsonised bacteria (S. epidermidis M7 or RP62A). 100μL of PMNLs (10^{7}/ml) suspended in Gel hanks solution were incubated with 50μl bacterial suspension (5×10^{8}/ml) and 50μl luminol (10^{-5} M) (Merck, Darmstadt, Germany). The activity was recorded continuously in a Bio-Orbit Luminometer 1251 (Bio-Orbit, Turku, Finland) connected to an IBM PS/1 computer terminal. The reaction temperature was 37^{0}C. Light emission was measured in triplicate at two-minute intervals for at least thirty minutes. The activity was expressed as the peak activity in mV. Efficacy of phagocytosis was

measured as a rate of ingestion over the first 10 minutes of the reaction. Rate of ingestion was measured from the slope of the curve obtained from the luminometer printout. This gave a reading of mV/ cell/ minute.
The basic experiment as described above was varied as follows-

2.2.5.1 Respiratory burst with various Bacteria: PMNL ratios

Determining the optimum ratio of PMNL: bacteria for maximal chemiluminescence were performed by varying the concentration of PMNLs and bacterial cells in the reaction mixture. Table 2 shows the combinations investigated.

Table 2 Bacteria: PMNL ratios investigated to induce maximum respiratory burst.

PMNL at 1×10^7	Bacteria at 1×10^5	Bacteria: PMNL Ratio
100μl	50μl	25: 1
100μl	25μl	12.5: 1

2.2.5.2 Induced CL in PMNL by opsonised and unopsonised *S. epidermidis*.

Opsonins are described as 'an antibody which renders bacteria and other cells susceptible to phagocytosis' (Dorlands Medical Dictionary, 24th Ed.) This experiment was designed to investigate whether opsonisation of bacteria with normal pooled serum (NPS) had any beneficial effect on the rate of ingestion.

Opsonising conditions were as follows

1 Bacteria (at 5 x 10^8) were incubated with an equal amount of pooled normal serum obtained from a group of healthy volunteers ≥ 3 in number at 37^0C for 15 minutes with shaking.

2 The bacteria/ serum mixture was then spun at 3000 rpm for 15 minutes.

3 The supernatant was removed and the bacteria were then resuspended in their original volume of gel - Hanks balanced salt solution. The bacterial preparation was used immediately.

Efficacy of phagocytosis was measured as a rate of ingestion calculated over the first ten minutes of each experiment. This was expressed as mV/cell/minute.

2.2.5.3 Ability of prosthetic joint material to induce the respiratory burst.

To investigate whether PMNLs were activated simply by the presence of prosthetic joint material, PMNLs and a sterilised disc of prosthetic joint material were placed in a luminometer tube and the activity monitored. Any activity observed was compared to control tubes containing PMNLs and bacteria and PMNLs alone.

2.2.5.4 Effect of incubation time on induction of PMNL respiratory burst by *S. epidermidis* adherent to prosthetic joint material

This experiment was designed to investigate the adherence mechanism of *S. epidermidis* to prosthetic joint material as assayed by its ability to induce the respiratory burst in PMNLs. The discs of prosthetic joint material were incubated with suspensions of *S. epidermidis* M7 or RP62A for 30, 60, 90 minutes or 24 hours. This would allow comparisons to be made between early on in the initial stages of bacterial attachment to a substrate and later on in the accumulation stage. The discs were sterilised as before. Using sterile forceps, the discs were then dropped into a suspension of bacteria, either M7 or RP62A at a concentration of 1 x 10^7 bacteria/ml and incubated for 30, 60, or 90 minutes or 24 hours at 37°C. After incubation, the discs were carefully removed to minimise disturbing the attached bacteria and gently rinsed in sterile PBS to remove any loosely attached bacteria. The bacteria adherent to the disc were then opsonised by adding 10%

NPS to each tube. After 15 minutes the serum solution was then drained off and the discs again rinsed gently in PBS. The discs were then removed from the NPS and placed in a luminometer tube and 100μl PMNLs and 50μl luminol added. The activity was then measured continuously for ≥ 30 minutes. Rate of ingestion was calculated over the first ten minutes of the experiment.

2.2.5.5 Effect of incubation conditions on adherence of *S. epidermidis* strains to prosthetic joint material and their ability to subsequently induce PMNL respiratory burst.

This experiment measured the ability of *S. epidermidis* to adhere to prosthetic joint material under static or shaking incubation conditions. The disc and the bacteria were prepared as before and were then incubated in the static or the shaking incubator (100 revolutions per minute) for 30, 60, or 90 minutes. After this time period had elapsed, the bacteria adherent to the disc were opsonised as before. The discs were then placed in luminometer tubes, and 100μl PMNLs and 50μl luminol added.

2.3 RESULTS

2.3.1 Respiratory burst with different Bacteria: PMNL ratios

Tables 3, 4 and 5 show the results obtained for peak chemiluminescence response, the time taken to reach this peak chemiluminescence and the rate of bacterial cell ingestion. Rate was calculated by measuring the mV of light produced per cell per minute over the first ten minutes of the experiment. Two different ratios of bacteria to neutrophils were investigated. In the first experiment there were twenty-five times as many bacterial cells as there were neutrophils, and in the second experiment the number of bacterial cells was reduced by fifty percent. It was hoped that these experiments would tell us the optimum ratios for maximal phagocytosis by having the maximum number of possible interactions between the bacteria and the neutrophils.

The figures shown are the mean values where n = 10 with the standard deviation for the ten experiments shown in brackets. The students t-test was used to determine if there were any significant differences in the two reaction mixtures.

Table 3 Peak chemiluminescent response (mV)

STRAIN	M7	RP62A	P VALUE BETWEEN STRAINS
12.5:1	4.5 (sd= 1.17)	8.60 (sd=2.15)	0.0000
25:1	9.16 (sd=2.12)	16.92 (sd=3.69)	0.0001
P value between ratios	0.0000	0.0000	******

Table 4 **Time to reach peak response (minutes)**

STRAIN	M7	RP62A	P VALUE BETWEEN STRAINS
12.5:1	14.91(sd=5.23)	15.48(sd=6.21)	0.76
25:1	18.81(sd=2.05)	15.61(sd=2.51)	0.0000
P value between ratios	0.0070	0.92	*******

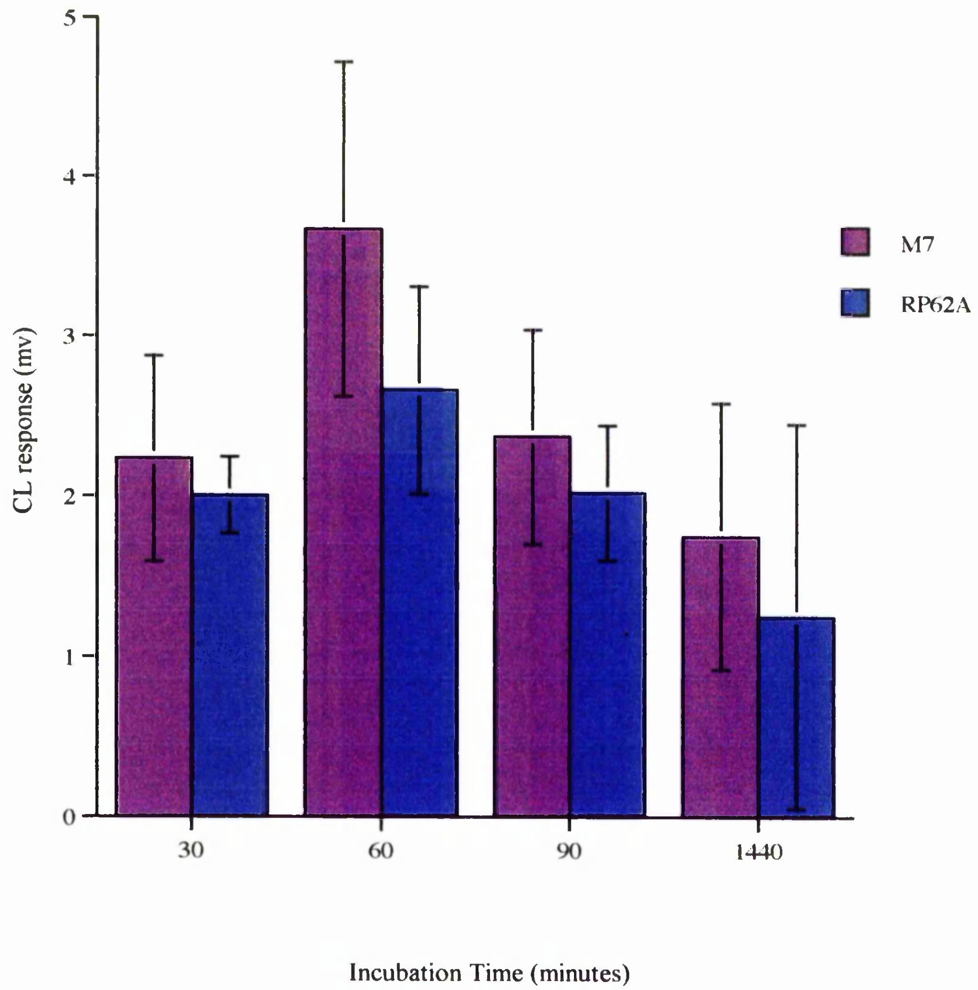
Table 5 **Rate of ingestion (mV/cell/minute)**

STRAIN	M7	RP62A	P VALUE BETWEEN STRAINS
12.5:1	0.327(s=0.2)	0.642(s=0.265)	P=0.0007
25:1	0.659(s=0.323)	1.11(s=0.660)	P=0.0028
P value between ratios	0.0000	0.0000	********

2.3.2 Effect of incubation time on induction of PMNL respiratory burst by *S. epidermidis* strains adherent to prosthetic joint material.

The graph in figure X shows the results obtained when discs of prosthetic joint material were incubated with suspensions of *S. epidermidis* M7 or RP62A in small test tubes for varying lengths of time. Incubation times varied from 30 minutes to 24 hours. The values shown are the peak chemiluminescent responses at each of the time points.

Figure X **Effect of incubation time on induction of the PMNL respiratory burst of *S. epidermidis* M7 and RP62A adherent to prosthetic joint material**



2.3.3 Effect of incubation time and conditions on the induction of the chemiluminescent response

Table 6 shows the chemiluminescent response obtained along with the rate at which light was released for incubation of prosthetic discs with bacteria for either 30, 60 or 90 minutes with the tubes containing the bacteria and discs being either shaken at 300 rpm or kept static.

Table 6 **Effect of incubation time and conditions on chemiluminescence**

STRAIN AND INCUBATION TIME (minutes)	PEAK CL RESPONSE (mV) (SD in brackets)			RATE OF LIGHT RELEASE (mV/cell/minute) (SD in Brackets)		
	STATIC	SHAKING	P value	STATIC	SHAKING	P value
M7 30	2.161 (0.631)	2.852 (0.315)	0.020	0.122 (0.126)	0.145 (0.045)	0.64
RP62A 30	2.005 (0.475)	2.187 (0.361)	0.38	0.09 (0.05)	0.11 (0.067)	0.66
M7 60	3.142 (0.561)	2.887 (0.823)	0.56	0.148 (0.05)	0.104 (0.057)	0.25
RP62A 60	2.402 (0.642)	2.543 (0.533)	0.71	0.146 (0.05)	0.077 (0.07)	0.25
M7 90	2.7 (1.18)	2.188 (0.062)	0.45	0.148 (0.16)	0.127 (0.110)	0.79
RP62A 90	2.063 (0.436)	3.218 (0.797)	0.064	0.547 (0.439)	0.212 (0.029)	0.32

2.3.4 Effect of opsonisation on the uptake of *S. epidermidis* strains adherent to prosthetic joint material.

Opsonisation is reported to facilitate the uptake of bacteria by neutrophils. The purpose of this experiment was to investigate whether opsonisation increased the uptake of bacteria. This was measured by the amount of light produced i.e the chemiluminescent response, or whether the time taken for this peak response to be reached was shortened by opsonisation or whether the rate of ingestion was faster. The results are shown in Tables 7, 8 and 9. The mean value is shown where n=16 with the standard deviation (SD) in brackets.

Table 7 Effect of opsonisation on peak chemiluminescent response (mV)

CONDITIONS	M7	RP62A
OPSONISED	10.46 (SD=6.01)	17.7 (SD=10.1)
UNOPSONISED	3.90 (SD=3.07)	15.0 (SD=11.7)
P value	0.0008	0.5

Table 8 Effect of opsonisation on the time taken to reach the peak CL response (minutes).

CONDITIONS	M7	RP62A
OPSONISED	20.19 (SD=2.81)	17.94 (SD=3.70)
UNOPSONISED	31.8 (SD=11.4)	24.3 (SD=11.4)
P value	0.0011	0.048

Table 9 **Effect of opsonisation on rate of bacterial cell ingestion (mV/cell/minute).**

CONDITIONS	M7	RP62A
OPSONISED	0.851 (SD=0.407)	1.606 (SD=0.896)
UNOPSONISED	0.274 (SD=0.251)	1.032 (SD=0.753)
P value	0.0001	0.059

2.3.5 Effect of prosthetic joint material on PMNL activation

Table 10 below shows the results obtained when PMNLs were incubated with prosthetic joint material.

Table 10 **Possible effect of prosthetic joint material on induction of the respiratory burst by PMNL.**

SAMPLE	PEAK CL (mV)	PEAKTIME (minutes)	RATE OF LIGHT RELEASE (mV/cell/minute)
PMNL	1.1	95.8	0
M7 + PMNL	30.1	19.4	2.85
PMNL + DISC	1.2	2.8	0
DISC	2.0	0.0	0
PMNL	1.0	2.8	0
RP62A + PMNL	30.4	16.7	3.03
PMNL + DISC	1.3	1.4	0
DISC	1.9	0.0	0

2.4 DISCUSSION

The phagocytic cell is very important in host defence. Without adequate numbers of neutrophils or properly functioning neutrophils, patients develop infections. The basic processes that are involved in eliminating invading microorganisms are chemotaxis, opsonisation, ingestion, digestion and killing-collectively known as phagocytosis. For optimal ingestion by PMNLs there should be an optimum PMNL: Bacteria ratio, i.e. enough circulating neutrophils to effectively clear the bacteria from the infection site. Opsonisation also facilitates uptake of the bacteria, if the bacterium is opsonised it is more readily ingested. In this chapter conditions for optimum phagocytosis of bacteria adherent to the surface of prosthetic joint material were investigated using the technique of chemiluminescence. Since it was first demonstrated in 1928, luminescence analysis has become widely used as a replacement or alternative method for many assays carried out using conventional techniques such as colorimetry, spectrophotometry and radioactivity. A flash of light is released when the phagocyte ingests material of some sort. The amount of light released and the speed at which it does so can give us both information about the bacterium in the way it is ingested and can also tell us how well the phagocyte is working. The advantages of luminescence include greater sensitivity (up to 1,000 times more sensitive than photometry) and that only a small amount of specimen is required, a very important factor test material is limited or invasive sampling is necessary.

2.4.1 Respiratory burst induced in PMNL by varying the ratio of Bacteria: PMNLs.

2.4.1.1 Amount of light produced.

Phagocytes have receptors on their cell surface which recognise the complement component C3bi or IgG which is found attached to opsonised bacteria. Insufficient white cells (known as neutropenia) may result in the development of infection whereas an excess of white cells (known as neutrophilia) working without cohesion might result in a process as autoimmune disease such as lupus or diabetes mellitus (Nepom & Erlich, 1991). Two different ratios of bacteria to PMNLs have been investigated, 25:1 and 12.5:1. This experiment is normally performed with a PMNL:bacteria ratio of 25:1. It was decided to investigate whether or not reducing the number of bacteria present in the reaction mixture would increase the rate at which they were ingested. The idea was that the neutrophils would come into contact with a more limited number of bacteria and there would be more neutrophil:bacterial interactions resulting in quicker ingestion. As can be seen from the results in Table 3, when there is twice the number of bacteria present in the reaction mixture the amount of light produced increases accordingly. This is in accordance with the theory that the number of bacterial cells present in the reaction mixture dictates the amount of light released.

There was a greater chemiluminescent response observed for strain RP62A. This is true regardless of the number of bacterial cells present in the reaction mixture. The amount of light produced is almost twice that observed for strain M7. On analysis this difference between the strains was found to be significant with p values of $p \leq 0.0000$ and $p \leq 0.0001$ being obtained for the different reaction mixtures (25:1 and 12.5:1).

2.4.1.2 Time taken to reach the peak CL response

The time taken to reach the peak chemiluminescent response differed for the two strains. With strain M7, when there were more bacteria present in the reaction mixture, the time taken to reach the peak CL response increased although unlike light production, did not double. The time difference observed was significant with a p value of $p \leq 0.007$. No such difference was observed for strain RP62A. When the number of bacteria present in the reaction mixture was actually doubled the time taken to reach the peak CL response only increased by 10-15 seconds. This difference was not significant ($p \leq 0.92$).

When the strains were compared to each other, a difference was only observed with the reaction mixture containing the "regular" ratio of bacteria:PMNLs. The reason for this is unclear as if there is a difference in how easily the bacteria are ingested according to their properties then this should be observed regardless of the number of bacteria present in the reaction mixture.

2.4.1.3 Rate of light release

A similar response to the amount of light produced by the bacteria is observed with the rate at which the light is released. By reducing the number of bacteria present in the reaction mixture the rate at which the light is subsequently released is reduced by fifty percent accordingly. RP62A cells are ingested at a rate which is twice that observed for M7 cells. This was true regardless of the number of bacteria present in the reaction mixture with significant differences of $p \leq 0.0007$ and $p \leq 0.0000$ being obtained for 12.5:1 and 25:1 ratios.

There does not seem to be a standard "recipe" for the optimum ratio of bacteria to PMNLs. Other authors have used ratios of as many as 200:1 (Kallman *et al.*, 1998) to as little as 10:1 (Taylor *et al.*, 1999). When studying neutrophil function all that matters is that differences in the phagocytic capacity of the neutrophils can be demonstrated regardless of the number of bacteria available for ingestion.

2.4.2 Effect of incubation time on induction of the respiratory burst.

The graph in figure X shows the results obtained when the incubation time to allow the bacteria to adhere to the surface of the prosthetic joint material was varied. Four different incubation times were investigated which fell into two categories, early and late. By definition, early refers to the initial stages of attachment when the bacteria are settling onto the surface and forming weak bonds of attachment. The greatest CL response was observed when the bacteria had adhered to the prosthetic joint material for 60 minutes. CL response fell at 90 minutes and at 24 hours which is surprising, as the longer the bacteria had to adhere, the greater the number of adherent bacteria there should be on the surface of the prosthetic joint. One possible explanation for this is that the bacteria were in the early stages of extracellular-slime (ESS) formation. ESS may have an inhibiting effect on the chemiluminescent respiratory burst by preventing ingestion of the bacteria. Most studies comparing adhesion of M7 and RP62A have been performed after 24 hours incubation (Konig *et al.*, 1998 and 1999) where the number of colony forming units have been counted after washing off the adherent bacteria. To the best of my knowledge no other research groups have studied the effect of incubation time on the ability of PMNLs to ingest adherent bacteria using the chemiluminescent method described in this study.

2.4.3 Effect of incubation conditions on the adherence of *S. epidermidis* strains to prosthetic joint material and their subsequent ability to induce PMNL respiratory burst.

Most *in vitro* studies between bacteria and neutrophils are held under shaking conditions (Gemmell, 1989). However, *in vivo* phagocytosis occurs both in suspension and from a static substrate. Several studies have shown differences between surface and suspension phagocytosis (Lee *et al.,* 1983 and Vas, 1983). Phagocytosis of bacteria free in suspension results in the emission of more reactive oxygen intermediates over a longer length of time than does phagocytosis of a biofilm of bacteria. Perhaps this is because there is more available surface area of the bacteria to come into contact with the white cells when the bacteria are in suspension and thus are more readily attacked by the white cells.

Table 6 shows the results obtained when bacteria and neutrophils were incubated for different lengths of time under both static and shaking conditions. It was thought that shaking the reaction mixtures would increase the number of bacteria:PMNL interactions and thus increase the CL response. At thirty minutes there is a significant difference observed in the rate at which light is released for strain M7 ($p \leq 0.020$) but not for strain RP62A when the bacteria are incubated with shaking compared to static conditions. The rate at which the light is released over the first ten minutes of the experiment however did not show any significant differences. There is no significant difference observed as the incubation time increases to sixty minutes. At ninety minutes there is a difference although again not highly significant ($p \leq 0.064$) for strain RP62A but not M7. The reason this difference between static and shaking conditions was not observed at sixty minutes is unclear. As the differences were not significant and were observed in both strains it was thought that they may be explained by differences in the neutrophils. The results are the mean values of many experiments where different preparations of neutrophils were used. The longer a neutrophil is stored isolated generally the more reactive it becomes in chemiluminescent experiments and often a greater response is observed. This is generally true of neutrophils isolated for more than 6 hours. These neutrophils were freshly harvested and used as soon as possible, so this could not be the reason.

2.4.4 The role of opsonisation in phagocytosis.

Opsonisation comes from the Greek word 'opsonein' meaning to sauce or season (Collins English Dictionary, XIX Edition). By coating a particle with an opsonin it makes it more palatable and more easily ingested by the phagocyte (Mims, 1995). Many bacteria have developed a defence against opsonophagocytosis and are thus able to escape phagocytosis by PMNLs. The most important anti-phagocytic defence of bacteria is an enveloping capsule. These capsules protect the microbes against PMNLs by interfering with opsonisation (Finlay & Falkow, 1989). A substantially thicker capsule may confer more protection against phagocytosis (Bruyn, Zegers & von Furth, 1992). For example, pathogens that cause pneumonia and meningitis, such as *H. influenzae, N. meningitidis, E. coli, S. pneumoniae, K. pneumoniae,* and group B streptococci, have polysaccharide capsules on their surface. Non-encapsulated derivatives of these organisms are less virulent. This is exemplified by pneumococci. Pneumococcal strains isolated from humans fall into two groups. The encapsulated group is referred to as "smooth" because of the smooth mucoid appearance of the colonies on microscopy. Unencapsulated strains are referred to as "rough". Smooth and rough strains differ greatly in their virulence where a million times the amount of an unencapsulated strain of pneumococci is required to kill a mouse compared to an encapsulated strain (Avery & Dubois, 1931).

When non-encapsulated and encapsulated strains of *Streptococcus uberis* were incubated with bovine mammary macrophages it was found that the presence of a capsule may protect the *S. uberis* from phagocytosis. It was postulated that this occurred through direct interaction of capsular material with the macrophages as there was 78% ingestion of unencapsulated *S. uberis* with a killing rate of 75% compared to 48% of encapsulated *S. uberis* with a killing rate of only 35%. When the strains were opsonised with homologous antiserum, only differences in the phagocytosis and intracellular killing of the encapsulated strain were observed (84% versus 48% and 52% versus 35% respectively). This increase was almost abolished when the antiserum was absorbed with purified capsule or when the macrophages were pre-treated with purified capsular material. Further investigation with electron microscopy showed that when the macrophages were incubated with the encapsulated *S. uberis* there was contact between the bacterium and the macrophage with no signs of membrane activation. However, incubation with the non-encapsulated strain induced the formation of pseudopods and membrane ruffling, both early stages in phagocytosis (Almeida & Oliver, 1993).

Strain differences within the coagulase-negative staphylococci in their susceptibility to opsonophagocytosis have been observed. These differences do not seem to be related to slime production although the presence of serum is essential for optimum phagocytosis (Gemmell, 1987). The cell surface hydrophobicity status of the bacterium is important in determining whether or not an opsonin is required as certain cell surface structures may be more hydrophobic or hydrophilic in nature. In a study by Kristensson, Hastings & Spencer (1988) of luminol-enhanced chemiluminescence of forty-three strains of *Staphylococcus epidermidis*, the presence of slime had no effect on the requirement for opsonisation as both slime and non-slime-producing strains exhibited an increase in their chemiluminescent response with increasing serum concentrations

The role of opsonisation in the phagocytosis of two strains of *S. epidermidis*, one slime producing, RP62A and its non-slime-producing mutant M7 was investigated. This was measured as the ability of the opsonised or unopsonised bacterium to induce the respiratory burst in PMNLs and was measured as the peak CL response, the time taken to reach the response and the rate at which the bacteria were ingested.

2.4.4.1 Peak CL response

As mentioned earlier the surface properties have a very important effect on whether bacteria need to be opsonised or not. The two strains investigated differ in that one strain is able to accumulate and produce slime whereas the other is not. There is the theory that producing slime may protect bacteria from ingestion by phagocytes. If this is true then the effect of opsonising the slime-producer, thus making it more favourable to the phagocyte may cancel out this protective effect of slime. The results in Table 7 show the CL response obtained for both bacterial strains. As can be seen there is a highly significant difference observed for the non-slime producing strain M7 between opsonised and unopsonised preparations. Opsonising M7 gives a peak reading of 10.46 mV of light whereas unopsonised M7 results in less light being produced- only a third of that seen with the opsonised strain only 3.9 mV ($p \leq 0.0008$). On the other hand, opsonising strain RP62A does not seem to have any effect at all. The difference in the amount of light produced was not significant with 17.7mV and 15 mV of light being released from opsonised and unopsonised preparations respectively. Thus opsonising a slime producing strain does not make it any more susceptible to ingestion. It may be that the presence of slime is more attractive to the phagocyte and makes the bacteria more easily ingested. The results shown here are in agreement with a study performed by Gemmell and Marshall in 1987 who showed that phagocytosis of slime-producing strains of *S. epidermidis* occurred in the presence or absence of human serum.

2.4.4.2 Time taken to reach the peak CL response.

This experiment was designed to investigate whether opsonised bacteria are ingested faster than unopsonised. The results are shown in Table 8. In both cases the length of time to reach the peak CL response was shorter if the bacteria had been previously opsonised regardless of whether they produced slime. However, the reduction in time was only highly significant for strain M7 ($p \leq 0.0011$). A p-value of ≤ 0.048 was obtained for slime-producing RP62A. RP62A was ingested faster than M7 regardless of opsonisation thus slime may have a greater effect than opsonisation in making the organism more palatable to the PMNL.

2.4.4.3 Rate of bacterial ingestion

Opsonising certainly has an effect on the rate at which bacteria are ingested. The most dramatic effect is observed with strain M7 where there is a three-fold increase in rate, which is highly significant ($p \leq 0.0001$). A less dramatic effect is observed with RP62A ($p \leq 0.059$). This is in agreement with the results observed for the amount of light produced and the time taken to do so. This work is therefore in contrast to the many reports stating that slime has a protective effect against ingestion by white cells by lowering ingestion rates (Shiau & Wu, 1998; Heinzelmann *et al.,* 1997 and Hancock, 1989). Slime may make the cell surface more hydrophilic, a factor which may interfere with the phagocytic response to infection (Rodgers, Phillips & Olliff, 1994). It would seem that in this case that the presence of slime in fact increases the affinity of the bacteria to the neutrophil. It may be that the slime anchors the white cell to the bacterium. Now firmly attached the polymorph can easily ingest the bacteria. Non-opsonised RP62A when ingested emits 15 mV of light. This is almost four times the amount emitted by non-opsonised M7.

2.4.5 Effect of prosthetic joint material on chemiluminescence

One of the most important points when designing and manufacturing any material for use on or in the human host is that it is non-toxic to human tissue. If the material itself were to induce an immune reaction with the host immune system this would be most detrimental to the well being of the host. Common indications of such a reaction are redness, swelling, heat and pain at the site. It is especially important in joint replacements (as well as heart valve replacements) that the body does not reject the new hip or knee and that the tissue integrates with the material to promote normal activity. The cellular response to particulate material from orthopaedic implants is well documented. Particles of prosthetic joint material are generated when wear occurs between the different components of the implant. Increased levels of metal or polyethylene particles have been found within synovial tissue, bone trabeculae and bone marrow as well as in the serum and urine of patients who have undergone joint replacements (Al-Saffar, Revell & Kobayashi, 1997). The biocompatibilty of the biomaterial itself is of great importance and this is also well documented. Most experiments have been performed using the *in-vivo* implanted chambers. In this situation the biomaterial of interest is placed inside a cage which is then placed into the animals body. This allows close monitoring of the local tissue response without leading to spread of disease. In a study by Marchant, Anderson & Dillingham in 1986, the components of the inflammatory response including white cells were monitored over the implantation of low-density polyethylene and polyvinylchloride. The polyvinylchloride stimulated an intense acute phase inflammatory response which led on to a chronic inflammatory response whereas in contrast the polyethylene promoted relatively small increases in the acute and chronic phases of inflammation. When polyethylene terephalate (PET) was implanted subcutaneously into rats for periods of two to four weeks minimal acute or chronic inflammation was observed (Chinn *et al.,* 1998). Such experiments would have required ethical consideration and appropriate approval from the Home Office and could not be developed in the time course of this study so it was decided to develop a relatively quick and easy method to establish whether the biomaterial in question could activate the immune response. The biomaterial was prepared and placed in a sterile test tube. The idea was that if white cells were activated by the biomaterial there would be an involuntary flash of light (chemiluminescence) from the white cells when they were added to the tube containing the biomaterial.

Four different reaction mixtures were tested as shown in Table 10. The positive controls for the experiment were the reaction mixtures containing bacteria, white cells and luminol. Negative controls were white cells and luminol, and discs and luminol chosen to investigate whether a CL effect was in fact due to the prosthetic disc material and was not spontaneous CL by the white cells.

From the results in Table 10 it can be seen that the prosthetic joint material itself does not have any effect on the white cells. There was very little luminescence which was almost the same from the white cells themselves, the prosthetic joint material and the combination of neutrophils and prosthetic joint material. There was no peak response as an almost flat line was observed on the chart readout. This shows that there is no activity from the white cells and more importantly the prosthetic joint material does not activate the cells causing damage to local tissue.

Thus optimum phagocytosis conditions include 25:1 bacteria: neutrophils with the bacterial preparation opsonised. The effect of shaking the reaction mixture was inconclusive. The prosthetic joint material used in these experiments was ideal as it did not activate the white cells which would have resulted in a false high chemiluminescence reading. The best results were obtained when the bacteria had been allowed to adhere to the prosthetic joint material for 60 minutes, which is ideal as it allows the full experiment to be completed in one day.

CHAPTER THREE

DEVELOPMENT OF AN *IN VITRO* MODEL TO STUDY PHAGOCYTOSIS OF BACTERIA ADHERENT TO BONE AND PROSTHETIC JOINT MATERIAL.

3.1 INTRODUCTION

3.1.1 The use of biomaterials in modern medicine

The use of metals and other materials to repair the human body has been recorded for centuries, dating back several millennia (Agrawal, 1998). The continuously increasing demand in modern medicine for biomaterials in tissue substitution makes biomedical devices one of the most important aspects of modern medical care (Tang & Eaton, 1995). Such biomaterials include sutures, indwelling catheters, internal fixation devices, joint replacement systems, dental materials, contact lenses and cardiac valves (Gristina *et al.*, 1985).

One of the more common uses of biomedical devices is in joint replacement. The modern era of total hip arthroplasty is over 30 years old, and during that time the procedure has proved to be highly effective in improving physical function, social interaction, and over-all health of millions of patients (Laupacis *et al.*, 1993). The major barrier to the extended use of biomaterials is bacterial adhesion, which causes biomaterial-centred infection (Gristina *et al.*, 1993). The introduction of a foreign material into living tissue-intentionally as in biomedical applications (implants, prostheses or drugs), or unintentionally when minerals or fibres are inhaled, results in the creation of interfaces between the biomaterial and the surrounding tissue (Kasemo & Lausmaa, 1994). Biomaterials are surrounded by an immune-incompetent, fibro-inflammatory, integration-deficient zone within which stimulation of cellular immune responses results in superoxide and cytokine-mediated tissue damage with increased susceptibility to infection or aseptic loosening (Gristina, 1994). Patients who have had a total joint replacement are particularly susceptible to infection because of the foreign body that has been implanted (Garvin & Hansen, 1995). Unfortunately the rise in the number of joint replacements being performed has resulted in a concomitant rise in infection rates. As a result, infection is recognised as one of the most serious complications of implanted devices (Boswald *et al.*, 1995) requiring complex and lengthy management (Brydon *et al.*, 1996). Prosthetic infection following total joint replacement can have catastrophic results both physically and psychologically for the patients. It may lead to complete failure of the arthroplasty, possible amputation, prolonged hospitalisation, and even death (An & Freidman, 1997). Initially the procedure was associated with notable rates of infection. In 1969, Charnley and Eftekar stated: "It is a paradox that simple adherence to Listerian principles has abolished virulent postoperative infections, but infection by mildly pathogenic staphylococci appears to be increasingly common", thirty years later, the coagulase-negative staphylococci and *Staphylococcus aureus* still dominate the list of pathogens. Infection rates have been reduced considerably with measures such as prophylactic antibiotics. Penicillinase-resistant penicillins and cephalosporins or broad-range antimicrobials such as cefamandole and cefuroxime are used because of the concern regarding organisms resistant to the spectrum of coverage provided by the first generation cephalosporins. Charnley attributed the decline in infection rates primarily due to air cleanliness and in particular the use of a laminar air-flow system and body exhaust attire (Charnley, 1972 and Charnley & Eftekar, 1969). More recently, in addition to antibiotics, laminar air-flow systems and body exhaust suits, the careful selection of patients along with the elimination of remote infections such as dental, cutaneous and urinary tract infections, the use of ultraviolet light and the limitation of traffic in the operating room have all contributed to an infection rate of less than 1 per cent (Garvin & Hanssen, 1995).

There are three main questions to be addressed in the use of biomaterials

1. What are the mechanisms that cause abnormal inflammatory responses in the absence of infection and result in interface cellular disorganisation and device failure?
2. What causes host defences to be compromised to the extent that normal flora organisms like *Staphylococcus epidermidis*, with little or no virulence potential, can cause life-threatening infections at the implant-host interface?
3. What is the nature of surface regions of biomaterials that facilitate bacterial adherence?

(Gristina, 1994)

3.1.2 Bacterial adhesion onto biomaterials

Microorganisms can attach to a wide variety of surfaces, ranging from inorganic materials such as rocks and soil particles to plant and animal tissues (Douglas, 1987). Bacterial adhesion onto biomaterials is an essential step in the pathogenesis of biomaterial-associated infection (Reed & Williams, 1978). Colonisation and infection of plastic medical devices involves the unique interaction of a microbe with a nonbiologic surface. The exact mechanism of prosthetic infection remains unclear. *In vitro* studies have shown the ability of bacteria to adhere onto many substrates such as suture materials (Chu & Williams, 1984), stainless steel (BoulangePetermann, 1997) the gastric mucosa (Clyne & Drumm, 1997), bone implants (Fischer *et al.*, 1996), and intravascular catheters (Galliani *et al.*, 1997). In all cases the ability to adhere varied depending on the nature of the biomaterials.

Pathogenic strains of *Staphylococcus epidermidis* and *Staphylococcus aureus* have been shown to have an affinity for biomaterial surfaces and are capable of initiating infection (Gristina, 1994). In a study by Chang & Merritt in 1994, adherence of *S. epidermidis* to three common implant materials, stainless steel, polymethacrylate and commercially pure titanium was investigated. Adherence was greatest for stainless steel followed by polymethylmethacrylate and titanium and was least when gentamicin was added to polymethylmethacrylate. The rates of infection and colonisation correlated with the propensity for *S. epidermidis* to adhere to the given material.

3.1.3 Factors affecting bacterial adhesion

The factors which affect the adherence of a bacterial cell to the surface of a biomaterial include the surface chemistry of the cell and the material, as well as the local environment. An important question to be answered in the investigation of infected prosthetic devices is, 'is the adhesion target the native plastic surface as it is machined and then purchased, or is it a plastic surface covered with adsorbed host-derived proteins from circulating blood, cells and tissue debris, or a combination of both?' (Gristina, 1994). Gristina suggested that biomaterials or prostheses could act as substrata that encourage bacterial adhesion and proliferation, causing resistant infection (Gristina, 1987; Gristina, 1994; Gristina & Costerton, 1985 and Gristina & Kolkin, 1983). They described this phenomenon as a "race for the colonisation of the biomaterial surface", in which bacteria compete with host-tissue cells for the ability to secrete proteins or carbohydrates and to integrate with or bind to the surface of the biomaterial (Gristina, 1994). If microbes win this race, then bacterial species are able to adhere to the surface and form a biofilm.

Biological recognition mediates cell and tissue interactions with biomaterials. These interactions are typically mediated by proteins that have been adsorbed to the biomaterial surface (Elbert & Hubbell, 1996). Much development is therefore aimed at modifying the surface of biomaterials to reduce protein adsorption. Blood-contacting biomaterials may activate the complement cascade, thus promoting leukocyte adhesion to the biomaterial surface. Investigators will often demonstrate microbial adhesion to a surface that has been treated with serum or plasma on the presumption that the substances coating the plastic will form the relevant target (Vaudaux *et al.*, 1995).

Staphylococcus aureus has been shown to attach to biomedical materials which have been coated in host proteins such as fibronectin, fibrinogen or fibrin. The interaction between the bacterium and the host protein is mediated by specific surface proteins called bacterial adhesins which recognise specific domains of the host protein (Vaudaux *et al.,* 1995). There are two surface fibronectin-binding proteins expressed by *Staphylococcus aureus* FnBPA and FnBPB. When insertion mutations conferring tetracycline and erythromycin resistance respectively were inserted into these genes singly there was no significant reduction in their adhesion to polymethylmethacrylate coverslips coated with fibronectin. When a double mutant of FnbA and FnbB was constructed it was completely defective in adhesion (Greene *et al.*, 1995) suggesting that both proteins are necessary. Tang & Eaton, (1995) have shown that fibrinogen has a particularly pro-inflammatory effect. It has been hypothesised that the extent of complement activation is modulated by biomaterial formulation and the presence of fluid shear stress (Kao *et al.*, 1996). It was found that adherent leukocyte densities decreased as the shear stress increased.

Of the cell population analysed the monocytes adhered in greater numbers than did the neutrophils, under both shear and static conditions. The addition of complement factor C3 and fibronectin was essential to maintain adhesion under conditions of shear stress.

For many pathogens, the interaction between bacterial cell and the host surface determines the ability of the microorganism to colonise and infect the host (Christensen *et al.*, 1985). In order for a bacterium to attach to a surface the "chemistry" of that surface must be suitable for the organism in order to ensure its survival. Adherence interactions must involve complementary components on the colonisable host surface and on the bacterial cell surface. The interactions can be split into two groups, long-range interactions and short-range interactions.

The long-range interactions between cells and solid surfaces are frequently described by the mutual force which is a function of the distance and the free energy. This interaction has been described in the DVLO theory (see Chapter One for details). The DVLO theory is composed of two additive terms, G_A, the interaction due to van der Waals forces and G_E, the interaction energy of electric double layers associated with charged groups present on a cell and on a solid surface. When the cell and the solid surface are coming into close contact (i.e $<$ 3nm) short-range interactions occur. These can be distinguished into chemical bonds, electrostatic bonds and hydrophobic bonds. The short-range interactions may be attractive or repulsive depending on the nature of the surfaces involved. One important surface property is surface hydrophobicity - of both the bacterium and the substratum. Hydrophobic interactions are involved in the mechanism of adhesion of a variety of bacteria to host tissues (Braga & Reggio, 1995).

3.1.4 Measurement of bacterial hydrophobicity

Cell surface hydrophobicity has been implicated in a wide variety of adhesion phenomena such as adhesion to biomaterials (Pasqual *et al.*, 1986), contact lenses (Klotz *et al.,* 1989), the fermentation and brewing industry (Amory & Rouxhet, 1988) and opsonic requirements and phagocytosis (van Bronswijk *et al.*, 1989). Numerous tests have been proposed to measure cell surface hydrophobicity, and in many cases cell surface components that promote or reduce hydrophobicity (hydrophobins and hydrophilins) have been identified (Rosenberg & Kelljeberg, 1986).

Microbial hydrophobicity has been studied since 1924, since then various techniques have become available for measuring hydrophobic surface properties of microbial cells (Rosenberg, 1991). These are listed in table 1. Most microbial cell surface hydrophobicity assays can be divided into two broad categories. The first category measures the hydrophobic properties of the outer cell surface as a whole. Such assays include Contact Angle Measurements (CAM), partitioning of cells into one or another liquid phase - the Two-Phase Partition system, (TPP), and adsorption of individual hydrophobic molecular probes at the cell surface. The second category measures hydrophobicity in terms of adhesion. Such techniques include microbial adhesion to hydrocarbons (MATH), hydrophobic interaction chromatography (HIC), and adhesion to polystyrene and to other hydrophobic solid surfaces. In such instances a hydrophobic “tip” may be sufficient to mediate adhesion, thus classifying a bacterium as hydrophobic (Marshall & Cruikshank, 1973 and Marshall, Cruikshank & Bushby 1975).

Any measurement of microbial hydrophobicity has inherent technical difficulties. Culture conditions can profoundly affect hydrophobic surface properties of a wide range of microorganisms. In some cases sequential subculturing of clinical isolates results in gradual disappearance of hydrophobic properties (Bandin *et al.,* 1989 and Westergren & Olsson, 1983), suggesting that fresh isolates should be used whenever possible. Also, adhesion of microorganisms to hydrophobic surfaces is, at least in some cases cell density dependent (van Pelt *et al.*, 1983).

3.1.4.1 Hydrophobic Interaction Chromatography (HIC).

Hydrophobic Interaction Chromatography (HIC) offers a method for the determination of relative values for surface hydrophobicity. HIC measures microbial adsorption to octyl- or phenyl- sepharose beads. Smyth and co-workers (1978) used this simple technique which was originally developed for protein separation (Hjertén, Rosenberg & Pahlman, 1974). It is based upon the theory that bacterial cells will bind to octyl or phenyl- sepharose beads (see figure XII). The percentage of bound cells can then be measured by the loss of turbidity, or, in this case radioactivity in the eluate as compared with the initial level. In some cases, salting-out agents such as ammonium sulphate are added to promote adhesion to the gel (although untreated beads should also be used as a control). One potential problem of this method is that mechanical entrapment within the gel matrix or between the gel and glass support. This may occur with the use of salting-out agents (which may cause aggregation) or in studies of chains or clumps of microorganisms or large cells. This problem may be overcome by using a batch procedure, i.e. by mixing the bacterial cells and beads freely in suspension (Rosenberg & Kelljeberg, 1986).

3.1.4.2 Microbial Adhesion to Hydrocarbons (MATH)

In 1980, Rosenberg, Gutnick & Rosenberg, showed that various bacterial strains thought to possess hydrophobic surface characteristics adhered to liquid hydrocarbons, whereas non-hydrophobic strains did not. Bacterial adhesion to hydrocarbons (BATH) was proposed as a simple, general technique for studying cell surface hydrophobicity (see figure XI). Expansion of this method to study eukaryotic organisms (Klotz, Drutz & Zajic, 1985; Mozes *et al.*, 1987, Mozes & Rouxhet, 1987) has resulted in this method being more appropriately named Microbial Adhesion to Hydrocarbons (MATH). MATH is generally considered to be a measure of the organism's cell surface hydrophobicity. The observation that the zeta potentials of hydrocarbons can be highly negative in the various solutions commonly used in MATH, has suggested that MATH may measure a complicated interplay of long range van der Waals and electrostatic forces and of various short range interactions. Thus using MATH as a method only holds when the MATH test is carried out under conditions where the interacting surfaces have no charge (Busscher, Bos & van der Mei, 1995). In a study by van der Mei, van de belt-Gritter & Busscher in 1995 of both intrinsically hydrophobic and hydrophilic bacteria (as determined by water contact angle measurements) it was observed that the hydrophobic strains were removed in a pH dependent manner. Maximal removal occurred when the pH value was at a zeta potential of zero for the organism or the hydrocarbon. The hydrophilic strains were not removed by the hydrocarbons to any significant extent, because the attractive forces between water and the organism were much stronger than the forces of attraction between the organism and the hydrocarbons. This observation questions the use of MATH as a hydrophobicity assay. For this reason, most researchers perform more that one test when determining the hydrophobicity of an organism.

3.1.5 Interaction of neutrophils with biomaterials

The adhesion of neutrophils and other leukocytes to biomaterial surfaces is an important phenomenon in the host response to biomaterials because the number of adherent leukocytes is often related to the inflammatory response after implantation (Yung *et al.*, 1996). Any differences in the expression of molecules by leukocytes on their adherence to the substrate can be detected by flow cytometry. Studies of the morphology of human neutrophils adherent to both hydrophilic (glass) and hydrophobic (FEP-Teflon, polyethylene and polypropylene) surfaces was performed in a parallel-plate flow chamber using scanning electron microscopy. They showed that under stress conditions the neutrophils adherent to the glass exhibited surface veils, ridges and ruffles, suggesting a high level of cell migration. Adhesion of activated PMNLs was also higher to glass (Granchi *et al.*, 1998). Once adhered, neutrophil functions such as phagocytosis and the resultant respiratory burst and protease release may occur resulting in the deterioration of the implanted biomaterial and injury to the surrounding tissue. From immunologic studies it is known that inflammatory reactions can be modulated by use of anti-growth factors (such as tryphostins or tristatins which inhibit epidermal growth factor) or anti-inflammatory drugs (NSAIDS such as Ibuprofen) (van Luyn *et al.*, 1998).

In 1996, Kaplan *et al.*, proposed that biomaterial-associated infections resulted in increased morbidity and mortality. This was thought to be because of non-productive premature activation of neutrophils resulting in impaired phagocyte function at the biomaterial surface in the event of bacterial challenge. The supernatants of biomaterial-associated neutrophils were studied and it was found that they contained chemoattractants, which therefore induced chemotaxis, by fresh neutrophils. When fresh neutrophils were added to a biomaterial surface that had contained a previous innoculum of neutrophils, the fresh cells did not become stimulated to release reactive oxygen intermediates suggesting that not only does the biomaterial surface activate the initial wave of neutrophils but that subsequent waves of neutrophils exhibit an impaired host-defence function. The effect of biomaterials on neutrophil superoxide release has been investigated (Kaplan, Kuna & Reddigari, 1994). Three biomaterials commonly used in surgical practice, polytetrafluoroethylene (PTFE), polyurethane and dacron were studied. It was found that polyurethane was activating, but the activation effect could be inhibited by serum whereas PTFE was non-activating and dacron was not activating unless serum was present, thus the activation mechanisms on one material cannot be extrapolated to other materials.

3.1.6 Abnormal tissue responses to implanted devices

One of the greatest problems affecting the success of an implanted device is when the host views the implant as "non-self" and mounts an immune response to it. The biomaterials used for joint replacement are not always well tolerated by the patient, and a host inflammatory response may well occur (van Wachem *et al,* 1998). To some degree all biomaterials generate an inflammatory response. Biomaterials are able to stimulate cytokines. Yourtree *et al.,* (1997) demonstrated that dental materials induced the release of tumour necrosis factor alpha (TNF-alpha) from mouse macrophages. The inducing source was methacrylic acid (MAA), the hydrolysis product of many methacrylate biomaterial monomers. Depending upon the mechanics of the system and the materials involved, the implant is normally well tolerated and is able to achieve its function. Evaluation of the host response to implanted materials requires systematic, objective investigations of both responses at both the cellular and molecular levels (Hunt, McLaughlin & Flanagan, 1997). The presence of cytokines may be readily assayed using bioassays and enzyme-linked immunoassays (ELISA). The measurement of such cytokines may provide both qualitative and quantitative indication of which cells are responding to a particular stimulus.

Any type of debris (generated by poor surgical technique, or wear products from the polyethylene/metal interface) may lead to the aseptic loosening process. Aseptic loosening is a common problem affecting the success and life-span of a prosthesis (Wooley, Nassar & Fitzgerald, 1996). Until recently, the hypotheses surrounding the causes of aseptic loosening have centred on surgical technique, the material properties of the actual prosthesis and it's design. Evidence has now come to light that some implanted materials may cause immune reactions in a number of individuals, and it is suggested that cellular responses to biomaterials should be considered during the selection of prosthesis composition (Larsson, Iscove & Coutinho, 1980). A pathological response consistent with an aggressive foreign body reaction to cemented arthroplasties and the presence of a cellular response in the surrounding tissue has been illustrated on examination of the bone/cement interface of loosened arthroplasties (Maguire, Coscia & Lynch, 1987). Some degree of wear of the bearing surfaces of total joint replacement systems is inevitable, and particulate products of wear may be formed from the bearing surfaces of both metal and plastic components.

Polyethylene in the form of a concave bearing surface at the hip wears very slowly, and clinical and histological studies suggest that the small amount of wear products generated by polyethylene is tolerated well by the body (Toumbis *et al.*, 1997). Evidence for the release of metals from prostheses in man was obtained by Coleman, Herrington & Scales (1973). Nine patients with prostheses composed of cobalt-chromium articulating with cobalt chromium had elevated levels of cobalt and chromium in both blood and urine, three patients with prostheses composed of cobalt-chromium articulating against high molecular weight polyethylene, had undetectable levels of cobalt-chromium in either blood or urine.

Wear debris from the prosthesis may result in an impressive infiltrate of macrophages (Pandey *et al.*, 1996). Macrophages are the pivotal cell types involved in the healing of an implant in the tissues. These cells, upon interaction with any foreign material, might initiate a spectrum of responses which could lead to acute and chronic inflammatory changes affecting the biocompatibility of the implant (Bhardwaj *et al.*, 1997).

The tissue reaction after implantation passes through three phases: (1) an initial phase which lasts from the time of implantation to about three weeks after the operation: (2) a reparative phase beginning during the fourth week after operation and lasting for up to two years: and (3) a stabilisation phase which begins six months to two years after operation (Willert, 1973). In the initial phase, the outer surface of the cement has uniform contact with surrounding body tissues. Histological examination of the implant bed in the initial stage reveals a zone of dead bone and bone marrow extending up to 5mm from the cement surface. In the reparative phase the dead tissues are progressively invaded by living vascular granulation tissue bringing with it scavenger cells which may remove dead bone and bone marrow, and connective tissue cells which lay down fibrous tissue and new bone. The dead bone is removed by the activity of the osteoclasts. The active repair process proceeds until all, or almost all, of the dead cells have been replaced by living bone and living soft connective tissues. After this has been achieved, a stable state is eventually established between the implant and its bed. The implant may therefore be considered to be healed into place during the stabilisation phase which begins one or two years after the operation (Willert, 1973).

3.1.7 Choosing a suitable prosthesis

The selection and application of synthetic materials for surgical implants has been directly dependent upon biocompatibility of specific prosthetic devices (Lemons, 1996). The early rationale for ceramic biomaterials was based upon the chemical and biochemical “inertness” of the material compounds. The development of biomaterials is an ever evolving process (Peppas & Langer, 1994). Many biomaterials in clinical use today were not originally designed for use as biomaterials but were ‘off-the-shelf’ materials that clinicians found useful in solving a problem. Examples include Dacron which is used in vascular grafts and was derived from textiles and commercial grade polyurethanes, the basis of artificial hearts. But the use of these materials also has drawbacks, the dacron-based grafts may only be used if their diameter exceeds 6mm, or occlusion may occur because of biological reactions at the blood-material and tissue-materials interfaces. Blood-materials interactions can also lead to clot formation in the artificial heart which gives the possibility of a stroke or further complications (Peppas & Langer, 1994).

Problems may also occur during the manufacture of the product. An example of this is ultra-high molecular weight polyethylene (UHMWPE) which is variable in its manufactured state. Polyethylene has little biological and chemical reactivity and its high molecular weight imparts resistance to wear. But the manufacture of implants of this polymer is difficult. Many of the normal processes such as injection moulding break down the long-chain molecules of UHMWPE and make it more liable to wear. The polymer is available as a base powder, which could be used to mould implants but problems with the cost and actual difficulties of this process means that most of the manufacturers obtain their stock as sheets or bars from specialist companies such as Hoechst. The prosthetic components are then machined from this stock. Less than 1% of the current world production of UHMWPE is used for implants hence there is little implant-related commercial pressure on the polymer producers. Thus changes may be made in the polymer without the knowledge of the end user or the manufacturer of the prosthesis. The material is still UHMWPE, but minor changes in the catalyst or the fusion process at fabrication can lead to changes in the final product (Amis, 1996).

An effective approach for developing a clinically applicable biomaterial is to modify the surface of a material that already has excellent biofunctionality and bulk properties. The major surface properties that should be modified include two kinds of biocompatibility, one the surface property that elicits the least foreign-body reaction and two, cell- and tissue-bonding capabilities. Cell culture studies have often been used in the determination of the suitability of biomaterials as surfaces for the attachment and growth of cells (Steele *et al.*, 1994). Most bacteria have evolved elaborate mechanisms for adhering to solid surfaces hence no prosthetic biomaterial currently available is able to resist bacterial adherence and subsequent colonisation.

The number of total hip replacement systems available in the UK, and presumably the rest of the world, is rapidly increasing, but there is little or no scientific evidence that the newer, more expensive implants are better than the more established designs. After follow-up tests some years after implantation some will undoubtedly prove to be unsatisfactory and will result in considerable patient suffering and expense (Murray, Carr & Bulstrode, 1995). Some designs have good results initially at five years but may develop high failure rates by ten years (Malchau, Herberts & Ahnfelt, 1993 and Owen *et al.*, 1994). The published results suggest that for most implants the revision rate is about 10% at ten years (Ritter & Campbell, 1987; Roberts, Finlayson & Freeman, 1987; Wilson-MacDonald & Morscher, 1990 and Schulte *et al.*, 1993). The choice of which system to use is really up to the surgeon, the choice being based on good clinical grounds, bearing in mind how long the implant may have to function. The current practice of modifying an implant every few years, without changing its name should cease, and modified implants should be regarded as new designs.

Of the utmost importance for the successful use of an implant is a good adhesion of the surrounding tissue to the biomaterial (Eisenbarth *et al.*, 1996). Surface composition and topography influence the properties of the adherent cells. Although these products appear smooth macroscopically, at the ultra-structural level there are numerous irregularities that may serve as a nidus for bacterial adhesion (Karlan *et al.*, 1980 and Peters, Locci & Pulverer, 1982). A tiny scratch on the surface of the prosthesis, invisible to the naked eye is all that a bacterium requires to anchor itself to the surface and “hide” from the flushing action of blood and fluids as they flow over the area.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains

Staphylococcus epidermidis strains M7 and RP62A were prepared as described in the methods section of Chapter Two. The radionucleotide [8 $^{-3}$H] -Adenine (specific activity 197 mCi/ml, Amersham International Plc, Buckinghamshire, England) was incorporated during growth by the addition of 100µl of the radionucleotide to 20ml broth containing 1 cfu of either M7 or RP62A.

3.2.2 Preparation of the bacteria

The bacteria were harvested by centrifuging (1000 x g_{max} for 10min at 15°C, IEC Centra-4X Centrifuge, International Equipment Company, Dunstable, Bedfordshire, England.), washed three times with phosphate-buffered saline (PBS) (Appendix A) and resuspended in PBS to a final concentration of 1 x 10^7 cfu/ml (OD = 0.025 at 620nm) (CE 272 Linear Readout Ultraviolet Spectrophotometer, Cecil Instruments Limited, Cambridge, England).

3.2.3 Opsonisation of bacteria

Standardised suspensions of both bacterial strains were opsonised in 10% normal human serum for 15 minutes at 37°C before being centrifuged as above and resuspended in Hank's balanced salt solution containing 0.1% gelatin (gel-Hanks) (Appendix A).

3.2.4 Production of slime

The ability of the strains to produce slime was tested using by Alcian blue staining. Alcian blue is a polysaccharide-specific stain which will stain any extracellular slime material (one of the main components of which is polysaccharide). Plastic test tubes (110 x 17 mm, Nunc, Nunc A/S, Kastrupvei 90, Denmark) containing 18 and 24 hour cultures of *S. epidermidis* M7 and RP62A in Mueller-Hinton broth were emptied and filled with Alcian blue (0.25% w/v R.A. Lamb, Waxes and General Laboratory Supplies, North Acton, London) solution for 2-3 minutes to stain the extracellular material (slime) adherent to the plastic test tubes.

3.2.5 Measurement of bacterial adhesion to plastic

This is a modified method of that developed by Christensen *et al.,* (1985). Bacteria were grown overnight in Mueller-Hinton broth, washed three times in PBS and resuspended in PBS to a final concentration of 1 x 10^7 bacteria/ml (OD = 0.025 at 620nm). 100µl of each bacterial suspension was added to the wells of a 96-well microtitre plate. The bacteria were allowed to adhere for varying lengths of time (15, 30, 45, 60, 90, or 180 minutes). The contents of the wells were emptied and the plate washed twice with PBS and inverted to remove excess PBS. The remaining attached cells were fixed with Bouin's fixative (Appendix A) for 30 minutes. The wells were emptied and washed with PBS to remove any traces of fixative and 100µl crystal violet (0.5%w/v, Appendix A) added to the wells and the plate allowed to stand for 30 minutes. The plate was then rinsed with tap water and read in an ELISA plate reader (details) at 570 nm.

3.2.6 Measurement of Bacterial Cell Surface Hydrophobicity

Two methods were tested-

3.2.6.1 Interaction with Hydrocarbons (MATH).

This assay was based on the method described by Rosenberg, Gutnick & Rosenberg (1980). The bacterial cell surface hydrophobicity was determined by measuring the affinity of the bacteria for an immiscible liquid hydrocarbon-in this case hexadecane, mixed with an aqueous suspension of bacteria. On mixing, hydrophobic bacteria will adhere to the hexadecane, and the two immiscible phases separate. Bacteria hydrophilic in nature will remain in the aqueous phase. Washed cells (late exponential growth phase) were suspended in PBS to an absorbance (A) of 1.0 at 660nm. 0.86ml of the bacterial suspension was placed in a round-bottomed plastic test-tube. 0.14ml hexadecane (Sigma) was carefully added down the inner wall of the test-tube so that mixing did not occur and the hexadecane formed a layer floating on the top of the bacterial suspension (see figure XI). The tubes were vortex-mixed for two minutes, and, after phase-separation (15 minutes), the lower aqueous phase was then carefully removed to a clean test-tube using a fine-tipped pasteur-pippette. After withdrawing the pipette, the first few drops of the aqueous suspension were discarded to avoid contamination by hexadecane. Tubes were then vortex mixed for a further five seconds to resuspend any cells that had aggregated or settled out. The final absorbance (A) of the aqueous phase was determined at a wavelength of 660nm in the CE 272 spectrophotometer. The strains were measured in triplicate. The mean hydrophobicity values and the standard errors of the means were calculated using standard statistical formulae (See appendix B).

ΔA was calculated as follows-

$$\Delta A = \text{Initial } A - A \text{ after phase separation.}$$

A change in OD 660nm of:

0-9%	indicated a very hydrophilic surface
10-49%	showed moderate hydrophilicity
50-79%	showed moderate hydrophobicity
80-100%	denoted a very hydrophobic surface

3.2.6.2 Hydrophobic Interaction Chromatography (HIC)

Glass pasteur pipettes (Bilbate Ltd., Daventry, England) were plugged with cotton wool and washed with ethanol and a 75% solution of Seven Salts Solution (SSS) (appendix A). The column (diam 5mm) was then filled to a height of 50mm with a

Figure XI Microbial adhesion to hydrocarbons (MATH)

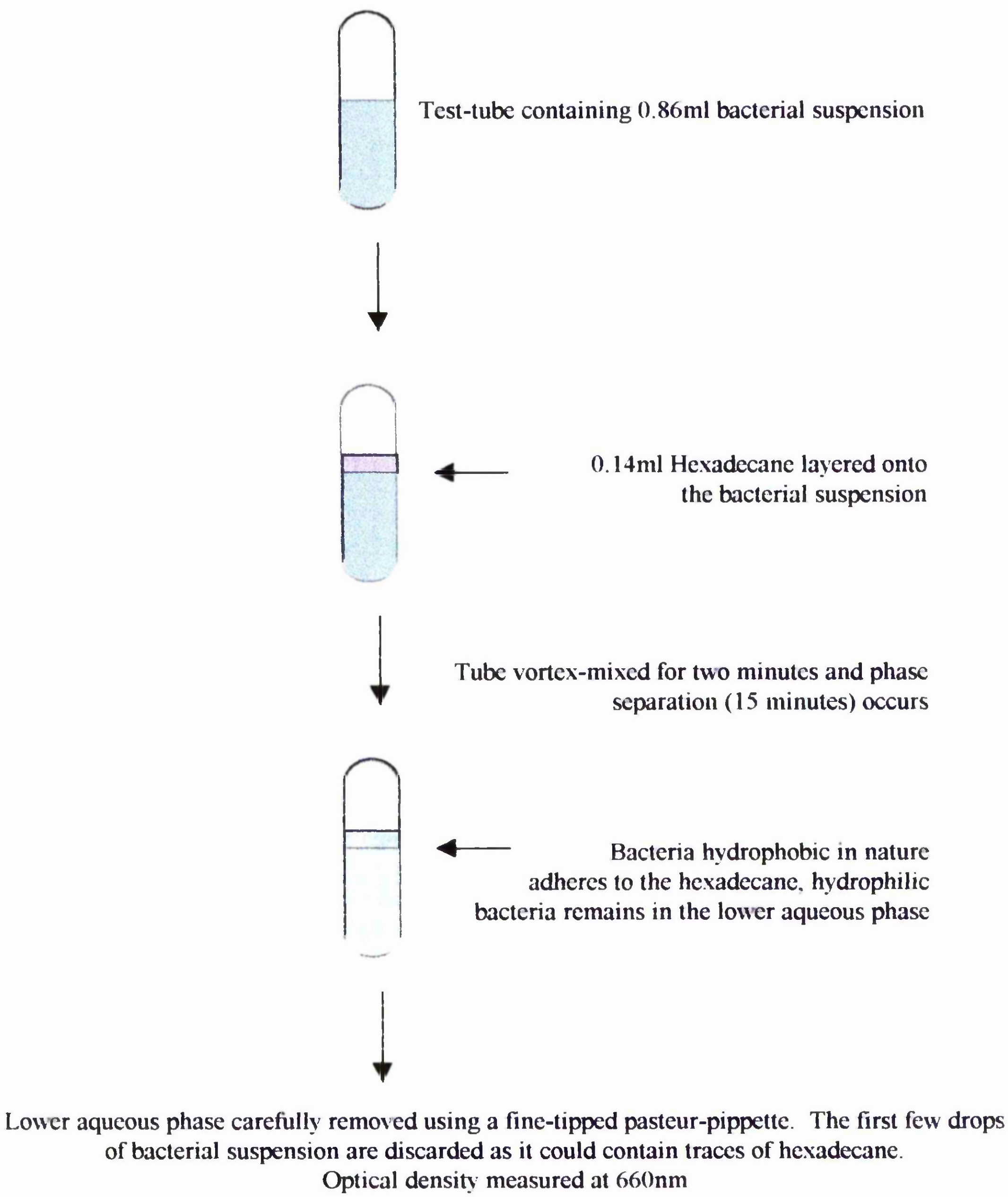
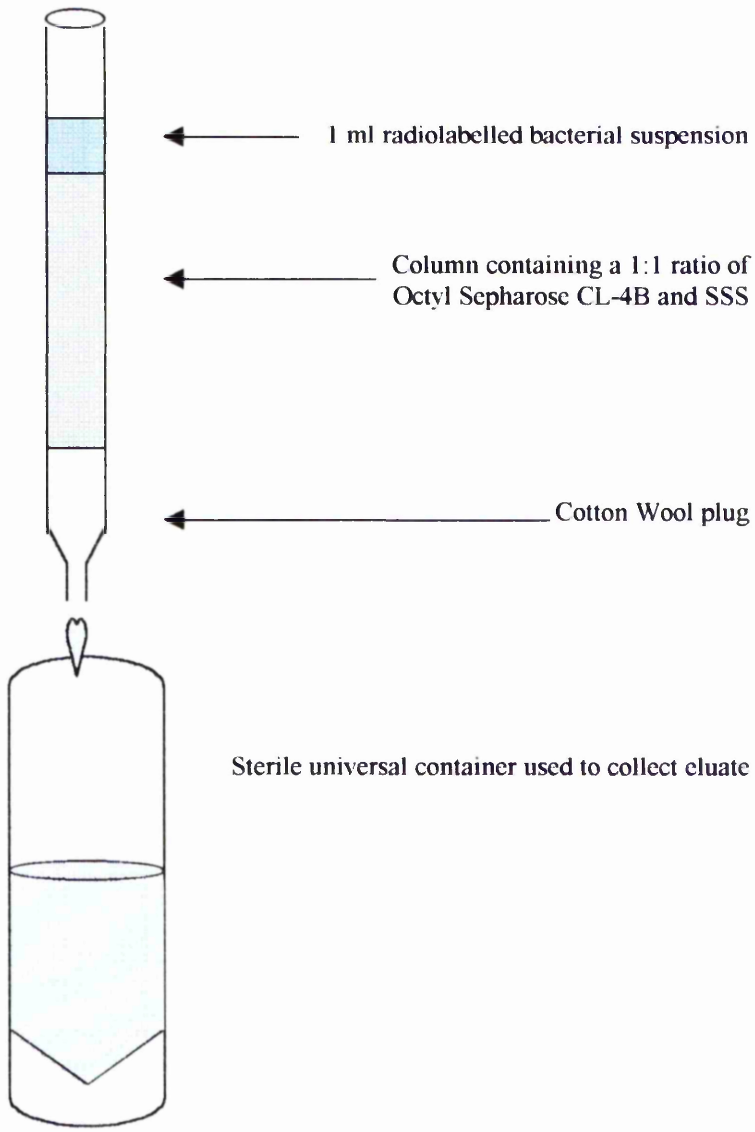


Figure XII Hydrophobic Interaction Chromatography (HIC)



1:1 ratio of Octyl Sepharose CL-4B (Pharmacia Biotech Ltd., St Albans, Herts) to 100% SSS. 1ml bacterial suspension radiolabelled with [8-^{3}H] Adenine (Amersham Ltd.) diluted in PBS to a concentration of 1 x 10^{8} cfu/ml (O.D = 0.115 at 620nm) was then added to the column (see figure XII). The column was then washed with 12ml 75% SSS. The affinity of the bacteria for hydrophobic Octyl Sepharose CL-4B gel is expressed as the ratio between the radioactivity of the gel fraction (g) and the corresponding eluate (g/e). Increasing g/e values indicate an increasing degree of bacterial surface hydrophobicity (Pedersen, 1980). Logarithmic values of (g/e) were interpreted as follows-

Log(g/e)	+0.51	-	+2.0	very hydrophobic
	+0.01	-	+0.5	moderately hydrophobic cell surface
	- 0.01	-	-0.5	moderately hydrophilic surface
	- 0.05	-	-0.2	very hydrophilic

3.2.6.3 Statistical analysis of the two methods

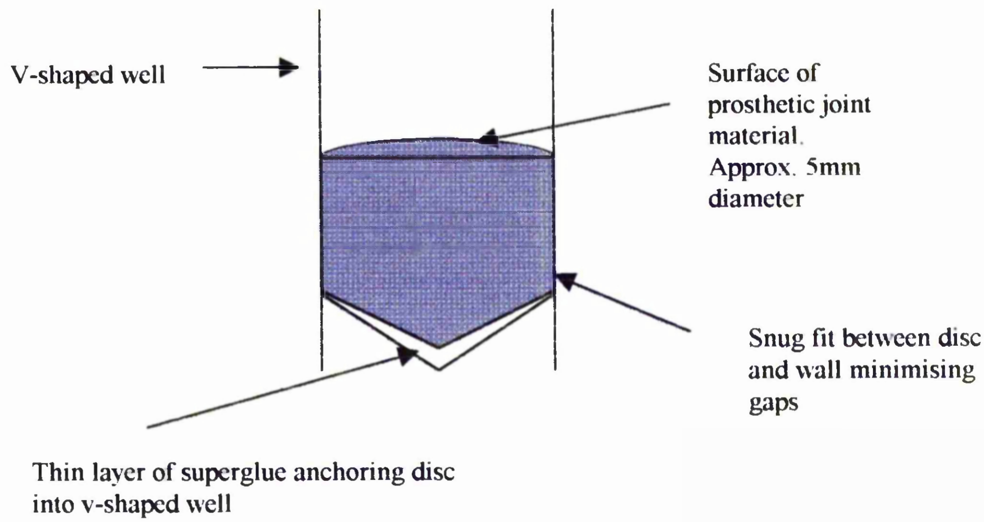
Correlation between the two methods was analysed using the rank correlation test (Wardlaw, 1985). An r-value of 1 indicates complete agreement between the methodologies whereas a value of –1 shows complete disagreement.

3.2.7 Substrate materials

3.2.7.1 Prosthetic joint material

The prosthetic joint material used in this study was Ultra High Molecular Weight Polyethylene (UHMWPE) obtained from 3M Healthcare Ltd., Rotherham, England in the form of mushroom-shaped discs approximately 5mm in diameter (see figure XIII) The discs were finished and polished to the standard equivalent to that used in the manufacture of hip replacement systems.

Figure XIII   Prosthetic joint material as used in the model



3.2.7.2 Polystyrene

Presterilised Sero-wel 96 v-well plates (Bibby Sterlin Ltd, Stone, Staffs, UK) were used as the source of polystyrene. They were individually wrapped and no further sterilising was required. All plates came from the same batch to prevent any manufacturer variation.

3.2.7.3 Bone

Bone specimens were obtained from patients undergoing either partial or total hip replacement at Glasgow Royal Infirmary. The bone was stored in alcohol (96% ethanol, Analar) at $4^{o}C$ for 24 hours to remove fat. The outer surface (2-3 mm depth) was carefully removed using a hand-held fret saw and the bone was punched into discs 5mm in diameter. The discs of bone were then stuck into the well of a 48-well microtitre plate (Falcon, Becton Dickinson Labware, New Jersey, USA) using superglue as before. Again individually wrapped, pre-sterilised plates from the same batch to prevent batch variation were used.

3.2.8 Isolation of peripheral blood cells

3.2.8.1 Isolation of polymorphonuclear leukocytes

PMNLs were isolated as described in the methods section of Chapter Two. The white cells were stored at $4^{0}C$ in gel-Hanks solution until use.

3.2.8.2 Isolation and purification of monocytes

Monocytes were isolated and purified as described in Chapter Two.

3.2.9 Cell Culture

A murine macrophage-like cell line J774 was obtained from the Department of Immunology, Western Infirmary, Glasgow (courtesy of Dr I. M^{c}Innes). The cells were grown and maintained in RPMI 1640 medium (Gibco BRL, Life Technologies Ltd, Paisley, Scotland) supplemented with 10 % fetal calf serum (FCS), 5mM glutamine, penicillin G (100IU/ml), and streptomycin (100μg/ml) (all from Gibco) at 37°C under 5% CO2. When the cells were at confluence the cell monolayer was detached by placing the cells on ice for five minutes and then using a cell scraper to remove the cells from the tissue culture flask surface. Cells were washed with RPMI 1640 and adjusted to 5×10^{5} cells/ml.

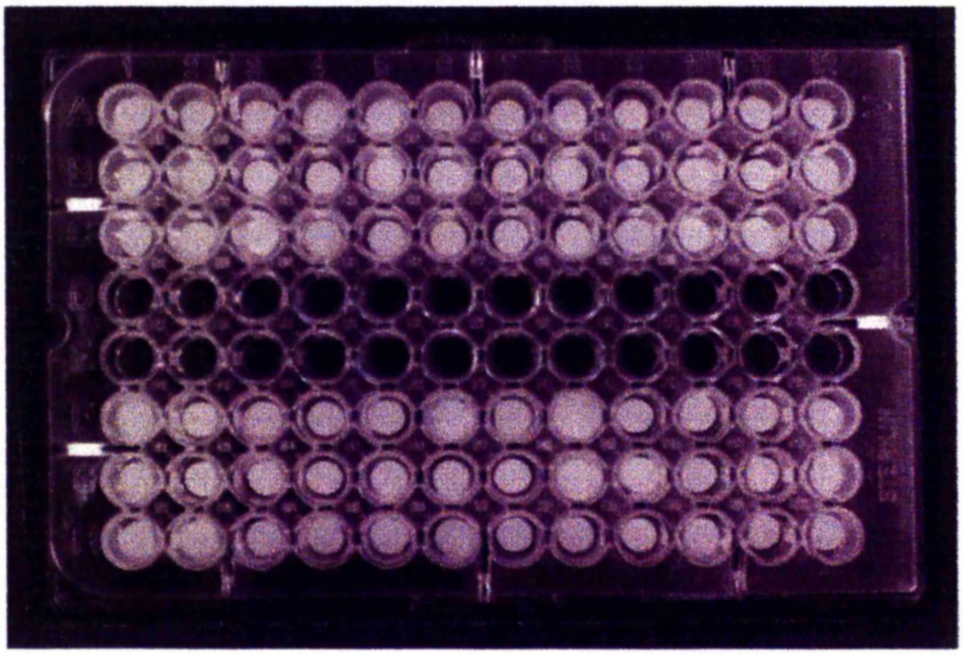
3.2.10 The Models Systems

3.2.10.1 The model for adherence to and phagocytosis from prosthetic joint material

Small discs (0.5cm in diameter) of PMMA were stuck into the wells of a 96-well microtitre plate (Falcon) using superglue. The plate was then sterilised by soaking overnight in a 10% solution of Decon-90 (Decon Laboratories Limited, England), rinsed in running water for 10 minutes to remove all traces of Decon-90 and then 96% ethanol (Analar) was added to the wells and allowed to evaporate off in a fume hood (see figure XIV)

100μl of bacterial suspension was then added to the wells. The bacteria were allowed to adhere for periods of 15, 30, 45, 60, 90 and 180 minutes. After this time period had elapsed the non-adherent bacteria were removed using a Pasteur pipette and the surface washed three times with saline (0.85% solution, Oxoid, Unipath Ltd, Basingstoke, Hampshire, England) and the percentage bacterial adherence measured by measuring the total radioactivity in the washings and subtracting this value from the initial level. Adherent bacteria on the surface of the prosthetic joint material were then opsonised by the addition of 10% NPS obtained from healthy

Figure XIV The Model System



Photograph shows 96-well microtitre plate with discs of prosthetic joint material stuck into each well volunteers for 15 minutes at 37°C. Excess serum was removed using a Pasteur pipette and the surface rinsed by gentle washing with Gel Hanks solution. Thereafter 5 x 10^5 PMNLs/ MN or J774 cells were then added to each well. They were allowed to act for 15, 30, 45, 60, 90 and 180 minutes at 37°C. After incubation, the PMNLs/ MN/ J774 cells containing ingested bacteria from the surface of the prosthetic joint were removed using trypsin/EDTA solution for 15 minutes. The wells were washed three times with saline, aspirating gently each time to remove all the bacteria and PMNLs from the well.

The contents of the wells were then spun down in a scintillation vial at 400 x g_{max} at 15 °C for 10 minutes (IEC Centra-4X, International Equipment Company, Dunstable, Bedfordshire, England) resulting in a pellet of cell-associated bacteria and a supernate of non-cell associated bacteria. The pellet was washed three times with saline, the supernate of non-cell associated bacteria being collected each time. The supernatants were all pooled together and spun down at 1000 x g_{max} for 15min at 15°C. 3ml scintillation fluid (Ecoscint A, National Diagnostics, Atlanta, Georgia USA) was then added to the vials. Radioactivity was measured using a Wallac 1409 Scintillation Counter (Wallac Oy, Turku 10, Finland).

The efficacy of phagocytosis (as a percentage) was calculated as follows-

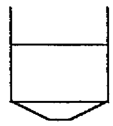
$$\%\ \text{Phagocytosis} = \frac{\text{Radioactivity of cell-associated bacteria}}{\text{Radioactivity of cell} \quad + \quad \text{non-cell associated bacteria}}$$

This procedure is summarised in figure XV.

3.2.10.2 Model for adherence to and phagocytosis from bone

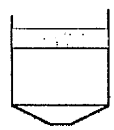
Bone was punched into discs 5mm in diameter using a hand-held punch. The discs of bone were then stuck into the wells of a 48-well plate (Falcon) using superglue as before. 300μl bacterial suspension was then added to the wells and the bacteria

Figure XV Development of model assay



100ul bacterial suspension added to the well

Bacteria allowed to adhere to the surface for 15-180 minutes



Bacterial suspension pipetted off and non-adherent bacteria removed by gentle washing 3 times with PBS.

Adherent bacteria opsonised by the addition of 100ul NPS. Incubated at 37°C for 15 minutes

NPS removed and 100ul PMNLs/MNs/J774 cells added to the wells

Adherent cells removed by trypsin/EDTA solution

Surface of prosthetic joint washed three times with saline to remove all cells containing bacteria

Well contents and washings pooled and spun down at 400 x g_{max} at 15°C for 15 minutes

Pellet of cell-associated bacteria (washed three times)

Washings containing non-cell associated bacteria pooled and stored

3ml scintillation fluid added to all vials and radioactivity measured using the Wallac 1409 Counter allowed to adhere for 180 minutes. Non-adherent bacteria were removed by gentle washing three times with PBS. The adherent bacteria were then opsonised by the addition of 300μl serum suspension. Excess serum was removed and 300μl PMNLs added to each well. PMNLs were removed from the surface using trypsin/EDTA solution and cell and non-cell associated bacteria collected and counted as described in section 3.2.10.1

3.2.11 The use of lysostaphin to prevent extracellular adhesion of bacteria to PMNLs.

Lysostaphin is a lytic enzyme produced by a strain of *Staphylococcus simulans* which attacks the pentaglycine bridge between peptidoglycan chains and is thus specific for staphylococci. Lysostaphin (10mg/l) was added to the phagocytic mixtures and allowed to act for 30 minutes to ensure that phagocytosis of the bacteria had taken place and was not due to mere attachment of the bacteria to the PMN. The lysostaphin would lyse all extracellular bacteria.

3.3 RESULTS

3.3.1 Production of slime by the bacteria

3.3.1.1 Qualitative test tube assay

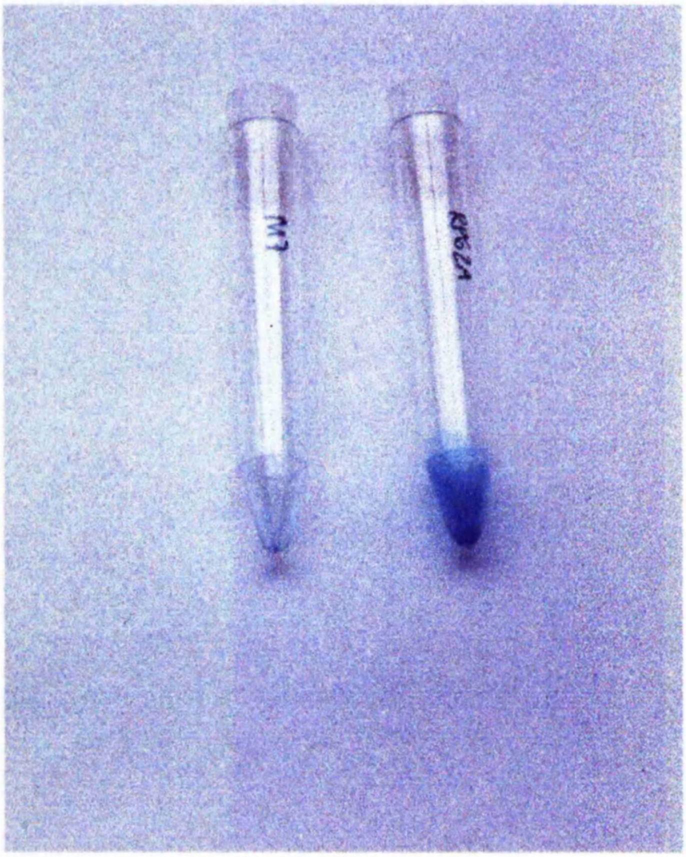
Slime production was measured according to the method of Christensen *et al.*, (1982). Strain RP62A stained to a much greater extent with Alcian blue than did strain M7 indicating the presence of slime. This is shown in figure XVI overleaf. Results were read as strong adherence (+ + + or + +), weak adherence (+), or negative (equivocal or no adherence), according to the density of the adherent film.

Strain RP62A was scored as + + + due to its highly dense presence of slime on the walls of the test tube. Strain M7 was scored as negative (-). There was no visible difference in the amount of slime material produced after 24 hours compared to 18 hours.

3.3.1.2 Estimation of bacterial adherence to polystyrene by spectroscopic analysis of crystal violet staining

Cell suspensions of *S. epidermidis* M7 and RP62A were allowed to adhere to the surface of a 96-well polystyrene plate for varying lengths of time between 15 and 180 minutes. After this time period had elapsed the adherent bacteria were stained with crystal violet. In order to obtain a more exact interpretation of the density of adherence the optical density of the wells were measured spectrophotometrically in an ELISA plate reader. The results are shown in table 11 overleaf

Figure XVI   Slime production as determined by staining with alcian blue



Photograph shows polystyrene test tubes stained with alcian blue stain after incubation with either *S. epidermidis* M7 or RP62A in Mueller-hinton broth overnight at 37°C. Slime production by strain RP62A is observed by blue staining on the walls of the test-tube. The absence of staining in the test-tube containing strain M7 shows a lack of slime production

Table 11 Optical density readings of *S.epidermidis* M7 and RP62A adherent to polystyrene after staining with crystal violet

INCUBATION TIME (minutes)	OPTICAL DENSITY READING 570nm				P value
	M7	(SD)	RP62A	(SD)	
15	0.106	0.013	0.099	0.015	0.34
30	0.123	0.038	0.132	0.045	0.95
45	0.128	0.019	0.177	0.069	0.04
60	0.128	0.026	0.177	0.019	0.00
90	0.141	0.021	0.217	0.065	0.00
180	0.219	0.097	0.255	0.084	0.26

The optical density corresponds to the thickness of the bacterial film and serves as an index of slime formation. The results above show an increase in optical density with increasing incubation time. The values obtained for strain RP62A are slightly higher than those obtained for strain M7 which suggests that RP62A adheres to a greater extent. Only after 45 minutes incubation is there a significant difference in the absorbance values measured for M7 compared to RP62A. At 180 minutes however, the difference is not significant which suggests that at this stage M7 has accumulated on the polystyrene surface to the same extent as RP62A

3.3.2 Determination of bacterial hydrophobicity.

3.3.2.1 MATH-Hexadecane adherence assay

To determine the hydrophobicity of the two strains, the percentage reduction in optical density of the test suspensions as compared with the control suspensions was calculated. These percentage values are proportional to the hydrophobicity of the test bacteria in any given suspending medium (Rosenberg, Gutnick & Rosenberg, 1980). The results are listed in table 12.

Table 12 Measurement of bacterial cell hydrophobicity by MATH

Bacterial strain	(%) Reduction in absorbance of aqueous phase after mixing with hexadecane Experiment number			Mean	Outcome
	1	2	3		
M7	41	39	40	39.9	Moderately hydrophilic
RP62A	40	36	41	39.0	Moderately hydrophilic

Taking the scoring system as described in the methods section, M7 having a reduction in absorbance value of 39.9% and RP62A with a similar value of 39 % are both determined to be moderately hydrophilic in nature. The set of results shown above are typical of the results obtained. A total of 15 sets of experiments were performed with the standard deviations of 39.9 +/- 3.3 and 39 +/- 2.8 obtained for M7 and RP62A respectively.

3.3.2.2 Measurement of bacterial cell hydrophobicity by Hydrophobic Interaction Chromatography.

The surface hydrophobicity of *S. epidermidis* M7 and RP62A was estimated by measuring the adsorption of each bacterium to octyl-sepharose. This is estimated by counting the number of radioactive bacteria which do not adhere to the octyl-sepharose column and pass through into the elute. The amount of radioactivity in the gel column (which is indicative of adherent bacteria) is also measured. The results are shown in Table 13 overleaf.

Table 13 Radioactive evaluation of cell surface hydrophobicity

Strain	Radioactivity associated with the gel fraction g	Radioactivity associated with the eluate e	% of radioactivity in gel fraction g/e	Log g/e	Outcome phobic / philic
M7	247964.8	414350	0.6	-0.22	philic
RP62A	667790.7	776253.1	0.86	-0.07	philic

Results are expressed as the mean count (H^3 counts per minute) where n = 22. The percentage of radioactive bacteria in the hydrophobic gel fraction of the column represents the degree of hydrophobicity of the bacterial strain. As both strains retained a small percentage of radioactivity in the gel fraction they are deemed moderately hydrophilic in their nature after calculation of their log g/e values fell in the range from –0.01 to -0.5.

3.3.2.3 Comparison of the two methods using Spearman's rank correlation test.

A correlation coefficient value (r) of 0.04 and 0.05 was obtained for strains M7 and RP62A respectively, showing that both tests did correlate. This is to be expected as the chemical compositions of both octyl sepharose and hexadecane are similar (van der Mei, Weerkamp & Busscher, 1987).

3.3.3 Bacterial adherence to the substrate

3.3.3.1 Determination of the optimal incubation time to allow maximal adherence of the bacteria to the substrate.

Optimal incubation times for adherence of bacteria to different substrates differ due to variations in the nature of the substrates being investigated. In this study,

Figure XVII Determination of the optimal incubation time to allow maximal adherence of *S. epidermidis* M7 to prosthetic joint materia

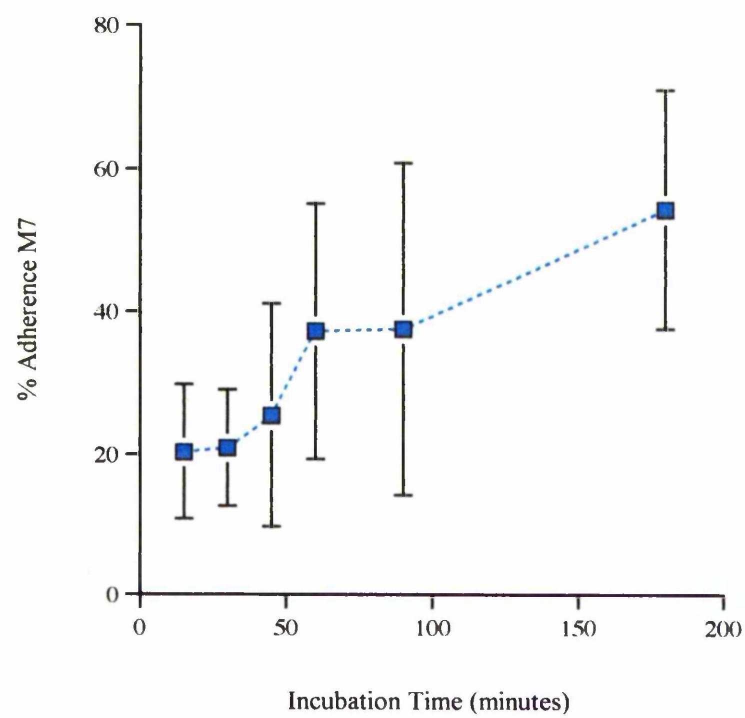
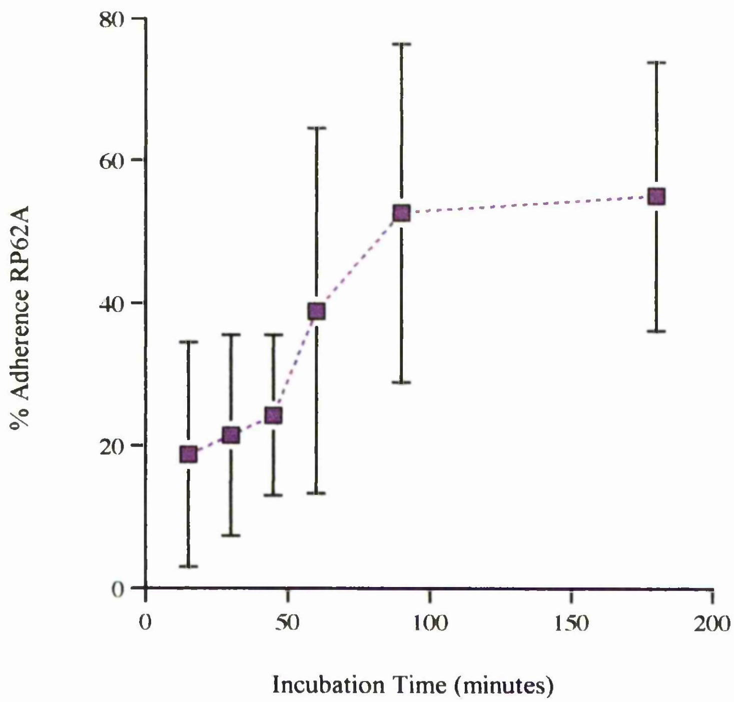


Figure XVIII Determination of the optimal incubation time to allow maximal adherence of *S. epidermidis* RP62A to prosthetic joint material

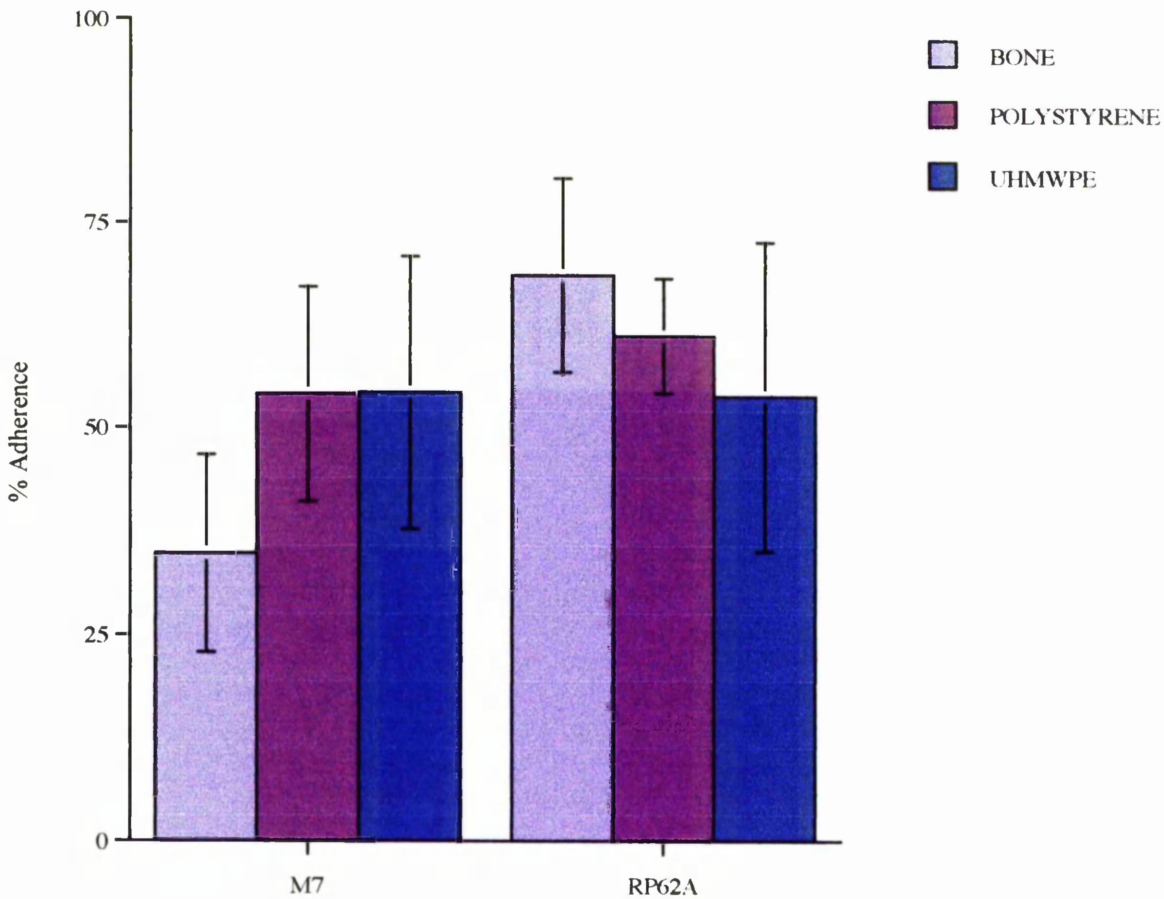


adherence to bone, prosthetic joint material and polystyrene was compared. The initial experiments to determine how easily the two bacterial strains would adhere was performed on prosthetic joint material (UHMWPE) [bone specimens were very limited in number]. Incubation times ranged from 15 to 180 minutes. The bacteria were in suspension (in PBS) and hence had no nutrient to sustain them or allow them to multiply, so an upper time limit of 180 minutes incubation was chosen for maximum bacterial cell viability and also to allow the experiments to be completed within the normal working day. As the results in figures XVII and XVIII show, maximal adherence was obtained after 180 minutes incubation at 37°C for both strains.

3.3.3.2 Comparison of adherence of bacteria to polystyrene, UHMWPE and bone.

Now that 180 minutes was determined as the optimal incubation time to allow maximal adherence to prosthetic joint material, adherence to different substrates was investigated. The ability of strains M7 and RP62A to adhere to the prosthetic joint material (in this case UHMWPE) was compared with polystyrene and bone. The graph overleaf (figure XIX) shows the percentage adherence calculated after 180 minutes at 37°C. The limited number of bone specimens available meant that the optimal incubation time to allow adherence to bone could not be determined but the optimal time from polystyrene and UHMWPE was used. A similar percentage adherence was calculated for all of the substrates for strain RP62A. The only substrate where adherence levels were low was bone with strain M7. There was a highly significant difference observed between the two strains ($p \leq 0.002$) for adhesion to bone but no significant difference was observed for the other substrates tested.

Figure XIX Comparison of adherence of *S. epidermidis* M7 and RP62A to polystyrene, UHMWPE and bone



3.3.4 Uptake of adherent bacteria

3.3.4.1 Determination of the optimal incubation time for ingestion of the bacteria.

As with the determination of the optimal incubation time for adherence, the optimal incubation time for ingestion of the bacteria by PMNLs was determined. PMNLs were used to determine the optimal incubation time as they were more readily available and it was easier to obtain a pure yield of PMNLs using the separation procedure described in the methods section.

Figures XX and XXI show the results obtained for incubation times ranging from 15 to 180 minutes as before. The upper time limit of 180 minutes was chosen as it was the maximum possible time for polymorph viability. Viability fell after 180 minutes from 95 % to 70 % as determined by exclusion of trypan blue dye. The ingestion rate remained fairly high over the time course investigated but maximum ingestion occurred at 45 minutes. After 45 minutes the percentage ingestion fell suggesting that the PMNLs were beginning to die, perhaps due to a lack of nutrients. PMNLs have their greatest activity early on when they have just been isolated from peripheral blood, which slowly decreases with time. The incubation time of 90 minutes was chosen as it allowed for any slower acting PMNLs to engulf the bacteria. Ingestion of both strains of bacteria was high suggesting, there is an absence of any protection of RP62A from the phagocytes, this may be that the bacteria has only adhered to the substrate and had not started to accumulate and produce slime.

3.3.4.2 Comparison of phagocytosis by PMNLs/ PBMN's and J774 cells

Now that the optimal incubation time for phagocytosis had been determined, it was decided to investigate the phagocytic efficacy of different cell types. Polymorphonuclear leukocytes were compared with peripheral blood monocytes and J774 cells (the methods of obtaining these cells are described in section 3.2.8). The graph in figure XXII overleaf shows the results obtained for each cell type and

Figure XX **Effect of incubation time on ingestion of *S. epidermidis* M7 by PMNLs**

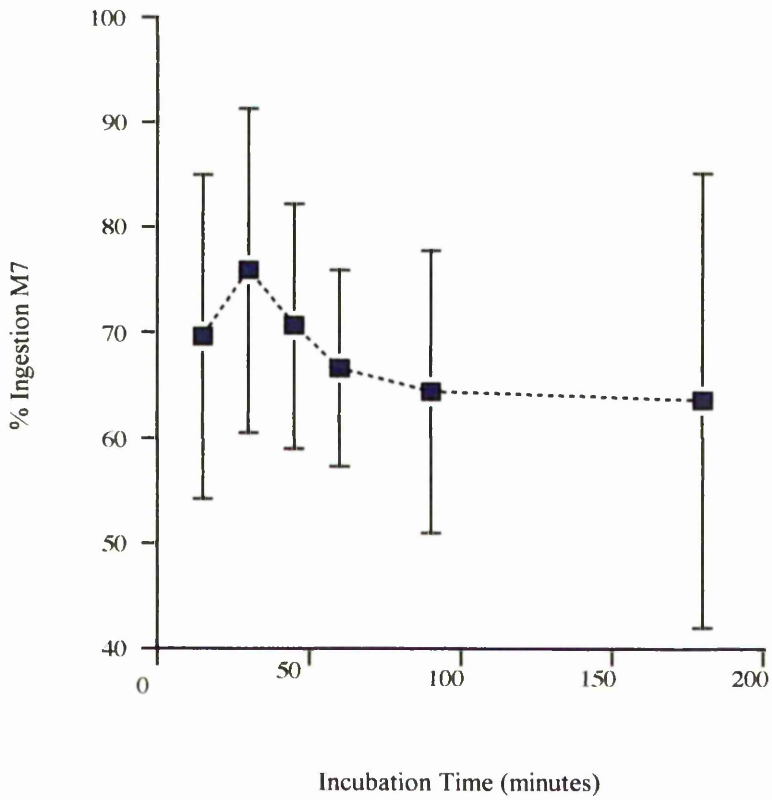


Figure XXI Effect of incubation time on uptake of *S. epidermidis* RP62A by PMNLs

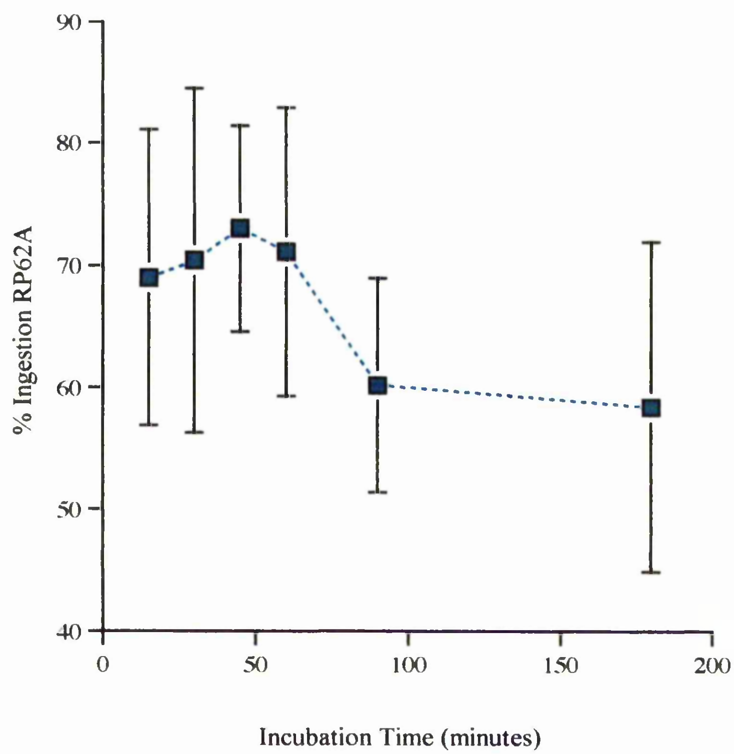
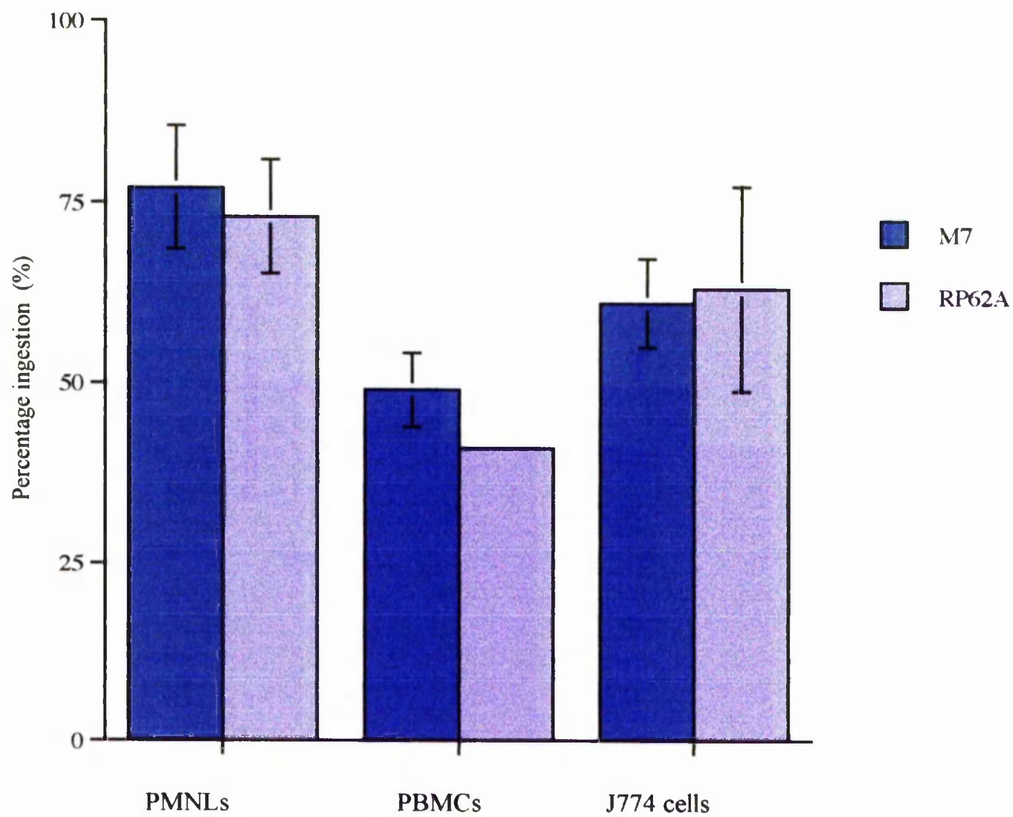


Figure XXII Comparison of ingestion of *S. epidermidis* M7 and RP62A by different cell types



bacterial strain. As can be seen uptake was greatest for PMNLs and lowest for MNs with J774 cells falling between the two. There was no significant difference between the two strains of bacteria in any of the cell populations tested. However, when each strain was compared by the different cell types significant differences became apparent in certain cell combinations. These are shown in table 14 below.

Table 14 Significant differences observed for the uptake of strains M7 and RP62A by different cell populations

Bacterial strain	% Uptake			P Value	Significance
	PMNLs	MNs	J774		
M7	67.9	49	***	0.020	S
M7	67.9	***	61.72	0.35	NS
M7	***	49	61.72	0.023	S
RP62A	64.8	41.07	***	0.018	S
RP62A	64.8	***	66.2	0.81	NS
RP62A	***	41.07	66.2	0.000	HS

S= significant ($p < 0.05$), NS= not significant ($p > 0.05$), HS= highly significant ($p < 0.01$)

3.3.4.3 Comparison of phagocytic uptake by PMNLs with and without the use of exogenous lysostaphin

The figures in table 15 show the results obtained when lysostaphin was added to the reaction mixture to kill any extracellular bacteria that may have adhered to the surface of the neutrophil resulting in an incorrect value for phagocytic ingestion.

Table 15 The effect of lysostaphin of phagocytic uptake of bacteria

STRAIN	% Uptake + LYSOSTAPHIN	% Uptake - LYSOSTAPHIN	P value
M7	49.75	48.8	0.98
RP62A	54.4	52.3	0.94

As can be seen from the results, the use of lysostaphin did not change the uptake of the bacteria significantly. It was decided to discontinue the lysostaphin step in the assay.

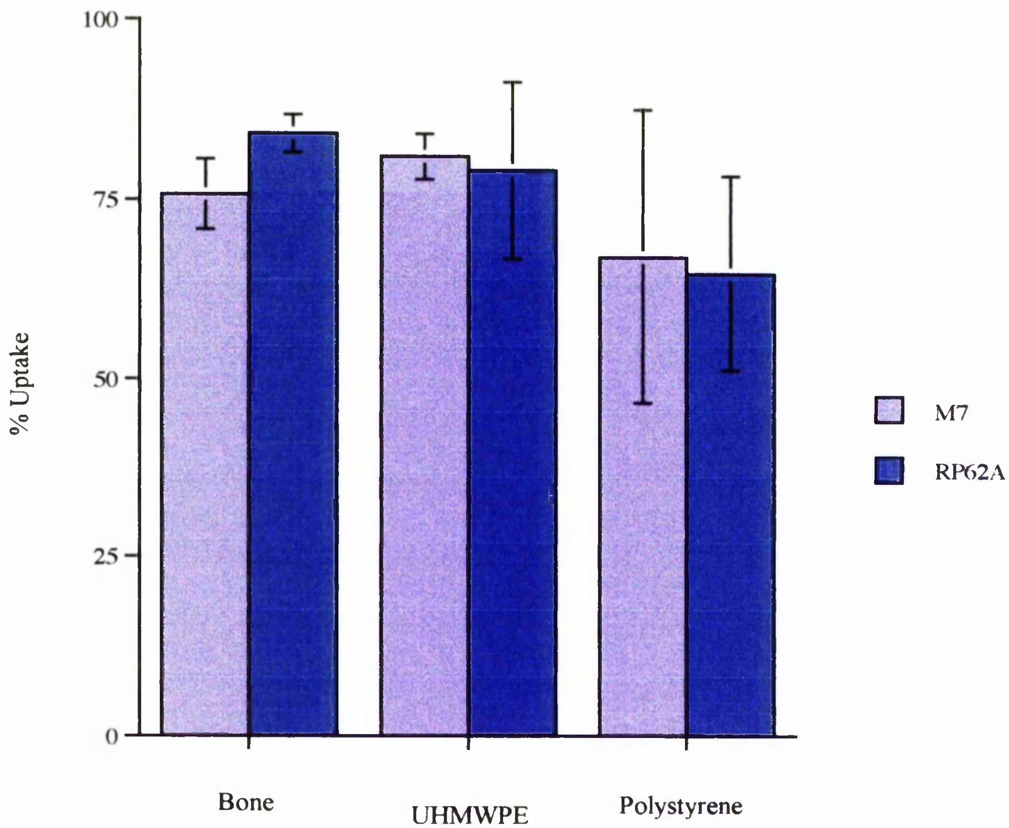
3.3.4 Comparison of phagocytosis from different substrates

Phagocytosis of *S. epidermidis* M7 and RP62A adherent to three different substrates was investigated. Substrates were polyethylene, UHMWPE and bone. The results are shown summarised in table 16 and graphed in figure XXIII.

Table 16 Significant differences observed for the uptake of strains M7 and RP62A from different substrates

Bacterial strain	% Uptake			P Value	Significance
	Bone	UHMWPE	Polystyrene		
M7	75.7 (4.86)	80.9 (3.16)	***	0.20	NS
M7	75.7 (4.86)	***	67 (20.4)	0.33	NS
M7	***	80.9 (3.16)	67 (20.4)	0.13	NS
RP62A	84.1 (2.65)	79 (12.2)	***	0.42	NS
RP62A	84.1 (2.65)	***	64.7 (13.5)	0.082	S
RP62A	***	79 (12.2)	64.7 (13.5)	0.0049	HS

Figure XXIII Comparison of ingestion of *S. epidermidis* M7 and RP62A from different substrates



3.4 DISCUSSION

In this study, bacterial adhesion to prosthetic joint material and its phagocytosis are investigated. The insertion of a prosthetic device results in many opportunities for the prosthesis to be contaminated by bacteria from the surrounding skin area. Although skin is pre-operatively sterilised by swabbing the area with antiseptic, it may still harbour commensal skin organisms such as *Staphylococcus aureus* and *epidermidis* and *Streptococcus viridans* and *Streptococcus pneumoniae* which may utilise the opportunity to become pathogenic.

3.4.1 Bacterial adhesion

Bacteria can be washed away by body fluids such as blood and urine hence adhesion to the substrate of interest is an essential step in the development of infection (Perrone *et al.*, 1997). As discussed previously, adhesion is a two-step process, and once initial attachment has occurred, many bacteria strengthen this attachment by producing an extracellular capsule or slime layer. Slime production has been defined as "the elaboration of a tenacious bacterial film on the walls of a culture vessel when a small inoculum of bacteria is allowed to reach stationary growth phase in a supportive medium" (Christensen *et al.,* 1990). Slime production by coagulase-negative staphylococci may be determined by either qualitative or quantitative tests. The qualitative test-tube assay described by Christensen *et al.,* (1982) depends on the visual assessment of the degree of adherence of staphylococci to the sides of glass or polystyrene test tubes. Although simple to perform and suitable for routine diagnostic use, the tube assay is subjective and not highly reproducible (Christensen *et al.,* 1985 and Deighton *et al.,* 1988). The quantitative test (Christensen *et al.,* 1985), where the adherent bacteria have attached and produced slime which is then stained, gives an objective measurement of the degree of adherence by the amount of slime produced as indicated by the optical density reading.

Both methods were investigated in this chapter. According to the test-tube method of Christensen *et al.*, strain RP62A is denoted as a slime producer due its heavy staining by Alcian blue (+++) whereas with M7, the mutant strain, the staining with alcian blue was negligible and M7 is thus denoted as a non-producer (-). In the spectroscopic analysis, the absorbance value obtained increased with increasing incubation time with maximum absorbance results after 180 minutes incubation. The optical density values obtained for strain RP62A were greater than those measured for M7 at all time points investigated although the difference in the optical density readings only became significant after 45 minutes incubation which suggest that it in quite early on in the initial stages of attachment that differences are observed between these bacterial strains. Because individual observers may disagree in their interpretation of test tube results, quantitative, spectroscopic analysis is more readily comparable when reading results.

3.4.2 Bacterial Hydrophobicity

While there are a number of methods available to characterise cells surface hydrophobicity (CSH) and cells surface charge (CSC) of microorganisms, there is still debate concerning the correlation of results between individual methods (Jones *et al.*, 1996). In this study the techniques of microbial adherence to hydrocarbons (MATH) and hydrophobic interaction chromatography (HIC) were used to measure the cell surface hydrophobicity of *S. epidermidis* M7 and RP62A. These methods have been shown to be compatible. In a study of isolates from *S. epidermidis* derived from microbial biofilm by Jones *et al.,* in 1996, good correlation of cell surface hydrophobicity measurement was observed (r = 0.89).

Hydrophobic Interaction Chromatography was first applied to the study of bacterial hydrophobicity by Stjernstorm *et al.,* in 1977. Subsequently, this method has come under investigation and a number of inaccuracies have been found. Of greatest importance was that the majority of the bacteria that had been retained by the sepharose gel were subsequently released if the gel was removed from the column and gently washed (Olsson & Westergren, 1982, and Wilson, Edgar & Leach, 1984). The number of cells retained on the column after several washes is considered indicative of the hydrophobic level of the bacterial cell population. This may lead to false low levels of bacteria thus determining the particular strain hydrophilic in nature. At the other end of the scale, the sepharose gel may mechanically trap the bacterial cell, preventing it flowing through the gel column although it has not bound to the beads leading to false high cell surface hydrophobicity levels.

The other method used (MATH) also has discrepancies. Problems have arisen with variations in the cell cycle in that bacteria give different results depending upon what stage they are in their cycle. Studies by Beck *et al.,* (1988) showed that stationary-phase cells expressed lower cell surface hydrophobicity determined by binding to hexadecane. Comparison of binding of a great variety of *S. aureus* isolates to various plastic polymers showed that exponential- phase cells of hydrophobic strains of *S. aureus* bound most efficiently to a number of plastics compared with encapsulated strains (Ludwicka *et al*., 1984). It seems very likely that carbohydrate surface polymers, such as capsular substances and slime materials are expressed under certain growth conditions and cause a decline in cell surface hydrophobicity.

The original two phase partition assay (also known as the hydrocarbon method) by Rosenberg, Gutnick & Rosenberg, (1980) used three hydrocarbons namely, hexadecane, octane and xylene. Nesbitt, Doyle & Taylor, (1982) used hexadecane and toluene. The majority of studies report the use of hexadecane. van Haecke & Pijck, (1988) have shown that hexadecane does not damage the intactness of microbial cells during the assay, similar results were obtained with octane. Xylene, on the other hand, resulted in cell lysis and it was for this reason that it's use as a test hydrocarbon was avoided. An important drawback of this assay is that steady-state conditions are practically unattainable. Experimental variables such as the suspending medium used, the choice of hydrocarbon, vortex mixing times, incubation temperature, growth rate, growth medium and the age of the culture can all affect the results (Rosan *et al.,* 1982). Repeated subcultures may cause a decrease in cell surface hydrophobicity after a few passages on blood agar (Wadström, 1990). This may explain some of the differences reported between studies. The main advantage with this assay is that it is easy to perform and requires no specialist equipment. Since it is a rapid test, there is little chance for the microorganisms to undergo cell surface changes as a result of ongoing metabolism or prolonged incubation. Secondly, the adherent cells rise rather than sediment following the assay, so there is little chance of confusing settling or clumping of cells with adhesion (Rosenberg & Doyle, 1990). Similarly, no washing steps (which can result in desorption or translocation of the cells, particularly if air: substratum interfaces are generated during the washing procedures (van Pelt *et al.,* 1985) are necessary.

The two methods employed in this study were in agreement that both strains of *S. epidermidis* were moderately hydrophilic in nature. Reports on the hydrophobicity of the coagulase negative staphylococci as a group are contradictory (Hogt *et al.*, 1986 & Pascual *et al.,* 1986). Heinzelmann *et al.,* (1997) found strain RP62A to be less hydrophobic in nature than it's non slime producing variant as determined by hydrophobic interaction chromatography. They correlated this decrease in hydrophobicity with a hydrophilic slime coat. Likewise, Baldassarri *et al.,* 1997 found the slime-negative strains of *S. epidermidis* to have greater surface hydrophobicity than their parent strains. Using the salt aggregation test (SAT) Mamo *et al.,* 1987 found ten strains of *S. epidermidis* to be hydrophilic. The differences observed are mainly due to the type of test employed.

It is not surprising that hydrophobicity tests sometimes fail to correlate. Certain types of bacteria cannot be expected to give the same results in different tests. For example a bacterium with a highly hydrophobic outer surface layer except for an adhesive hydrophobic tip or fimbriae might be hydrophobic as measured by MATH or HIC methods, but hydrophilic when measured using CAM or TPP methods. Similarly, bacteria that tend to autoaggregate may give disproportionately high hydrophobicity values in SAT compared with other techniques. External factors such as antibiotics are also known to affect hydrophobicity. Vancomycin and ciprofloxacin are known to affect the surface properties of *S. epidermidis*. Bacteria often grow at sub-inhibitory concentrations during antibiotic treatment. This may lead to altered adhesive cell surface properties and to disruption of the indigenous microflora, in addition to the creation of a more pathogenic biofilm (Cuperus *et al.*, 1995).

3.4.3 Hydrophobicity, slime production and adherence

Adherence has been associated with hydrophobicity (Rosenberg *et al.,* 1983) but there is also evidence to the contrary (Rosan *et al.,* 1988). Further work by Galliani *et al* in 1994, hypothesised that slime production in *S. epidermidis* was not correlated with initial adhesion regardless of the method used to measure adhesion (qualitative or quantitative). Hydrophobicity, on the other hand was found to correlate well with adhesion. Thus, the relationship between hydrophobicity, slime production and adherence has come under considerable debate.

Slime production, adhesion and hydrophobicity is highly strain dependent (Galliani *et al*., 1994). Most of the reports have obtained their data using dissimilar buffers to measure hydrophobicity and adherence (Rosenberg, 1981 and Gibbons & Etherden, 1983). Both adherence (Yamazaki *et al.,* 1981, and Eifert, Rosan & Golub, 1984) and hydrophobicity (Rogers, Pilowski & Zilm, 1984) have been shown to be affected by the composition of the buffer used to suspend the test bacteria which may affect the associations suggested. For bacteria to demonstrate a high affinity to a particular surface, specific interactions between the bacterial cell surface and the substrate occur. This may be enhanced by, but does not require hydrophobic structures on the bacterial cell surface. Early adhesion interactions are thought to depend mainly on hydrophobic interactions, slime production is thought to interpose secondarily, after the initial attachment (Galliani *et al*., 1994). Hydrophobicity assays may therefore be used to identify isolates that are most likely to adhere well to surfaces.

3.4.4 Bacterial adherence to plastic

A number of investigators have used a radiolabelling technique to assay bacterial adherence to plastic (John *et al*., 1995; Gorman *et al*., 1997; Schloricke *et al*., 1997; Bryden *et al*., 1996 and Bos *et al*., 1996). Studies of adhesion using radiolabelled bacteria allow a more accurate quantification of bacterial adhesion to biomaterials.

The graphs in figures XVII and XVIII show that adherence is time dependent. The time scale under which slime production takes place is very important. As discussed earlier, coagulase-negative staphylococci adhere to surfaces in two phases by different mechanisms. Initially there is rapid attachment which is then followed by a second stage of accumulation. Christensen *et al.*, 1985 reported that the initial stage of attachment occurred maximally after two hours and the second accumulation stage reached a maximum after six hours incubation. Adhesion of *S. epidermidis* to polyurethane catheters reached a maximum after two hours incubation (John *et al.*, 1995). Ludwika *et al.*, 1984 used a bioluminescent technique and found that adherence of *S. epidermidis* to polyethylene occurred within a few minutes with a maximal plateau being reached within an hour.

Hence it is important in the design of studies involving bacterial adhesion to decide whether attachment or accumulation is the end point. As expected, there was no difference between the two strains in their ability to adhere to the prosthetic material. This is due to both strains being adherence positive. After three hours incubation at 37°C both strains exhibited ~50% adherence. Production of slime by RP62A did not in any way enhance adherence to either polystyrene or UHMWPE. A longer incubation time to allow the bacteria to accumulate was not possible without supplementing the bacteria with nutrient broth which would have resulted in an unknown bacterial concentration in the wells which may have differed from well to well. Both strains adhered well to all the substrates tested with the polystyrene and the prosthetic joint material being colonised by both strains to almost the same extent. Only with bone was there a difference in the adhesion of the two strains. RP62A adhered almost twice as much as M7-the amount of adherence being similar to the values obtained for polystyrene and prosthetic joint material. The surface of the bone was much more irregular in its appearance and it is thought that this would enhance the adherence of an organism as it is easier for a bacterium to attach to a rough surface rather than a smooth one.

Surface treatment of biomaterials is becoming an increasingly popular method of improving device function and biocompatibility without the expense and time required developing new materials. Surface sensitive methods such as X-ray photoelectron spectroscopy and infrared spectroscopy as well as surface energy measurements are useful for surface characterisation. Ion- beam-based processes such as implantation and ion-beam-assisted deposition (IBAD) have proven particularly successful in this area because they offer a wide array of beneficial bulk surface property modifications without adversely affecting bulk properties (Sioshansi & Tobin, 1996). For instance orthopaedic implants are made harder and more wear resistant by ion-implanting their articulating surfaces. Many other devices such as venous catheters are treated to improve friction fretting resistance and biocompatibility. To determine the bioresponse to the polymer surface, enzyme-linked immunosorbent assays, cell growth and full blood tests may be used (Hocker & Klee, 1996). The final solution for a biomaterial is a material, which in the course of degradation constantly presents a new biocompatible surface.

3.4.5 Phagocytic uptake of the bacteria

Polymorphonuclear leukocytes and monocytes play a fundamentally important role in the host defence against bacterial infection (Peterson *et al.,* 1977). Patients with defects of these phagocytic cells suffer recurrent and severe infection with a wide variety of microorganisms (Baehner & Johnston, 1972; Cline, 1973; Quie, 1973 and Stossell, 1974). In an early study by Peterson *et al.*, 1977 where ingestion of *S. aureus* Cowan 1, *E. coli* ON2 and *L. monocytogenes* by polymorphonuclear leukocytes and monocytes were compared, significantly fewer bacteria were found to be associated with the monocytes compared to the phagocytes. After 15 minutes incubation the monocytes had taken up a mean of 40%, 47% and 52% fewer of *S. aureus* Cowan 1, *E. coli* ON2 and *L. monocytogenes* respectively than had the polymorphonuclear leukocytes. In order to efficiently phagocytose a substance, the phagocytic cell must first locate the substance to be ingested. Once located (in response to the various chemotactic factors generated by the invading bacteria), the neutrophils engulf the invading organism by extending a pseudopodium around the bacterium until it is enclosed in a phagosome. Pseudopodium formation occurs through polymerisation of microfilaments of actin monomers in the cytoskeleton of the neutrophil (Zigmond, 1989).

The use of exogenous lysostaphin did not decrease the percentage uptake for any of the strains significantly so it was decided to remove this step from the procedure. Phagocytosis from prosthetic joint material in each of the cell populations tested differed. Polymorphonuclear leukocytes were the most successful at phagocytosing the bacteria with strain M7 being slightly easier ingested than RP62A (77.2 % and 73.55 respectively although this difference was not significant, $p \leq 0.68$). Monocytes were the least successful in their ingestion of the bacteria with a phagocytosis rate of 50 and 46.4 %. (again no significant difference, $p \leq 0.12$). The isolation procedure may have contributed to this lower ingestion capacity as it is fairly harsh on the monocytes. Although cell viability was not affected as determined by trypan blue staining, isolation may have affected the phagocytic capacity of the cells. Since the phagocytosis mixtures containing monocytes also contained contaminating lymphocytes (a drawback of the separation procedure performed), it may be that the presence of the lymphocytes impaired phagocytosis by the monocytes. J774 cells are murine monocyte macrophage cells. These cells were included in the study because they provided a reference monocyte cell line to compare neutrophil phagocytosis with that had not been subjected to a harsh isolation procedure. The ingestion rates for the J774 cells fell in between the rates for PMNLs and MNs with 61.7% of M7 cells being ingested and 63.5% of RP62A cells being ingested. This strengthens the theory that the human monocytes may have been damaged by the isolation procedure. No significant difference between the two strains was observed for this cell line, $p \leq 0.26$.

If we compare uptake of the same strain of bacteria by different cell populations then differences are apparent. When PMNLs are compared with monocytes, there is a highly significant difference in the uptake of both M7 ($p \leq 0.020$) and RP62A ($p \leq 0.0018$). But, when PMNLs are compared with J774 cells, the difference is significant (for M7, $p \leq 0.35$ and for RP62A, $p \leq 0.81$) but not as highly significant as before. A very significant difference is observed again when monocytes are compared with J774 cells where a p value of $p \leq 0.023$ is obtained for M7 but an even more significant difference p value of ≤ 0.000 is obtained for RP62A.

A study investigating entry and intracellular survival of group B streptococci in J774 cells, showed that entry into the cells (or uptake of the bacteria by the cells) occurred within the first five minutes of incubation (Valentin-Weigand *et al*, 1997). When a similar study investigated invasion of *Mycobacterium tuberculosis* into human alveolar pneumocytes (cultured), J774 cells and fresh human derived peripheral macrophages, of the three cell populations investigated, the initial intracellular level of *M. tuberculosis* six hours post infection was greatest for the J774 cells (13% compared to 9% for the peripheral blood macrophages and only 2 % alveolar pneumocytes). Thus it may be easier for J774 cells to ingest bacteria than monocytes or macrophages, but not PMNLs. The chemotactic factors for PMNLs and J774 cells differ. This was shown in a study of culture filtrates of *Candida albicans* and *Saccharomyces cerevisiae* by Edens *et al.,* in 1999. The differences observed in the uptake rates for strains M7 and RP62A by PMNLs/MNs and J774 may be due to the differences in chemotactic factors released by the bacteria for these different cell types.

3.4.6 Phagocytosis from different substrates

There is much literature concerning the adhesion of *S. epidermidis* to the many different biomaterials used today. However, literature concerning phagocytosis of the adherent bacteria is not so well documented. Kaplan *et al*., 1996 reported that biomaterial-associated infection may be linked to non-productive premature activation of neutrophils which resulted in impaired phagocyte function. PMNL adhesion and subsequent respiratory burst to two commercial polymers were investigated using both radiolabelling and chemiluminescent techniques (Lim & Cooper, 1996). It was found that when PMNLs are incubated with biomaterials, oxygen radicals are produced in the early stages. When the neutrophils are subsequently stimulated (in this case with phorbol myristate acetate), a reduced chemiluminescent response was observed.

The phagocytosis of both strains of bacteria from bone, UHMWPE and polystyrene was compared. For all three substrates there was no significant difference in the uptake of each strain. However, when the uptake of RP62A from

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UHMWPE was compared to the uptake of the same strain from polystyrene a highly significant difference occurred ($p \leq 0.0049$). The reason for this one difference is unclear as it was not shown with strain M7. Both strains are able to adhere to this surface with no significant difference so the amount of adherent bacteria did not differ. The same supply of neutrophils was used in both tests so the difference is not due to variation in neutrophil function.

Previous work in this laboratory compared the effects of antibiotics on the adherence of *Staphylococcus aureus* and *epidermidis* to plastic and their subsequent uptake by PMNLs (Gemmell, 1989). Exposure to vancomycin at low concentrations prior to contact with the polystyrene surface reduced the adherence and increased the susceptibility of *S. epidermidis* Tait, whereas exposure to sub-MIC clindamycin had no effect at all. When *S. aureus* was exposed to vancomycin in a similar way strain to strain differences were observed. *S. aureus* Cowan 1 experienced reduced adherence and increased susceptibility to phagocytosis but *S. aureus* Morris was not affected. This is thought to be because of differences in the surface topography of the two strains as Cowan 1 is rich in protein A and Morris is encapsulated. Changes in adherence do not correlate with changes in susceptibility to surface phagocytosis. This work also concluded that *S. epidermidis* was more adherent than *S. aureus* to plastic surfaces.

Prosthetic biomaterials have made a profound impact on reconstructive surgery but complete biocompatibility remains elusive (Kaplan *et al.,* 1994). An increasing demand for biomaterials corresponds to the deficiency in the knowledge of biocompatibility (Hocker & Klee, 1996). Before effective preventative or therapeutic measures can be achieved, the process, characteristics or mechanism of bacterial adhesion to biomaterials have to be studied (An & Friedmann, 1997). Although a prosthetic joint may have an early record of success, deterioration due to mechanical failure or deleterious host responses to the implant may compromise long term function. The eventual retrieval and detailed analysis of the implanted structure provides an invaluable opportunity to determine the characteristics of implant success or failure and to provoke the development of still better implants.

CHAPTER FOUR

COMPARISON OF ISOLATED POLYMORHONUCLEAR LEUKOCYTES FROM DIFFERENT PATIENT GROUPS WITH RESPECT TO THEIR ABILITY TO INGEST ADHERENT COAGULASE-NEGATIVE STAPHYLOCOCCI.

4.1 INTRODUCTION

The immune system is part of a general defence system that has evolved to protect humans from harmful invasion of microorganisms (Puck, 1997). The integrity of the immune system is essential for defence against infectious organisms and their toxic products and, therefore for the survival of all individuals (Abbas, Lichtman & Pober, 1994). Defects in one or more components of the immune system can lead to serious and often fatal disorders which are called immunodeficiency diseases.

Immunodeficiencies may be classified into two broad groups, primary immune deficiencies and secondary immune deficiencies. Primary immune deficiencies (also known as congenital immunodeficiencies) are rare disorders, most of which are due to genetic defects that affect cell maturation or function at different levels during haematopoesis. Most diseases are manifested early in infancy and childhood but some may not be detected clinically until later in the third or fourth decade of life (Ten, 1998). Diseases include X-linked agammaglobulinemia, Hyper-IgM Syndrome, Common Variable Immunodeficiency, Severe Combined immunodeficiency and Wiskott-Aldrich Syndrome (Rosen, Cooper & Wedgwood, 1995). The result is an inadequate immune response to microorganisms, self antigens and tumour cells which leads to increased susceptibility to infections, autoimmunity or malignant disease (Ten, 1998). Secondary immune deficiencies are also known as acquired immune deficiencies and they develop as a consequence of malnutrition, disseminated cancers, autoimmune disease, treatment with immunosupressive drugs or infections of immunocompetent cells. Diseases include diabetes, HIV and AIDS.

While severe primary defects are rare, patients with secondary or acquired immunodeficiency disorders are more commonly encountered, particularly in hospital medicine. The immunodeficiency seen in several of the secondary immunodeficient conditions may be considered as iatrogenic, either directly as a consequence of modern treatment regimens, or indirectly, as a consequence of increased survival in diseases with depressed host defences. Infections associated with many of these conditions are frequent, serious and often represent an important part of the clinical problems of the patient.

In most immunodeficiency disorders, multiple defects are usually present in three major antimicrobial defence systems the B and T -cells, the neutrophil granulocytes and monocytes/macrophages, and the complement system (Dale, 1981 and Froland, 1981). Many of the defects compromise important interaction mechanisms between the various components of the antimicrobial defences such as secretion of cytokines by the T cells. These cytokines in turn recruit and activate the inflammatory leukocytes (the macrophages and granulocytes) causing a persistent inflammatory state. Phagocytes and complement participate in the effector phase of specific immunity therefore congenital disorders of phagocytes may have disastrous consequences. An example is chronic granulomatous disease, where there is a defect in phagocyte killing due to the absence of one of the enzymes involved in triggering the oxidative burst. Leukocyte adhesion deficiency describes a rare defect in which neutrophils fail to mobilise and migrate to sites of tissue injury. The original gene defect associated with the majority of cases is in the gene encoding CD18, the beta chain common to several types of leukocyte surface integrin complexes composed of CD18 and various types of CD11. A second defect in the gene encoding CD11c has been associated with leukocyte adhesion deficiency in conjunction with developmental and growth retardation, in which natural killer (NK) cell function is also compromised (Etzione, 1994).

The complement system involves more than 30 proteins encoded throughout the genome. Deficiencies in the complement system are rare (less than 1 percent of primary immunodeficiencies). Usually complement deficiencies of component C1 to C4 clinically manifest during childhood with pyogenic infections and autoimmune disorders such as glomerulonephritis or systemic lupus erythematosus. Chediak-Higashi syndrome is an autosomal recessive disorder characterised by giant lysosomal granules in phagocytes, melanocytes and other cells. Patients have unusual light pigmentation and recurrent pyogenic infections that do not respond well to conventional therapy. Phagocyte defects are known to account for 9 % of primary immunodeficiencies and manifest with recurrent pyogenic and fungal infections (Rosa, 1998).

In this study it is the defects in the phagocytic cell system of two groups of immunocompromised patients, those with rheumatoid arthritis and those with diabetes mellitus that come under review. These patient groups were chosen as they were a readily accessible group of patients at Glasgow Royal Infirmary. Diabetic and rheumatoid arthritis patients are subject to infection due to their immune status. They also may require joint replacement therapy at some time - the rheumatoid arthritis patients due to the nature of their disease and the problem of mobility and the diabetic patients due to bone loss. Both diseases are discussed in greater detail.

4.1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects the synovial tissue surrounding the joints. "Itis" literally means inflammation. This inflammation is associated with swelling and pain. It was first recognised in the mid 18th Century and has since been described world-wide (Hochberg & Spector, 1990) and now it has a prevalence of one per cent (Spector, 1990). RA is one of a range of rheumatic and musculo-skeletal disorders that includes osteoarthritis, ankylosing spondylitis, various conditions resulting in back pain, arthritis, fibromyalgia and lupus erythrematosus. Inflammation of the synovial membrane (which creates the lubricating fluid for the joint) is recognised by warmth, redness, tenderness, or swelling (Fries, 1979). Any of the 187 synovial joints of the body may become affected. The course of rheumatoid arthritis can vary in form from a mildly disabling condition to a severe progressive disease. At the very beginning the disease is usually silent and without symptoms, but there is evidence that the early stage of disease is a unique period, often characterised by profound inflammation, severe symptomatology and a likelihood of progression (Emery, 1994; Young, Aarons & Toon, 1991 and Scott *et al.,* 1984). For a minority of 10-15 per cent, onset may be quite rapid with quite severe symptoms developing over a few days or weeks, for the majority the onset is gradual and insidious. Initially symptoms are more likely to affect the hands and the wrists. In these stages individuals experience the pain, swelling and stiffness that are cardinal characteristics of the disease, but may also feel fatigue and general malaise (Liang *et al.,* 1992). When RA progresses, the disease invades the cartilage, tendons and bone and irreversible destruction of the tissue begins. At later stages of the disease, progressive destruction of joints occurs. The combined effects of inflammation and joint damage result in increased disability.

RA has a major impact on the patient and is associated with significant levels of pain as well as functional disability and has a wider psychosocial impact on exercise and recreation, relationships and psychological well-being and ability to work (Carr, 1996). Most pain arises from pressure on the joint capsule and adjacent ligaments. As there is no current cure for RA, there is much emphasis on the management and the treatment of the disease. Treatment of RA is concerned with arresting the development of the disease and associated disability and reducing the severity of symptoms. As the causes of RA have yet to be identified, therapies are somewhat indirect in their mode of action, aimed at reducing joint inflammation and swelling and consequently pain, stiffness and joint deterioration. Analgesics are most often used to control pain. Drug treatments are either 'symptomatic' or 'slow-acting antirheumatic' (SAARDs). Symptomatic drugs therapies are not designed to have any impact on underlying disease processes. It requires the use of one or more of the large number of non-steroidal anti-inflammatory drugs (NSAIDs) available such as aspirin or ibuprofen which are intended to reduce the severity of inflammation and stiffness experienced by the patient. One major problem with the use of NSAIDs is that they are one of the most common sources of adverse reactions or "side-effects" (Brookes, 1993). The most common side-effect is gastrointestinal upset but other problems such as renal or skin damage may occur. There seems to be no clear evidence to support the use of any particular NSAID in preference to others (Joint Working Group, 1992). If there is no improvement with the use of NSAIDs then SAARDs are used. SAARDs have no direct analgesic properties but have a wide range of modes of action that may result in halting the progression of disease. As with NSAIDs there is a large choice of SAARDs which all have potential adverse reactions.

The most common forms of therapy are pharmacological, although pharmacological therapies have a short life span therapeutically and most are withdrawn due to their side-effects. In a study in 1990, Wolfe estimated that one and a half to two years was the recommended maximum that a patient could remain on SAARD therapy due to side-effects. This affects the long-term outlook to this therapy due to a lack of longer clinical trial evidence (Rasker & Cosh, 1992). Therefore increasing attention has been given to alternative forms of treatment including physical therapy, wearing splints or paraffin baths that will reduce the consequences of the disease. Surgery for joint replacement is performed where the damage to the joint is irreversible and severely disabling. Surgery itself may cause problems and patients with RA have a higher risk of joint infections than others. Corticosteroids and immunosuppressors also contribute to the added risk of infections in RA patients. Some of these infections are due to common micro-organisms such as *Staphylococcus aureus*, but infections with opportunistic micro-organisms such as *Staphylococcus epidermidis* or *Escherichia coli* have been reported (Bannwarth *et al.,* 1994 and Cornelissen *et al.,* 1991). The long term course of the disease differs greatly amongst individuals. There are three general patterns of disease (Scott & Huskisson, 1992). The most common response, found in 70% of individuals is progressive disease with periods of flare-ups where the disease is more active and the symptoms more severe and periods of remission. A second less common pattern (only 25% of individuals experience this) is intermittent with periods of flares and brief remission. The third group (only 5%) experience what is termed malignant disease- an extremely severe form of the disease. The long term outcome of the disease also varies. Overall there is a common pattern of increased disability which varies in the picture presented of the rate at which such deterioration occurs, the extent of disability at outcome and whether there is accelerated deterioration early in the history of the disease (Wolfe & Cathey, 1991; Rasker & Cosh, 1992 and Leigh & Fries, 1991). The variation in endpoint across the studies may be due to differences in sample recruitment, length of follow-up time and observer differences. It used to be commonly observed that RA, whilst a very disabling disease, was never fatal but it is now clearly recognised to be capable of significantly shortening the expected survival of affected individuals (Pinals, 1987). Overall, the disease decreases the life expectancy from three to ten years in both men and women. In general, patients with RA die of expected causes (e.g., cardiovascular or malignant disorders) but at a younger age than normal individuals (Markensen, 1991).

In order to aid in the diagnosis of this disease increasingly precise definitions and clinical criteria have been sought to distinguish RA from related disorders (Rigby

& Wood, 1990). The criteria for diagnosis involve clinical signs of disease activity in the joints, such as swelling and partial dislocation together with evidence from x-rays and in most cases the presence of rheumatoid factor in serum. Rheumatoid factor is a form of antibody to type II collagen which was discovered in the mid-1950's. It is suggested that these antibodies are evidence of auto-immune responses that bring about the disease. Assessing for the presence of rheumatoid factor is the most helpful immunological test in diagnosing RA. However in some studies it has become clear that as many as 72% of those who have a positive test for rheumatoid factor may not have the disease (Shmerling & Delbanco, 1992). This is especially true of elderly patients where a false-positive result is most often observed, as this group is prone to develop weakly positive tests for a variety of auto-antibodies, few of which emerge as being clinically significant. Therefore as a stand-alone test for RA, that for Rheumatoid Factor needs to be approached with caution. Another test is anti-nuclear antibody also known as anti-nuclear factor) which is positive in 32% of all arthritis tests but shows greater sensitivity in testing for juvenile-onset RA where it is positive in 76% of cases. X-rays give a clearer clinical picture showing soft tissue thickening, juxta-articular osteoporosis, a loss of joint space or bone erosion. X-rays should be used in conjunction with laboratory tests for a positive disease identification.

Although significant advances have been made in understanding the pathogenesis of RA, the cause and pathogenesis of the disease are complex, involving both genetic and environmental factors (Harris, 1990). It is generally considered an auto-immune disease, but there is no clear evidence of what factors trigger this destructive response of the body's immune system. It is not known whether RA is one disease or a syndrome caused by different agents (Alarcon, 1995). Some research evidence supports the view that a virus such as Epstein-Barr may be involved in the initial triggering of the auto-immune response that leads to RA. However it is also possible that such phenomena are secondary to the onset of RA (Sewell & Trentham, 1993). It does appear that, however triggered, T-cells, which are normally part of the body's adaptive immune system and which normally respond to foreign bodies, are induced to attack the individual's joints (Harris, 1990).

There is also evidence that these auto-immune responses more commonly occur in individuals who are genetically susceptible. Autoantigens expressed on human leukocytes are known as human leukocyte antigens or HLAs. HLA typing of large groups of patients with various autoimmune diseases has shown that some HLA alleles occur at higher frequency in these patients than in the general population. Approximately 90 per cent of patients with severe RA have HLA-DR halotypes that contain the sequence motif glutamine-arginine/lysine-arginine-alanine-alanine (QK/RRAA, according to the one-letter amino-acid code) in the DRB1 chain (Ollier & Thomson, 1992 and Winchester, Dwyer & Rose, 1992). This has been referred to as the "rheumatoid epitope". Predisposition to the disease and its severity resides within this epitope and it's presence and dose has been shown to be associated with very destructive arthropathy and organ system involvements in some RA patients. Thus it is felt that the epitope not only confers susceptibility to the disease but is also a marker for disease severity (Alarcon, 1995). Only 30% of causes of RA can be attributed to genetic factors; the rest remain unexplained (Alarcon, 1995). The only other inherited factor that is consistently associated with RA is female gender. It is reported that women are more likely to develop RA than men; the ratio is 2:1(or 3:1) (Lawrence *et al.,* 1989) (Markensen, 1991).This may be due to the possible modulatory effect of sex hormones, hormone replacement therapy, fertility and pregnancy. A number of observations support a role for sex hormones in the aetiology and pathogenesis of disease. Both genetic and environmental studies have separately identified predisposing disease factors but it is most likely a combination of both.

4.1.2 Septic arthritis

Septic arthritis (also referred to as infective arthritis) is a common complication of RA, occurring in about 3% of cases (Goldenberg, 1989). The commonest causes of disease are *Staphylococcus aureus* (80%), *Haemophilus influenzae*, and goncocci. Other less common causes are TB, *Brucella* and *Salmonella typhi* (Oxford Handbook of Clinical Medicine, 1997 Edition). Patients with damaged joints (e.g. with RA) or on steroids are at greater risk. Pyogenic infection of a joint is usually spread haematogenously, but may develop from adjacent osteomyelitis. Prosthetic joints can be easily involved as they become infected by haematogenous bacterial spread. Only RA has been shown to be a risk factor for the development of deep sepsis after hip and knee arthroplasty surgery (Fitzgerald *et al.,* 1977; Stinchfield *et al.*, 1980; Surin, Sundholm & Backman, 1983 and Wilson *et al.,* 1990). Factors such as underlying illnesses, the use of corticosteroids and other immunosuppressants play an important role in septic arthritis. The mortality rate is high at around 15-20% making septic arthritis a medical emergency. The mortality rate is exacerbated by the delay in diagnosing septic arthritis. The disease is often confused with an exacerbation of rheumatoid arthritis, and clinical signs such as fever may be absent in 10% of patients or there may be a low grade temperature in 40% (Canvin *et al.*, 1997). Although this delay has not been shown as yet to cause increased mortality (Yu *et al.*, 1992) delayed diagnosis can cause extensive joint destruction (Canvin *et al.*, 1997). This usually results in removal of the infected prosthesis. Leaving the infected prosthesis *in situ* was shown to be effective in only 40% of cases, as compared to removing the prosthesis, as a two-step procedure or arthrodesis, where it controlled the infection in 85% of cases. Patients with RA also have had higher rates of infection after joint replacement. Post-operative infection is a frequent complication of joint replacement therapy resulting in a longer term of hospitalisation for the patient resulting in increased costs which have to be met by the hospital therefore much research goes into its prevention.

Although septic arthritis occurs infrequently (Kaardorp *et al.*, 1995) its serious consequences have led to recommendations for its prevention (Maderazo, Judson & Pasternak, 1988). Prevention is especially aimed at patients with prosthetic joints who are at an increased risk of acquiring septic arthritis. Other risk factors for septic arthritis in patients with joint disease include an age of 80 years or older, diabetes mellitus, RA, recent joint surgery, and a skin defect (Kaardorp *et al.*, 1995).

4.1.3 Osteoarthritis

Osteoarthritis is far more common than rheumatoid arthritis, in fact it is the commonest joint condition. It was not distinguished from rheumatoid arthritis until

the beginning of the twentieth century. In large part, this distinction resulted from studying the pathology of the two conditions, especially under the microscope. "Osteo" was included in the word because of the changes observed in the joints. Bone is not a principal feature of the condition, and the joints are not primarily inflamed but instead are worn as a result of joint failure, to which a degree of secondary inflammation may have been added. The presence of osteoarthritis can be detected in over 80% of individuals over the age of 75 years. The mean age of onset is 50 years and it is symptomatic three times more often in women. It used to be commonly referred to as 'wear and tear' arthritis because it is often induced by injury or excessive use of joints.
It is a disease that primarily affects joint cartilage but changes occur in all joint structures. It initially affects smaller joints beginning as minute fissures on the surface of the cartilage, which eventually result in a thinning of the cartilage, leaving the ligaments long and in some cases the joint unstable. At the same time the underlying bone is affected becoming denser, growing out at the sides in response to the damage. In contrast to the inflamed joint in the RA patient where the inflamed synovium is thickened, initially synovial inflammation in the osteoarthritis patient is minimal but later as fragments of cartilage and bone are shed into the synovial fluid and are absorbed by the synovium. This results in a secondary inflammatory response in and around the synovium. As part of the response, the capsule and surrounding ligaments become loose or, alternatively thickened and more rigid. It eventually involves major weight-bearing joints such as the hip or knee. Most individuals experience pain on movement which is worse at the end of the day. They have background pain on resting and there is joint stiffness. Most joint surgery is performed on individuals with osteoarthritis. Like many disease OA may be hereditary (or at least have a hereditary component), this was first reported by Robert Stecher (1965) who established a hereditary link in Herberden's nodes (small nodules near the ends of the fingers). In families where the evidence of hereditary disease is strong osteoarthritis is linked to the gene on the twelfth chromosome controlling type 2 collagen.

Unlike RA there are no specific blood tests to diagnose OA but in many cases the diagnosis is obvious clinically. Aspiration of the joint will yield a clear fluid unlike that observed in the case of septic arthritis where the synovial fluid is turbid or purulent and has a low viscosity. Radiology of the involved joints shows narrowed joint space, osteophytes, sclerosis of sub-chondral bone, subchondral cysts, irregularity of the joint surface and disorganisation of the joint.

4.1.4 Diabetes mellitus

Diabetes is a metabolic disease characterised by hyperglycaemia, glycosuria and a wide spectrum of clinical and pathological manifestations that occur because of either a lack of insulin or because of the presence of factors that oppose the action of insulin. It may present in a variety of ways, ranging from abrupt onset of coma to asymptomatic glycosuria discovered on routine urine testing. Presenting clinical features depend upon the degree of insulin lack, manifesting by the degree of hyperglycaemia, ketosis and wasting, and on the possible presence of tissue damage. Thirst and polyurea are the main presenting symptoms and are often associated with polyphagia, weight loss, ocular refractive change and infection.

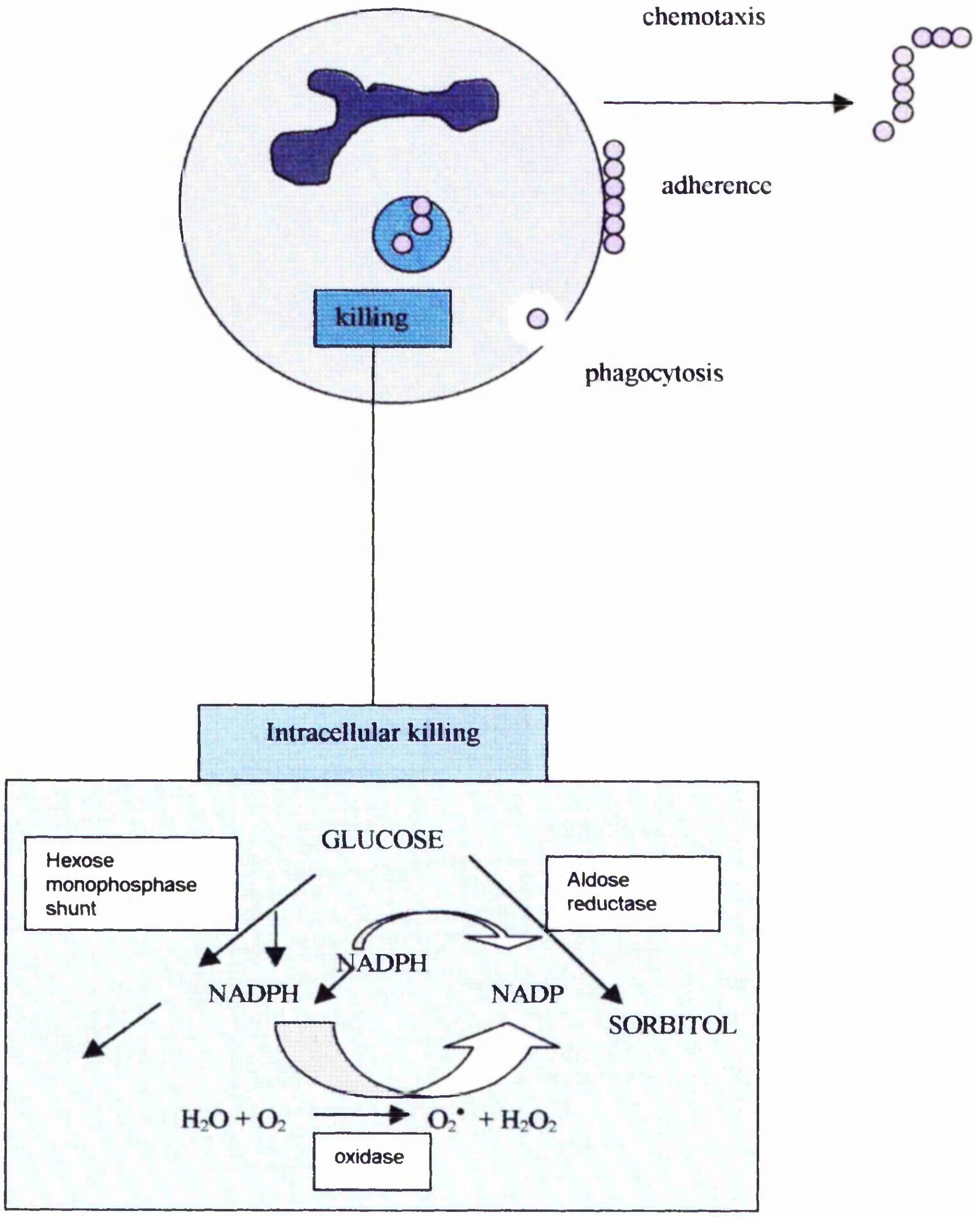
The discovery of insulin and its life-saving property by Banting and Best in 1921, marked the start of the therapeutic era in the history of diabetes. It confirmed the concept of an insulin deficiency as the basic abnormality in the diabetic and gave rise to definition between the two types of diabetes mellitus. Type-1 diabetes, which is insulin-dependent (IDDM) is also called juvenile diabetes. It affects about 0.25 % of the population, with a peak age onset of 11 to 12 years and may be associated with other autoimmune diseases. It is normally diagnosed when patients present with typical symptoms such as thirst, polyuria and weight loss. These patients have a deficiency of insulin resulting from destruction of the insulin-producing β cells of the islets of Langerhans in the pancreas, and continuous hormone replacement therapy is needed. The prolonged survival of younger diabetics due to the introduction of insulin revealed the delayed complications of the disease, affecting the small vessels of the retina and the renal glomeruli, damaging the peripheral nerves and accelerating atherosclerotic change in the larger arteries supplying the heart, brain and lower limbs. Type II diabetes is less acute, more prevalent, clinically non-lethal and is termed as non-insulin dependent

(NIDDM) and is also known as maturity-onset diabetes. This affects the older age group and is due to impaired insulin secretion and insulin resistance. Patients with type II diabetes may at some time require insulin, especially those with ketonuria or those who have had a sudden onset. Diabetes is associated with, and is sometimes secondary to other endocrine and metabolic disorders which may present with diabetic symptoms or may be discovered when investigating patients with the primary disorder (e.g. chronic pancreatitis).

Diabetes may increase general susceptibility to infection (Textbook of Diabetes, 1992). The reasons why diabetic patients present with an increased susceptibility to frequent and protracted infections remains unclear (Moutschen, Scheen & Lefebvre, 1992). This increased risk is likely to be multi-factorial. Infections cause hyperglycaemia and may precipitate diabetic ketoacidosis. This is due to increased counter-regulatory hormone secretion, which stimulates gluconeogenesis, and also to sympathetically mediated inhibition of insulin release. Insulin resistance may be induced in peripheral tissues by cytokines released in response to infection (Johnston, 1997). Hyperglycaemia can interfere with crucial activities of phagocytic cells notably the respiratory burst, responsible for intracellular killing of microorganisms which is attenuated by polyol pathway activation. The respiratory burst is dependent on nicotinamide adenine dinucleotide phosphate (NADPH) which is normally generated by glucose through the hexose monophosphate shunt. In diabetes, more glucose enters the phagocytes and is metabolised by the polyol pathway. Aldose reductase, the rate-limiting enzyme of this process, requires NADPH and this is consumed when flux going through the polyol pathway increases. This competition for NADPH is thought to account for the reductions in the respiratory burst and in intracellular killing (see figure XXIV overleaf).

Impairment of the respiratory burst has been demonstrated in both human and experimental diabetes (Marhoffer *et al.*,1992; Sato *et al.*, 1992 and Ueta *et al.*, 1993). This impairment was correlated with a reduction in the intracellular killing of the microorganisms which worsens with increasingly poor diabetic control.

Figure XXIV Ingestion and killing of microorganisms by polymorphonuclear leukocytes in patients with diabetes mellitus



When treated with ponalrestat, an aldose-reductase inhibitor, intracellular killing by neutrophils from diabetic patients was improved (Boland *et al.*, 1992).

Hyperglycaemia can also affect neutrophil chemotaxis, adherence and phagocytosis (Wilson & Reeves, 1986). In a study of 61 diabetic patients Delmaire *et al.*,1997 showed that there was no isolated impairment of any single PMNL function in diabetes. Global dysfunction occurs where there are abnormalities at baseline level due to PMNL hyperfunctioning. There are also abnormalities after stimulation of the PMNLs where various functions are no longer enhanced and some are reduced. These defects may have been the consequence of the combined action of several factors. The role of adhesion of PMNLs in the diabetic patient is one area of controversy. Some authors have reported a decrease in adherence (Bagdade *et al.,* 1978 and Tater *et al.,* 1987) whereas others have reported an increase (Delmaire *et al.*, 1997 and Wautier *et al.,* 1990). Phagocytosis studies have also given conflicting results. These studies have used a variety of techniques and agents and perhaps this explains the ambiguity in the analysis of the data. Moutschen, Scheen & Lefebvre, 1992; Wilson & Reeves, 1986 and Dziatowiack *et al.,* 1982 found no alteration of phagocytosis in diabetics whereas Marhoffer *et al.*, 1992 and Nolan, Beaty & Bagdade, 1978 reported a phagocytosis deficiency with Staphylococci. A subtle improvement in phagocytosis was found where there was improved diabetic control (Kjersem *et al.*, 1988).

The question as to whether or not the diabetic has a greater risk of requiring joint replacement therapy has also come under review. There is disagreement about whether bone disease is a complication of diabetes (Heath, Melton & Chu, 1980; M^{c}Nair, 1988; Selby, 1988; Melchior *et al.*, 1990; Bouillion, 1991 and Delbridge *et al.*, 1988). Among patients with fractures there is an increased prevalence of diabetes (Kelsey *et al.,* 1992 and Meyer, Tverdal & Falch, 1993), but among patients with diabetes there is no increase in risk of fracture (Heath, Melton & Chu, 1980 and Melchior *et al.,* 1990). There are several possible explanations for this paradox, and if the association is genuine there are several reasons why diabetes might increase fracture risk, including osteopenia and an increased likelyhood of falling. Bone mineral density has come under scrutiny in a bid to end this controversy. Although evidence exists that bone mass could be decreased in insulin-dependent diabetes (type-1 disease), contradictory results have been observed in patients with non insulin-dependent (type 2) diabetes (Mathiassen *et al.,* 1990 and Okuno *et al.,* 1991). The differences observed in the two groups of diabetes may explain the ambiguity in the literature. This group of patients was chosen for this study as there seems to be an increase in the number of patients undergoing joint-replacement therapy who are also diabetic.

4.2 MATERIALS AND METHODS

4.2.1 Patient and control group demography

4.2.1.1 Patients with Rheumatoid arthritis

These patients were recruited either as inpatients in Wards 14 and 15 of Glasgow Royal Infirmary or as outpatients attending the Centre for Rheumatic Diseases. A total number of 16 patients (of which 11 were female and 5 were male) between the ages of 30 and 80 years of age (mean age = 59.25 years) were included in the study. No selection process was employed, and there were no inclusion/exclusion criteria for entry into the study. The patients were divided into groups, Group A were male and Group B were female.

4.2.1.2 Patients with Diabetes mellitus

The patients were recruited from the diabetic clinic at Glasgow Royal Infirmary over a 4-month period. All were diagnosed as having juvenile-onset diabetes (or type-1, insulin-dependent diabetes). In total 22 subjects were included, of which 10 were and 12 were female with a mean age of 55.9 years (range 33-69 years). The patients were divided into two groups, group A male and group B female.

4.2.1.3 Control group

The control group consisted of members of the laboratory and secretarial staff of the Department of Bacteriology, GRI. Like the patient groups they were divided into two groups, male and female. Control subjects, by definition consisted of those currently receiving no antibiotic therapy and not outwardly showing any signs of infection including coughs or colds.

Table 17 Clinical data of the diabetic patients

CLINICAL DETAILS	TOTAL (n = 21)
Sex (M/F)	10/11
Mean Age (years)	59.25 (30-80)
Mean Diabetes duration	11 years 4 months
Mean Glucose level (mmol l^{-1})	14.1 (range 7-23.5) (normal ≥ 6mmol/l)
Mean HbA_{1c} (%)	11.6 (range 3.4-5.2)

Table 18 Rheumatoid Arthritis patients

CLINICAL DETAILS	TOTAL (n=16)
Sex (M/F)	5/11
Age (years)	55.9 (33-69)
Mean RA Duration	17 years 2 months
Rheumatoid factor (+ve/-ve)	73 % +ve: 27% -ve
CRP levels	63.25 mg/L (normal ≤ 10mg/L)
ESR	36.6 (normal >10)

Table 19 Control group

DETAILS	TOTAL (n = 11)
Sex (M/F)	3/8
Age (years)	40.1 (24-61)

4.2.2 Isolation of polymorphonuclear leukocytes.

Polymorphonuclear leukocytes were isolated according to the method of Bøyum *et al.*, 1968 as described in the methods section of Chapter Two. The method was modified slightly for the samples from rheumatoid arthritis patients as they were spun for a longer period of time at 3000rpm, 35-40 minutes to allow for optimal band separation and distinction. This was also found to prevent separation of the red cell pellet with accompanying loss of the polymorphonuclear band. The reason for this was not clear but it is believed that some of the medication the patients were receiving at the time of sampling to manage their RA may have affected the separation of the blood. This was not observed with the blood samples from the diabetic patients.

4.2.3 Serum source and opsonisation of bacteria.

Blood was obtained from normal healthy volunteers and allowed to clot in sterile universal containers as before. Equal volumes of each bacterial strain were then opsonised with 10% serum for 15 minutes at $37^{O}C$. The strains were then washed with gel-Hanks solution and diluted to a concentration of 1×10^7 bacteria/ml.

4.2.4 Preparation of multi-well plates containing discs of prosthetic joint material.

Sterilised discs of prosthetic joint material (polymethylmethacrylate) were stuck into the wells of a 96-well microtitre plate using superglue as described in the methods section of Chapter Three.

4.3 RESULTS

4.3.1 Neutrophilia and neutropenia

The hallmark of a chronic inflammatory lesion is an inflammatory infiltrate which is predominately composed of macrophages and lymphocytes and a few PMNLs (Brown, 1988). In RA, this is observed in the inflamed joint but is also accompanied by high PMNL levels in the synovial fluid (Finbloom, Martin & Gordon, 1987). Patients with Rheumatoid arthritis generally have a greater number of circulating phagocytes in their white cell population due to their constant inflammatory state. The yield of polymorphonuclear leukocytes was calculated (number of PMNLs x 10^7/ml) for each patient, and cell viability >95% checked. In some of the patients a low yield of polymorphonuclear leukocytes was obtained after the separation procedure. This may not be a reflection of the patients immunological status but may be due to the separation procedure not working maximally due to the patients concurrent medication.

Unlike those with Rheumatoid Arthritis, patients with Diabetes Mellitus do not normally have a high neutrophil count. Diabetes may in fact increase susceptibility to infection. A higher infection rate is often associated with a low neutrophil count (neutropenia).

Table 20 PMNL yield of the two patient groups compared to the control group

Group	ml PMNL at 1 x 10^7 cells /ml isolated from 20ml whole blood.
Rheumatoid Arthritis	3.2
Diabetes Mellitus	3.25
Control	2.8

At a first glance there does not appear to be any differences between the patient groups and the controls but, when the data is further analysed and further divided into gender groups differences are then observed. With the RA group there is a highly significant difference between the males and the females. The mean PMNL yield was 2.42 ml/20ml whole blood for males but 4.16ml/20ml for females ($p \leq 0.064$). There was also a significant difference between the female patients and the controls (4.16 ml for the patients compared to 2.59ml for the controls, $p \leq 0.059$). No such gender difference was observed for the diabetic patients (males 3.17ml/20ml, females 2.99ml/20ml, $p \leq 0.75$). This is observed in the graphs overleaf.

As there was a significant difference in the yield of PMNLs separated from male patients and female patients, the phagocytic activity of the two groups of patients was compared in this way.

4.3.2 Phagocytic activity of patients with Rheumatoid Arthritis.

This has been split into two groups, male and female. The larger number of female subjects included compared to male subjects reflects the incidence of this disease which is two to three times more prevalent in the female sex than the male. Due to the number of patients recruited into the study there were not sufficient numbers in the younger age group to allow age comparisons to be made, again this reflects incidence of disease which has a peak onset of 40 years. The phagocytic activity of the male patients and the female patients were compared to the control group and also to each other.

4.3.2.1 Table 21 Phagocytic activity of male patients with rheumatoid arthritis compared to a control group expressed as a percentage ingestion of strain M7 or RP62A

MALE RA PATIENTS				CONTROL GROUP			
STUDY NUMBER	AGE	% M7	% RP62A	CONTROL NUMBER	AGE	% M7	% RP62A
RAM-1	58	93.8	88.6	RAC-1	23	86.0	80.4
RAM-2	52	87.1	84.1	RAC-2	23	72.9	63.6
RAM-3	60	52.3	61.3	RAC-3	23	54.6	63.5
RAM-4	62	64.3	63.5	RAC-4	48	73.8	61.4
RAM-5	72	71.9	75.4	RAC-5	40	66.8	72.5
MEAN	**60.8**	**73.9**	**74.6**	**MEAN**	**31.4**	**70.8**	**68.3**
SD	**7.29**	**16.8**	**12.1**	**SD**	**11.8**	**11.4**	**8.01**

4.3.2.2 Table 22 Phagocytic activity of female patients with rheumatoid arthritis compared to a control group expressed as a percentage ingestion of strain M7 or RP62A

FEMALE RA PATIENTS				CONTROL GROUP			
STUDY NUMBER	AGE	% M7	% RP62A	CONTROL NUMBER	AGE	% M7	% RP62A
RAF-1	66	76.4	82.4	RAC-1	46	66.8	72.5
RAF-2	69	52.1	59.2	RAC-2	34	42.6	28.9
RAF-3	66	66.2	72.7	RAC-3	34	42.6	28.9
RAF-4	33	57.9	71.1	RAC-4	27	72.7	81.2
RAF-5	48	39.1	68.4	RAC-5	27	72.7	81.2
RAF-6	69	51.6	55.1	RAC-6	27	72.7	81.2
RAF-7	40	67.2	57.7	RAC-7	24	64.8	64.4
RAF-8	68	79.8	80.5	RAC-8	24	64.8	64.4
RAF-9	57	73.0	86.9	RAC-9	24	64.8	64.4
RAF-10	43	59.1	61.9	RAC-10	24	54.6	63.5
RAF-11	89	61.3	54.1	RAC-11	24	54.6	63.5
MEAN	**58.9**	**62.2**	**67.3**	**MEAN**	**28.6**	**61.2**	**59.1**
SD	**16.4**	**12.0**	**10.7**	**SD**	**6.9**	**11.2**	**20.8**

Duplication of control subjects arose when there were two or more patients tested at the same time. Limited numbers of control subjects available meant that a separate control was not available for each patient.

4.3.3 Phagocytic activity of patients with Diabetes Mellitus.

As with the rheumatoid arthritis patients this has been split into two sections, male and female. The incidence of Diabetes in the population is equal for both sexes and this is reflected in the number of both male and female subjects recruited into the study. The results are shown in tables 23 and 24 overleaf.

4.3.3.1 Table 23 Phagocytic activity of male patients with Diabetes Mellitus compared to a control group.

MALE DM PATIENTS				CONTROL GROUP			
STUDY NUMBER	AGE	% M7	% RP62A	CONTROL NUMBER	AGE	% M7	% RP62A
DMM-1	16	66.4	54.1	DMC-1	35	60.6	45.1
DMM-2	30	23.1	29.5	DMC-2	53	39.4	43.5
DMM-3	30	64.4	64.0	DMC-3	35	57.5	61.7
DMM-4	32	41.4	47.4	DMC-4	53	55.5	39.7
DMM-5	47	40.8	42.6	DMC-5	53	55.5	39.7
DMM-6	42	69.5	63.9	NONE	***	***	***
DMM-7	51	57.9	61.6	DMC-6	23	59.3	66.5
DMM-8	35	48.8	45.4	DMC-7	61	57.1	54.1
DMM-9	73	73.6	63.1	DMC-8	45	76.7	71.9
DMM-10	30	78.4	62.9	DMC-9	45	76.7	71.9
MEAN	**38.6**	**56.4**	**53.5**	**MEAN**	**40.3**	**59.8**	**54.9**
SD	**15.7**	**17.5**	**11.8**	**SD**	**18.1**	**11.4**	**13.5**

4.3.3.2 Table 24 Phagocytic activity of female patients with Diabetes mellitus compared to a control group.

FEMALE DM PATIENTS				CONTROL GROUP			
STUDY NUMBER	AGE	% M7	% RP62A	CONTROL NUMBER	AGE	% M7	% RP62A
DMF-1	39	46.7	51.2	DC-1	61	57.1	54.1
DMF-2	18	74.1	66.4	DC-2	35	60.6	45.1
DMF-3	26	76.9	77.0	DC-3	35	60.6	45.1
DMF-4	20	70.0	56.6	DC-4	28	87.0	72.6
DMF-5	28	64.1	63.1	DC-5	28	87.0	72.6
DMF-6	51	61.8	63.7	DC-6	48	78.3	57.1
DMF-7	45	67.3	63.7	DC-7	48	78.3	57.1
DMF-8	57	62.9	70.6	NONE	***	***	***
DMF-9	35	76.5	74.3	DC-8	23	66.7	69.4
DMF-10	32	61.3	61.0	DC-9	23	70.2	50.6
DMF-11	34	54.9	54.8	DC-10	61	57.1	54.1
MEAN	**35**	**65.14**	**63.9**	**MEAN**	**35.5**	**70.3**	**57.7**
SD	**12.3**	**9.23**	**8.0**	**SD**	**18.2**	**11.7**	**10.4**

When the above sets of data from both patient groups are compared graphically the following trends are observed (figures XXV and XXVI).

Figure XXV Phagocytic ingestion of *S. epidermidis* M7 and RP62A by PMNLs of Rheumatoid Arthritis patients compared to a control group

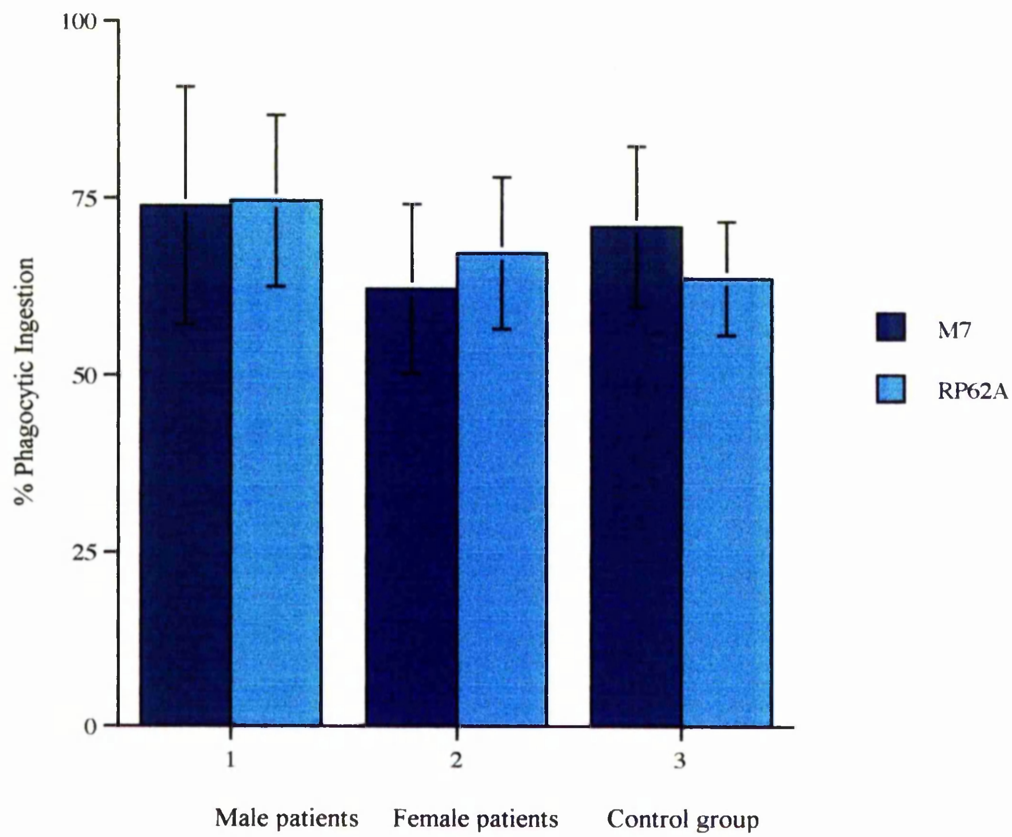
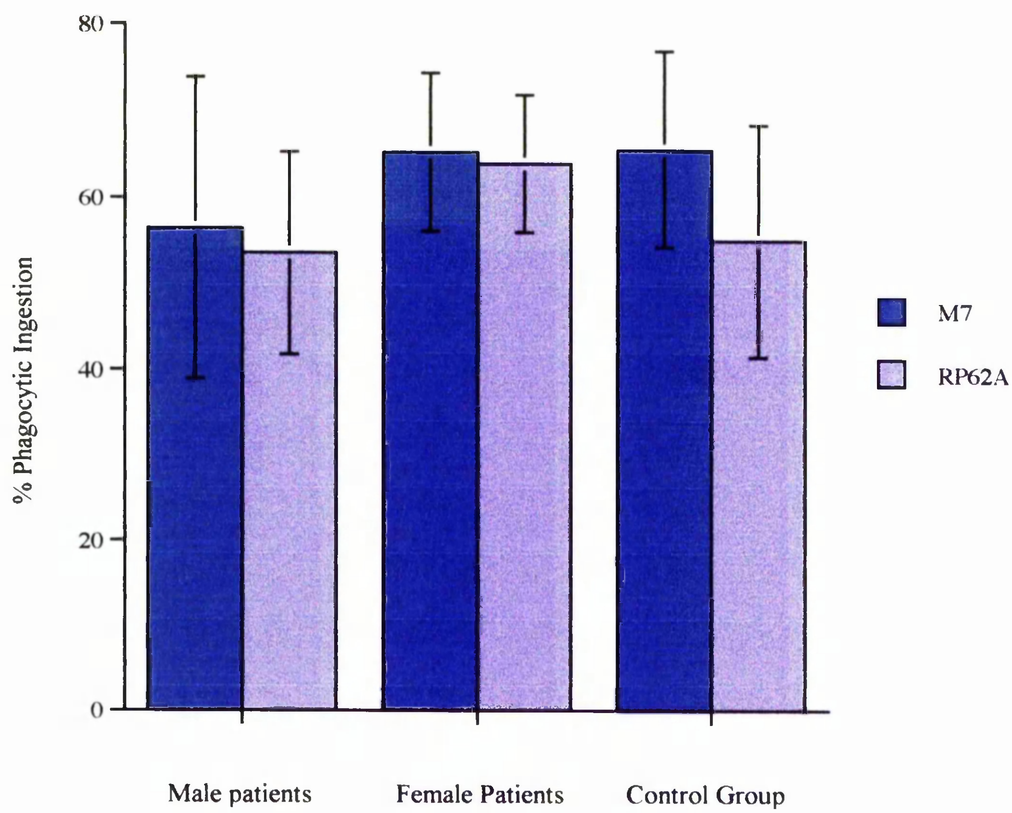


Figure XXVI Phagocytic Ingestion of *S. epidermidis* M7 and RP62A by PMNLs of Diabetes Mellitus patients compared to a control group



4.4 DISCUSSION

4.4.1 Polymorphonuclear cell yield in the immunosuppressed host compared to the normal control

Immunodeficiency which is a deficiency of the immune response due to hypoactivity or decreased numbers of lymphoid cells is not a rare occurrence. The immune system, though very intelligent can cause great harm when it suddenly reverses its role and instead of being beneficial is detrimental to the host. This results in auto-immune diseases where the ability to remain non-reactive to self components breaks down. Rheumatoid arthritis is a relatively common disorder that affects both men and women at the prime of their lives. It has the potential to alter an individuals survival, comfort, function, and ability to remain gainfully employed. Diabetes mellitus may strike at any point in life. Juvenile-onset diabetes is unfortunately not a rare occurrence. Whilst it may be difficult for a child or young adult to accept that their life is very different from that of their "normal" peers, they may adapt to the changes in life such as routine blood monitoring, injection of insulin and careful control over glucose intake better than an adult who has to change their way of life due to mature-onset diabetes.

Patients with autoimmune diseases such as rheumatoid arthritis or diabetes mellitus have their immune system carefully monitored by routine blood analysis. It is noted that RA patients in particular have a raised white cell count-neutrophilia. DM patients although susceptible to infection due to their immunsuppressed state do not generally have a raised white cell count. As the ability of the PMNLs to phagocytose adherent bacteria of these patient groups was under review it was decided to monitor the initial PMNL count. This would tell us whether having exceptionally high or low numbers of neutrophils affected their phagocytic capacity-i.e. if you have low numbers of neutrophils, do they simply work harder to clear infection?

PMNLs were isolated from patients with rheumatoid arthritis and diabetes mellitus using a modification of the original method developed by Bøyum, 1968. The yield of PMNLs, calculated as the ml of cells at a concentration of 1×10^7 cells /ml from an original whole blood volume of 20 ml was measured. Patient yield was compared to a control group consisting of members of staff who were in apparent good health. Of the two groups tested against the control group only the RA patients had raised white cell count with an average yield of 3.2ml per 20ml compared to 2.6 per 20ml with the control. This raised count whilst not significant reflects the constant pro-inflammatory state of the RA patient. No significant difference was found with the diabetic patients compared to the controls. This is perhaps strange as most authors are in agreement that that the diabetic patient is susceptible to infection (Molenaar *et al.*, 1976; Marhoffer *et al.,* 1992 and Boland *et al.*, 1992). This would lead us to suspect that increased infection prevalence is thus due to poor neutrophil activity and cannot be explained by a decrease in the number of circulating neutrophils in the patients blood.

4.4.2 Phagocytic activity of patients with rheumatoid arthritis or diabetes mellitus compared to a control group.

4.4.2.1 Rheumatoid arthritis

A number of investigators have sought to determine whether or not the function of blood PMNLs is impaired in patients with RA when compared with PMNLs from normal subjects. Early reports stated that both chemotaxis and phagocytosis were impaired (Mowat & Baum, 1971; Roberts-Thomson *et al.,* 1976; Bodel & Hollingsworth, 1966; Corberand *et al.,* 1977; Wilton, Gibson & Chuck, 1978 and Attia *et al.,* 1982). Breedveld *et al.,* 1985; Wandall, 1985 and King *et al.*, 1986, found that there was no major dysfunction of circulating PMNLs in the blood of patients with RA. In fact PMNLs might be considered hyperactive because of their increased chemiluminescent response when compared to a normal control group. In a study by Eggleton *et al.,* (1995), PMNLs isolated from RA patients had higher rates of superoxide anion production than cells from healthy individuals in response to stimulation by FMLP. Rheumatoid blood may contain inflammatory mediators which may 'prime' circulating neutrophils *in vivo* making them more responsive to a second stimulus.

From this study it was found that there was no significant difference in the phagocytic ability of the rheumatoid arthritis patients compared with the control group. The percentage of bacteria ingested was slightly higher for both M7 and RP62A compared to the control but was not significant (73.9% c.f. 70.8 for males, 62.2% c.f. 61.2% for females and 74.6 c.f. 68.3 male and 67.3 c.f. 59.1 females). When the phagocytic ability of male patients was compared to female patients again no significant difference arose ($p \leq 0.22$ for M7 and $p \leq 0.35$ for RP62A). So, although the disease is more prevalent in the female there is no difference in the neutrophil kinetics. It may be that the increased numbers of circulating neutrophils in the rheumatoid arthritis patient are working harder, though not significantly more than the control neutrophils. The joints of RA patients contain large numbers of neutrophils and are often red and "hot" to the touch when the patient is undergoing a disease flare. Most of the patients in this group were actually in-patients receiving therapy for a current flare. The patient data collected on inclusion of the patients into the study shows that there were increased CRP levels. This is indicative of an ongoing inflammatory reaction, hence the increased numbers of neutrophils with increased function.

4.4.2.2 Diabetes mellitus

As with rheumatoid arthritis patients there is much literature concerning neutrophil function in the diabetic patient. Immune abnormalities have been demonstrated using *in vitro* models in both patients with insulin-dependent and non-insulin dependent diabetes (Alexiewicz *et al.,* 1995 and Delmaire *et al.,* 1997). Receptors for insulin may be found on PMNLs, monocytes and activated lymphocytes. The binding of insulin to the receptor determines it's metabolic and growth effects, both of which are crucial to the normal functioning of the cells involved in immunity. This has been shown to be impaired in the cells of diabetic patients (Moutschen, Scheen & Lefebvre, 1992).

Adherence, chemotaxis and phagocytosis are all neutrophil functions reported to be affected in the diabetic. In this study only phagocytosis was investigated. When the diabetic patients were compared to the control group, no significant difference in the uptake of either M7 or RP62A could be demonstrated. The uptake was marginally lower for both M7 and RP62A in the male patients and M7 in the females. When males and females were compared to each other a significant difference did occur. Female neutrophils ingested significantly more bacteria than male neutrophils ($p \leq 0.034$). Unlike rheumatoid arthritis, diabetes mellitus has equal prevalence in both sexes. Why female neutrophils are better at ingesting the bacteria than male neutrophils is at first unclear. Age is known to affect neutrophil function but there is no significant difference in the mean ages of the two groups. Male diabetic patients are just as likely to attend clinic appointment as females (this is reflected in the even numbers of male and female patients recruited into the study) and manage their diabetes accordingly. The total number of patients recruited into the study was rather small ($n = 22$). If the study was extended the difference in males and females may not be observed. On the other hand , the increase in phagocytic capacity of the female group may be similar to that observed in the rheumatoid arthritis patients in that more females had either a mild infection or were recovering.

Thus like the patients with RA increased prevalence to infection in the diabetic would not seem to be caused by impaired neutrophil function. Most studies of phagocytosis have been performed on *Staphylococcus* or *Pneumococcus*. Several reports have considered the number of intracellular bacteria evaluated by both microscopic and culture (cfu) methodologies (Bybee & Rogers, 1964; Crosby & Allison, 1966 and Miller & Baker, 1972). In this study bacteria were radioactively labelled and the intracellular bacteria counted in a scintillation counter. The advantage of this radioactive method is that any extracellular, but attached bacteria were removed by centrifugation and washing steps. The afore-mentioned authors did not find any difference in the phagocytic ability of the diabetic patients compared to the controls. However, the above assays may not have differentiated between engulfment and killing. When the total number of bacteria killed is investigated, reduced phagocytosis of opsonised *Pneumococcus* and *Staphylococcus aureus* has been demonstrated in both type 1 and type 2 diabetes (Bagdade, Root & Bulger, 1974; Tan, Anderson & Watanakunakorn 1975 and

Nolan, Beaty & Bagdade, 1978). Bagdade, Root & Bulger, 1974 showed that the effect could be reversed after metabolic control was maintained.

4.4.3 Further research and treatment

Patients with diabetes mellitus (especially type 1) often present with immunologic abnormalities and an increased incidence of other autoimmune manifestations. Specific studies of the host defence mechanisms in these patients may therefore help us to define the importance of auto-immunity related factors (Moutschen, Scheen & Lefebvre, 1992). Conventional drug therapy in RA has failed to control long-term morbidity and mortality associated with RA (Blackburn, 1996). In the treatment of RA, the challenge is to prevent the progression of early disease to the stage of severe erosion and deformity of the joints. As they are currently used, available therapies have limited potential to attain this goal. A study by Wolfe & Hawley, (1985) showed that few patients receiving disease-modifying drugs actually went into remission, and only 2 % remained in remission for more than three years. The relentless progression of RA is reflected in the loss of functional capacity and disability (Makisara & Makisara, 1982).

This was a pilot study using a newly developed model. A larger study with more patients and age-matched controls may yield the answers we require. In this study the control blood samples could not be age-matched. It is very difficult to obtain control bloods from a healthy elderly population.

CHAPTER FIVE

DISCUSSION

Advances in molecular biology, cell biology, and other areas of science have changed the way we understand the mechanisms in which microbial pathogens interact with their hosts (Mitchell, 1998). Despite the powerful advances made possible by molecular biology techniques, the basic concept devised by Smith in 1968, that pathogenicity or virulence is a multi-factorial property that consists of five stages is still valid today. These five stages come under the following headings; attachment to the host, entry to the host, multiplication within the host, interference with host defence systems and damage to the host. Successful infection requires completion of all five stages. Human host defence against bacterial invasion is based on a complex of specific and non-specific defence mechanisms. These include the ability of skin and mucous membranes to resist bacterial penetration, anti-bacterial environments within the host, such as the highly acidic nature of the gut and the phagocytic cells of the immune system that locate, engulf and destroy invading pathogens. One of the major problems of today is nosocomial infection. Such infections are caused by bacteria that are present on, or in, the human host at all times and generally live harmlessly. Problems arise when these bacteria suddenly become detrimental to the host. This is especially true of the coagulase-negative staphylococci. CoNS, and in particular *Staphylococcus epidermidis,* have emerged in recent years as major nosocomial pathogens due to frequent infections associated with implanted medical devices (Mack *et al.,* 1999). Clinical reports have clearly shown that the presence of a biomaterial makes the adjacent tissues susceptible to both immediate and delayed infection (Gristina and Costerton, 1985). The purpose of the present study was to develop a model to study bacterial adherence to different substrates such as bone, prosthetic joint material and polystyrene. The role of the immune systems ability to deal with such adherent bacteria was investigated, by studying phagocytosis of the adherent bacteria. In order to determine the cells with the most efficient phagocytosing capacity, neutrophils were compared with monocytes and macrophages.

5.1 Bacterial adhesion

Bacterial adherence to and growth on biomaterials contribute to the pathogenesis of *Staphylococcus epidermidis* infection (Karamanos *et al.,* 1997). Adhesion is the term used for the immediate binding of a microorganism to a surface, following exposure for one to two hours (Christensen, Baldassarri & Simpson, 1995). The term adherence reflects not only the adhesion of bacteria to a surface, but also the residence time and the stability of the interaction (Linton, Sherriff & Miller, 1999). Bacterial adhesion has been the subject of many studies. Early reports were confusing and there was a lack of any distinction between adherence and accumulation (Rupp & Hamer, 1997). To address this problem several authors have developed models of bacterial adhesion. The first reported technique was developed by Fletcher (1976), when the effect of proteins on the adherence of marine organisms to polystyrene petri dishes was studied. The adherent bacteria were stained with crystal violet. The number of adherent bacteria was then determined indirectly by measuring the extinction of the stained plates at 590nm against clean plates. As the importance of bacterial infections with increasing use of biomedical materials grew, the original method of Fletcher was further developed to study bacterial infection of medical polymers.

Early models of adhesion were rather crude. In 1977 Maki *et al.,* developed a model to study adhesion of coagulase-negative staphylococci to intravascular catheters. Later, Sheth *et al.,* (1983)modified Maki's method, in that sections of catheter material were cut into segments and immersed in bacterial suspensions for various lengths of time. The catheter segments were then rinsed and then the number of adherent bacteria calculated by the colony forming units (CFU) which were counted after the catheter segment had been rolled out onto the agar plate (Maki, 1977). The problem with this method is that it did not account for the number of adherent bacteria that would be found in the lumen of the catheter thus lower results would have been reported than was actually true.

Although there are advantages and disadvantages to every model, slime-producing coagulase-negative staphylococci raise special problems regarding enumeration. When grown in broth to encourage slime production, these microorganisms coagglutinate into an indispersible mass of bacteria. Consequently, slime-producing bacteria cannot be centrifuged or washed, as required in the methods of Sheth *et al.,* (1983) and Ludwicka *et al.,* (1984). Slime production also interferes with counting procedures that rely in colony forming units (CFU's), such as that used by Sheth *et al.,* (1983), since a single CFU may represent a single organism or a cluster of many organisms. Hogt avoided these problems by selecting for study three strains that were not slime producers. The remaining investigators, either raised their organisms under conditions that did not encourage overt slime production, or simply did not address these issues at all. If an investigation into the pathogenesis of infection by coagulase-negative staphylococci regards slime production as a virulence factor, then the method employed to evaluate it should avoid the problems of washing and CFU's. Ideally, the method should also include a time scale that allows for both attachment and accumulation. The current study avoided this problem by developing a model of adherence, where only adherence and not accumulation was measured.

This study employed a radiolabelled method to measure adhesion. Radiolabelled bacterial adhesion assays have the potential to accurately quantify bacterial adhesion to biomaterials. However, initially methods using radiolabelled bacteria had several limitations that impeded accuracy and reproducibility. One particular problem was the use of relevant control strains and the lack of replicates in each experiment. In my study the number of replicates included in each assay performed was three; the mean data in each case was reported. The experiments were repeated several times, especially in the initial stages when the optimal incubation times were being elucidated. There were no problems with unusually high background readings; both strains of bacteria incorporated the radiolabel well and controls were included in each experiment. The design of the experiment was such that only adherent bacteria were measured. The number of non-adherent bacteria was initially measured by counting the number of bacteria in the supernatant and washings. This ensured that there were only adherent bacteria on the surface and not simply bacteria that had settled during the incubation period. This was further checked by the use of lysostaphin, which lysed any extracellular bacteria. As there was no significant difference with the use of lysostaphin this extra step was discontinued. This particular technique was modified from one previously developed in our laboratory to study bacterial adhesion to catheter material (Marshall & Gemmell, 1987).

As biomaterials are often coated with blood proteins in the *in vivo* situation, the role of such proteins promoting bacterial adhesion to biomaterials has also been investigated. The effects of human blood, serum and purified blood proteins on the adherence of both slime-producing and non-slime producing strains of coagulase-negative staphylococci was investigated by Muller *et al*., in 1991. They found that the deposition of blood factors onto plastic biomaterials did not play a significant role in promoting the adherence of coagulase-negative staphylococci. This was in contrast to earlier work reported by Herrmann *et al.,* in 1988; who found that fibronectin, laminin and fibrinogen acted as mediators of adherence of coagulase-negative staphylococci. However, Muller *et al.,* (1991) did find that host proteins in blood may enhance adherence after the initial attachment phase. In my study, the role of complement in mediating phagocytosis of adhered bacteria was investigated. Both strains of *S. epidermidis* were coated with complement. It is known that *S. epidermidis* requires opsonisation in order for efficient phagocytosis to take place. From my results, the effect of opsonising strain M7 was far greater than the effect of opsonising RP62A, although both strains did benefit from complement. The model I developed in this study will allow further investigations, such as the role of blood proteins and fibrinogen in mediating adherence of different strains of bacteria, with scope for many further applications still.

The nature of the surface to which bacterial attachment is studied is of great importance. Many studies are performed on segments of catheter, stainless steel or polystyrene. The surfaces of these products have thus been modified, albeit unknowingly, and this may affect results. This is especially true for catheters. Locci, Peters & Pulverer (1981) showed by SEM that both the internal and external surface of such catheters had intrinsic irregularities that provided convenient sites for bacteria to adhere to and proliferate. Adherence to catheters can occur either internally or externally. An external infection is rarely caused by the coagulase-negative staphylococci and more often represents infection of the surrounding tissue (Bayston, 1984).

This study was performed using discs of prosthetic joint material that were polished, to the state ready for implantation. This was an important fact, as bacterial adhesion had to be quantified to the actual final product. Careful handling of the discs minimised any damage, and gloves and fine forceps were used in the preparation of the multi-well plates containing the discs. This ensured that commensal bacteria did not contaminate the discs during handling. When designing the shape of the discs, it was important to devise a shape that would ensure a snug fit into the wells of the microtitre plate and not leave any gaps. This is why the mushroom shape was developed. The plug (or stalk) of the mushroom angled the disc correctly into the v-shaped well. It also allowed room for the glue that was required to anchor the disc. If too much glue was used, it spilled over the edges and onto the surface of the disc, thus lessening the possible surface area. It was also very important to get the diameter of the disc correct. This ensured the snug fit and therefore minimised the gap between the disc and well wall. A large gap would have resulted in bacteria collecting in this "niche", enabling them to escape phagocytosis by the neutrophils. The height of the disc was chosen carefully so to allow enough space for the bacterial suspension to be placed in the wells. Again, if too much glue was placed in the wells there was not enough room for the bacterial suspension, or the suspension was in contact with the adhesive plastic cover used in the incubation to prevent evaporation. An important benefit in using this model was the ease of its preparation. The plates were set up 24 hours in advance to ensure that the glue was completely dry and the discs were anchored properly. Before any bacteria were added, alcohol was placed in the wells and allowed to evaporate in a fume hood. This was to provide as sterile an environment as possible, and to mimic what occurs in the *in vivo* situation. When a hip, or any other prosthetic joint, is being inserted into the body it is only removed from its sterile protective packaging at the last possible moment.

5.1.1 Bacterial strains used to study adherence

Strain M7 is indistinguishable from strain RP62A in all phenotypic and genetic characteristics, except that it fails to accumulate when attached to glass or plastic (Schumacher-Perdreau *et al.*, 1994). Strain M7 lacks a 140kDa protein that has been shown to be essential for cumulative growth. This protein could only be isolated when the bacteria were grown under sessile cultivation conditions. Under these conditions the bacteria are inoculated onto a membrane which is laid over medium, known to support production of the slime in a petri dish (Hussain *et al.,* 1997). This shows an important property of staphylococci, in that they can rapidly change specific phenotypic features (Christensen, Baddour & Simpson, 1987). It also suggests that the protein essential for cumulative growth is only switched on or activated when in contact with a substrate. Adherence conditions were varied, with both the length of time for maximal adherence and either; under static or shaking conditions. The length of time for bacteria to adhere varies greatly, with some authors reporting attachment almost instantaneous and others reporting that it can take several hours before bacteria are actually attached to the substrate. Ludwicka *et al.*, stated that an incubation time of one hour was required. John *et al.*, (1995) reported that a two-hour incubation time was required for maximal adhesion of *S. epidermidis* NCTC 11047 to catheter material. Then at the other end of the time scale: Pascual *et al.*, (1986) showed that both hydrophilic and hydrophobic strains of *S. epidermidis* were still adhering to teflon catheters well after eighteen hours, whereas Lopez-Lopez, Pascual and Perea (1991) stated that maximum adherence of *S. epidermidis* HUS41 was only attained after 24 hours incubation time. For the purpose of this study, bacteria was incubated for up to three hours for adherence to occur. This allowed for 60% of the initial bacterial innoculum to adhere. This incubation time was selected as we were only interested in the initial stages of adhesion and not secondary accumulation. The percentage of adhered cells did not increase significantly from 90 to 180 minutes for strain RP62A, but did so for strain M7. The incubation time of 180 minutes was therefore chosen, as both strains of bacteria had adhered in equal amounts by this time point.

Rupp & Hamer (1998) have criticised the various, and sometimes poorly, defined strains of bacteria used for adhesion studies. *S. epidermidis* RP62A has been extensively used for adhesion studies (Muller *et al.*, 1991; Karamanos *et al.,* 1997; Schwank *et al.,* 1998 & Linton, Sherriff & Millar, 1999) and it was for this reason that this particular strain and its non-biofilm forming mutant M7 was chosen to compare adhesion to bone, polystyrene and ultra-high molecular weight polyethylene. Recent studies have identified important proteins in attachment and accumulation. This has been elucidated using mutants created by the insertion of plasmid DNA. Two distinct classes of biofilm-negative mutants have been characterised by the use of *Tn*917 insertion sequences. *S. epidermidis* O-47 affected in initial attachment were named as class A mutants and those affected in intracellular adhesion and cell aggregation were named class B mutants (Heilmann *et al.,* 1997). When the mutant was complemented with the appropriate stretch of DNA, it was able to attach to a polystyrene surface and form a biofilm. Further study of the important factors of why certain strains are able to accumulate led to the discovery of a genetic locus known as the intracellular adhesion locus.

5.1.2 The genetics of biofilm formation

Biofilm formation has been shown to be mediated by the *ica* operon. This operon consists of *ica*A, *ica*D, *ica*B and *ica*C genes (Heilmann *et al.,* 1996 & Gerke *et al.*, 1998). The actual function of the individual genes is as yet not understood, but it is though that co-expression of *ica*A, *ica* D and *ica* C is required for production of PIA (Gerke *et al.,* 1998). When this operon is activated, a polysaccharide adhesin is synthesised. This adhesin is named polysaccharide intracellular adhesin (PIA). The purpose of this adhesin is to mediate cell to cell contact and trigger the production of the biofilm (Ziebuhr *et al.*, 1999). Thus, the presence of the *ica* operon significantly contributes to the virulence of *Staphylococcus epidermidis*. So, it is not the ability to produce slime that affects virulence, but the presence or absence of the *ica* gene cluster. The knowledge of which strains possess this locus would therefore indicate the more virulent and therefore more dangerous strains of *S. epidermidis*. The ability to engineer a vector that would knock out this locus may prevent some of the more severe *S. epidermidis* infections occurring in our hospitals

5.2 Hydrophobicity

The last two decades has witnessed extensive research into the hydrophobic properties of microbial cells. Consequently, cell surface hydrophobicity has been implicated in a number of adhesion phenomena (Rosenberg & Doyle, 1990). Numerous tests have been proposed to measure cell surface hydrophobicity and in many cases the cell structures that either promote or inhibit hydrophobicity have been identified. The first experiments were performed by Mudd & Mudd in 1924, who demonstrated bacterial partitioning at the oil-water interface. Reed and Rice then demonstrated that nonwettable mycobacteria were able to pass from the aqueous to the oil phase (Reed & Rice, 1931). Experiments were sporadic in the following years until the 1970's, when bacterial hydrophobicity emerged once again and was the topic of many papers (Tanford, 1973; Marshall & Cruikshank, 1973 and Hjerten, Rosenberg & Pahlman, 1974). The first work investigating the role of hydrophobicity in phagocytosis came from van Oss and Gillman in 1972. Many techniques to measure bacterial hydrophobicity were introduced in the late 1970's and early 1980's. Such techniques included: two-phase partitioning (TPP), hydrophobic interaction chromatography (HIC), microbial adherence to hydrocarbons (MATH) and the salt aggregation test (SAT). Correlation between the tests is sometimes difficult to prove. In some cases researchers present data from two different methods but fail to show any correlation between them (Rosenberg & Doyle, 1990).

Two methods were used in this study: HIC and MATH. Both methods are completely adhesion based and their outcome is determined by all parameters involved in adhesion, including not only hydrophobicity, but also the presence of surface appendages and localised groups. This is in contrast to techniques such as contact angle measurements that measure the overall cell surface hydrophobicity. MATH and HIC correlate well, as was shown here with Spearman's rank correlation coefficient scores of 0.04 and 0.05 for M7 and RP62A. They are simple to perform and did not require any specialised equipment. HIC is recommended for detecting hydrophilic carbohydrate capsules, microcapsules, or other carbohydrate surface structures. MATH has been used before in the comparison of encapsulated and non-encapsulated coagulase-negative staphylococci (Hogt *et al.,* 1986). Hogt *et al.,* found that the encapsulated strains were more hydrophobic than the unencapsulated strains. When van der Mei, van de Beltgritter and Busscher, (1995) used water contact angle measurements they could not demonstrate any difference in the hydrophobicity of either encapsulated or unencapsulated strains. One potential limitation of the BATH technique is that there is the possibility that cells are damaged during the vortexing procedure in the presence of the hydrocarbons. However, van Haecke *et al.,* showed that performing the technique in the presence of hexadecane has no effect on cell intactness, whereas when performed in the presence of xylene, cell lysis occurred. It was for this reason that the tests were performed with hexadecane. It has been shown that for tests of hydrophobicity of coagulase-negative staphylococci, BATH has the highest positive predictive value (Martin *et al.,* 1989).

A major problem in studying the adherence of *S. epidermidis* is the enormous amount of strain to strain variation that occurs. Most strains are able to attach to polymer surfaces, albeit with quantitative differences between the strains (Espersen *et al.*, 1990; Hogt, Dankert & Feijen, 1986 and Pascual *et al.*, 1986). As previously mentioned; there are two stages in adherence. The first stage is mediated by hydrophobic interactions, whereas the second stage is concerned with accumulation, which is where slime is important. The findings of different investigators are that extracellular slime does not play an important role in the early phase of attachment to the biomaterial surface (Hogt, Dankert & Feijen, 1986; Timmermann *et al.*, 1991 and Pascual *et al.*, 1986). Hydrophobicity appears to be correlated to cell surface proteins, since both hydrophobicity and adherence to the biomaterial are reduced by protease treatment (Nilsson *et al.,* 1998). The two strains of *Staphylococcus epidermidis* tested here were both found to be hydrophilic in nature. Evidence has shown that the presence of a capsule results in decreased hydrophobicity and adhesion for *Staphylococcus aureus,* (Reifsteck, Wee & Wilkinson, 1989), *Streptococcus pyogenes* (Ofek, Whitnack & Beachey, 1983) and *Klebsiella pneumoniae* (Benedi, Ciurana & Thomas, 1989). Rosenberg and Doyle (1990) suggested that the coagulase-negative staphylococci could be excluded from this group of organisms. My results agreed with the observation that the presence of a capsule results in reduced hydrophobicity, due to the hydrophilic nature of strain RP62A. However, the non-encapsulated M7 was also hydrophilic. It may be that these two strains are indeed hydrophilic and that strain RP62A is simply more hydrophilic than strain M7, due to the presence of extracellular slime (or capsule). Growth conditions are also known to influence hydrophobicity. Both strains were grown under the same conditions. They were maintained on Columbia

Blood agar (CBA) plates and grown in Mueller-Hinton broth at 37°C for 18 hours. None of these conditions are reported to influence hydrophobicity in any way. Such strain variation is also observed with *Streptococcus pneumoniae.* Most strains of pneumococci are encapsulated, and bind poorly to hexadecane or plastic (Courtney, Hasty & Ofek, 1990). This suggests that the organisms are not hydrophobic, but in fact hydrophilic.

These two strains were chosen because they differed in their ability to accumulate i.e form a biofilm on a substrate. The fact that they are both hydrophilic shows that hydrophobicity is not affected by slime production. If hydrophobicity was affected by slime production, then we may have seen a difference in the hydrophobicity of the two strains. Both strains were grown at 37^0C for 18 hours, by which time slime production by RP62A had started.

The bacterial surface can be considered to be a mosaic of molecules possessing different physical attributes: such as being positively or negatively charged, hydrophilic, hydrophobic, or amphipathic. It is the balance of these attributes that confers upon the bacterium its net surface charge, or renders the organism hydrophobic (Courtney, Hasty & Ofek, 1990). Investigators have demonstrated the hydrophobic nature of most of the organisms that colonise medical devices (Klotz, 1990). The coagulase-negative staphylococci are the microbes associated with infections of plastic devices at almost any site of the body, and hence have come under great scrutiny. Plastic medical devices are generally hydrophobic and thus non-wettable in nature. As a rule they generally favour the adherence of organisms to their surfaces (Klotz, 1990).

The importance of hydrophobicity and adherence to substrates such as prosthetic joint implants, contact lenses and teeth has increased in importance. The microfouling of these plastic devices following adhesion to the surfaces results in inefficiency and destruction of the underlying materials (Klotz, 1990). This has led researchers to try to develop mechanisms to prevent adhesion. Surface-active agents reduce hydrophobic interactions, and by doing so reduce microbial adhesion to plastic (Klotz, 1990). The decrease in adhesion can be explained by a reduction in the surface tension of the suspending medium caused by the surfactant. Creating a situation where the interfacial tensions between the bacterium, the surface and the suspending medium of the bacterium are such that adhesion cannot occur. Hydrogel is a hydrophilic gel that is used to coat the surface of catheters. This gel was developed primarily for its haemocompatibility, which reduces the incidence of device-related thrombophlebitis-inflammation of a vein associated with thrombus formation. The hydrogel coating is very hydrophilic which indicates a high surface energy. Such negatively charged, hydrophilic surfaces discourage bacterial adhesion and it was hoped that this hydrophilic polymer might reduce the adhesion of bacteria to its surface by electrostatic repulsion. A study by Kristinsson (1989) failed to demonstrate a statistically significant reduction in the adhesion of either *S. aureus* or *S. epidermidis.* Yet both Denyer *et al.,* (1990) and John *et al.*, (1995) reported reduced adhesion to catheters coated with hydrogel. The differences may be explained by differences in the strains used to investigate adherence.

5.3 Phagocytosis

Cells have evolved a variety of strategies to internalise particles and solutes, including pinocytosis, receptor-mediated endocytosis and phagocytosis (Aderem & Underhill, 1999). Pinocytosis refers to the uptake of fluid and solutes and is closely related to receptor-mediated endocytosis. By contrast, phagocytosis is concerned with the uptake of larger particles ($\geq 0.5\mu m$ in diameter). Most cells have some sort of phagocytic capacity, but neutrophils are referred to as professional phagocytes and are very efficient at internalising particles (Rabinovitch, 1995). The non-professional phagocytes (such as retinal epithelial cells) have intermediate phagocytic ability. The major difference with respect to phagocytic capacity and efficiency of professional and non-professional phagocytes, can probably be ascribed to the presence of an array of dedicated phagocytic receptors that increase particle range and phagocytic rate. The transfection of fibroblasts and epithelial cells with cDNAs encoding Fc receptors (FcRs) dramatically increases the phagocytic rate of the cells (Metchnikoff, 1905).

Phagocytosis is extremely complex, and no single model can account for the diverse structures and outcomes associated with particle internalisation. This complexity is, in part, due to the diversity of receptors capable of stimulating phagocytosis, and in part, due to the capacity of a variety of microbes to influence their fate, as they are internalised (Aderem & Underhill, 1999). In the present study, the ability of neutrophils to phagocytose adherent *S. epidermidis* from the surfaces of prosthetic joint material, bone and polystyrene was investigated.

Many microorganisms have developed strategies, which may reduce the efficacy of phagocytosis. The most straightforward anti-phagocytic approach is to kill the phagocyte. Some bacteria, as they multiply in tissues, release soluble materials that are lethal for phagocytes. The coagulase-negative staphylococci release haemolysins, proteinases, DNAases, and lipases (Gemmell, & Schumacher-Perdreau, 1986). Other bacteria produce substances that paralyse neutrophils, and hinder the directional movement of polymorphs to the vicinity of the bacteria. As a result of inhibiting chemotaxis, the host is less able to focus polymorphs and macrophages into the exact site of infection. Many virulent microbes are equipped with cell wall structures that enable them to resist opsonisation, e.g. *H. influenzae.* Capsules also prevent complement activation and reaction between IgG and cell wall epitopes. The capsule may also prevent the polymorphs from intracellular killing of bacteria. Once ingested, bacteria utilise several mechanisms to escape toxic events in the phagolysosome. Some of these avoidance mechanisms impact upon the host's immune response in such a way that opportunistic infections can occur.

The phagocytosis of two strains of bacteria by three different phagocytic cells was investigated. The strains differed in that M7 lacks a 140kD surface protein. This protein is required for M7 to accumulate on a surface. The incubation time for adherence to occur was three hours. If the bacteria were able to both adhere, and start to accumulate in this time period, then it was thought that there would be a difference observed between the two strains. This difference would be in the phagocytosis of the bacteria. Slime may affect phagocytosis. It may either enhance ingestion, and if so, then we may observe an increase in the phagocytosis of RP62A, or, it may protect the bacteria from the neutrophils, resulting in a decrease in ingestion. This study demonstrated no difference in either the uptake of M7 or RP62A. From this we can assume either, that at three hours slime production by RP62A is minimal, or that slime has no effect on phagocytosis. In order to test this hypothesis we would have to incubate the bacteria for longer time periods. This study used washed cell preparations resuspended in PBS. This does not provide the requisite nutrients required for outgrowth of the bacteria. The cells did not gain any nutrients from the surface of the prosthetic discs themselves. In order to perform longer experiments the bacteria would need to be incubated in some sort of nutrient broth. This would involve constant nutritious environment, and so may not have been able to produce slime. It was postulated that slime might have two effects on phagocytosis. It may protect bacteria for the scavenging effect of neutrophils, or it may actually enhance phagocytosis. As slime was not produced by strain RP62A, this question could not be answered. In order to answer the question, the timing of the experiments would have to be redesigned, which was not possible.

The different cell types investigated were neutrophils, monocytes and a macrophage like cell line, J774. All of these cells are professional phagocytes and are very efficient at internalising particles (Aderem & Underhill, 1999). Both macrophages and monocytes are thought important in the pathogenesis of chronic inflammatory disease such as rheumatoid arthritis, and have been shown to have increased phagocytic capacity (Steven *et al.,* 1984). The cells with the best phagocytic capacity were the neutrophils, followed by the J774 cells and then the monocytes. Of those cells, neutrophils are the first to arrive at the site of infection or injury. This may be due to their superior phagocytic capacity.

5.4 Phagocytosis by different patient groups

Neutrophils differ in their functional capacity from individual to individual. These differences are subject to underlying clinical condition, infection and the possible effect of medication. It was decided to investigate whether there was any difference in the phagocytic capacity of neutrophils from two groups of patients.

Diabetic patients are reported to have a decreased neutrophil activity. Rheumatoid arthritis patients on the other hand are reported to have an increased activity.

5.4.1 Rheumatoid Arthritis Patients

The Hollander hypothesis (1965) states that large numbers of PMNLs migrate from the blood into the articular synovial fluid, wherein the cells phagocytose immune complexes, and in doing so release synovial enzymes which mediate cartilage erosion. A number of workers have sought to differentiate whether PMNL function is impaired in rheumatoid arthritis patients compared to normal controls. The reasoning behind this hypothesis is that an impaired neutrophil function is the cause for increased infection observed in these patients (Baum, 1971; Uddin, Krause & Kelly, 1970 and Mohr & Wessinhage, 1978). Initial reports stated that both chemotaxis (Mowat & Baum, 1971 and Roberts-Thomson *et al.*, 1976) and phagocytosis (Bodel & Hollingsworth, 1966; Corberand *et al.,* 1977; Wilton *et al.*, 1978 and Attia *et al.*, 1982) were impaired. Later reports did not agree with these theories (Turner, Schumacher & Myres, 1973; Hallgren *et al.*, 1978; Hanlon, Panayi & Laurent, 1980; Sheehan, Brown & Dumonde, 1984 and King *et al.*, 1986). An even more recent report stated that neutrophils from rheumatoid arthritis patients were in fact more responsive to priming agonists and stimulation than those from controls (Eggleton *et al.,* 1995). Thus, instead of being impaired in function, the trend has now completely reversed and much of the problems in rheumatoid arthritis, such as tissue degradation and exacerbation of the immune response may now be attributed to overstimulation of neutrophils. The original chemotaxis studies may have been impaired by experimental design, as it is difficult to relate laboratory conditions to the *in vivo* situation, where vast numbers of neutrophils are migrating into the joint space (Brown, 1988). The problem in evaluating the phagocytic ability of neutrophils from patients with RA is that although the neutrophils are perfectly able to ingest the bacteria, they contain immune complexes that may interfere with the digestion of the internalised substances.

Chemiluminescence studies suggest that neutrophils of RA patients are not impaired in their function, but are quite the opposite. An increased chemiluminescent response has been observed in RA patients when they were compared to normal controls (Wandall, 1985). Unfortunately, due to the problems recruiting patients, chemiluminescent studies could not be performed with the cohort studied here. From the radioactive phagocytosis studies performed in the present study, there were no significant differences between the uptake of bacteria by both RA patients and controls. Therefore, my results are in agreement with those more recent studies; that neutrophil function is not impaired in the rheumatoid arthritis patient. Rheumatoid arthritis is unusual, in that the large-scale movement of the neutrophils into the synovium is more characteristic of a persistent acute inflammatory reaction, and not a chronic inflammatory disease. This is perhaps why we do not observe a decrease in their phagocytic capacity when compared to normal controls, as they have simply adapted to their environment. In addition, RA patients are a very diverse group in the nature of their disease, there are many different therapies available, and all of which may affect results.

Microorganisms have been implicated as the cause of many rheumatic diseases (Goldenberg, 1998). Bacterial arthritis is the most rapidly destructive joint disease, with an incidence of 30-70 per 100,000, in patients with rheumatoid arthritis and those who have undergone joint replacement surgery (Kaandorp *et al.*, 1995). Bacteria may be introduced into the joint during surgery or joint aspiration. The most common causative agents of bacterial arthritis are staphylococci, with *Staphylococcus aureus* being the causative agent in 40% of cases in a recent study performed in England and Wales (Ryan *et al.*, 1997). *Staphylococcus aureus* causes 80% of joint infections in patients with concurrent rheumatoid arthritis and in those with diabetes (Goldenberg, 1998). Host factors, which predispose to bacterial arthritis, include the patients age, decreased immunocompetence and pre-existing joint disease. In a study performed in the Netherlands, aged greater than 80 years; diabetes mellitus and rheumatoid arthritis were found to be important independent risk factors (Kaandorp *et al.*, 1995). Septic arthritis is most likely in patients with long-standing rheumatoid arthritis. In some cases, treatment is often delayed due to a mis-diagnosis of exacerbation of the underlying rheumatoid arthritis. If one or two joints suddenly become inflamed in a patient with rheumatoid arthritis, then septic arthritis should be suspected until proven otherwise (Goldenberg, 1998). Treatment usually requires antibiotics and drainage of the infected joint. The outcome of bacterial arthritis has not changed significantly. Permanent joint damage develops in about 50% of cases and the mortality is 10-16% (Ryan *et al.*, 1997). The large number of joint arthroplasties performed has resulted in joint prostheses being the most important risk factor for septic arthritis. About 50,000 hip replacements are performed in the UK annually and about 300,000 world-wide. Two –thirds of these patients are over 65 years of age. Although the majority of patients with arthritis undergoing hip replacement have osteoarthritis (85%), the quality of life for those with rheumatoid arthritis can be dramatically improved by hip replacement (Crawford & Murray, 1997). Deep infection occurs in around 0.5-2% of total hip replacements, and is the cause for revision surgery in 7.5% of cases. Infection is more common in patients with rheumatoid arthritis, diabetes mellitus, chronic renal failure, patients taking corticosteroid treatment and high risk surgical patients (Crawford & Murray, 1997). Female rheumatoid arthritis patients who are under 55 years of age are a high-risk group for failure, with a 25% failure rate by 15 years. This may be due to the changed biological activity of the host bone, although it is not clear (Havdrup, Hulth & Telhag, 1976).

5.4.2 Diabetic patients

Neutrophil dysfunction has been hypothesised as the reason behind recurrence and increased frequency of infection in the diabetic patient. Several studies have elicited abnormalities of at least one of the functions of neutrophils, but few have investigated the full functioning of the cells (Delmaire *et al.*, 1996). The causes for the abnormalities have remained inconclusive. A study by Delmaire *et al.,* in 1996 investigated neutrophil function in 61 diabetic patients. This group showed that there was no impairment of a single neutrophil function, but more of a global dysfunction of all. They hypothesised that this could be the result of the combination of several factors. When they studied phagocytosis by internalisation of latex beads, they found no difference in the phagocytic capacity of the neutrophils from the diabetic patients, compared with the control population. Authors have reported conflicting results over the study of phagocytosis. Like

Delmaire *et al.*, Wilson & Reeves (1986) found no difference in the uptake of *Candida albicans* by patients with diabetes compared to controls, nor did Dziatowiack *et al.,* in 1982 when they investigated the phagocytosis of *Candida albicans, Staphylococcus aureus* and mycobacteria. On the other hand, Marhoffer *et al.,* 1992 and Nolan *et al.,* 1978 reported a decrease in the phagocytic capacity of diabetic neutrophils. In the present study, the phagocytic capacity of 21 patients with insulin-dependent diabetes was compared to a control population.

The results of the present study agree with those of Delmaire *et al.,* (1996); Wilson & Reeves (1986) and Dziatowiack *et al.,* (1982), in that no significant difference in the phagocytic uptake of *Staphylococcus epidermidis* M7 or RP62A from the surface of prosthetic joint material between patients and controls could be demonstrated. This is in accordance with a study performed in Glasgow. In the Glasgow study, the chemiluminescent response of PMNLs of patient and control subjects to *Staphylococcus aureus* cowan were compared. No significant difference between patient and controls could be demonstrated (Gallacher *et al.,* 1995). The patients in this particular study all had very good glycaemic control, with levels near that of controls. In my study, the glucose control levels were poor, with the mean level being 11.6 (range 8.6-13.6mmol/l). Glucose tolerance is traditionally classified by the 1985 World Heath Organisation (WHO) criteria into three categories; normal, impaired and diabetic (Weyer, Borgardus & Pratley, 1999). Currently, diabetes diagnosis depends on a venous plasma glucose concentration that exceeds 7.8mmol/l (Keen & Barnes, 1997). A level below 6.0mmol/l means that diabetes mellitus can be excluded. A value in-between these ranges indicates glucose intolerance, and that further testing is required. Therefore, the similarity between patients and controls in my study was not due to differences in glucose control. When the data was taken one step further and the patients were compared in two groups, split by sex, then a significant difference was observed between males and females ($p \leq 0.034$). The reason for this difference is unclear. It is not due simply to there being a greater number of females screened, as the groups were split evenly, with the ratio of females to males being 11:10. The other studies discussed above did not split their patients into groups based on sex. This would have perhaps given us the backup we require to state whether or not sex has an effect on neutrophil function. Unlike rheumatoid arthritis, diabetes mellitus does not have a greater prevalence in the female population.

Priming neutrophils from diabetic patients is known to increase their chemiluminescent response (Delmaire *et al.*, 1996). CRP is a valuable tool in determining the immunological status of a patient. This would tell us whether the patient was fighting infection at the time of blood sampling, and thus had primed neutrophils. CRP levels were not measured at the time of patient recruitment into the study. The patients were attending the diabetic drop-in clinic for monitoring of their blood glucose levels. CRP levels would not be measured unless infection was suspected. The case notes would need to be recalled for each of the patients, with special attention paid to C-reactive protein (CRP) levels if they were available. This study was based on a total of twenty-one patients, if the study was to be continued and a larger number of patients recruited into the study, then the difference observed between male and female diabetic patients may be lost. Diabetics are a very heterogeneous group of patients and this phenomenon may not be observed in a larger, more widespread study.

The key benefit of this study was the development of a model which gave good reproducible results, and enabled bacterial adhesion and neutrophil function studies. Further studies could investigate the following factors:

- Temperature-the bacteria were allowed to attach at 37°C. Decreasing the incubation temperature from 37°C to room temperature (15-22 °C) may have an inhibitory effect on initial adhesion.
- Incubation time-increase the time from three hours to allow slime production
- Incubation media- i.e. nutrient broth cf. PBS or the addition of blood to nutrient broth to investigate blood products and how they affect slime production
- Different strains of bacteria - not only CoNS, but other bacteria found in joint infection such as *Staphylococcus aureus,* and the role of protein A in mediating attachment
- Other substrates used in the development of prosthetic joints, such as titanium, ceramics and PMMA.

- Use substrates of known hydrophobicity. The strains of *S. epidermidis* investigated in this study were both hydrophilic and adhered well to the substrates investigated. The surface hydrophobicity of the substrates in the present study was not determined, so investigating known hydrophobic or hydrophilic substrates may determine those best at resisting adherence by hydrophilic strains of *S. epidermidis*
- The effect of coating the discs of prosthetic joint material with antibiotics, and/or antiseptic agents- silver sulphadiazine and chlorhexidine, have been shown to inhibit adherence and biofilm formation on central venous catheters, the effect of such agent on hip material needs to be investigated
- Studying antibiotic resistant strains (MRSA, MRSE). This is a very important fact for current medicine, as isolation of these antibiotic resistant strains is increasing and they are known to increase mortality
- Further study of susceptible patient groups, such as those with osteoarthritis
- The effect of disease therapy i.e first and second line drugs for rheumatoid arthritis and insulin for diabetes mellitus

This study has shown that of the two strains of bacteria investigated both adhered well to discs of prosthetic joint material. When adherence was compared to bone and polystyrene, the greatest adherence occurred to bone, with similar values obtained for prosthetic joint material and polystyrene. Both strains proved to be hydrophilic in nature. When phagocytosis of the bacteria was investigated, opsonising the adhered bacteria significantly increased their ingestion. In the comparison of the different cells of the immune system that are able to phagocytose particles, the neutrophils were the most efficient at ingesting the adhered bacteria. When phagocytosis of bacteria from bone, UHMWPE and polystyrene were compared, there was no significant difference in the ingestion of adherent bacteria from native bone, to either UHMWPE or polystyrene. Thus neutrophils do not see plastic medical devices as “foreign”, and are able to attach and internalise bacteria as normal.

APPENDIX A

Bouin's Fixative

Saturated aqueous picric acid	75ml
40% Formaldehyde	25ml
99% Acetic acid	5ml

Columbia Blood Agar (CBA) Gibco

Columbia Agar:

Peptone Special	23g
Corn Starch	1g
Sodium Chloride	5g
Agar	15g

Dissolve in 1 litre distilled water. Add defribrinated horse blood (5% vol/vol)
Sterilise by autoclaving at 121°C for 15 minutes.

Gel-hanks

Distilled water	80ml
1% Gel	10ml
Hanks Balanced salt solution	10ml

Adjust pH by adding 0.1M NaOH

Luminol

Luminol (5-amino-2,3-dihydro-1,4 phthalazinedione)	1.77mg
Dimethyl sulphoxide	1ml

Dissolve luminol in dimethyl sulphoxide to give a concentration of 10^{-2}M.
Dilute to 10^{-5}M with sterile PBS before use.

Mueller Hinton Broth

Beef, dehydrated infusion from	300g
Casein hydrolysate	17.5g
Starch	1.5g

Dissolve 21g in 1 litre of distilled water. Sterilise by autoclaving at 121°C for 15 minutes.

Octyl sepharose

Octyl-Sepharose CL-4B
Pharmacia
Fine Chemicals AB
Uppsala
Sweden
Particle size: 40-190μm
Ligand concentration: Approximately 40μmole/ml gel bed

Wash ten times with distilled water to remove all traces of ethanol. Dissolve in 100% Seven salts solution. Store at 4°C

Phosphate Buffered Saline

NaCl	8.0g
KCl	0.2g
Na_2HPO_4	1.15g
KH_2PO_4	0.2g

Dissolve in 1 litre of distilled water. Sterilise by autoclaving

Seven Salts Solution (NSS)

NaCl	23.48g
Na_2So_4	1.96g
$NaHCO_3$	0.10g
KCl	0.33g
$MgCl_2.2H_2O$	2.49g
$CaCl_2.2H_2O$	0.55g
H_3BO_3	0.01g

Dissolve in 1000ml double-distilled water. Adjust pH to 8.2

Trypan blue vital stain

Trypan Blue (Vital stain C I 23850)	0.40g
NaCl	0.81g
K_2HPO_4	0.06g
Methyl p-hydroxybenzoate	0.05g
Water	900ml

Heat mixture to boiling, cool and adjust pH to 7.2-7.3 with 1 molar NaOH (approximately 8 drops). Adjust volume to 1000ml.

APPENDIX B

STATISTICAL FORMULAE

All statistical evaluations were performed using MINITAB software. Tests performed were

1. Student T-test

$$t = \frac{(\bar{Y}_2 - \bar{Y}_1)\sqrt{N}}{\sqrt{(s_1^2 + s_2^2)}}$$

The student's t-test was used to evaluate the following:

- Strain to strain differences in the chemiluminescent response, the time taken to reach the peak CL response and the rate of the reaction
- The effect of opsonisation on the CL response, the time taken to reach the peak CL response and the rate of the reaction
- The difference in optical density values between the strains after staining with alcian blue
- Differences between strains in the adherence of M7 and RP62A to different substrates
- Differences in the phagocytic uptake of the two strains form different substrates
- Differences in the uptake of M7 and RP62A by different cell types (PMNLs, MNs and J774 cells)
- Differences between patient groups and a control group in the uptake of M7 and RP62A by PMNLs.

2. Spearman's Rank Correlation Coefficient Test

Spearman's Rank correlation coefficient test was used to determine whether the two methods employed to determine the hydrophobicity levels of the two strains correlated or not.

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