SURFACE PROPERTIES OF *ENTEROCOCCUS FAECALIS* IN RELATION TO INFECTIVE ENDOCARDITIS

Submitted by

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Summary

The effect of growth conditions on both the appearance and the antigenic profile of cells of *Enterococcus faecalis* was investigated using electron micrographs of ruthenium red stained and sectioned cells and SDS-PAGE and blotting techniques respectively.

Three specific antigens of molecular weights 73, 40 and 37 kdaltons were of particular interest being expressed most strongly after growth in serum. This medium was deemed to most closely mimic *in vivo* growth conditions reflecting an environment similar to that which the microorganisms would encounter during bacteraemia, preceding the colonisation of the endocardium and the development of infective endocarditis.

The 40 and 37 kdalton antigens were shown by immunogold labelling to be exposed on the surface of the cells although they did not appear to be connected with the fimbriae shown to exist on some of the *E.faecalis* cells examined by negative staining.

The 73, 40 and 37 kdalton antigens were crudely purified using sarkosyl and ammonium sulphate precipitation, and used as the basis of a serodiagnostic test for *E.faecalis* endocarditis using an ELISA system. This was tested in a blind trial and the success rates were 94% for positives, 90% for negatives with endocarditis caused by other organisms and 80% for *E.faecalis* infections other than endocarditis.

The binding of *E.faecalis* cells to the serum proteins fibronectin and albumin was investigated using ^{125}I labelled proteins, followed by Scatchard analysis. This showed that *E.faecalis* cells do loosely bind large amounts of both of these proteins, thus surely affecting the way in which the host's immune system perceives the cells. The *E.faecalis* receptor for fibronectin was partially characterised and appeared to involve protein and/or carbohydrate containing components, but did not involve LTA or the 40 and 37 kdalton species specific antigens.

Dedicated to my parents, Joan and Albert,

and to all my friends from Aston

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ABBREVIATIONS

AHA	American Heart Association
AMPS	Ammonium persulphate
BATH	Bacterial adherence to hydrocarbon
Bis	N,N'methylene bisacrylamide
BSA	Bovine serum albumin
BSAC	British Society for Antimicrobial Chemotherapy
°C	Degrees centigrade
CDM	Chemically defined medium
CIA	Clumping inducing agent
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
Fab	Antigen binding fragment of immunoglobulin
Fc	Crystallizable fragment of immunoglobulin
FN	Fibronectin
HS	Heat-inactivated horse serum
IE	Infective endocarditis
Ig	Immunoglobulin
IV	Intravenously
K	Thousand
L	Litre
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
m	Metre
M	Moles per litre
µ	Micro
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
NBTE	Non-bacterial thrombotic endocarditis
NHS	Normal human serum
NVS	Nutritionally varient streptococci
OD	Optical density
PBS	Phosphate buffered saline
PG	Peptidoglycan
PMSF	Phenylmethylsulphonyl fluoride
PS	Polysaccharide
PVE	Prosthetic valve endocarditis
RF	Retardation factor
RNA	Ribonucleic acid
rpm	Revolutions per minute
Sarkosyl	Sodium lauryl sarcosinate
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TA	Teichoic acid
TU	Teichuronic acid
UTI	Urinary tract infection
V	Volts
v/v	Volume per volume
w/v	Weight per volume

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1 SHORROCK P J, LAMBERT P A (1989). Binding of fibronectin and albumin to *Enterococcus (Streptococcus) faecalis*. Microbial Pathogenesis; 6: 61-67.

2 SHORROCK P J, LAMBERT P A, SMITH E G, FARRELL I D, GUTSCHIK E, AITCHISON E J. The serological response in *Enterococcus faecalis* endocarditis using an enzyme-linked immunosorbent assay. Journal of Clinical Microbiology. Currently in press.

3 LAMBERT P A, SHORROCK P J, AITCHISON E J, DOMINGUE P G, POWER M E, COSTERTON J W. Effect of *in vivo* growth conditions upon expression of surface protein antigens in *Enterococcus faecalis*. FEMS Microbiol. Immunol. Currently in press.

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1 AITCHISON E J, SHORROCK P J, LAMBERT P A, SMITH E G, FARRELL I D. Serodiagnosis of *Streptococcus faecalis* endocarditis by western blotting of surface protein antigens. 3rd European Congress of Clinical Microbiology, 11-14 May 1987, p142.

2 SHORROCK P J, LAMBERT P A. Binding of fibronectin and albumin to an endocarditis isolate of *Streptococcus faecalis*. Society for General Microbiology, 111th Meeting, Warwick, 11-14 April 1988, p24.

3 SHORROCK P J, LAMBERT P A, SMITH E G, FARRELL I D, GUTSCHIK E. Serodiagnosis of *Enterococcus faecalis* endocarditis using an enzyme linked immunosorbent assay. American Society for Microbiology Annual Meeting 1989, New Orleans, LA, 14-18 May 1989.

1 INTRODUCTION

1.1 Streptococci: history and classification

The present *Bergey's Manual of Systematic Bacteriology* lists seven genera of facultatively anaerobic, Gram positive cocci. Five of these including Streptococcus do not contain cytochrome enzymes, and are thus catalase negative (Schleifer, 1985).

Streptococci were first described by Billroth (1874) as chain-forming organisms he observed in infected wounds. In 1884 Rosenbach gave the same *Streptococcus pyogenes* to cocci that grew in chains and had been isolated from superative lesions in man. In 1919 Brown introduced the terms alpha (α), beta (ß) and gamma (δ) to describe the three types of haemolytic reactions observed on blood agar plates by streptococci. The ß-haemolytic streptococci produce a wide clear zone of complete haemolysis in which no red cells are visible on microscopic examination. α-haemolytic streptococcus colonies are surrounded by a nearly colourless zone of haemolysis resulting from destruction of erythrocytes, and a zone of discoloured but intact erythrocytes close in around the deep colonies. These erythrocytes have a green or brownish green colour. δ-haemolytic streptococci produce no haemolysis either on the surface or within the agar. To be absolutely certain of the haemolytic characteristics of a strain it is necessary to examine colonies located beneath the surface of a pour plate as surface colonies sometimes produce deceptive appearances or growth spreading over and hiding haemolytic zones. Another important and useful method of differentiating species is the use of serological typing which followed the detection of the

group specific antigens by Lancefield (1933). In 1924 Hitchcock first observed a soluble specific substance upon which the serological grouping of streptococci came to be based (Hitchcock, 1924a). In 1933 Lancefield discovered the serological specificity of the substance which led to the development of her grouping system for streptococci. This divided the genus into approximately 20 groups, and each was assigned a letter of the alphabet (Lancefield, 1933).

The group specific antigens of streptococci are polysaccharides (for example as in groups A,B,C,E,F,G,H,K,L, P and U) or teichoic acids (as in groups D and N). Members of each Lancefield group are generally biochemically uniform (with the exception of group C which contains recognisably different biotypes) and cause similar diseases in a characteristic host range (Parker, 1975).

The chemical nature of the antigen has now been established for some streptococcal groups. The group antigen in group A and B streptococci for example is composed of L-rhamnose and N-acetyl galactosamine (Hammond et al, 1984) and the amino sugar is responsible for the antigenic specificity of the polysaccharide. The group D and N streptococci possess a teichoic acid group specific antigen (Elliott, 1960; Wicken et al, 1963) and further studies have indicated that the group antigen is a membrane or lipoteichoic acid (Archibald and Baddiley, 1966; Wicken and Knox, 1975).

The group specific antigen was detected serologically by precipitin techniques using antisera which were prepared by injecting whole cells into rabbits (Hitchcock, 1924a and 1924b;

Lancefield, 1928 and 1933). However cross reactions may occur between certain species of streptococci and indeed recently strains of *E.faecalis* have been reported that react with group G serum as well as group D (Birch et al, 1984; Harvey and McIllmurray, 1984). Thus the sole use of Lancefield groups could lead to misidentification and it should be used in conjunction with biochemical tests when classifying or identifying streptococci.

In terms of classification the genus has traditionally been divided into four major groups after the most widely accepted classification of streptococci by Sherman (1937). These groups are; the pyogenic streptococci, the enterococci, the lactic streptococci and the viridans streptococci. These divisions were based on properties such as reducing abilities, tolerance to salt and temperature, limits of growth, production of ammonia from peptone and the type of haemolysis produced on blood agar. More recently Jones (1978) and Bridge and Sneath (1982) extended and modified the original proposals of Sherman (1937). The grouping Jones proposed was very similar to that of Sherman and was based on physiological and biochemical reaction. The terms "viridans" and "enterococci" were replaced by the terms "oral" and "faecal" respectively. Three additional groupings "pneumococci", "anaerobic" and "other" streptococccci were also included. The grouping proposal by Bridge and Sneath (1982) is based on data from a numerical taxonomic study. They divided each of the pyogenic and the viridans groups into two or three further groups, but once again these groups largely correspond to the traditional partitioning of the streptococci based on the proposal of Sherman (1937).

In 1984 the genus Streptococcus was split into three genera, *Enterococcus, Lactococcus* and *Streptococcus sensu stricto* (Schleifer and Kilpper-Balz, 1984). Properties used to categorise the organisms included serological (Lancefield, 1933) groupings, peptidoglycan type, guanosine plus cytosine content of deoxyribonucleic acid, cell wall polysaccharides on menaquinone content (Scleiffer and Kilpper Balz, 1987).

The genus *Streptococcus sensu stricto* comprises the majority of known species, in particular the pyogenic and oral streptococci, including the pneumococci, but not the anaerobic streptococci such as *S.parvulus, S.hansenii, S.pleomorphus* or *S.morbillurum.* Members of the genus *lactococcus* which encompasses all the lactococci are catalase negative, Gram-positive, facultatively anaerobic cocci. The spherical or ovoid cells occur singly, in pairs or in chains, and are often elongated in the direction of the chain. Such elongation resulted in some cases in a rod-like appearance and gave rise to the previous classification of some lactococci as lactobacilli. The lactococci are non-motile and usually non-haemolytic with only some strains of *L. lactis* showing a weak α-haemolytic response. All lactococci usually grow in 4% w/v NaCl, the exception being *L.lactis* subsp. *cremoris* which tolerates only 2% w/v NaCl. The ability of lactococci to grow at 10°C but not at 45°C is a characteristic feature that distinguishes them from both streptococci and enterococci. The enterococci are also catalase negative Gram-positive facultatively anaerobic cocci, but can be distinguished by their ability to grow usually both at 10°C and 45°C in 6.5% w/v NaCl and at pH 9.6 DNA-DNA hybridization studies have confirmed that the following are valid *Enterococcus* species: *E.avium, E.casseliflavus, E.durans,*

E.faecalis, *E.faecium*, *E.gallinarum*, *E.hirae*, *E.malodoratus*, and *E.mundti* (Collins, Farrow and Jones, 1986; Collins et al, 1984; Knight and Sclaes, 1986; Schleifer and Kilpper - Balz 1984). The serological grouping, biochemical and chemical characteristics differentiating the genus *Enterococcus* are shown in table 1.

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Table 1 Biochemical, serological and chemical characteristics differentiating species of the genus *Enterococcus*

Species	Serological group	Hydrolysis of Arginine	Hippurate	Acid production from Arabinose	Arbutin	Melezitose	Melibiose	Sorbitol	Sorbose	Tagatose	Glycerol	Major Menaquinones[a]	Motility	Yellow Pigment	H_2S prod.
E.avium	D & Q	-	v	+	+	+	-	+	+	+	+	-	-	-	+
E.casseliflavus	D	(-)	-	+	n.d.	-	+	-	-	-	-	MK7, MK8	+	+	-
E.durans	D	+	v	-	n.d.	-	-	-	-	-	-	-	-	-	-
E.faecalis	D	+	+	-	-	(+)	-	(+)	-	+	+	DMK9, DMK8	-	-	n.d.
E.faecium	D	+	+	+	+	-	(+)	-	+	-	+	-	-	-	n.d.
E.gallinarum	D	+	+	+	+	(-)	+	+	-	+	+	MK8	+	-	-
E.hirae	D	+	-	-	+	v	+	-	-	v	v	-	-	-	n.d.
E.malodoratus	D	-	v	-	n.d.	-	+	+	+	-	v	-	-	-	+
E.mundtii	D	+	-	+	+	-	+	v	-	-	v	n.d.	-	+	n.d.

Adapted from Schleifer and Kilpper - Balz, 1987.
Abbreviations n.d. not determined; v, variable; +, positive; (+) most strains positive; -, negative; (-) most strains negative.
a; abbreviations according to Collins and Jones, 1981.

1.2 Structure, function and composition of the Gram-positive cell wall

The cell wall of the Gram-positive cell is responsible for maintaining its shape and integrity since the protoplasmic substance of bacteria exerts such a high osmotic pressure that if not for the tensile strength of the cell wall, the bacterial cell would be ruptured. In electron micrographs the Gram-positive wall is seen as a reasonably amorphous layer lying immediately external to the cytoplasmic membrane which is 20 to 50nm thick. Approximately 50% of the weight of Gram-positive bacteria is comprised of the highly cross linked polymer peptidoglycan, compared to just 10% in Gram-negative walls. The structure of the Gram-positive cell is shown in figure 1a and that of the Gram-negative cell is represented in figure 1b. The remaining 50% of the weight of Gram-positive bacteria is made up of a variety of accessory polymers, the most important of which are teichoic and teichuronic acids. These and other components of the wall will now be discussed.

1.2.1 Peptidoglycan

Surrounding the cytoplasmic membrane of the Gram-positive bacterial cell is a complex polymer called peptidoglycan or mucopeptide (Baddiley, 1972; Wicken and Knox, 1980). A three dimensional network surrounding the cell is formed by the peptidoglycan and this gives the cell both strength and rigidity (Baddiley, 1972; Wicken and Knox, 1980, Rogers, 1970; Shockman and Barrett, 1983). The structure consists of glycan chains which are composed of N-acetylglucosamine alternating with N-acetyl muramic

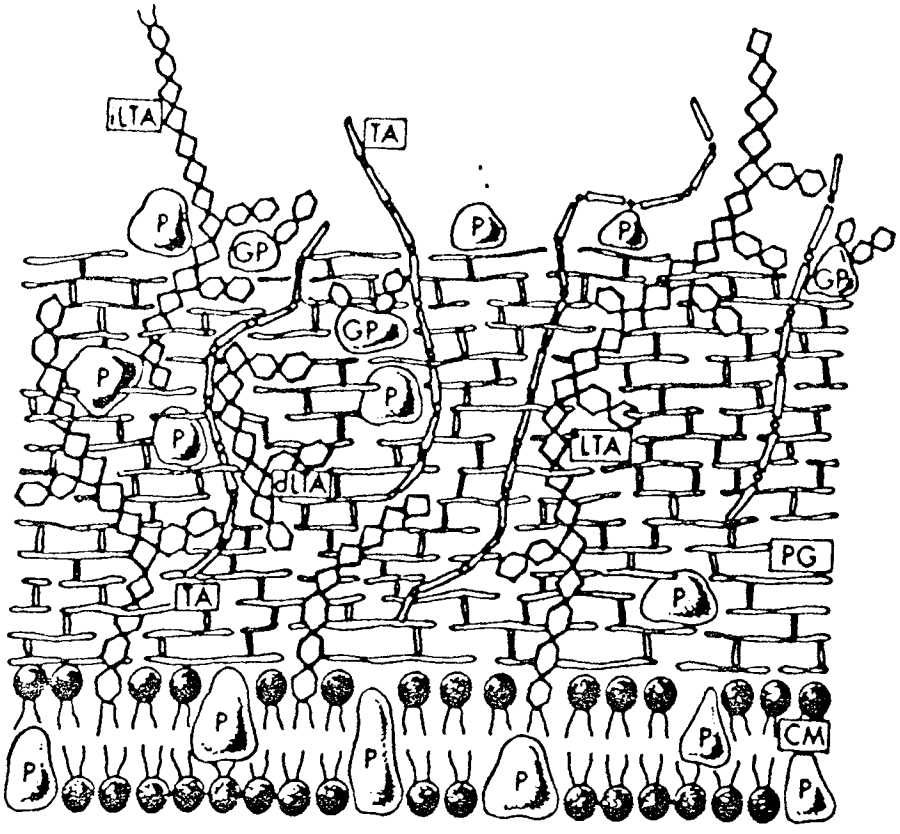

Figure 1a Schematic representation of a cross section of a Gram-positive bacteria.

CM, cytoplasmic membrane; PG, peptidoglycan; P, protein; GP, glycoprotein; TA, teichoic acid or teichuronic acid; LTA, lipoteichoic; dLTA, deacylated lipoteichoic acid; iLTA, inverted lipoteichoic acid (glycolipid protruding away from the cell surface).

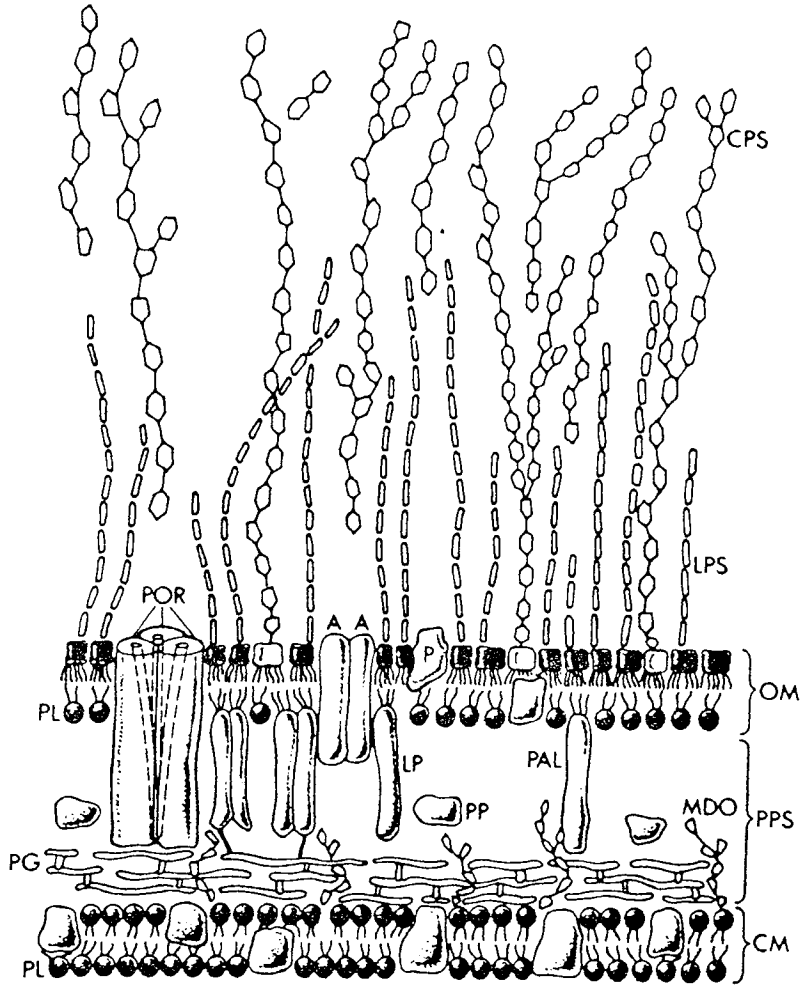

Figure 1b Schematic representation of a cross section of a Gram-negative bacteria.

CM, cytoplasmic membrane; PPS, periplasmic space; OM, outer membrane; PG, peptidoglycan; P, protein; PP, periplasmic protein; LP, lipoprotein; CPS, capsular polysaccharide; LPS, lipopolysaccharide; MDO, membrane-derived oligosaccharide; A, other outer membrane protein; PL, phospholipid.

acid. These two units are joined by a 1,4-ß-glycosidic linkage (Ghuysen, 1968; Schleifer and Kandler, 1972). The glycan chains themselves are cross-linked by peptide chains. (Baddiley, 1972; Penn, 1983). These are usually composed of L-alanine (which is joined to the acetylmuramic acid residue) linked in turn to D-glutamic acid which is then linked by its
ɣ-carboxyl group to an L-diamino acid (although in the case of *E.faecalis* this is L-lysine) followed by D-alanine (Schleifer and Kandler, 1972). The peptide chains themselves are cross-linked by interpeptide bridges, the composition and sequence of which varies greatly among different Gram-positive bacteria. The composition in *E.faecalis* peptidoglycan is L-lysine-L-alanine-L-alanine-L-alanine (Deibel and Seeley, 1974). Thus in *E.faecalis* the distal amino group of the L-lysine in the peptide chain is involved in the formation of the interpeptide bridge with D-alanine on adjacent peptide subunit (Ghuysen 1968, Schleifer and Seidl, 1977). Species to species variation in the chemical structure of peptidoglycan is seen for example in the nature of the amino acids joined to muramic acids and forming the cross links between glycan strands. Such variations are useful for taxonomic purposes (Schleifer and Kandler, 1972), but the roles of such chemical variations in peptidoglycan function are as yet unknown.

Peptidoglycan composition may, exceptionally be affected by the media in which growth takes place as demonstrated by Shockman and Barrett, (1983). An example can be seen in *E.faecalis* where instead of lysine, hydroxylysine can be incorporated into the peptidoglycan. This only happens however if lysine content is limited and a high concentration of hydroxylysine is added to the growth medium (Smith and Henderson, 1964).

Peptidoglycan, being accessible at the cell surface is a possible candidate for a streptococcal virulence factor, since the primary stage in bacterial infections is usually attachment to host tissues and thus it is likely that bacterial surface structures would be involved (Gibbons and van Houte, 1975; Smith, 1977). Streptococcal peptidoglycan has indeed been shown to inhibit phagocytosis by rabbit polymorphs (Jones and Schwab, 1970). This inhibition was shown to be caused by a toxic effect on the phagocytes. Peptidoglycan has been shown to be capable of lysing red blood cells and platelets, and also of producing fever, dermal and cardiac necrosis (Parker, 1975, Davis et al, 1980).

1.2.2 Teichoic acids

Teichoic acids (TA's) are polymers of glycerol or ribitol units joined together by phosphodiester linkages. The name can also be applied to polymers in which an integral part of the chain of glycerol or ribitol phosphate units are sugar residues. Teichoic acids in the Gram-positive cell wall are covalently linked to peptidoglycan through a phosphodiester linkage with the 6 position of muramic acid residues of the peptidoglycan (Ghuysen et al, 1965; Knox and Hall, 1965). The chain length of the polymers in intact cells is thought to be in the order of 35 to 40 units. These chains normally bear a number of substituents which have an important influence on their molecular properties. Examples are D-alanyl groups that may be present found attached by an ester linkage between the carboxyl group of D-alanine and a free hydroxyl group on either glycerol or ribitol units of the teichoic acid chain (Baddiley, 1972; Beachey, 1980). This allows many variations on the basic TA structure. Teichoic acids and acidic

polysaccharides are believed to be concerned with cation balance and maintainance of high, controlled concentrations of bivalent cations in the region of the cytoplasmic membrane (Baddiley, 1972). Teichoic acid has not been conclusively linked to streptococcal virulence (Knox and Wicken, 1973).

1.2.3 Teichuronic acids

Teichuronic acids (TU's) are a group of wall polymers which have properties similar to the teichoic acids. Like TA's they are covalently attached to one end to peptidoglycan. Little is yet known about the precise way in which teichoic acids are linked to the peptidoglycan, although it is probably by phosphodiester linkage (Hammond et al, 1984). Unlike teichoic acids the repeating unit of teichuronic acids does not however contain phosphate, their negative charge being due to the carboxyl groups of the uronic acid residues. The distribution of TU's among the Gram-positive bacteria is complex, and depends upon the nutritional conditions in which the cells are grown (Hammond et al, 1984).

1.2.4 Lipoteichoic acid

All Gram-positive bacteria which have a teichoic acid as part of their cell wall also contain a lipoteichoic acid which can be found associated with the cytoplasmic membrane (Hammond et al, 1984) and are thus known as membrane teichoic acids. Unlike wall teichoic acids which exhibit considerable structural diversity and occur as either ribitol or glycerol phosphate chains bearing a variety of substituents, membrane teichoic acids are chemically more specialised and possess exclusively poly (glycerol phosphate)

chains (Lambert et al, 1977). Thus lipoteichoic acid (LTA) as the membrane teichoic acid is also known, is composed of repeating units of glycerol phosphate joined through a 1-3 phosphodiester linkage (Coley et al, 1978). These glycerol phosphate units are covalently linked by a phosphodiester bond to glycolipid molecules located within the cell membrane (Hay et al, 1963; Toon et al, 1972). The glycolipid consists of a diglyceride unit, and a number of sugars and the nature of the sugar unit varies from organism to organism (Hammond, et al 1984). In *E.faecalis* this is a phosphatidyl kojibiosyl diglyceride (Toon et al 1972). The LTA molecule thus extends from the glycolipid which anchors it to the cytoplasmic membrane with the fatty acids embedded in the membranes outer leaflet, through the cell wall to the surface of the bacterial cell.

In 1975, Joseph and Shockman discovered that LTA was found outside the bacterial cell, having been lost either naturally from the cell due to cell wall turnover during growth and division or actively secreted. Such extracellular LTA was shown by Wicken and Knox (1977) to exist in either a micellar form or in a deacylated form. As a result of the discovery by Joseph and Shockman (1975), Wicken and Knox (1975) proposed a new model for the cellular location of LTA. This stated that during the transportation of LTA to the environment external to the cell LTA becomes detached from the cytoplasmic membrane giving a transient stage where LTA exists solely in the cell wall. Such transient LTA either keeps its fatty acid tail or is deacylated. In 1983 whilst studying group A streptococci Beachey et al reported that in transit through the cell wall some of the LTA molecules become reorientated, thus exposing their lipid end at the cells surface. This is shown in figure 1a as iLTA (inverted LTA). Beachey et al

(1980) showed that a stable complex was formed with this reorientated LTA, the hydrophilic region of which interacts with the charged areas of protein molecules in the bacterial cell wall. The surface of group A streptococci have been shown by Tylewska et al, (1979) to be very hydrophobic, and this could be explained by the presence of inverted LTA on the surface. This reorientation of LTA could possibly occur in other Gram-positive bacteria. One important property of lipoteichoic acids is the fact that they can bind to autolytic enzymes, thus inhibiting their actions and in intact cells it is most likely the wall teichoic acids which control the autolytic enzymes present in the wall (Hammond et al, 1984). Lipoteichoic acids like wall teichoic acids tightly bind magnesium ions and are believed to help maintain an adequate supply of these ions to the organism from the surrounding medium. This magnesium can then be used by membrane bound enzymes requiring magnesium to function. Thus the main function of LTA within the cell is related to this ability to bind bivalent cations that are required in order to maintain the correct physical functions and enzymatic activities of the cytoplasmic membrane (Toon et al, 1972). One theory put forward concerning this is that LTA and wall teichoic acids combine to channel magnesium ions from the outer cell surface to the cytoplasmic membrane (Lambert et al, 1977).

Yet another important property of LTA involves the adhesion of bacteria to surfaces. In 1976 Beachey and Ofek demonstrated that LTA as well as M protein was a component of group A streptococcal fimbriae, and that these fimbriae when denuded of M protein still had the ability to adhere to human buccal epithelial cells suggesting that the LTA component of the fimbriae was acting as an

adhesin. Since that time yet more evidence has been accumulated which indicates that LTA does mediate bacterial adherence to human tissues which possess LTA receptors (Beachey et al, 1980; Beachey, 1981; Beachey et al, 1983). Simpson et al (1980) suggested these were possibly albumin like proteins, but evidence was also seen that LTA might be involved in the adherence to mammalian cells by binding to the high molecular weight glycoprotein fibronectin (see section 1.5).

1.2.5 Polysaccharides

Streptococci of groups A,B,C,E,F,G,H,K,L,O,P and U contain peptidoglycan bound group specific polysaccharide antigens (Krause, 1963; Krause and McCarty, 1961 and 1962) and even though in group D and group N streptococci the group specific antigens are teichoic acids, polysaccharides are the antigens' immunodeterminants (Wicken and Knox, 1975; Knox and Wicken, 1977). Thus as well as group specific antigens covalently attached to peptidoglycan polysaccharide may also exist in the streptococcal cell wall as substituents on the hydroxyl groups of teichoic acid (Baddiley, 1972; Toon et al, 1972) or sometimes in the form of exopolysaccharides which are associated with the outer surface of the cell (Costerton et al, 1978, Costerton et al, 1981).

The group specific antigen of the group D streptococci is cytoplasmic membrane associated, whereas in contrast the type specific polysaccharide antigens of the group D streptococci are cell wall associated. Group and type specific antigens are accessible to antibodies in grouping/typing sera, which indicates

that these polysaccharides may also be accessible to host molecules, and thus could be part of pathogenic processes and act as receptors (Linzer et al, 1984). The group specific antigens of groups A and B streptococci for example have been implicated in arthritis (Schwab, 1979) and in *Streptococcus sanguis* the serotype I polysaccharide antigen may participate in the attachment of this bacteria to tooth surfaces (Okahashi et al, 1983).

A high molecular weight exopolysaccharide referred to as a "glycocalyx" has been shown to be synthesised from simple sugars by some bacteria (Costerton et al, 1978; Cheng et al, 1981; Costerton et al, 1981). For example oral viridans streptococci synthesise dextrans from sucrose (Gibbons and van Houte, 1975). Certain strains of bacteria in infective endocarditis have been shown to produce exopolysaccharides both *in vitro* and *in vivo* (Ramirez-Ronda 1978 and 1980; Mills et al, 1984).

Larger cardiac vegetations are formed by dextran producing bacteria (Mills et al, 1984) which may be because of an increase in the number of bacteria adhering to the vegetation or to an increased stimulation of fibrin and platelet deposition (Hook and Sande, 1974; Sullam et al, 1985). The dextran producing organisms in the larger vegetations are more resistant to the action of antibiotics than dextran negative strains in smaller vegetations (Yersin et al, 1982; Pulliam et al, 1985). This has been shown to be due to the relative metabolic inactivity of these bacteria in the larger vegetations (only bacteria growing near the vegetation surface are metabolically active (Durack and Beeson, 1972) rather than due to an inability of the antibiotic to penetrate the vegetation (McColm and Ryan, 1985).

The production of dextran was shown to be a virulence factor for *Streptococcus sanguis* in infective endocarditis by Scheld et al (1978) who demonstrated the increased adherence of those organisms to artificial fibrin platelet vegetations when they were grown in sucrose and were thus able to produce dextrans, hence demonstrating that the production of this exopolysaccharide is important in the adherence of oral streptococci to non bacterial thrombotic endocarditis, and is thus a factor in the pathogenesis of infective endocarditis.

1.2.6 Cell wall proteins

Several proteins are found in association with the Gram-positive cell wall one of the best characterised being protein A, which is found in certain strains of *Staphylococcus aureus*. This 42 kdalton molecular weight protein is covalently linked to a site on peptidoglycan by its carboxy terminus. Protein A is able to bind immunoglobulin G (IgG), each molecule of protein A being able to bind two or more IgG molecules, and binding occurs at the Fc portion of IgG rather than at the site responsible for the specific antigen-antibody interaction. Groups A,B,C and G streptococci have been shown to possess surface proteins which are similar to protein A of *Staphyloccocus aureus* (Forsgren and Sjonquist, 1966) and these act as receptors for the Fc portion of immunoglobulins (Kronvall, 1973; Chhatwal and Blobel, 1987). These streptococcal Fc receptors are able to bind all four IgG subclasses and those of group A streptococci are able to bind to IgA (Myhre and Kronvall, 1977; Bjorck and Kronvall, 1984). No link between the streptococcal Fc receptor proteins and pathogenicity has as yet been established although it has been

suggested that IgG Fc receptors may inhibit streptococcal phagocytosis by means of interfering with complement's classical pathway of action (Burova, 1982).

Other well documented streptococal proteins are the M,T and R proteins of groups A, C and G streptococci. Streptococcal M protein was first identified over 60 years ago in group A streptococci (Lancefield, 1928), and its structure in group A streptococci is that of a fibrous coiled dimer which protrudes from the cell surface (Phillips et al, 1981), forming fine hair like fimbriae, along with LTA (Beachey and Ofek, 1976). M protein or M-like proteins or group A streptococci have been reported in group B (Maxted, 1948) C (Maxted, 1948; Woolcock, 1974), E (Daynes and Armstrong 1973) and G (Maxted, 1948; Maxted and Potter, 1967).

The streptococcal M protein is now probably one of the best defined molecules of the known bacterial virulence determinants, having a direct role in streptococcal virulence (Davis et al, 1980). M protein has been shown to protect group A streptococci from phagocytosis (Lancefield, 1962; Fox, 1924), thus enabling the bacteria to persist in infection. Yet another virulence factor of M protein is the fact that it appears to provide the organism with an adherence advantage and as such may be considered as an attachment factor (Fischetti, 1989). Ellen and Gibbons (1972) first showed that streptococci bearing the M-protein molecule on their surface can adhere better to epithelial cells *in vitro* than M-deficient organisms. M protein has also been shown in group A streptococci to complex with LTA, with reorientation of the LTA to expose its glycolipid end, thus increasing cell surface hydrophobicity (Ofek et al, 1982; Beachey et al, 1983).

T proteins, like M proteins may be used to type the group A streptococci (T antigens may give cross-reactions when using M proteins in typing streptococci). These proteins are also found in group C, G and L streptococci.

R proteins are found in the cell walls of streptococci of groups A, B, C, G and L streptococci (Parker, 1984), but are not associated with virulence and are not generally used in typing systems.

Other cell wall/surface proteins which have been studied are those of oral streptococci. These cell wall/surface proteins are implicated in the adhesion of oral streptococci to buccal epithelial tissue, salivary components or tooth surfaces for example Russell (1979) showed that proteins associated with the cell wall of *Streptococcus mutans* are likely attachment factors to salivary components and host epithelial cells. Like M protein in *S.pyogenes*, surface associated proteins of streptococci may be carried on hair like fibrillar structures. (Handley et al, 1985; Weerkamp et al, 1987). Weerkamp et al (1986) investigated antigen C, a glycoprotein involved in *S.salivarius* attachment to host surface and reported it to be mainly located in a fibrillar layer, external to the cell wall. Protein B however, which is responsible for bacterial aggregation (Weerkamp and McBride, 1981) is located within the cell wall.

In *Enterococcus faecalis* surface protein antigens have been reported which aid in the spread of multiple antibiotic resistance amongst strains (Franke and Clewell, 1981). In *E.faecalis* the multiple antibiotic resistances are plasmid-mediated (Clewell and

Franke, 1974; Dunny et al, 1978). Production of a sex pheromone or "clumping inducing agent" (CIA) by the *E.faecalis* donor cells in response to the plasmid (s) carried by recipient cells is believed to mediate bacterial conjugation which subsequently leads to transference of plasmids.

Other cell proteins are autolytic enzymes which are responsible for modifying the wall structure. Several bacterial species are known to possess autolytic cell wall peptidoglycan hydrolases or autolysins (Conover et al, 1966; Cornett et al, 1979). Autolysins have a very high affinity for the cell wall (Shockman and Cheney, 1969). They act by breaking covalent links in the peptidoglycan at specific points, which is necessary for the walls to expand during growth (Hammond et al, 1984). Control of these enzymes is exerted in part by the teichoic and/or teichuronic acids associated with the peptidoglycan of the Gram-positive cell wall. The autolysins bind tightly to teichoic and teichuronic acids and in this way enzymatic activity is regulated (Hammond et al, 1984), though the precise nature of this mechanism is as yet unknown.

1.3 *Enterococcus faecalis*: a group D enterococcus

Enterococcus faecalis (*E.faecalis*) according to the recent revision of the genus *Streptococcus* into three genera by Schliefer and Kilpper-Balz (1984) and Schleifer (1985), belongs to the genus *enterococcus*. *E.faecalis* is found in the mouth and throughout the small intestine of normal adults in small numbers (eg $10-10^3$ cfu/g) and is found in the faeces of normal adults in higher concentrations (eg 10^7 cfu/g) (Noble, 1978; Kager et al 1981). This group D enterococcus is responsible for 10-15% of cases of infective endocarditis (Wilson and Geraci, 1983). *E.faecalis* IE seems to be especially prevalent in young women, elderly males and intravenous drug abusers (Lerner and Weinstein, 1966; Mandell et al, 1970; Kaye, 1982; Parker 1984; Arbulu and Asfaw, 1987). Regarding entry into the bloodstream, young women are a high risk group for gynaecological procedures, elderly males are a high risk group for genitourinary procedures, such as those involving the prostate gland and the infection of intravenous drug users could possibly reflect a lack of hygiene involved. Endocarditis caused by *E.faecalis* has several characteristics which distinguish it from endocarditis caused by the viridans streptococci. Patients with enterococcal endocarditis for example show few peripheral symptoms such as oslers nodes, Janeway lesions and petechiae (Mandell et al, 1970). *E.faecalis* adheres to heart valve tissue more avidly than do viridans streptococci and staphylococci and far more so than facultative Gram-negatiue bacilli (Gould et al, 1975). Indeed enterococcal endocarditis has been shown to be able to affect heart valves in which no previous underlying damage is evident (Mandell et al, 1970, Wesby, 1978). However the most important difference between endocarditis caused by *E.faecalis* and

that caused by organisms such as the viridans streptococci is that enterococci are insensitive to the antibiotic regimen used against the penicillin-sensitive viridans streptococci (Wilson and Geraci, 1985; Kim and Bayer, 1987). The multiple antibiotic resistances of *E.faecalis* are plasmid-borne and these are transferred between cells by conjugation. The treatment of *E.faecalis* thus usually involves a synergistic combination of antibiotics such as penicillin plus an aminoglycoside (Watt, 1978; Indrelie et al, 1984).

1.4 Infective endocarditis (IE)

1.4.1 Natural history, classification, and epidemiology of Infective endocarditis

In March 1885 William Osler, Professor of Clinical Medicine at the University of Pennsylvania, delivered his three Gulstonian Lectures on "Malignant Endocarditis" to the Royal College of Physicians. This was the first comprehensive account in English of the disease and they were soon published in the British Medical Journal (Osler 1885). The subject was of medical importance both then, and now over a century later.

The understanding of the pathogenesis of infective endocarditis has greatly increased from Osler's time, as has the ability to diagnose the disease and the capacity to treat what was then almost invariably a fatal infection. However after the introduction of penicillin, which resulted in a fall in mortality, the overall mortality from infective endocarditis has remained steady at between 20 to 30% of cases (Hayward, 1973a; Wilson and Geraci, 1983; Newsom 1984, Anonymous 1984). It is the elderly who today constitute the majority of patients with endocarditis and the mortality ranges from 40% to 70% (Robbins, et al, 1980). Thus endocarditis is still a significant and life threatening medical and surgical problem.

By definition infective endocarditis is the result of the infection by a microorganism of a platelet fibrin vegetation that is located on the endothelial surface of the heart (either the valvular or mural endocardium).

In the past the disease has been classified as "acute" or "subacute" bacterial endocarditis. The term "bacterial endocarditis" however, is not always appropriate since not all infecting organisms are bacteria, and thus the term infective endocarditis (IE) is now more frequently used. The acute infection usually develops on normal heart valves, progresses rapidly causes severe destruction and frequently leads to metastatic foci of infection. In the untreated patient it is fatal in days to weeks and even with appropriate treatment the mortality is high. It is classically associated with infection caused by *Staphylococcus aureus, S pyogenes. S pneumoniae* and *Neisseria gonorrhoeae.* The subacute infection occurs on damaged heart valves, and is characterised by the slow progression of the disease (additional damage develops slowly and metastatic foci of infection are less common), low grade fever, night sweats, weight loss and vague systemic complaints. The results of treatment are good, and even without treatment the infection may take years to become fatal. This form of the disease is classically caused by the viridans streptococci. Although this classification is useful however the correlation of the infecting organism, the rapidity of the course of the disease and the presence of underlying disease is not perfect. For example *Staphylococcus aureus* infection can cause subacute disease and viridans streptococci can lead to the development of endocarditis on a normal valve and run an acute course. For this reason a classification based on the etiologic agent responsible is preferable as it has implications for the course usually followed, the likelihood of pre-existing heart disease and the appropriate antimicrobial agent(s) to employ. Thus the importance of isolating the causal organism in a patient with endocarditis cannot be over emphasised. Indeed evidence

exists that patients with endocarditis have a better chance of survival (mortality rate, 15%) where their infecting organism has been identified (Hayward, 1973b).

Another distinction with both clinical and therapeutic implication is whether the infection involves a natural valve, a prosthetic valve or has occurred in a patient who abuses intravenous drugs as the therapy and prognosis of these three situations are quite different.

It is difficult to determine the incidence of IE, as the criteria for diagnosis and the methods of reporting vary with different series (Van Reyn et al, 1981). Despite these difficulties however it appears that IE accounts for approximately 1 case per thousand hospital admissions with a range of 0.16 to 5.4 cases per thousand admissions in a review of ten large surveys (Van Reim et al, 1982; Kaye, 1976). This incidence has not changed in the past thirty years (Durack and Petersdorf, 1977). An autopsy study in the United Kingdom showed that there was no change in the yearly number of IE cases from 1939 to 1967 (Hayward, 1973). Endocarditis is more common in men than women, at a ratio of 2:1 (Anonymous, 1984) and the average age at presentation is now over 50 (Durack and Petersdorf, 1977; Garvey and Neu, 1978; Bayliss et al, 1983). The mean age of women presenting with the disease is six to seven years younger than that of men (Kaye, 1982). The mean age of patients with IE has gradually increased in the antibiotic era compared to that in pre-antibiotic times when the mortality was greatest in young persons. The incidence of rheumatic heart disease is declining in Western Societies (Besterman, 1970) and the changing pattern in age of IE patients

is likely the result of this with the increasing incidence of IE in older patients being due to the longer survival of a generation with such rheumatic disease. Another change that has taken place since pre-antibiotic times is the proportion of acute cases which has increased from approximately 20% in the pre-antibiotic era to 33% at the present time (Kaye, 1976; Durack and Petersdorf, 1977). However despite these changes in the spectrum of the disease IE remains in the antibiotic era, a prevalent disease with a significant mortality (Anonymous, 1981).

1.4.2 Clinical presentation, diagnosis and associated complications of IE

The interval between an event likely to produce bacteraemia (for example dental extraction) and the onset of IE is actually quite short, the "incubation Period" in 84% of 76 cases of streptococcal endocarditis for example having been shown to be less than 2 weeks (Starkebaum et al, 1977). However the time from onset of symptoms to diagnosis in subacute IE is often much longer than this. IE should be suspected when a patient known to have a congenital or valvular heart disease develops a fever or complains of unusual tiredness. Often however IE occurs in patients with no heart lesions or whose heart disease has been hitherto unsuspected and it is in such cases that the diagnosis is often dangerously delayed because it has not been considered.

Infective endocarditis can affect the patient by four mechanisms (Weinstein and Schlesinger, 1974). (i) Constant bacteraemia, splenomegaly (especially with prolonged illness) and metastatic infection. (ii) local invasion which may result in disturbances of cardiac conduction, mitral incompetence, valve-ring abscesses and pericarditis, mycotic aneurysms of the sinus of valsalva and valve perforation. (iii) peripheral embolization, bland or septic which may effect virtually any organ. Major embolic episodes occur in 30% to 40% of patients with infective endocarditis (Hayward, 1973a). Fragments of infected vegetations break off and lead to mycotic aneurysms and septic infarction. About 40% of these emboli occur in the abdomen, and chest (spleen, kidney and myocardium) 50% in the brain, and 10% in the bones of the limbs (Kaye, 1982). These abscesses may be a source of reinfection of

the cardiac vegetations and consequently lead to failure of treatment; (IV) circulating immune complexes which is a feature of IE despite it being unusual to find viable bacteria and specific antibodies simultaneously in circulating blood. The presence of both antigen and antibody leads to the formation of circulating immune complexes. In IE as a result of sequestering of organisms, the presence of antibody and neutrophils does not block the shedding of bacteria into the bloodstream as happens in other infections. Gram-negative organisms are destroyed by antibody and complement without a requirement for polymorphs and as a result can only rarely establish a persistent intravascular infection.

As a result of these four different processes being involved the clinical presentation of patients with IE is highly variable. Clinical manifestations and the approximate frequency with which they occur are summarised in table 2.

The classical peripheral manifestations noted in the table are found in up to one half of cases. Osler's nodes are small, painful, nodular lesions which are usually found in the pads of fingers or toes. In size they are 2-15mm and they disappear in hours to days. The nodes are rare in acute IE cases but occur in 10-25% of all cases. They are not however specific for IE as they can be seen in lupus erythramatosus, gonococcal infections, haemolytic anaemia and in extremities with cannulated radial arteries.

Roth spots when seen are oval, pale retinal lesions surrounded by haemorrhage and usually located near the optic disk. They are also found in leukaemia, systemic lupus erythromatosus and anaemia

and actually occur in less than 5% of cases of IE. Splinter haemorrhages which are linear red to brown streaks in the finger or toe nails are however commonly found in IE. Petechiae may result from either local vasculitis or emboli and usually appear in crops on the conjunction, buccal mucosa, palate and extremities. The lesions which are found in 20-40% of cases are initially red but within 2-3 days become brown and barely visible. Regarding diagnosis of endocarditis, a clinical diagnosis needs a recognition of patients who are considered to be most "at risk" such as those with damaged valves or heart murmurs. It also requires a history of precipitating procedures such as dental treatment, intravenous therapy and cytoscopy. In particular it is essential to consider the fact that there is often a lack of classical physical signs (Bain et al 1987). Anaemia without a concurrent reticulocytosis is usually present. As a result of concomitant focal glomerulonephritis it is also necessary to be aware that blood urea levels are elevated and about 50% of endocarditis partient have haematuria. Echocardiography, especially two-dimensional echocardiography, can be useful in confirming the diagnosis of IE (Hickey et al, 1981 and Effron et al 1983), however the demonstration of vegetations on a valve on echocardiography does not by itself constitute definitive evidence of endocarditis.

The single most important investigation in the diagnosis of endocarditis is the isolation of the infecting organism from blood cultures, as the definitive diagnosis depends upon this. Once the causative organism has been identified appropriate treatment can be commenced. However sterile blood cultures have been noted in 2.5 - 31% of cases of IE (Cannady and Sanford, 1976; Pesanti and

Smith, 1979; Van Scoy, 1982; Bayliss et al, 1983). There are several possible reasons for culture-negative endocarditis such as cultures being taken towards the end of a chronic course (longer than 3 months), fungal endocarditis, endocarditis caused by obligate intracellular parasites such as rickettsia, chlamydiae and possibly viruses and the most common theory, the previous exposure to antimicrobial agents. Although in the past it was suggested that there was no significant difference between the numbers of patients who had received antibiotic agents in culture-negative and culture positive groups (Hampton and Harrison, 1967; Anonymous, 1977), more recent studies have indicated that the administration of antibiotic agents is the single significant difference between the groups (Pesanti and Smith, 1979).

The importance of a precise microbial diagnosis of IE cannot be overemphasised and the mortality of patients with such a precise diagnosis is approximately 15% compared with 30% in those in whom the infecting organism is now known (Shanson, 1981).

Thus there is a need to develop new and rapid testing methods for use not just in culture negative cases but also to increase the rapidity with which a precise microbial diagnosis can be given since with blood culture techniques it may take a matter of days to identify the infecting agent.

TABLE 2 Clinical Manifestations of infective Endocarditis

Symptoms	Percent	Physical Findings	Percent
Fe			
Ch			
We			
Dy			
Sw			
An			0
We			3
Ma			
Co			0
Sk			
St			7
Na			
He			
My			
Oe			2
Ch			
Ab			5
De			
Her			
Ba			

Adopted from Scheld and Sande, 1985

1.4.3 Pathogenesis of Infective Endocarditis

The pathogenesis of infective endocarditis is a complex process requiring at least three crucial elements, firstly preparation of the cardiac valve for bacterial colonization, secondly production of a bacteraemia followed by adherence of circulating bacteria to the prepared valvular surface and finally survival of the adherent bacteria with propagation of the endocardial vegetation. Each of these events will now be considered in detail, whilst a proposed scheme for the pathogenesis of infective endocarditis is shown in Figure 2.

1.4.3.1 Preparation of the valve for colonisation: formation of non-bacterial thrombotic endocarditis (NBTE)

Early theories on the development of endocarditis proposed that the endocardial infection occurred secondary to the entrapment of septic emboli within the capillaries of valves (Luschka, 1852). However subsequent detailed anatomic studies revealed that the valve tissue is avascular which led to this theory being discounted. Since that time other work has shown that endocardial infection is initiated by the adherence of circulating microoganisms to the valve surface (Durack and Beeson, 1972). Bacteraemia itself however is not sufficient to produce an endocardial infection. In experimental animals for example it is nearly impossible to produce IE with intravenous injections of even large inocula of bacteria unless the valvular surface is first damaged or otherwise altered (Durack and Beeson, 1972). When a polyethylene catheter is passed across the aortic valve of a rabbit thus producing direct valve injury however, endocarditis

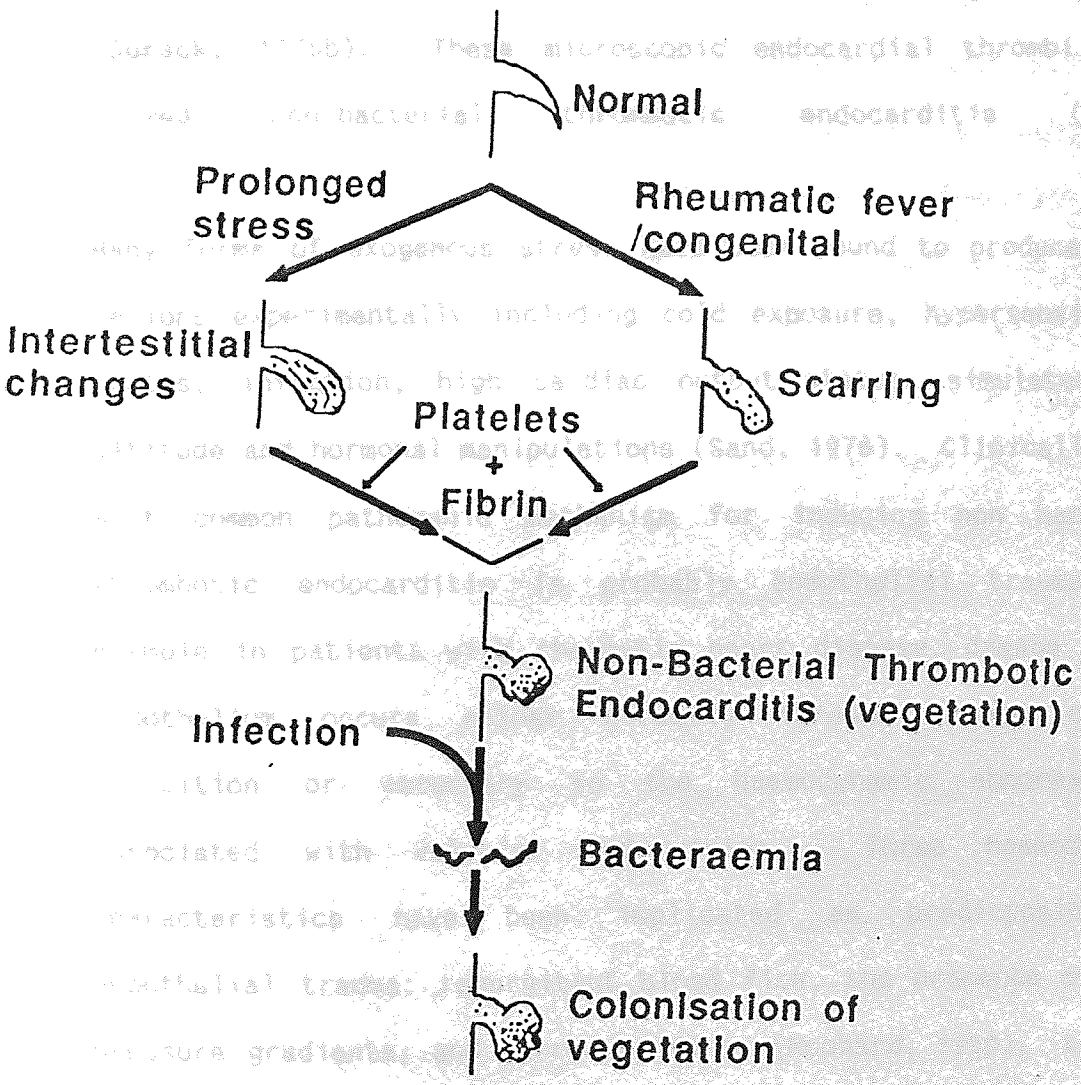

Figure 2 Proposed pathogenesis of bacterial endocarditis.

(Adapted from Angrist and Oka, 1963)

is readily produced as shown for example with Streptococcus viridans. (Durack and Beeson, 1972) and fungi (Durack and Beeson, 1973). When these early lesions were microscopically examined, it was demonstrated that the organisms were intimately adherent to fibrin platelet deposits overlying interstitial oedema and mild cellular distortion that had formed in areas of valvular trauma (Durack, 1975b). These microscopic endocardial thrombi, were termed non-bacterial thrombotic endocarditis (NBTE).

Many forms of exogenous stress have been found to produce these lesions experimentally including cold exposure, hypersensitivity states, infection, high cardiac output states, simulated high altitude and hormonal manipulations (Sand, 1976). Clinically, the most common pathogenic mechanism for inducing non bacterial thrombotic endocarditis is probably endothelial trauma, for example in patients with rheumatic heart disease, damage to the endothelium occurs either directly through immune complex deposition or secondary to the haemodynamic abnormalities associated with valvular deformities. Three haemodynamic characteristics have been implicated as predisposing to endothelial trauma: regurgitant blood flow, the presence of high pressure gradients, and narrow orifices (Roobard, 1960). Lesions which meet these criteria produce turbulent non-laminar blood flow which results in endothelial trauma. Examples of such lesions are aortic stenosis or a ventricular septal defect, and clinically such lesions are associated with a high incidence of bacterial endocarditis. These and other pre-disposing factors are listed in table 3.

1.4.3.2 Production of a bacteremia and adherence to the NBTE

After the formation of non-bacterial thrombotic endocarditis, the valve surface has become pre-disposed to bacterial colonization, however in the clinical situation, before organisms lodge in the heart, bacteraemia must occur. Transient bacteraemia occurs whenever a mucosal surface, heavily colonised with bacteria is traumatised, for example with dental extractions and other dental procedures. They have also been shown to occur after gastroscopy, liver biopsy, sigmoidoscopy, barium enema examination and many procedures that are carried out on the urogenital tract (Everett and Hirschmann, 1977). The degree of bacteraemia is proportional to the trauma produced by the procedure and the number of organisms inhabiting the surface. The organisms isolated reflect the resident microbial flow and bacteraemia is usually low grade ($\leq$ 10 cfu/ml) and transient. However certain bacteria adhere to the fibrin-platelet mass than others. For example after routine dental or medical procedures bacteraemia is frequent (Everett and Hirschmann, 1977) and blood cultures taken after such events as sigmoidoscopy, tonsillectomy, tooth extraction or even tooth-brushing usually grow organisms such as *Propionbacterium acnes*. *Actinomyces viscosus*, diphtheroids or enteric bacteria but these organisms rarely cause endocarditis (Rogosa et al, 1960). Viridans streptococci however frequently cause endocarditis, despite being isolated in only one third of such blood samples (Rogosa et al, 1960). *Streptococcus bovis*, enterococci and staphylococci also frequently cause endocarditis and controlled experiments using animal models confirms the fact that marked species variability exists in the ability of bacteria to initiate endocardial infection (Gould et al, 1975 Baddour et al, 1984).

The explanation for this most likely depends on several factors, such as the ability of the circulating bacteria to avoid immunoglobulin mediated clearance and the ability of the organism to adhere to valve surface in order to initiate the endocardial infection.

One of the most well characterised mechanisms promoting adherence to non bacterial thrombotic endocarditis is the bacterial synthesis of extracellular polysaccharides such as dextran. A role for dextran in the mediation of bacterial adherence was shown by Scheld et al, (1978) and Ramirez-Ronda, (1978). However although dextran may mediate the adhesion of some bacteria to valve surfaces, other mechanisms of adherence certainly exist as well. Other substances suggested as a mediator of adhesion include mannans which was investigated by Maisch and Calderone (1980) who looked to a fibrin-platelet matrix formed *in vivo*. Fibronectin, a plasma glycoprotein which is also a major surface constituent of mammalian cells has been shown to be a mediator of adherence in infective endocarditis. Immunofluorescent techniques have revealed the presence of fibronectin on the surface of traumatised rabbit heart valves but not on the surface of undamaged valves (Scheld et al, 1983). Work by Simpson and Beachey (1983) and Abraham et al (1983) showed the binding *in vitro* of several organisms such as *S.aureus*, Group A and viridans streptococci to fibronectin. Indeed the presence of a specific fibronectin binding protein has been demonstrated in *S.aureus* (Espersen and Clemmensen, 1982).

The role of fibronectin in the pathogenesis of endocarditis will be further considered in section 1.5.1.

Laminin, another glycoprotein has also attracted attention as a possible adhesive factor which could be of importance in the pathogenesis of infective endocarditis. Laminin is also a constituent of the basement membrane of the epithelium, which could become exposed in endocardial lesions (Switalski et al, 1984 and 1987). Indeed it has been demonstrated that some strains of oral viridans streptococci do possess high affinity proteinaceous laminin receptors (Switalski et al, 1987). Another adhesive human plasma protein, complement S protein (vitronectin) has also been shown by immunofluorescence to be present in endothelial cells (Preissner et al, 1988). Chhatwal et al (1987) have described specific interactions of streptococci with vitronectin and Valentin-Weigand et al (1988) have described the mediation of adherence of streptococci to human endothelial cells (from human umbilical vein) by vitronectin. Thus the possibile role for vitronectin in endocarditis cannot be ignored.

TABLE 3

Major predisposing factors in endocarditis

Rheumatic heart (valvular) disease. Represents 25%-60% of cases. The mitral valve is affected more often than the aortic valve, but infections of the aortic valve have increased since the introduction of antibiotics from 5.2% to 38.6% of cases. Right-sided infection occurs in fewer than 10% of cases.

Congenital heart disease. Represents 10% to 20% of cases (for example,ventricular septal defect, tetralogy of Fallot, infundibular pulmonary stenosis, bicuspid aortic valve, patent ductus arteriosus, and coarctation of the aorta). Secondum atrial septal defects are rarely affected.

Mitral valve prolapse. Represents 10% of cases.

Idiopathic hypertrophic subaortic stenosis

Peripheral arteriovenous fistulas (endocarditis can occur on fistula or in the heart)

Indwelling intravenous or intra-arterial plastic cannulas, or pacemakers

Cardiac and prosthetic valve surgery. Represents 10% to 20% of cases

Prosthetic aortic grafts

Degenerative heart disease (calcified mitral annulus, degenerative valves, calcified aortic stenosis)

Alcoholism

Chronic haemodialysis. Endocarditis occurs in 5% of patients on long-term haemodialysis

Intravenous drug abuse

Syphilitic aortic disease

Immunosuppression

Severe burns

Adapted from King and Harkness, 1986

1.4.3.3 Survival of bacteria and propagation of the endocardial vegetation

Once the colonisation of the valve occurs and a critical mass of adherent bacteria develops, the vegetation enlarges further by platelet-fibrin deposition and continued bacterial proliferation. Thus dense colonies of micro-organisms are often sandwiched between layers of platelets and fibrin and this laminar structure is thought to represent reseeding of the vegetation by circulating bacteria, and may be one method of vegetation enlargement (Durack, 1975b).

As stated, one of the major components of the vegetation is fibrin and the clotting cascade may be activated by more than one route. An example of this is *S.aureus* which is able to induce thrombin activity directly because of its ability to synthesise staphycoagulase, which is an enzyme that activates prothrombin (Hendrix et al, 1983).

However the majority of bacteria do not possess direct procoagulant activity. In 1984 Drake et al showed that bacteria adherent to valve surfaces stimulate the expression of tissue factor (tissue thromboplastin) by the underlying valvular tissue. Thus this activates coagulation by the extrinsic pathway. Exactly how bacteria stimulate such local host cells to express tissue factor is still under investigation, but when Drake et al (1984) looked at valve leaflets from rabbits with *Streptococcus faecalis* endocarditis they did express significantly more tissue factor activity than normal leaflets, although the bacteria alone had no procoagulant activity.

1.4.4 Treatment of infective endocarditis

Today, with effective antibiotic therapy at least 85% of patients with IE can be cured compared with the pre-antibiotic era when IE invariably proved fatal. (Wilson and Geraci, 1983). However the management of IE requires careful considerations of choice, dose and duration of antimicrobial therapy. In each case of bacterial endocarditis the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) must be determined for the usual antibiotics. The antibiotic agents most active against the isolated organism are then chosen for therapy with the aim of achieving serum levels of bactericidal antibiotics in excess of the MBC. In urgent cases in which antimicrobial therapy is necessary before the causative agent has been identified or in cases where blood cultures are repeatedly negative the regimen used must include a combination of antibiotics which are effective against the most resistant organisms likely to be encountered such as *E.faecalis* and *Staphylococcus aureus* (Hayward, 1973; Scheld and Sand, 1985; King and Harkness, 1986).

In 1983 and 1985 Wilson and Geraci published their recommendations for the treatment of IE based upon their experience of managing the disease and that of other physicians. Their regimens for staphylococcal, streptococcal and culture negative endocarditis are summarised in tables 4 and 5 and those for other causes of IE are summarised in tables 6a and 6b.

Table 4 Treatment of staphylococcal endocarditis

Microorganism	Antimicrobial therapy	Duration of treatment (weeks)	Alternative therapy	Duration of treatment (weeks
Staphylococcal endocarditis				
Penicillin-sensitive staphylococcal (MIC less than 0.1µ/mL) (*Staph.aureus* or *Staph. epidermidis*)	Aqueous penicillin G (20 mill.units/day IV)	4-6	Cephalothin (2g IV every four hours	4-6
Penicillin-resistant (MIC greater than 0.1 µg/mL) (methicillin-sensitive) *Staph. aureus* or *Staph. epidermidis*)	(Flu)cloxacillin (2g IV every four hours plus Gentamicin (1mg/kg IV every eight hours+	4-6 1-2	Cephalothin (2g IV every four hours or Vancomycin (7.5 mg/kg IV every six hours)+	4-6
Methicillin-resistant *Staph. aureus* or *Staph. epidermidis*	Vancomycin (7.5 mg/kg IV every six hours) plus Gentamicin (1mg/kg IV every eight hours)+	4-6	Rifampicin (600mg/day by mouth) plus Fusidic acid (500 mg IV or by mouth every eight hours)	4-6

Adapted from Wilson and Geraci 1983. + final dosage is dependent on the results of serum antimicrobial assays and of renal function tests. Mill = million; IV = intravenous route

Table 5 Treatment of streptococcal endocarditis

Microorganism	Antimicrobial therapy	Duration of treatment (weeks)	Alternative therapy	Duration of treatment (weeks)
Streptococcal endocarditis				
Penicillin-sensitive streptococci (MIC less than 0.2µg/mL); non-enterococcal group D	Aqueous penicillin G (20 mill.units/day IV)	4	Cephalothin (1.5g IV every four hours)	4
	or		or	
	Aqueous penicillin G (20 mill.units/day IV) plus	2	Vancomycin (7.5 mg/kg) every six hours)+	4
	Gentamicin (1mg/kg IV every eight hours)+	2		
	or			
	Aqueous penicillin G (20 mill.units/day IV) plus	4		
	Gentamicin (1mg/kg IV every eight hours)+	2		
Relative penicillin-resistance(MIC greater than 0.2µg/mL); nutritionally variant viridans streptococci	Aqueous penicillin G (20 mill.units/day IV) plus	4	Vancomycin (7.5mg/kg IV) every six hours)+	4
	Gentamicin (1mg/kg IV every eight hours)			
Enterococcal endocarditis	Aqueous penicillin G (20-40 mill.units/day IV)	4-6	Vancomycin (7.5mg/kg IV every six hours)+	4-6
	or			
	Ampicillin (12 g/day IV) plus	4-6	plus	
	Gentamicin (1mg/kg IV every eight hours)		Gentamicin (1mg/kg IV every eight hours)+	

Adapted from Wilson and Geraci 1983 + final dosage is dependent on the results of serum antimicrobial assays and of renal function tests. Mill = million; IV = intravenous route

TABLE 6a Treatment of miscellaneous causes of endocarditis

Microorganism	Antimicrobial therapy	Duration of treatment (weeks)	Alternative therapy	Duration of treatment (weeks)
Enterobacteriaceae				
Esch. coli Klebsiella Proteus, etc	Ampicillin (12g/day IV or Gentamicin (1.7mg/kg IV every eight hours)+ can add Ticarcillin (3g IV every four hours or Piperacillin (4g IV every six hours	4-6	Cephalothin (8-12g/day IV) or Cefotaxime (2g IV every eight hours)	4-6 4-6
Ps.aeruginosa	Tobramycin (1.7mg/kg IV every eight hours)+ plus Ticarcillin (3g IV every four hours) or Piperacillin (4g IV every six hours)	6		
HACEK organisms				
Haemophilus, Actinobacilus Cardiobacterium Eikenella, Kingella	Ampicillin (12 g/day IV) can add Gentamicin (1.7mg/kg IV every eight hours)+	3	Desensitize patient and treat with ampicillin	
Corynebacterium spp.				
Penicillin-sensitive	Aqueous penicillin G (20 mill. units/day IV) plus Gentamicin (1mg/kg IV every eight hours)+	4-6	Vancomycin (7.5 mg/kg IV every six hours)+	4-6
Penicillin-resistant	Vancomycin (7.5 mg/kg IV every six hours)+	4-6		

Adapted from Wilson and Geraci 1983. +Final dosage is dependent on the results of serum antimicrobial assays and of renal function tests. Mill = million; IV = intravenous

Table 6b Treatment of miscellaneous causes of endocarditis

Microorganism	Antimicrobial therapy	Duration of treatment (weeks)	Alternative therapy	Duration of treatment (weeks
Fungi				
Candida spp.	Amphotericin B (1-1.5mg/kg/day IV plus 5-flucytosine (150 mg/kg/day by mouth in divided doses)	6-8	None	
Culture-negative endocarditis				
Normal valve	Penicillin G (20-40 mill units/day IV plus Gentamicin (1.7mg/kg IV every eight hours)+	4-6	Vancomycin (7.5mg/kg IV every six hours)+ plus Gentamicin (1.7mg/kg IV every eight hours)+	4-6
Prosthetic valve	Vancomycin (7.5 mg/kg IV every six hours)+ plus Gentamicin (1.7mg/kg IV every eight hours)+	4-6		

Adapted from Wilson and Geraci 1983. + Final dosage is dependent on the results of serum antimicrobial assays and of renal function tests mill = million; IV = intravenous

In the case of IE caused by enterococci such as *E.faecalis* or *E.faecium* a separate antibiotic treatment regimen is necessary as detailed in table 5. Enterococci are insensitive to the antibiotic treatment regimen used for penicillin sensitive viridans streptococci (Wilson and Geraci 1985; Kim and Bayer, 1987) and are thus not killed by penicillin alone. The efficacy of penicillin or ampicillin in combination with streptomycin for the treatment of enterococcal endocarditis has been shown in several studies (Kaye et al, 1961; Lernerand and Weinstein, 1966; Mandell et al, 1970; Wilson et al, 1982). However some strains of enterococci show high level resistance to streptomycin *in vitro* (MIC to streptomycin greater than 2000 mg/ml) (Moellering et al 1971; Gutschik, 1982; Indrelie et al, 1984). Penicillin and streptomycin synergism occurs neither *in vitro* or *in vivo* against these strains and the administration of streptomycin for four weeks to patients infected with these organisms results in vestibular toxicity in up to 20% of them (Wilson and Geraci, 1983). Thus treatment with penicillin and gentamicin which act synergistically against most streptomycin-resistant strains of enterococci is recommended (Weinstein and Moellering Jr, 1973; Soriano and Greenwood, 1979; Simmons et al, 1986). A typical example of an antienterococcal regimen is aqueous penicillin G 20-40 million units I/V daily plus gentamicin 1mg/kg I/V every eight hours, continued for four weeks (Wilson and Geraci, 1983). The optimal duration of therapy is in fact debated, with some workers believing that four weeks of antimicrobial therapy is adequate and other suggesting a minimum of six weeks (Mandell et al, 1970; Tompsett and Berman, 1977). If symptoms of infection have been present for more than three months, six weeks treatment is usually recommended (King and Harkness, 1986). In combatting enterococcal

endocarditis, ampicillin, piperacillin or for penicillin-allergic patients, vancomycin may also be used in conjunction with an aminoglycoside (Wilson and Geraci 1983, Newsom 1984; Simmons et al, 1986). Resistance was found to be shown by enterococcal strains to combinations of penicillin with other aminoglycosides (Moellering Jr et al, 1979). All enterococcal strains investigated were killed with a penicillin-gentamicin combination, but *E.faecium* strains were shown to be more resistant to penicillin plus kanamycin, netilmicin or tobramycin than *E.faecalis* strains. If a particular enterococcal isolate is not killed by a penicillin-gentamicin combination then other penicillin-aminoglycoside combinations should be tried (Parker, 1984).

In recent years strains of enterococci which show high-level gentamicin resistance have been noted (Horodicineau et al, 1979; Mederski-Samoraj and Murray, 1988). These were all reported in the USA. Recently however an *E.faecalis* isolate from a patient with infective endocarditis in a UK hospital who did not respond to treatment with penicillin and gentamicin was shown to have an MIC of more than 2000 µg/ml (the usual range being 4-64 µg/ml). (Smyth and Holliman 1988). Such resistance has only rarely been seen before in the UK, but because of capacity to spread and because of their association with previous therapy with cephalosporins or gentamicin (Zervos et al, 1987) it appears likely that such isolates will become more common in the UK, as they have in the USA. Thus Smyth and Holliman, 1988, recommended that all *E.faecalis* isolates from patients with serious sepsis be tested with a 200µg gentamicin disc or have MIC's measured.

Such resistance indicates that in future alternative antibiotic therapy may be necessary, but looking at the newer antibiotics such as ciprofloxacin or imipenem used in syngergistic combinations with penicillins or aminoglycosides, studies have shown the anti-enterococcal activity to be no better than with traditional synergistic combinations (Fernandez-Guerrero et al, 1987; Indrelie et al, 1984).

The resort to operative intervention in treating a patient with acute bacterial endocarditis that would not respond to antibiotics was reported in 1965 by Wallace et al. Their patient had endocarditis which was caused by *Klebsiella* and although the organism was sensitive to kanamycin and polymixin B, the patient did not respond to antibiotic treatment alone. The excision of the aortic valve and the soft vegetations that were confined to the valve leaflets left the patient bacteriologically and clinically cured. In the case of prosthetic valve endocarditis (PVE) which may occur at the time of surgery or through other routes at a later date aggressive chemotherapy is required to give a favourable outcome. In all cases six to eight weeks of appropriate therapy is recommended, and surgical replacement of the infected valve is the rule (King and Harkness, 1986). In the case of a non responsive case of culture negative endocarditis surgical removal of the valve and microbiological examination of it may also be necessary. Thus it can be seen that in some cases of IE the removal of the infected heart valve and its replacement with a prosthetic device is indicated.

1.4.5 Prophylaxis of infective endocarditis

Transient bacteraemia is recognised as a fundamental event in the pathogenesis of bacterial endocarditis (Hook and Kaye, 1962). Dental treatment, surgical procedures or instrumentation involving mucosal surfaces or contaminated tissue may all cause such as transient bacteremia. Although infective endocarditis occurs in patients without evidence of pre-existing heart disease, the majority of cases occur in patients with rheumatic, congenital or other cardiovascular diseases (Weinstein and Robin, 1973; Lerner and Weinstein, 1966). Patients who have had a prosthetic replacement of a damaged valve are also at risk. The rationale for prevention of endocarditis is clear since it carries a significant mortality and all authorities agree that it is important to provide antibiotic prophylaxis to cover certain procedures associated with predictable bacteraemia in patients known or suspected to have susceptible heart lesions (McGowan, 1987; Oakley, 1987).

Since viridans streptococci are still the most frequent cause of infective endocarditis, it is important to consider the use of antibiotic prophylaxis to cover dental procedures. It has been estimated that there is between a 1 in 533 (Kelson and White 1945) and a 1 in 115,000 (Pogrel and Welsby, 1975) chance of endocarditis complicating a dental procedure carried out on a susceptible patient. The two most frequent dental procedures implicated in the development of endocarditis are scaling and extraction but antibiotic prophylaxis is needed for any procedure causing predictable bleeding of the gums. Rational antibiotic prophylaxis recommendations depend largely on indirect data from

bacteraemia studies (Shanson, 1981). The reason for this being that it would be difficult, if not impossible to arrange to test a particular antibiotic prophylaxis regimen in a multicentre trial (Durack, 1975a) and even if such a trial were possible it would almost certainly not be carried out on ethical grounds. There is thus no good evidence that prophylaxis prevents bacterial endocarditis (Petersdorf, 1978). Taking this fact into account it is especially important to avoid causing serious toxicity associated with prophylactic drugs.

As most susceptible patients present to dental practitioners outside of hospital, it is essential that there are simple recommendations to be followed by dentists and patients in order to ensure maximum compliance and thus obtain the most benefit from prophylaxis. In 1977 the American Heart Association (AHA) recommended parenteral penicillin plus aminoglycoside as its primary regimen and for patients allergic to penicillin, vancomycin by intravenous infusion. Because overall these recommendations were extremely complex they were largely ignored in the USA (Brooks, 1980) as well as in the UK (Gould, 1984). In 1982 the British Society for Antimicrobial Chemotherapy (BSAC) published new guidelines (Working Party of BSAC, 1982) which appeared more reasonable for use in general dental practice and because they depend largely on the use of oral antimicrobial regimens which are more likely to be complied with. Indeed work by Scully et al (1987) showed that compliance with the BSAC recommendation appeared to exceed the low compliance with other regimens) and concluded that the BSAC recommendations appear to have increased patient protection during dental treatment. In

1986 revised recommendations of the BSAC Working Party were issued (Simmons et al, 1986) and the recommendation for antibiotic prophylaxis of IE for dental procedures are summarised in table 7.

The BSAC recommendations for antibiotic prophylaxis of IE for non dental procedures are summarised in table 8. Surgery or instrumentation at a site that supports a commensal flora may cause a transient bacteraemia. The incidence of endocarditis after any such procedure is unknown but is believed to be small, and thus the need to administer prophylactic antibiotics to prevent endocarditis had to be carefully considered for each type of procedure. In the case of genitourinary surgery or instrumentation, for example cytoscopy, urethral dilation and prostatectomy and transrectal biopsy of the prostate the antibiotic regimen to prevent endocarditis is directed at faecal streptococci (eg *Streptococcus (Enterococcus) faecalis).* In the event of infected urine however prophylaxis should be adjusted to encompass the urinary pathogen as well. In the case of obstetric and gynaecological procedures (for example uncomplicated vaginal delivery, cervical dilation, curettage of the uterus and insertion or removal of intrauterine contraceptive devices) routine antibiotic prophylaxis is only recommended in patients with prosthetic valves and is once again directed against faecal streptococci. Gastrointestinal procedures such as gastro-intestinal endoscopy, colonoscopy, proctoscopy, sigmoidoscopy or barium enema, may also cause a bacteraemia, but since they rarely appear to cause endocarditis, prophylaxis is once again recommended only for those patients with prosthetic valves and is again directed at faecal streptococci.

Table 7 Working party of the BSAC: recommendations (1986) for antibiotic prophylaxis of infective endocarditis for dental procedures

Adapted from Shenson, 1987

	Not allergic to penicillin	Allergic to penicillin or have had a penicillin within the previous month
(i) Local or no anaesthesia	Amoxycillin 3g single oral dose[b] 1h before dental work (taken under supervision)	Erythromycin stearate 1.5g orally (under supervision) 1-2h before dental work plus 0.5g 6h later
(ii) General anaesthesia (no prosthetic valve)	Amoxycillin 1g in 2.5ml 1% lignocaine hydrochloride approx. 15 min before induction plus 0.5g orally 6h later or (a) Amoxycillin 3g oral dose 4h before anaesthesia and a further 3g oral dose as soon as possible post-operatively or (b) Amoxycillin, 3g oral dose together with probenecid 1g orally 4h before anaesthesia	Vancomycin 1g by iv infusion by gentamicin 120 mg iv immediately before induction (or 15 min before the surgical procedure).
(iii) 'Special risk' patients[a] who should be referred to hospital	Amoxycillin 1g im (see (ii) above) plus 120mg gentamicin im plus 0.5g amoxycillin orally 6h later	Vancomycin, iv infusion plus gentamicin (see (ii) above)

[a] Patients who have had a previous attack of infective endocarditis, or have a prosthetic valve and are to be given general anaesthesia.

[b] Doses above are for adults. The prophylaxis dose is reduced to half the adult dose for children under ten years and quarter the adult dose for children under five years. The dose of vancomycin for children is 20mg per kg.

Table 8 Working Party of the BSAC: recommendations for antibiotic prophylaxis of infective endocarditis for non-dental procedures

Adapted from Shanson, 1987

(1) *Surgery or instrumentation of the upper respiratory tract*
Same prophylaxis as for dental procedures (Table 7) but post-operative antibiotic may have to be given im if swallowing is painful[a]

(2) *Genitourinary surgery or instrumentation*
For patients with sterile urine the suggested cover should be directed against faecal streptococci and is as for 'special risk' cases in Table 7 (including the use of gentamicin in combination with either amoxycillin or vancomycin). If the urine is infected prophylaxis should in addition encompass the pathogen isolated[a]

(3) *Gastrointestinal procedures*
Cover is suggested for patients with prosthetic valves undergoing instrumentation of the gastrointestinal tract and is as for 'special risk' cases in Table 7[a]

(4) *Obstetric and gynaecological procedures*
Cover is suggested only for patients with prosthetic valves and is directed against faecal streptococci. The prophylaxis is as recommended for 'special risk' cases in Table 7[a]

[a] Patients who have had a previous attack of infective endocarditis or have a prosthetic valve and are to be given general anaesthesia.

1.5 Binding of serum proteins to *E.faecalis* and their possible role in the pathogenesis of endocarditis

Gram positive cocci are known to possess receptors for a variety of plasma proteins. (Kuusela, 1978; Bjork et al, 1987; Myhre and Kronvall, 1980a. Myhre and Kronvall, 1980b). At present however the nature of only a few of the receptors has been characterised. Little is known of the biological significance of such binding, although it is presumed to play a role in microbial pathogenicity. The binding of two serum proteins, fibronectin and albumin will now be considered in more detail.

1.5.1 Fibronectin

Fibronectin is a high molecular weight glycoprotein (approximately 440,000 DA). It occurs in both a soluble and insoluble form. It is found in plasma in a normal range of 250-600 µg/ml (Mosher and Williams, 1978), although women have higher plasma concentrations than men with an average of 400 µg/ml compared to 300µg/ml for men. Soluble fibronectin is also found in amniotic fluid (Chen et al, 1976) seminal fluid (Vuento et al, 1980; Gressner and Wallraff, 1981), joint fluid (Carsons et al, 1981) and cerebro-spinal fluid (Gressner and Wallraff, 1981). Fibronectin is found in an insoluble form in tissue where it is covalently cross linked into multimeric fibres. Soluble fibronectin can be incorporated into these fibres in the extracellular matrix or on the surface of cells (McKeown-Longo, 1987). Many cell types synthesise and secrete fibronectin, but most circulating fibronectin is produced by hepatocytes (Akiyama and Yamada, 1983).

A current model for the structure of fibronectin is shown in figure 3. Plasma fibronectin is a dimer of two polypeptides of approximate molecular weight 220,000 Da, each joined by disulphide bonds at their extreme carboxyl ends (Mosesson et al, 1975; Iwanga et al, 1978). Cell surface fibronectin exists both as disulphide-bonded dimers and multimers (Yamada et al, 1977). Thus fibronectin displays many interesting structural and functional properties and plays important roles in wound healing (Mosher, 1980; and Grinnel 1984); as an opsonin in mediating the clearance of tissue debris by phagocytic cells (Blumenstock, 1981); in clot stabilisation (Mosher, 1980; and Mosher 1984) and cell adherence to plastic or basement membrane (Mosher, 1980; Mosher, 1984; Akiyama et al, 1981).

Fibronectin is also able to bind to bacteria as first reported by Kuusela (1978), who looked at the binding of *Staphylococcus aureus* to fibronectin. Since that time the binding of fibronectin to streptococci has also been investigated and at least three binding mechanisms have been postulated. Thus two of the most well known pathogenic bacteria, *Streptococcus pyogenes* and *Staphylococcus aureus* adhere to fibronectin on epithelial cell surfaces (Beachey and Courtney, 1987; Proctor, 1987). LTA has been suggested as the major receptor for group A streptococci in binding to fibronectin (Courtney et al, 1983). Although LTA is anchored to the streptococcal cell by intercalation of its glycolipid end into the cytoplasmic membrane, some of the LTA is constantly excreted by the streptococci into the growth media (Alkon et al, 1977; Philips et al, 1981) and therefore LTA must be in constant transit through the cell wall. Beachey et al had postulated that during this transit some of the LTA molecules become complexed through their

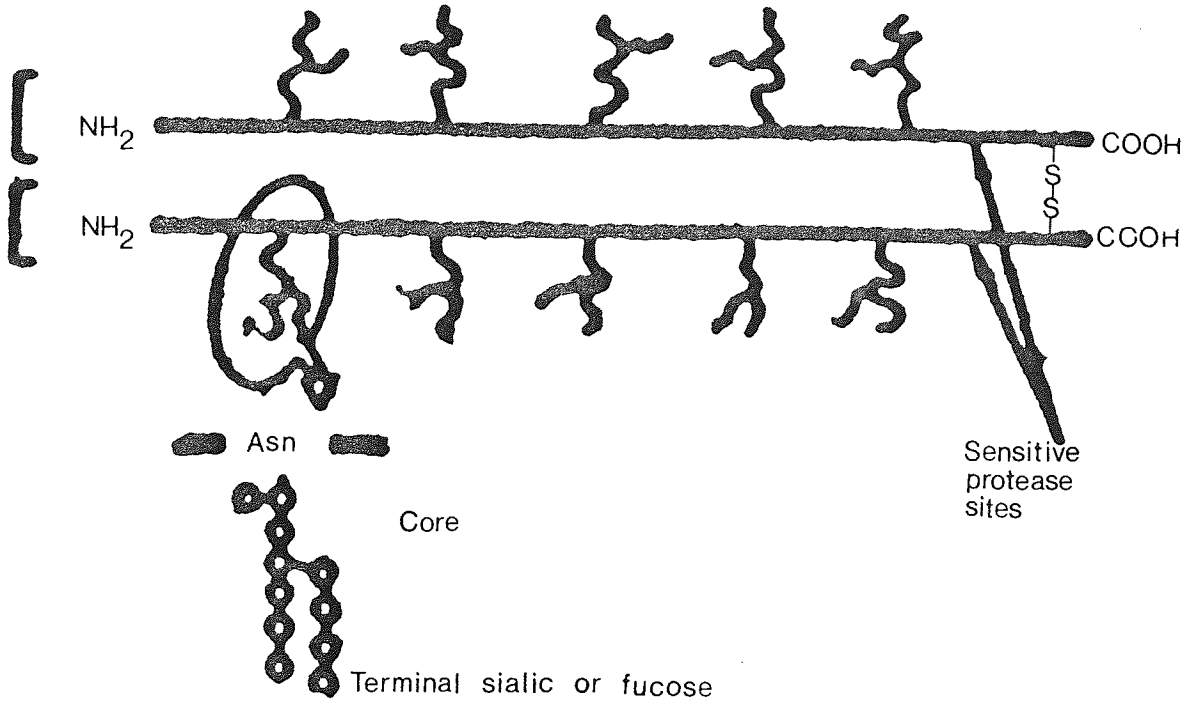

Figure 3 Basic structure of fibronectin showing the elongated shape and inter-subunit disulphide bonds, with the adjacent sites that are sensitive to the proteases plasmin and trypsin. The oligosaccharides consist of a core of N-acetylglycosamine and mannose linked to asparagine residues with distal galactose residues that may be linked to terminal sialic acid or fucose residues. (Adapted from Yamada and Olden, 1978).

polyionic backbones to clusters of positive charges on surface proteins and this re-orientation would leave the lipid moieties of LTA free and exposed. In 1982 Ofek et al showed that LTA is able to form complexes with certain surface proteins including the M protein of group A streptococci and thus mediate the attachment of these bacteria to the amino acid terminus of fibronectin through its glycolipid end (Beachey and Courtney 1987; Beachey et al, 1983; Courtney et al, 1988). Findings of these studies suggest that LTA plays a major role in the binding of fibronectin to group A streptococci, however the possibility that other surface components may be involved cannot be entirely ruled out and indeed Speziale et al (1984) reported a trypsin-sensitive receptor for fibronectin in *S.pyogenes* in contrast to the LTA mediated adhesion described above. *Staphylococcus aureus* also binds to the amino terminus of fibronectin, but the binding of *S.pyogenes* to fibronectin is not inhibited by *S.aureus* thus suggesting unique receptor sites for these organisms (Courtney et al, 1986). LTA does not appear to be involved in the adherence of *S.aureus* to LTA, but a large fibronectin binding protein has been identified (Espersen and Clemmensen, 1982; Froman et al, 1987) and cloned into *E.coli* (Flock et al, 1987).

Fibronectin, because of several of its properties is a likely candidate for a role in infective endocarditis. These properties include the facts that:

(a) It has the capacity to bind platelets and fibrin (thus it can continue the thrombogenicity of surfaces.

(b) It has adhesive properties for a variety of cell types and bacteria.

(c) It has multiple functional domains (thus allowing simultaneous binding to micro-organisms as well as cells or collagenous materials).

(d) Its ubiquitous distribution due to its existing as both a soluble form which circulates in the blood, and as an insoluble form found in the extracellular matrices of tissues.

The work by Scheld et al (1983) showing the presence of fibronectin on the surface of traumatised rabbit valves but not on the surface of undamaged valves also suggested a role for FN in endocarditis as a component of non bacterial thrombi. Proctor, et al, (1984) have shown that the number of FN receptors found on various *S.aureus* isolates directly correlates with invasiveness, and fibronectin has also been shown to bind to candidal species in the relative order of their pathogenicity (*Candida albicans* and *Candida tropicalis* bound much more than other species). (Skerl et al, 1984). Looking at *S.aureus* a model for the role of FN in the pathogenesis of infective endocarditis was put forward by Hamill, (1987), and this involved the idea that sometimes circulating bacteria became coated with soluble fibronectin, and may adhere to intact endothelium. This is probably not a frequent *in vivo* occurrence but could account for the development of endocarditis in the absence of a prior valve injury. It was also proposed that circulating bacteria could adhere at the site of an NBTE to fibronectin coated platelets, and that fibrin in the NBTE binds circulating bacteria, with fibronectin-fibronectin interactions in the NBTE allowing the adherence of still more bacteria. Other proposed roles for FN included the binding of

either FN coated or uncoated bacteria to fibroblasts at the base of the wounded endothelium, binding and FN coated bacteria to matrix collagen exposed by damage and phagocytosis of FN coated bacterial cells by endothelial cells, after which the bacteria, which may multiply intracellularly kill the cell, and expose the thrombogenic underlying extracellular matrix, resulting in the development and propagation of an NBTE. As stated by Hamill (1987), further studies are needed to find whether these *in vitro* observations do indeed correlate with the situation *in vivo* in endocarditis and to investigate their role of FN in endocarditis caused by other bacteria.

As well as *Staphylococcus aureus* and *Streptococcus pyogenes* streptococci of groups C and G as well as *Streptococcus sanguis* and *Streptococcus pneumoniae* have also been shown to bind fibronectin (Myhre and Kuusela, 1983; Courtney et al, 1985, Lipoteichoic acid has been implicated as the receptor for fibronectin on these streptococci using hydrophobicity studies (Courtney et al, 1985b) and the release of lipoteichoic acid fibronectin complexes by penicillin treatment (Nealon et al, 1986).

1.5.2 Serum albumin

Serum albumin is a protein which has been extensively studied. It is a single protein consisting of only one peptide chain of 580 to 585 amino acid residues (according to species). The polypeptide chain lacks a carbohydrate moiety and is also characterised by a low content of tryptophan (1 to 2 residues) and methionine (4 to 6 residues). The secondary structure meanwhile is a unique pattern of double loops which are held together by disulfide bridges (Rothschild et al, 1988). Serum albumin has several functions. It can transport ligands such as fatty acids, sterols, drugs and hormones (McMenamy, 1977 and Ockner et al, 1983). It also has the function of maintaining osmotic pressure. Albumin is synthesised in the liver as shown by Peters and Anfinsen (1950).

Wideback and Kronvall (1980) have shown that group C and G streptococci possess specific albumin binding sites. These have recently been identified as part of the protein G component of the wall. (Sjubring et al, 1988). However, the bearing of this on streptococcal pathogenicity is as yet unknown.

1.6 Aims and Objectives

The initial aim of this research project was to investigate further the species specific protein antigens of *Enterococcus faecalis* first described by Aitchison et al (1987). *E.faecalis* was of particular interest because of the avidity with which it has been shown to adhere to host endothelium, and also because of the stringent antimicrobial regimen required to treat endocarditis caused by this organism.

Identification of the causative organism isolated from the blood of patients with infective endocarditis is of paramount importance for the selection of appropriate treatment and because of the rigorous antibiotic therapy needed to treat *E.faecalis* endocarditis, a new rapid technique which might reliably confirm or exclude *E.faecalis* endocarditis would be of great clinical value. Thus it was aimed to use the antigens as the basis of a rapid serodiagnostic test, using an ELISA assay tested in a blind trial after the partial purification of the antigens had been achieved.

During the study it was deemed desirable to mimic as closely as possible *in vivo* growth conditions, and thus bacteria were generally grown in serum where possible rather than just complex laboratory media. This was in order to reflect the growth of bacteria in the bloodstream during bacteraemia which precedes endocarditis. The appearance of the cells using ruthenium red stained and sectioned cells examined by electron microscopy was thus investigated with both serum grown and brain heart infusion

grown cells. The expression of surface protein antigens of cells grown in different media was also investigated using immuno-blotting techniques.

The final section of this project looked at the binding of the serum proteins fibronectin and albumin to *E.faecalis*, considered the role of such binding in the pathogenesis of endocarditis and, in the case of fibronectin, attempted to characterise the receptor molecule.

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2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial Strains

Eight strains *Enterococcus faecalis (E.faecalis)* were used: three strains cultured from the blood of a patient with *E.faecalis endocarditis (EBH1, EBH2 and EBH3): three strains isolated from patients with urinary tract infections (SFBG, 741 and 777); a laboratory strain of E.faecalis* subspecies Zymogenes (Facklam, 1972; Deibel and Seeley, 1974) kindly donated by Dr N J Parsons at Birmingham University (SFZ) and a reference strain, *E.faecalis* subspecies zymogenes NCTC 5957, obtained from the National Collection of Type Cultures, Central Public Health Laboratory, Collindale, London (deposited in the NCTC in 1940 from the American Type Culture Collection; originally isolated from pasteurized milk by J M Sherman, 1937).

2.1.2 Growth media

Brain-heart infusion agar was obtained from Oxoid Ltd; Basingstoke, Hants and brain-heart infusion broth (BH1) from Difco, East Moseley, Surrey and from Lab M, Salford, Manchester. When reconstituted, Difco BH1 contained per litre; infusion from calf brains 200g, infusion from beef heart 250g, protease peptone 10g, dextrose 2g, NaCl 5g and di-sodium phosphate 2.5g pH 7.4. Lab M BH1 contained, per litre brain and heart infusion solids 17.5g, "Lab M" tryptose 10g, dextrose 2g, NaCl 5g and di-sodium phosphate 2.5g, pH 7.4. Heat-inactivated, mycoplasma tested horse serum (HS) was obtained from Gibco, Paisley, Glasgow. HS was used neat. These media and nutrient agar and broth were prepared according to manufacturers instructions and sterilised by

autoclaving at 121°C for 15 minutes. Blood agar was prepared by adding defibrinated horse blood (Gibco) 7% v/v to sterile blood agar base (Lab M).

A chemically defined medium (CDM) based upon the minimal salts medium used by Shockman et al (1961) was also used.

In some experiments the CDM was supplemented with 1% v/v horse serum. The minimal salts medium contained per litre: KH_2PO_4 400mg; K_2HPO_4 300mg; Na_2HPO_4 26.65g; NaH_2PO_4 16.45g; glucose 20.0g; sodium acetate 6.0g; ammonium sulphate 600mg; magnesium sulphate 200mg; sodium chloride 10mg; ferrous sulphate 10mg and manganese sulphate 10mg. The minimal salts medium was autoclaved at 121°C for 15 minutes. Glucose and ferrous sulphate were sterilized separately and then added aseptically together with the HS to the minimal salts medium.

2.1.3 Chemicals

The chemicals used were of Analar grade or equivalent obtained from British Drug Houses (BDH) Chemical Ltd, Poole, Dorset or Fisons Laboratory Reagents, Loughborough, Leics or Sigma Chemical Company, Poole, Dorset.

2.1.4 Glassware

Glassware was Pyrex brand (Corning Glass Ltd, Sunderland, Tyne and Wear). For cleaning it was immersed in 5% w/v Extran 300 (BDH) at room temperature for 12 hours. It was then rinsed once in

distilled water, once in 1% v/v hydrochloric acid. Six times in distilled water and finally twice in double distilled water. Glassware was dried at 60°C, covered with aluminium foil and sterilised by dry heat at 160°C for 3 hours.

2.1.5 Human sera

Serum was collected by venopuncture from patients at East Birmingham Hospital, Bordesley Green, East Birmingham, England, Bispebjerg Hospital, Copenhagen, Denmark and Hospital A Calmette, Lille, France. Solid matter was removed from the sera by low-speed centrifugation. Sera were stored at -20°C until required.

2.1.6 Equipment

Equipment used in this study and not specified in the text was supplied by:-

Autoclave - Astell, Astell Hearson, Catford, London.

Automatic Pipettes - Gilson Pipetman, P-200 and P-1000. Anachem, Luton, Beds.

Balances - 1. Oertling HC22, Oertling, Orpington, Kent

2. Sartorius Type 1702, Sartorius Instuments Ltd, Belmont, Surrey.

Blood Collecting Tubes - Polypropylene stoppered tubes, Sterilin Ltd, Teddington, Middx.

Centrifuges - 1. Beckman J2-21 high speed centrifuge, Beckman R11C Ltd, High Wycombe, Bucks.

2. Eppendorf centrifuge S412, Anderman & Co Ltd East Moseley, Surrey.

Coating Unit - Nanotech 300S, Nanotech thin films Ltd, Manchester, England.

ELISA plate reader - Titertek Multiscan model 3/0C; Flow Laboratories Ltd.

Freeze dryer - Edwards Modylo Freeze dryer, Edwards High Vacuum Ltd, Crawley, Surrey.

Gamma counter -	ICN Gamma Set 500, ICN Tracelab Division, Cleveland, Ohio, USA.
Gel electrophoresis apparatus	Mini Protean II cell, Bio-Rad Laboratories Ltd, Watford, Herts.
Gel Drier -	Bio-Rad model 224 gel slab drier, Bio-Rad Laboratories Ltd, Watford Herts.
Immunoblotting tank -	Trans-blot cell, Bio-Rad Laboratories Ltd, Watford, Herts.
Immunoelectrophoresis equipment	Flat bed 2117 multiphor II electrophoresis unit, LKB instruments Ltd, Croydon, Surrey.
Incubators -	Gallenkamp orbital shaking incubator, Gallenkamp, London.

Microscope slides - Hendley - Essex multispot microscope slides, PTFE-coated, C A Hendley Ltd, Loughton, Essex.

pH meter - Pye Unicam 290 pH meter, Cambridge, Cambs.

Photography equipment - Nikon camera FM, Nippon kogaku kk, Tokyo, Japan with Ilford Pan F film or Kodak technical pan film 2415.

Power packs - Bio-Rad Model 500-200 (electrophoresis; Bio-Rad Model 250-2.5 (immunoblotting)

Sealable tubes - Screw-top culture tubes with teflon lined caps, Sterilin, Teddington, Middx.

Sonicator - MSE Soniprep 150 ultrasonic disintegrator. Measuring and Scientific Equipment, Crawley, Surrey.

Spectrophotometers -	LKB Biochrom ultrospec 4050, LKB Biochrom, Cambridge, England.
-	SP6-400 UV spectrophotometer. Pye Unichem Ltd, Cambridge, England.
Syringes -	Gillette Surgical, Isleworth, Middx.
Transmission Electron Microscope	Hitachi H-600 Electron microscope, Hitachi Ltd, Tokyo, Japan
U.V Lamp -	Hanovia Lamps, Slough, Berks.
Vortex mixer -	Whirlimixer, Fisons Scientific Apparatus, Loughborough, Leics.

All addresses are in UK unless otherwise stated.

2.2 EXPERIMENTAL METHODS

2.2.1 Growth Conditions

2.2.1.1 Growth Experiments

The bacteria were grown in the chosen medium at 37°C with agitation at 160 revolutions per minute (rpm) in an orbital shaker - *E.faecalis* cells did not produce any pigment which may have interfered with optical density (OD) measurements. A wavelength of 470nm was chosen to monitor subsequent growth curves, an OD of 1.0 at 470nm indicating a concentration of approximately 10^9 cells/ml. Medium constituents did not interfere with measurements at this wavelength, and none of the organisms produced extracellular products which adsorbed light at 470nm.

Bacterial strains were maintained on nutrient agar slopes at 4°C and subcultured monthly.

2.2.1.2 Growth measurements

Growth was followed by measuring changes in the optical density of the medium with time, a method considered the most appropriate for following changes in bacterial number during growth (Greenwood, 1977). At low cell concentration on the OD is directly proportional to cell concentration (Kenward, 1975). This relationship is expressed by Beer-Lamberts law.

$OD \propto \log_{10} (Io/I)$

where Io = incident light

I = emergent light

The relationship obeys the Beer-Lambert law up to an OD of 0.3 (Kenward, 1975). Above this absorbance this relationship does not apply, due to the secondary scattering of light and the OD increases less than proportionally with increasing cell concentration. If the cell suspension is diluted with fresh media to an absorbance less than 0.3 proportionality is restored.

Media prewarmed to 37°C were incubated with stationary phase *E.faecalis* cells grown in corresponding media. Samples were removed aseptically as 30 minute intervals, and the OD measured, diluting samples appropriately if the OD_{470} was greater than 0.3 Time versus $\log_{10} OD_{470}$ was plotted to give growth curves. Growth in several media was monitored.

2.2.2 Preparative techniques

2.2.2.1 Preparation of whole cells

Bacteria were grown to early stationary phase. Cells were harvested by centrifugation at 11,000 rpm. Washed three times and resuspended in 10mM Tris-HC1 buffer, pH 7.4 to OD_{470} = 5.0. The resuspended bacteria were stored at -20°C until required.

2.2.2.2 Extraction of lipoteichoic acid

The method used was a modification of that used by Coley et al (1972). Stationary phase cells of *E.faecalis* strain EBH1 were harvested by centrifugation at 11,000 rpm for 10 minutes, washed three times and resuspended in 0.01M Tris-HC1 buffer (pH 7.4). The cell suspension was then lyophilized 3.5g of dried cells were added to 150mls of a mixture of chloroform: methanol (2:1 v/v) and stirred overnight at room temperature. This solution was then passed through filter paper and the residue left to allow the solvent to evaporate. The residue was then suspended in 40mls of distilled H_2O and added to 40mls of a solution of 80% aqueous phenol. This was stirred at 4°C for 40 minutes. The solution was then centrifuged at 10,000 rpm for 10 minutes, and the upper aqueous phase (containing LTA, RNA, and DNA) was removed using a pasteur pipette and dialysed extensively against distilled H_2O to remove any remaining phenol. The resulting solution was then incubated with DNA ase from bovine pancreas (Sigma), 10µg/ml and RNA ase from bovine pancreas (Sigma), 10µg/ml in 5mM Tris buffer pH8.0 containing 1mM $MgCl_2$ for 12 h. The aqueous phenol extraction

then repeated to remove the enzymes, and the aqueous layer was lyophilized and then resuspended in distilled H_2O to a concentration of 1mg/ml.

The authenticity of the LTA preparation was confirmed by double diffusion immunoprecipitation (section 2.2.3.3) using group D antisera (Wellcome) in the central well (2), which produced a single precipitation band by well 3 (Figure 4). The LTA used in well 1 was donated by Dr Eileen Aitchison and was prepared by the method of Hamada et al (1985). The two precipitin bands seen probably represent both acylated and de-acylated LTA.

2.2.2.3 Solubilization of bacterial components

Bacterial cell components were solubilized for crossed-immunoelectrophoresis and before antigen purification for ELISA assays by digestion with mutanolysin and treatment with N-lauroyl sarcosinate (Sarkosyl), respectively. Mutanolysin is a muramidase isolated from *Streptomyces globisporus* and has been used to digest the cells of *S.mutans* (Siegel et al, 1981), *S.sanguis* (Morris et al, 1985) and *S.salivarius* (Weerkamp et al 1986). This enzyme has also been used by Williamson et al (1986) to lyse enterococci to obtain penicillin-binding proteins. Sarkosyl is a detergent which has traditionally been used to isolate outer membranes of Gram-negative organisms (Filip et al 1973) and has more recently been used by Jenkinson (1986) in the extraction of surface proteins of *S.sanguis*.

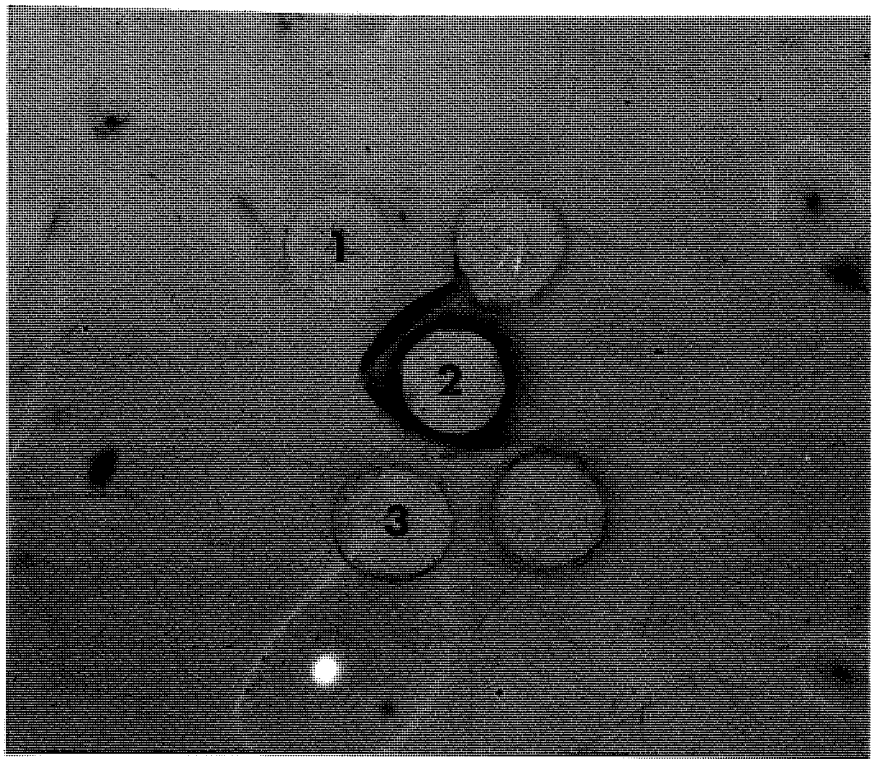


Figure 4 Double diffusion immunoprecipitation against group D antisera to confirm the authenticity of lipoteichoic acid (LTA) produced by two methods.

Key

Well No 1 contains LTA produced by the method of Hamada et al (1985).

Well No 3 contains LTA produced by a method based on that of Coley et al (1972).

Well No 2 contains group D antisera (Wellcome).

2.2.2.3.1 Mutanolysin digestion

The method used was that of Morris et al (1985). Whole cells were suspended to an OD_{470} = 10.0 in 0.05M HEPES. (N-2 hydroxyethylpiperazine-N' 2 ethanesulphonic acid) buffer (Sigma), pH 7.2 containing 0.02% w/v sodium azide. 1mM phenylmethylsulphonyl fluoride (PMSF; Sigma), was included to inhibit proteolysis. Mutanolysin (Sigma) was then added to give a final concentration of 50 units/ml, and the mixture was incubated overnight at 37°C. Digestion of all strains (as determined by the reduction in turbidity) was usually approximately 80% in this time.

A control without mutanolysin was incubated. The suspension was centrifuged at 15,000rpm for 10 minutes and the supernatant was dialysed against water and lyophilized.

2.2.2.3.2 Sarkosyl Extraction

Early stationary phase cells were harvested from a 1L culture. These were washed three times in 10mM Tris-HCl, pH 7.4 and resuspended in 5ml of a 1% w/v solution of Sarkosyl (Sigma) in 10mM Tris-HCl, pH 7.4 containing 1mM Na_2EDTA. The suspension was vortex-mixed and incubated for 20 minutes at room temperature, without lysis occurring. Centrifugation at 5,000rpm for 20 minutes removed unbroken cells. This procedure removed surface proteins, polynucleotides, LTA and PSs from the bacterial cell (Jenkinson 1986). The Sarkosyl extracts were stored at -20°C until required.

2.2.2.4 Immunisation of rabbits

2.2.2.4.1 Preparation of anti-whole cell antisera

Cells of strain EBH1 were freshly harvested, washed and resuspended in 25ml of 0.9% w/v NaCl. The bacteria were killed by exposure to UV light for 10 minutes. Antiserum was raised by injecting pairs of rabbits (3kg, half-lop males) with 1ml of the killed-cell suspension at three sites in the neck. After eight weekly injections, a small quantity of blood was obtained from the rabbits' ears and the anti-EBH1 antibody titre was determined by slide agglutination tests. A titre of 128 or more was taken to indicate that the rabbit was hyperimmune to EBH1. The hyperimmune rabbits were bled by cardiac puncture and the sera stored at -20°C until required.

2.2.2.4.2 Preparation of monospecific antisera

Monospecific polyclonal antibodies were prepared using the method of Knudson (1984). Whole cells of strain EBH1 were separated by SDS-PAGE (see section 2.2.3.1) on a 12% single-track polyacrylamide gel. The separated cell components were then electrophoretically transferred onto a nitrocellulose membrane (see section 2.2.3.2). Narrow strips were removed from both ends, and the middle of the blot and developed with antiserum in order to reveal the antigenic profile. The nitrocellulose shown to bear the required antigens was then excised from the corresponding undeveloped region of the blot. This section was then macerated and dissolved in 500µl of dimethyl sulphoxide (DMSO). An equal volume of Freund's incomplete adjuvant was added to the remainder.

100ul of the antigen suspensions were injected weekly for eight weeks at three sites in the neck of 3kg, half-lop male rabbits, starting with the suspension made with complete adjuvant, with the 7 subsequent injections being made with the suspension in the incomplete adjuvant. Blood was obtained from the rabbits by cardiac puncture, and the sera were stored at -20°C until required.

2.2.2.5 Preparation of IgG from antiserum

In order to give as clean a background as possible, and give a reagent of as high a specific activity as possible for immunonegative staining (see section 2.2.3.7) the IgG was extracted from the antiserum. Different methods exist for the isolation of IgG, based upon gel filtration or precipitation ion exchange chromatography, and affinity chromatography. However the method chosen was a simple method based on ammonium sulphate precipitation as used by Hancock and Poxton (1984). 1ml of serum was placed in an ice-bath and stirred gently on a magnetic stirrer, 0.67ml of a saturated solution of ammonium sulphate was added dropwise at the rate of one drop per second. The serum was stirred for 15 minutes in the ice bath and then centrifuged at 5,000rpm for 10 minutes at 4°C where the IgG formed a pellet. This was resuspended in 1ml of 0.05 M Tris buffer (pH 8.0) and dialysed against 2 litres of the same buffer for 24 hours. The IgG was preserved by the addition of sodium azide to a final concentration of 0.1% w/v, and was then stored at -20°C until required.

2.2.3 Experimental techniques

2.2.3.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Whole cells were subjected to SDS-PAGE using the system of Lugtenberg et al (1975) with a 12% w/v acrylamide concentration in the separating gel. The solutions and chemicals used to prepare the gels and buffers required for SDS-PAGE are shown in table 9.

The separating gel solutions were mixed in a 250ml beaker and stirred with a magnetic stirrer. Polymerisation of the gel was started with addition of TEMED (N,N,N'N',tetra-methylene diamine, BDH) then AMPS (ammonium persulphate, BDH) and the solution was poured in between the glass plates of the mini-protean gel electrophoresis unit. (Bio-Rad Min Protean II gel apparatus, gel dimensions 10cm x 7cm x 0.75mm) separated by plastic spacers. After setting of the separating gel the stacking gel was prepared in the same way and poured in between the plates on top of the running gel. A comb with 12 wells was inserted and the stacking gel left to set, after which the comb was removed. The depth of the stacking gel was at least 5mm greater than that of the wells. The gel was then fitted onto an electrophoresis tank and electrode buffer was added to the tank. Whole cell suspensions (OD_{470} = 5.0) were added to an equal volume of sample buffer and boiled for 10 minutes at 100°C. On cooling, the sample solutions were loaded into the wells of the stacking gel and electrophoresis was carried out at constant voltage of 200 volts, until the tracking dye had run to the edge of the gel (about 1 hour).

Table 9 Solutions and Chemicals used to Prepare the Gels and Buffers required for SDS-PAGE

	Separating gel 12%	Stacking gel	Sample denaturing buffer	Electrode buffer
Stock I	5ml			
Stock II		2·5ml		
SDS 10% w/v (sodium dodecyl sulphate specially purified for biochemistry BDH)	0.5ml	0·15ml	5ml	20ml
Tris 1.5M pH 8.8 (Tris [hydroxymethyl] aminomethane, Sigma)	6.17ml			
Tris 0.5M pH 6.8		3.75ml	2.5ml	
Distilled water	7.92ml	8.0ml	5ml	to 2L
TEMED (N,N,N¹,N1, tetra-methylene diamine, BDH)	46.7µl	40µl		
AMPS 10% w/v freshly prepared (Ammonium persulphate, BDH)	66.7µl	50µl		
Glycerol (BDH)			2.5ml	
2-mercaptoethanol (Sigma)		0.25ml		
5% Bromophenol blue (BDH)		0.2ml		
Tris (Sigma)				6g
Glycine (Biochemical grade, BDH)				28.8g

Stock I - Solution of 44% w/v acrylamide (Sigma) and 0.8% w/v Bis(N,N1 methylene bisacrylamide, Sigma)

Stock II - Solution of 30% w/v acrylamide and 0.8% w/v Bis

Gels were either stained overnight (0.1% w/v coomassie brilliant blue R-250 L Sigma) in 50% v/v methanol - 10% v/v glacial acetic acid solution) and then destained to reveal proteins (5% v/v methanol - 10% v/v glacial acetic acid solution) or else they were subjected to immunoblotting.

Molecular weights of proteins separated by SDS-PAGE were determined by the construction of a calibration curve using commercially available molecular weight markers (Sigma) which were subjected to SDS-PAGE. Their Rf values were then determined (Rf value = distance moved by protein through the running gel ÷ distance moved by bromophenol marker dye through the running gel). A plot of $\log_{10}$ value gives a calibration curve, from which the molecular weight of unknown proteins may be determined.

2.2.3.2 Immunoblotting

The cell components separated by SDS-PAGE were transferred onto nitrocellulose membranes (0.45µm pore size, Bio-Rad laboratories Ltd, Watford, Herts) by the Western blotting method of Towbin et al (1979). Transfer was carried out in an ice-cooled transfer buffer containing 25mM Tris, 192mM glycine and 20% v/v methanol, pH 8.3. The polyacrylamide gel and nitrocellulose membrane were rinsed briefly in transfer buffer and sandwiched between chromatography paper (Whatman Ltd, Maidstone, Kent) the Scotch brite pad (Bio-Rad) (likewise pre-soaked) and lastly perforated plastic support grids. The sandwich was placed in the transblot cell (Bio-Rad Transblot apparatus) containing transfer buffer. Blotting was performed at 30V for 18-24 hours at 4°C. Efficiency of transfer is known to be a function of molecular weight

(Burnette 1981; Vaessen et al 1981) and transfer was found to be high under these electrophoretic conditions (comparing Coomassie blue stains of gels before and after transfer showed virtually complete transfer). Initial blots were also stained with 0.8% w/v amido black (naphthol blue black, Sigma) in 0.7% w/v glacial acetic acid (Fisons) in order to determine completely the transfer of proteins to the nitrocellulose. The rest of the blots were immunodetected. Initially blots were washed by gentle agitation for 30 minutes at 37°C in TTBS (Tween-Tris buffered saline containing 0.3% w/v Tween 20, 0.9% w/v NaCl in 10mM Tris-HCl, pH 7.4). This blocked unbound sites on the nitrocellulose (Batteiger et al, 1982). The blot were then rinsed three times in TBS (Tris-buffered saline, containing 0.9% w/v NaCl in 10mM Tris-HCl pH 7.4). The blots were subsequently probed by gentle agitation for 3 hours at 37°C in either hyperimmune rabbit anti *E.faecalis* serum (see section 2.2.2.4.1), serum from patients with 1E or serum from control patients each diluted 1:50 in TTBS. The blots were then rinsed three times in TBS and soaked in TTBS containing staphylococcal protein A-horse radish peroxidase conjugate (Sigma) 0.25 µg/ml. The serum incubated blots were visualised after rinsing three times in TBS by adding a freshly prepared solution containing H_2O_2 0.01% v/v (Thornton and Ross Ltd, Huddersfield, Yorks) and 4-chloro-1-napthol (Sigma) (25 µg/ml) in 10mM Tris-HCl (pH 7.4). The 4-chloro-1-napthol was first dissolved in a small amount of methanol and then added to the Tris-H_2O_2 solution. After 10-15 minutes at room temperature the colour reaction was stopped by flooding the blots with distilled water. A photographic record of results was then made.

The reaction of antigens from a particular organism with several sera was carried out by a technique known as strip-blotting. As before whole cells were separated by SDS-PAGE on a 12% polyacrylamide gel, and the separated components were transferred electrophoretically onto a nitrocellulose membrane as described above. The nitrocellulose was then cut into 5mm wide strips which were developed in plastic trays, divided into compartments (9cm x 1cm) holding a volume of 5-10ml. This procedure enabled strips bearing replicate patterns of antigens to be reacted with a range of sera.

2.2.3.3 Double diffusion immunoprecipitation

The Ouchterlony double diffusion technique was performed (Ouchterlony, 1958) 1% w/v agarose M (LKB, medium electroendosmosis, gelling temp 42°C) was prepared using Tris-barbiturate buffer, (barbitone 22.4g, Tris 44.3g calcium lactate 0.533g and sodium azide 0.65g to 1 litre of distilled water pH 8.6). The mixture was boiled to dissolve the agarose, and was then cast onto the hydrophilic side of gel bond support film (LKB Ltd, Croydon, Surrey) cut to squares of 6cm x 6cm. The volume to be cast to give a suitable depth of gel was determined as shown below.

Volume (ml) = area of gel bond (cm^2) x 0.132.

After setting, wells were punched in the gel, using a number 2 cork borer, (5mm diameter) and double diffusion immunoprecipitation was carried out by adding 10-30µl of antiserum to the central well and similar volumes of antigen preparations to

the laterally surrounding wells. The gel was then incubated in a moist chamber at 4°C for 18-24 hours. Non-precipitated proteins were removed by pressing and washing the gel (Weeke, 1977). The gel was placed between wet filter paper over which was placed a thick layer of dry, absorbent paper tissues. This was then sandwiched between glass plates and was evenly pressed with a weight for 10 minutes to reduce the gel to a thin film. The paper was then removed and discarded and the gel bond was transferred to a solution of 0.9% w/v NaCl for 10 minutes to allow the saline to both wash and reconstitute the gel. These processes (the pressing and the washing) were repeated three more times, with the last wash being in distilled water to remove NaCl from the gel. After the final pressing of the gel it was dried in hot air. The gel was then stained for protein to reveal precipitated antibody-antigen complex. Coomassie blue solution was then added to the gel for 10 minutes (Coomassie brilliant blue R-250, Sigma, 1g, ethanol 96% v/v 90ml, glacial acetic acid 20ml, distilled water 90ml). Excess staining solution was then removed from the gel with destaining solution (ethanol 96% v/v 90ml, glacial acetic acid 20ml, distilled water, 90ml).

2.2.3.4 Partial Purification of *E.faecalis*- specific antigens using ammonium sulphate precipitation

The *E.faecalis* specific 73, 40 and 37k antigens were partially purified in order to develop an enzyme linked immunosorbent assay (ELISA) method for the serodiagnosis of *E.faecalis* endocarditis. The method of ammonium sulphate precipitation was used. This method was based on the fact that at high salt concentrations proteins differ in solubility (Scopes, 1982).

Early stationary-phase cells of EBH1 grown in 1 litre of HS were harvested, washed and resuspended in 10mM Tris-HCl, pH 7.4. The cells were extracted with sarkosyl as described in section 2.2.2.3.2.

To 5ml of digested cells was added 100mg of streptomycin sulphate (Glaxo laboratories Ltd, Greenford, Middx). The suspension was incubated for 30 minutes at room temperature and was then centrifuged at 15,000 rpm for 10 minutes. The pellet was then reconstituted in distilled water and dialysed for 48 hours against distilled water and stored at -20°C. An amount of $(NH_4)_2 SO_4$ equivalent to a 30% saturated solution (see calculation below) was added to the supernatant remaining after streptomycin precipitation. The suspension was then vortex mixed, incubated at room temperature for 30 minutes and then centrifuged at 15,000 rpm for 10 minutes. The pellet was reconstituted in distilled water and dialysed against distilled water. Using saturated concentrations of $(NH_4)_2SO_4$ of 60 and 90% the precipitation was repeated. The pellets obtained at each precipitation stage were resuspended and dialysed against distilled water. Also dialysed

was the final 90% supernatant. Following dialysis the pellets and final supernatant were lyophilized, resuspended in 200µl of distilled water and stored at -20°C.

The amount of solid $(NH_4)_2SO_4$ to be added to a litre of a solution already at S_1% saturation to take it to S_2% saturation (Scopes 1982) was calculated as follows.

$$\text{Weight g} = 533\ (S_2 - S_1) \div (100 - 0.3S_2)$$

2.2.3.5 Enzyme linked immunosorbent assay (ELISA)

An enzyme linked immunosorbent assay using the 73,40 and 37 kdalton specific antigens was developed. This was used in a blind trial during which sera from 36 patients were investigated. 16 with *E.faecalis* endocarditis (confirmed by positive blood cultures); 10 with endocarditis caused by a range of Gram-positive organisms and 10 with *E.faecalis* infections other than endocarditis.

The test was also used to measure IgG, IgM and IgA levels in serial serum samples from four patients with *E.faecalis* endocarditis. Wells of the microtitre plates (Immulon, Dynatech) were coated with the specific antigens. These had been shown by SDS-PAGE, immunoblotting and detecting with an IE patients sera (see sections 2.2.3.1 and 2.2.3.2) to be present in the 90% ammonium sulphate precipitate of a sarkosyl extract of cells grown in horse serum. 100µl of the resuspended, dialysed precipitate was diluted with 80ml of 0.05 M carbonate buffer, pH 9.6. 100µl of this diluted antigen solution was added to the wells of the

microtitre plate and left overnight at 4°C. The wells were washed twice and blocked for 1 hour with phosphate buffered saline containing 0.05% v/v Tween 20 (PBST). After two further washes in PBST, doubling dilutions of antisera in PBST (100µl) were added to the wells and the plates incubated at 37°C for 2 hours. The wells were washed twice in PBST and 100µl of antibody detection conjugate added to each well. For IgG detection in the trial of sera from 36 patients, protein A peroxidase (Sigma) was used at 1.25µg/ml in PBST. In the longitudinal study of different antibody levels of 4 patients, goat antihuman IgG, IgM or IgA (Sigma) diluted 1:1000 in PBST was used. In all cases the plates were incubated for 2 hours at 37°C. After two washes in PBST, 100µl of chromogenic substrate solution was added to each well. The solution was prepared by adding 10mg of 3,3',5,5' tetramethylbenzidene (dissolved in 1ml of dimethyl sulphoxide) to 100ml of 0.1M sodium acetate/citrate buffer, pH 6.0 followed by 8µl of hydrogen peroxide (30% v/v). The colour reaction was stopped by addition of 35µl of 2M sulphuric acid to each well, and the absorbance at 450nm measured using an ELISA plate reader.

2.2.3.6.1 Negative staining

Using the technique of negative staining, the surface morphology of *E.faecalis* cells was investigated. Nickel grids (Agar Aids) covered with carbon coated Formvar (Emscope laboratories Ltd, Ashford, UK) were plasmaglowed in a Nanotech 300S coating unit to produce a hydrophilic surface. A turbid drop of a water washed cell suspension was placed on the grid and to this was added a drop of 1% w/v methylaminetungstate (Emscope). Excess liquid was

dried off with filter paper, and the grid was examined on an Hitachi H600 transmission electron microscope. Cells taken from early log, mid-log and stationary phase of growth were examined.

2.2.3.6.2 Determination of the percentage of fimbriate cells

The cells settled on the grids as dense clumps of various sizes, with some isolated cells apparent. At an instrument magnification of 40,000, cells around the edges of the clumps were examined for fimbriation. For each sample, approximately 100 cells were examined to determine the percentage of fimbriate cells.

2.2.3.7 Immunonegative staining

To demonstrate the presence of antigenic determinants on the surface of *E.faecalis* immunonegative staining was used. The method used was a modification of the method of Beesley et al (1984). Tris buffer (0.05M Tris-HC1, pH 8.6) containing 0.1% gelatin, 1% ovalbumin and 0.01% Tween 20 was used for all cytochemical labelling. The ovalbumin in the buffer is a non-specific blocker, the gelatin is a spreading agent and the Tween 20 helps to clean the background. A stock solution of the tris, gelatin and tween was made and stored at -4°C until required for use, at which time the ovalbumin was added.

A washed cell suspension was placed on carbon-formvar nickel grids. These were then air dried and inverted onto drops of buffer. After 1 minute the grids were transferred to a drop of either buffer (for the no serum control) or a dilution of antisera.

In this case the antisera used was purified monospecific rabbit IgG's to the 40 and 37 kdalton antigens, and purified non-immune rabbit IgG (see section 2.2.2.5). Several dilutions of antiserum were used and grids were incubated at room temperature for 30 minutes. The grids were washed five times in buffer and reincubated at room temperature for 30 minutes in goat anti-rabbit serum conjugated to 10nm colloidal gold (Auroprobe, GAR G10, Janssen Pharmaceutics, Beerse, Belgium) diluted in buffer (two dilutions were chosen of 1:10 and 1:20). The grids were washed five times in water and negatively stained in 1% methylamine tungstate and examined by TEM.

2.2.3.8 Ruthenium red staining

Ruthenium red staining has been widely used in electron microscopy to locate acidic and polysaccharide like material.

A modification of the method used by Luft (1971) was used. A solution of ruthenium red (Johnson and Mathey Chemicals) was made up in water to a final concentration 10mg ml^{-1} (1500 p.p.m.). Treated or untreated cells were washed three times in 0.2M cacodylate buffer (pH 7.3) and initially fixed in a mixture of 0.5ml 3.6% v/v glutaraldehyde 0.5ml 0.2m-cacodylate buffer pH 7.3 and 0.5ml ruthenium red stock solution for 1 hour at room temperature. After three washings in 0.2M-sodium cacodylate buffer, cells were fixed in a mixture of 0.5ml 4% w/v osmium tetroxide in distilled water, 0.5ml 0.2M-cacodylate buffer (pH 7.3) and 0.5ml ruthenium red stock solution. The cells were fixed overnight in this mixture. Controls were prepared by substituting distilled water for the ruthenium red stock solutions. Cells were

finally washed three times in 0.2M-cacodylate buffer, dehydrated in a graded series of ethanol solutions and embedded in LR white resin (London Resin Co). Section were then cut on a Reichert OMU4 microtome and these were then photographed on a Hitachi H600 electron microscope.

2.2.3.9 Bacterial adherence to hydrocarbon

The technique developed by Rosenberg et al (1980) was used. 200µl of hexadecane (Sigma) was added to 1ml of strain EBH1 resuspended to OD_{470} = 1.0 in PUM buffer ($K_2HPO_4 3H_2O$, 22.2g; KH_2PO_4 7.26g; urea 1.8g; $MgSO_4.7H_2O$, 0.2g distilled water to 1000ml, pH 7.1). After 10 minutes pre-incubation at room temperature the mixture was vortex mixed for two minutes. When separation of the layer was complete (20 minutes), 100µl of the aqueous layer was removed using a Hamilton syringe, and after diluting this with 900µl of PUM buffer, the OD_{470} of the layer was measured. Each experiment was performed in triplicate. The absorbance reading of the suspension after vortex mixing was expressed as a percentage of the original absorbance, the figure obtained giving an indication as to the hydrophobicity of the cells.

2.2.3.10 Iodination of serum proteins

There are several established methods for radio iodination of proteins, for example enzymatic iodination using the enzyme lactoperoxidase. This method, first described by Marchalonis (1969) is an extremely "gentle" method and minimizes damage to labelled proteins, but gives a relatively low yield of iodination, thus making it difficult to obtain proteins of high specific

radioactivity. For these reasons, and also because of the ease of use, Iodobeads (Pierce Chemical company) a relatively new solid state reagent which can be used to iodinate proteins were used in iodination of the serum proteins fibronectin and albumin. The iodobead itself consists of a uniform non porous plastic bead covalently modified with an oxidising agent (N-chlorobenzene sulfonamide (sodium salt) that facilitates the iodination of tyrosines in peptides and proteins. Work by Markwell (1982) indicated a much greater efficiency of incorporation of radioiodide into protein and also recovery of initial protein than with the lactoperoxidase method.

2.2.3.10.1 Iodination of fibronectin

Human FN (Sigma) was iodinated using the catalyst, N-chlorobenzene sulphonamide immobilised on plastic beads (Iodobeads, Pierce), 20µl of $Na^{125}I$ (5mCi/ml, 0.115mCi/µmole, Amersham) in water was added to 200µ of FN (1mg/ml in water); two iodobeads were added and the iodination allowed to proceed for 20 minutes at 20°C with gentle mixing. The reaction was terminated by removal of the beads, 0.5ml of 0.5M KI was added and the solution dialysed against distilled water for 3 days at 5°C to remove all unreacted iodide. The solution of iodinated FN was lyophilised and redissolved in 2ml water to give 8 x 10^4 dpm/10µl.

2.2.3.10.2 Iodination of albumin

Human albumin (Sigma) was iodinated using the above method 100µl $Na^{125}I$ (5mCi/ml, 0.115mCi/µmole, Amersham) in water was added to 1ml albumin (1mg/ml in PBS). The dialysed solution of iodinated albumin was lyophilised and redissolved in water to give 4.45 x 10^5 dpm/10µl.

2.2.3.11 Binding assays

2.2.3.11.1 Binding assay for fibronectin

Binding assays were performed in 1.5ml plastic eppendorf centrifuge tubes which had been immersed in 1% bovine serum albumin overnight at 37°C and then washed in phosphate buffered saline (PBS; 0.1M, pH 7.2) before use to suppress binding of FN. 1ml of cell suspension was added to each of a series of tubes

followed by ^{125}I-labelled FN to give a range of concentrations from 2.5 to 45µg/ml (5.7×10^{-6} to 1×10^{-4} µmoles/ml). Cell suspensions were vortex mixed for 1 minute and incubated at 20°C for 1 hour, with mixing every 15 minutes. Cells were pelleted by centrifugation for 5 minutes (16,000g). The pellet was resuspended in 1ml PBS and centrifuged again. After two further washes the labelled FN bound to the cell pellet was measured (ICN Gamma set 500). Separate time course experiments showed that binding equilibrium was established in 1 hour contact.

2.2.3.11.2 Binding assay for albumin

Binding assays for albumin followed the same method, but a solution of Tris-Tween buffered saline (0.3% Tween 20, 0.9% NaCl in 10mM Tris-HCl, pH 7.4) was used to block binding of the ^{125}I labelled albumin to the plastic tubes. Concentrations of albumin added to construct an isotherm ranged from 500 to 4000µg/ml OD 5.0 cells (75×10^{-3} to 5.9×10^{-2} µmoles/ml).

2.2.3.11.3 Pre-treatments of cells and ^{125}IFN

In order to partially characterise the FN receptor of the cells the effect of pre-treatments of the cells or FN upon binding were investigated. Before addition of 20µl I-FN, 1ml cell suspensions were either:

a) Heated at 60, 80 or 100°C for 15 minutes

b) Incubated with trypsin immobilised on acrylamide beads (Sigma) for 1hr at 37°C, followed by removal of the beads.

c) Incubated with 80µg/ml of protease k (Sigma) in PBS at 60°C for 1 hr or

d) 0.1M sodium periodate, pH 5.0 for 1hr at 37°C followed by washing three times and resuspension in PBS,

e) Incubated for 1hr at 20°C with 20µl of a solution of bovine serum albumin (Sigma), 200mg/ml or

f) 40µl/ml group D antiserum (Wellcome) or

g) 40µl/ml monospecific antiserum raised in rabbits against the 40 and 37 k dalton *E.faecalis* specific surface protein antigens (see section 2.2.2.4.2) for 1hr at 20°C.

In one experiment ^{125}I-FN was incubated with 5-40µg/ml of lipoteichoic acid (LTA) extracted from whole cells.

3 RESULTS AND DISCUSSIONS

3.1 Immunochemistry and surface properties of *Enterococcus faecalis*

3.1.1 Antigenic composition of *E.faecalis* and the effect of growth conditions

Work by Lambert et al, (1986) and Aitchison et al (1987) has previously shown that three surface proteins of molecular weights 73, 40 and 37 kdaltons are prominant antigens of *E.faecalis*, which are expressed strongly following growth in serum. They appear to be specific to *E.faecalis* and antibodies toward them are found in patients with *E.faecalis* endocarditis, but not with endocarditits due to other streptococci or in patients with other *E.faecalis* infections (Aitchison et al, 1987). For this reason the expression of these proteins in particular was investigated. It is known that surface properties related to pathogenicity are influenced by the growth conditions (Smith, 1977; Brown and Williams 1985). Thus the effect of growth media upon antigen expression by *E.faecalis* EBH1 was investigated using sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Two methods of sample preparation were used. Whole cell suspensions were boiled in sample denaturing buffer for 10 minutes and then subjected to SDS-PAGE on 12% gels, followed by western blotting. This procedure released some protein but did not lyse the cells (cell debris remained in the sample wells at the top of the stacking gel).

Alternatively, cell suspensions were first treated with the muramidase, mutanolysin to lyse the cells then boiled in denaturing buffer and subjected to SDS-PAGE. This method enabled all the cellular proteins to be released and separated (section 2.2.3.1). The suspensions were boiled in sample denaturing buffer and subjected to SDS-PAGE and western blotting.

The samples were grown in a variety of media: a chemically defined medium (CDM); the chemically defined medium + 0.1% horse serum; Difco brain heart infusion; Lab M brain heart infusion; and horse serum. The growth rates in the different media had previously been investigated and showed considerable differences. The growth curves for horse serum and Difco brain heart infusion grown cells obtained *in vitro* can be seen in figure 5. The growth curve for labM grown cells was almost identical to that of the Difco grown cells. The doubling time for the cells grown in both lab M and Difco brain heart infusion was approximately 25 minutes, the growth of cells in serum was much slower although the doubling time was difficult to estimate. Cells grown in CDM and CDM + 0.1% horse serum likewise grew very slowly. Previous results had shown that the antigenic profile and doubling time for cells grown in horse serum and pooled normal human serum were identical (E J Aitchison, PhD thesis, Aston University, 1987) and thus horse serum was deemed a suitable medium to mimic growth in human serum. Peak expression of the 73, 40 and 37 kdalton antigens is achieved at late stationary phase (Dr S J Prior, 1987, CAMR, Porton Down, Salisbury, Wilts personal communication). The cells in each case were thus harvested in stationary phase, washed three times and resuspended to an OD_{470} = 5.0 in 0.01M Tris-HCl buffer pH 7.2.

The western blots obtained were probed with sera from a high titre *E. faecalis* endocarditis patient (patient 1, table 11) and protein A-peroxidase.

The results can be seen in Figure 6. The 73,40 and 37 kdalton antigens were strongly expressed by cells grown in serum or in either of the brain heart infusions. They were present in the mutanolysin digests and the whole cell preparations, indicating the effective release from the cells on boiling with denaturing buffer (containing 2% SDS and 5% ß-mercaptoethanol). In contrast the CDM-grown cells did not release any detectable antigen on boiling with denaturing buffer (lane 1) although mutanolysisn treatment showed a range of antigens to be present. The notable difference in the antigen profiles of the CDM-grown cells compared with the other media was the virtual absence of the 40 and 37 kdalton bands and the increased expression of the 73 kdalton antigen complex. Addition of 0.1% horse serum to CDM rendered the cells susceptible to release of antigen with SDS and increased expression of the 73 kdalton antigen, but not the 40 and 37 antigens. Cells grown in horse serum expressed the 40 and 37 kdalton antigens most strongly.

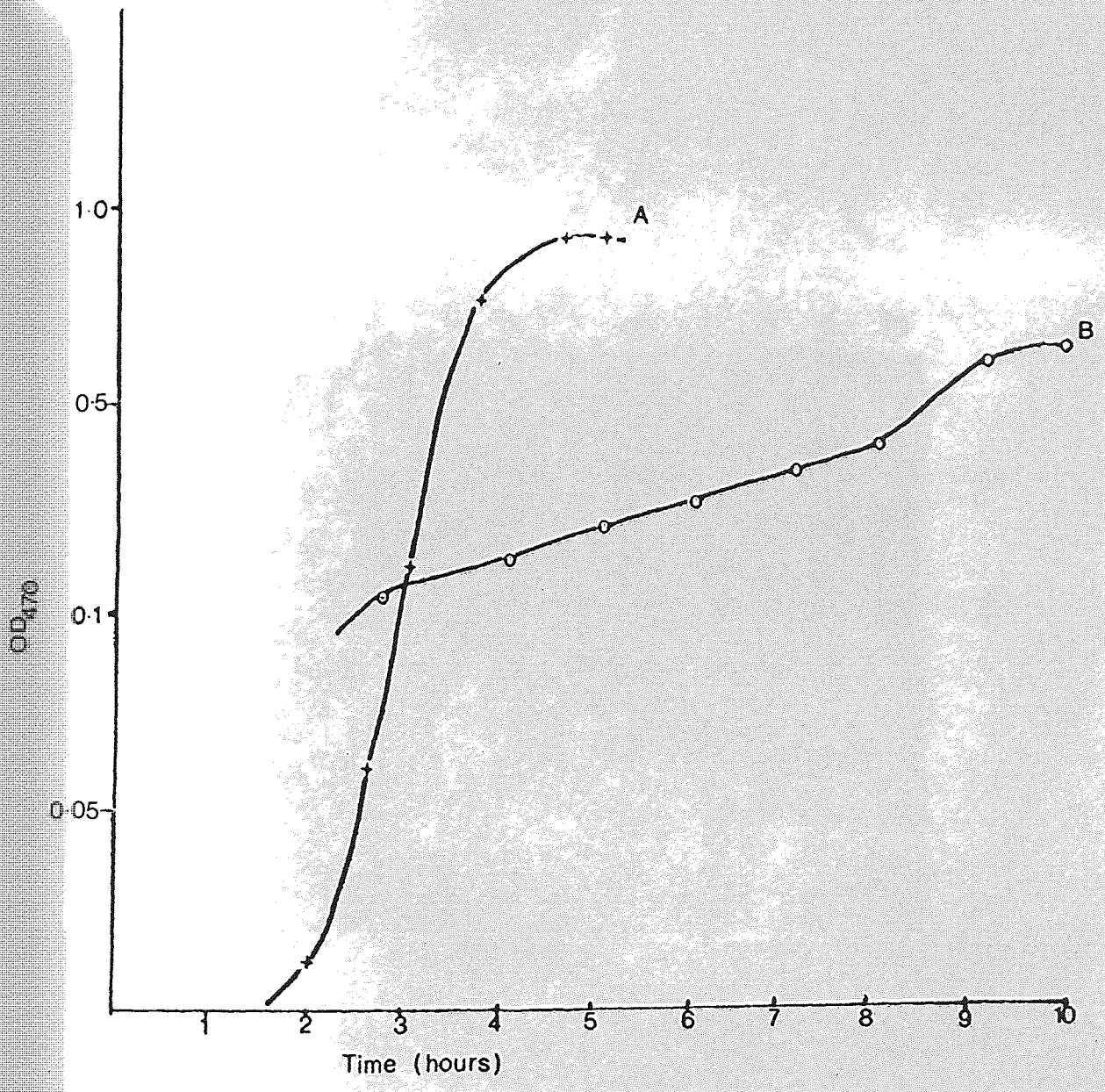


Figure 5 Growth curves of *E.faecalis* in brain heart infusion or horse serum at 37°C in batch culture at 180 rpm on orbital shaker.

Key

A Brain heart infusion (Difco)

B Horse serum

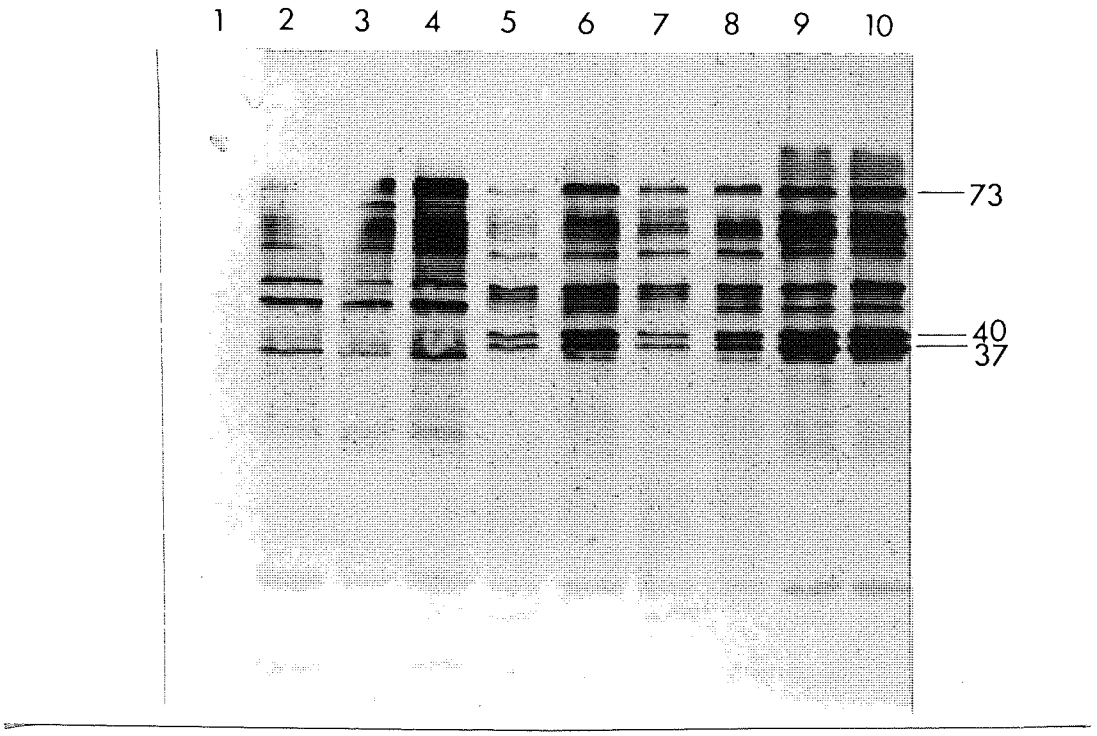


Figure 6 Western blot of *E.faecalis* whole cell preparations and mutanolysin digests probed with sera from a high-titre *E.faecalis* endocarditis patient (patient 1 in Table 11) and protein A-peroxidase. Cells were grown in: CDM (1,2); CDM + 1% horse serum (3,4); Difco brain heart infusion (5,6); lab m brain heart infusion (7,8); horse serum (9,10). Lanes 1,3,5,7,9 are whole cell suspensions boiled in sample denaturing buffer for 10 mins; lanes 2,4,6,8,10 are mutanolysin digests boiled in sample denaturing buffer for 10 min.

3.1.2 Investigation into the degree of fimbriation of an infective endocarditis isolate of *E.faecalis*

In order to investigate whether the infective endocarditis isolate EBH1 possessed fimbriae, the technique of negative staining was used. Despite the large number of Gram-negative species known to possess fimbriae as yet, relatively few Gram-positive species have been shown to carry them. Handley and Carter, (1979) reported peritrichous fimbriae on a small proportion of *Streptococcus mitior* strains which had been isolated from peridontal abscesses, and Handley and Jacob (1981) showed the presence of fimbriae on the cell surface of three strains of *E.faecalis* Fimbriae can vary from 3 to 14nm in width with their length reaching up to 20µm (Ottow, 1975). The fimbriae shown on the *E.faecalis* strains by Handley and Jacob were comparatively thin and short in relation to other fimbriae being up to 0.5µm long and 4.5nm in diameter.

The technique of negative staining makes use of the principle of surrounding or "embedding" the cells with an electron dense material, in this case 1% methylamine tungstate. The negative staining took place on Nickel grids covered with carbon coated Formvar, which had previously been plasmaglowed to make them more hydrophilic.

The investigation of fimbriation of EBH1 was carried out on cells grown in both Difco brain heart infusion and horse serum, and the effect of the phase of growth at which the cells were harvested was also investigated using difco grown cells (due to the difficulty in accurately pinpointing early and mid log phase in horse serum grown cells). The fimbriation of cells in early and

mid log phase, and stationary phase was thus investigated. Figure 7shows a typical fimbriated EBHI cell grown to mid log phase in Difco brain heart infusion.

Investigation of stationary phase cells grown in both Difco brain heart infusion and horse serum revealed very little fimbriation of cells. Figures 8 and 9 show non-fimbriated EBHI cells grown in Difco brain heart infusion and horse serum respectively. The cells settled on the grids as dense clumps of various sizes with some isolated cells apparent and at an instrument magnification of 20,000 cells around the edges of the clumps were examined for fimbriation. Approximately 100 cells per sample were examined to determine the percentage of fimbriate cells. For both the Difco and horse serum grown cells in stationary phase less than 5% of cells showed any degree of fimbriation. The cells taken in early log phase showed approximately 35% fimbriation and the cells taken in mid log phase 25%. The density of fimbriation seemed to vary little from cell to cell.

Figure 7 Negatively stained cells of *E.faecalis* (Strain EBH1) grown in Difco brain heart infusion to mid-log phase showing fimbriation (magnification x 60,000).

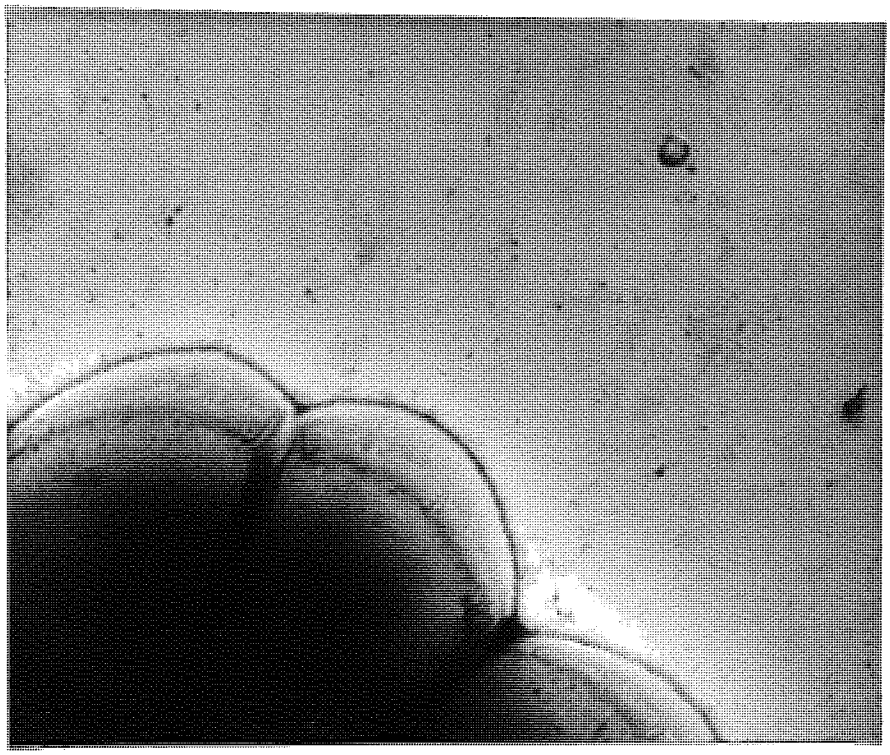


Figure 8 Negatively stained cells in *E.faecalis* (Strain EBH1) grown in Difco brain heart infusion to stationary phase showing no fimbriation (magnification x 50,000).

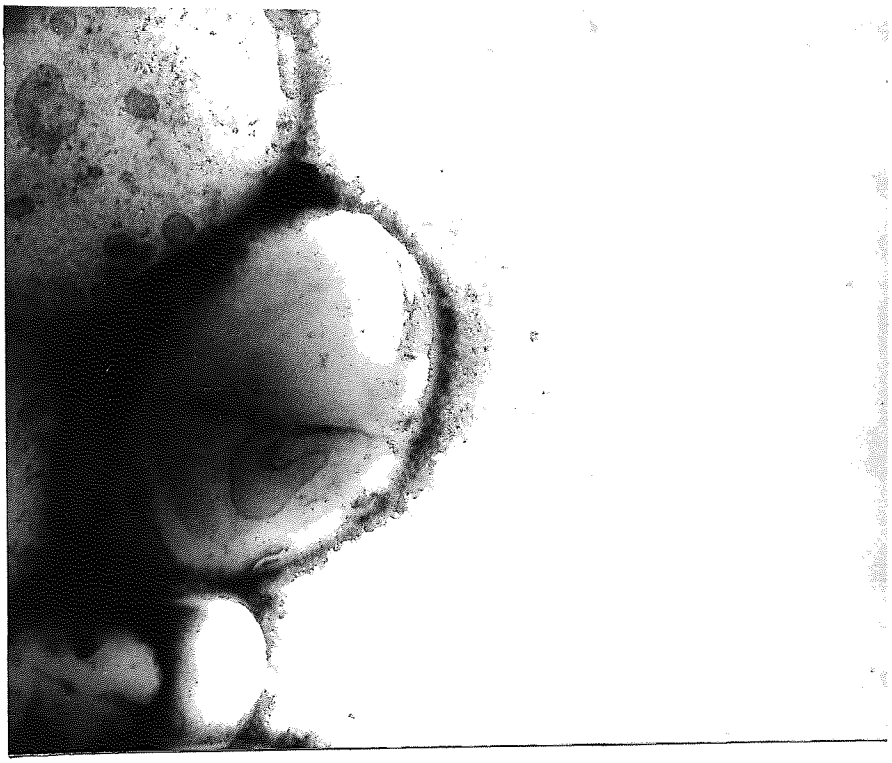


Figure 9 Negatively stained cells of *E.faecalis* (Strain EBH1) grown in horse serum to stationary phase showing no fimbriation (magnification x 40,000).

3.1.3 Location of the 40 and 37 kdalton species specific antigens of *E.faecalis* using immunonegative staining

The immunonegative stain technique is a very simple procedure. The antigen is dried down onto carbon coated electron microscope grids, then sequentially immunolabelled with antibody and gold probe "on the grid". Antibodies to the 40 and 37 kdalton antigens were raised in rabbits and the resulting monospecific antisera was purified as detailed in the methods (section 2.2.2.5). The monspecific antisera and purified IgG's were reacted against a western blot of serum grown *E.faecalis* EBH1 cells. The resulting strip blot can be seen in Figure 10 and indicate that the purified monospecific reacts with only the 40 and 37 kdalton antigens.

The immunonegative stain technique was used in order to help determine the location of the 40 and 37 kdalton antigens, the technique being selective for identification of proteins/antigens exposed on the surface of the bacterial cell. As a positive control pre-immune serum from the rabbit in which the monospecific antiserum was raised was used. EBH1 cells were grown in both difco brain heart infusion and serum to stationary phase. Cells taken at early log, mid-log were also investigated in the Difco grown cells (because of the difficulty in accurately pinpointing early and mid-log phase in the horse serum grown cells).

The results showed a surface covering of gold with the monospecific serum for both the difco and horse serum stationary phase cells. Figures 11 and 12 show cells grown in Difco brain heart infusion to stationary phase probed with neat and 1:5 diluted monospecific antiserum, and figure 13 shows cells grown in

horse serum to stationary phase probed with 1:5 diluted monospecific antiserum. Both Difco and horse serum grown cells showed no such surface labelling with the pre-immune serum, with only occasional particles of gold seen in the background (Figures 14 and 15 respectively). Early and mid log phase difco grown cells like the stationary phase cells also showed moderate surface labelling which was fairly uniform amongst all the cells examined (Figures 16 and 17). The pre-immune sera for both growth phases once again showed no such labelling.

The results suggest that the 40 and 37 kdalton antigens are present on the surface of *E.faecalis*. The fact that the cells showed almost identical labelling at whichever phase of growth they were examined, and the fact that all cells examined showed the labelling to a greater or lesser degree, implies that the 40 and 37 antigens are not associated with the *E.faecalis* fimbriae described in section 3.1.3.

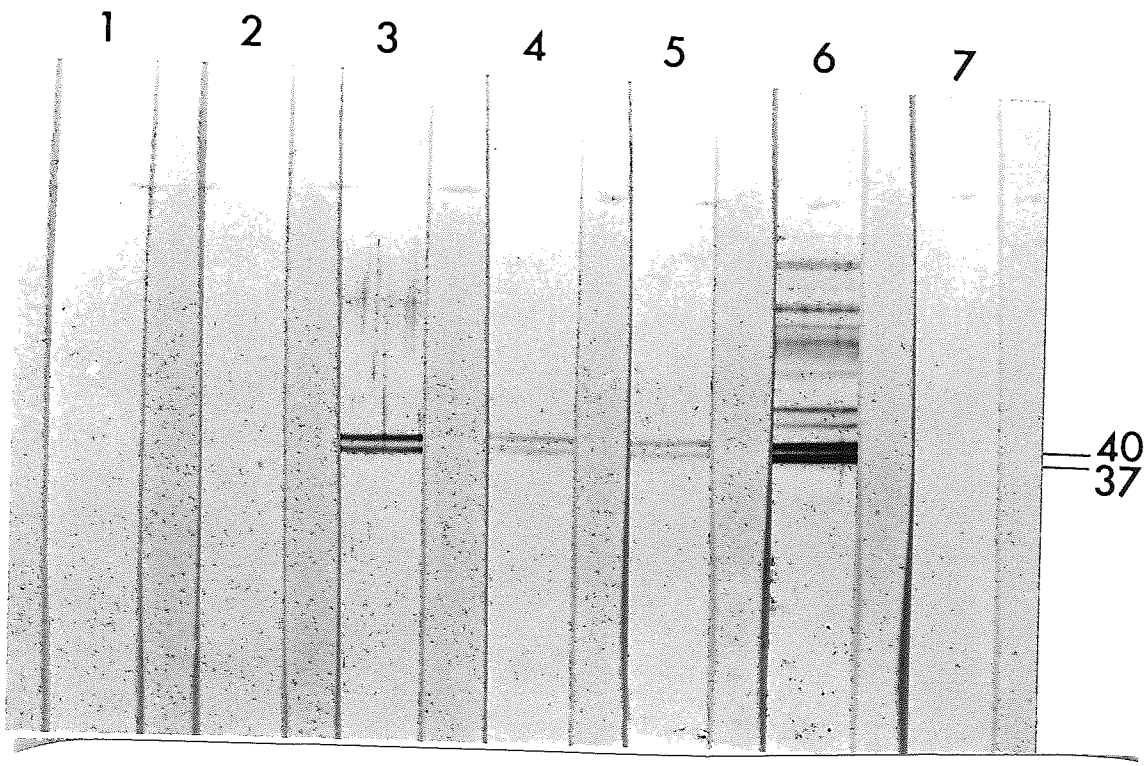


Figure 10 Strip blot of whole cells of *E.faecalis* grown in horse serum probed with (1) pre-immune rabbit serum; (2) purified IgG from pre-immune rabbit serum; (3) immune serum to the 40 and 37 kdalton antigens raised in rabbits; (4) and (5) purified IgG fraction to the 40 and 37 kdalton antigens, batch a) and b) respectively; (6) serum from an *E.faecalis* IE patient; (7) no antibody added.

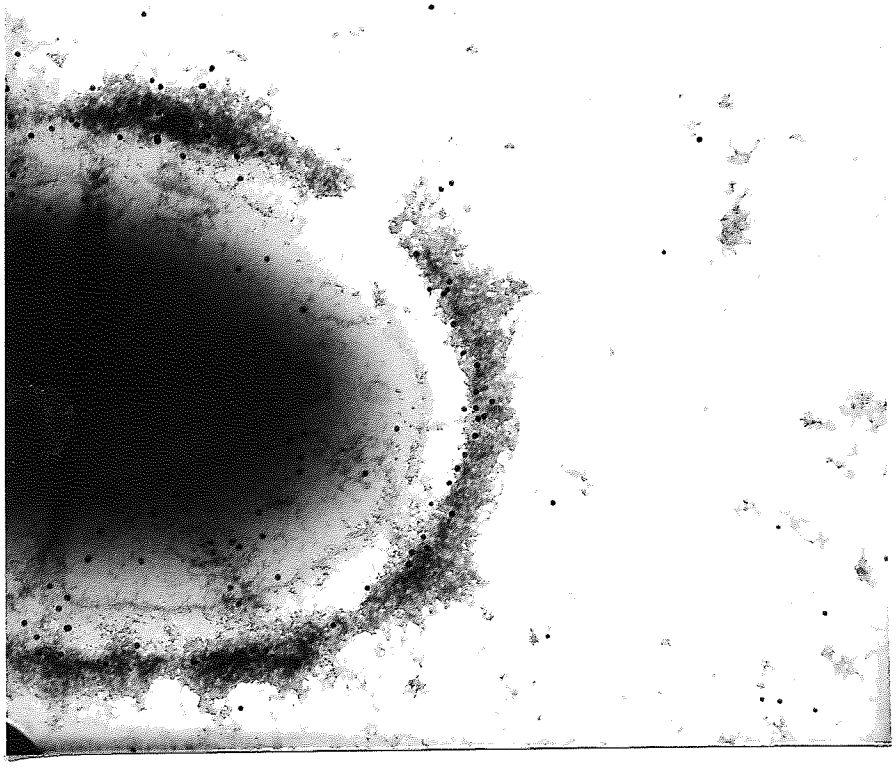


Figure 11 Immunonegative staining of *E.faecalis* cells (strain EBH1) grown to stationary phase in Difco brain heart infusion, labelled with neat monospecific antiserum raised in rabbits to the 40 and 37 kdalton species specific antigens and probed with goat anti-rabbit serum conjugated to 10nm colloidal gold (magnification x 60,000).

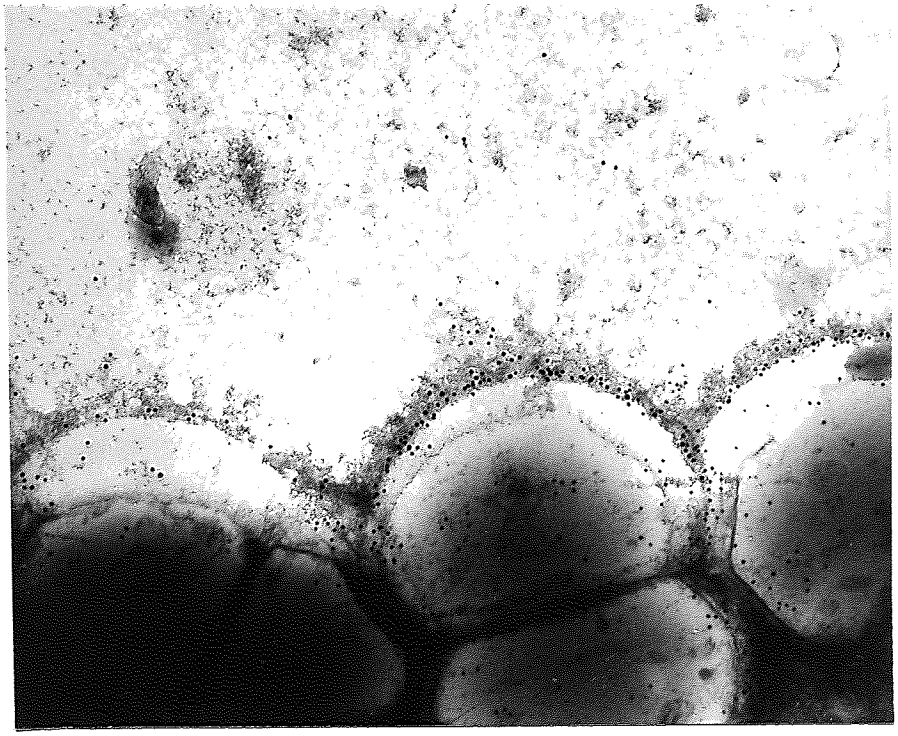


Figure 12 Immunonegative staining of E faecalis cells (strain EBH1) grown to stationary phase in Difco brain heart infusion, labelled with monospecific antiserum raised in rabbits to the 40 and 37kdalton species specific antigens diluted 1:5 and probed with goat anti-rabbit serum conjugated to 10nm colloidal gold (magnification x 40,000)

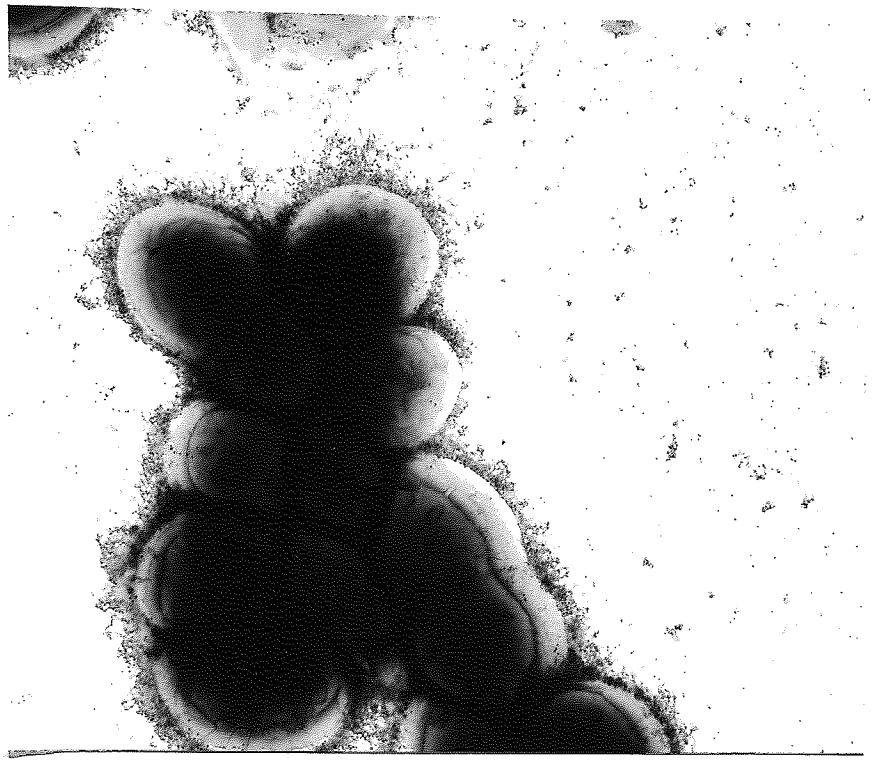


Figure 13 Immunonegative staining of *E.faecalis* cells (strain EBH1) grown to stationary phase in horse serum (Gibco), labelled with monospecific antiserum raised in rabbits to the 40 and 37 kdalton species specific antigens diluted 1:5 and probed with goat anti-rabbit serum conjugated to 10nm colloidal gold (magnification x 35,000)

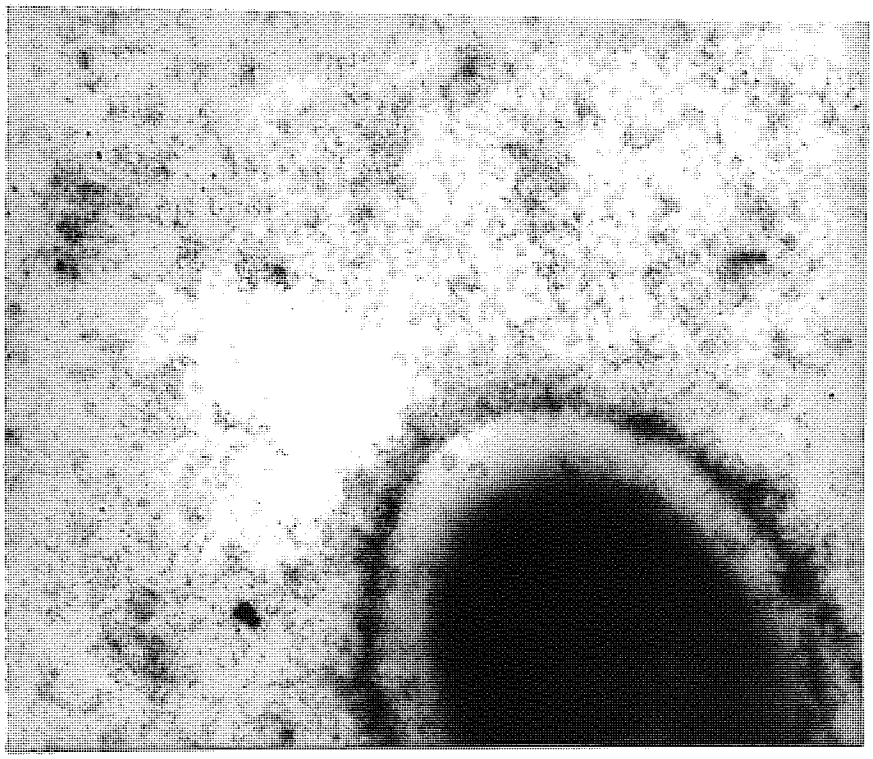


Figure 14 Immunonegative staining of *E.faecalis* cells (strain EBH1) grown to stationary phase in Difco brain heart infusion, labelled with neat pre-immune rabbit antiserum and probed with goat anti-rabbit serum conjugated to 10nm colloidal gold (magnification x 50,000).

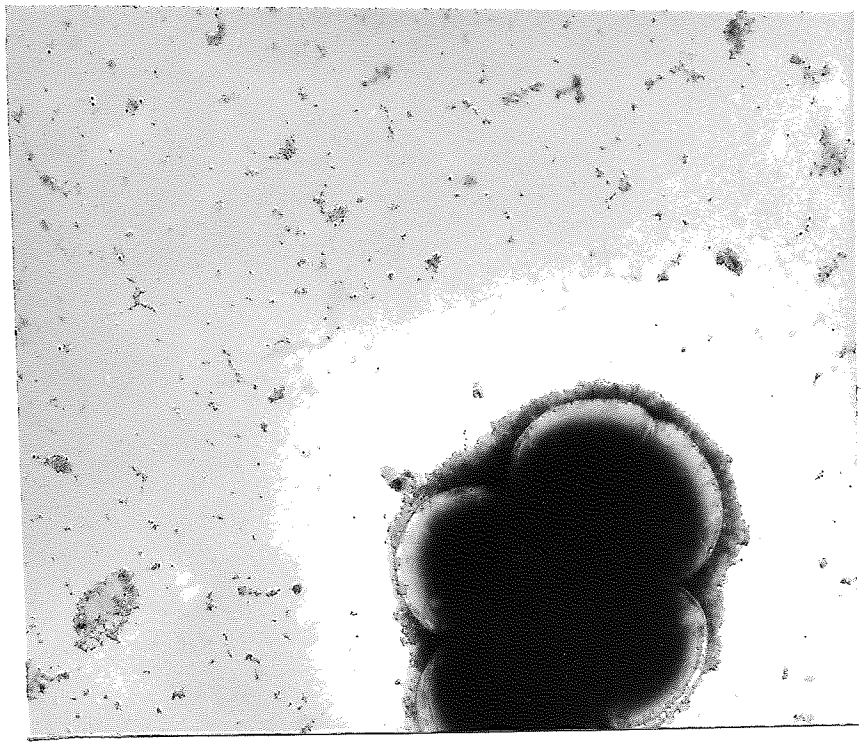


Figure 15 Immunonegative staining of *E.faecalis* cells (strain EBH1) grown to stationary phase in horse serum (Gibco) labelled with pre-immune rabbit antiserum diluted 1:5 and probed with goat anti-rabbit serum conjugated to 10nm colloidal gold (magnification x 20,000).

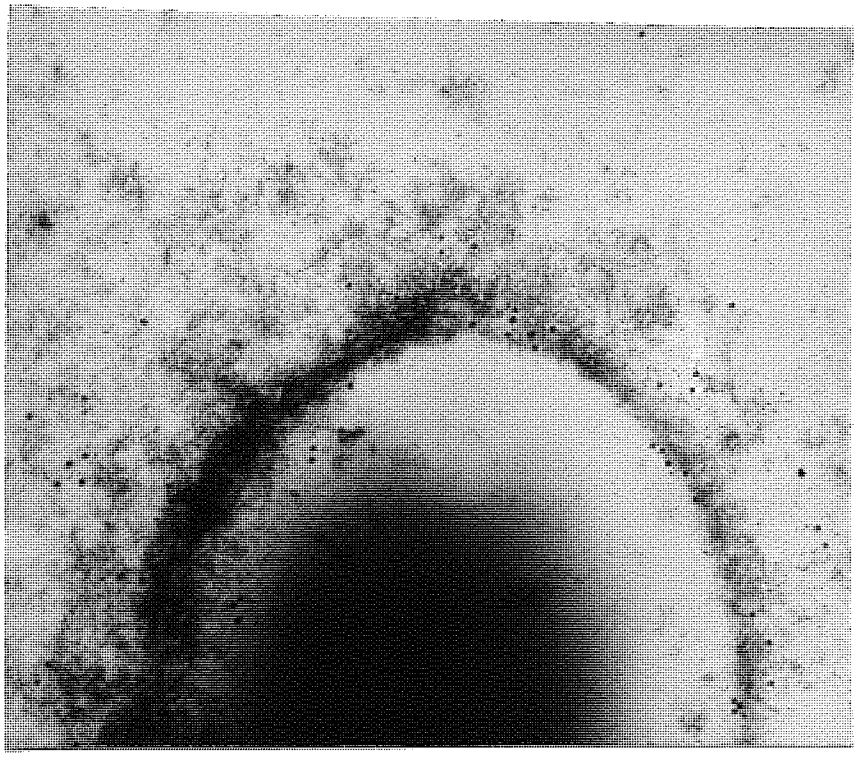


Figure 16 Immunonegative staining of *E.faecalis* cells (strain EBH1) grown to early log phase in Difco brain heart infusion, labelled with monspecific antiserum raised in rabbits to the 40 and 37 kdalton species specific antigens diluted 1:5 and probed with goat anti-serum conjugated to 10nm colloidal gold (magnification x 60,000).

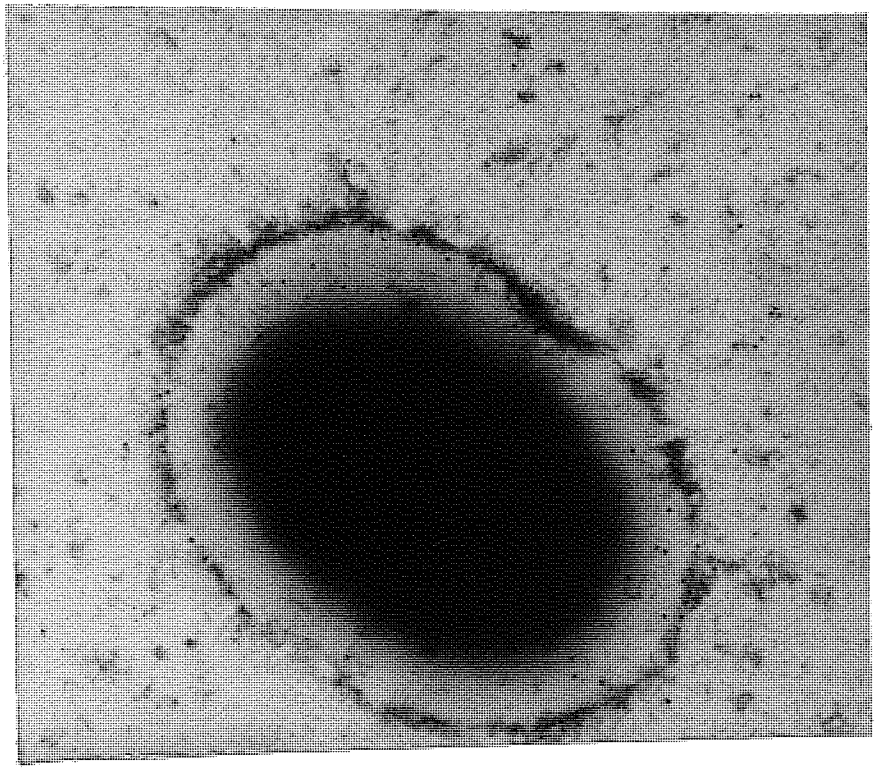


Figure 17 Immunonegative staining of *E.faecalis* cells (strain EBH1) grown to mid log phase in Difco brain heart infusion labelled with monospecific antiserum raised in rabbits to the 40 and 37 kdalton species specific antigens diluted 1:5 and probed with goat anti-rabbit serum conjugated to 10 nm colloidal gold (magnification x 40,000).

3.1.4 Examination of the surface of *E.faecalis* using ruthenium red staining

The technique of ruthenium red staining is one which has been widely used in electron microscopy to locate acidic and polysaccharide like material. In a catalytic reaction ruthenium red complexes with carboxyl or hydroxyl groups in the presence of osmium tetroxide, giving an electron dense precipitate. In this way polymers which carry a high charge density (usually acidic polysaccharides) are stained (Luft, 1971), although the staining of the neutral polysaccharide dextran on the surface of *Leuconostoc mesenteroides* has also been reported. (Brooker, 1979). After conventional fixation polysaccharides are not detected. The technique was first studied in detail by Luft (1971) and has since been used in several studies on surface structure and composition. It has frequently been used to describe adhesive components (Costerton and Irvin, 1981; Brooker, 1979 and Fletcher and Floodgate, 1973).

It was decided to investigate the surface of *E.faecalis* using the ruthenium red staining technique, and cells grown to stationary phase in Difco brain heart infusion were stained, fixed, embedded and sectioned as detailed in the methods (section 2.2.3.8). The results can be seen in Figure 18. These show a fine layer uniformly surrounding the cell. A similar ruthenium red staining layer has recently been described in *Streptococcus salivarius* (Handley et al, 1988).

Little is known about such ruthenium red layers, and it is not known if they should be considered to be part of the cell wall, however there are many biochemical observations which show that neutral and acidic polysaccharides covalently linked to the peptidoglycan are common in Gram-positive bacteria and are considered to be components of the cell wall (Shockman and Barrett, 1983 Rogers et al 1980). In the case of *S.salivarius* it was suggested that lipoteichoic acid (LTA) may be present in the ruthenium red layer (Handley et al, 1988) and this could possibly also be the case with *E.faecalis*. It was found by Brooker (1979) that different *L.mesenteroides* strains showed a variety of different surface layer patterns outside the cell wall and that growth in different media influenced the appearance of the ruthenium red staining layers. It was thus decided to investigate *E.faecalis* cells grown to stationary phase in horse serum. The results can be seen in Figure 19. They show that in addition to a thin stained layer like that shown in the Difco grown cells, large irregular patches of stained material are apparently attached to the cells. The precise nature of this layer is not known. It could possibly be a serum component/s bound to the cells. If this is the case such molecules might shield surface antigens from the host immune system and thereby account for the simple antigenic profiles reported by Dalhoff (1985) for *in vivo* grown cells.

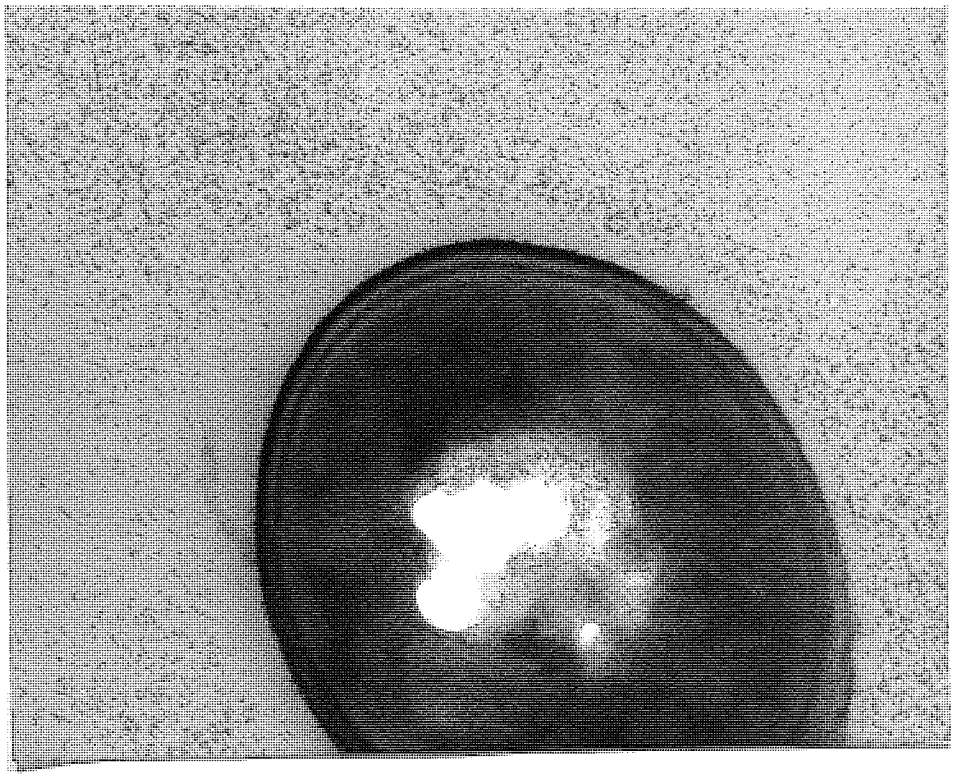


Figure 18 *E.faecalis* (strain EBH1) grown in Difco brain heart infusion, sectioned and stained using ruthenium red (magnification x 80,000).

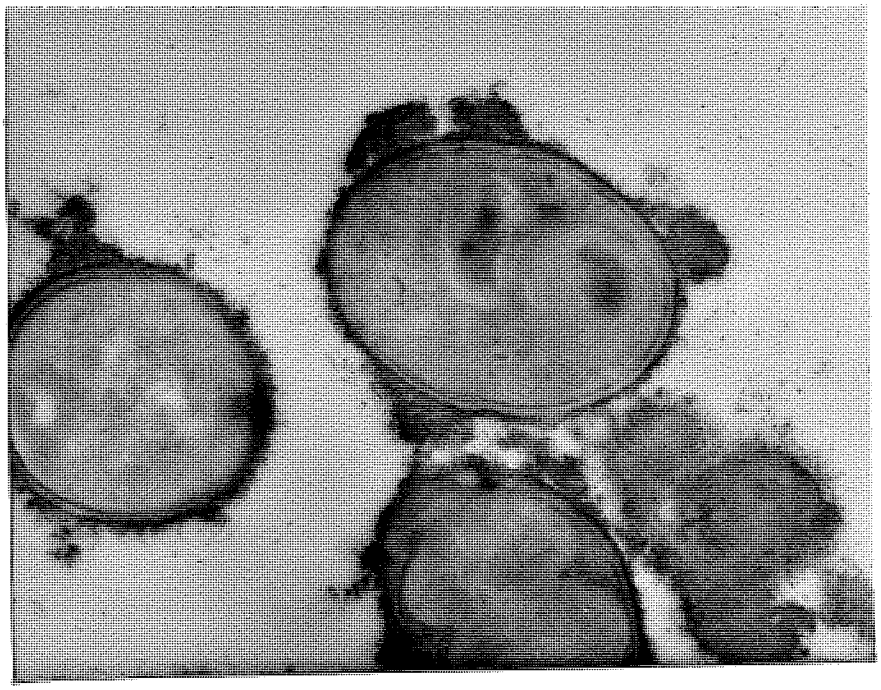


Figure 19 *E.faecalis* (strain EBH1) grown in horse serum, sectioned and stained using ruthenium red (magnification x 30,000).

3.1.5 Discussion of section 3.1

Using a western blotting approach it has previously been shown that patients with *E.faecalis* infective endocarditis display a strong IgG response to three protein antigens of 73, 40 and 37 kdaltons (Aitchison et al, 1987). Their specificity towards *E.faecalis* and their association with endocarditis suggested an application in the serodiagnosis of *E.faecalis* to infection in endocarditis. Relatively little is known about the nature of these *E.faecalis* specific antigens however apart from their reaction with lectins on western blots (Aitchison et al, 1986; Aitchison et al, 1987) when both the 73 and 37 kdalton antigens were shown to be glycosylated antigens by their action as receptors for wheat germ agglutinin, asparagus pea lectin, concanavalin A and soybean agglutinin. The location of the 40 and 37 kdalton antigens in whole cells was thus investigated using electron microscopy with immunonegative staining using purified monospecific antinserum prepared from excised regions of nitrocellulose sheets to which the 40 and 37 kdalton antigens had been transferred by western blotting. It was not possible to prepare monospecific antiserum to the 73 kdalton antigen because of the presence of other bands in this region of the blot. The polyclonal serum obtained for the 40 and 37 kdalton antigens gave a strong reaction to these bands on the strip blot (Figure 10) but it was not however possible to isolate monospecific antiserum to the individual 40 and 37 kdalton proteins because of their close proximity as bands on the preparative blots.

Work using western blotting has shown that the *E.faecalis* specific antigens are expressed when the cells are grown in both brain heart infusion and horse serum. Thus for the immunonegative staining technique, cells grown in both brain heart infusion (Difco) and horse serum were investigated. The results showed that in both cases the purified IgG from the monospecific antiserum to the 40 and 37 kdalton antigens bound to the surface of *E.faecalis* (Figures 11, 12 and 13) and controls using the pre-immune rabbit IgG in place of the monospecific IgG gave no labelling of cells or background (Figures 14 and 15). This showed that the antigens are accessible to antibodies on the cell surface. The labelling pattern of antibody on the horse serum grown cells was slightly different to that in the brain heart infusion grown cells in that more gold particles were deposited some distance away from the cell surface (Figure 13). As no background labelling was seen in the pre-immune IgG controls or in cells treated with conjugate alone it is presumed that some antigen is released from the cells when they are applied to the grids.

The presence of fimbriae on *E.faecalis* strain EBH1 was also investigated using the technique of negative staining. Fimbriae were seen on cells grown in both brain heart infusion and serum but their expression depended on the phase of growth in which the cells were taken to be investigated. A significant number (approximately 35%) of cells early logarithmic phase were fimbriate with the value falling to 25% in mid log phase and as low as 5% in stationary phase cells. These results agreed with

work performed by Handley and Jacob (1981), who also noted a decrease in the percentage of fimbriate cells of two *E.faecalis* strains in stationary phase.

No variation in the immunogold labelling of the 40 and 37 kdalton antigens according to phase of growth was seen however, thus suggesting that the antigens are not part of the fimbriae.

Ruthenium red staining has previously been used to detect surface fibrils in bacteria such a *Streptococcus salivarius* (Handley et al, 1988) and has revealed different lengths of surface fibril on these bacteria. Indeed a variety of surface structures have been described on oral Gram-positive bacteria. Negative staining has shown that peritrichous fibrils and tufts of fibrils occur in different strains of *Streptococcus sanguis* (Handley et al, 1985) and *Streptococcus salivarius* (Weerkamp et al, 1986). Some of these structures might be involved in adhesion (Weerkamp et al, 1986) or the coaggregation with other oral genera (Handley et al, 1985).

Staining of *E.faecalis* with ruthenium red failed to reveal any surface fibrils, but did reveal a ruthenium red staining layer surrounding the cell wall. The technique showed that there was a considerable difference between horse serum and brain heart infusion grown cells. The brain heart infusion cells produced a smooth uniform ruthenium red staining layer overlying the cell wall, whereas by contrast horse serum grown cells showed an irregular layer with large patches of stained material seen on the surface and between adjacent cells. This material is probably polysaccharide as ruthenium red is known to form electron dense

precipitates with carboxyl and hydroxyl groups in the presence of osmium tetroxide and acidic (Luft, 1971) and neutral polysaccharides (Brooker, 1979) are stained in this way. It is possible that the dense material adhering to the horse serum grown cells originates from serum, but this seems unlikely given the vigorous washing the cells received prior to their use. Further studies on the sensitivity to proteases and periodate are needed to confirm the layers polysaccharide nature.

3.2 Serodiagnosis of *E.faecalis* endocarditis using an enzyme linked immunosorbent assay (ELISA)

Despite the advent of antimicrobial chemotherapy infective endocarditis (I.E.) remains a difficult condition to both diagnose and treat and still carries a mortality rate of 30% (Hayward 1973a, Wilson and Geraci 1983 and Newsom 1984). There has been a decline in developed countries in the number of cases due to haemolytic ("Viridans") streptococci which carry a low mortality of 6% (Bayliss et al, 1983) and an increase in the number of cases due to enterococci with a mortality of about 14% (Bayliss et al, 1983). The increase in the number of cases due to enterococci is especially prominent in the elderly (Wesby, 1978). Current trends indicate a rise in the mean age of endocarditis patients, which is partly due to the increased age of the population but also due to a decrease in the occurrence of rheumatic heart disease in the young and the earlier surgical correction of congenital heart defects. Thus the incidence of *Enterococcus faecalis* endocarditis is becoming more prevalent, and is especially difficult to treat requiring a combination of penicillins, aminoglycosides and/or glycopeptide antibiotics (for example vancomycin) for several weeks, with attendant side effects (Wilson and Geraci, 1985). An increasing threat to successful therapy is the emergence of resistance to these agents (Mederski-Samoraj and Murray, 1983, Uttley et al, 1988).

Thus identification of the causative organism isolated from the blood of patients with I.E. is of paramount importance for the selection of appropriate treatment (Hayward, 1973b). Confirmation is made by cultures of blood samples, but in up to 10% of cases

these may be negative (Bayliss et al 1983) thus new rapid techniques to reliably confirm or exclude *E.faecalis* in I.E. and which might also be useful in assessing therapeutic response would be of great clinical value. Three species specific protein antigens have previously been described, with molecular weights of 37, 40 and 73 kdaltons, which are strongly expressed following growth in serum (Lambert et al 1986 and Aitchison et al 1987). These antigens appear to be specific to *E.faecalis* and looking at the IgG response, antibodies towards them are found in patients with *E.faecalis* endocarditis, but not with endocarditis due to other streptococci (Aitchison et al 1987). However the western blotting methods used to establish the specificity of the antibodies towards these antigens are not convenient for application as a routine serodiagnosic test for *E.faecalis* endocarditis. It was therefore decided to develop an enzyme-linked immunosorbent assay (ELISA) based on the 37 40 and 73 kdalton antigens.

3.2.1 Extraction and partial purification of the 73, 40 and 37 kdalton antigens

Since the antigens had been shown to be most strongly expressed when grown in serum and also because this was considered to most closely mimic the *in vivo* growth situation, the EBH1 cells were grown in horse serum. Previous experiments had shown that the antigenic profile of strain EBH1 grown in horse serum was identical to that of EBH1 grown in normal human serum (data not shown). In order to extract the 73, 40 and 37 kdalton antigens from the cells they were treated with the detergent sodium lauroyl sarcosinate (sarkosyl). This procedure had been shown by Jenkinson (1986) to remove surface proteins from the cell as well as LTA, polynucleotides and polysaccharides.

To partially purify the 73, 40 and 37 kdalton antigens the method of ammonium sulphate precipitation was used. This method is based on the fact that at high salt concentrations proteins differ in solubility (Scopes, 1982). Before precipitation with ammonium sulphate nuclear material was precipitated by the addition of 1% w/v streptomycin sulphate (Glaxo). Precipitation with ammonium sulphate was then carried out using progressive partial saturation concentrations of 0, 30, 60 and 90%.

The salting-out of the proteins from the sarkosyl extract was investigated by subjecting the dialysed precipitates and also the 90% supernatant to SDS-PAGE followed by blotting onto nitrocellulose, with detection of the antigen by probing with the serum of an *E.faecalis* I.E. patient with a high titre to the 40 and 37 kdalton antigens. The results can be seen in Figure 20. As

can be seen the sarkosyl extract from whole cells shows a complex pattern of proteins. It would be expected that the addition of the streptomycin sulphate would precipitate out much high molecular weight material plus a large proportion of antigens, made up of the characteristic antigenic pattern of *E.faecalis.* As can be seen from the blot the 73 40 and 37k antigens were deposited mainly in the 90% precipitate. Thus it was decided that these partially purified antigens would be used as the basis for the ELISA test for serodiagnosis of *E.faecalis* endocarditis.

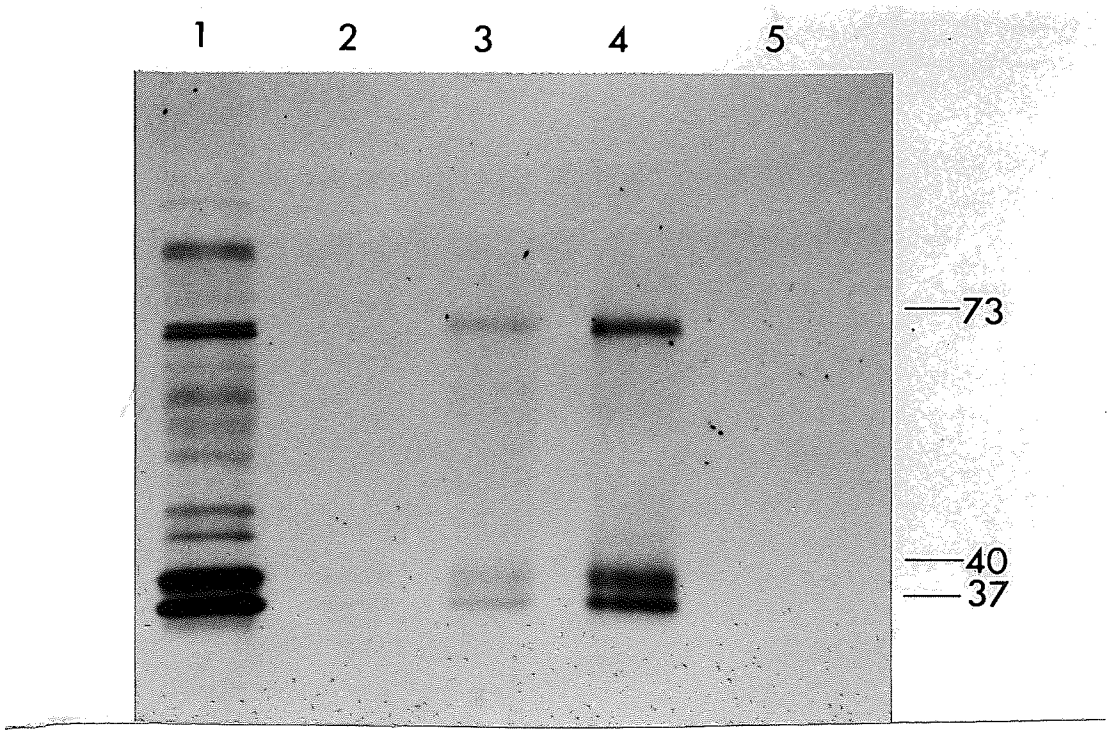


Figure 20 Western blot of *E.faecalis* antigen preparations probed with sera from a high titre *E.faecalis* endocarditis patient and protein A - peroxidase. Lanes are: sarkosyl extract from whole cells, (1), ammonium sulphate precipitates from the sarkosyl extract at 30% (2), 60% (3), and 90% saturation (4); supernatant remaining after 90% ammonium sulphate precipitation (5).

3.2.2 Development of an enzyme linked immunosorbent assay (ELISA)

The antigen solution produced was used as the basis of the ELISA test. In order to optimise the ELISA, a concentration of antigen was decided upon by using a checkerboard test on ELISA microtitre plates (Dynatech Immulon). The antigen solution was tested in a range of dilutions from the concentrated solution and then diluted with carbonate buffer down to a concentration of 1:1000. The solutions were then tested against dilutions of the serum of an endocarditis patient, pooled normal human serum (the patients pre-immune serum was not available and so the negative control was the pooled normal human serum) and monospecific antiserumraised to the 40 and 37 kdalton antigens. Other controls used included wells with no sera added to them, and also wells with no antigen solution coating them. The dilution of the conjugate to be used was also varied in order to optimise the assay results.

3.2.3 Endocarditis serodiagnosis trial

The trial which consisted of the investigation of sera from 36 patients was conducted completely blind, so that even the number of *E.faecalis* endocarditis samples present was unknown.

In fact of the 36 patients, 16 had *E.faecalis* endocarditis (confirmed by positive blood cultures); 10 had endocarditis caused by a range of Gram-positive organisms and 10 had *E.faecalis* infections other than endocarditis. The precise details of the samples are summarised in table 10.

The trial investigated the level of IgG in the serum samples using a titre of serum from 1:50 to 1:3200. IgG is the most abundant species of immunoglobulin (Ig) in normal human serum, comprising 80% of the total Ig content, and is probably the major immunoglobulin to be synthesised during the secondary response (Roitt, 1971).

Interpretation of ELISA results varies from study to study. Studies with Gram-positive bacteria have used E_{450} 0.1 (van de Rijn et al, 1986) and E_{450} 0.2 (Jacob et al, 1985). Here the serum titre to reduce the absorbance to 0.1 was used. The titre taken to give a positive result was 1:100 and was determined by investigation of the sera of a range of patients with *E.faecalis* endocarditis, and noting the weakest response. A summary of the results of the blind trial can be seen expressed as a scatter plot in Figure 21. The serum titres of the patients can be seen plotted in Figures 22 to 40. In some cases the readings after the first dilution of the serum of 1:50 were so low as to be

unreadable and a titre to give an E_{450} of 0.1 was estimated as no graph was plottable (patients 4, 13, 14, 15, 16, 17, 20, 21, 22, 25, 27, 28, 39, 30, 34 and 35). Background colour developed for pooled normal human serum was also <0.05 for a 1:100 dilution 15/16 serum samples from *E.faecalis* endocarditis patients gave positive results, with titres ranging from 1:100 to 1:10,000 (only patient 31 gave a false negative result) 2/10 patients with *E.faecalis* infections other than endocarditis gave false positive results (patients 18 and 36). Patient 18 had a chronic urinary tract infection and patient 36 had osteomyelitis. 1/10 patients with endocarditis due to organisms other than *E.faecalis* gave a positive result, (patient 23) the infecting organism in this case being a nutritionally variant streptococcus (NVS).

Table 10

Serum samples used in the ELISA trial

Sera from Patients with *E.faecalis* not associated with IE

1 x *E.faecalis* from drainage site

1 x *E.faecalis* bronchial washings

1 x *E.faecalis* from nephrostomy fluid

2 x *E.faecalis* from peritoneal dialysis fluid

1 x *E.faecalis* wound swab

2 x *E.faecalis* urinary tract infection (UTI)

1 x *E.faecalis* osteomyelitis

Sera from patients with IE caused by organisms other than *E.faecalis*

1 x *S.mitis* IE

1 x *S.bovis* IE

2 x *S.sanguis* IE

1 x *S.aureus* IE

1 x *S.epidermidis* IE

1 x *S.mitior* IE

1 x ß-haemolytic strep IE

1 x nutritionally variant streptococcus (NVS)

Sera from patients with *E.faecalis* IE

16 x *E.faecalis* IE patients

Control serum

a negative control of pooled normal human serum was used

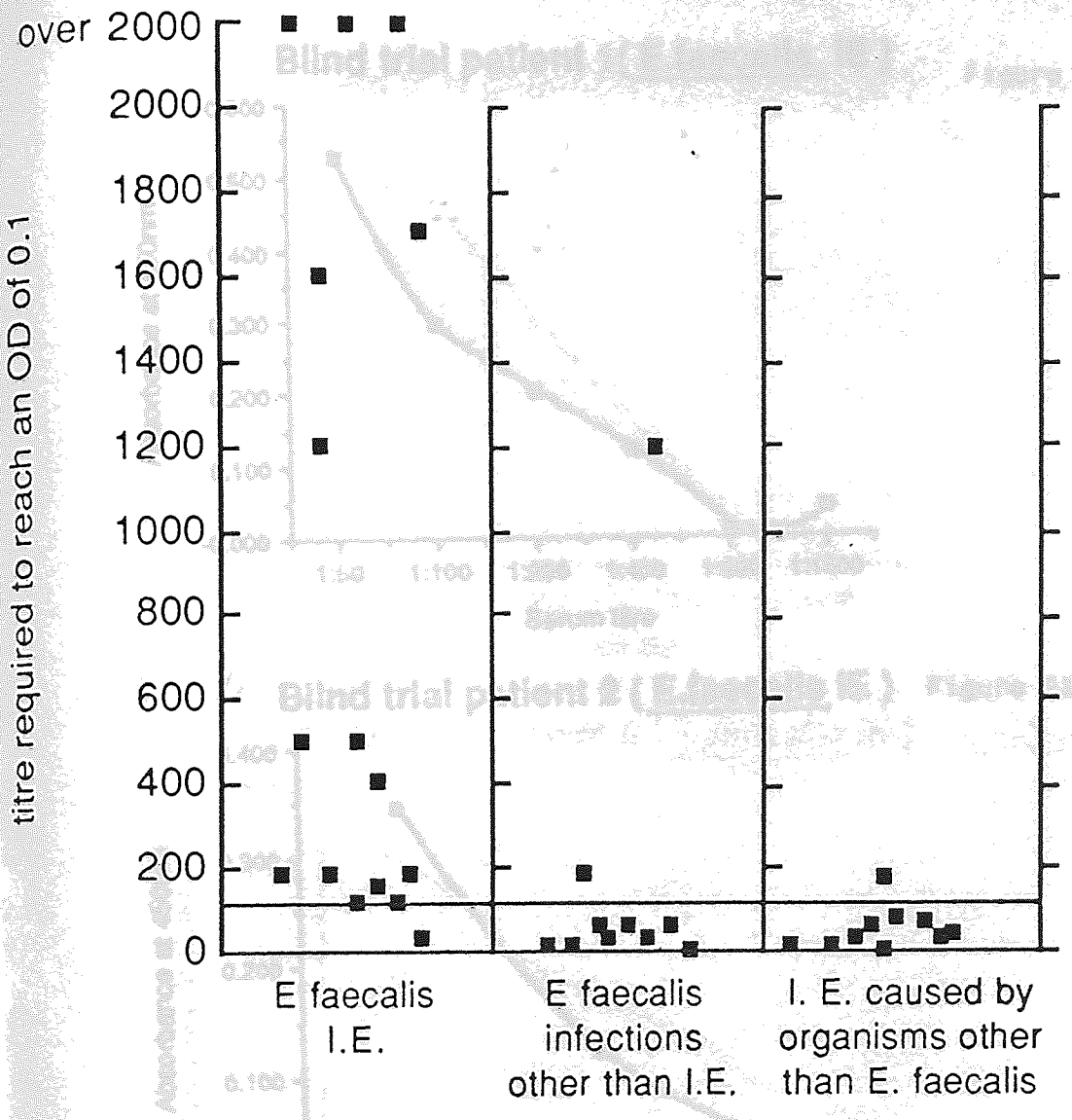
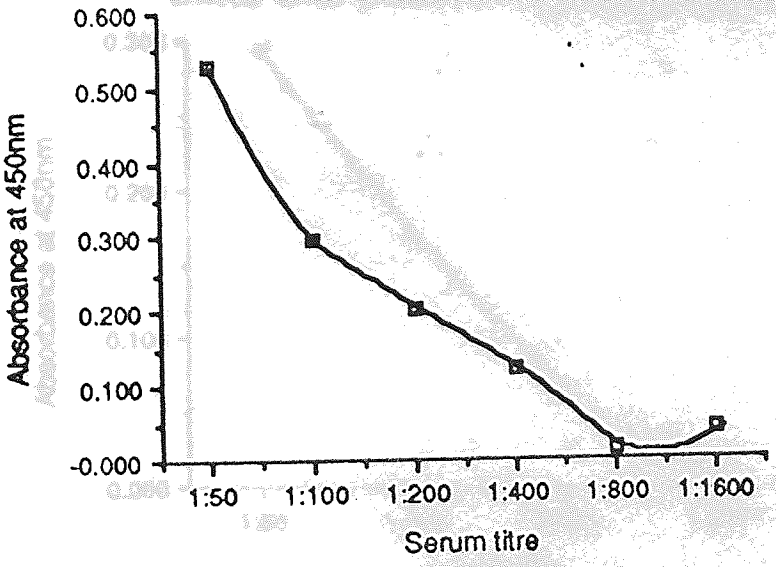


Figure 21 IgG levels to *E.faecalis* specific antigens in sera from patients with: A, *E.faecalis endocarditis; B, E.faecalis* infections other than endocarditis; and C, endocarditis caused by organisms other than *E.faecalis*. Reciprocal titres are the dilutions of sera required to reduce the colour to an absorbance at 450 nm of 0.1. Values greater than 100 are considered positive.

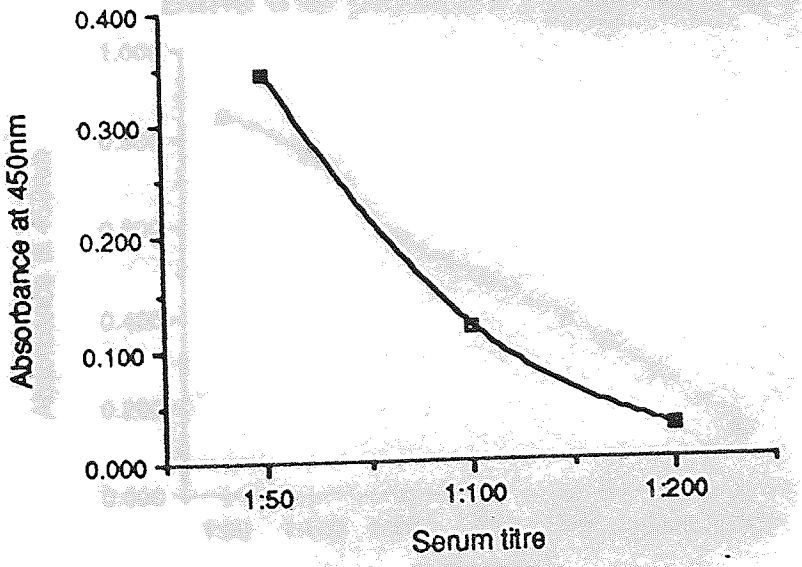
Blind trial patient 1(E.faecalis IE)

Figure 22



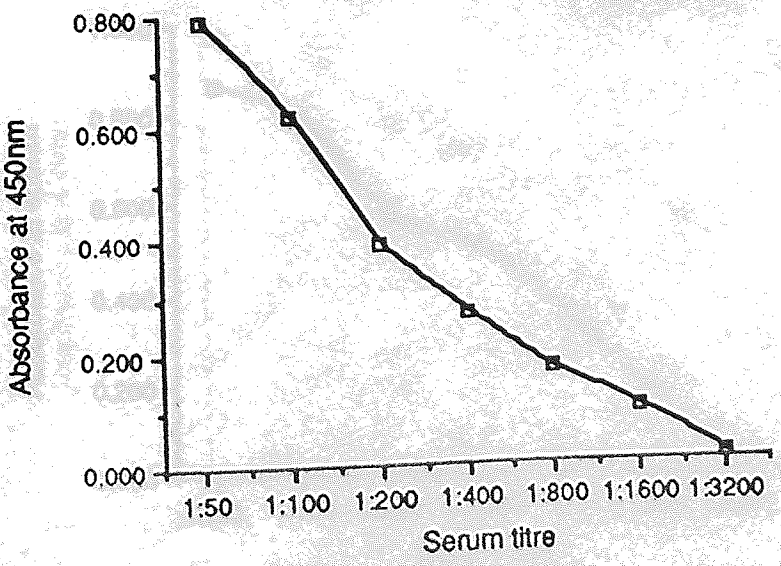
Blind trial patient 2 (E.faecalis IE)

Figure 23



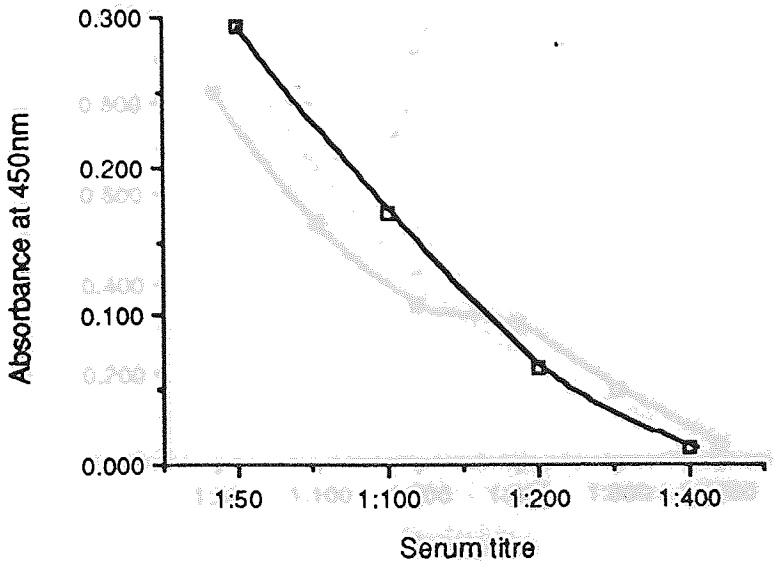
Blind trial patient 3 (E.faecalis IE)

Figure 24



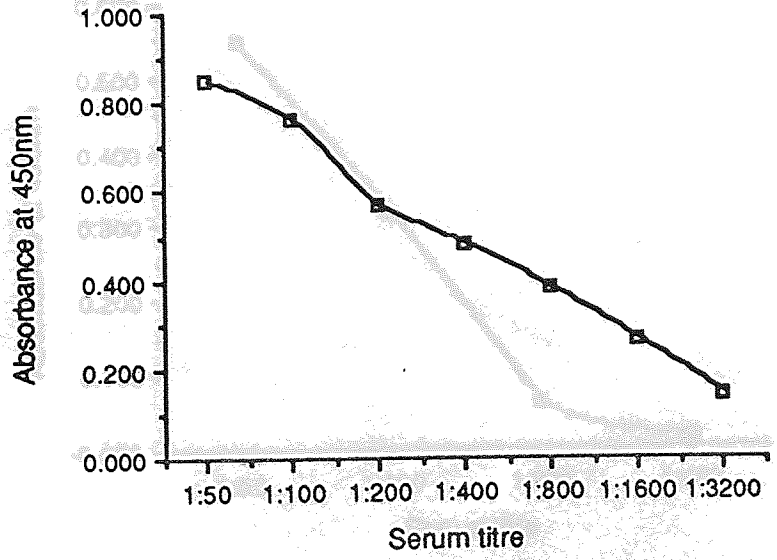
Blind trial patient 5 (E.faecalis IE)

Figure 25



Blind trial patient 6 (E.faecalis IE)

Figure 26



Blind trial patient 7 (E.faecalis IE)

Figure 27

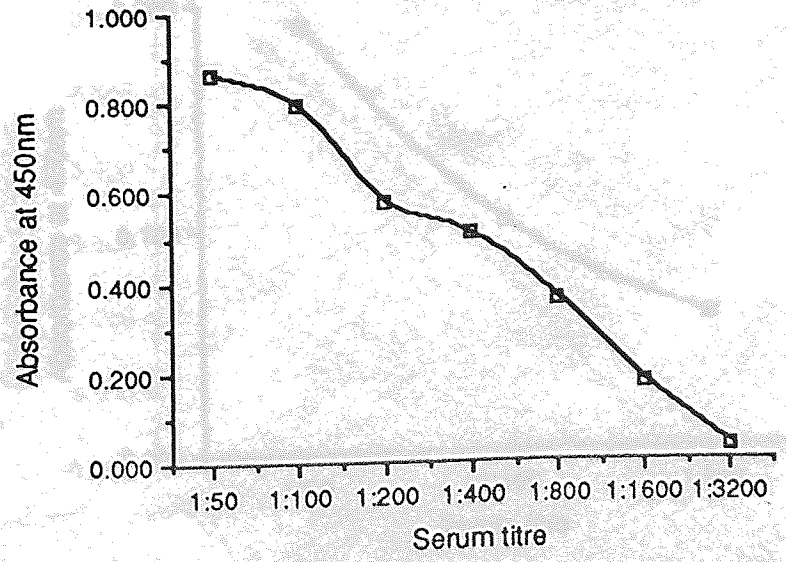


Figure 28

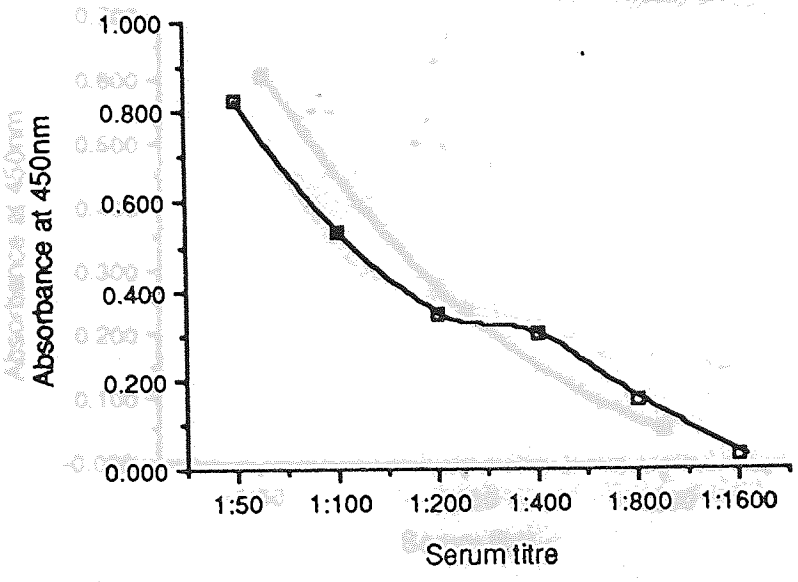


Figure 29

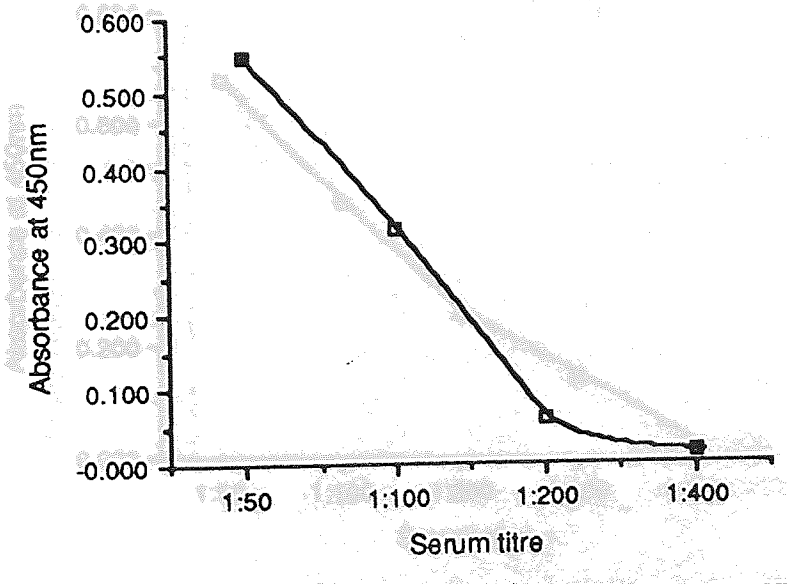
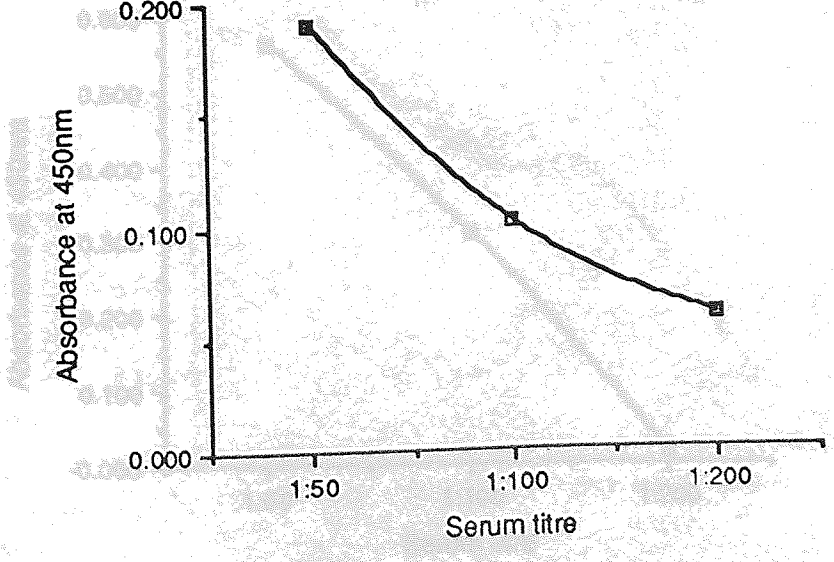
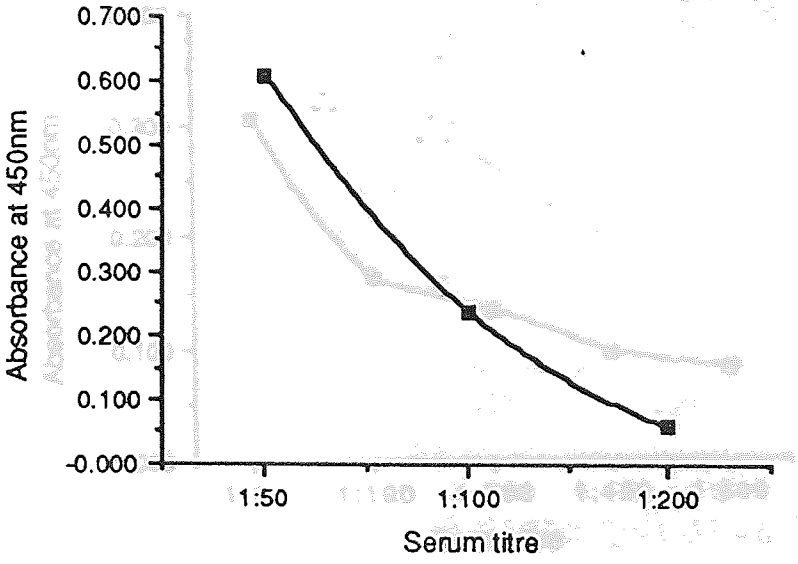


Figure 30



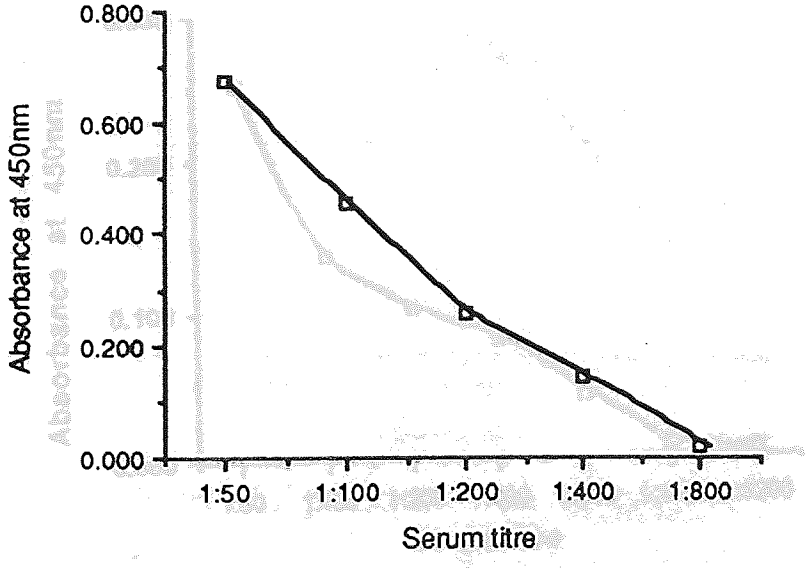
Blind trial patient 11 (E.faecalis IE)

Figure 31



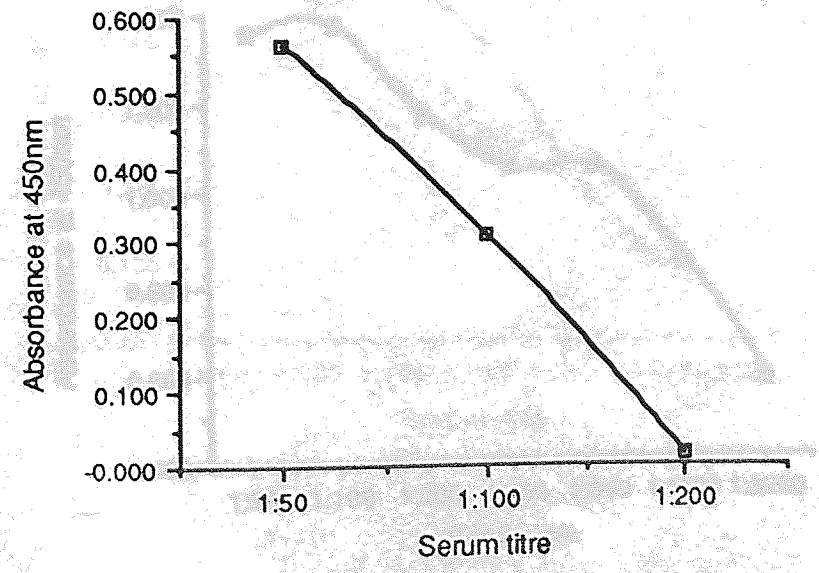
Blind trial patient 12 (E.faecalis IE)

Figure 32



Blind trial patient 18 (E.faecalis UTI)

Figure 33



Blind trial patient 19 (E.faecalis IE)

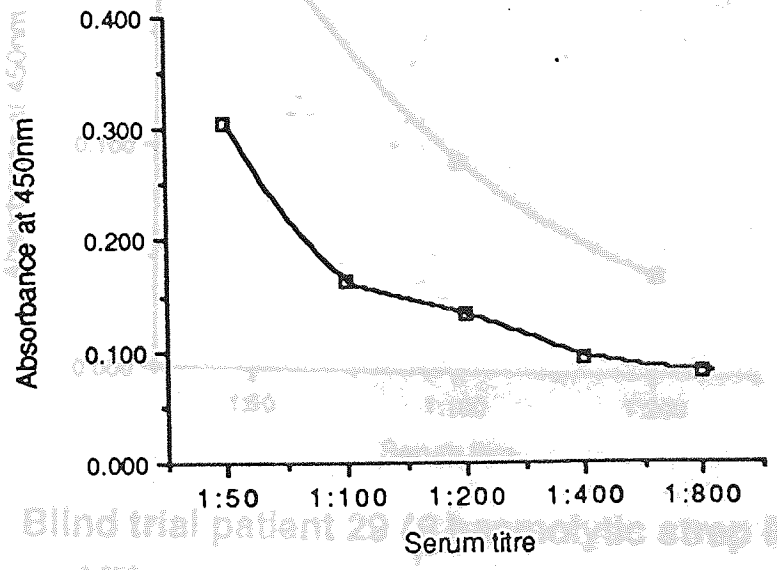


Figure 34

Figure 35

Blind trial patient 23 (NVS IE)

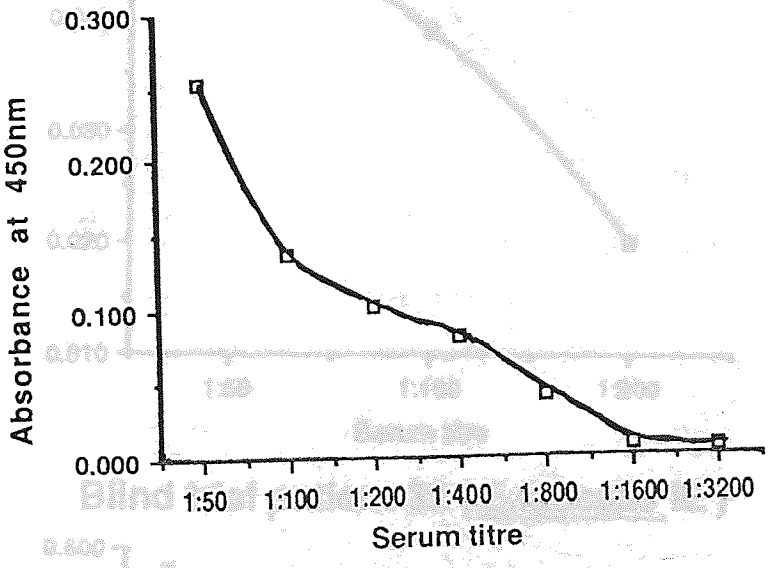
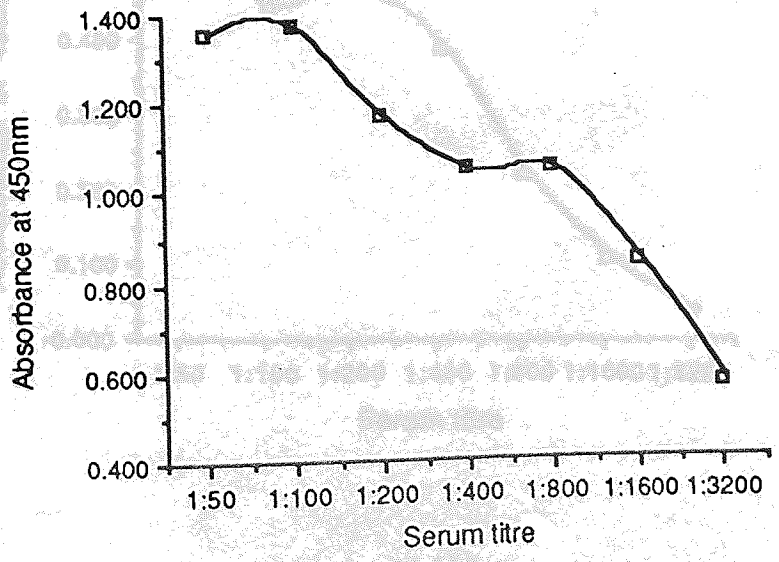


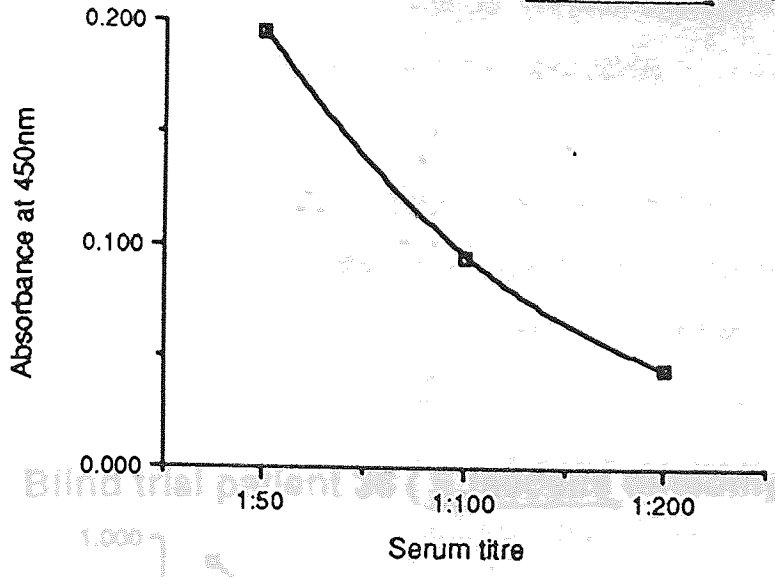
Figure 36

Blind trial patient 24 (E.faecalis IE)



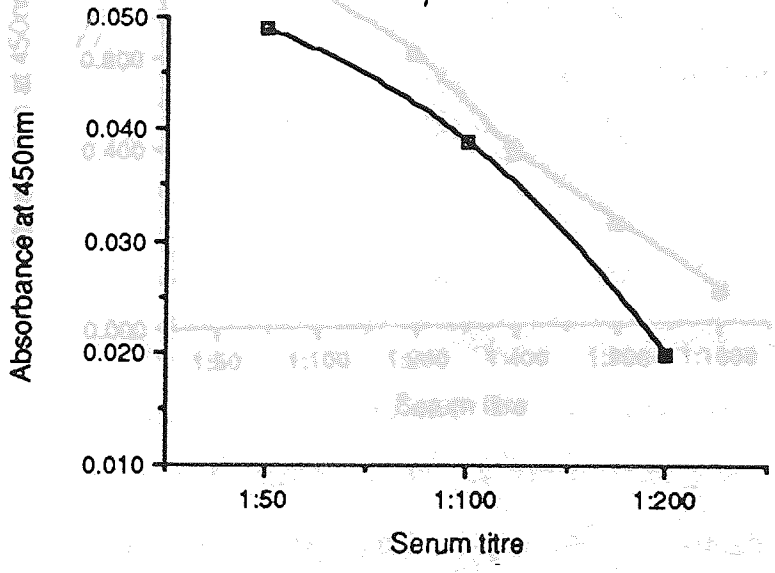
Blind trial patient 26 (E.faecalis IE)

Figure 37



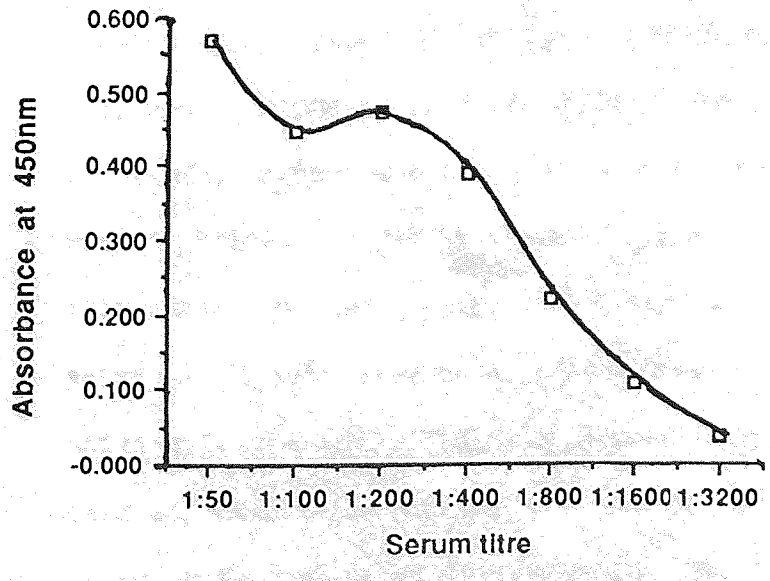
Blind trial patient 29 (β haemolytic strep IE)

Figure 38

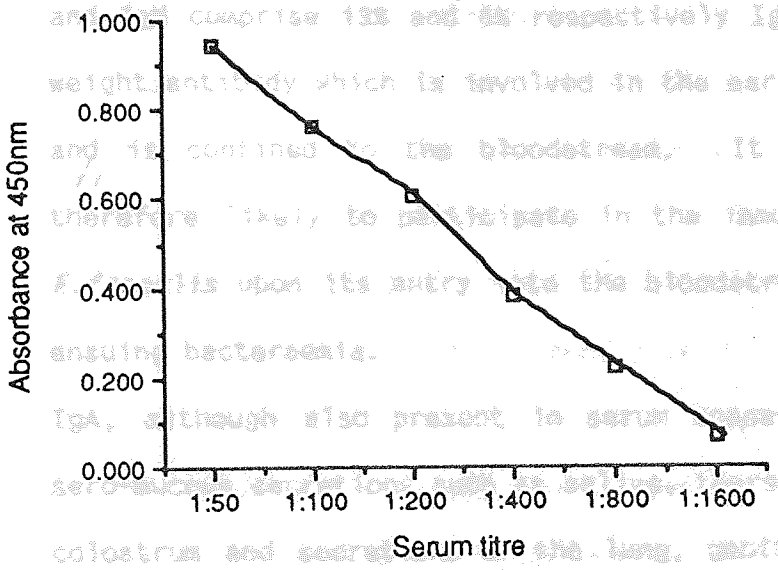


Blind trial patient 32 (E.faecalis IE)

Figure 39



Blind trial patient 36 (E.faecalis osteomyelitis) Figure 40



3.2.4 Longitudinal study of IgG, IgM and IgA levels in four *E.faecalis* endocarditis patients

The production of human immunoglobulins (Ig's) in response to infection with *E.faecalis* was studied using the same ELISA system as was used in the blind trial. In normal human serum IgG is the most abundant immunoglobulin (comprising 80% of the total Ig content), (Roitt, 1971). Two other major human Ig classes, IgA and IgM comprise 13% and 6% respectively IgM is a high molecular weight antibody which is involved in the early stages of infection and is confined to the bloodstream. It is this Ig which is therefore likely to participate in the immunological response to *E.faecalis* upon its entry into the bloodstream and throughout the ensuing bacteraemia. IgA, although also present in serum appears selectively in the sero-mucous secretions such as saliva, tears, nasal fluids, sweat, colostrum and secretions of the lung, genito-urinary and gastro-intestinal tracts. It is thus likely to come into contact with *E.faecalis* in its natural habitat of the gut. IgG is the major Ig to be synthesised during the secondary immune response. For these reasons IgG, IgA, and IgM were considered as being important in the human response to *E.faecalis*. *The longitudinal study of 4 E.faecalis* endocarditis patients are shown in table 11. These serial titres of the different immunoglobulin types show some interesting features. The serum samples in the study were taken after clinical diagnosis of endocarditis and the start of antibiotic therapy. The long exposure to antigen released from a cardiac vegetation during the course of endocarditis would be expected to induce an IgG response, and indeed the levels of IgG for the 4 patients were generally far higher than IgM or IgA.

Samples taken earlier in the course of the infection might well show higher IgM responses. For patient 1, IgG levels remained high for several months, with low but detectable levels of IgM and IgA. This patient had had *E.faecalis* endocarditis on two previous occasions and appeared to have retained a high IgG titre throughout. The reductions in months 9 and 25 coincided with the clinical deterioration of the patient. Patient 2 showed a dramatic rise in IgG with corresponding rises in IgM and IgA around 8 months after diagnosis of *E.faecalis* endocarditis. This may be caused by a major release of antigen from the site of infection, giving a subsequent immune response. Patient 3 produced a significant IgM response in month 2, a correspondingly large increase in IgG, and a slightly lower IgA response. In contrast to this, patient 4 showed a very low IgM response, and no detectable level of IgA. There are no obvious explanations for the different patterns of antibody response in the four patients studied. Presumably major influences upon the amount of antigens released into circulation from the site of infection are the duration and extent of the infections, and the outcome of antibiotic therapies.

3.2.5 Discussion of section 3.2

The results of the blind trial using the 73, 40 and 37 kdalton antigen in an ELISA system, confirm the serodiagnosic potential of these antigens in *E.faecalis* endocarditis. The results showed a 94% success rate for positives (15/16), a 90% success rate (9/10) for negatives with endocarditis caused by other organisms and an 80% success rate (8/10) for negatives with *E.faecalis* infections other than endocarditis. The *E.faecalis* endocarditis patient who failed to give a positive result had transitional cell carcinoma of the bladder and this malignancy and/or therapy with adriamycin could have contributed to the poor immune response to the infection. Of the three false positives shown in the trial, the highest titre (1:1200) was found in serum from a patient with *E.faecalis* osteomyelitis. This site of infection and its protracted nature are analogous to the conditions of infective endocarditis, where prolonged release of antigen from the infection site could be responsible for the high IgG response of the patient.

The second false positive was given by a serum sample from a patient with an *E.faecalis* urinary tract infection, which gave a titre of 1:180. The infection had persisted for several months. Western blotting showed that the reaction was against the 73 and 37 kdalton antigens (personal communication, E G Smith, East Birmingham Hospital, Birmingham, England). Once more it seems likely that in this case prolonged release of antigen from the site of the infection had given a detectable IgG response. No sera from the other *E.faecalis* infections (drainage site, bronchial washings, peritoneal dialysis fluid, nephrostomy fluid,

wound swab, blood culture and urinary tract gave positive titres of 1:100 or greater. This could be because these infections were not of a sufficiently long duration to elicit an IgG response or that the antigens are produced particularly strongly by organisms when they infect specific sites (although this is not supported by the fact that only the chronic urinary tract infection showed a positive result, whilst a negative result was given with the serum of a patient with another UTI not of a chronic nature). Previous work has shown that all strains of *E.faecalis* from whatever source are capable of expressing the antigens (Aitchison et al 1987) with no major differences between western blot profiles between *E.faecalis* strains from endocarditis and those from other infections. Guzman et al (1989) however have recently reported significant differences in adhesive properties between endocarditis strains of *E.faecalis* and those from urinary tract infections. The endocarditis strains adhered better to Girardi heart cells than did the strains from urinary tract infections, but associated less efficiently with human neutrophils. The association with neutrophils was further reduced following growth in serum but as this change was cancelled by subculture in brain heart infusion it would not appear to involve expression of the specific antigens, which are expressed in both these growth media. It seems more likely to be related to the binding of serum proteins such as fibronectin or albumin.

The third false positive was an endocarditis caused by a nutritionally variant streptococcus (NVS) which gave a titre of 1:180. NVS are a collection of viridans streptococci requiring supplementation of media with pyridoxal for laboratory recovery (Carey et al, 1975; Reimer and Reller, 1981). NVS strains were

found as pathogens causing endocarditis and otitis media. They are responsible for 5-10% of case of streptococcal endocarditis (Roberts et al 1979; Bouvet et al 1982; Wilson and Geraci, 1985). Van de Rijn and George (1984) showed that there are several serological types of NVS grouped according to expression of surface proteins, and that as well as possessing serotype specific proteins the serotypes of the NVS share several common antigens with other viridans streptococci. It is possible that the NVS possessed some similar antigenic determinants to *E.faecalis*. The NVS strains studied by van De Rijn and George appeared to possess surface proteins in the 73 and 37 kdalton region. Western blotting studies showed that the NVS giving the false positive result produced a number of antigens, including bands of 73 and 40 kdalton which were detected by sera from the NVS patient, but not by sera from an *E.faecalis* endocarditis patient (personal communication; E G Smith, East Birmingham Hospital, Birmingham, England).

Some workers believe the penicillin G sensitivity of NVS to be similar to that of other viridans streptococci (Wilson and Geraci, 1985). Others however believe these organisms to be more difficult to treat than usual viridans streptococci and that either combination therapy with an aminoglycoside and penicillin (Carey et al, 1977; Feder et al 1980; Roberts et al 1979) or vancomycin with an aminoglycoside (Bouvet et al, 1985) should be used. If the latter is in fact the case then a false positive for an NVS in a clinical situation would have no ill effect on the patient as the treatment selected for the *E.faecalis* endocarditis would be equally appropriate against resistant NVS.

The value of a rapid serodiagnostic test for diagnosis of *E.faecalis* IE is indisputable. The ideal way to diagnose streptococcal endocarditis is to isolate the organism from the blood stream (Shafi and Heimer, 1982) but this is not always the most appropriate or most rapid way. Traditional blood culture techniques may take a matter of days to identify the infecting agent and also fall short in culture negative cases of endocarditis or those where organisms are isolated from only one of several blood cultures. A serological method of diagnosis is thus an obvious progression. A test involving the use of a fluorescent antibody for diagnosis of viridans streptococcal endocarditis was used by Shansun and Hince, (1978), but although it had advantages in terms of rapidity it was found of little value in culture negative cases. The serodiagnosis of *E.faecalis* endocarditis using the technique of immunoblotting was first attempted by Aitchison et al, (1987) using the serological response of patients to the 73, 40 and 37 kdalton antigens. Whilst this method gave promising results it is not a convenient method for routine use, also no attempt was made to quantify the amount of antibody to the antigens present in patients sera (this could have been measured using the technique of strip blotting and increasing dilutions of sera, but would be quite time consuming). Thus this technique, whilst showing the obvious serodiagnostic potential of the species specific antigens had severe limitations regarding routine use. Having partially purified the antigens it was obviously more convenient to use them as the basis of an enzyme linked immunosorbent assay (ELISA). As can be seen from the results this has great potential for the serodiagnosis of *E.faecalis* endocarditis, and could possibly be even further improved by more rigorous purification of the antigens for example

by FPLC. It can also be used to investigate serial serum samples of patients taken throughout the course of their infection. There are no obvious explanations for the different patterns of antibody response shown in the four patients studied here although extent and duration of infection and outcome of antibiotic therapy would almost certainly influence this. Further longitudinal studies are obviously needed to correlate antibody levels with patient response to therapy. It would also be useful to measure antigen levels or immune complexes. Espersen et al, (1988) have shown that detection of staphylococcal antigen in urine offers a rapid method for diagnosis of *Staphylococcus aureus* endocarditis.

Independent studies on enterococcocal endocarditis by Burnie et al (1987) have identified a number of other *E.faecalis* protein antigens which show promise for exploitation in serodiagnosis. Using the immunoblotting technique they found strong IgM responses in endocarditis patients to bands of 112, 88-90 and 45-47 k dal and strong IgG responses to the 88-90 and 45-47 antigens. The relationship of these antigens to the 73, 40 and 37 kdalton antigens remains to be established. Allowing for different estimates of molecular weights, the 45-47 bands might be equivalent to the 40 and 37 bands. The 112 and 88-90 bands found by Burnie et al (1987) are not seen on the immunoblotting system used here, possibly reflecting different growth conditions or method of antigen preparation.

TABLE 11 Levels of IgG, IgM and IgA levels in sera from *E.faecalis* endocarditis patients at intervals shown after diagnosis of infection

Patient 1

	Reciprocal ELISA titre								
Month/day	1/6	1/7	7/3	7/6	7/11	7/15	7/22	7/24	9/1	25/8
IgG	6000	6000	6500	5500	6000	7500	6000	6500	2000	1600
IgM	400	250	360	300	300	200	200	200	30	30
IgA	125	60	85	150	150	100	100	110	75	150

Patient 2

	Reciprocal ELISA titre						
Month/day	1/11	2/3	3/26	8/14	9/3	9/7	10/3
IgG	700	750	800	10000	8000	9000	7000
IgM	120	90	75	500	500	350	200
IgA	20	25	75	420	500	350	220

Patient 3

	Reciprocal ELISA titre			
Month/day	1/17	2/7	5/16	11/5
IgG	3500	4000	1200	800
IgM	650	3200	800	550
IgA	900	1200	600	800

Patient 4

	Reciprocal ELISA titre			
Month/day	1/30	6/26	10/30	19/31
IgG	3000	1200	3000	1500
IgM	85	65	85	25
IgA	0	0	0	0

[a] Reciprocal titres are the dilutions of sera required to reduce the colour to an absorbance at 450 nm of 0.2.

3.3 Binding of plasma proteins to *Enterococcus faecalis*

3.3.1 Binding of Fibronectin (FN) to *Enterococcus faecalis*

Gram-positive cocci possess receptors for a variety of plasma proteins; for example vitronectin (complement S protein) to groups A and G streptococci (Wiegand et al 1988); laminin to *Staphylococcus aureus* (Lopes et al, 1985); albumin to group C and G Streptococci (Myre and Kronvall, 1980b); and Fibronectin to *Staphylococcus aureus* (Kuusela, 1978). At present the nature of only a few of the receptors has been characterised and little is known of the biological significance of such binding, although it is presumed to play a role in microbial pathogenicity.

The group D enterococcus *E.faecalis* is implicated in 10-15% of cases of endocarditis (Wilson and Geraci, 1983) and has a particular affinity for heart valve tissue compared with other streptococci and staphylococci (Scheld and Mandell, 1984). A crucial step in the pathogenesis of infective endocarditis involves adherence of circulating bacteria to components of the damaged cardiac valve surfaces. Binding of serum proteins to the bacteria could influence adherence to the valve and endothelium. Fibronectin (FN) is a 440,000 dalton glycoprotein found in a soluble form in blood plasma in the extracellular matrix of connective tissue and basement membranes of mammalian cells (Yamada and Olden, 1978). Recent attention has been given to its role as an adhesin in the pathogenesis of infective endocarditis (Scheld et al, 1985).

Evidence supporting this includes the fact that FN is exposed on the surface of damaged heart endothelial tissue and thus provides a potential site at which bacteria possessing specific FN receptors can bind (Kerenyi et al, 1985). In a previous study (Myrhe and Kuusela, 1983) on binding of human FN to streptococci of groups A, C and G it was reported that group D streptococci also bind FN, although to a lesser extent than the other groups studied. No attempt was made however to quantify the degree of binding with respect to the number of molecules bound per cell and the affinity of binding or to characterise the binding site. Here the binding of FN to an endocarditis isolate (EBH1) of *Enterococcus faecalis* was investigated, using a direct binding assay.

The direct binding assay is a well established technique which provides reproducible quantitative data. It has been used extensively in the study of binding between human serum proteins and microorganisms (Myhre and Kronvall, 1977 and 1980a). The protein to be investigated is labelled with a suitable isotope (in this case the γ-emmitting ^{125}I was used). The test was carried out with trace amounts of labelled FN mixed with E.faecalis (strain EBH1). After an incubation step the intact bacterial organisms were separated from the non bound protein by centrifugation and the radioactivity associated with the pellet was determined. Thus a binding isotherm of bound FN against free FN was plotted (Figure 41) and from this using Scatchard analysis (Scatchard, 1949) a Scatchard plot was constructed (Figure 42).

The Scatchard plot obtained was linear (correlation coefficient = 0.97) indicating the existence of one type of binding site on the cell surface and no co-operativity in the binding process. From the intercept of the Scatchard plot, the number of molecules FN bound per bacterial cell was calculated and from the gradient the association of the binding was determined.

Calculation of the number of molecules of FN bound per cell

By regression analysis the line of best fit for the Scatchard plot was found, and when y = 0, x was predicted to equal 9.58

ie 9.58 µgFN/10^{10} cells

M.wt of FN = 440,000

from Avagadro's number, 440,000g contains 6 x 10^{23} molecules

$$1g = \frac{6 \times 10^{23}}{4.4 \times 10^{5}} \text{ molecules of FN}$$

$$1\mu g = \frac{6 \times 10^{23}}{4.4 \times 10^{11}} \text{ molecules of FN}$$

$$9.58\mu g \text{ FN/cell} = \frac{9.58 \times 6 \times 10^{23}}{4.4 \times 10^{11}} \times \frac{1}{10^{10}} \text{ molecules/cell}$$

= 1,306 molecules/cell

ie. each bacterial cell binds approximately 1,300 molecules of FN.

Calculation of the association constant

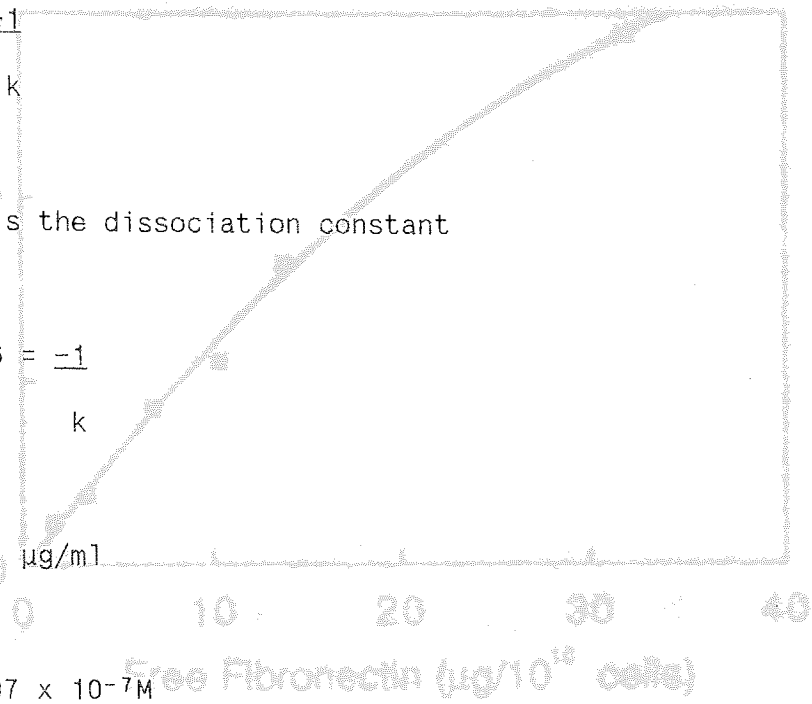
This can be calculated from the slope of the Scatchard plot.

Slope = $\frac{-1}{k}$

where k is the dissociation constant

$-0.01086 = \frac{-1}{k}$

$k = 90.91\ \mu g/ml$



$k = 2.07 \times 10^{-7} M$

The association constant = $\frac{1}{k}$

$= 4.8 \times 10^{6} M^{-1}$

Thus it was determined that the cells could bind a maximum of 1,300 molecules of FN per cell, with an association constant of $4.8 \times 10^{6} M^{-1}$.

Other strains of *E.faecalis* investigated showed a similar level of binding (see table 12).

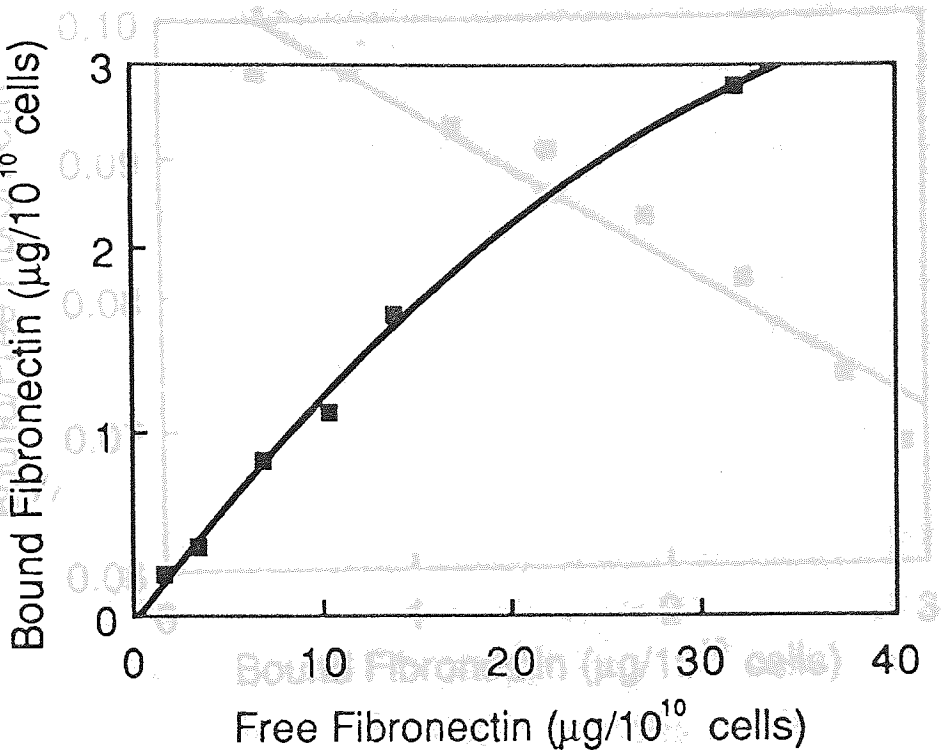


Figure 41

Binding of FN to whole cells of *E.faecalis* EBH1 in PBS, pH 7.2, 1hr contact at 20°C expressed as an isotherm.

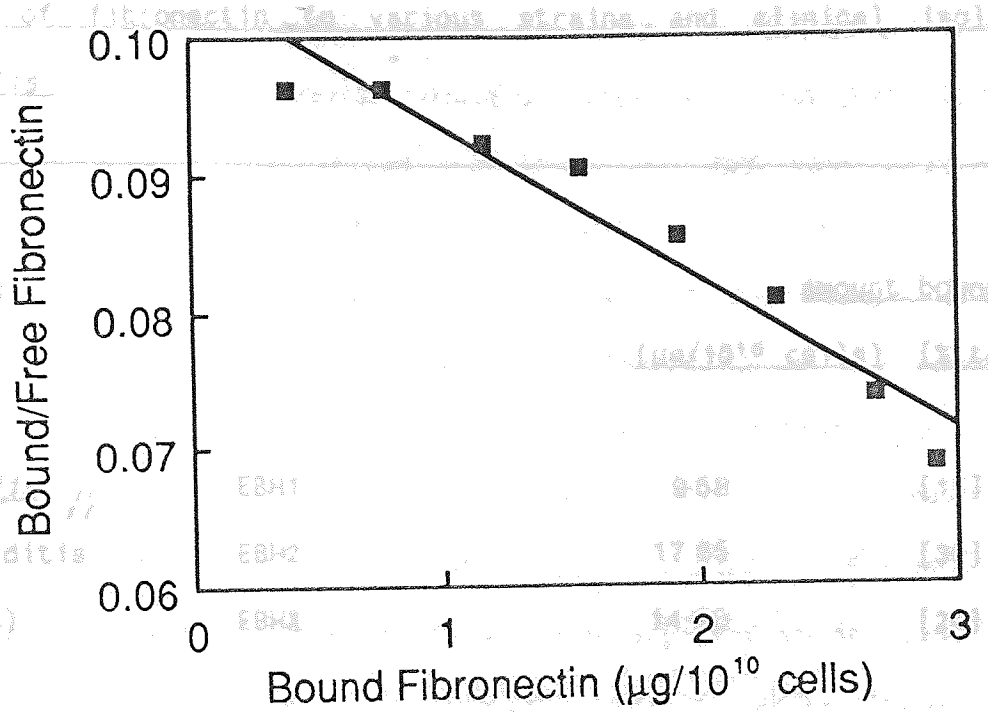


Figure 42

Binding of FN to whole cells of *E.faecalis* EBH1 in PBS, pH 7.2, 1 hr contact at 20°C expressed as a Scatchard plot.

Table 12

Binding of fibronectin to various strains and clinical isolates of *E.faecalis*

Organism		amount bound ($\mu g/10^{10}$ cells)	[% total]
E.faecalis	EBH1	9·58	[16]
(endocarditis	EBH2	17·85	[30]
isolates)	EBH3	14·00	[24]
E.faecalis	SFBG	10·69	[18]
(urinary tract	777	14·31	[24]
isolates)	741	13·15	[22]
E.faecalis	NCTC 5957	12·00	[20]
(reference	SFZ	9·00	[15]
strains)			

Binding was measured after 1hr exposure of 1ml of OD 5 cells (1.3×10^{10} cells) of each strain to 2µg ^{125}I FN. Results are expressed as the amount of FN bound and the % of the total amount which bound [% total].

3.3.2 Partial characterisation of the FN receptor of *E.faecalis*

The effects of several pretreatments of cells or FN upon binding were investigated in order to partially characterise the FN receptor of *E.faecalis*. These pre-treatments and their effects on binding are summarised in table 13 and the pretreatments themselves are detailed in method section 2.2.3.11.3. In order to determine if there was any involvement of lipoteichoic acid (LTA) in the binding process the ^{125}I labelled FN was pretreated with LTA before its addition to the organism in the binding experiment. Several concentrations of LTA were used and the results are summarised in table 13. If FN was binding to *E.faecalis* LTA, pretreatment of FN with LTA would be expected to decrease binding to the cells. The LTA pretreatment however had no effect upon the binding of the FN. The experiment was also repeated using LTA prepared by the alternative extraction method of Hamada et al (1985) (donated by E J Aitchison) and this too showed no significant decrease in binding of the FN (data not shown). The effect of pretreating the *E.faecalis* cells with group D antiserum (Sigma) was also investigated. The Lancefield group specific antigen of the group D streptococci is now recognised as membrane teichoic acid (LTA) (Wicken et al 1963) and thus this pretreatment of the cells with group D antiserum before addition of ^{125}I FN also characterised the possible involvement of LTA in the binding process. Once again as can be seen in table 13 no effect was seen indicating that binding of FN to *E.faecalis* does not appear to involve LTA.

In order to discover if the 40 and 37 kdalton species specific protein antigen of *E.faecalis* were involved in the binding of FN, purified monospecific antisera raised to these antigens was added to the cells before the addition of the fibronectin. If these proteins were significantly involved in FN binding then pretreatment of the cells with antisera to them would be expected to decrease the binding but this was not in fact the case and no effect was seen.

The pretreatment of the cells with trypsin also showed no effect on the binding process, and bound FN was not displaced by addition of excess FN. However, heating to 80 or 100°C, exposure to protease or periodate did reduce the FN binding. (see table 13).

Table 13

The effects of pretreatments of cells or FN upon binding of FN to *E.faecalis* EBH1, 1 hr contact in PBS at 20°C.

Pretreatment of cells:	%FN Binding
none	100
trypsin	92
protease K	62
BSA	107
periodate	83
60°C	90
80°C	81
100°C	52
group D antiserum	93
40/37Kdal antiserum	100
LTA pretreatment of FN:	
10µg LTA/20µl labelled FN	105
20µg LTA/20µl labelled FN	97
30µg LTA/20µl labelled FN	98
40µg LTA/20µl labelled FN	106
20µg/ml unlabelled FN added to cells after binding of labelled FN	10

Pretreatment conditions as described in Materials and Methods. FN binding was measured after exposure of 1ml of OD 5.0 cells to 2µg ^{125}I-FN for 1hr. Results are the means of 3 separate experiments with standard deviations ≤ ± 5%.

3.3.3 Binding of human serum albumin to *E.faecalis*

Albumin is present in high concentrations in serum (40mg/ml). Previous studies have shown that group C and G streptococci possess specific albumin binding sites (Myhre and Kronvall 1980b).

Binding of albumin to *E.faecalis* strain EBH1 was investigated by addition of ^{125}I labelled albumin to cell suspensions, thus facilitating the construction of a binding isotherm (Figure 43). Scatchard analysis was used and a Scatchard plot constructed (Figure 44) The linear Scatchard plot obtained for the albumin binding site on *E.faecalis* showed that there was no co-operativity in the binding process. Calculations from the Scatchard plot showed that each *E.faecalis* cell bound a maximum of 50,000 molecules of albumin with a much lower affinity than that of the FN binding (association constant of 10.8×10^3 M^{-1}.

3.3.4 Effect of LTA on binding of *E.faecalis* to serum albumin

It has previously been demonstrated that streptococcal LTA binds to serum albumin (Simpson et al, 1980) and so the effect on binding of pre-incubation of albumin with LTA was investigated (Table 14).

The results showed that although LTA did reduce the binding it did not completely inhibit it, thus indicating the involvement of other sites in the binding process.

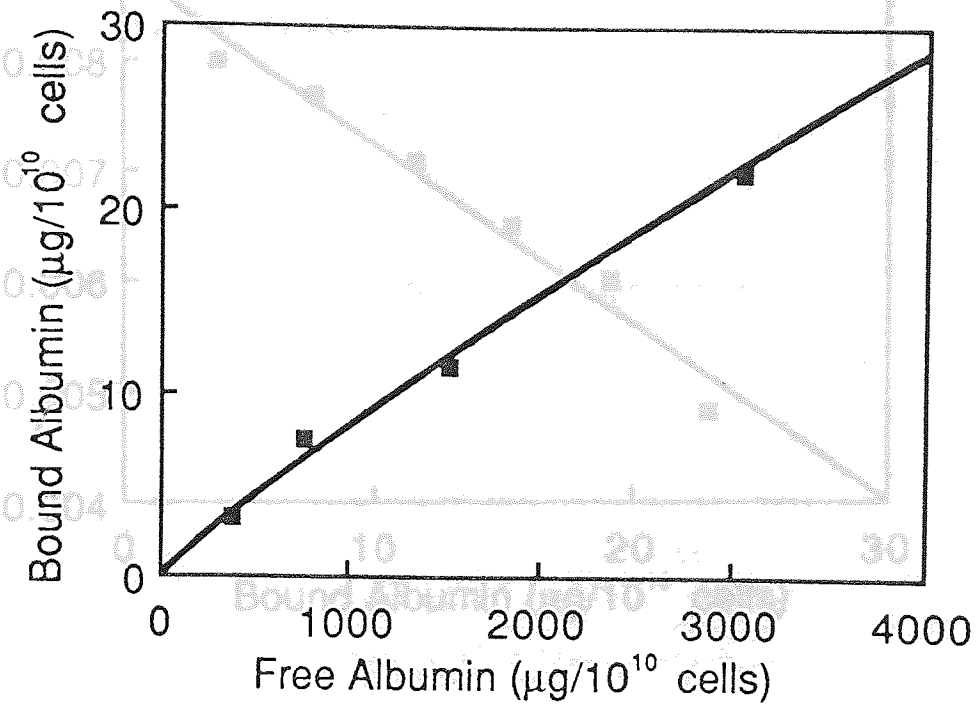


Figure 43

Binding of albumin to whole cells of *E.faecalis* EBH1 in PBS pH 7.2, 1 hr contact at 20°C expressed as an isotherm.

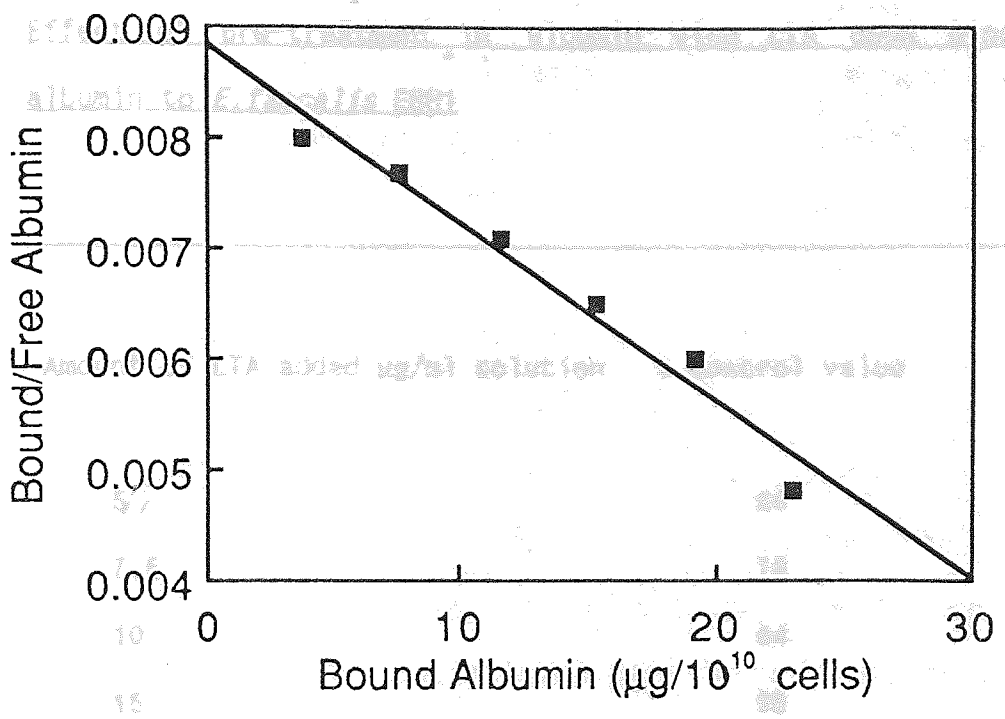


Figure 44

Binding of albumin to whole cells of *E.faecalis* EBH1 in PBS pH 7.2, 1 hr contact at 20°C expressed as a Scatchard plot.

Table 14

Effect of pre-treatment of albumin with LTA upon binding of albumin to *E.faecalis* EBH1

Amount of LTA added µg/ml solution	% control value
5	86
7.5	79
10	64
15	58

Binding was measured after 1hr exposure of 10µl ^{125}I albumin (0.1µg/ml) to LTA, before addition of 1ml *E.faecalis* EBH1 (1.3×10^{10} cells/ml). Results are expressed as a percentage of a control value (no LTA added to the albumin) which was taken as 100%.

3.3.5 Investigation of cell surface hydrophobicity of *E.faecalis* using bacterial adherence to hydrocarbon

Bacterial adherence to hydrocarbon (BATH) is a method of measuring the hydrophobicity of bacterial cells. The more hydrophobic the cells the greater the partitioning into the hydrocarbon layer (Rosenberg et al, 1980). The hydrocarbon used here was hexadecane, and the effect of several pretreatments of 1ml of EBH1 cells (OD_{470} 1.0) for 30 minutes as 37°C was investigated. The pretreatments were as follows: FN (Sigma) at a concentration of 20nM and also 30nM ; bovine serum albumin (Sigma) at 4 and 40mg/ml cells; group D antiserum (Wellcome) at 40 µl/ml cells; guinea pig serum at 40µl/ml cells and guinea pig serum previously heated to 56°C for 30 minutes at 40µl/ml cells. The effect on surface hydrophobicity of these pretreatments is summarised in Figure 45.

As can be seen the addition of fibronectin had no effect on the surface hydrophobicity of the cell. Work by Courtney et al (1985a) showed that human plasma fibronectin inhibited the adherence to hydrocarbon of the group A streptococci, *Streptococcus pyogenes* in a dose dependent manner. They also showed that pretreatment of the fibronectin with LTA blocked the binding of FN to *S.pyogenes* cells and abolished its ability to inhibit the adherence in a dose dependent manner. This data pointed to the fact that fibronectin binds to the LTA of group A streptococci, preventing it from interacting with the hexadecane phase. The pretreatment of fibronectin with LTA obviously abolished this effect. Looking at the group D streptococci, concentrations of 20nM and 30nM of FN were incubated with the cells for 30 minutes at 37°C. Courtney et

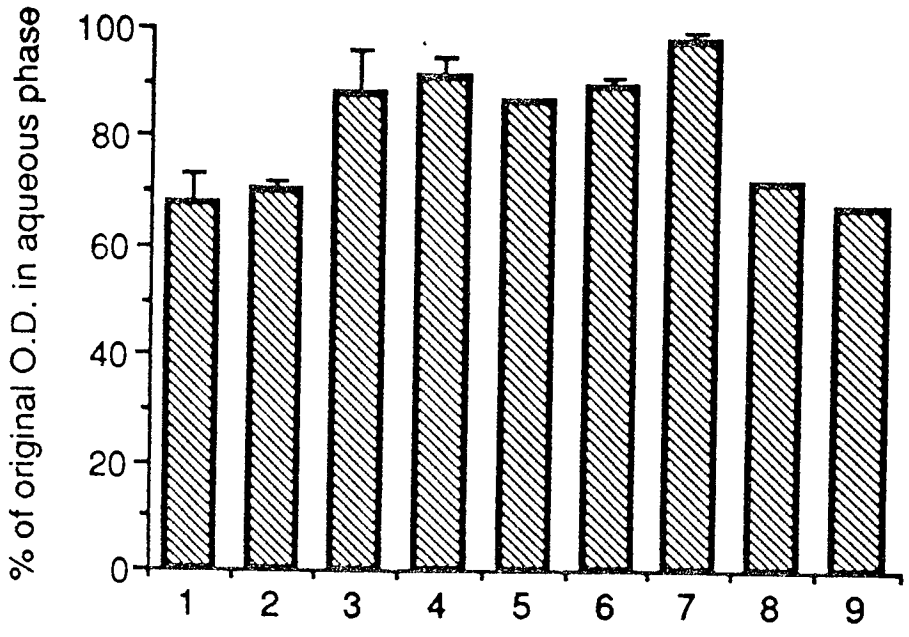


figure 45

Surface hydrophobicity of *E.faecalis* EBH1 measured by BATH. Results are expressed as % of original OD of cells in the aqueous phase after vortex mixing with hexadecane (see Materials and Methods). Cells in 1 and 3-9 were grown in Difco brain heart infusion, cells in 2 were grown in Lab M brain heart infusion. Cells were pretreated with: 3, guinea pig serum; 4, heat-inactivated guinea pig serum; 5, group D antiserum; 6, bovine serum albumin (0.4mg/ml); 7, bovine serum albumin (4mg/ml); 8, fibronectin (10µg/ml); 9, fibronectin (13.3µg/ml).

al (1985a) saw a significant decrease in the surface hydrophobicity of *S.pyogenes* cells with concentrations of FN above 15nM. However, in the case of the *E.faecalis* EBH1 cells no effect could be seen, indicating that binding of FN to group D streptococci does not involve LTA.

When the cells were pretreated with group D antiserum (Wellcome) a decrease in surface hydrophobicity was seen. The hydrophobicity of group A streptococci is thought to be due to inverted surface LTA (Miorner et al, 1983), thus if this is also the case for group D streptococci pretreating the cell with group D antiserum would be expected to "block" LTA and thus decrease hydrophobicity.

Any possible effects by complement upon surface hydrophobicity were investigated by pretreating the cells with guinea pig serum for 30 minutes at 37°C. A control was set up using guinea pig serum pretreated for 30 minutes at 56°C to destroy any complement activity. The results show a decrease in hydrophobicity in both cases indicating that whatever the reason for this it was not mediated by complement.

The effect of albumin on hydrophobicity was also investigated at concentrations of 0.4mg/ml and 4mg/ml cells. Previous findings by Simpson et al (1980) showed that albumin binds to streptococcal LTA, and the pretreatment did indeed give results showing a decrease in hydrophobicity. The concentration of albumin in human serum is 40mg/ml and thus it can be seen that even in much smaller concentrations than those seen physiologically, albumin has a definite effect on the hydrophobicity of *E.faecalis*. The concentration of albumin showing the effect is so small that it is

possibly this which was having the effect when the guinea pig sera was added to the cells, and could also be what caused the decrease in surface hydrophobicity when the cells were pretreated with group D antiserum.

3.3.5 Discussion of section 3.3

The results show that *E.faecalis* binds significant amounts of FN which might contribute to the pathogenicity of the organism in endocarditis. The fact that heating to 80 or 100°C, exposure to protease K or periodate reduce FN binding suggests that FN binds to sites composed of protein and carbohydrate. The maximum number of FN molecules bound per cell (1,300) and the association constant (4.8×10^6 M^{-1}) compare with values of 7,500 molecules/cell and 5.6×10^9 M^{-1} respectively for *Staphylococcus aureus* , which posesses a specific FN binding protein (Proctor, Mosher and Olbrantz, 1982). LTA alone does not appear to be involved, since pre-incubation of the labelled FN with LTA or of the cells with group D antiserum (anti-LTA) had no effect. *E.faecalis* therefore differs from group A *Streptococcus pyogenes* which binds FN via LTA associated with M protein on the cell surface, thus mediating adhesion to mammalian cells (Beachey et al, 1983). The binding did not appear to involve the 40 and 37 kdalton species specific antigen, although recent reports have indicated a 41 kdalton protein in *S.pyogenes* which binds plasmin (personal communication, C C Broder, University of Florida). It is possible that this could be related to the 40 kdalton *E.faecalis* protein, and future work looking at the binding of *E.faecalis* to the serum protein, plasmin, and its possible involvement in the pathogenesis of endocarditis would be of considerable interest.

Binding of albumin was of a much lower affinity then binding of FN (association constant 10.8×10^3 M^{-1}). Previous studies have shown that group C and G streptococci possess specific albumin

binding sites (Myhre and Kronvall, 1980b), recently identified as part of the protein G component of the wall (Sjobring et al, 1988). By comparison, the binding of albumin to group D streptococci has been considered to be insignificant (Wideback and Kronvall, 1982), however the binding of up to 50,000 molecules of albumin per cell of *E.faecalis*, must be of significance *in vivo* especially with respect to cells causing a bacteraemia prior to adherence to heart endothelial tissue. In addition to altering the surface properties of the cells, these loosely bound molecules might shield surface antigens from the host immune system, and thereby account for the simple antigenic profiles reported for *in vivo* grown cells (Dalhoff, 1985).

The linear Scatchard plot obtained for albumin binding indicated the existence of one type of binding site on *E.faecalis*. It has previously been demonstrated that LTA binds to serum albumin (Simpson et al, 1980). Pre-incubation of albumin with LTA did reduce the binding, but did not completely inhibit it.

BATH measurements show that albumin has a great effect upon surface hydrophobicity, causing a considerable decrease when present in only very small concentrations, probably by binding to the LTA on the cell surface. Binding of FN however had no effect upon cell surface hydrophobicity. It seems likely that the FN-bacteria interaction involves a specific receptor, rather than non-specific hydrophobic interactions.

The number of FN receptors on *S.aureus* has been shown to correlate with invasiveness (Proctor et al, 1984). Work has also been performed with *S.sanguis* where an *S.sanguis* mutant, lacking FN

binding ability was shown in an animal model to be far less virulent than a strain that could bind FN. The number of animals developing the disease with the mutant strain was negligible (at a low inoculum no disease was shown) and when the vegetations were examined far fewer mutant strain bacteria were bound than FN binding bacteria (personal communication, J H Lawrence, Truman VA Hospital).

Further work is needed to determine the role of FN, albumin and other plasma proteins in the pathogenesis of *E.faecalis* endocarditis and to characterise specific bacterial receptors of those involved in the pathogenesis of the disease. Use of mutant strains of *E.faecalis* lacking the ability to bind FN and/or albumin and work with animal models as in the *S.sanguis* study would establish their relative contributions to the virulence of *E.faecalis* in endocarditis.

3.4 Concluding remarks

Surface proteins of Gram-positive bacteria have been recognised for some time as being of considerable importance in infection and the initial main aims of this project were:

a to investigate the localisation of species specific protein antigens of *Enterococcus faecalis*;

b to purify them and look at their potential as the basis for a rapid serodiagnostic test for *E.faecalis* endocarditis, and to consider their possible role in the pathogenesis of the disease, along with other factors.

The growth of *E.faecalis* in different media and its effect on both the cells' appearance (as examined by electron micrographs of ruthenium red stained and sectioned cells) and the protein antigen profile (as investigated using SDS-PAGE and immunoblotting techniques) was studied and both were shown to be markedly influenced by the growth environment. Growth in serum was deemed to most closely mimic *in vivo* growth conditions, reflecting an environment similar to that which the microorganisms would encounter during bacteraemia, preceding colonisation of the endocardium.

The localisation of the 40 and 37 kdalton species specific antigens of *E.faecalis* was investigated by electron microscopy with immunonegative staining using purified monospecific antiserum. The technique showed that the purified IgG from the

monospecific antiserum to these antigens bound to the surface of *E.faecalis*, the amount binding being unaffected by the phase of growth at which the cells were taken.

Negative staining of cells with methylamine tungstate showed the presence of fimbriae on the surface of some *E.faecalis* cells, grown in both serum and brain heart infusion, although the degree of fimbriation was shown to depend on the phase of growth at which the cells were taken to be investigated. A significant number (approximately 35%) of cells taken in early logarithmic phase were fimbriate, with the value falling to around 25% in mid-log phase and as low as 5% in stationary phase. Due to the fine structure of the *E.faecalis* fimbriae they were not visible after incubation with antiserum in the immunonegative staining techniques. However, no variation in labelling of cells with monospecific antiserum was seen according to their phase of growth (in fact all cells examined showed surface labelling whatever their growth phase), thus strongly implying that the 40 and 37 kdalton antigens are not part of the fimbriae, although they are clearly exposed on the surface of intact cells.

Attempts were made to detect surface fibrils, similar to those seen in certain oral streptococci in thin sections by ruthenium red staining. Although no surface fibrils were detected, considerable differences were seen between cells grown in horse serum and those grown in brain heart infusion. In the horse serum grown cells, large irregular patches of stained material were seen on the cell surface and between adjacent cells, compared to a smooth uniform layer surrounding the BHI grown cells. The nature of the material adhering to the horse serum grown cells is as yet

unknown. It is unlikely to have originated from the serum due to the rigorous washing the cells underwent before applying to the grids and during staining, but this cannot totally be discounted.

Regarding *E.faecalis* in infective endocarditis the potential of using the 73, 40 and 37 kdalton antigens was investigated as the basis of a serodiagnostic test for *E.faecalis* IE. Infective endocarditis is both a difficult condition to diagnose and treat and carries a mortality of up to 30%. *E.faecalis* endocarditis is particularly difficult to treat, being insensitive to the antibiotic regimen used against the penicillin sensitive viridans streptococci. Treatment requires a combination of penicillins, aminoglycosides and/or glycopeptide antibiotics such as vancomycin for several weeks with attendant side effects. Thus identification of the causative organism from the blood of the patient is of the utmost importance for the selection of appropriate treatment. The incidence of culture negative cases from blood cultures and the length of time involved in such cultures show the obvious value of a rapid test to either confirm or exclude *E.faecalis* in infective endocarditis, especially if this might also be useful in assessing therapeutic response.

To this end the partial purification of the antigens was achieved by precipitation with 90% ammonium sulphate after the major antigens were released from horse serum grown cells by 1% sarkosyl and 1mM EDTA. Based on these extracted and partially purified antigens, an ELISA assay was developed and its performance evaluated in a blind trial. The results of the trial were very encouraging, giving a 94% success rate for positives (15/16), a 90% success rate (9/10) for negatives with endocarditis caused by

other organisms and an 80% success rate (8/10) for negatives with *E.faecalis* infections other than endocarditis. These results might possibly be further improved by more rigorous purification of the antigens, thus giving a rapid and convenient serodiagnostic test for *E.faecalis* endocarditis. The ELISA assay was also used in a longitudinal study investigating IgG, IgA and IgM levels in serial serum samples of four endocarditis patients, and whilst further longitudinal studies are obviously needed this may in the future be useful in monitoring patient response to therapy.

Regarding the pathogenesis of *E.faecalis* endocarditis, the binding of the serum proteins fibronectin and albumin to the bacteria was investigated, and in the case of fibronectin the receptor was partially characterised. The 40 and 37 kdalton antigens did not appear to be involved in the binding of FN, and likewise LTA also appeared to play no role. Binding for both fibronectin and albumin was of reasonably low affinity, thus meaning that the material surrounding horse serum grown cells revealed by ruthenium red staining is almost certainly not either of these serum components. However, it showed that many molecules of these proteins bind to cells and amongst other effects these loosely bound molecules might shield surface antigens from the host immune system.

Thus in conclusion, of the protein antigens investigated the 40 and 37 kdalton antigens have been shown to be exposed on the surface of the cells, although they are apparently not part of the *E.faecalis* fimbriae seen on some cells. The partially purified 73, 40 and 37 kdalton antigens showed great success as the basis of a serodiagnostic test for *E.faecalis* endocarditis although no

link between these antigens and pathogenesis has yet been established. To take this work a stage further it would be of great clinical benefit to produce an ELISA kit comprising species specific antigens from a range of Gram-positive organisms, if such antigens could be found. This test could then be used to discover quickly and efficiently the causal organism in endocarditis.

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