PATHOGENICITY FACTORS OF THE 'STREPTOCOCCUS MILLERI GROUP'

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Index of Commonly used Abbreviations

BEC	Buccal Epithelial Cells
BHIB	Brain Heart Infusion Broth
CL	Chemiluminescence
EM	Electron Microscopy
HIC	Hydrophobic Interaction Chromatography
PBS	Phosphate Buffered Saline
PMNL	Polymorphonuclear Leukocytes
RBCs	Red Blood Cells
SLO	Streptolysin O
SLS	Streptolysin S
SMG	"Streptococcus milleri group"
YE	Yeast Extract

Acknowledgements

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

To Dr C.G. Gemmell, my supervisor, for his assistance and guidance throughout the period of my study and in the preparation of this thesis.

To Dr J Bagg, for assuming the role of my supervisor one year into the project, and for his help and encouragement throughout both the practical work and the preparation of this thesis.

To Prof. T.W. MacFarlane for overseeing the whole project and his helpful comments and ideas throughout.

To Dr I Montgomery in the Institute of Physiology, for undertaking the electron microscopic work in this thesis, and for his time in showing me the techniques involved.

To Mr D MacKenzie, the chief MLSO in Microbiology, Department of Oral Sciences, for his help and advice in use of equipment and techniques involved in the practical work.

To Mr W Marshall, senior chief MLSO in the Department of Oral Sciences, for his help in various aspects of this work.

To all my colleagues in the Department of Bacteriology, Glasgow Royal Infirmary, for assistance with techniques and for their generous donation of blood when required.

To the Medical Research Council for the award of a postgraduate studentship.

To all the postgraduate students at the Dental Hospital for their continued support and advice throughout this work.

To my family and friends for their support and encouragement throughout the years.

Preface

The work described in this thesis was undertaken in the Department of Oral sciences and the Department of Bacteriology, University of Glasgow. This work was supported by a postgraduate studentship from the Medical Research Council.

Parts of this work have been presented as posters at scientific meetings :

(1) Effect of capsule on adherence of the SMG to BEC.

BRITISH SOCIETY FOR DENTAL RESEARCH, LONDON, 1993.

Abstract published : Journal of Dental Research **72(4)** p735.

(2) Opsonic requirements of strains of the SMG.

BRITISH SOCIETY FOR DENTAL RESEARCH, SHEFFIELD, 1994.

Abstract published : Journal of Dental Research **73(4)** p850.

(3) Susceptibility of the SMG to phagocytosis.

BRITISH SOCIETY FOR DENTAL RESEARCH, SHEFFIELD, 1994.

Abstract published : Journal of Dental Research **73(4)** p850.

Declaration

This thesis is the original work of the author

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SUMMARY

The "Streptococcus milleri group" (SMG) are part of the commensal flora, inhabiting the mouth, respiratory and gastrointestinal tracts. They are now recognised as significant pathogens with a proclivity for pyogenic infections such as dental, liver and brain abscesses, as well as being involved in endocarditis and other diseases. Much confusion had surrounded the nomenclature and taxonomy of the group until recently, when three distinct species *S.anginosus*, *S.constellatus* and *S.intermedius* were identified. This led to greater characterisation of the group, but its pathogenicity is less well understood. The aim of the work in this thesis was therefore to examine some of the factors of the SMG which contribute to causation of infection.

A group of strains for analysis were collected by isolating organisms from dental abscesses, clinical infections of non-oral source and dental plaque. These represented the three species and were tested for Lancefield grouping, haemolysin, hyaluronidase and DNase production and ability to agglutinate red blood cells. The majority of strains were Lancefield non-groupable, but where a grouping could be determined, group F predominated. Toxin and enzyme activity varied and could not be attributed to species or isolate type, and no strains agglutinated RBCs. Therefore, a heterogenous

group of isolates was collected for further investigation.

Encapsulation had been suggested as a virulence factor for the SMG by previous investigators (Brook and Walker 1985 : Lewis et al 1988), although the mechanism was unclear. Therefore, the capsule of the SMG was investigated by negative staining and electron microscopy. All but one strain were encapsulated with small and large capsule types revealed by both methods. Hyaluronic acid was shown to be a component of the capsule as treatment with hyaluronidase removed capsule to varying degrees. Bacterial cell surface hydrophobicity was measured using two methods, with results of each showing good correlation. Strains exhibited both hydrophobic and hydrophilic characteristics, with possession of capsule or species and isolate type having no bearing on this property.

Willcox and Knox (1990) found that dental abscess isolates adhered to BEC to a greater extent than other SMG organisms, and this was investigated. This was indeed found to be true, with dental abscess isolates adhering more readily than both clinical and plaque isolates. It was found that capsule hindered the adherence of the SMG to BEC, as treatment of strains with hyaluronidase caused increased adherence. Cell surface hydrophobicity had no effect on ability to adhere to BEC.

It was suggested that the capsule masked specific adhesins on the SMG surface which adhered to receptors on the BEC.

The opsonic requirements of the SMG were investigated as was their ability to elicit a chemiluminescent response from PMNL. Opsonization was found to increase chemiluminescence, with serum concentrations of 15% or more proving optimum. A range of chemiluminescence values were obtained, but these could not be related to either species or isolate type. In addition, capsule and cell surface hydrophobicity played no part in either opsonization or chemiluminescence.

In addition, phagocytosis of the SMG was further studied to determine if they were able to resist ingestion. Capsule was found to be antiphagocytic,strains with large capsules being ingested less readily than those with small capsules. This property was confirmed, as treatment with hyaluronidase to remove capsule resulted in increased ingestion efficacy. Again no relevance could be attributed to either isolate type or species in the level of ingestion produced, and cell surface hydrophobicity again appeared to play no role.

Therefore the aims of the work were achieved, with a greater understanding of some of the pathogenicity factors of the SMG having been obtained. However, these

are still only a small part of the whole picture as to why the SMG frequently cause purulent infection, either as the sole infecting organism or within a mixed infection, and much further work is required. It is hoped that an increased understanding of the group will in turn lead to better disease management of patients infected with this clinically significant group of bacteria.

CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Organisms of the *"Streptococcus milleri group"* (SMG) are part of the commensal flora of the mouth, nasopharynx, gastrointestinal and urogenital tracts of man. They have been shown to cause serious pyogenic infections such as brain, liver, lung and dental abscesses as well as having a role in other infections such as endocarditis. Although the fact that the SMG cause a series of infections is well documented, the virulence factors of the group are less well studied. The purpose of this work was to examine a range of factors which may aid in the ability of the SMG to colonise and damage host tissues and to evade the host's defence mechanisms.

A review of the current information on the SMG relating to taxonomy, infections, therapy and pathogenesis will serve as an introduction to the practical work.

1.2 CHARACTERISATION OF THE SMG

1.2.1 TAXONOMY OF THE STREPTOCOCCUS MILLERI GROUP

The name *Streptococcus milleri* is not included in the Approved List of Bacterial Names (Skerman et al 1980) and there has been much confusion and debate over the nomenclature and taxonomy of organisms of the Streptococcus milleri group (SMG). There have been a multitude of names used to describe such organisms, the first reference being by Andrews and Horder (1906) who described *S.anginosus* as a variant of *S.pyogenes* associated with sore throats. The original isolates are no longer available for comparison with the type strain (Hardie 1984). The terms intermedius and constellatus have been used since the 1920's by many authors to describe viridans streptococci isolated anaerobically (Facklam and Carey 1985). The SMG have also frequently been described as minute haemolytic streptococci, as first described by Long and Bliss (1934). Streptococcus MG was used by Mirick et al (1944) to describe a non-haemolytic organism isolated from the respiratory tract of humans. The title *Streptococcus milleri* was introduced by Guthoff (1956) to describe a group of non-haemolytic streptococci isolated from dental abscesses. Finally the names *S.anginosus-constellatus* and *S.MG-intermedius* have also been used (Facklam 1977).

Such disarray within the classification of the SMG was addressed in a series of studies by Coleman and Williams as summarized in 1972. Within the *S.milleri* cluster were included the following; *S.milleri* (Guthoff), Streptococcus MG (Mirick), all group F strains of streptococci, certain non-haemolytic streptococci of groups A,C and G, strains possessing an Ottens and Winkler typing antigen but no Lancefield group, minute haemolytic streptococci (Long and Bliss 1934). This clarified the situation, but the scheme was not universally accepted, particularly in the USA.

Facklam (1977) found similarities between Streptococcus-MG and *S.intermedius*, and *S.constellatus* and *S.anginosus*, and also considerable physiological and serological similarity between these two clusters. He therefore divided them for epidemiological purposes into three types: *S.MG-intermedius* (non-β haemolytic,lactose fermenter), *S.anginosus-constellatus* (non-β haemolytic, lactose nonfermenter) and *S.anginosus* (β haemolytic). Thus a universally accepted taxonomy for *S.milleri* was not apparent. Facklam (1984) illustrated the differences between American and British taxonomic schemes and changed *S.anginosus-constellatus* to *S.constellatus* with *S.MG-intermedius* becoming *S.intermedius* and *S.anginosus* remaining unchanged.

Several workers have further pursued this question, attempting to find additional or alternative means by which to clarify SMG taxonomy. Workers have disagreed on the homogeneity of the SMG, some finding it to be homogeneous in phenotypic tests (Lutticken et al 1978; French et al 1989) and DNA-DNA hybridisation (Ezaki et al 1986; Coykendall et al 1987). Others have reported heterogeneity. Aluyi and Drucker (1983) found variation in whole cell trimethylsilyl-sugar profiles of 39 strains of *S.milleri* from various body sites. Cookson et al (1989) performed qualitative and quantitative analysis of the cellular fatty acids of 21 strains finding no qualitative difference, but quantitative data analysis revealed three groups. *S.anginosus* and *S.constellatus* were indistinguishable but separate from the other two groups, these being *S.intermedius* with a wide fermentation pattern and *S.intermedius* with a narrow fermentation pattern. Yakushiji et al (1988) found serological variation in 71 oral isolates of *S.milleri*, obtaining 10 serotypes (a-j) on the basis of cell-surface carbohydrate antigens, with only 4 strains untypable. Drucker and Lee (1983) calculated DNA base pair ratios (mol % guanine+cytosine) for 18 strains of *S.milleri* and found values ranging from 36.6-42% G+C, indicating genetic heterogeneity.

Whiley and collaborators have undertaken a series of studies in an attempt to clarify the position of

heterogeneity within SMG. Whiley et al (1981) found heterogeneity after SDS-polyacrylamide gel electrophoresis of 23 strains of *S.milleri*. DNA-DNA hybridisation studies by Whiley and Hardie (1989) revealed 4 homology groups from 25 strains. Groups 1-3 corresponded to *S.constellatus*, *S.anginosus* and *S.intermedius*, with group 4 being named *S.parasanguis*. SDS-PAGE analysis of these strains confirmed the identification of 3 distinct groupings within the SMG. This led in 1990 to development of a scheme for phenotypic differentiation of the 3 DNA homology groups by enzymic action on fluorogenic compounds, which allowed a collection of 157 strains to be identified (Whiley et al 1990). The culmination of this work saw Whiley and Beighton (1991) publishing amended descriptions recognising *S.anginosus*, *S.constellatus* and *S.intermedius* as distinct species.

Winstanley et al (1992) used a combination of conventional biochemical tests and pyrolysis mass spectrometry, after a similar combination had proved successful in identifying coryneform bacteria (Hindmarch et al 1990). This work found 5 clearly distinct groups, three corresponding to the DNA homology groups suggested by Whiley and Hardie (1989) representing *S.anginosus*, *S.constellatus* and *S.intermedius*, with group 4 containing 3 Lancefield group C β haemolytic strains, and group 5 possibly representing a second biotype of *S.anginosus*.

This wealth of evidence supporting 3 groupings within the SMG has led to the general acceptance of three species, *S.anginosus*, *S.constellatus* and *S.intermedius*, ending the controversy over taxonomy of the group which has hindered the study of pathogenicity.

1.2.2 ASSOCIATION OF SMG WITH INFECTION AND BODY SITE

Acceptance of the existence of three species within the SMG gave rise to two areas of investigation, the first being assessment of the available test systems for identifying these species , and secondly, as a result of the three species identification, the relation of these to specific sites of isolation or infections. Ahmet, Warren and Houang (1995) compared the commercially available Rapid ID 32 Strep test with the fluorogenic identification system of Whiley et al (1990) and found them to yield similar identifications. Flynn and Rouff (1995) found an 88%-98% agreement in identification between traditional tests and the Flou-card Milleri system. Further to the development of the fluorogenic system of Whiley et al (1990), where discrimination between *S.anginosus* and *S.constellatus* was problematic, Whiley et al (1995) examined the 16S-23S rRNA intergenic spacer region of the genome of SMG strains. This has been suggested as being useful in determining phylogenetic relationships between bacteria, aiding

strain identification (Greisen et al 1994). This readily distinguished between *S.anginosus* and *S.constellatus*, although *S.intermedius* and *S.anginosus* were not reliably distinguished by this method. Jacobs et al (1996) found 42 of 399 *"Streptococcus milleri"* strains reacted with the 16S rRNA gene sequence of both *S.constellatus* and *S.intermedius* probes and classified this as a distinct type within the group. This supports the suggestion by Jacobs (1997) that although the phylogenetic validity of the current taxonomy has been confirmed there is likely to be further investigation. The ability to accurately identify three species of SMG has led to greater understanding of species distribution and production of clinical conditions. Whiley et al (1992) found *S.intermedius* to be associated with brain and liver abscesses, possibly due to possession of a range of tissue degrading enzymes. *S.anginosus*, which are of two biotypes, were isolates from the gastrointestinal and urogenital tracts (broad fermentation pattern) and oropharyngeal infections, with *S.constellatus* mostly associated with dental abscesses. Gomez-Garces, Alos and Cogollos (1994) found that *S.anginosus* predominated in abdominal areas, while *S.intermedius* was isolated mainly from hepatic abscesses. Jacobs et al (1995) found *S.anginosus* to be recovered more often from the genito-urinary tract and *S.constellatus* from the thorax. *S.intermedius* was mostly found in the head and neck area,

but was also isolated from the abdomen, skin,bones and soft tissue.

1.2.3 BACTERIOLOGICAL CHARACTERISTICS

The SMG are commensals commonly isolated from the mouth, oropharynx, gastrointestinal tract and vagina, and are responsible for a variety of infections. They form minute colonies (less than 0.5 mm diameter), may require CO_2 for isolation (Kambal 1987) and have a distinct caramel odour (diacetyl), which has been suggested as a presumptive identification method (Chew and Smith 1992), when cultured on agar plates. Gram staining reveals spherical cells forming chains or pairs.

They are a heterogeneous group exhibiting various haemolytic, Lancefield and biochemical reactions. Kambal (1987) found 54% non haemolytic, 29% beta haemolytic and 17% alpha haemolytic *S.milleri* among 80 strains, while Jacobs et al (1995) found one third of 518 SMG isolates to be β haemolytic. Lancefield serological reactions are of little use in identifying the SMG, as a variety of antigens can be expressed, mainly groups A, C, F and G. Whiley et al (1990) found that in strains of *S.constellatus* and *S.anginosus* belonging to Lancefield group F, the former were nearly all beta haemolytic and the latter were all non haemolytic. Similarly Jacobs et al (1995) found that

the majority of *S.anginosus* strains were non haemolytic and carried Lancefield group F or were nongroupable, while most of the *S.constellatus* isolates were β haemolytic, along with 16.6% of *S.intermedius* strains.

1.3 THE STREPTOCOCCUS MILLERI GROUP AS PATHOGENS

1.3.1 CARIES

Unlike *Streptococcus mutans*, *Streptococcus milleri* is not recognised as a primary agent in production of dental caries, despite being reported in high numbers in deep areas of carious dentine by Edwardsson (1974). However, Poole and Wilson (1979) found *S.milleri* in similar numbers on normal and carious teeth,though they probably sampled whole teeth, not specific lesions as described by Edwardsson (1974).

S.milleri strains have been used in attempts to induce caries in gnotobiotic rats. The first published investigation showed an endocarditis strain of *S.intermedius* to cause more severe caries than *S.mutans* 6175 (Rosen and Kolstad 1977). Other studies have similarly found organisms of the SMG to be cariogenic, but less severely than *S.mutans*. Drucker and Green (1978) showed four plaque isolates of *S.milleri* to produce moderate (4-8

lesions) fissure caries in gnotobiotic rats, with two of these also causing moderate approximal caries. Two isolates from dental abscesses and another plaque isolate induced little or no caries (less than 4 lesions). A series of studies on the cariogenicity of SMG organisms have been reported by investigators from Aichi-Gakuin University in Japan. Yoshizaki (1983) and Hosoi (1985) both consider *S.intermedius* ATCC 27335 to cause caries in rats and hamsters, with Bessho (1985) showing that it was capable of producing lesions in extracted human teeth *in vitro*. Caries produced in this fashion was shown to be less severe than that caused by *S.mutans* in the presence of sucrose, but significantly more severe than that found in uninoculated controls with an indigenous oral flora. Caries development was independent of sucrose or any other carbohydrate, suggesting that SMG may be responsible for some sucrose-independent caries in humans. Horton et al (1985) found *S.milleri* NCTC 11169 produced fissure caries in gnotobiotic rats fed on diets high in sucrose, glucose and maize starch, with ranking of caries incidence in relation to diet in that order. Willcox, Drucker and Green (1987) found *S.oralis* and *S.milleri* induced similar levels of caries in gnotobiotic rats fed a high sucrose diet. Willcox, Drucker and Hillier (1988) found that *S.milleri* was less cariogenic than mutans streptococci and *S.salivarius* but induced more fissure caries than *S.sanguis*, *S.faecalis*, *S.mitis* and

S.lactis. Some investigators have tried to assess the properties of SMG organisms which allow them to induce caries. Ogawa (1989) found that *S.intermedius* OE-1 produced fissure caries (scored 22.3 using the Konig method), having chondroitinase activity and producing lactic acid as a metabolic end product. Kobayashi et al (1992) inoculated gnotobiotic rats fed on a diet containing 25% sucrose with a strain of *S.intermedius* and found the destruction of the dentine was similar to that caused by *S.mutans*. They suggested that this was due to proteolytic activity of the test strain rather than their acidogenic activity. Smalley et al (1994) investigated the mucin-sulphatase activity of oral streptococci, suggesting this activity may contribute to the destruction of salivary mucins and therefore may aid in the development of dental caries. However, little or no such activity was found in 4 strains of *S.constellatus* and 3 *S.anginosus* strains tested.

1.3.2 PERIODONTAL DISEASE

A series of studies by Moore et al found SMG organisms isolated frequently in association with experimental gingivitis (1982a), severe periodontitis (1982b) and severe generalized periodontitis (1983). Further to this Moore et al(1983) suggested that *S.anginosus* may be

implicated in a model of episodic destructive activity in periodontal disease, caused by periods of aggressive pyogenic activity. No pathological significance could be attributed to *S.milleri* by Crawford and Russell (1983) who found no significant differences in numbers of viridans streptococci isolated from healthy sites,or from cases of gingivitis, early or moderate periodontitis, or severe periodontitis.

Haffajee et al (1985) examined the flora of periodontal pockets of 16 people undergoing surgical and antibiotic treatment for periodontal disease. In the group showing poor response to treatment there were significantly elevated proportions of *S.intermedius* as well as *F. nucleatum* and *P.micros*. Topoll, Lange and Muller (1990)found that patients with advanced periodontal disease, in whom *S.intermedius* was a prevalent isolate from subgingival plaque samples, developed multiple periodontal abscesses after taking a course of broad spectrum antibiotics. They suggested that with advanced periodontal disease, systemic antibiotic therapy without subgingival debridement may change the composition of the subgingival microbiota, favouring the development of multiple abscesses. Socransky et al (1991) found 25% of 2299 sites sampled to be colonised with *S.intermedius* amongst 13 other organisms, while Haffajee et al (1992) found *S.intermedius* in 80% of the same 90 subjects. Magnusson

and Walker (1996) reported on refractory periodontal disease, which is characterised by low plaque scores and low responsiveness to periodontal therapy. They stated that in this case the subgingival microflora can be either predominantly Gram positive with elevated levels of *S.intermedius* or Gram negative, containing classical periodontal pathogens.

Periodontal disease has also been implicated as a predisposing factor for other SMG infections. Christiensen et al (1993) reported a septic pulmonary embolism where *S.intermedius* was isolated from the pus. The only predisposing factor was toothache and periodontal disease, and the authors suggested the gingiva as the source of *S.intermedius*, leading to bacteraemia, seeding of the lungs and abscess formation. This is supported by Wong, Donald and MacFarlane (1995) who found predisposing factors, including periodontal disease, in 80% of 25 cases of pulmonary disease.

1.3.3 ODONTOGENIC ABSCESSES

Originally *S.milleri* was described by Guthof (1956) as an organism isolated from dental abscesses, illustrating its association with purulent lesions. This has been confirmed by more recent studies, with *S.milleri* being frequently isolated from dental abscesses, often in pure culture (Sabiston et al 1976; Bartlett and O'Keefe 1979;

Aderhold et al 1981). A recent study by Lewis et al (1986) to quantify bacteria in pus aspirated from 50 acute dento-alveolar abscesses found *S.milleri* to predominate, isolating it from 20 cases, in two of which it was the sole organism. This correlated with Von Konow and Nord (1983) and Fisher and Russell (1993) who similarly found *S.milleri* to be the commonest facultative anaerobe in orofacial infections. With the recognition of three species of SMG, *S.constellatus* has been reported as the most common of the group associated with dental abscess formation (Whiley et al 1992). Although the pathogenic role of *S.milleri* in abscess formation is not clear, Aderhold et al (1981) suggested that the early phase of abscess formation involves a mixed aerobic-anaerobic infection where streptococci utilise oxygen and prepare the environment for anaerobic growth. This is supported by Lewis et al (1986) whose pure cultures of *S.milleri* were obtained when samples were obtained from patients on the first day of clinical symptoms, with an anaerobic flora predominating in 2-3 day old infections.

S.milleri isolates have been used to induce abscesses in mice with some success. Flynn and Lynes (1986) used *S.milleri* type 1 injected into the subcutaneous and submandibular spaces of Swiss white mice and examined the inoculation sites at different time intervals. A 72% incidence of abscess formation was obtained,

submandibular space abscesses 62%, subcutaneous space abscesses 78%, with abscesses resolving at 19.2 (+/- 1.1 SD) days. Lewis et al (1988) also used *S.milleri*, both as the single inoculating organism and in combination with another species, to induce abscesses in male albino mice. *S.milleri* alone produced either a Type A reaction, a diffuse inflammatory infiltrate, or Type B, a localized abscess without peripheral necrosis. Combination with *Bacteroides intermedius* (now *Prevotella intermedia*) gave a more severe group C reaction in most cases (localized abscess with peripheral necrosis), indicating synergistic interaction with the anaerobic Gram negative bacilli which may be important in abscess development in man.

1.3.4 ENDOCARDITIS

Like other dental plaque bacteria, those belonging to the SMG can cause infective endocarditis. Parker and Ball (1976) found 5.4% of 317 isolates from patients with endocarditis to be *S.milleri*. They further suggested that SMG are a less common cause of endocarditis than other dental plaque bacteria because they do not produce dextran, which allows adherence to heart valves. This may be discounted for two reasons. First, a study by Facklam (1977) showed that the proportions of *S.milleri* in plaque and endocarditis were almost identical i.e. low. Secondly, extracellular dextrans require sucrose for synthesis and since this is not present in blood it seems

unlikely to be of importance (Gosling 1988). It may be more likely that fimbriae present on some SMG bacteria are involved in attachment (Handley et al 1985). In a study of streptococci from the blood and internal organs of patients with specified systemic infections, Parker and Ball (1978) found 21% of 173 patients from whom *S.milleri* was isolated to have endocarditis, but only in those over 35 years old. Seven of the patients had previous valvular damage. Herzberg et al (1992) found that the SMG accounted for between 9-15% of all infective endocarditis cases. Glauser and Francioli (1982) successfully induced endocarditis in rats using *S.intermedius*. Willcox et al (1994) attempted to explain some pathogenic determinants of the SMG which may be involved in endocarditis, namely the ability to aggregate human platelets. Endocarditis results from infection by a microorganism of a platelet/fibrin vegetation that is located on the endothelial surface of the heart (Whiley and Hardie 1989). The ability of bacteria to aggregate platelets is considered to be important in the induction and progression of endocarditis, with Willcox and investigators finding that Lancefield group C SMG strains did aggregate platelets (Willcox et al 1994). In a further study, Willcox (1995) found that the SMG bound fibronectin and bound to platelet/fibrin or fibrin clots and fibrinogen, while *S.constellatus* species produced thrombin-like activity. He suggested that these *in vitro* pathogenic properties may be involved in both production

of abscesses and endocarditis.

S.milleri has also been implicated in cases of myocardial abscesses associated with endocarditis (Wallis et al 1986) and has been isolated from cases of purulent pericarditis (Akashi et al 1988).

1.3.5 BACTERAEMIA

Viridans streptococci may enter the circulation after vigorous toothbrushing, with more significant bacteraemia resulting from trauma, surgical procedures or purulent lesions in various body sites. Parker and Ball (1976) found 14% of patients with bacteremia to have serious underlying disease such as leukaemia, Hodgkin's disease and diabetes mellitus. Bacteremia due to *S.milleri* following dental extraction is well reported (Shanson et al 1978; Barnham et al 1989). Bacteraemic infections due solely to *S.milleri* are very infrequent and Murray et al (1978) and Shlaes et al (1981) concluded that *S.milleri* seldom infects an otherwise healthy individual in the absence of trauma. Jacobs et al (1994) analysed 19 cases of bacteraemia involving the SMG, where eleven patients had underlying disease, with local trauma to the mucosal barrier an important risk factor. *S.anginosus* was found to be the most common (16/20) while 8 of 20 strains carried the Lancefield group C antigen.

1.3.6 CENTRAL NERVOUS SYSTEM INFECTIONS

Reports of *S.milleri* isolated from infections of the central nervous system and meninges are numerous. Indeed this gives it its reputation for pathogenicity. Of streptococci associated with intracranial pus, *S.milleri* is the commonest of all isolates (De Louvois 1980). De Louvois et al (1977) carried out a multicentre study of organisms isolated from abscesses of the central nervous system and found 20 of 36 streptococcal isolates were *S.milleri* Lancefield group F, Ottens and Winkler type O III. Fourteen were from brain abscesses and 6 from subdural or extradural empyemas. Melo and Raff (1978) reported 3 cases of brain abscess in which *S.MG-intermedius* was isolated in pure culture. Where studies considered location of infection or underlying disease, it was shown that SMG organisms did not have a strong predilection for any area of the brain or that any particular infection or condition preceded formation (Shlaes et al 1981;Hendrix et al 1982;Puthecary and Rapport 1982). Considering the rarity of brain abscesses, the evidence of their association with SMG organisms suggests that it may be one of the commonest bacterial agents of such infections. Indeed animal experiments have shown that *S.milleri* has a well defined affinity for the central nervous system of mice (De Louvois et al 1974). Whiley et al (1990) found *S.intermedius* to be the most common of SMG organisms in

brain abscesses (21/25- 84%),and suggested that as *S.intermedius* is the most common of SMG isolates in plaque this may be the source. Homer, Whiley and Beighton (1994) found that an isolate of *S.intermedius* from a brain abscess produced specific glycosidase activities and suggested that these may allow the persistence and growth of *S.intermedius* at sites anatomically removed from its normal habitat.

Other than brain abscesses, SMG organisms are infrequently associated with other CNS infections, often requiring a history to show trauma or antecedent purulent infection. Parker and Ball (1976) found *S.milleri* in 8 of 65 (12%) cases of streptococcal meningitis. Two patients in a study by Shlaes et al (1981) infected with Group F streptococci developed meningitis following cervical osteomyelitis, one developing an epidural abscess. Blayney et al (1984) list 3 cases of subdural empyema complicating sinusitis. Meningitis following tooth extraction has been reported (Wu and Tsung 1983) and following nasal malignancy and sinusitis (Plotkin 1982). These indicate infection secondary to trauma or infection, but Tescon-Tumang et al (1982) reported a fatal case of purulent meningitis which showed presence of *S.MG-intermedius* where there was no preceding trauma or infection.

1.3.7 ABDOMINAL INFECTIONS

This is another area where the SMG has gained a reputation for pathogenicity. A review of clinical isolates of the SMG by Gosling (1988) showed that between 7% and 40% of clinical isolates were from the abdomen, with liver abscesses, purulent appendicitis and post-surgical infections most frequently reported.

SMG organisms have been associated with appendicitis by many workers (Murray et al 1978; Madden and Hart 1985; Kambal 1987). Poole and Wilson (1977) cultured 121 appendices and found a significantly higher level of isolation of *S.milleri* in inflamed appendices than normal (58% vs 26%). There was a pronounced connection between the presence of *S.milleri* in the appendix and purulent manifestations of appendicitis, and it was also isolated from other abdominal sites associated with appendicitis. Madden and Hart (1985) reviewed appendectomy cases over 1 year in a childrens' hospital. Preoperative cultures from unknown sites from 33 patients gave growth of *S.milleri* in 9 cases (27%). Postoperative cultures from the infected site where an abscess or purulent wound discharge developed gave a 69% isolation rate of *S.milleri*. McAllister et al (1988) administered cefotaxime in an attempt to reduce postoperative sepsis following appendicectomy. Sixteen wound infections occurred among 401 patients, with *S.milleri* being a

predominant isolate.

There are several case reports of liver abscess associated with the SMG (Bateman et al 1975;Allison et al 1984). Chua et al (1989) reviewed 33 cases of *S.milleri* liver abscess, 15 of which had detailed case notes. Among them, males predominated (only 1 woman) and single and multiple abscess cavities were observed, 4 of 7 with single sites being under 40 years of age,and 6 of 8 with multiple sites over 40 years. Nine (60%) had an underlying illness, predominantly gastrointestinal disease, but also present were dental infections and diabetes. Another review by Moore-Gillon et al (1981) similarly found predisposing factors, 10 of 16 having had abdominal surgery or disease.

Other abdominal infections which have yielded *S.milleri* include peritonitis (McCue 1983), subphrenic abscess (Ball and Parker 1979), cholangitis, splenic abscess and pelvic abscess (Murray et al 1978) and infected aortic aneurysm (Fox 1979). In these reports one or few cases are detailed and other intestinal bacteria are involved.

1.3.8 LUNG INFECTIONS

A significant proportion of SMG-associated infections are thoracic infections, empyema most commonly, though lung

abscesses and purulent pneumonia are also reported (Shlaes et al 1981;Frankish and Kolbe 1984). Parker and Ball (1976) studied streptococci from 22 purulent thoracic infections. Six (27%) isolates were *S.milleri* with only *S.pyogenes* found more frequently (7). Of 23 cases of empyema or lung abscess studied by Waitkins et al (1985) 13 (57%) yielded *S.milleri*, with 8 strains isolated in pure culture. Wong, Donald and MacFarlane (1995) reviewed 25 cases of pulmonary disease due to the SMG, where 16 had empyema, 5 had a lung abscess and 4 had both. There was a strong male predominance (84%) and predisposing factors were present in 80% of patients. The mouth and upper respiratory tract have frequently been regarded as the source of *S.milleri* and it is thought that infections are preceded by aspiration (Shlaes et al 1981). The SMG has also been isolated from purulent pericarditis (Reder et al 1984) and from myocardial abscesses (Wallis et al 1986).

1.3.9 EAR, NOSE AND THROAT INFECTIONS

Bannatyne and Randall (1976) isolated only 8 strains of group F streptococci in mixed culture from the external auditory canal of patients at a childrens' hospital from 1970-1975, suggesting *S.milleri* is rare in ear infections.

The SMG has been repeatedly isolated from cases of sinusitis both acute and chronic, sometimes in pure

culture (Wort 1975;Blayney et al 1984). Van Der Auwera (1985) found 7 isolates of *S.milleri* from acute maxillary sinusitis, with 4 of these in pure culture. Whitworth (1990) suggested the role of the SMG in sinusitis to be similar to its suggested role in dental abscesses i.e. autogenic succession. The major complication of SMG sinus infections is CNS infection.

SMG organisms have been found in the tonsils (Brook et al 1980) and aspirates of peritonsillar abscesses (Brook 1981) in children. They have also been reported in throat swabs from adults and children (Wort 1975),but their pathogenic significance is disputed. Poole and Wilson (1976) showed a trend of heavier growth from throats of those with symptoms than from asymptomatic carriers. Of 25 patients with pharyngitis and tonsillitis, 21 (84%) had heavy growth compared to only 5 (31%) symptomless carriers. Yamashiro (1991) also found a higher incidence of SMG in patients with purulent respiratory disease (24.7%) compared to healthy controls (11.7%), whereas Fox, Turner and Fox (1993) found similar levels of isolation from controls (11%) and patients with pharyngitis (11.1%). This may support the previous suggestion that *S.milleri* is not generally regarded as an important pathogen in the throat, with studies by Bucher and Von Gravenitz (1984) and Ruoff et al (1985) stressing the importance of distinguishing β-haemolytic SMG of Lancefield Groups A,C and G from the more pathogenic,

large zone β-haemolytic members of the group. However, Mitchelmore et al (1995) found *S.milleri* one of the most common isolates from pus aspirated from peritonsillar abscesses.

1.3.10 SKIN AND SUBCUTANEOUS INFECTIONS

Isolates of the SMG from superficial abscesses and wound infections have been reported by a series of investigators. Skin and subcutaneous isolates made up 8% of Poole and Wilson's (1976) SMG isolates from a reference laboratory and 30% of a collection at the Mayo Clinic Hospital (Libertin et al 1985) amongst others. Miller et al (1983) reported a very high incidence of subcutaneous sepsis caused by *S.milleri* following human bites. A similarly high incidence of small distal extremity abscesses after trauma was found by Libertin et al (1985). There have also been isolations of *S.milleri* from cellulitis (Murray et al 1978; Flanagan and Mills 1994) and rarer conditions of necrotising fasciitis (Shlaes et al 1981), where Imamura et al (1995) found *S.intermedius* as the causative species, and hydradenitis suppurativa (Highet et al 1980). Brook and Walker (1985) amongst others have been successful in inducing subcutaneous abscesses in experimental animals using *S.milleri* alone and in combination with other bacteria. Lewis et al (1988) investigated the capacity of bacterial

species isolated from human acute dentoalveolar abscesses to induce subcutaneous tissue reactions in mice. Injection of *S.milleri* alone produced either type A (diffuse inflammatory infiltrate) or type B (localised abscess without peripheral necrosis) reactions. When combined with *B.intermedius*, a type C (localised abscess with peripheral necrosis) reaction occurred, and there was a high incidence of recovery of the 2 organisms. This suggests a synergistic effect in abscess formation.

1.3.11 OBSTETRIC AND NEONATAL INFECTIONS

As the SMG are common inhabitants of the vagina it is hardly surprising that they have been found in these infections. Keay et al (1995) examined the prevalence of *S.constellatus* in urine of controls and patients with interstitial cystitis, with significantly greater isolation in IC patients (6/11) versus controls (0/7). With neonatal and obstetric infections, onset was associated with premature rupture of the membranes and consequent ascending infection, but it is unclear whether infection follows rupture or causes it. *S.intermedius* was found in 30% of cervical cultures from patients with premature membrane rupture but from only 7% of healthy controls (Evaldson et al 1980). Cox et al (1987) reported 2 cases of fatal infection of neonates of 26 weeks gestation due to *S.milleri*, suggesting in these

cases that an inadequately functioning cervix allowed ascending infection. *S.intermedius* endocervically inoculated into five pregnant ewes spread to the amniotic fluid and placenta (Evaldson et al 1983) illustrating that ascending infection can occur while the membranes are intact. Bergman et al (1995) described a strain of *S.anginosus* associated with neonatal infection, which exhibited a gliding type of motility, expressed as spreading growth on certain agar, which may aid in ascending infection.

1.3.12 INFECTION OF THE BONES AND JOINTS

There have been reports of the association of *S.milleri* with septic arthritis (Lever et al 1982; Ortel, Kallianos and Gallis 1990) and osteomyelitis (Shlaes et al 1981). In many cases these have been mixed infections and in an immunocompromised host or an individual with a number of predisposing factors present. Balantine, Papasian and Burdick (1989) reported a case of arthritis due to *S.anginosus* where the only predisposing factors were osteoarthritis of the knee and an acute meniscal tear, this case being the fifth found due to *S.anginosus*. Furthermore, Mateo et al (1991) described a case of pyogenic arthritis due to *S.anginosus* in a non-immunocompromised host, and suggested that problems with identifying the SMG may account for the rarity of reports

on its involvement in articular infections.

1.4 TREATMENT OF SMG INFECTIONS

Surgical drainage is central to the management of abscesses, augmented by antibiotics (Whitworth 1990). All but a few strains of *S.milleri* are very sensitive to penicillin, with Jacobs and Stobberingh (1996) finding only 1.4% of 423 strains of SMG to be of intermediate susceptibility to penicillin. However, Bantar et al (1996) found penicillin resistance rates of 12.5% and suggested emerging resistance to antibiotics may present a potential problem in therapeutic management of infections caused by the group. Alternatives are erythromycin, clindamycin and the cephalosporins. However, sensitivity to tetracyclines is variable (Tillotson and Ganguli 1984). Sulphonamides are inactive against the SMG, possibly promoting their growth (Mirick et al 1944), and they have been used as a selective agent for isolating these organisms from the mouth (Mejare and Edwardsson 1975).

Certain other antibiotics have been implicated in contributing to the pathogenicity of the SMG, specifically metronidazole and gentamicin in combination. Tresadern et al (1983) reported the development of 13 post-operative SMG infections in patients after colorectal surgery. In 6 of the 13 cases metronidazole

and gentamicin had been administered as prophylaxis for surgery, and the author suggested that the suppression of anaerobic and enteric Gram negative organisms, and the lack of activity of these two antibiotics against the SMG, allowed their expression as a pathogen. Piscitelli et al (1992) also suggested that suppression of the rest of the normal flora in this way can give an ecological advantage to the SMG with resultant disease.

1.5 POTENTIAL VIRULENCE FACTORS OF SMG ORGANISMS

From the preceding evidence it is clear that the SMG is capable of producing infection in a range of sites, and that compared with other viridans streptococci these organisms have an enhanced ability to produce purulent disease (Table 1.1). An important question is whether this ability is possessed uniformly by all members of the group or whether there are well defined subgroups with individual sites and modes of action. Possible virulence factors have been rarely studied hitherto.

1.5.1 HAEMOLYSINS

The fact that streptococci can cause several types of haemolysis on blood agar has long been known, and served as the basis for early classification of these

Table 1.1 Frequency of Isolation of streptococcal species in purulent disease

MICROORGANISM	% FREQUENCY OF ISOLATION FROM PURULENT DISEASE	
	CLINICALLY RECOGNISED	BACTERIOLOGICALLY CONFIRMED
GROUP A	15.1%	12.9%
GROUP B	22.4%	25.9%
GROUP C	3.3%	1.7%
GROUP G	3.3%	1.7%
S.sanguis	5.3%	3.4%
S.mitior	2.6%	3.4%
Unclassified 'viridans'	2.6%	1.7%
S.salivarius	2.6%	3.4%
S.milleri	28.3%	29.3%
S.mutans	0%	0%
S.faecalis	2.0%	2.6%

Adapted from Parker and Ball (1976)

microorganisms. Many strains causing human infections produce β-haemolysis. Todd (1932,1938) and Weld (1934) described the production of two distinct haemolytic toxins - Streptolysin O and Streptolysin S.

STREPTOLYSIN O (SLO)

SLO is an oxygen labile, antigenic bacterial toxin produced by most Group A and some Group C and G streptococci, with all strains producing an immunochemically and functionally identical toxin (Halbert 1970). It can be found in culture filtrates of complex media such as Todd Hewitt broth and also may be synthesised in a chemically defined medium as shown by Bernheimer (1982). Peak SLO synthesis usually occurs between 6 and 12 hours incubation, with titres falling rapidly after this time. SLO oxidised to its inactive form may be reactivated by a variety of sulphydryl compounds which restore the initial high activity (Herbert and Todd 1941). Hewitt and Todd (1939) showed that its activity could be irreversibly inhibited by low concentrations of cholesterol. Cholesterol was found to be the binding site for the toxin, being illustrated by the activity of SLO on the membranes of mycoplasma. Parasitic strains whose membranes contained cholesterol were sensitive to SLO, whereas saprophytic strains containing carotenol instead of cholesterol were insensitive (Bernheimer and Davidson 1965). Alouf and

Raynaud (1968) showed that the first step in SLO activity involves irreversible binding of the reduced form to membrane cholesterol. Further insight into the mode of action was gained when Dourmashkin and Rosse (1966) examined, by electron microscopy, the effect of SLO on erythrocyte membranes, and reported scattered holes of 500 Å diameter which allowed rapid release of haemoglobin. More recent investigations using electron microscopy showed that SLO forms arc or ring-shaped structures on erythrocyte membranes after binding to cholesterol molecules (Bhakdi et al 1985). Two haemolytic forms of SLO have been reported. The first is an acidic type with a molecular weight of 69,000 +/- 3,000 with a pI of 6.0 to 6.4 (Bhakdi et al 1984). The second is a neutral and degraded type of molecular weight 57,000 +/- 5,000 and a pI of 7.0 to 7.5 (Bhakdi et al 1984). Sekiya et al (1993) detailed a ring shaped structure with a crown formed by SLO on erythrocyte membranes, observed by electron microscopy. Numerous researchers have reported a multiplicity of toxic effects of SLO on animals as reviewed by Halbert (1970), while Hamburger and Lemon (1953) and Quinn (1957) confirmed these in man. SLO has been shown by Halbert et al (1961a, 1961b) to be the streptococcal factor responsible for the pathogenesis of rheumatic fever. It has been shown to stimulate light emission by human neutrophils (Andersen and Duncan 1980), to suppress chemotaxis and mobility of neutrophils (Andersen and van Epps 1972), to

inhibit phagocytosis by macrophages (Ofek et al 1972) and suppress lymphocyte responses to phytohemagglutinin (Andersen and Cone 1974).

STREPTOLYSIN S (SLS)

Streptolysin S is the oxygen stable, non-antigenic haemolysin responsible for β haemolysis around colonies on aerobic blood agar plates. It is produced by streptococci of Groups A,C,G and others, especially pyogenic strains. Expression of SLS activity depends upon contact between the microorganism and one of many agents like serum albumin, ribonucleic acid and Tween and Triton detergents. These different inducers give rise to different forms of SLS, acting also as carrier molecules for the haemolytic moiety, allowing it to be transferred from one carrier to another, as shown by Ginsburg and Harris (1963). SLS is sensitive to streptococcal proteinase (Wannamaker 1983) and its activity is inhibited by several phospholipids (Elias et al 1966) and trypan blue. Haemolysis is also inhibited by zinc ions, apparently by interfering with the attachment of SLS to erythrocyte membranes (Avigad and Bernheimer 1978). It is sensitive to heat and acid.

Koyami and Egami (1963) purified SLS and found it to consist of an oligonucleotide and polypeptide in the

ratio 3.3 to 1, with amino acids glycine, glutamic acid and serine in large amounts. A stable preparation of RNA-core SLS was reported by Lai et al (1978). Both moieties of the polypeptide-oligonucleotide complex were in a dimeric form, with the molecular weight of the complex 15,000 and that of the oligonucleotide carrier 7,100.

SLS has a broader spectrum of action than SLO. It is lytic or toxic to all those cells being sensitive to SLO, and has activity against wall-less forms of some bacteria and L forms of two strains of *Streptococcus pyogenes*. SLS is also responsible for the leukotoxic property of group A streptococci, where PMNLs are killed after ingestion of the streptococci (Ofek et al 1972), while lower concentrations of SLS inhibit phagocytosis.

Dourmashkin and Rosse (1966) found that,unlike SLO, SLS does not cause holes in erythrocytes, but subtly alters the lipid and/or protein organization in the membrane which allows free passage of ions. The retained haemoglobin exerts osmotic pressure, drawing water into the cell and causing it to rupture.

Weissmann et al (1965) showed that repeated intra-articular injection of Streptolysin S caused chronic osteoarthritis in rabbits, while Ginsburg (1970) showed repeated SLS injections to cause myocarditis. Ofek et al

(1972) illustrated that sublethal amounts of SLS suppressed phagocytosis of mouse peritoneal macrophages, suggesting that this contributed to the invasiveness and pathogenicity of microorganisms. Studies on human and mouse lymphocytes indicate that T cells are more sensitive than B cells to SLS (Hyrniewicz and Pryjma 1977). In vivo studies indicated that SLS suppressed T cell-dependent antibody responses, and suggested that SLS can suppress helper T cells (Hyrniewicz and Pryjma 1979).

1.5.2 INTERMEDILYSIN

Nagamune et al (1996) detailed a novel cytotoxin specific for human cells produced by *S.intermedius* UNS46 isolated from human liver abscess. This toxin, named intermedilysin, consisted of two proteins of 54 and 53 kDa, and its pattern of activity caused the investigators to distinguish it from all known bacterial cytolysins.

1.5.3 HYALURONIDASE

Poole and Wilson (1979) showed a positive association between hyaluronidase production in the SMG and haemolysis, production being most marked in β haemolytic strains of Lancefield groups A,C and F. Steffen and Hentges (1981) failed to find hyaluronidase production whereas Unsworth et al (1980) found 4 distinct serotypes which correlated with the source of the strain. Isolates

from dental plaque and purulent lesions produced type IV hyaluronidase suggesting an oral origin for abscess strains. High titres of antibodies in blood donor sera to type III and IV hyaluronidase also suggest a greater invasiveness for strains producing these types (Unsworth 1980). Unsworth (1989) showed significant hyaluronidase production in 50%-100% of *S.milleri* strains isolated from abscess sites, with higher frequency in deeper sites. In contrast, strains from normal flora sites showed low frequency of production (4%-52%). Jacobs and Stobberingh (1995) also found that hyaluronidase activity correlated significantly with infection-related strains when they examined 518 isolates of SMG. Homer et al (1993), using a sensitive spectrophotometric assay, showed that hyaluronidase was produced only by *S.intermedius* and *S.constellatus* in accordance with previous observations by Killper-Balz et al (1984). This was also found by Jacobs and Stobberingh (1995) and Willcox, Patrikakis and Knox (1995) and has been suggested as a further point of species identification within the group. This strong association between hyaluronidase production and *S.milleri* from abscesses suggests that the enzyme has an important role in their formation.

It has also been suggested by Homer et al (1994) that production of hyaluronidase may play a role in nutrition and replication of *S.intermedius* at abscess sites. They detected growth of a strain of *S.intermedius* when

hyaluronic acid was supplied as the sole carbohydrate source, due to depolymerisation of the substrate by hyaluronidase and internalisation of the resultant disaccharides.

1.5.4 NUCLEASES

Extracellular DNAse is found in about one half of strains tested (41%-67%) and RNAse in about two thirds (67%-80%) (Marshall and Kaufman 1981;Drucker and Lee 1983). Jacobs and Stoberringh (1995) found RNAse activity to be equally distributed among the 3 species, while DNAse activity was more frequently present in *S.intermedius* and *S.constellatus* and was associated with infection-related strains ($p < 0.001$).

1.5.5 CAMP FACTOR

The CAMP phenomenon was first reported in 1944 by Christie, Atkins and Munch-Peterson, providing the acronym. The haemolytic activity of staphylococcal β-lysin is enhanced by an extracellular factor produced by group B streptococci, called the CAMP factor, and this is routinely used in laboratory identification of these streptococci (McFadden 1980).

1.5.6 PROTEOLYTIC ENZYMES

Some researchers have failed to find the tissue destroying enzymes collagenase (Ottens and Winkler 1962), gelatinase, caseinase, (Lutticken et al 1978) fibrinolysin, chondroitin sulphatase and lecithinase (Steffen and Hentges 1981). Steffen and Hentges (1981) did find gelatinase and collagenase activity in one of two clinical isolates examined. A strain of *S.MG-intermedius* from infective endocarditis produced several extracellular proteins with proteolytic activity (Strauss et al 1977). Growth of this strain in conditions mimicking the conditions in a fibrotic heart lesion gave a 4-8 fold increase in these proteins, sufficient to damage tissues. Willcox, Patrikakis and Knox (1995) investigated the degradative enzymes of oral streptococci and found that strains of the SMG had arylamidase activity and degraded casein. No strains were able to degrade bovine serum albumin or produced chondroitin sulphatase, glycosyltransferase or dextranase (Willcox, Patrikakis and Knox 1995).

Homer et al (1993) found that strains of *S.intermedius* produced chondroitin sulphate depolymerase when the organisms were grown in the presence of glycosaminoglycan, suggesting that the enzyme is inducible. As well as a role in tissue degradation, Shain et al (1994) suggest an additional role of

nutrition at sites of infection, after showing that a strain of *S.intermedius* grew on chondroitin sulphate A. In further investigation of this strain of *S.intermedius* UNS 35, an isolate associated with brain abscess, Shain, Homer and Beighton (1996a) found it produced chondroitin sulphate (CS) depolymerase A and C. This property was inducible with little activity produced in the presence of glucose, which was consistent with the low level of production of polysaccharide degrading enzymes by other bacteria when a more readily catabolisable substrate is available (Salyers and Kotanski 1980). As chondroitin sulphate is distributed throughout connective tissues, in most cases as a hybrid structure, Shain et al (1996) suggested the ability of *S.intermedius* UNS35 to produce CS depolymerase A and C may play a role in destruction of host connective tissues and bacterial nutrition at an otherwise nutritionally compromised infection site. On further investigation Shain et al (1996b) purified this glycosaminoglycan depolymerase with SDS-PAGE analysis yielding a single band of molecular weight 83 000. However, their previous assumption that the enzyme was a chondroitin sulphate depolymerase was revised as it exhibited greatest activity against hyaluronic acid, with degradation rates of CS-A and CS-C at 8% and 2% of the rate with HA. It could therefore be considered a hyaluronidase, rather than a CS depolymerase. The pH optimum was around neutral similar to peptostreptococcal hyaluronidase (Tam and Chan 1985), but it was unusual in

having a high pI of 9.3.

Byers, Homer and Beighton (1996) reported strains of *S.anginosus*, *S.constellatus* and *S.intermedius* were able to use sialic acid efficiently as a sole carbon source independently of production of sialidase. They suggested this may play a role in survival *in vivo* in extra-oral diseases.

1.5.7 RUTINASE

Some *S.milleri* strains from the mouths of healthy individuals were shown by Parisis and Pritchard (1983) to release carcinogenic substances by the hydrolysis of common foodstuffs. Hydrolysis of rutin, a common component of food and drink, liberated quercetin, a genotoxic substance which may be involved in production of epithelial carcinoma. The rutinase was constitutive, partly inducible, cytosolic and most active at pH 6.5. The authors suggested that the reaction is likely to occur *in vivo* due to the constitutive nature of the rutinase, the intra-oral availability of rutin and the pH range of activity which closely resembled that of the mouth.

1.5.8 BACTERIOCINS

The ability of the SMG to colonize in the presence of a

mixed flora may be aided by production of substances antagonistic to other microorganisms. Dajani et al (1976) demonstrated bacteriocin-like activity in 78% of alpha haemolytic streptococci. Drucker and McKillop (1982) described widespread production of antagonistic substances in the form of H_2O_2 production and bacteriocin-like activity among *S.milleri* strains from various sources. Virtually all of these isolates were in turn sensitive to the bacteriocin-like activity of *S.mutans* NCTC 10832. Dajani (1991) suggested a low rate of bacteriocin-like activity among α haemolytic *S.milleri* strains. Kanamoto et al (1996) found strains of *S.intermedius* produced a bacteriolytic substance which lysed *M.luteus*.

1.5.9 SYNERGY

Shinzato and Saito (1994) have suggested synergy between *S.constellatus* and *Prevotella intermedia*. Whilst each alone caused pneumonia in mice, in combination they resulted in more severe pneumonia, often associated with lung abscess formation or empyema. Acute pneumonia with mixed infection resulted in a 60% mortality rate, while there was only 10% mortality and mild pneumonia in each separate infection. Additionally, enhancement of growth of *S.constellatus* occurred when cultured with *P.intermedia*, with growth also stimulated by a culture filtrate of

P.intermedia. This filtrate also inhibited the bactericidal activity of human PMNL, and was shown *in vivo* to be responsible for a 20% mortality rate and delayed clearance of *S.constellatus*. The investigators suggested that *P.intermedia* may act with *S.constellatus* in the production of pulmonary infections by stimulating its growth and suppressing bactericidal activity of the host.

1.5.10 INTERACTION WITH THE HOST IMMUNE SYSTEM

Higerd et al (1978) found that gentle washing of *S.intermedius* liberated a crude extracellular product named EP-Si which suppressed fibroblast proliferation and altered lymphocytic responses in vitro. Further work by Arala-chaves et al (1979) revealed a strongly immuno-suppressive, non-cytotoxic substance in this crude extract. When purified, it gave a 90 Kd protein named F3' EP-Si which was shown to induce T-suppressor lymphocytes and to have B-cell mitogenic activity (Arala-chaves et al 1981). Lima et al (1992) further studied F3'EP-Si (renamed P90) and its role in the survival of *S.intermedius* in the host. They demonstrated that P90 treated mice were 50 times more susceptible to infection with *S.intermedius* than untreated mice. This was believed to be due to mitogenic and immunosuppressive effects of P90 on B cells. Experiments showed that B cells obtained from P90 treated mice were less able to respond to

antigenic challenge, even in the presence of normal T cells, and T cells obtained from P90 treated mice could actively suppress the specific immune response of normal B cells. The authors suggested that even though these experiments were performed using a larger amount of P90 injected into mice than *S.intermedius* can produce *in vivo*, the effect on the survival of *S.intermedius* in the host would still be marked.

Toyoda, Kusano and Saito (1995) found a virulent strain of *S.constellatus* exerted a substantial inhibitory effect on phagocytic killing compared to an avirulent strain from the normal flora, and suggested it was mediated by a structural component of the organism. This would also contribute to delayed bacterial clearance.

1.5.11 CAPSULE

Encapsulation is a well established virulence factor for other bacteria, conferring varying degrees of resistance to phagocytosis. For example in Group A streptococci capsule has for many years been investigated for its role in virulence of these organisms via resistance to phagocytosis (Kass and Seastone 1943, Whitnack, Bisno and Beachey 1981, Dale et al 1996). Encapsulation has been suggested as playing a role in the pathogenesis of infections caused by a mixed bacterial flora such as acute dentoalveolar abscesses. Lewis et al (1993a)

isolated capsulate bacteria from acute dentoalveolar abscesses, having previously shown *S.milleri* to be particularly prevalent among facultative anaerobes in such infections (25/43 isolates) (Lewis et al 1986). Of the *S.milleri* isolates 2 were acapsulate and 25 were capsulate, with the authors suggesting a role in pathogenicity (Lewis et al 1993a). Brook and Walker (1985) demonstrated capsule on some *S.milleri* strains and found that only those strains possessing capsule could cause subcutaneous abscesses when injected alone into mice. However, when the unencapsulated strains were injected together with other capsulated organisms, encapsulation and pathogenicity were often restored. Lewis et al (1988) also demonstrated the ability of encapsulated *S.milleri* strains to produce abscesses in pure culture. Therefore, if SMG bacteria are to cause purulent disease, a polysaccharide capsule may be a pre-requisite. Lewis et al (1993b) investigated a possible role of SMG capsule in pathogenicity, that was resistance to phagocytosis. Of 10 *S.milleri* strains (8 capsulate, 2 non-capsulate) there was no significant difference in the percentage uptake by human PMNL. As resistance to phagocytosis did not appear to be a function of capsule the authors suggested it may play a role in pathogenicity by influencing functioning in cells or acting in synergy with other species in mixed infections.

The nature of the capsular material of the SMG is not

known but the typing antigen present in Group F and related streptococci has been regarded as antiphagocytic (Huis int Veld and Linnssen 1973). It may be similar in structure to the capsule of other streptococci, but this has not been investigated.

1.5.12 SURFACE APPENDAGES

M protein fimbrial structures have been studied on *S.pyogenes* and similar structures have been observed on some oral strains of the SMG (Handley et al 1985). These may be important in adherence to surfaces or other organisms of a mixed mucosal flora, although no work has been done to indicate their role in the SMG. Bergman et al (1995) found isolates of *S.anginosus* exhibited a gliding type of motility, expressed as spreading growth on certain types of agar media. These strains lacked any observable organelles of motility and were negative when tested in conventional motility test stab cultures. Electron microscopy revealed that motile strains produced more extracellular glycocalyx than non-motile strains.

1.5.13 CELL WALL ASSOCIATED PROTEINS

Lutticken et al (1978) found one or two protein antigens (sm antigens) in hydrochloric acid extracts of *S.milleri* strains from pyogenic infections. These sm antigens were thought to be situated at or near the cell surface and a

role in virulence similar to M protein of *S.pyogenes* was suggested.

1.5.14 SURFACE ASSOCIATED PROPERTIES

Willcox and Knox (1990) studied a variety of characteristics of the SMG that could be associated with pathogenicity and compared these with type strains of *S.sanguis*, *S.mutans* and *S.pyogenes*. Recent abscess isolates had a significantly greater ability to adhere to buccal epithelial cells than did other strains, with values for abscess strains similar to those of *S.salivarius*, which preferentially binds to epithelial surfaces. Compared to *S.sanguis* the SMG were generally not aggregated by human saliva. They co-aggregated weakly with strains of *Veillonella* spp. and actinomycetes. The generally low level of adherence to saliva-coated hydroxyapatite correlated with its observed low levels in dental plaque.

It has been suggested that ability of *S.pyogenes* to bind fibrinogen enables it to escape the host defence mechanisms (Whitnack et al 1984). In the study of Willcox and Knox (1990), 22% of the fibronectin added to the assay bound to test strains of *S.pyogenes*. SMG strains had a corresponding value of >2%, which although not high still represented a significant level of binding (10^7 molecules per bacterial cell) and, therefore, may aid

pathogenicity. Binding of fibronectin may also aid pathogenicity, as it is found on the surface of many mammalian cells. Conversely, Yang et al (1988) suggested that bacteria coated in fibronectin are more readily opsonized and ingested by polymorphonuclear leucocytes. Fresh isolates from infections bound fibronectin to a significantly greater extent than others, suggesting that binding of fibronectin may contribute to pathogenicity.

Hydrophobicity and surface charge may also play a role in pathogenicity. Absolom (1988) stated that the more hydrophobic the bacterial cell the more likely it is to be phagocytosed. SMG strains varied in the degree of their hydrophobicity with no relation to source (Willcox and Knox 1990). The authors suggested localized regions of charge on the cell surface, as strains with a high cell surface hydrophobicity were also able to possess a high cell surface charge. Alternatively, ability to display both hydrophobicity and negative cell surface charge may indicate that molecules such as lipoteichoic acid are involved. In addition, cell surface proteins may undergo conformational changes dependent on environment, which expose different regions. A cell may, therefore, have a charged cell surface and be hydrophobic. Later, Willcox,(1995) found that cell surface hydrophobicity of the SMG increased by 3% - 36% after binding fibronectin, but not proportionally.

Willcox et al (1994) found that Lancefield group C SMG strains could aggregate human platelets mediated by cell surface protein(s). The ability of bacteria to aggregate platelets is considered to be important in the induction and progression of endocarditis. It also may play a role in abscess formation where bacteria are localized in a platelet/fibrin clot. Lancefield group C SMG aggregated human platelets within 10 minutes of mixing (Willcox et al 1994). Addition of substances which chelated cations, inhibited the cycloxygenase pathway in platelets, reduced the availability of ADP or disrupted platelet membrane stability reduced this aggregation. Willcox et al (1994) suggested the platelet interacting substance on the surface of the SMG to be proteinacious, as treatment with protease inhibited aggregation, whereas treatment with lipase, periodate and antisera to Lancefield group C polysaccharide had no effect. Group C SMG strains had previously been shown by Willcox et al (1993) to bind large amounts of albumin to their cell surface. A cell surface protein of M_r 24000 which was liberated by lysozyme treatment was shown to be the cell-surface receptor on *S.intermedius* strain C5. This receptor was physically dissimilar from protein G, an albumin and IgG-binding protein of "large colony" Lancefield group C and G streptococci. Combination of these findings led them to suggest a potential role in pathogenesis for albumin binding and a novel mechanism of platelet aggregation for SMG Group C isolates.

Willcox (1995) examined potential pathogenicity features of the SMG which may be involved in production of endocarditis and abscesses. All strains were able to bind fibronectin, ranging from 12-198 mol/cell, with strains within each species showing wide variation. Binding of fibronectin increased the cell surface hydrophobicity of strains by 3-36%, though the amount of fibronectin bound did not correlate with the increase in hydrophobicity. Organisms bound to platelet-fibrin or fibrin clots and fibrinogen, with maximum adhesion values of 16.5%, 21.8% and 151 mol/cell respectively, with the two former relating to two strains of *S.constellatus* associated with abscesses and the latter a strain of *S.intermedius* isolated from the throat . *S.constellatus* strains produced thrombin-like activity, and Lancefield group C SMG aggregated rat platelets. Binding of fibronectin has previously been shown to be related to adhesion of SMG to saliva-coated hydroxyapatite and the isolation of strains from infections (Willcox and Knox 1990). Binding of fibronectin may aid the ability of an organism to invade the body and can also help bacteria to adhere to fibronectin associated with fibrin clots, therefore contributing to endocarditis (Manning et al 1994). Fibronectin may also promote the ingestion of bacteria by host PMNL as it has been shown that bacteria coated with fibronectin are more readily phagocytosed (Yang et al 1988). Willcox et al (1995) characterised the cell-surface receptor for fibronectin on strain *S.anginosus* F4

and found it to be a protein of M_r 14000 which was released from cells after mutanolysin digestion. The binding was specific, with cells having a maximum number of binding sites per cell of 770. They reported that fibronectin binding to cells increased the association of the bacteria with PMNL, but did not increase killing. The production of thrombin-like activity may also be involved in causing endocarditis and abscesses as thrombin converts fibrinogen to fibrin and is one of the major enzymes in the blood coagulation pathway. Therefore coagulation of plasma can seal off bacteria in a platelet-fibrin clot, and this may protect bacteria from elements of the host defence system (Willcox 1995). Willcox (1995) found that these *in vitro* pathogenic factors did not correlate with each other, except for a correlation between binding of large amounts of fibrinogen and the ability to aggregate platelets, suggesting fibrinogen binding may aid in platelet aggregation.

1.6 INTRODUCTION TO THE PRACTICAL WORK

It is therefore clear that the SMG possess a wide variety of pathogenicity factors which help them to cause disease. Although there have been more studies into the pathogenic determinants of the SMG since the clarification of taxonomy of the group and improved isolation and identification methods have been employed, much further work is required in order to fully characterise the group.

The aim of the practical work was therefore to address some of the issues suggested by the literature which may contribute to the pathogenicity of the SMG. In light of the ability to identify the three species of the group, could any significance be attributed to their site of isolation or toxin and enzyme profiles, allowing certain species to predominate in specific infections? Cell surface characteristics were investigated, especially possession of capsule, as these were implicated in the literature as possible virulence factors. Furthermore does the possession of capsule affect the interaction of the SMG with host cells? Willcox and Knox (1990) found dental abscess isolates of SMG had a proclivity for adherence to buccal epithelial cells, and their work was expanded in the current investigation. Finally the reaction of the body's immune response to the SMG was investigated via phagocytosis by human polymorphonuclear leukocytes. Were any species or isolate type resistant? Is capsule antiphagocytic?

It is hoped that in addressing these questions the understanding of the virulence of the SMG may be forwarded a stage and further work will be stimulated.

The following chapter is concerned with the isolation, identification and characterisation of a range of SMG isolates, in order that a heterogeneous group was available for further study.

Chapter Three examines the surface properties of the SMG, namely possession of capsule and cell surface hydrophobicity. Capsule plays an important role in the pathogenicity of other streptococcal strains, and therefore this was examined for the SMG. Similarly, cell surface hydrophobicity can contribute to ability to cause disease, for example preventing opsonisation and therefore phagocytosis, and so this was also examined.

Chapter Four investigates adherence of the SMG to buccal epithelial cells. This expands on the experimental work of Willcox and Knox (1990) which showed that abscess isolates adhered more readily than other SMG isolates to BEC. This finding was examined and the results related to species. Further to this the data were also analysed with respect to influence of capsule or cell surface hydrophobicity.

Chapters Five and Six are both concerned with ability of the SMG to resist phagocytosis. The interaction of SMG with PMNL was examined using chemiluminescence and the effect of serum opsonisation on this process was investigated. Chapter 6 explored this interaction further by measuring directly, using a radiometric assay, the number of organisms ingested by PMNL. In both chapters the influence of species or isolate type on phagocytosis was assessed, as well as the possible conferring of resistance by either capsule or hydrophobicity.

CHAPTER TWO ISOLATION, IDENTIFICATION AND CHARACTERISATION OF 'STREPTOCOCCUS MILLERI GROUP' (SMG) STRAINS

2.1 INTRODUCTION

2.1.1 AIMS OF THE INVESTIGATION

The aim of the work in this thesis was to investigate whether there are specific virulence factors which allow the SMG to cause disease. In order for any organism to be considered pathogenic it must be able to : 1] adhere to the host, 2] multiply within the host, 3] produce damage to the host and 4] avoid the host's defence mechanisms long enough to cause disease. The oral streptococci have often been considered opportunistic pathogens, as certain of these pathogenicity factors were absent. The following chapters will examine some of these features in relation to the SMG.

The first stage was to gather a collection of strains representative of the three species. These were collected from dental abscesses, clinical infections and supragingival plaque. This allowed re-examination of the evidence described in the literature review which suggested that isolates of the SMG from different infections have different properties. These were then characterised for Lancefield grouping, toxin production and degradative enzymes, to determine whether

distribution of these attributes correlated with either isolate type or species. Indeed throughout the experimental work results were analysed for any relationship with species or isolate.

Therefore, the identification of specific virulence factors of the SMG which may explain its ability to cause purulent infections provided an underlying rationale for the research. In the absence of these factors, SMG may still be considered an opportunistic pathogen requiring the correct environmental conditions or interaction with other organisms to be effective in causing disease.

2.1.2 STRAIN SELECTION

To allow for meaningful comparisons, three groups of isolates were collected. These were isolates from dental abscesses, non-oral isolates from unspecified clinical infections and a commensal group of plaque isolates from healthy mouths. NCTC strain 11325 was also included to represent a type strain of *S.constellatus*, isolated from a case of purulent pleurisy (Holdeman and Moore 1974). These groups allowed examination of the distribution of the three species, *S.anginosus*, *S.constellatus* and *S.intermedius*, in different ecological situations. Similar studies have revealed an association of *S.constellatus* species with dental abscesses (Whiley et al 1992). It also allowed investigation of factors which may aid

proliferation and spreading, as discussed earlier (P28-39), indicating whether these are peculiar to a particular species or isolate type. For example, production of certain enzymes such as sialidase, hyaluronidase and chondroitin sulphate depolymerase by specific species may account for their increased prevalance in certain infections, such as the association of *S.intermedius* with brain abscesses (Beighton 1994).

2.2 MATERIALS AND METHODS

2.2.1 CONFIRMATION OF PURITY OF STRAINS

Both the strains from dental abscesses and the isolates from non-oral infections had been isolated previously by other workers and were stored in a lyophilized state. These were reconstituted with 0.5 ml of Anaerobic Blood Broth (ABB), then plated out onto blood agar plates to check for viability and purity. They were then Gram-stained and confirmed as Streptococcus milleri group organisms by API 20 Strep kits (API Laboratory Products, Hampshire, England), which gave a biochemical profile and identified the SMG as Biotypes I,II or III. This was later replaced by the API RAPID 32 STREP (API Laboratory Products, Hampshire, England) test kit as this identified the SMG as either *S.anginosus*, *S.constellatus* or *S.intermedius*.

2.2.2 ISOLATION OF THE SMG FROM PLAQUE

Samples of supragingival plaque were taken from 20 trainee dental hygienists, all of whom had excellent oral hygiene and healthy mouths. The plaque was transferred into Transport Medium (Difco, Detroit, Michigan) and dispersed in the medium by mechanical agitation (Whirlimixer, Fisons, Leicestershire, England). A loopful of the suspension was plated out for single colonies on a semi-selective medium consisting of Sensitivity Test Agar (STA Lab 12, Lab M, Amersham) 40 g/l, sulfamethazine (4-amino-N-[4,6-dimethyl-2-pyrimidinyl]-benzene-sulphonamide) (Sigma, Poole, Dorset, England) 1000 µg/ml, nalidixic acid (Sigma, Poole, Dorset, England) 30 µg/ml and defibrinated horse blood 5% v/v. This allowed growth of *S.anginosus*, *S.constellatus*, *S.intermedius* and *S.mutans*, but no other oral streptococci (Whiley et al 1993). Single colonies were further plated out for purity on blood agar and identified using the Rapid ID 32 STREP test (API Laboratory Products, Hampshire, England).

2.2.3 STORAGE OF CULTURES

Pure cultures of all strains were stored on Protect beads (Technical Service Consultants, Bury, England) at - 70°C and - 20°C. Those stored at - 70°C were kept as a back up culture, while those held at - 20°C were routinely used

as starter inoculae. When strains were required for testing, a bead was removed and plated onto blood agar, incubated and cultures used from this plate. This allowed for minimal subculturing of strains and ensured properties as close to the original isolate as possible.

2.2.4 PLATE HAEMOLYSIS

Haemolysis produced on Columbia base agar + 5% sterile defibrinated horse blood was recorded as alpha (greening of blood around colonies), beta (lysis of blood) and no haemolysis (no change in blood). Colonial morphology was also noted at this point, particularly in reference to rough or smooth appearance.

2.2.5 LANCEFIELD GROUPING

This was determined using the Oxoid Streptococcal Grouping Kit (Oxoid), a latex agglutination test for groups A,B,C,D,F and G.

2.2.6 PHENOTYPIC DIFFERENTIATION

Strains were assigned to species *Streptococcus anginosus*, *Streptococcus constellatus* or *Streptococcus intermedius* by the method of Whiley et al (1990). Ten milligrams of the 4

methylumbelliferyl (4MU)-linked substrates, 4 MU-β-D fucoside, 4 MU-N-Acetyl β-D galactosaminide, 4 MU-α-D glucoside and 4 MU-β-D glucoside (Sigma) were each dissolved in 0.15 ml dimethylsulphoxide (DMSO) and diluted in 100 ml of 50 mM TES buffer (Sigma), giving a final concentration of 100 µg/ml. Strains were grown on blood agar for 2 days in 5% CO_2 at 37° C, harvested by swabbing the plate surface and suspended in TES buffer. This suspension was adjusted to an optical density at 620 nm of 0.1, representing approximately 1 x 10^8 organisms per ml, and 50 µl of this suspension was mixed with 20 µl of each substrate solution in the wells of a flat bottomed microtitration plate (Titertek, Flow Laboratories Ltd, Irvine, Scotland). This was incubated at 37° C for 3 hours, after which presence of enzyme activity could be visualised by use of an ultraviolet transilluminator; a positive result was indicated by blue fluorescence.

The three species reacted thus :

Substrate	S.anginosus		S.constellatus	S.intermedius	
	OR			OR	
1	-	-	-	+	+
2	-	-	-	+	+
3	+	+	-	-	+
4	-	+	+	+	+

(From Whiley et al 1990)

Where degradation of numbered substrates indicated :

1= β-D fucosidase activity 2= β-D galactosaminidase activity

3= β-D glucosidase activity 4= α-D glucosidase activity

2.2.7 STREPTOLYSIN O MEASUREMENT

Production of streptolysin O was tested in 20 ml cultures grown in Todd-Hewitt Broth (Oxoid) and Brain-Heart Infusion Broth (Oxoid) using the method of Abdul-Amir (1980)

Organisms were grown overnight at 37°C, after which SLO could be recovered from the culture supernate. The supernatant was saturated with sodium thioglycollate to provide -SH groups and act as a reducing agent to prevent SLO from being oxidized to its inactive state.

Assay Procedure

Serial doubling dilutions of the supernate were prepared with isotonic phosphate buffered saline (PBS). Red blood cells were washed three times with saline and resuspended to give a 2% w/v solution. One millilitre of each dilution was mixed with 1ml of 2% RBC (either sheep, horse or human) and incubated for 1 hour in a 37°C water bath along with a control tube containing PBS + RBC. The tubes were agitated to resuspend sedimented RBC's and then centrifuged for 2 minutes at 1 000g. The absorbance of the supernatant at 540 nm was measured and the value

of the control subtracted. The level of SLO activity was determined, where 1 haemolytic unit was defined as the amount of haemolysin in 1ml of PBS which caused 50% haemolysis of 1ml of RBC after 60 minutes, the titre being the highest dilution showing activity.
Positive samples were confirmed by adding anti-streptolysin O (Wellcome, Dartford, England) to show specific neutralization of the haemolysin.

2.2.8 STREPTOLYSIN S SCREENING

Horse serum (20 % w/v) was added to 100 ml of THB and BHI broth and organisms incubated as before. Cells were then harvested by high speed centrifugation (15 000 g for 30 minutes at 4°C) and the resultant pellets were resuspended in 8 ml of undiluted inactivated horse serum (heated at 56°C for 30 minutes) and shaken for 60 minutes with large glass beads (ballotini 12) in a Mickle disintegrator (Mickle Engineering, Gomshall). The resulting suspension was centrifuged (15 000g for 10 minutes) and the supernatant retained. Cell contents were further extracted by suspension in 8 ml of horse serum and centrifugation (15 000g for 30 minutes) after which the supernatants were pooled and filtered to provide the assay fluid. The assay procedure was the same as that described for SLO.

Anti-streptolysin O was added to positive samples and

they were then assayed. This tested that the haemolytic effect was not neutralized, and thereby confirmed that the haemolytic activity was due to SLS.

2.2.9 SCREENING FOR PROTEASE

A 250µl aliquot of a 1% solution of Azocoll (Sigma) was mixed with 250µl of culture supernate and the mixture was incubated at 37°C for 30 minutes. Two volumes of ice-cold 5% TCA were added and mixed and the solution centrifuged for 5 minutes at high speed in a centrifuge (Micro centrifuge, MSE). The resulting supernatant was mixed with an equal volume of 0.5M NaOH and the absorbance at 440 nm was read after a 5 minute development time at room temperature. 1 protease unit = change in OD 440 of 0.1. A known positive strain of *Clostridium perfringens* was used as a control.

2.2.10 SCREENING FOR HYALURONIDASE

The method of Unsworth (1989) was used. Strains were grown overnight in 20ml of Todd-Hewitt broth with added Neopeptone 2% w/v, $Na_2H_2PO_4.2H_2O$ 0.13 g/l and $Na_2HPO_4.2H_2O$ 0.74 g/l, which prevented proteinase production. The broth cultures were centrifuged until the supernate was clear (1 000g/15mins) and 0.025ml was added to the first and second wells of one row of a microtitre plate

(Titertek, Flow Laboratories Ltd, Irvine, Scotland). From these first two wells, doubling dilutions were made with cold distilled water. A further 0.025 ml of cold distilled water was added to each well followed by 0.05ml of potassium hyaluronate (Bacto AHT Substrate,Difco) which was reconstituted with 8ml of cold diluted India ink (4%). The tray was shaken to mix its contents and incubated for 20 minutes at 37°C, after which it was cooled at 4°C for 30 minutes. Finally, 0.025ml of cold 1 N acetic acid was added to each well and the tray again shaken to allow mixing. Presence of a black clot indicated intact hyaluronate substrate which clotted by addition of acid. Absence of a clot showed presence of hyaluronidase activity, as the hyaluronate substrate had been digested. The titre of hyaluronidase activity was the lowest dilution showing a negative reaction.

2.2.11 SCREENING FOR DNAse

The method of Porschen and Sonntag (1974) was used. DNAse agar (Oxoid) was supplemented with 0.3% yeast extract and cultures grown overnight. Plates were then flooded with 1 N HCl. Production of DNAse was demonstrated by a clear zone surrounding the colonies due to hydrolysis of DNA into nucleotide fractions which were not precipitated by the acid. Intact DNA formed a cloudy precipitate in the medium.

2.2.12 CAMP TEST

A single streak of the test strain was made perpendicular to a streak of beta lysin-producing *Staphylococcus aureus* on sheep blood agar plates, and the plate incubated in CO_2 at 37° C. A positive result, enhancement of the staphylococcal beta lysin by CAMP factor, was indicated by the development of an arrowhead at the junction of the two streak lines. A positive control of group B streptococcus, *S.agalactiae* was included.

2.2.13 HAEMAGGLUTINATION

Strains were grown overnight in BHIB + 0.3% YE then washed with PBS and suspended to an OD 660 nm of 1.0 (5 x 10^9 cells/ml). Suspensions (2 % w/v) of sheep, horse and human RBC's in PBS were used to measure haemagglutination. The PBS was supplemented with 1% BSA to act as a non specific protein blocker which prevented autoagglutination of the RBC's. Phosphate buffered saline (50 µl) was placed into the wells of a round bottomed microtitration tray. An equal volume of bacterial suspension was placed in the first well and double diluted across the tray. To each well was added 25 µl of the suspension (2%) of RBCs. The tray was then shaken for 30 seconds and left at room temperature for 3 hours, after which the trays were examined. Haemagglutination appeared as a mat of erythrocytes

covering the bottom of the wells, while absence of haemagglutination was indicated by a red button of sedimented RBCs. A negative control of RBCs plus PBS was included, while *Bacteroides fragilis* strain NCTC 9344 acted as a positive control. The titre of activity was indicated by the last dilution showing a positive reaction.

2.3 RESULTS

2.3.1 STRAIN IDENTIFICATION AND CHARACTERISATION

Tables 2.1, 2.2 and 2.3 show the strain number, species, pattern of haemolysis and Lancefield grouping of the dental abscess, clinical and plaque isolates. The letters O, C and P have been used in the strain numbering system to denote oral, clinical or plaque origin of the strain.

All three species were represented within each of the three groups of isolates. Dental abscess isolates comprised *S.anginosus* 13/26, *S.constellatus* 8/26 and *S.intermedius* 5/26. Of the clinical isolates, 5/20 were *S.anginosus*, 9/20 *S.constellatus* and 6/20 *S.intermedius*. Among the plaque isolates, 5/13 were *S.anginosus*, 2/13 *S.constellatus* and 6/13 *S.intermedius* respectively.

TABLE 2.1 DENTAL ABSCESS ISOLATES

STRAIN	HAEMOLYSIS	LANCEFIELD	SPECIES
002 (R)	α	F	S.constellatus
003	α	F	S.intermedius
006	α	NT	S.anginosus
007	NONE	F	S.intermedius
008	α	NT	S.anginosus
009	NONE	F	S.intermedius
010	NONE	C	S.anginosus
016 (R)	NONE	NT	S.constellatus
018	β	F	S.constellatus
019	α	F	S.anginosus
020	β	F	S.constellatus
021	α	NT	S.anginosus
023	NONE	NT	S.anginosus
024	NONE	A	S.anginosus
025 (R)	β	NT	S.constellatus
025	β	NT	S.constellatus
030	α	NT	S.anginosus
031	α	NT	S.anginosus
034	β	A	S.anginosus
035	NONE	NT	S.intermedius
039	NONE	F	S.anginosus
040	NONE	F	S.anginosus
041	NONE	NT	S.anginosus
042	NONE	NT	S.constellatus
043	β	NT	S.constellatus
044	β	NT	S.constellatus
045	β	F	S.constellatus
046	NONE	NT	S.intermedius

Lancefield grouping NT = non-typeable with antigens A,B,C,D,F and G.

(R) = Rough "star shaped" colony appearance as opposed to smooth pinprick colonies common to the other strains.

TABLE 2.2 CLINICAL ISOLATES

STRAIN	HAEMOLYSIS	LANCEFIELD	SPECIES
C01	β	NT	S.constellatus
C02	α	NT	S.intermedius
C03	α	NT	S.intermedius
C04	NONE	A	S.anginosus
C05	β	F	S.constellatus
C06	β	F	S.intermedius
C07	β	C	S.intermedius
C08	β	F	S.constellatus
C09	β	NT	S.constellatus
C10	NONE	C	S.anginosus
C11	NONE	C	S.anginosus
C12	β	NT	S.constellatus
C13	NONE	NT	S.intermedius
C14	α	NT	S.intermedius
C15	β	F	S.constellatus
C16	β	F	S.constellatus
C17	β	NT	S.constellatus
C18	NONE	F	S.anginosus
C19	NONE	F	S.anginosus
C20	β	NT	S.constellatus

Lancefield grouping NT = non-typeable with antigens A,B,C,D,F and G.

(R) = Rough 'star shaped' colony appearance as opposed to smooth pinprick colonies common to the other strains.

TABLE 2.3 PLAQUE ISOLATES

STRAIN	HAEMOLYSIS	LANCEFIELD	SPECIES
P01	α	C	S.anginosus
P02	α	F	S.anginosus
P03	α	F	S.anginosus
P04	β	NT	S.constellatus
P05 (R)	NONE	NT	S.anginosus
P06 (R)	NONE	NT	S.anginosus
P07 (R)	NONE	NT	S.intermedius
P08	NONE	NT	S.intermedius
P09	NONE	NT	S.intermedius
P10	NONE	NT	S.intermedius
P11	NONE	NT	S.intermedius
P12	NONE	NT	S.intermedius
P13	β	NT	S.constellatus
NCTC 11325	NONE	NT	S.constellatus

Lancefield grouping NT = non-typeable with antigens A,B,C,D,F & G.

(R) = rough 'star shaped' colony appearance as opposed to smooth pinprick colonies common to the other strains.

Colonies were of two appearances, the most common being the typically reported small, pinpoint, smooth mucoid colonies. The second type were rough "star shaped" pinpoint colonies which were less common and indicated by (R) in Tables 2.1 - 2.3. All gave the distinctive caramel odour of diacetyl on blood agar plates incubated for 18 hours at 37°C.

Haemolytic strains, either α or β type haemolysis, and non haemolytic strains were found in each group of isolates, with a fairly even spread of the three types in dental abscess isolates. Beta haemolysis dominated in clinical isolates and an absence of haemolytic activity was common amongst plaque isolates.

Lancefield Groups A, C and F were found, with group F predominating, but most commonly there was no reaction to Lancefield grouping sera.

2.3.2 STREPTOLYSINS

Results of streptolysin O and S screening are shown in Tables 2.4, 2.5 and 2.6. Positive strains gave similar results and titres with sheep, horse and human red blood cells, with the exception of strain C13 which was SLO positive only with human RBC. As was expected most Group A strains were SLO positive (2/3) along with two Group F and one non-typable strain. All beta-haemolytic strains

TABLE 2.4 STREPTOLYSIN AND ENZYME TITRES OF ABSCESS ISOLATES

STRAIN	SLO (TITRE)	SLS (TITRE)	HYALURONIDASE (TITRE)	DNASE
O02	-	-	-	-
O03	-	-	+(1/8)	+
O06	-	-	-	-
O07	-	-	+(1/4)	+
O08	-	-	-	-
O09	-	-	+(1/16)	+
O10	-	-	-	-
O16	-	-	+(1/2)	+
O18	+(1/64)	+(1/8)	+(1/4)	+
O19	-	-	-	-
O20	+(1/64)	+(1/8)	+(1/8)	+
O21	-	-	-	-
O23	-	-	-	-
O24	-	-	-	-
O25R	-	+(1/32)	+(1/16)	+
O25S	-	+(1/16)	+(1/8)	+
O30	-	-	-	+
O31	-	-	-	+
O34	+(1/4)	+(1/8)	-	+
O35	-	-	+(1/4)	+
O39	-	-	-	+
O40	-	-	-	+
O41	-	-	-	+
O42	-	-	+(1/16)	+
O43	-	+(1/4)	+(1/16)	+
O44	-	+(1/16)	+(1/16)	+
O45	-	+(1/4)	-	+
O46	-	-	+(1/32)	+

TABLE 2.5 STREPTOLYSIN AND ENZYME TITRES OF CLINICAL ISOLATES

STRAIN	SLO (TITRE)	SLS (TITRE)	HYALURONIDASE (TITRE)	DNASE
C01	-	+(1/16)	+(1/64)	+
C02	-	-	-	-
C03	-	-	-	-
C04	+(1/16)	-	-	+
C05	-	+(1/16)	+(1/16)	+
C06	-	+(1/32)	+(1/8)	-
C07	-	+(1/64)	+(1/128)	+
C08	-	+(1/8)	+(1/32)	+
C09	-	+(1/16)	-	+
C10	-	-	-	-
C11	-	-	-	-
C12	-	+(1/4)	+(1/128)	+
C13	+(1/128)	-	+(1/8)	+
C14	-	-	-	-
C15	-	+(1/32)	+(1/64)	+
C16	-	+(1/8)	+(1/16)	+
C17	-	+(1/8)	+(1/16)	+
C18	-	-	-	-
C19	-	-	-	-
C20	-	+(1/16)	+(1/32)	+

TABLE 2.6 STREPTOLYSIN AND ENZYME TITRES OF PLAQUE ISOLATES

STRAIN	SLO (TITRE)	SLS (TITRE)	HYALURONIDASE (TITRE)	DNASE
P01	-	-	-	-
P02	-	-	-	-
P03	-	-	-	-
P04	-	+(1/4)	+(1/8)	-
P05	-	-	+(1/16)	-
P06	-	-	-	-
P07	-	-	+(1/8)	+
P08	-	-	-	+
P09	-	-	-	+
P10	-	-	-	+
P11	-	-	-	-
P12	-	-	+(1/4)	-
P13	-	+(1/8)	-	+

showed SLS activity.

2.3.3 PROTEASE ACTIVITY

All strains failed to show protease activity. This activity had been found by Steffen and Hentges (1981), but not by others (Mergenhagen, Thonard & Scherp, 1958 , Ottens & Winkler, 1962).

2.3.4 HYALURONIDASE ACTIVITY

Tables 2.4, 2.5 and 2.6 illustrate hyaluronidase activity, which was exhibited by 16/28 dental abscess isolates, 11/20 clinical and 2/13 plaque isolates of species *S.constellatus* and *S.intermedius*. One strain of *S.anginosus* was positive for hyaluronidase (P05). Titres ranged from ½ to 1/128 with no recognisable pattern of distribution of theses titres.

2.3.5 DNASE ACTIVITY

DNase activity is illustrated in Tables 2.4, 2.5 and 2.6. This activity was shown by a variety of strains, consisting of 71% of dental abscess isolates, 60% of clinical and 38% of plaque isolates.

2.3.6 CAMP FACTOR

All strains tested negative for CAMP factor. Therefore the test strains did not produce the extracellular CAMP factor which enhances the haemolytic activity of the staphylococcal β lysin.

2.3.7 HAEMAGGLUTINATION

No strain was able to haemagglutinate sheep, horse or human RBC. Ability to haemagglutinate RBCs is often used as an indicator of whether bacteria can attach to host cells, as it is a simple experiment to perform. Attachment to eukaryotic cells can be an important virulence determinant in a number of bacteria, and the inability of the SMG to haemagglutinate may indicate an incapacity to adhere well to certain host cells.

2.3.8 SIGNIFICANCE OF SPECIES IN DISTRIBUTION OF HAEMOLYSIS, HYALURONIDASE AND DNASE ACTIVITY

Table 2.7 illustrates the distribution of selected characteristics in relation to species. *S.anginosus* strains showed a low incidence of β-haemolysis, SLS, hyaluronidase (4.3% for all three) and SLO activity (8.7%), while they were moderately positive for α-

Table 2.7 ASSOCIATION OF HAEMOLYSIS, TOXIN AND ENZYME CHARACTERISTICS WITH SPECIES

	SPECIES		
Characteristic	S.anginosus	S.constellatus	S.intermedius
α-haemolysis	9/23 (39%)	1/21 (4.7%)	4/17 (23.5%)
β-haemolysis	1/23 (4.3%)	18/21 (85.7%)	2/17 (11.7%)
No haemolysis	13/23 (56.5%)	2/21 (9.5%)	11/17 (64.7%)
SLO	2/23 (8.7%)	2/21 (9.5%)	1/17 (5.9%)
SLS	1/23 (4.3%)	18/21 (85.7%)	2/17 (11.7%)
HYALURONIDASE	1/23 (4.3%)	17/21 (80.9%)	10/17 (58.8%)
DNase	7/23 (30.4%)	19/21 (90.5%)	11/17 (64.7%)

haemolysis (39%), no-haemolysis (56.5%) and DNase activity (30.4%). In contrast, *S.constellatus* strains showed high incidence of β-haemolysis and SLS activity (85.7%),hyaluronidase (80.9%) and DNAse (90.5%) production. Strains of *S.intermedius* showed average hyaluronidase (58.8%) and DNase (64.7%) activity, tended to be either α-haemolytic (23.5%) or non-haemolytic (64.7%) and had a low incidence of β-haemolysis and SLS activity (11.7%) and SLO activity (5.9%).

2.4 DISCUSSION

2.4.1 METHODS OF IDENTIFICATION

Use of the API 20 Strep test kit was an unsatisfactory method of identification of the SMG, as species were not distinguished, only Biotypes I, II or III which did not directly correspond to the three species. The Rapid ID 32 Strep kit was a far more useful system, as it identified the three species. In most cases the reaction of the test strain produced an identification number corresponding to only one species, but on several occasions the identification was not definite, with a resulting ID of, for example, 96.3% likely as *S.anginosus* with 3.7% possibility of *S.intermedius*. Therefore, to provide a conclusive yet rapid identification, the Rapid API 32 Strep test was used in conjunction with the fluorescence test of Whiley et al (1990). The

fluorescence test provided good identification of *S.intermedius* strains, but as the essential difference between *S.anginosus* and *S.constellatus* is presence of β-D glucosidase activity in the former and absence in the latter, the discrimination between these two was occasionally inconclusive. Thus, combination of the two methods provided excellent identification to species level of the SMG. This was in agreement with the findings of Jacobs and Stobberingh (1994) who compared the Rapid ID 32 Strep kit with the fluorescence method of Whiley et al (1990). They found the systems agreed on 70.2% of identifications, with the best agreement occurring for *S.anginosus* and most discrepancies with *S.intermedius* strains, despite the latter being the most readily distinguished by the fluorogenic system.

From the biochemical profiles obtained, it can be stated that in general most strains fermented lactose, sucrose, trehalose and salicin, were Voges Proskauer positive (formed acetoin) and hydrolysed arginine and aesculin. They tended not to ferment raffinose, mannitol, sorbitol, inulin or arabinose or to hydrolyse starch or hippurate.

Kellens et al (1994) suggested another method for rapid characterisation of the SMG, namely agglutination by lectins. They found that after boiling at pH 2, all 218 isolates of SMG tested were agglutinated with 25 lectins, giving 45 agglutination patterns. They suggested that

this could be an important assay in epidemiological and ecological studies of the SMG, potentially more useful than Lancefield grouping which only distinguishes 4 groups (A,C,F & G),leaving almost 50% of strains ungroupable.

2.4.2 SPECIES DISTRIBUTION

Until recently, disagreement over the taxonomy and nomenclature of the SMG had led to the use of various naming systems for the group and different systems of identification being employed by American and European microbiologists. This made it impossible to illustrate any relationship between isolate type and the disease caused. However, with the recent classification of the SMG into 3 species, some reports have detailed significant differences in the distribution of these species. Whiley et al (1990 & 1992) found *S.intermedius* to be the most common of the SMG causing brain abscesses and also the most frequent isolate from supra gingival plaque, suggesting a plaque origin. They also noted that *S.constellatus* was the most common species isolated from dental abscesses, with *S.anginosus* tending to be found in the gastrointestinal and urogenital tracts and oropharyngeal infections. In the current work *S.intermedius* was the most common plaque isolate (46% recovery), and of limited distribution in the other two groups (20% & 30% for abscess and clinical respectively).

S.constellatus was not the most common isolate from dental abscesses, but this may have been due to insufficient numbers. These results suggest that particular species of the SMG do have a proclivity for certain infections.

2.4.3 CHARACTERISATION

When the 61 isolates are considered as a whole group they follow the trends noted by a number of previous studies performed before the SMG were divided into three species (Poole & Wilson 1979 ; Ball & Parker 1979 ; Shlaes et al 1981 ; Ruoff, Kunz & Ferraro 1985). Most isolates were non-haemolytic (26/61 ; 43%) and many did not possess Lancefield antigens (34/61 ; 54%). Among those that were haemolytic, β haemolysis was more frequent (21/35 ; 60%) than α haemolysis (14/35 ; 40%). Where Lancefield grouping was determined, Group F was most common (19/27 ; 70%), followed by Group C (5/27 ; 19%), then Group A (3/27 ; 11%). No isolates of group G were found. There was a positive association between β haemolysis and Group F strains, with 8/19 (42%) showing β haemolysis, 5/19 (26%) showing α haemolysis and 6/19 exhibiting no haemolysis. Whiley et al (1990) noted that Group F *S.constellatus* strains were nearly all β haemolytic, a result which is supported by the present work. They also reported that Group F *S.anginosus* strains were non haemolytic, though this was not shown conclusively in the present study.

Therefore, the isolates collected represented a typical selection of SMG strains with characteristics similar to those reported previously.

2.4.4 POSSESSION OF TOXIN AND ENZYME ACTIVITY

Both the abscess and clinical isolates had similar numbers of strains possessing SLO, SLS, hyaluronidase and DNAse activity. SLO activity was present in 11% of abscess and 10% of clinical isolates, SLS in 29% of the former and 55% of the latter, hyaluronidase in 46% and 55% and DNAse in 71% and 60% of the respective groups. Plaque isolates had a lower prevalence of all properties tested. Thus, none possessed SLO activity, 15% had SLS activity, 31% produced hyaluronidase and 38% produced DNAse. This illustrated that in the true commensal flora fewer strains possessed virulence factors. The few strains which did possess degradative and invasive activity may be important in initiating potential disease if the appropriate conditions arise.

2.4.6 HYALURONIDASE ACTIVITY

Whiley et al (1990) found that hyaluronidase activity was possessed by *S.constellatus* and *S.intermedius* but not *S.anginosus* and used this as a factor in identification. This was also found in the current study, with the

exception of one *S.anginosus* strain, P05, which was positive for hyaluronidase at a titre of 1/16. The ability of this *S.anginosus* strain to exhibit hyaluronidase activity, when it is unreported by others, may be due to mis-identification of the species, although the identification was confirmed by both the API Rapid 32 Strep test and the fluorogenic system reported earlier. It may also have been caused by a contaminant, although the result was obtained on two separate occasions using fresh inoculum, and on plating-out, only one colony type was present. Finally it may have been a mutant strain which does have hyaluronidase activity. Although the method used here was sufficient for a rapid screening, a more sensitive assay method for detecting hyaluronidase activity (for example the spectrophotometric assay used by Homer et al 1993) would be useful to help clarify the picture.

Poole and Wilson (1979) found a positive association between hyaluronidase production and haemolysis, which was also found in this study. Amongst those strains exhibiting hyaluronidase activity, 64% were α- or β-haemolytic.

CHAPTER 3 SURFACE PROPERTIES OF THE "STREPTOCOCCUS MILLERI GROUP"

3.1 INTRODUCTION

3.1.1 GENERAL INTRODUCTION

The bacterial cell surface is perhaps the most obvious site of interaction between the pathogen and host, and is important in many aspects of virulence. Attachment to host cells and the extracellular matrix is a necessity for bacteria colonising sites such as mucosal surfaces which are constantly being washed with host secretions (Beachey 1981). Attachment may also be advantageous for the bacterium which obtains nutrients by lysing host cells or digesting the extracellular matrix, where contact, then lysis, delivers nutrients directly accessible to the organism. Similarly, attachment may be required in order for the organism to deliver toxins to the host in order to cause disease. This was found by Satterwhite et al (1978), where a non-adherent strain of enterotoxigenic *E.coli* was unable to produce diarrhoea, despite retaining its toxin-producing ability. The authors suggested that attachment to the intestinal epithelium was essential for the toxin's effectiveness. At other stages of infection interaction of pathogenic bacteria with host cells can be a disadvantage, such as the need to avoid binding of complement and immunoglobulin molecules, and therefore retention of

resistance to attachment to phagocytic cells. Silverblatt and Ofek (1978a) showed that fimbriated *P.mirabilis* adhered readily to urinary tract cells, causing pyelonephritis, but that these fimbriated cells also adhered well to phagocytic cells and were therefore more readily ingested and killed than their non-fimbriated counterparts (Silverblatt & Ofek 1978b). It can therefore be seen that the bacterial cell surface has a dual role to play in pathogenicity, the first being positive interaction with the host cell which aids the infection process, and the other in disguising its surface in order to avoid recognition by the host defence mechanisms.

This chapter will examine some surface characteristics of the SMG which may aid their pathogenicity, namely possession of capsule and bacterial cell surface hydrophobicity. The following two sections outline the possible significance of these.

3.1.2 RELEVANCE OF CAPSULE WITHIN THE SMG

The possibility that possession of capsule may be a pathogenic determinant for the SMG has been suggested by a number of investigators. Brook and Walker (1985) found that only strains of SMG in which > 50% of cells were encapsulated could cause subcutaneous abscesses in pure

culture in mice. In addition, when unencapsulated strains, which previously could not induce abscesses, were injected into mice along with a capsulate strain, they could subsequently cause abscesses themselves (Brook and Walker 1985). Lewis et al (1988) also found pure cultures of SMG strains to induce abscesses in mice. These results suggest that a polysaccharide capsule may be required by the SMG for production of pyogenic infections, though it is not entirely clear how it contributes to their pathogenicity. Both papers mentioned suggested that capsule may aid virulence by inhibiting phagocytosis, and this was investigated by Lewis et al (1993b). They found no difference in the degree of uptake of capsulate and non-capsulate strains of the SMG.

3.1.3 BACTERIAL CELL SURFACE HYDROPHOBICITY

The hydrophobic/hydrophilic nature of the bacterial cell surface affects subsequent interactions with other surfaces and particles, depending on their nature. In the absence of opsonin, the physico-chemical aspects of adherence are involved in phagocytosis of particles. The more hydrophilic the bacterial cell surface is in relation to the hydrophobicity of the phagocytic cell, the more likely it is to resist phagocytosis (Patrick and Larkin 1995) (Table 3.1).

During the course of an infection, opsonins will override these physico-chemical interactions, but cell surface hydrophobicity should not be disregarded in this instance. Absolom (1988) stated that the more hydrophobic the bacterial cell the more likely it was to be opsonised with IgG and phagocytosed. This suggests that hydrophilicity may contribute to pathogenicity by facilitating evasion of the host defences.

Table 3.1 The hydrophobicity/ hydrophilicity of bacterial surfaces in relation to phagocytic cells

	BACTERIUM/CELL	CONTACT ANGLE	
Hydrophilic; resistant to phagocytosis by human PMNL	S.aureus	16.5	Resistant to phagocytosis by guinea pig macrophages
	S.typhimurium	17.0	
	S.pneumoniae	17.0	
	H.influenzae	17.6	
	Human PMNL	18.0	
	S.pyogenes	21.3	
	Guinea pig macrophages	21.3	
	E.coli 07	23.0	
	N.gonorrhoeae	26.7	
Hydrophobic	B.abortus	27.0	

Figure adapted from Patrick & Larkin (1995) Ch 7 p141.

3.2 MATERIALS AND METHODS

3.2.1 CAPSULE STAIN

The presence of capsule was assessed microscopically by negative staining using a modified India Ink wet mount method. India Ink (Pelican Black 9150,Osmiroid, England) (1 ml) was added to 1 ml of sterile water and centrifuged at 22 000 g for 20 minutes at 10° C. The pellet was then washed twice by suspension in 2ml of sterile water and centrifugation at 11 000 g for 15 minutes at 10° C. The pellet was finally resuspended in sterile water and mixed by sonication and mechanical agitation to give an aqueous preparation which was stored at 4° C.

Colonies were picked off an 18hr blood agar plate and mixed in a loopful of India Ink on a glass slide. A coverslip was applied and tapped into position using a wooden stick.

Preparations were examined under oil at x 1000 magnification using a standard light microscope. The degree of encapsulation was recorded as capsule (+), small capsule + (s) and no capsule (-).

3.2.2 EXAMINATION OF CAPSULE BY TRANSMISSION ELECTRON MICROSCOPY

Test strains of the SMG were grown overnight in BHIB +YE then centrifuged at 1 000 g for 15 minutes. The resulting pellets were fixed for electron microscopy in Ruthenium red (Luft 1964 & 1971) using the following method. Primary fixative (Appendix ii) was added to the samples and incubated for 1 hour at 0° C, followed by a rinse with buffer wash (Appendix ii). Post fixative was then added and the samples incubated for 3 hours at room temperature. Following fixation the specimens were dehydrated with graded alcohol and embedded in Araldite (Glauert 1965). Ultrathin sections were cut with a Diatome diamond knife and mounted on Formvar coated 1000 µm aperture grids. The sections were examined using a Zeiss E.M. 109 electron microscope at 50 kV and electron micrographs taken on Ilford FP4 film.

All electron microscopy work was kindly performed by Dr Ian Montgomery of the Institute of Physiology, Glasgow University.

3.2.3 REMOVAL OF CAPSULE

(A) Physical

Initial attempts were made to remove the capsule by sonication. Bacterial suspensions were subjected to

sonication of varying power and duration with a Microson XL ultrasonic cell disruptor (Heat Systems, New York). This machine is used within the Dental Hospital to emulsify plaque samples without damaging the organisms, and therefore was thought to be mild enough to remove capsular material, whilst leaving the organism intact.

(B) Chemical

Capsule was removed by treatment with hyaluronidase, a method which has been used to strip other streptococcal species of their capsule. 5 mg of hyaluronidase (Type 1-S from bovine testes / 340 units per mg) (Sigma) was added to 1 ml of sterile distilled water. This solution (100 µl) was added to 1 ml of bacterial culture and incubated at 37°C for 20 minutes. After incubation a loopful was subjected to India ink capsule staining as previously described to check for absence of a capsule halo. A sample was also spun down into a pellet and processed for electron microscopy as detailed in the preceding section.

3.2.4 BACTERIAL CELL SURFACE HYDROPHOBICITY

The following two methods were used to measure cell surface hydrophobicity :

(A) Hexadecane Partition Assay

Relative hydrophobicities were assessed by the method of Rosenberg et al (1980). Bacterial suspensions were adjusted to an OD 660 nm of 1.0 (5 x 10^9 cells/ml). An aliquot (0.86 ml) of bacteria was mixed with 0.14 ml of n-hexadecane (Sigma) and incubated for 10 minutes at 30°C, after which the suspension was vortex mixed for 2 minutes. The mixture was left for 15 minutes to allow the suspension to separate, the aqueous phase was removed with a pastette and the OD 660 nm recorded. Triplicate samples were set up in this way and the experiments were repeated three times. The percentage reduction in OD provided a measure of cell surface hydrophobicity.

A change in OD 660 nm of :
0% - 9% indicated a very hydrophilic surface
16% - 49% showed moderate hydrophilicity
50% - 79% showed moderate hydrophobicity
80% - 100% denoted a very hydrophobic surface.

(B) Hydrophobic Interaction Chromatography

A modification of the method of Dahlback et al (1981) was used. Octyl Sepharose CL 4B (Pharmacia) was washed ten times with distilled water and then suspended 1:1 in 75%

seven salts solution (Appendix I). This was then packed to a height of 50 mm into glass Pasteur pipettes (diameter 5 mm) which had been washed with ethanol and plugged with cotton wool. Bacterial suspension (1 ml) labelled with tritiated adenine (2 µCi/ml)(Amersham, England) at OD 620 nm 0.45 (1 x 10^9 cells/ml) was added to the column and then eluted with 12 ml of 75% seven salts solution. The eluate and gel were collected in scintillation vials, 3 ml of Ecoscint A (National Diagnostics) was added and counts obtained. The degree of hydrophobicity was indicated by the ratio g/e, representing the radioactivity retained by the hydrophobic gel(g) and that present in the eluate(e). The higher the g/e value the more hydrophobic the interaction, i.e. log g/e of less than 0 indicates a hydrophilic nature.

log g/e values of :

+0.51 - +2.0 very hydrophobic
+0.01 - +0.5 indicated a moderately hydrophobic surface
-0.01 - -0.5 denoted a moderately hydrophilic surface
-0.51 - -2.0 very hydrophilic

3.2.5 STATISTICAL ANALYSIS

Correlation between the two methods of assessing cell surface hydrophobicity was tested using a rank

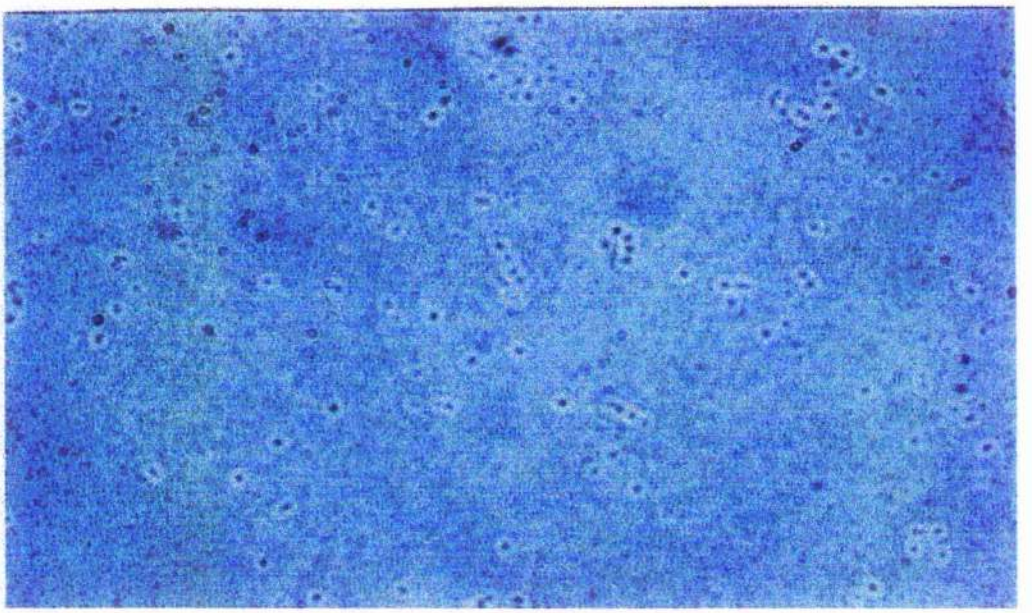
correlation test according to Spearman (Clarke 1980) which yielded a rank correlation coefficient, r_s within the limits ($-1 < r_s < 1$)where 1 indicates exact agreement and -1 complete disagreement.

3.3 RESULTS

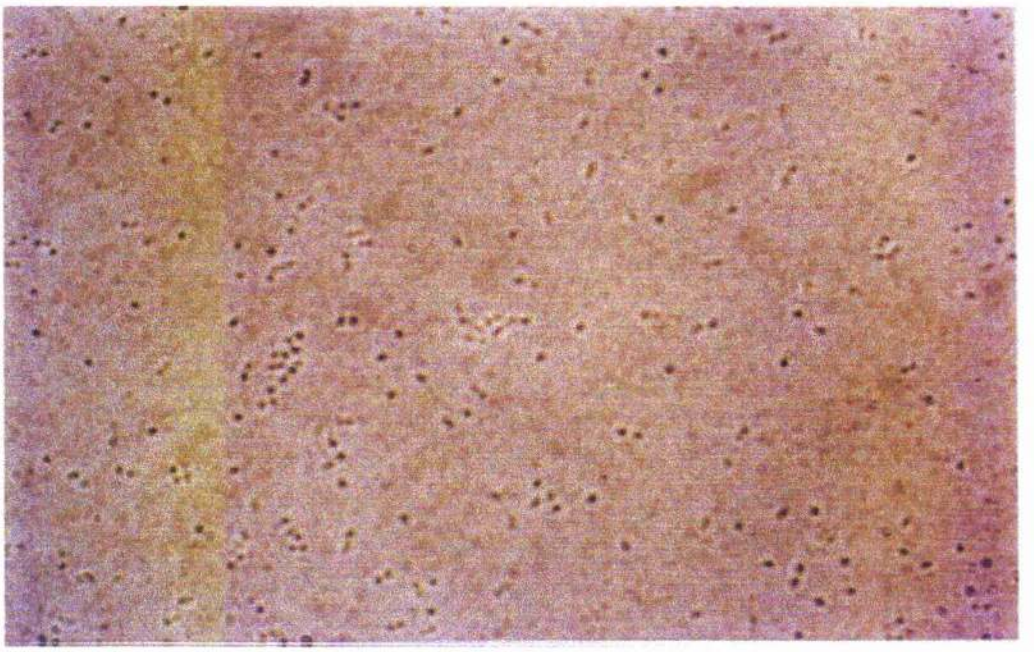
3.3.1 DISTRIBUTION OF CAPSULE

The test strains of SMG showed varying capsule size, which was characterised as + denoting large and +(s) representing small capsule as determined visually by staining with India ink and shown in Figure 3.1. Characterisation of capsule in this way for the three groups of isolates is illustrated in Tables 3.2, 3.3 and 3.4. Of the 61 strains tested, possession of + and +(s) capsule was evenly distributed, with 30/61 +, 30/61 +(s) and 1/61 having no capsule (O25R). As would be expected, passage through an animal model increased capsule size. Strains used here were previously used by Lewis et al (1988) for abscess induction in mice and strains before and after animal passage retained. It was found that capsule size increased (pairs O16 & O20 and O18 & O21 representing strains before and after passage), indeed O16 changed from a rough to smooth colony appearance. An unexpected result was the change of O18 *S.anginosus* to O21 *S.constellatus* after animal passage.

FIGURE 3.1 INDIA INK STAINING OF + AND +(S) CAPSULE TYPES



(A) + CAPSULE (Mag. X500)



(B) +(S) CAPSULE (Mag. X500)

TABLE 3.2 ENCAPSULATION OF DENTAL ABSCESS ISOLATES

STRAIN	CAPSULE	SPECIES
O02	+	S.constellatus
O03	+	S.anginosus
O06	+(s)	S.anginosus
O07	+	S.intermedius
O08	+	S.anginosus
O09	+	S.intermedius
O10	+(s)	S.anginosus
O16	+(s)	S.constellatus
O18	+(s)	S.constellatus
O19	+	S.anginosus
O20	+	S.constellatus
O21	+	S.anginosus
O23	+	S.anginosus
O24	+	S.anginosus
O25R	-	S.constellatus
O25S	+	S.constellatus
O30	+	S.anginosus
O31	+	S.anginosus
O34	+	S.anginosus
O35	+	S.intermedius
O39	+(s)	S.anginosus
O40	+	S.anginosus
O41	+(s)	S.anginosus
O42	+	S.constellatus
O43	+	S.constellatus
O44	+	S.constellatus
O45	+	S.constellatus
O46	+	S.intermedius

Capsule : + = large capsule present
+(s) = small capsule present
- = no capsule present

TABLE 3.3 ENCAPSULATION OF CLINICAL ISOLATES

STRAIN	CAPSULE	SPECIES
C01	+(s)	S.constellatus
C02	+	S.intermedius
C03	+(s)	S.intermedius
C04	+(s)	S.anginosus
C05	+(s)	S.constellatus
C06	+(s)	S.intermedius
C07	+(s)	S.anginosus
C08	+	S.constellatus
C09	+(s)	S.constellatus
C10	+	S.anginosus
C11	+	S.anginosus
C12	+(s)	S.constellatus
C13	+(s)	S.intermedius
C14	+(s)	S.intermedius
C15	+(s)	S.constellatus
C16	+	S.anginosus
C17	+(s)	S.constellatus
C18	+(s)	S.anginosus
C19	+	S.anginosus
C20	+	S.constellatus
NCTC 11325	+	S.constellatus

Capsule : + = large capsule present
+(s) = small capsule present
- = no capsule present

TABLE 3.4 ENCAPSULATION OF PLAQUE ISOLATES

STRAIN	CAPSULE	SPECIES
P01	+	S.anginosus
P02	+(s)	S.anginosus
P03	+(s)	S.anginosus
P04	+(s)	S.constellatus
P05	+(s)	S.anginosus
P06	+(s)	S.anginosus
P07	+(s)	S.intermedius
P08	+(s)	S.intermedius
P09	+(s)	S.intermedius
P10	+(s)	S.intermedius
P11	+	S.intermedius
P12	+(s)	S.intermedius
P13	+(s)	S.constellatus

Capsule : + = large capsule present
+(s) = small capsule present
- = no capsule present

When capsule distribution was considered in relation to species (Figure 3.2a), each species had similar proportions of large and small capsules. As table 3.5(a) shows, *S.anginosus* had 11/26 (42%) of strains with +(s) and 15/26 (58%) with + capsule, *S.constellatus* had 1/21 with no capsule, 10/21 (48%) +(s) and 10/21 with + capsular type, while *S.intermedius* strains showed 9/15 (60%) +(s) and 6/15 (40%) + capsule. However, in relation to source of isolates, both clinical and plaque isolates tended to have smaller capsules, while abscess isolates possessed more extensive capsular material (Figure 3.2b). Twenty one out of 28 abscess isolates had + capsular type, while only 7/20 clinical and 2/13 plaque isolates had larger capsule type.

3.3.2 EXAMINATION OF CAPSULE BY TRANSMISSION ELECTRON MICROSCOPY

TEM photographs of test strains (Table 3.5b) revealed two types of capsular material stained with ruthenium red. The first was a dense, dark- staining, thick layer of material and the second a more wispy, lightly-stained material (Figure 3.3). These corresponded respectively to strains showing + and +(s) encapsulation from light microscopy. The exception was strain 025R which was negative for capsule with India ink staining, but which showed a large, deep-staining capsule-like mass under TEM (Figure 3.4).

Figure 3.2 (a) Frequency of Encapsulation Amongst SMG - According to Species

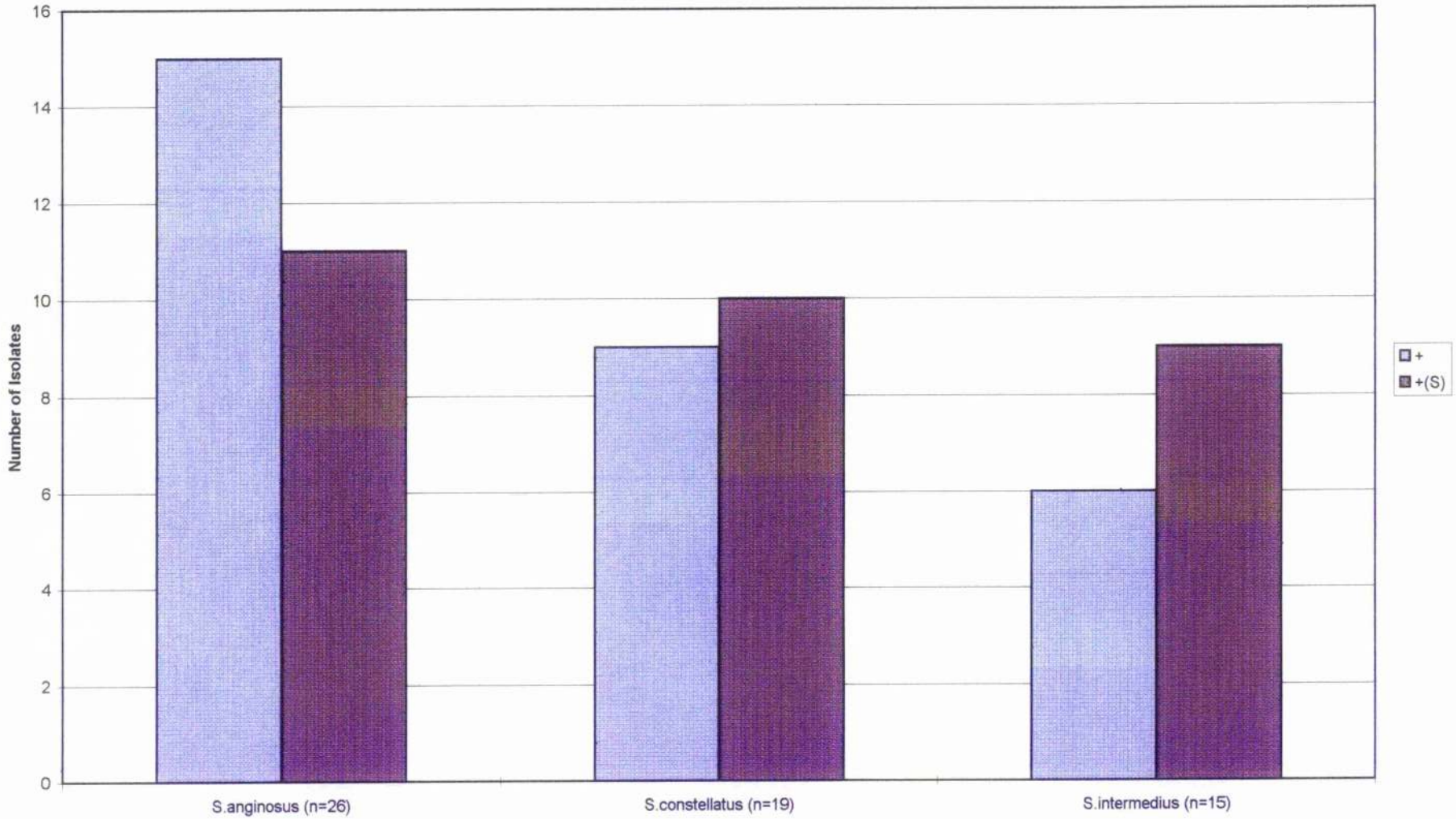


Table 3.5(a) Distribution of Capsule in Relation to Species

SPECIES	CAPSULE		
	-	+(S)	+
S.anginosus	0/26 (0%)	11/26 (42%)	15/26 (58%)
S.constellatus	1/21 (4%)	10/21 (48%)	10/21 (48%)
S.intermedius	0/15 (0%)	9/15 (60%)	6/15 (40%)

Capsule : + = large capsule present
+(s)= small capsule present
- = no capsule present

Table 3.5(b) Strains Subjected to Transmission Electron Microscopy

STRAIN	CAPSULE	STRAIN	*EM (NT &T)
C08	+	S.anginosus	YES
C09	+	S.intermedius	YES
C16	+(s)	S.constellatus	YES
C25R	-	S.constellatus	YES
C25S	+	S.constellatus	YES
C01	+(s)	S.constellatus	YES
C18	+(s)	S.anginosus	YES
NCTC 11325	+	S.constellatus	YES
P02	+(s)	S.anginosus	YES
P11	+	S.intermedius	YES

*EM (NT & T) = electron micrographs of strains both before and after treatment with hyaluronidase to remove capsule.

Figure 3.2 (b) Frequency of Encapsulation Amongst SMG - According to Clinical Source

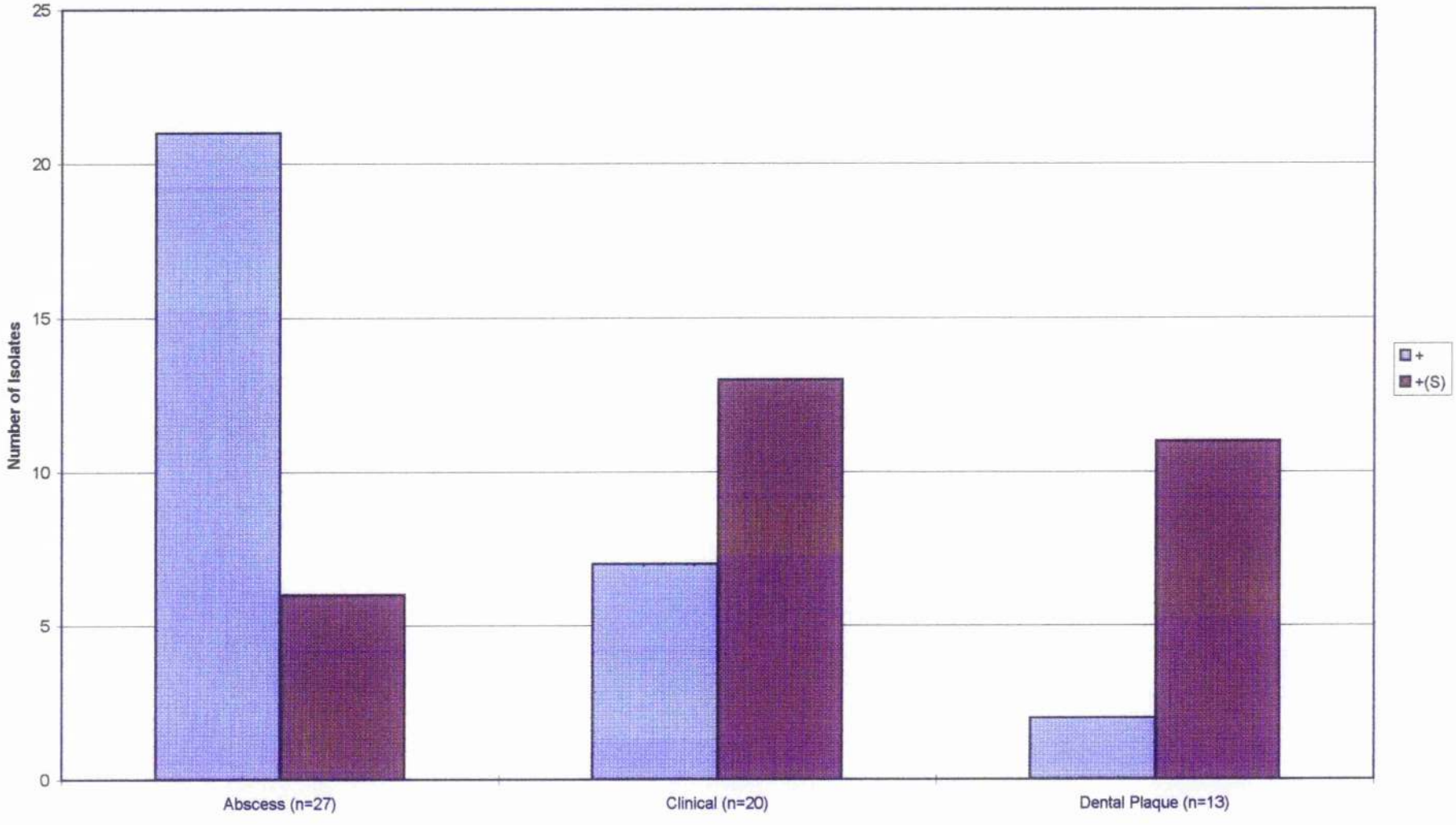
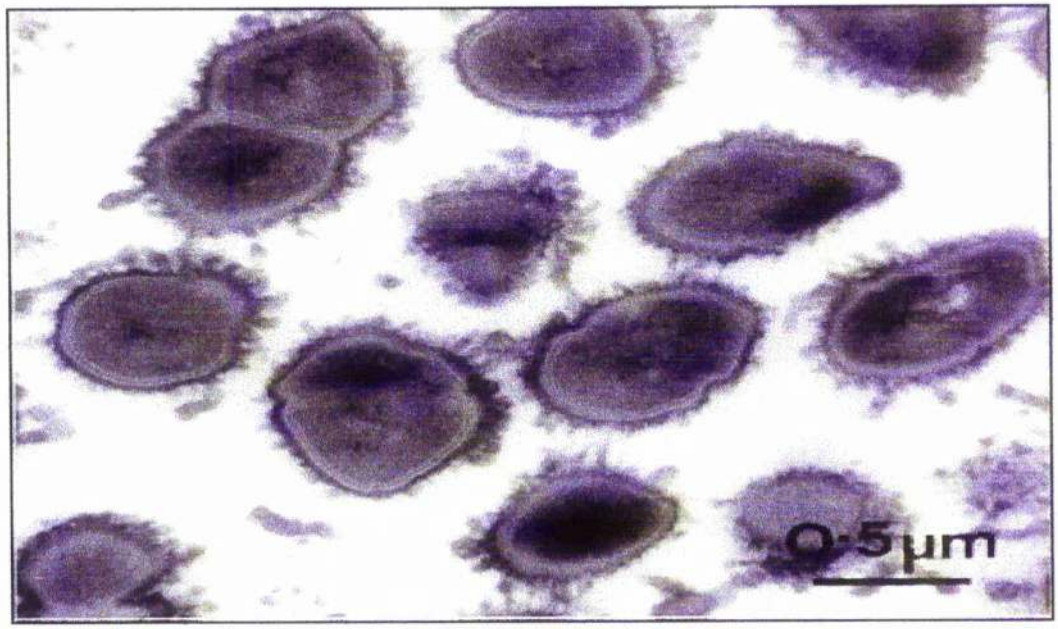
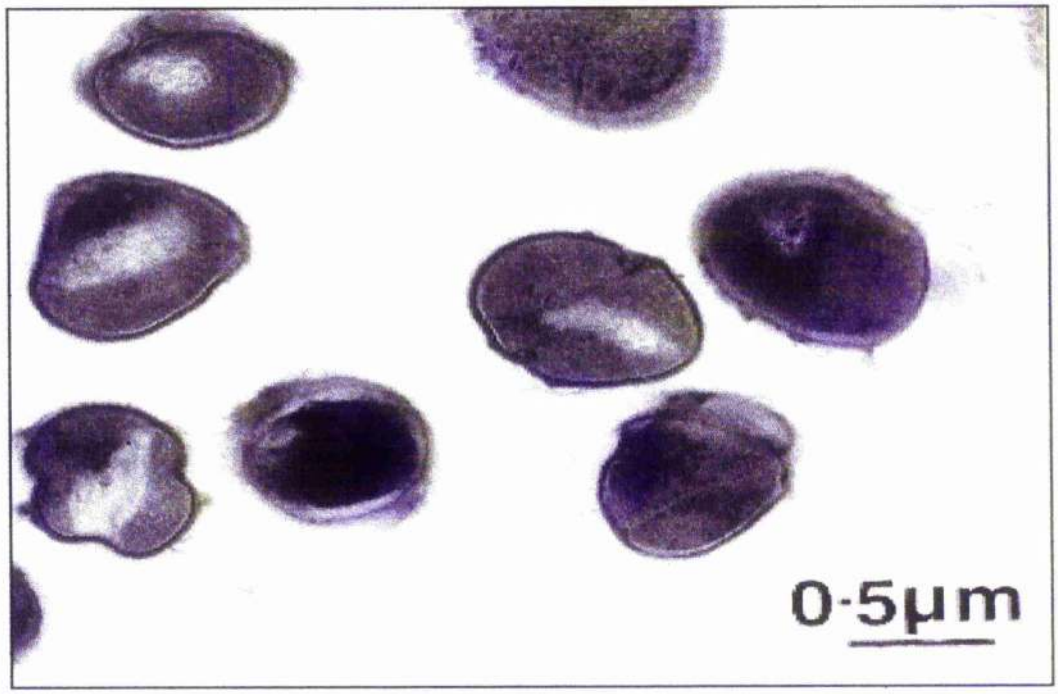


FIGURE 3.3 ELECTRON MICROSCOPY OF CAPSULAR MATERIAL
Micrographs show two capsule types : The first being thick capsule (+) and the second smaller capsule (+(S)).



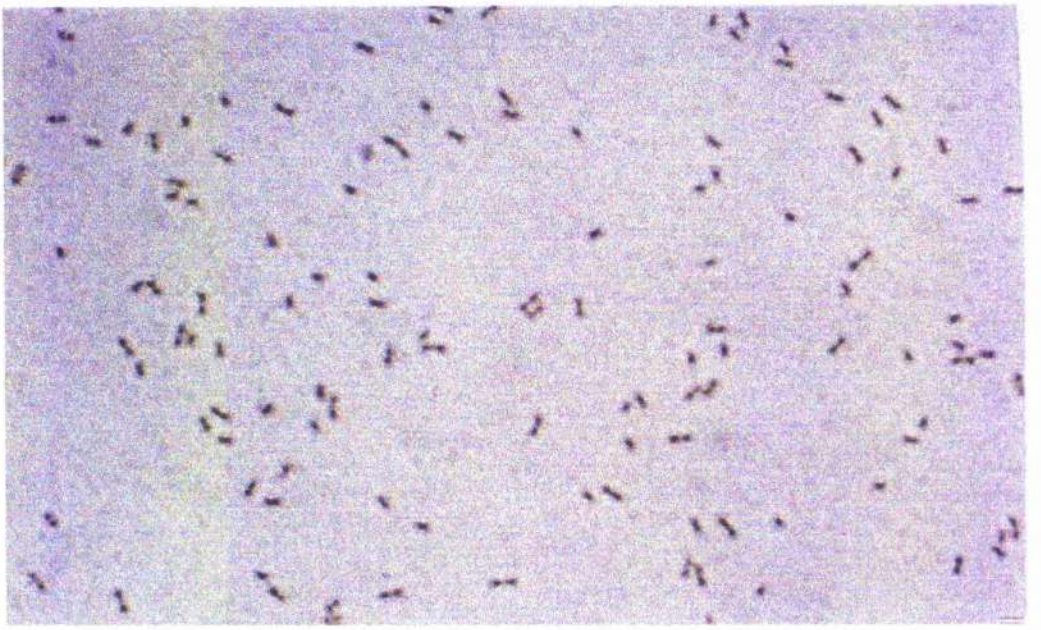
(A) LARGE CAPSULE : STRAIN 025S



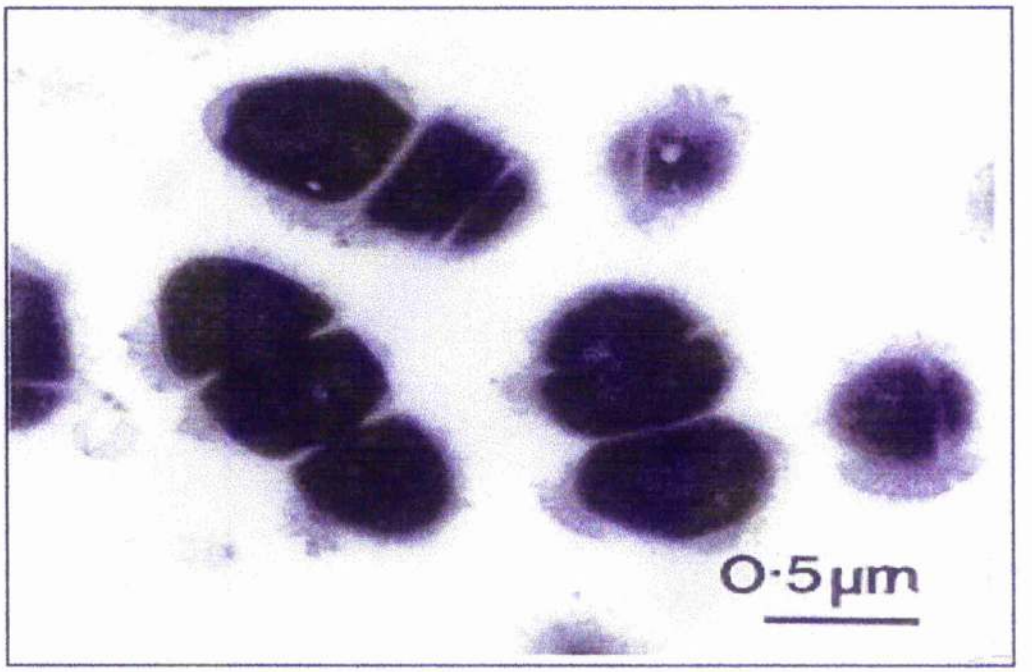
(B) SMALL CAPSULE : STRAIN C01

FIGURE 3.4 CAPSULE REACTION OF STRAIN 025R

Photograph (A) shows the negative capsule stain with India ink, while Micrograph (B) shows capsular material.



(A) STRAIN 025R SHOWING NEGATIVE CAPSULE STAIN (Mag. x 500)



(B) 025R SHOWING CAPSULAR MATERIAL WITH RUTHENIUM RED STAIN UNDER TRANSMISSION ELECTRON MICROSCOPY

3.3.3 REMOVAL OF CAPSULE (A) BY SONICATION AND (B) BY TREATMENT WITH HYALURONIDASE

(A) Sonication

Electron microscopy performed on test strains which had been sonicated as described showed that removal of capsular material was patchy and left some areas of the cell stripped of capsule, while others retained a thick capsular layer.

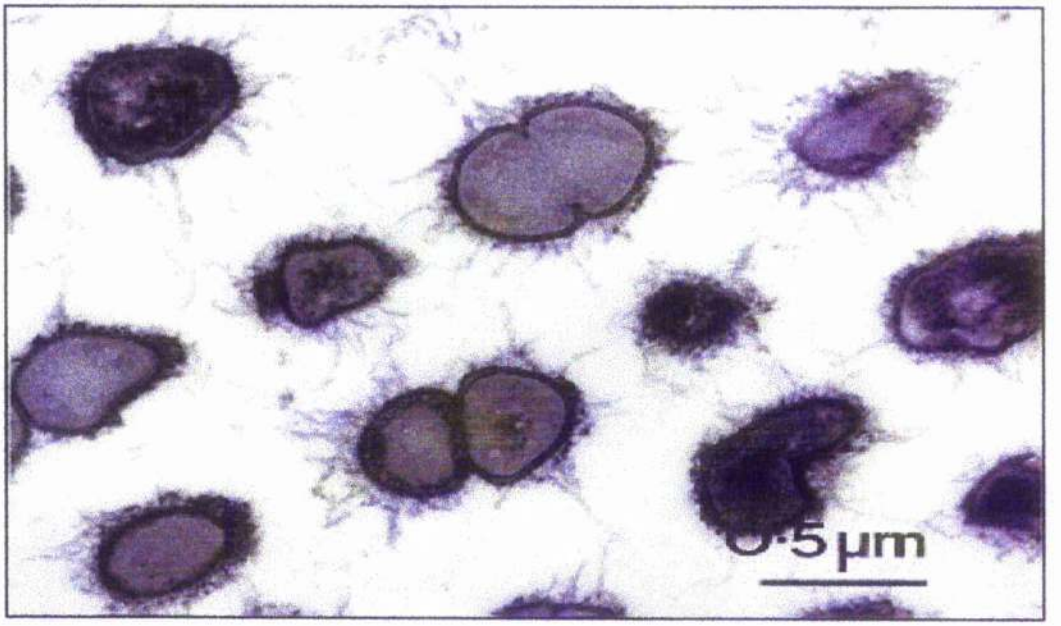
(B) Hyaluronidase

Treatment of strains of the SMG with hyaluronidase gave rise to three different outcomes. The first resulted in the test strain being completely denuded of capsule. This occurred with those strains exhibiting wispy extracellular material (Figure 3.5). The second outcome was partial removal of capsular material from those strains with the thicker gelatinous material (Figure 3.6). Finally, in the case of strain O25R,designated non-encapsulated due to India ink staining, no change took place (Figure 3.7).

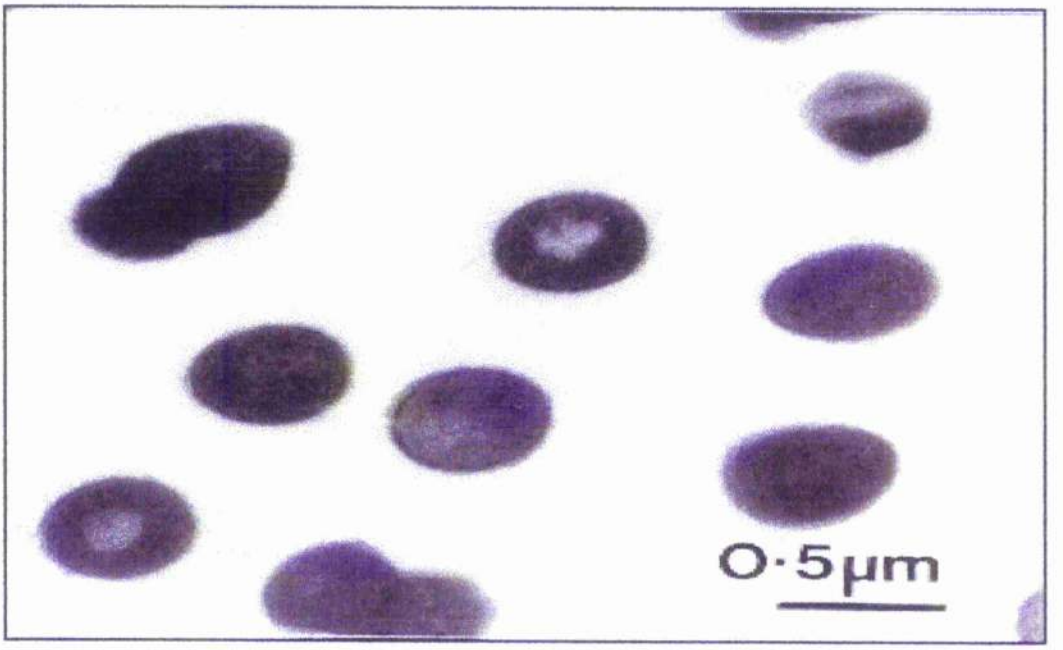
3.3.4 HYDROPHOBICITY

The selected SMG strains exhibited both hydrophobic and hydrophilic characteristics as shown in Tables 3.6, 3.7, 3.8 and 3.9. There was a statistically significant correlation between the two methods used to determine the

FIGURE 3.5 ALMOST COMPLETE REMOVAL OF CAPSULE BY TREATMENT WITH HYALURONIDASE

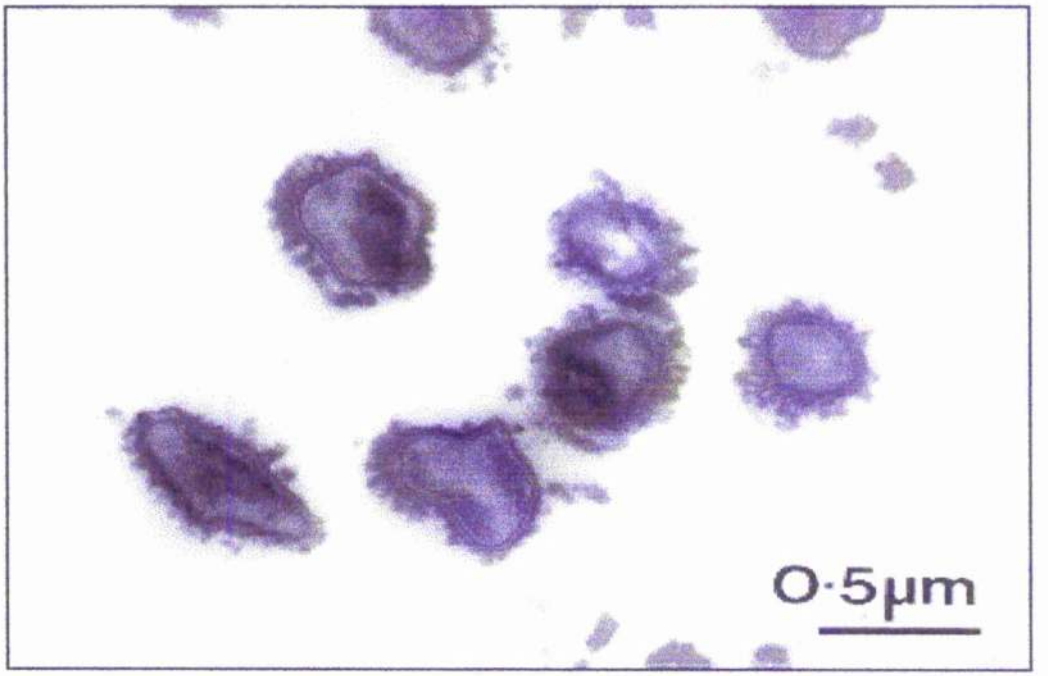


(A) STRAIN NCTC 11325 WITH CAPSULE (UNTREATED)

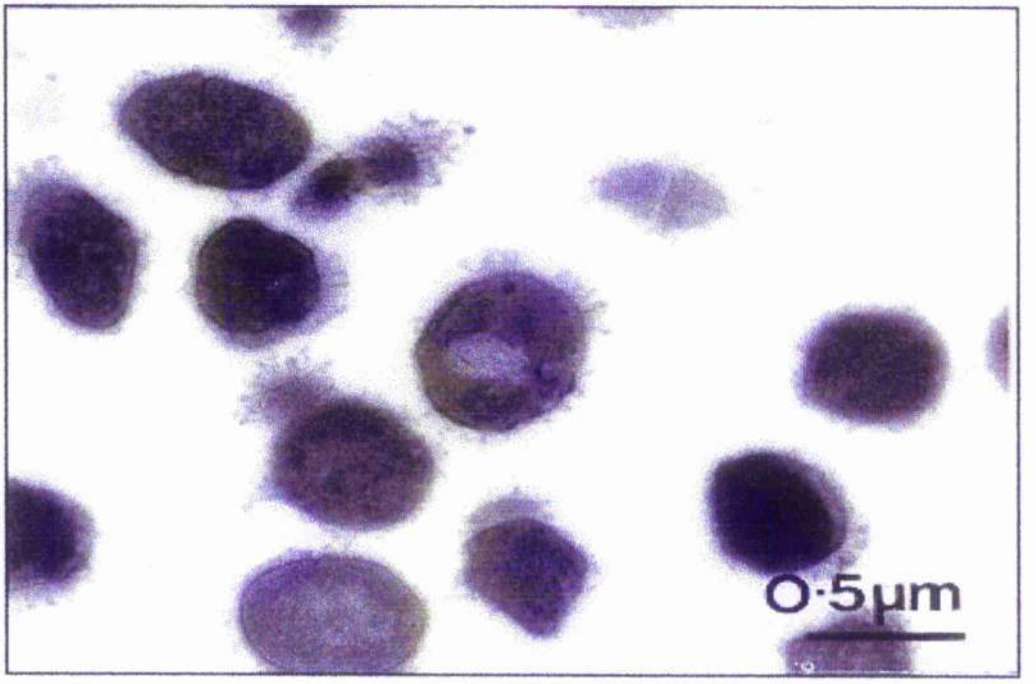


(B) STRAIN NCTC 11325 WITHOUT CAPSULE (HYALURONIDASE TREATED)

FIGURE 3.6 PARTIAL REMOVAL OF CAPSULE BY TREATMENT WITH HYALURONIDASE

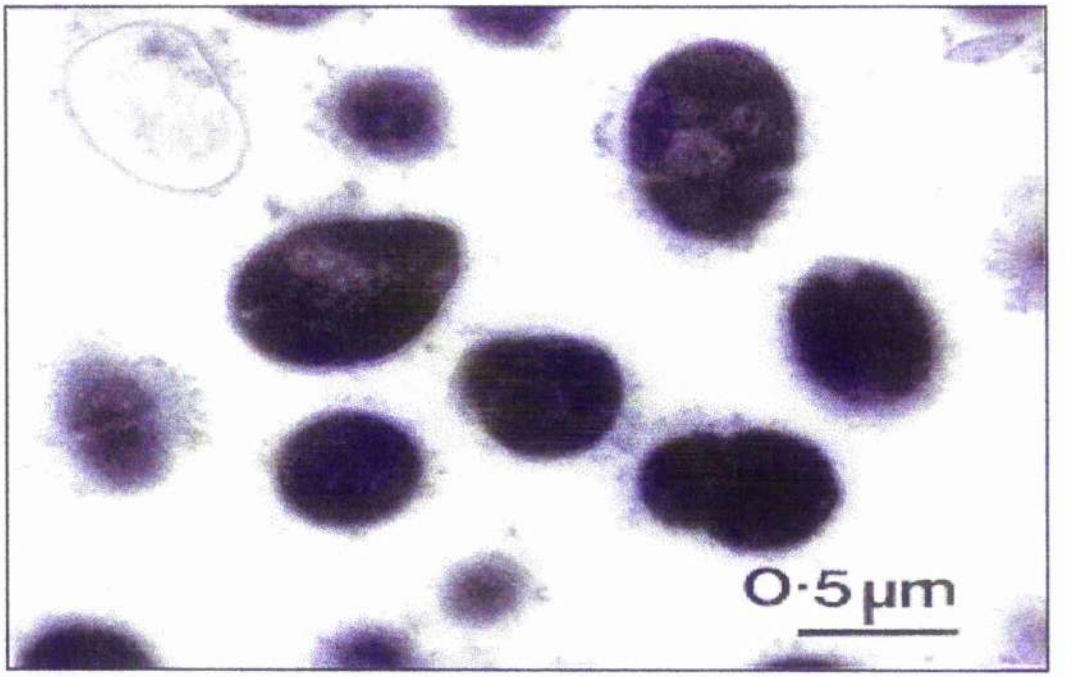


(A) UNTREATED STRAIN 025S

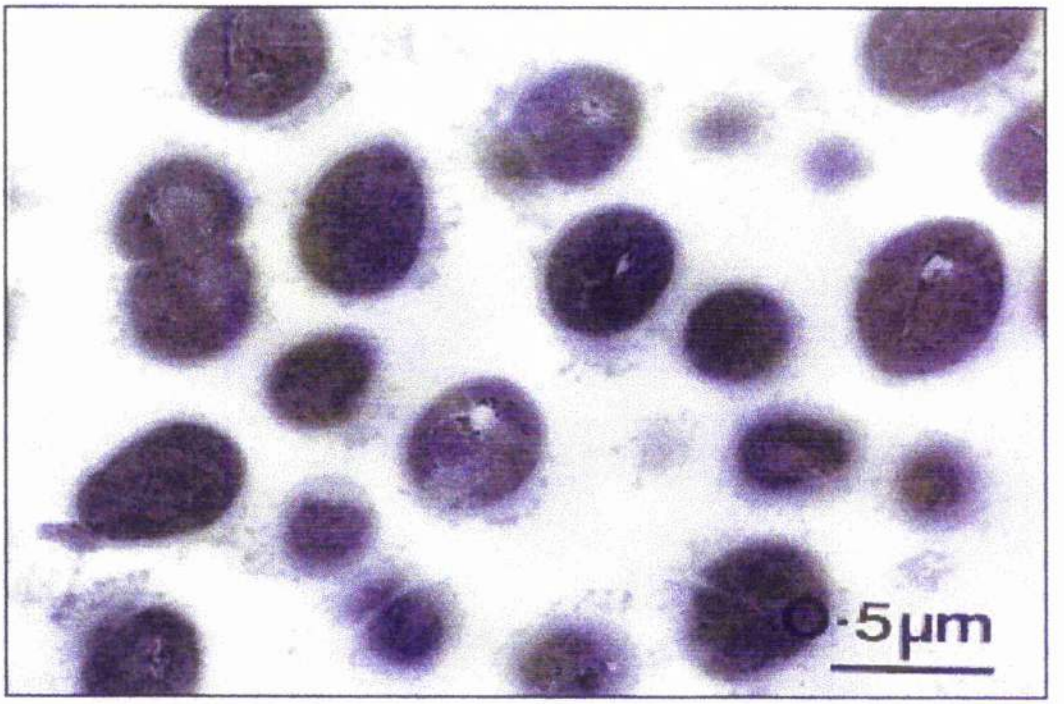


(B) STRAIN 025S TREATED WITH HYALURONIDASE

FIGURE 3.7 FAILURE TO REMOVE CAPSULE BY TREATMENT WITH HYALURONIDASE



(A) UNTREATED STRAIN 025R



(B) STRAIN 025R TREATED WITH HYALURONIDASE

TABLE 3.6 HYDROPHOBICITY OF SMG USING THE HEXADECANE PARTITION ASSAY

% CHANGE IN OD 660 nm					
STRAIN	RUN 1	RUN 2	RUN 3	MEAN	CATEGORY
O08	72%	61.6%	64%	65.8%	HYDROPHOBIC (M)
O09	56.3%	54.5%	60%	56.9%	HYDROPHOBIC (M)
O16	81.5%	94%	91%	88.8%	HYDROPHOBIC (V)
O25R	50%	87.5%	63.2%	66.9%	HYDROPHOBIC (M)
O25S	55.3%	46%	60.3%	53.8%	HYDROPHOBIC (M)
O46	84%	78%	81%	81%	HYDROPHOBIC (V)
C01	87%	87.6%	80.6%	85%	HYDROPHOBIC (V)
C02	40.3%	34.6%	33.3%	36%	HYDROPHILIC (M)
C06	33.3%	26.6%	38.3%	32.7%	HYDROPHILIC (M)
C08	44.6%	24%	24.3%	31%	HYDROPHILIC (M)
C10	5%	7.3%	2.3%	4.8%	HYDROPHILIC (V)
C18	94%	78.6%	92%	88.2%	HYDROPHOBIC (V)
NCTC	48%	42%	46.6%	45.4%	HYDROPHILIC (M)
P01	15%	7%	10.3%	10.7%	HYDROPHILIC (M)
P02	21%	42%	58.4%	40.4%	HYDROPHILIC (M)
P04	62%	57%	69.3%	62.7%	HYDROPHOBIC (M)
P08	74%	64%	84.1%	74%	HYDROPHOBIC (M)
P11	86%	38.6%	86.9%	87.1%	HYDROPHOBIC (V)
P13	64%	56.6%	74.3%	64.9%	HYDROPHOBIC (M)

Change in OD of
0%-9% very hydrophilic = HYDROPHILIC (V)
10%-49% moderately hydrophilic = HYDROPHILIC (M)
50%-79% moderately hydrophobic = HYDROPHOBIC (M)
80%-100% very hydrophobic = HYDROPHOBIC (V)

TABLE 3.7 HYDROPHOBICITY OF ABSCESS ISOLATES USING HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)

STRAIN	RUN 1	RUN 2	RUN 3	RUN 4	MEAN	CATEGORY
O08	0.63	1.46	1.017	1.02	1.031	HYDROPHOBIC
	-0.2	0.165	0.0073	0.008	0.0135	(M)
O09	0.114	0.179	0.0706	0.057	0.105	HYDROPHILIC
	-0.944	-0.746	-1.15	-1.24	-0.978	(V)
O16	0.476	3.24	0.943	1.226	1.47	HYDROPHOBIC
	-0.322	0.51	-0.025	0.088	0.167	(M)
O25R	-	3.27	0.323	0.6	1.397	HYDROPHOBIC
	-	0.515	-0.49	-0.22	0.145	(M)
O25S	0.14	0.225	0.218	0.53	0.278	HYDROPHILIC
	-0.85	-0.646	-0.66	-0.28	-0.555	(V)
O46	0.814	1.114	1.755	2.04	1.43	HYDROPHOBIC
	-0.089	0.047	0.244	0.42	0.155	(M)

First row figures represent value of g/e
Second row represent log g/e.
A log g/e value of less than 0 indicates hydrophilicity.

CATEGORY : Hydrophobic (V) = log g/e 0.51 - 1.5
Hydrophobic (M) = log g/e 0.01 - 0.5
Hydrophilic (M) = log g/e -0.01 - (-0.5)
Hydrophilic (V) = log g/e -0.51 - (-1.5)

TABLE 3.8 HYDROPHOBICITY OF CLINICAL ISOLATES USING HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)

STRAIN	RUN 1	RUN 2	RUN 3	RUN 4	MEAN	CATEGORY
C01	1.4	1.52	0.478	0.422	0.955	HYDROPHILIC
	0.146	0.18	-0.32	-0.375	-0.02	(M)
C02	0.41	0.995	0.128	0.516	0.512	HYDROPHILIC
	-0.38	-0.002	-0.89	-0.287	-0.29	(M)
C06	0.03	0.29	0.235	0.135	0.172	HYDROPHILIC
	-1.5	-0.54	-0.629	-0.87	-0.764	(V)
C08	0.027	0.152	0.038	0.048	0.066	HYDROPHILIC
	-1.57	-0.81	-1.42	-1.31	-1.18	(V)
C10	0.011	0.272	0.106	0.092	0.12	HYDROPHILIC
	-1.96	-0.566	-0.97	-1.03	-0.92	(V)
C18	1.18	3.145	0.932	0.806	1.515	HYDROPHOBIC
	0.07	0.497	-0.03	-0.094	0.18	(M)
NCTC	0.76	0.526	0.758	0.887	0.733	HYDROPHILIC
	-0.135	-0.274	-0.12	-0.052	-0.135	(M)

First row figures represent value of g/e

Second row represent log g/e.

A log g/e value of less than 0 indicates hydrophilicity.

CATEGORY : Hydrophobic (V) = log g/e 0.51 - 1.5

Hydrophobic (M) = log g/e 0.01 - 0.5

Hydrophilic (M) = log g/e -0.01 - (-0.5)

Hydrophilic (V) = log g/e -0.51 - (-1.5)

TABLE 3.9 HYDROPHOBICITY OF PLAQUE ISOLATES USING HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)

STRAIN	RUN 1	RUN 2	RUN 3	RUN 4	MEAN	CATEGORY
P01	0.635	0.57	0.37	0.525	0.525	HYDROPHILIC
	-0.19	-0.244	-0.49	-0.288	-0.28	(M)
P02	0.136	0.236	0.32	0.23	0.2305	HYDROPHILIC
	-0.86	-0.62	-0.49	-0.656	-0.637	(V)
P04	0.234	0.188	0.078	0.166	0.1665	HYDROPHILIC
	-0.63	-0.72	-1.1	-0.81	-0.778	(V)
P08	0.907	0.373	0.98	0.75	0.752	HYDROPHILIC
	-0.04	-0.42	-0.008	-0.156	-0.123	(M)
P11	0.193	0.242	0.68	0.37	0.371	HYDROPHILIC
	-0.71	-0.61	-0.16	-0.49	-0.43	(M)
P13	0.358	0.08	0.22	0.219	0.219	HYDROPHILIC
	-0.44	-1.08	-0.65	-0.72	-0.659	(V)

First row figures represent value of g/e

Second row represent log g/e.

A log g/e value of less than 0 indicates hydrophilicity.

CATEGORY : Hydrophobic (V) = log g/e 0.51 - 1.5

Hydrophobic (M) = log g/e 0.01 - 0.5

Hydrophilic (M) = log g/e -0.01 - (-0.5)

Hydrophilic (V) = log g/e -0.51 - (-1.5)

cell surface hydrophobicity of the SMG (r_s = 0.7193). Abscess isolates were hydrophobic in nature (all 6 strains with hexadecane partition assay and 4/6 with HIC), while clinical isolates were predominantly hydrophilic (4/6 with the hexadecane partition assay and 5/6 with HIC). However, plaque isolates gave conflicting characteristics between the two methods in 4/6 strains, making characterisation difficult.

Possession of capsule provides the cell with a hydrophilic surface. As table 3.10 shows, the extent of capsular material does not correlate with the cell surface hydrophobicity. Five of seven hydrophilic strains do show + type capsule, but 5/12 hydrophobic strains also exhibit a greater extent of capsular material.

3.4 DISCUSSION

3.4.1 DISTRIBUTION OF CAPSULE

Dental abscess isolates had larger capsules than the other two groups. This may have been expected as previous work showed that possession of a capsule was necessary for dental abscess isolates to cause a purulent infection (Lewis et al 1993a). As all these strains were isolated from the pus of dental abscesses a large capsule was to be anticipated. Successful pathogens must be

Table 3.10 Effect of Capsule on Hydrophobicity

STRAIN	CAPSULE	HYDROPHOBICITY*
O08	+	Hydrophobic
O09	+	Hydrophobic
O16	+(s)	Hydrophobic
O25R	-	Hydrophobic
O25S	+	Hydrophobic
O46	+	Hydrophobic
C01	+(s)	Hydrophobic
C02	+	Hydrophilic
C06	+(s)	Hydrophilic
C08	+	Hydrophilic
C10	+	Hydrophilic
C18	+(s)	Hydrophobic
NCTC 11325	+	Hydrophilic
P01	+	Hydrophilic
P02	+(s)	Hydrophilic
P04	+(s)	Hydrophobic
P08	+(s)	Hydrophobic
P11	+	Hydrophobic
P13	+(s)	Hydrophobic

* Hydrophobicity as determined by HIC

Capsule : + = large capsule present
+(s)= small capsule present
- = no capsule present

adaptable in order to survive and produce disease, exhibiting surface adhesins in order to adhere, but on invasion of deeper tissues they may produce capsules to mask their surface in order to avoid attachment to phagocytic cells. This situation occurs in *S.pneumoniae* and *S.pyogenes* (Beachey 1981) and may occur in the SMG, thus going some way to explaining the differing extent of possession of capsule within the SMG. There is no evidence in the literature to suggest capsule is needed for clinical isolates to cause infection, but results here, where all isolates possessed a degree of capsular material, may indicate that it is important, perhaps in avoiding phagocytosis. Plaque isolates may have smaller capsules to allow their surface structures to interact with other bacteria in the plaque mass, allowing them to persist in the mouth.

3.4.2 VISUAL AND ELECTRON MICROSCOPIC INVESTIGATION OF CAPSULE

Examination of India ink stains under light microscopy showed capsular halos which could be distinguished by size. Wilkinson (1958), described capsule as having a thickness of over 200 µm, which allows it to be visualised under light microscopy .

There was a good correlation between the capsule viewed via negative staining with India ink and electron

microscopic examination of ruthenium red stained sections. Two varieties of capsule appeared to be present. The first type showed a small halo stained with India ink which appeared as a corresponding wispy material under EM. The second gave a larger halo under light microscopy and correspondingly thicker material under EM. However, strain 025R showed no halo with India ink, but did have a layer of material stainable by ruthenium red. This may be a similar situation to that suggested by Patrick et al (1986), who found that a strain of *B.fragilis* (A334) showed no halo by the India ink method, but possessed a layer of material which stained with ruthenium red, designated the Ruthenium Red layer (RRL). They suggested that the two techniques do not detect the same structure. In this case strain 025R may possess a RRL and not capsule.

3.4.3 CAPSULE REMOVAL

As hyaluronidase randomly cleaves β-N acetyl hexosamine (1 -> 4) glycosidic bonds in hyaluronic acid, chondroitin and chondroitin sulphates, it can be suggested that the SMG capsule as for Group A and C streptococci, consists of hyaluronic acid which was removed by treatment with hyaluronidase (Figures 3.5, 3.6 and 3.7). Strains with the wispy material were completely denuded after treatment, while in the case of the denser material only partial removal occurred. This may have been because

more material was present and therefore either a longer exposure to hyaluronidase was needed to completely digest it, or a more concentrated solution of the enzyme was required. Another explanation may be that all the hyaluronic acid capsular material present had been removed and the residual material was of a different composition, remaining undigested, and so termed, as before, the RRL. In the case of strain 025R the fact that little change occurred after treatment with hyaluronidase supports the suggestion that the material here is RRL and not capsule as defined in the other strains, perhaps having a constitution other than hyaluronic acid. This could be investigated by stripping the capsule and assaying it for hyaluronic acid content. Preliminary investigations like this were carried out, but the assay system used was not specific for hyaluronic acid, just pentoses and hexoses which encompasses a number of substances. Another explanation for the patchy nature of removal may be impurity of the hyaluronidase enzyme, thus being contaminated with other enzymic activity. Confirmation of a purely hyaluronic nature for capsule of SMG strains would negate this problem.

3.4.4 HYDROPHOBICITY

A number of techniques are currently used to estimate cell surface hydrophobicity, with the two methods used here relying upon binding to a hydrophobic ligand. The

chemical composition of both octyl-sepharose and hexadecane leads us to expect that both assays would correlate well (Van der Mei et al 1987), which indeed was the case. Investigators have also found that other methods of assessing cell surface hydrophobicity correlate well, both when comparing different strains of the same species and with Gram positive and Gram negative species (Lindahl et al 1985 ; Clark et al 1985). However, others have found a lack of correlation between the methods (Dillon et al 1986 ; Mozes & Rouxhet 1987) and have suggested that it is not possible to define the surface 'hydrophobicity' of a bacterium other than on a comparative level with closely related strains of similar surface structure (Van der Mei et al 1987).

As this work only characterised SMG organisms, and therefore a similar cell surface composition would be expected, it was likely that the two methods would correlate with each other. However, this does not address the discrepancies obtained with the two methods used to characterise the plaque isolates. There may be some aspect of their composition which is peculiar to plaque isolates which makes these strains react differently in the two tests. Perhaps the possession of smaller amounts of capsular material amongst plaque isolates allows other surface structures which may be present such as pili to interact in a different manner than in the well encapsulated strain where similar

structures may be masked. There were also variations in results between runs, although every effort was made to standardise both growth and assay conditions. Expression of cell surface hydrophobicity can be affected by growth conditions and nutrient availability which may explain differences between runs.

Capsule provides the cell with a hydrophilic surface, yet some strains of SMG with large capsules were hydrophobic. It may be the case that these organisms have other surface structures, such as fimbriae, responsible for hydrophobicity, as occurs in the case of *E.coli* (Sherman et al 1985). Fimbriae have been reported in the SMG (Handley et al 1985), but their presence or absence in the present study cannot be commented upon as the sectioning procedure for EM examination makes it highly unlikely that these would remain visible. Negative staining with whole bacterial preparations would be necessary instead.

CHAPTER 4 ADHERENCE OF THE "STREPTOCOCCUS MILLERI GROUP" TO BUCCAL EPITHELIAL CELLS

4.1 INTRODUCTION

4.1.1 GENERAL INTRODUCTION

Many bacterial infections are initiated by adherence of the microorganism to the mucosal surfaces of the respiratory, gastrointestinal or urogenital tracts (Gibbons 1977). For colonisation to result the organism must adhere strongly in order to avoid being swept away by mechanical forces and cleansing mechanisms such as sneezing, coughing and peristalsis. For colonisation of mucosal surfaces organisms must not only adhere, but multiply rapidly in order to recolonise newly exposed epithelial cell surfaces as old ones and their adherent bacteria are exfoliated and swept away (Beachey and Courtney 1987). Certain bacteria adhere more readily in particular locations, where they are likely to cause infection, for example streptococci causing pharyngitis (Stollerman 1975) and coliform bacteria causing urinary tract infections (Silverblatt 1974). Indeed amongst the SMG, Willcox and Knox (1990) found that isolates from dental abscesses adhered more effectively than other strains to buccal epithelial cells (BEC).

Colonisation of the oral cavity requires that in order to

avoid being removed by saliva flow and the mechanical action of chewing, microorganisms adhere to the surfaces of the mouth. The strength of adhesion to various oral surfaces differs widely among oral bacteria and is a major reason for their different intraoral sites of localisation (van Houte 1983). Most pathogenic bacteria are capable of expressing multiple adhesins, each with a distinct receptor specificity and with an independent mechanism of regulating adhesin expression (Ofek & Doyle 1994). Hogg and Manning (1988) found that lipotechoic acid (LTA) inhibited adherence of viridans streptococci, including *S.milleri*, to fibronectin coated hydroxyapatite, suggesting it may be one adhesin for the group. Eifuku et al (1990) found that 60% of *S.milleri* strains tested could coaggregate with some actinomycetes. This was not inhibited by lactose and the patterns of inhibition of coaggregation suggested this reaction belonged to streptococcus coaggregation group 2, as detailed by Kolenbrander and Williams (1983). Three basic kinds of coaggregation are distinguishable on the basis of the assortment of heat or protease sensitivities with six groups of streptococci (coaggregation groups 1-6) and six groups of actinomyces (groups A to F) having been identified (Kolenbrander, Inouye and Holdeman 1983). Further work by Eifuku et al (1991) found some *S.milleri* strains exhibited lactose-inhibitable coaggregation with actinomycetes and streptococcus coaggregation groups 3, 4 or 5, suggesting that a number of adhesins may be

involved. Willcox et al (1994) found that Lancefield group C SMG strains aggregated human platelets due to a proteinaceous substance on the surface of SMG. Douglas et al (1990) reported on the ability of other oral streptococci to aggregate platelets, as this is one proposed mechanism in the pathogenesis of infective endocarditis. They found that among *S.sanguis*, a common causative agent of infective endocarditis, the ability to aggregate platelets was confined to certain biotypes, while other oral streptococci such as *S.mitior* and *S.mitis* did not aggregate platelets. They concluded that ability to aggregate platelets is not essential for an organism to cause infective endocarditis, but may still be an important virulence factor.

Lancefield group C SMG were also shown to bind albumin, with the receptor of *S.intermedius* C5 characterised as a cell surface protein of M_r 24000 dissimilar from protein G (Willcox et al 1993). The SMG were also found to bind fibronectin via a cell surface receptor of M_r 14000, and binding increased the association of strains with PMNL (Willcox et al 1995). These studies clearly indicate that the SMG has a number of adhesin molecules on its cell surface which participate in the adherence of strains to both host cells and other microorganisms, which can aid in pathogenicity in a number of ways.

The SMG regularly occur in the oral cavity, being found

in plaque, saliva and on the surfaces of the tongue and cheek (Mejare & Edwardsson 1975). Although more numerous in plaque than on the mucosal surfaces of the mouth, Willcox and Knox (1990) found that isolates of the SMG from dental abscesses adhered more effectively than other strains to buccal epithelial cells (BEC). As enhanced ability to adhere in the mouth would aid pathogenicity, the adherence to BEC for a range of isolates derived from oral abscesses, non-oral infections and dental plaque (commensal isolates) was measured. Viridans streptococci, including the SMG, as part of the normal oral flora can enter the bloodstream as a result of dental procedures. This may lead to infective endocarditis with reports implicating dental procedures in 14 to 19 percent of cases (Mostaghm & Millard 1975, Bayliss et al 1986). Therefore, dental procedures can produce a bacteraemia leading to infective endocarditis in at risk patients, and strains which adhere to surfaces in the mouth like BEC may be able to enter the body and cause disease in such a manner.

4.1.2 MICROBIAL ADHESION

The process which results in microbial adherence to a host cell can be considered as two distinct events. First, when the two bodies are situated at a distance from each other, non-specific attractive forces act between the two in order for them to approach close

enough for the second stage of adherence to occur, that is, specific interaction between adhesins and receptors on the two surfaces. Each of these stages will be discussed in the following sections.

4.1.2.1 Physico-chemical Aspects of Microbial Adhesion

Prior to the specific interactions between host cell and organism, interaction of a number of non-specific factors will have resulted in the two surfaces becoming close enough for specific adhesins to operate. The physical chemistry underlying these phenomena is complex, and this section will outline some of the basic principles to be considered in the analysis of microbial adherence. Figure 4.1 shows a diagrammatic representation of some of the aspects discussed.

Contact between two immiscible bulk phases creates an interface, which in terms of microbial adhesion is that between a solid collector and the aqueous system in which it is immersed. In a liquid milieu, the solid acquires a surface charge, either by ionisation of surface groups or by adsorption of ions. Once the charge is acquired the surface attracts counter-ions from the surrounding aqueous medium, this process being opposed by the thermal motion of the counter-ions. The net effect of these two opposing processes is the formation of a zone next to the

FIGURE 4.1 PHYSICO-CHEMICAL ASPECTS OF MICROBIAL ADHERENCE

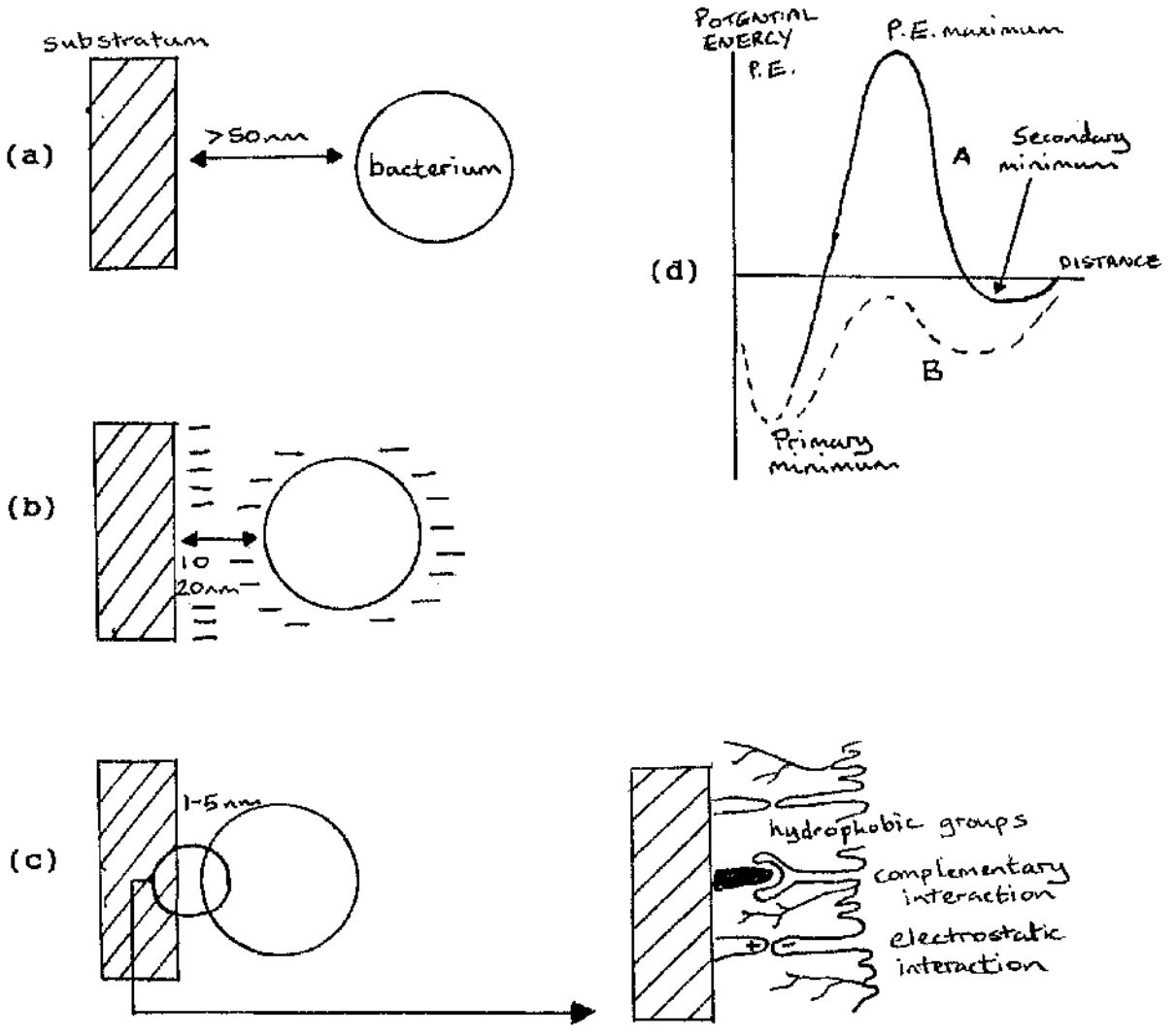


Figure shows the effect of the electrical double layer on bacterial adhesion, with 3 distinct interaction regions depicted. (a) >50 nm reflects van der Waals attractions. (b) a closer region, 10-20 nm, involves both van der Waals and Coulombic forces. The diameter of the electric double layer is 1-5 nm in physiological buffers, so bacteria are generally repulsed from substrata at distances of < 8-12 nm. (c) At < 2 nm complementary binding sites which may involve hydrophobin-hydrophobin, lectin-carbohydrate and charge-charge interaction. The presence of hydrophobic sites may stabilize other interacting sites, with the result being a virtually irreversible adhesion. (d) illustrates 2 extreme cases, A where there is a sufficiently high energy barrier preventing deposition and B where such a barrier has dissappeared.

charged surface, referred to as the "Gouy-Chapman diffuse electric double layer", where the concentration of counter-ions is greater than in the rest of the aqueous phase. It is usual for both the bacterial and collector surfaces to be negatively charged in aqueous environments, and therefore a bacterial cell will experience a repulsive force when its diffuse double layer approaches that of a collector (Fletcher et al 1980).

Analysis of long range forces between cells and between cells and surfaces is based on the DLVO theory of colloidal stability (Derjaguin & Landau 1941, Verwey & Overbeek 1948). For the model of microbial adhesion, contact interactions between bacteria and a collector may be considered in terms of the balance of electrostatic repulsion, and attractive forces of the van der Waals type.

At short distances between surfaces there is a very high potential energy barrier which must be crossed to allow direct interaction. This requires an energy supply, either from the particle itself or from bombardment by other molecules (producing Brownian motion of bacteria). Both produce insufficient energy (Brooks et al 1967), and it appears cells are incapable of passing the barrier. However, an attractive secondary energy minimum exists at a greater distance between bodies, which is relatively

weak. Although it does not account for adhesion, it may play a role in halting a cell long enough to allow other adherent interactions to take place.

4.1.2.2 Specific Interactions

Once an organism has penetrated these nonspecific forces, the interaction at the mucosal surface is highly selective, depending on whether or not the surface presents the receptors required for the adhesive structures on the surface of the invading organism (Beachey and Courtney 1987). The preference of particular bacteria for certain tissues over others (tissue tropism) was illustrated by Gibbons (1977). For example *Streptococcus mutans* was found in high numbers in dental plaque, but sparsely on the epithelial cells of the tongue. In contrast, *Streptococcus salivarius* attached in large numbers to tongue epithelial cells, but was absent from plaque.

The specific binding between unique adhesins on the bacterial cell and receptors of the host cell is common to all bacteria and the interaction can be compared to the combination which occurs between an enzyme and its substrate (Ofek and Beachey 1980). In general, bacterial adhesins are composed of proteins in the form of fimbriae or fibrils, while host cell receptors consist of

carbohydrate moieties of glycolipids or glycoproteins (Beachey, Giampapa and Abraham 1988).

Characterisation of adhesin and receptor molecules involved in adherence illustrates the highly specific nature of binding, and *S.pyogenes* will be considered here as an example. *S.pyogenes* initially adheres to and colonises the epithelial cells of the skin and pharyngeal mucosa (Wannamaker 1970). Exogenous fibronectin was shown to inhibit this adherence, and the organism adheres preferentially to fibronectin-coated epithelial cells (Abraham, Beachey and Simpson 1983), suggesting fibronectin binding has a major role to play in the adherence of this organism. Identification of the surface structure responsible for binding fibronectin has been problematic. Lipotechoic acid (LTA) has been implicated (Simpson and Beachey 1983), while Ofek, Simpson and Beachey (1982) suggested M protein played a role by forming a complex with LTA. This binding oriented LTA to expose the lipid moiety on the streptococcal cell surface and thus facilitated binding of the bacterium to fibronectin (Simpson, Courtney and Ofek 1987).

More recent studies have demonstrated that the high affinity fibronectin-binding property of *S.pyogenes* is mediated by a surface protein, named protein F (Hanski and Caparon 1992). They illustrated that when protein F

production was lost, *S.pyogenes* strains lost their ability to bind fibronectin. Sela et al (1993) identified two binding domains and suggested these bind to two different regions on the fibronectin molecule.

4.2 MATERIALS AND METHODS

ASSAY OF ADHERENCE OF SMG TO BEC

A modification of the method of Willcox and Knox (1990) was used, where incubation time was cut from 120 minutes to 45 minutes, and incubation was carried out in a titration plate of diameter 1 cm which allowed for greater mixing than that allowed in the microtitration plate used by the aforementioned authors.

4.2.1 COLLECTION OF BUCCAL EPITHELIAL CELLS

Buccal epithelial cells (BEC) were collected by agitating 10 ml of PBS in the mouth for 1 minute and returning it to a sterile universal. This was left to stand for 10 minutes to allow large particles to sediment, after which the suspension was decanted off into fresh universals. BEC from 4 donors were pooled and washed twice in PBS by centrifugation (110 g / 10 minutes) to remove loosely attached commensal flora. These were then resuspended to an OD 550 nm of 0.2 (1-2 x 10^6 BEC/ml).

In order to minimize interference of the normal flora with adherence, the BEC were screened in two ways. After washing, BEC from each donor were smeared onto a glass slide, the preparation Gram stained and examined under oil at x1000 magnification, where a count of bacteria adherent to 20 BEC was made. Any donor with levels of colonisation greater than a mean of 20 bacteria per BEC was not included in the pool of donors. On the day of collection and use of BEC a check was again made on the levels of adherent commensal bacteria after washing. Levels of colonisation of > 20 bacteria per BEC excluded that sample.

Variation was minimized by collecting BEC at the same time of day and pooling cells from four suitable donors.

4.2.2 PREPARATION OF BACTERIA

Bacteria were grown overnight in BHI + 0.3% w/v yeast extract, to which 3H adenine at 2 µCi/ml (Amersham, Buckinghamshire, England) was added. These cultures were incubated at 37° C for 18 hrs in an anaerobic jar, after which they were washed three times with PBS (1 000g for 15 mins) and resuspended to give an OD 660 nm of 1.00 (5×10^9 cfu /ml). The %efficiency of labelling (measured automatically by the LSC) with 3H ranged from the lowest value of 31.9% to the highest of 63.2%, but in most instances the efficiency of labelling was around 45%.

4.2.3 ASSAY PROCEDURE

Bacteria (0.1 ml) + 0.1 ml BEC were mixed in round bottomed wells (1 cm diameter)of a micro-titration plate and incubated at 37° C in an orbital incubator (Gallenkamp, East Kilbride) at 100 rpm for 45 minutes. Cells were then harvested by means of a vacuum pump onto polycarbonate filters of 8.0 µm pore size (Nucleopore) which were supported on a funnel in a conical flask. Unattached bacteria were removed by washing with 30 ml PBS in 5 ml aliquots. The filter was then placed into a scintillation vial and 3 ml of Ecoscint A (National Diagnostics, Atlanta, Georgia) added, after which the radioactive emission was measured on a Wallac 1409 liquid scintillation counter (Wallac, Turku, Finland). Controls of bacteria alone were also filtered and these background counts subtracted from counts of the samples. An unfiltered sample count (5 x 10^{8} cfu) was also made to represent 100% adherence. Each strain was tested in quadruplicate on four occasions.

The formula used to determine adherence was as follows :

$$\% \text{ Adherence} = \frac{\text{Sample count} - \text{Background count}}{\text{Total count}} \times 100$$

4.2.4 TIME COURSE OF BACTERIAL ADHERENCE

In order to define the optimum incubation time, a poorly adherent strain and a strain which bound well (found by following the exact method of Willcox and Knox, 1990) were incubated in the assay system for two hours, and sampled every 15 minutes up to 1 hour and 30 minutes thereafter. This was repeated three times and the optimum time for adherence recorded.

4.2.5 ROLE OF CAPSULE IN ADHERENCE TO BEC

Capsule was removed from test strains by treatment with hyaluronidase as detailed in Section 3.2.3. In brief, 300 µl of hyaluronidase solution (5 mg of type 1-S hyaluronidase from bovine testes added to 1 ml sterile water) was added to 3 ml of bacterial culture and incubated at 37° C for 20 minutes. Untreated samples had 300 µl of distilled water added and were incubated in the same way, after which the bacteria were used in the adherence assay.

4.2.6 VISUAL COUNTS OF ADHERENCE

To confirm the results of the radiometric assay the adherence of a few strains was checked visually. After

incubation and washing as normal the filter was smeared across a glass slide and once dry the preparation was Gram stained. The slide was examined microscopically at x 100 magnification under oil with a light microscope and the number of bacteria adherent to 20 BEC counted.

Photographs were taken using a Nikon FX-35 A camera connected to a Nikon optiphot microscope and attachments.

4.2.7 Statistical Analysis

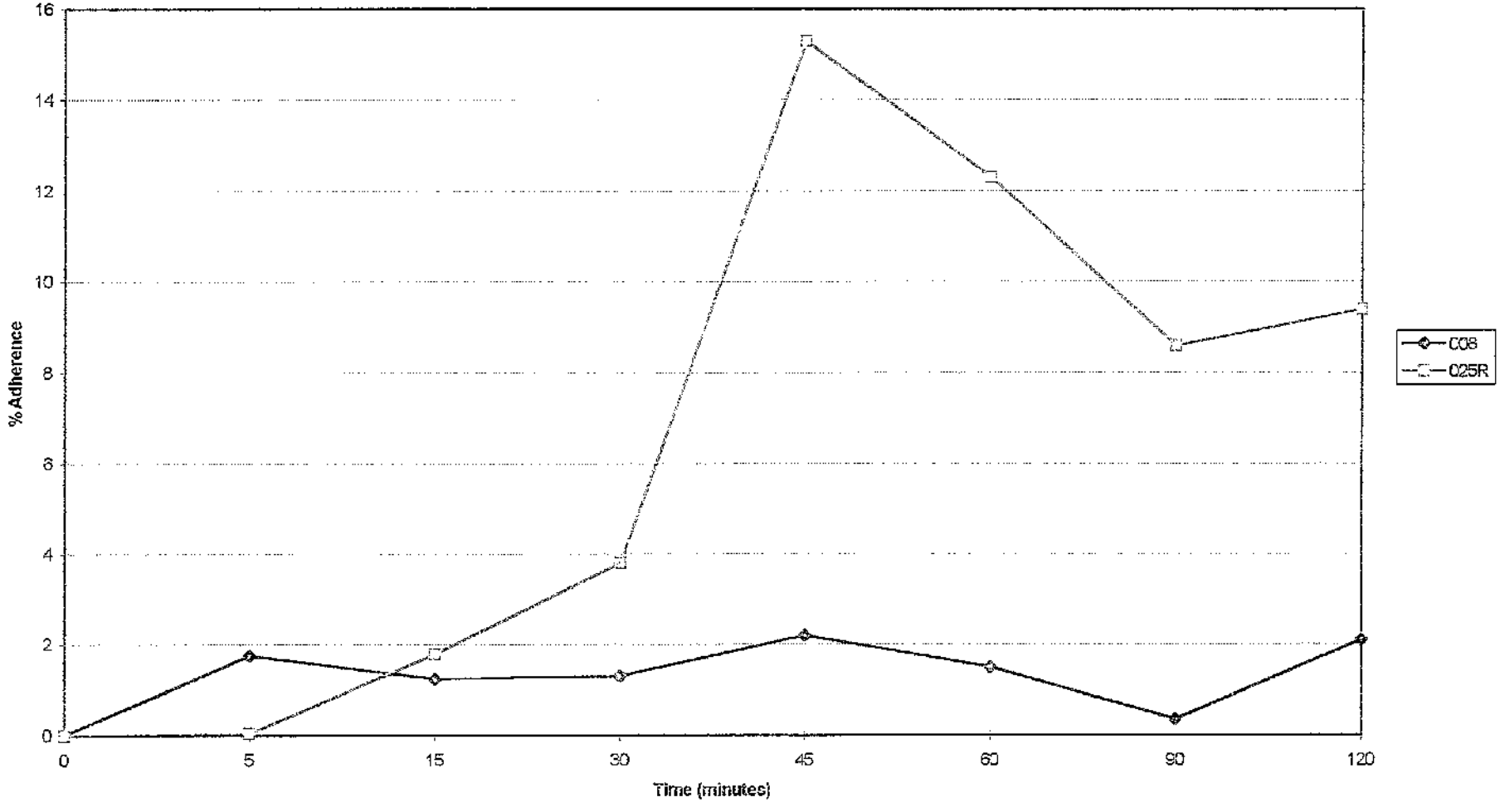
A non parametric statistical test, the Mann-Whitney U test, was used for comparisons between data, with a value of $p < 0.05$ being considered significant.

4.3 RESULTS

4.3.1 KINETICS OF ADHERENCE TO BEC

The kinetics of adherence of two strains of SMG are illustrated in Figure 4.2. Strain 008 adhered well after 5 minutes and stayed at a similar or lower value throughout, whereas 025R initially adhered poorly, but gradually increased over time. Strain 008 gave a peak adherence value of 2.2% (+/- 1.5%) and 025R gave one of 15.4% (+/- 6.5%), both peaking at 45 minutes. Both then showed decreasing adherence over 45 to 90 minutes and then rose at 120 minutes, but not to the peak value.

Figure 4.2 Adherence Kinetics of Strongly Adherent (025R) and Poorly Adherent (008) Abscess Isolates of SMG. (Figure Represents Mean of Three Separate Runs)



Therefore, instead of the 120 minutes incubation time used by Willcox and Knox (1990), 45 minutes was employed throughout.

4.3.2 ADHERENCE OF THE SMG TO BEC

Values for adherence to BEC of strains from the three test groups, those from dental abscesses (9), those from clinical infections elsewhere (6) and plaque isolates (6), are shown in Tables 4.1, 4.2 and 4.3 respectively. Dental abscess isolates gave mean % adherence values ranging from 0.76% to 26.8%, clinical isolates 0.11% to 0.95% and plaque isolates 0.16% to 3.55%. There is evidence that dental abscess isolates had a higher affinity for BEC than clinical and plaque isolates.

Indeed, there was a statistically significant difference between mean % adherence to BEC of dental abscess isolates compared to both clinical ($p=0.0027$) and plaque isolates ($p=0.029$), with no significant difference between the latter two. This may indicate that abscess isolates have different adhesin molecules on their surface than the other two species, which allow them to selectively bind to BEC.

4.3.3 ADHERENCE CHARACTERISTICS OF DIFFERENT SMG SPECIES

The ability of different species of streptococci of the

TABLE 4.1 VARIATION OF ADHERENCE OF DENTAL ABSCESS ISOLATES OF SMG TO BEC

% ADHERENCE OF STRAINS OF SMG							
STRAIN	RUN 1	RUN 2	RUN 3	RUN 4	MEAN	S.D.	SEM
O08	8.1%	4.1%	1.9%	7.74%	5.46%	2.98	1.49
O09	0.39%	1.37%	1.19%	0.11%	0.76%	0.61	0.3
O16	9.4%	37.2%	8.6%	29.6%	21.2%	14.3	7.15
O18	3.27%	1.5%	3.3%	-	2.47%	1.06	0.61
O20	1.05%	1.1%	1.3%	-	1.15%	0.13	0.07
O21	2.3%	2.5%	3.08%	-	2.63%	0.40	0.23
O25R	25.4%	37.8%	12.4%	31.5%	26.8%	10.8	5.42
O25S	8.4%	10.4%	3.4%	5.68%	6.97%	3.07	1.53
O46	5.7%	2.95%	0.85%	4.83%	3.6%	2.13	1.06

SD = STANDARD DEVIATION SEM = STANDARD ERROR OF MEAN

TABLE 4.2 VARIATION OF ADHERENCE OF CLINICAL ISOLATES OF SMG TO BEC

% ADHERENCE OF STRAINS OF SMG							
STRAIN	RUN 1	RUN 2	RUN 3	RUN 4	MEAN	S.D.	SEM
C01	0.34%	0.29%	0.17%	0.83%	0.4%	0.29	0.14
C02	0.33%	0.6%	0.11%	0.24%	0.32%	0.2	0.1
C06	0.98%	0.22%	0.36%	0.17%	0.43%	0.37	0.18
C08	0.24%	0.22%	0.21%	0.25%	0.23%	0.02	0.01
C10	0.004	0.065	0.28%	0.102	0.11%	0.12	0.06
C18	3.05%	0.29%	0.2%	0.27%	0.95%	1.4	0.7
NCTC 11325	1.06%	0.7%	1.15%	0.6%	0.88%	0.27	0.13

S.D. = STANDARD DEVIATION SEM = STANDARD ERROR OF MEAN

TABLE 4.3 VARIATION OF ADHERENCE OF PLAQUE ISOLATES OF SMG TO BEC

% ADHERENCE OF STRAINS OF SMG							
STRAIN	RUN 1	RUN 2	RUN 3	RUN 4	MEAN	S.D.	SEM
P01	0.43%	0.13%	0.24%	0.46%	0.32%	0.16	0.08
P02	0.25%	0.01%	0.35%	0.04%	0.16%	0.17	0.08
P04	1.06%	0.33%	0.46%	0.19%	0.51%	0.38	0.19
P08	2.55%	1.9%	1.67%	5.78%	2.98%	1.9	0.95
P11	2.18%	3.44%	3.69%	4.89%	3.55%	1.11	0.55
P13	0.15%	0.82%	0.19%	0.51%	0.42%	0.31	0.15

S.D. = STANDARD DEVIATION SEM = STANDARD ERROR OF MEAN

SMG to adhere to BEC is shown in Figure 4.3. From this graph it appears that *S.constellatus* strains have a higher mean % adherence to BEC than the other two species. However, there were insufficient numbers of each species used to draw any conclusions and there was no statistically significant difference between them. The two strongly adherent strains, O16 and O25R, resulted in an elevated mean % adherence for the *S.constellatus* group, whereas the other *S.constellatus* strains had similar adherence values to the other species. It is likely that the high adherence of these two strains is due to a factor other than difference in biotype (species).

4.3.4 ROLE OF CAPSULE IN ADHERENCE TO BEC

Adherence values of selected strains, representing members of the three species, with varying degrees of capsule, were examined before and after capsule removal, with results shown in Table 4.4. Treatment to remove capsule resulted in increased adherence, which was statistically significant ($p < 0.05$). The two strains showing comparatively high adherence (strains O16 & O25R) both had a rough colony appearance with no or little capsule surrounding individual cells.

4.3.5 VISUAL MEASUREMENT OF ADHERENCE TO BEC

Visual counts made of selected strains adherent to BEC

FIGURE 4.3 ADHERENCE CHARACTERISTICS OF DIFFERENT SMG SPECIES. Showing mean % adherence (mean of 4 runs) to BEC according to species. Numbers in brackets indicate number of isolates. Bars indicate plus one standard deviation.

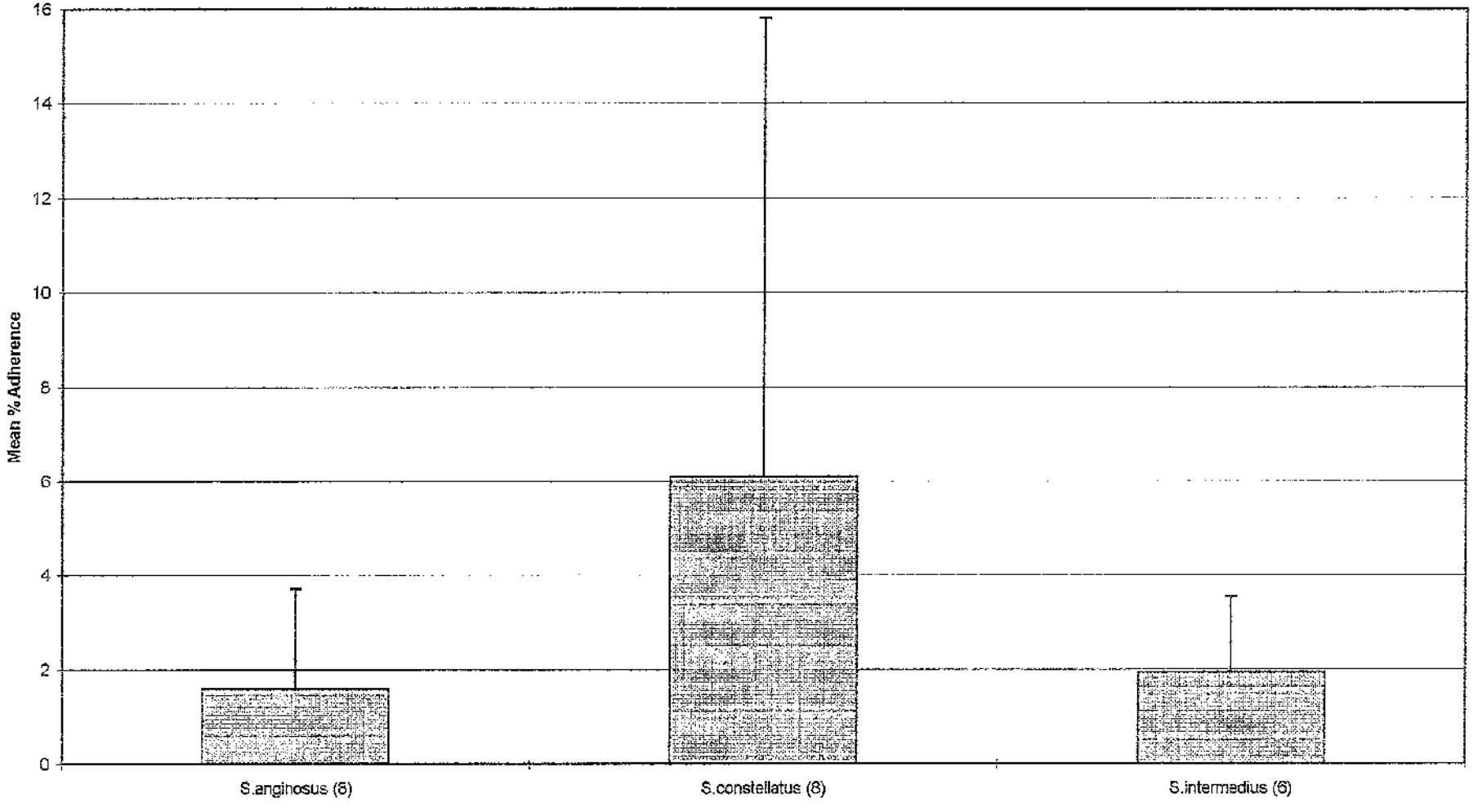


TABLE 4.4 % ADHERENCE OF SMG TO BEC AFTER REMOVAL OF CAPSULE

STRAIN (SPECIES)	RUN 1	RUN 2	RUN 3	RUN 4	MEAN
O09 (SI)	0.55%	0.007%	0.58%	0.57%	0.43%
O09 T [p]	0.65%	0.28%	0.58%	1.92%	0.86%
C01 (SC)	0.29%	0.46%	1.32%	0.21%	0.57%
C01 T [f]	0.4%	0.47%	1.63%	1.3%	0.95%
C18 (SA)	0.34%	0.2%	0.38%	0.1%	0.255%
C18 T [f]	0.55%	0.44%	0.53%	0.26%	0.445%
P02 (SA)	0.75%	0.6%	0.385%	0.72%	0.61%
P02 T [f]	1.2%	0.96%	0.89%	1.3%	1.09%
P11 (SI)	0.01%	2.15%	5.4%	9.3%	4.2%
P11 T [p]	1.43%	3.58%	5.9%	10.7%	5.4%
O25R (SC)	12.2%	13.5%	28.86%	26.3%	20.2%
O25R T [n]	13.67%	19.76%	37.95%	31.1%	25.6%
O25S (SC)	10.28%	5.62%	9.75%	11.5%	9.3%
O25S T [p]	19.04%	8.01%	12.14%	18.9%	14.5%
NCTC (SC)	2.7%	0.39%	0.69%	0.24%	1.005%
NCTC T [f]	4.71%	0.59%	1.86%	1.24%	2.1%

T indicates treated with hyaluronidase to remove capsule.

SA = S.anginosus SC = S.constellatus SI = S.intermedius

[p] indicates partial removal of capsule after hyaluronidase treatment

[f] indicates full removal of capsule after hyaluronidase treatment

[n] indicates no removal of capsule after hyaluronidase treatment

shown in Table 4.5 confirmed the results obtained in the radiometric assay. Photographs (Figures 4.4 A & B, 4.5 A & B and 4.6 A & B) show a poorly, moderately and strongly adherent strain before and after capsule removal, confirming that adherence increases when capsule is removed.

4.3.6 EFFECT OF CELL SURFACE HYDROPHOBICITY ON ADHERENCE OF SMG TO BEC

Adherence to BEC related to hydrophobicity as determined by HIC (Section 3.2.3) is shown in Table 4.6. Four of seven strains showing moderate to high adherence were hydrophobic, while 11/12 poorly adherent strains were hydrophilic.

4.4 DISCUSSION

4.4.1 ADHERENCE OF SMG TO BEC

SMG organisms exhibited varying abilities to adhere to BEC, and examination of the adhesion kinetics (Fig. 4.2) suggests two types of interaction. First, the poorly adherent strain adhered after only five minutes incubation at a level which fluctuated throughout the assay, but was close to the maximum adherence achieved. This suggests that adherence is occurring as a result of

TABLE 4.5 VISUAL COUNTS OF SMG ADHERENT TO BEC

STRAIN	MEAN (S.D.)[1]	COUNT AS % ADHERENCE[2]	% ADHERENCE[3]
O25R NT	459.5 (268.6)	9.2 - 18.4%	13.5%
O25R T	679.95 (512.3)	13.6 - 27.2%	19.75%
O25S NT	257.45 (163.1)	5.14 - 10.3%	5.615%
O25S T	320.25 (237.1)	6.4 - 12.8%	8.005%
NCTC NT	28.8 (18.99)	0.58 - 1.15%	0.3925%
NCTC T	36.85 (18.07)	0.74 - 1.47%	0.585%

1. MEAN Average number of organisms adherent to one BEC (mean count from 20 BEC)

2. COUNT EXPRESSED AS % ADHERENCE
The mean count of one BEC could be calculated as a percentage using the following formula :

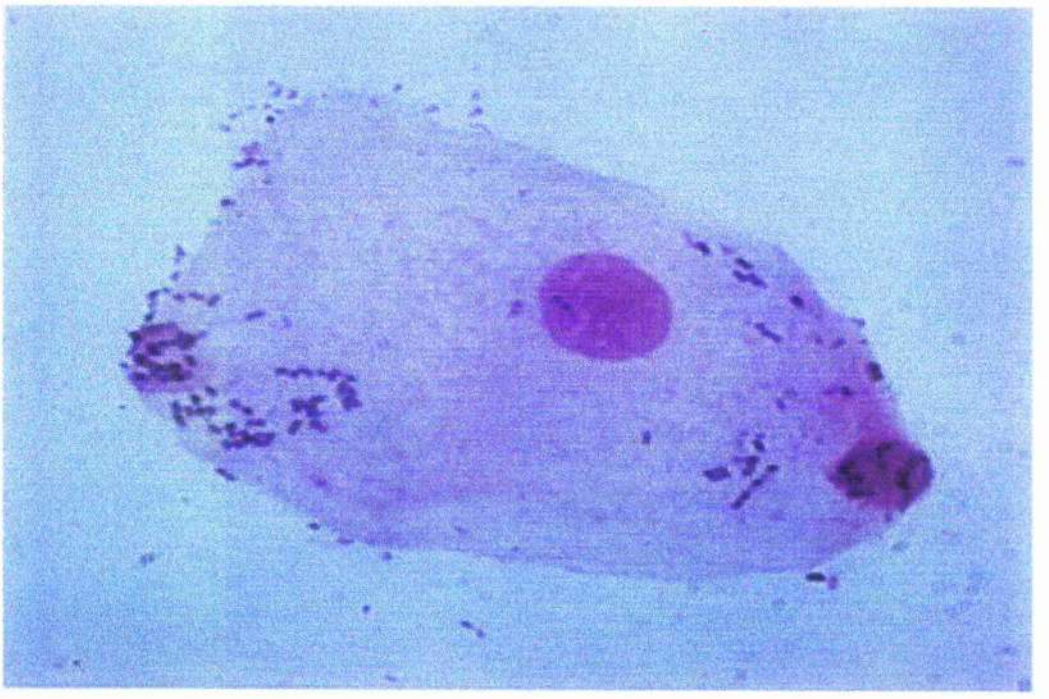
$$\% \text{ Adherence} = \frac{\text{MEAN COUNT} \times 1 - 2 \times 10^5}{5 \times 10^6} \times 100$$

Where $1 - 2 \times 10^5$ is the concentration of BEC used in the assay, providing the range of the two values in the table, and 5×10^6 represents the concentration of SMG test strains in the assay.

3. % ADHERENCE as found using radiometric assay.
NT = not treated with hyaluronidase (capsule intact)
T = treated with hyaluronidase (capsule removed)

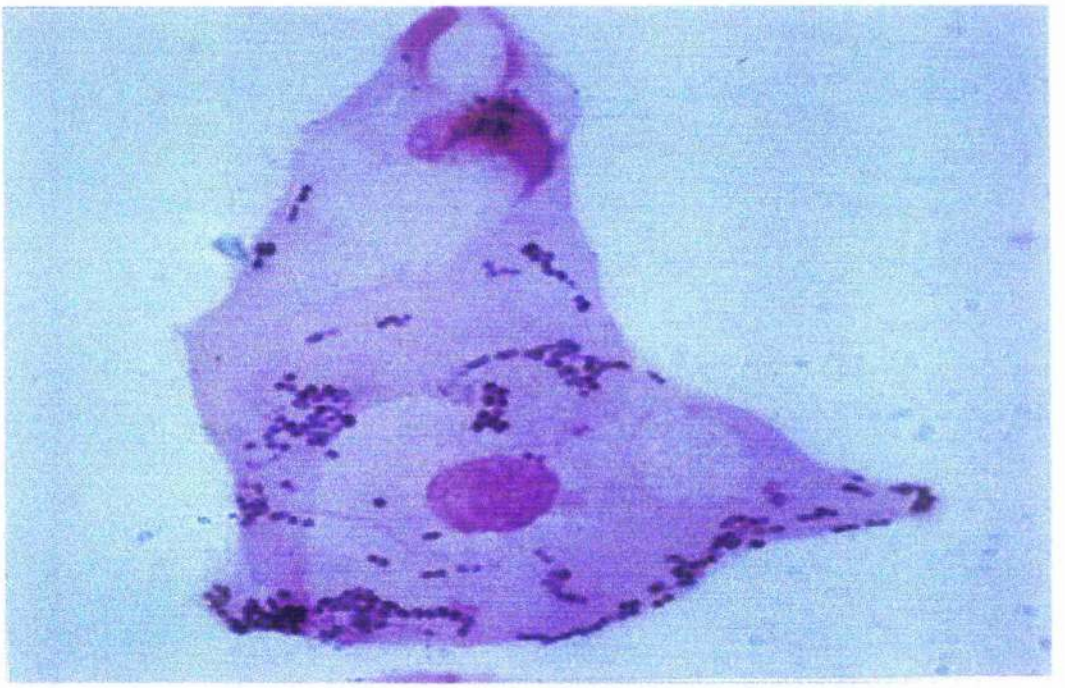
FIGURE 4.4 STRAIN NCTC 11325 ADHERENT TO BEC

(A) WITH CAPSULE (Mag. X 500)

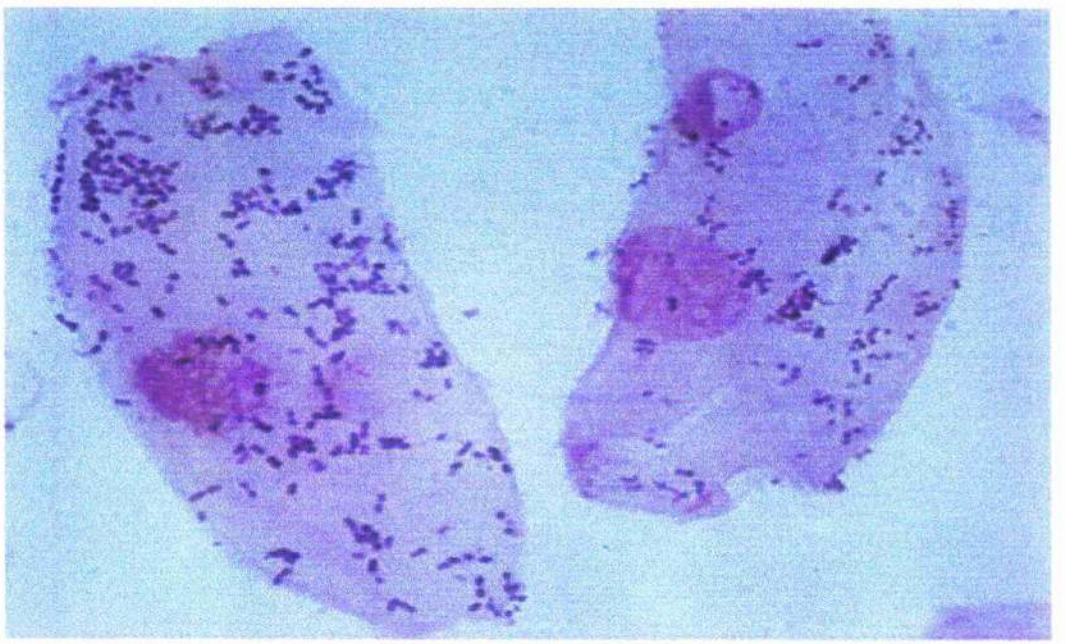


(B) WITHOUT CAPSULE (Mag. x 500)

FIGURE 4.5 STRAIN 025S ADHERENT TO BEC

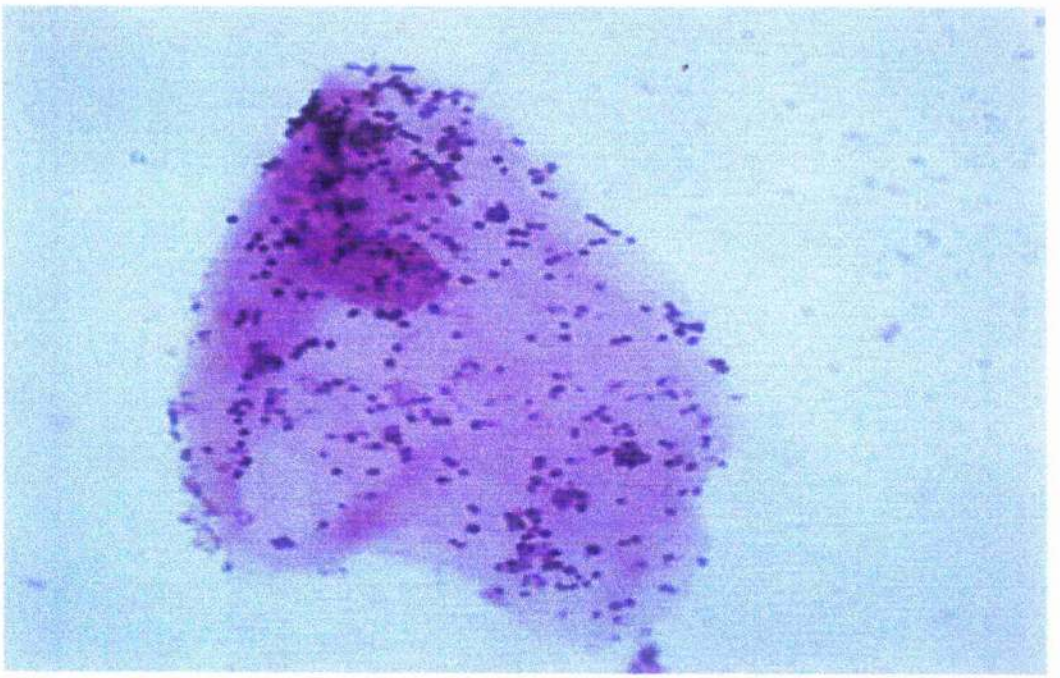


(A) WITH CAPSULE (Mag. x 500)

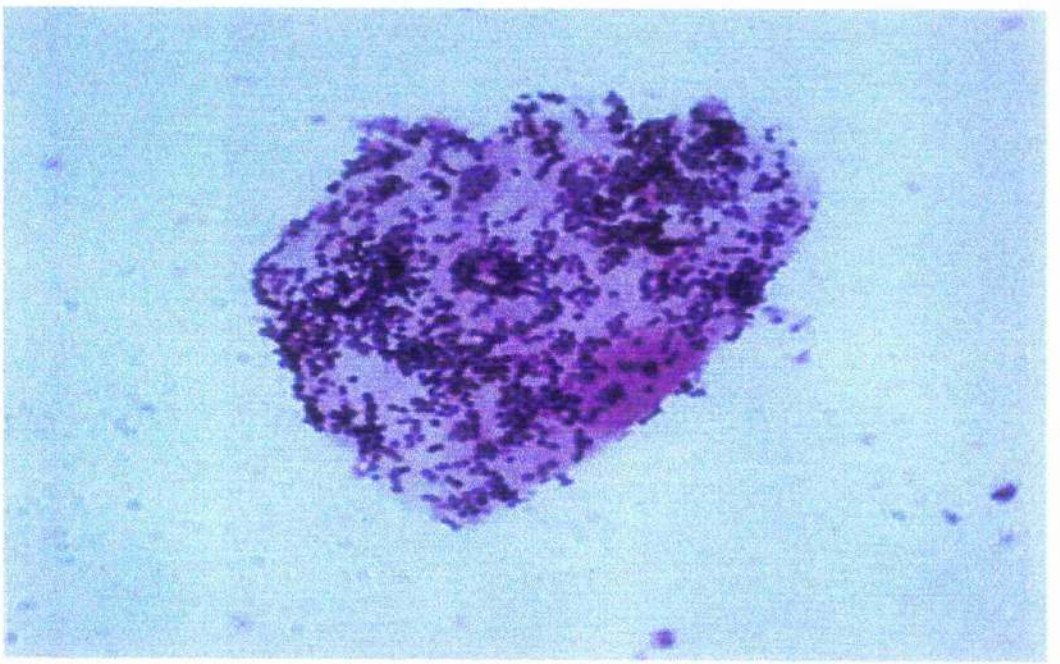


(B) WITHOUT CAPSULE (Mag. x 500)

FIGURE 4.6 STRAIN 025R ADHERENT TO BEC



(A) WITH CAPSULE (Mag. x 500)



(B) WITHOUT CAPSULE (Mag. x 500)

TABLE 4.6 RELATIONSHIP OF ADHERENCE TO CELL HYDROPHOBICITY

STRAIN	ADHERENCE	HYDROPHOBICITY
O08	MODERATE	HYDROPHOBIC (M)
O09	LOW	HYDROPHILIC (V)
O16	HIGH	HYDROPHOBIC (M)
O25R	HIGH	HYDROPHOBIC (M)
O25S	MODERATE	HYDROPHILIC (V)
O46	MODERATE	HYDROPHOBIC (M)
C01	LOW	HYDROPHILIC (M)
C02	LOW	HYDROPHILIC (M)
C06	LOW	HYDROPHILIC (V)
C08	LOW	HYDROPHILIC (V)
C10	LOW	HYDROPHILIC (V)
C18	LOW	HYDROPHOBIC (M)
NCTC 11325	LOW	HYDROPHILIC (M)
P01	LOW	HYDROPHILIC (M)
P02	LOW	HYDROPHILIC (V)
P04	LOW	HYDROPHILIC (V)
P08	MODERATE	HYDROPHILIC (M)
P11	MODERATE	HYDROPHILIC (M)
P13	LOW	HYDROPHILIC (V)

ADHERENCE : 0.01% - 2.0% = LOW
2.1% - 15% = MODERATE
15.1% - 30% = HIGH

HYDROPHOBICITY : categorised as stated in Table 3.6.

Change in OD of :

0% - 9% very hydrophilic
10% - 49% moderately hydrophilic
50% - 79% moderately hydrophobic
80% - 100% very hydrophobic

ionic and other physical forces of low specificity, which allow bacteria to become loosely associated with surfaces. This initial loose association results from attraction by van der Waal's forces, after which organisms are repelled by negative electrostatic charges as the two surfaces draw closer together. In this loose association bacteria are frequently located approximately 10 nm from the surface, at which point hydrogen, hydrophobic and other bonding can occur (Gibbons 1984). In contrast, the strongly adherent strain does not adhere well at first, but binding steadily rises over time to a maximum and then tails off. This indicates specific permanent bonding between complementary receptor and adhesin molecules located on the two cell surfaces.

As can be seen in the tables of results there is a large standard deviation in some cases, due to large variation between runs using the same strains. This was due to variation in BEC caused by factors out with experimental control. Although BEC were collected and processed in a way that minimised variation, BEC may be affected by factors such as donor health and diet which may influence receptors on their surface (Gibbons 1984). The indigenous flora present on BEC may affect the adherence of test strains, and this has been shown to vary between different donors and samples from the same donor over a time period (Pichichero 1984). However the screening process used allowed elimination of heavily colonised BEC

samples and Samaranayake, Hamilton and MacFarlane (1994) showed that the indigenous flora did not inhibit adherence of a strain of SMG to BEC. Therefore, comparisons between data are still valid as uniform BEC were used in each run and trends for levels of adherence can be observed. Tissue culture may have been used to provide uniform cells of oral epithelium, but this was not used for a number of reasons. First, as the aim was to compare results with the findings of Willcox and Knox (1990) it was important to use the same target cells. Secondly, the assay was intended to be a fairly quick and easy to run screening method which tissue culture would have complicated. Finally, as outlined previously, BEC can be affected by diet and health of the donor and therefore collecting them freshly for use in the assay would give results more reflective of the situation occurring in the mouth.

Visual counts of the number of bacteria adherent per BEC were significantly higher than those reported by other investigators, for example 10.6 per cell as found by Samaranayake et al (1994) and 11.6 - 12.8 per cell observed by Schollin (1988). When the counts were converted to % adherence as detailed in Table 4.5 there was a good correlation between these values and those found with the radiometric assay system, and these in turn were comparable with values found by Willcox and Knox (1990) who used a similar assay system.

4.4.2 THE POSSIBLE ASSOCIATION OF DIFFERENT SPECIES OF SMG OR THEIR SOURCE OF ISOLATION AND THEIR ADHERENCE TO BEC

In agreement with the findings of Willcox and Knox (1990), dental abscess isolates were found to adhere more readily to BEC than either extra-oral clinical or plaque isolates. They also stated that the values for adherence to BEC of these abscess isolates were similar to those found for *S.salivarius*, which is preferentially associated with epithelial surfaces (Sklavourion and Germaine 1980). In common with Willcox and Knox (1990), other strains of SMG were found to have a low level of adherence to BEC, which may have been expected as SMG are seldom cultured from the cheek (Mejare and Edwardsson 1975). Based on the evidence from other studies on adherence in the mouth it is likely that different strains of SMG have different surface structures which account for the range of adherence values. When considering a role for this in relation to pathogenicity of the SMG, it may be that enhanced adherence of abscess isolates allows proliferation, in order to allow sufficient numbers to be present to pass through the root canal to the tooth apex. Alternatively, colonisation of BEC may allow the SMG to persist in the mouth ready to take advantage of any local damage and enter the tissues, rather than being bound up in the plaque matrix.

This work supports the suggestion of Willcox and Knox (1990) that the SMG have specific target cells to which they adhere depending on the infection they cause. The clinical and plaque isolates here adhered poorly to oral cells, but are likely to adhere well to others. For example Gosling (1988) suggested that fimbriae which have been observed on some SMG and other plaque streptococci (Handley et al 1985) may be important for attachment to heart valves, in the pathogenesis of endocarditis. The ability of streptococcal cells to interact with specific target cells can be illustrated by the ability of *S.sanguis* to aggregate human platelets, a virulence factor in infective endocarditis. Ford et al (1993) found *S.sanguis* strain NCTC 7863 aggregated platelets after incubation with platelet-rich plasma after a lag phase of 7-19 minutes. Aggregation was prevented by aspirin and EDTA, and blocking the glycoprotein 1b receptor with monoclonal antibody inhibited aggregation. Further work by Ford et al (1996) revealed platelet aggregation depended upon plasma constituents binding to the bacterial surface during the lag phase, and aggregation could be inhibited by depleting/inhibiting complement. The rate of assembly of the C5b-9 complex on *S.sanguis* NCTC 7863 bacterial surface correlated with the lag time, leading the authors to suggest a role for the complement pathway in platelet aggregation by the type strain of *S.sanguis*. Other investigators (Chen et al 1994) had reported complement receptors on the surface of platelets, suggesting a

specific interaction between *S.sanguis*, complement activation and aggregation of platelets. In the SMG, Willcox et al (1994) found Lancefield group C strains aggregated platelets, with substances reducing aggregation which chelated cations, inhibited the cycloxygenase pathway in platelets, reduced the availability of ADP and disrupted platelet membrane stability. Willcox (1995) found a correlation between binding large amounts of fibrinogen and the ability to aggregate platelets, suggesting it aids platelet aggregation.

There were no significant differences between the different species and their ability to adhere to BEC. As *S.constellatus* is the most common SMG isolated from dental abscesses it may have been expected that this species would adhere to a greater extent. Of all the isolates, 3/5 *S.constellatus* abscess strains had the highest adherence values. Therefore, although all *S.constellatus* species cannot be said to adhere to BEC to a significantly greater extent than the other two species, it can be noted that a few *S.constellatus* strains do have a higher affinity for BEC, and this may be one aspect of their pathogenicity in dental abscess formation. Another reason for isolation of *S.constellatus* species most frequently from dental abscesses may be the finding of Toyoda et al (1995) that a strain of *S.constellatus* was

resistant to ingestion and killing by PMNL due to a structural component which was not identified. They suggested that this may be a virulence factor of *S.constellatus*, predisposing it to survival at foci of infection.

4.4.3 ROLE OF CAPSULE IN ADHERENCE

Bacterial capsules may participate in adherence in two ways. They provide a means for bacteria and target cell to bind together owing to the capsule's gelatinous nature, but alternatively they may hinder adherence by masking specific adhesins on the bacterial cell surface. As removal of capsule increased adherence of SMG to BEC it is likely that specific binding occurs between adhesins and receptors, and that these have been masked by the presence of capsule. A similar situation was reported for *S.pyogenes*, where only after fully encapsulated strains were treated with hyaluronidase to remove their capsules did they adhere well to oral epithelial cells (Beachey 1981). On regeneration of the capsules, ability to adhere was lost. As detailed in the introduction to this Chapter, adherence of *S.pyogenes* to epithelial cells is a complex process involving specific interactions of adhesin and receptor molecules. A similar situation can be suggested for SMG adherence, with capsule hindering these interactions.

As capsule removal did not increase adherence of all strains to the same extent it is clear this is not the only factor affecting adherence. This may also support our previous supposition that strains of SMG have specific target cells, as non-uniform levels of adherence to BEC may indicate either a difference in number of adhesins on the cell surface or different types of adhesins specific for other target cells.

By reference to the electron micrographs of strains after capsule removal illustrated in Chapter 3, an explanation may be found as to why treatment with hyaluronidase increased adherence at different rates. The isolates responded to hyaluronidase treatment in three ways : 1. virtually complete capsule removal, 2. partial removal and 3. no visible difference in surface structure. Where full capsule removal occurred, this presumably allowed all adhesins to be available to receptors on the BEC. When partial or little capsule removal occurred, parts of the cell surface, and therefore adhesins, remained masked by capsule, thus reducing the adherence.

As most of the test strains possessed capsular material, it is likely to make a positive contribution to pathogenesis in some way, which outweighs the negative effect on adherence found here. This may well be an involvement in resistance to phagocytosis. Whitnack et al (1981) demonstrated that the hyaluronate capsule of

S.pyogenes interfered with the attachment step of phagocytosis, and capsule has also been shown to interfere with the opsonic process as detailed in Chapter 6. Therefore, as stated by Beachey (1981), pathogens must be highly adaptable to survive and produce disease. On mucosal surfaces they must possess surface adhesins in order to adhere, then immediately after invasion they must either shed adhesin or produce masking capsules in order to avoid attachment to phagocytic cells. This may explain why the plaque isolates had smaller capsules as a group than the other two types of isolate. The latter, having been isolated from infections, will have generated capsules to avoid phagocytosis as suggested by Beachey (1981), while the plaque isolates, having yet to invade the tissues, have small capsules in order that they may adhere effectively without their surface structures being masked with capsule.

4.4.4 ROLE OF CELL SURFACE HYDROPHOBICITY IN ADHERENCE TO BEC

Most of the strains which adhered poorly were of a hydrophilic nature, which may contribute to their poor attachment. As discussed in section 4.1.2, before specific interaction between adhesins and receptors of a bacterium and its target cell can occur, they must be able to approach sufficiently close to one another. The ability to do this is affected by the relative surface

forces of each cell, and owing to the net negative charges on both cells, repulsion occurs. However, this is overcome by the attraction of hydrophobic molecules on the bacterial surface to the hydrophobic phospholipid molecules in the lipid bilayer membrane of the target cell (Beachey 1981). This allows the two cells to approach and thus for stronger specific interaction to occur. In the case of hydrophilic strains the repulsive forces cannot be overcome by counteractive attractive forces between hydrophilic molecules and therefore these strains may be unable to approach sufficiently closely to allow specific adhesin/receptor interactions to occur. Also, Absolom (1988) found that the early attachment phase in bacterial adhesion depends on the relative surface tension of the interacting phases, and that the most hydrophobic bacteria adhered to the greatest extent to all surfaces when suspended in a liquid with a high surface tension.

Strongly adherent strains showed both hydrophobic and hydrophilic characteristics. In the case of hydrophobic cells the above scenario is likely, i.e. attraction between hydrophobic molecules precedes specific interaction. In hydrophilic strains other attractive forces (such as those outlined in the DLVO theory : Section 4.1.2.1) may be involved in drawing the two surfaces close enough to interact. Whether hydrophobic or hydrophilic in nature, it is likely that highly

adherent strains of the SMG have more adhesins on their surfaces complementary to the receptors present on BEC than do poorly adherent strains. Those strains which adhere poorly to BEC may in turn have a proclivity for other cells of the body, having adhesins of a different specificity present on their surface.

CHAPTER 5 OPSONIC REQUIREMENTS OF THE SMG AND INDUCTION OF RESPIRATORY BURST (CHEMILUMINESCENCE) OF PMNL

5.1 INTRODUCTION

5.1.1 GENERAL INTRODUCTION

The preceeding chapters have examined aspects of the SMG which assist the organism in causing infection. These aspects have included adherence to target cells in order to persist in the host, examination of toxin and enzyme production which may help provide nutrition for the organism or damage host tissues to allow spreading, and characterisation of surface structures which may aid virulence. In response to bacterial invasion the body will initiate its own defence mechanism, the immune system. The host has developed three major mechanisms for killing bacteria : complement-mediated lysis, phagocytosis and cell mediated cytotoxicity, all of which the successful pathogen must evade. This and the following chapter will investigate the ability of the SMG to evade the host's first line of defence against bacterial invasion, that is, phagocytosis by white blood cells. This was examined by measuring the respiratory burst (chemiluminescence) induced when bacteria and PMNL interact. The effect of serum opsonization on chemiluminescent response was also investigated. The data were then analysed in order to ascertain whether any

significance could be attached to either species or source of isolate. The influence of both capsule and cell surface hydrophobicity on respiratory burst induction was also examined, as these factors have both been implicated in conferring resistance to phagocytosis as discussed previously (Chapter 3 : Surface Structures).

5.1.2 POLYMORPHONUCLEAR LEUKOCYTES

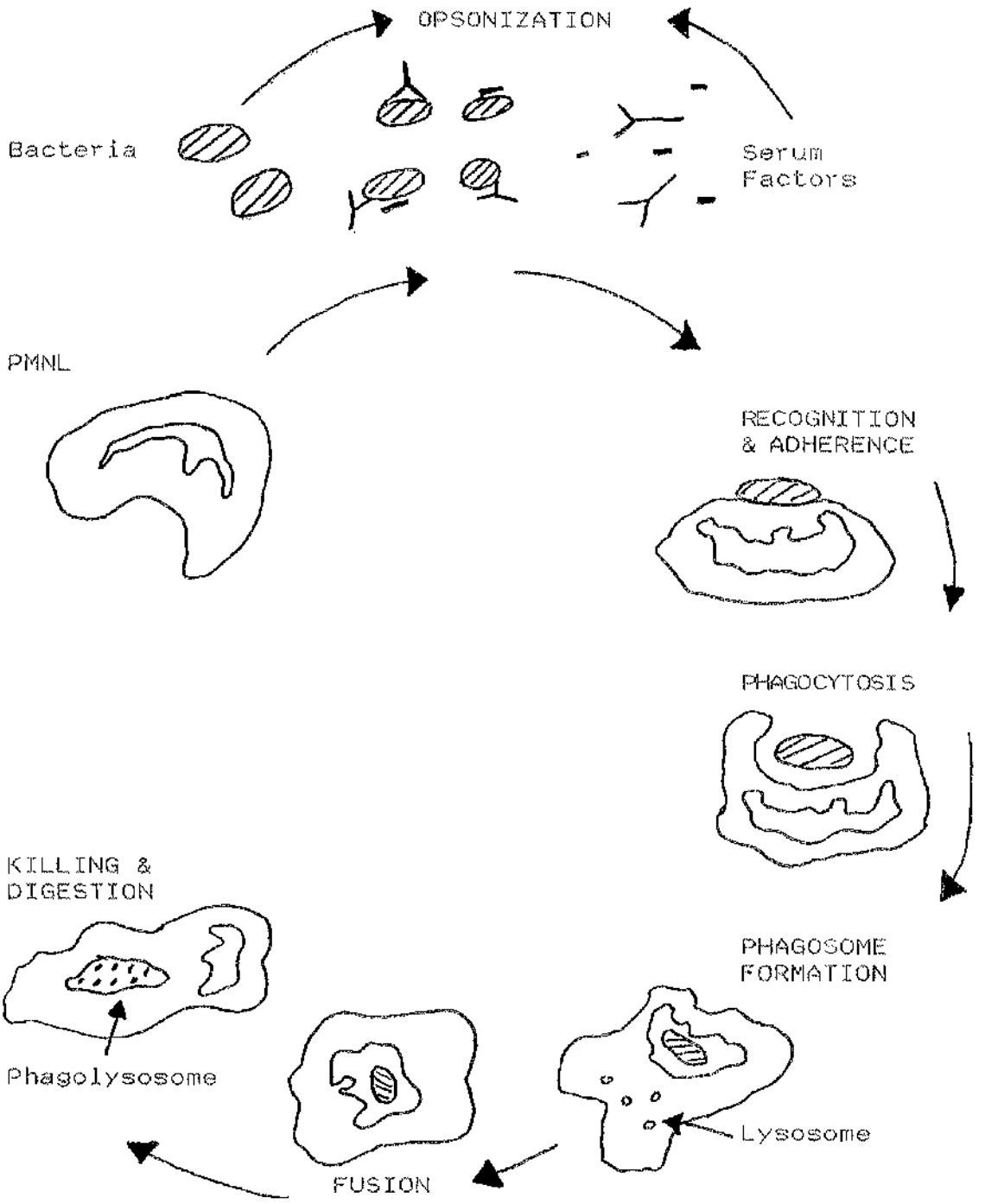
PMNL are characterised by a large nucleus with 3-5 lobes and presence of numerous granules in the cytoplasm. They develop in the bone marrow from multipotential stem cells over a period of 12-13 days, after which they are released into the bloodstream. Half remain adherent to the vessel walls and the other half enter the tissues within 12 hours. PMNL make up 40 % - 70 % of circulating white blood cells.

5.1.3 THE PHAGOCYTIC PROCESS

The phagocytic process can be divided into 3 stages, recognition, ingestion and killing (Fig. 5.1)

1. Recognition : in response to chemotactic stimuli from tissues, PMNL arrive at the site of infection and contact the invading organism. This only initiates ingestion if the PMNL recognise the intruders. Opsonization of the bacterium with immunoglobulin G (especially IgG1 & IgG3) or complement factor C3b allows recognition. This leads

FIGURE 5.1 THE PHAGOCYTIC PROCESS



to adherence of the bacterium to the surface of the PMNL due to interaction of Fc and C3b receptors on the surface of the phagocyte corresponding to the opsonins.

2. Ingestion : after adherence, the PMNL surrounds the particle with pseudopodia which fuse around the object and internalise it in a phagolysosome.

3. Killing : cytoplasmic lysosomes present in the phagocyte migrate to the phagosome and fuse with it, forming a phagolysosome into which the lysosomes' contents, including enzymes and other chemicals which degrade bacteria, are released. Subsequent killing of the bacterium occurs due to production of superoxide anion, hydrogen peroxide, singlet oxygen and hydroxyl radicals.

5.1.4 CHEMILUMINESCENCE

The production of chemiluminescence, the emission of light by phagocytic cells while ingesting microorganisms and other particles, was first described by Allen, Stjernholm and Steele (1972). When PMNL come into contact with opsonized bacteria or other particles there is an increase in hexose-monophosphate shunt activity giving an increase in NADPH. In the presence of NADPH-oxidase and O_2 this produces O_{2^-} and/or HOOH which then produce singlet oxygen in the presence of myeloperoxidase and Cl^-. It is this $^1O_2$ which is responsible for light emission and bactericidal activity. This native

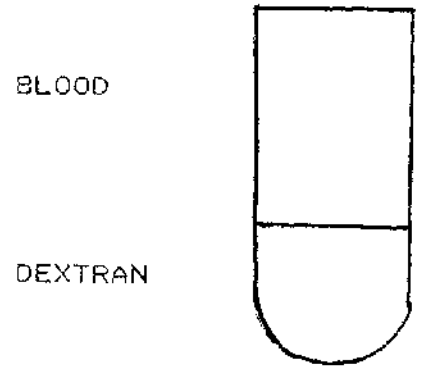
chemiluminescence is very weak, requiring large numbers of cells and a sensitive light detector. However, the sensitivity of the technique can be increased by addition of the cyclic hydrazide 5-amino-2,3 dihydro-1,4-phthalazinedione (luminol). Oxidation of this compound by reactive oxygen species produced during phagocytosis gives increased light production at 425 nm (Allen and Loose 1976), due to the oxidising species raising the energy of the peripheral electrons of the molecule to an excited state, which on returning to the ground state release the energy as a photon of light. Monitoring of the chemiluminescence response allows measurement of opsonic activity and cellular function, although it is an indirect method of assessing bactericidal function as it does not directly differentiate between defects of ingestion and intracellular killing (Easmon, Cole, Williams & Hastings 1980). Incorporation of luminol to reduce the number of cells required allows investigation of phagocytic function in neonates and neutropenic patients (Stevens, Winston & Van Dyke 1978).

5.2 MATERIALS AND METHODS

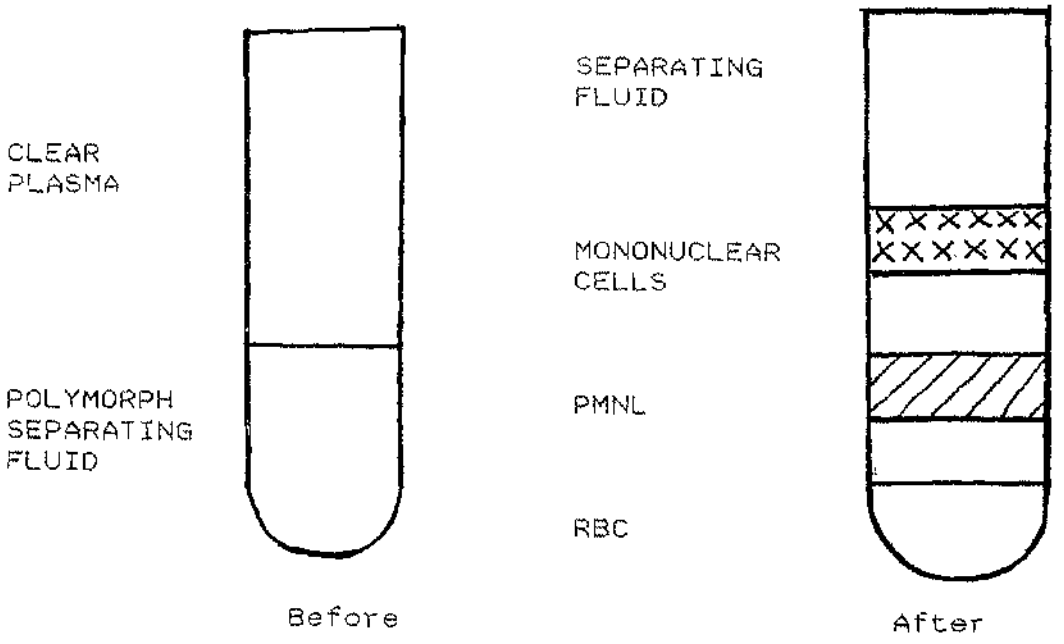
5.2.1 PREPARATION OF POLYMORPHONUCLEAR LEUKOCYTES

Normal human blood was collected by venipuncture and added to tubes containing 10 units of heparin (Multiporin) per ml to prevent clotting. A 5% solution

FIGURE 5.2 SEPARATION OF PMNL FROM WHOLE BLOOD



RBC SEDIMENTATION



Before and after centrifugation.

of dextran in saline (2-3 ml) was added to conical tubes, the heparinised blood layered onto this and left to separate for 30 minutes. After this time the leucocyte-rich top layer was removed and carefully layered onto 2-3 ml of Lymphocyte Separating Fluid (Tech Gen, London), maintaining two layers. This was then centrifuged for 30 minutes at 160 g, after which time four fractions were apparent (Figure 5.2). The layer of PMNL was removed and washed in gel Hanks (Appendix I) by centrifugation at 160g for 15 minutes, the supernatant discarded and the resultant pellet was resuspended in gel Hanks. From this suspension 10 µl of the PMNL were added to 90 µl of white cell diluting fluid (2% acetic acid) and 10 µl of this suspension was counted in a haemocytometer (Depth 0.2mm 1/16 mm^2) (Weber, Weber Scientific, Sussex, England). The yield of PMNL was calculated from the equation : Cell count (in 16 small squares) x 5 x 10^3 x 10 . PMNL were used at a concentration of 1 x 10^7 PMNL/ml.

5.2.2 PREPARATION OF BACTERIA

Bacteria were grown overnight in BHIB + 0.3% YE then washed 3 times in PBS before being suspended to an OD at 620 nm of 0.45 (5 x 10^8 cfu/ml).

5.2.3 SERUM PREPARATION

Normal pooled human serum was prepared by collecting blood from four volunteers and allowing this to clot for 2-3 hours. A wooden stick was then run around the edge of the tube to loosen the clot and the tubes centrifuged at 220 g for 15 minutes. The serum was removed from the top and the clot subjected to centrifugation two more times, after which the serum was pooled, aliquoted into 0.5 ml volumes and stored at - 70°C.

5.2.4 OPSONIZATION

Bacteria used were opsonized with serum concentrations of 1%, 5%, 10%, 15% and 20% v/v diluted in gel Hanks. Equal volumes of serum and bacteria were mixed and incubated at 37° C for 15 minutes with shaking at 100 rpm. They were then centrifuged at 1 000g for 15 minutes, the supernatant discarded, and the bacteria resuspended in the original volume of gel Hanks.

5.2.5 ASSAY PROCEDURE

Opsonized bacterial suspension (100 µl) + 100 µl of PMNL + 50 µl of Luminol (Appendix I) were added to polystyrene microcuvettes (Clinicon, Life Sciences UK Ltd., Basingstoke, England), mixed by shaking and quickly loaded into a Bio Orbit 1251 Luminometer (Bio Orbit,

Turku, Finland). This was connected to a PC running a Bio Orbit Multiuse 1251-130 V 2.0 program in Windows. This measured chemiluminescence (mV) proceeding with time (seconds), and produced a graphical representation of these variables, from which peak values could be determined.

5.2.6 EFFECT OF HYALURONIDASE TREATMENT OF SMG ON THEIR CHEMILUMINESCENT RESPONSE TO PMNL

Cells of SMG were treated with hyaluronidase in an attempt to determine the role of capsule on bacterial susceptibility to phagocytosis. Capsule was removed by treatment with hyaluronidase as described in Chapter 3 (3.2.3) and the effects of such treatment are fully examined there. In brief, hyaluronidase treatment resulted in three scenarios. First, strains with small amounts of capsular material were almost completely denuded of material, secondly, those with a more densely staining mass under TEM had a proportion of the material removed, and finally, strain O25R showed no change in surface material after treatment. Treated and untreated strains were included in the assay

5.2.7 STATISTICAL ANALYSIS

The Mann-Whitney U test was used to compare data, with a value of $p < 0.05$ being considered significant.

5.3 RESULTS

5.3.1 SERUM Opsonization

Initial tests with 3 strains showed that the SMG were phagocytosed without serum opsonization, but the chemiluminescent response was enhanced by opsonization (Table 5.1). This also dictated the use of serum concentrations between 1% and 20% for further work, as the optimum response was usually within these limits.

Mean peak chemiluminescent responses for the 20 test strains opsonized with serum concentrations 1%-20% are illustrated in Tables 5.2, 5.3 and 5.4. A range of values were found, with chemiluminescence increasing with % serum, indicating that the SMG are efficiently opsonized. Opsonization with 20% normal pooled human serum, an effective serum concentration of 10%, tended to give optimum response with most organisms, the exceptions being O16, P08, P13 and NCTC 11325, all of which peaked at 15% (7.5% final concentration).

From the data obtained it can be seen that there may be a large variation in chemiluminescent response between runs performed with the same strain. Rather than this reflecting a change in the bacterium, it is due to the different donors of PMNL, among whom there may be high and low responders. The factors affecting the response

TABLE 5.1 EFFECT OF OPSONIZATION ON ABILITY OF SMG TO INDUCE A CHEMILUMINESCENT RESPONSE IN HUMAN PMNL

	CHEMILUMINESCENCE (mV) AFTER STRAINS OPSONIZED WITH SERUM SOLUTION					
STRAIN	UNOPSONIZED	1%	5%	10%	15%	20%
O25R	42.4	43.3	61.9	73.9	71.3	74.9
O25S	50.9	71.9	96.8	123	131	132
NCTC 11325	53.2	61.9	85.4	105	111	95.5

Figures represent mean peak chemiluminescent response of strains without opsonization and with opsonization at serum concentrations of 1% - 20%.

TABLE 5.2 CHEMILUMINESCENCE OF PMNL IN RESPONSE TO ORAL (ABSCESS) ISOLATES

STRAIN	SPECIES	MEAN PEAK CHEMILUMINESCENCE (mV) AT SERUM CONCENTRATIONS: 1%	5%	10%	15%	20%
008	S.anginosus	20.46	36.63	50.7	62.76	80.43
009	S.intermedius	39.96	41.66	55.2	68.83	72.46
016	S.constellatus	63.0	99.24	139.3	159.3	142.4
025R	S.constellatus	43.3	61.9	73.9	71.3	74.9
025S	S.constellatus	72.0	97.2	123.6	131.0	133.0
041	S.anginosus	28.1	39.56	49.2	58.6	72.16
046	S.intermedius	7.14	7.4	11.18	13.04	14.1

Values are the mean of three separate experiments performed with three different PMNL donors.

TABLE 5.3 CHEMILUMINESCENCE OF PMNL IN RESPONSE TO CLINICAL ISOLATES

STRAIN	SPECIES	MEAN PEAK CHEMILUMINESCENCE (mV) AT SERUM CONCENTRATIONS: 1%	5%	10%	15%	20%
C01	S.constellatus	191.6	237.0	236.0	266.0	274.6
C02	S.intermedius	26.63	35.4	43.16	49.96	60.9
C06	S.intermedius	22.3	27.1	44.6	59.56	68.46
C08	S.constellatus	21.2	28.34	34.5	36.38	39.03
C10	S.anginosus	1.95	2.86	5.9	8.5	11.23
C18	S.anginosus	27.86	37.04	39.1	43.05	43.6
NCTC 11325	S.constellatus	62.0	85.4	105.1	111.0	95.4

Values are the mean of three separate experiments performed with three different PMNL donors.

TABLE 5.4 CHEMILUMINESCENCE OF PMNL IN RESPONSE TO COMMENSAL (PLAQUE) ISOLATES

STRAIN	SPECIES	MEAN PEAK CHEMILUMINESCENCE (mV) AT SERUM CONCENTRATIONS: 1%	5%	10%	15%	20%
P01	S.anginosus	3.68	5.63	6.7	10.9	13.77
P02	S.anginosus	45.8	57.56	62.4	64.76	67.9
P04	S.constellatus	50.56	58.06	66.8	81.9	84.2
P08	S.intermedius	10.28	12.6	13.08	13.61	13.25
P11	S.intermedius	4.16	5.25	7.7	8.37	9.94
P13	S.constellatus	12.84	27.96	36.0	45.13	42.1

Values are the mean of three separate experiments performed with three different PMNL donors.

of PMNL to bacterial stimuli are numerous. For example, Olinescu et al (1994) found that levels of testosterone and oestrone lower than 10^{-5} M in donors increased the chemiluminescent response of their PMNL, while higher levels decreased it. Despite this variation between experiments, comparisons are still valid as each run was performed with PMNL from the same donor and therefore high and low chemiluminescence inducers amongst the SMG will be evident on each occasion.

5.3.2 RATE OF INDUCTION OF RESPIRATORY BURST (CHEMILUMINESCENCE)

As shown in Figures 5.3, 5.4 and 5.5 the rate of chemiluminescence starts slowly, building to a peak generally between 10-25 minutes and thereafter begins to tail off as PMNL and bacterial concentrations become rate-limiting. Measurement of the initial rate of chemiluminescence is equivalent to quantification of the rate of phagocytosis (Allen 1977), therefore a less sharp incline to peak may indicate resistance to phagocytosis in that strain.

Fredlund (1993) calculated a chemiluminescence index (CI) in order to standardise results, having found, as we did, that differing PMNL donors can cause large variation in peak values. This method uses the maximal rate constant

Figure 5.3 (a) Chemiluminescent Response of PMNL to Abscess Isolates of SMG. Traces represent a typical result obtained in a single run.

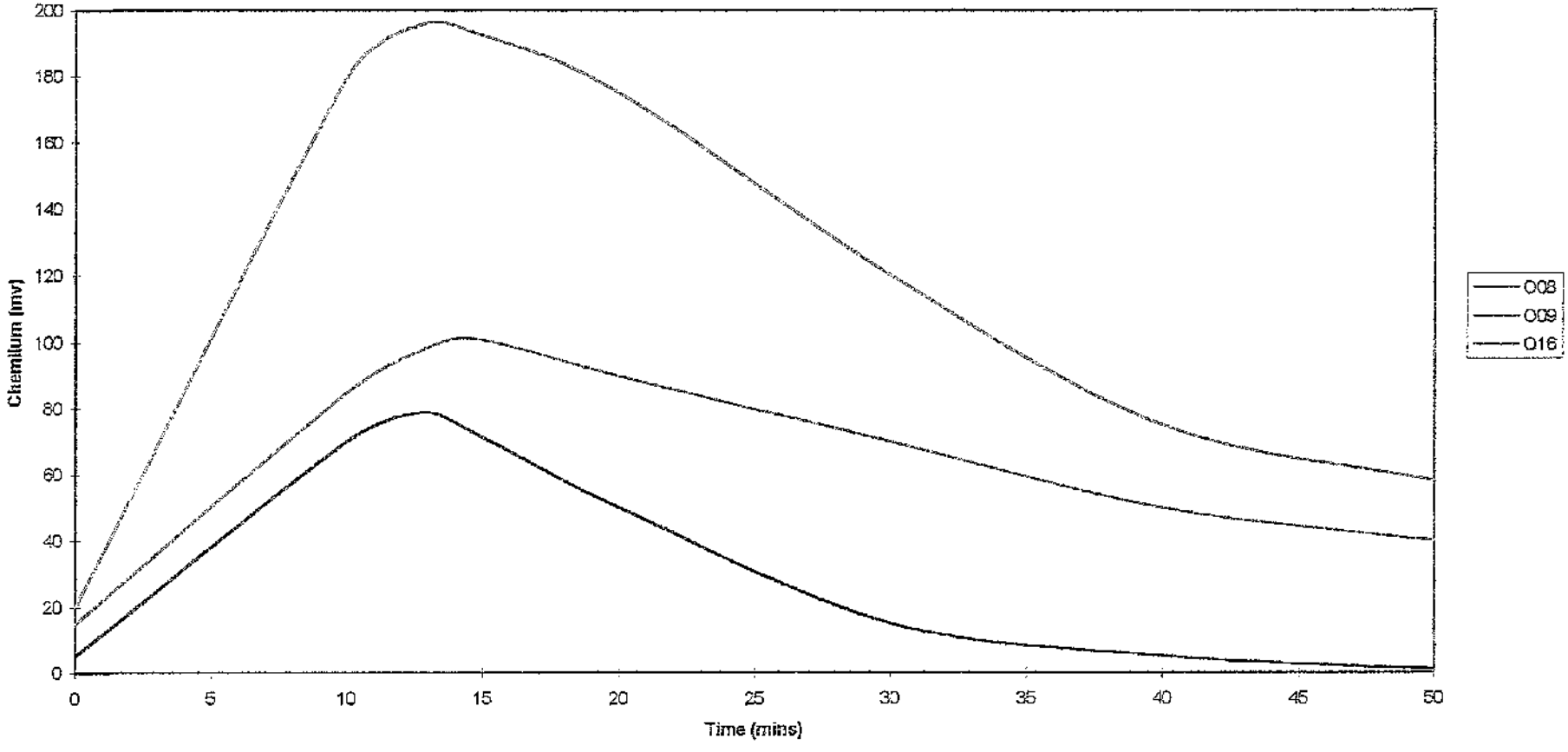


Figure 5.3 (b) Chemiluminescent Response of PMNL to Abscess Isolates of SMG. Traces represent a typical result obtained in a single run.

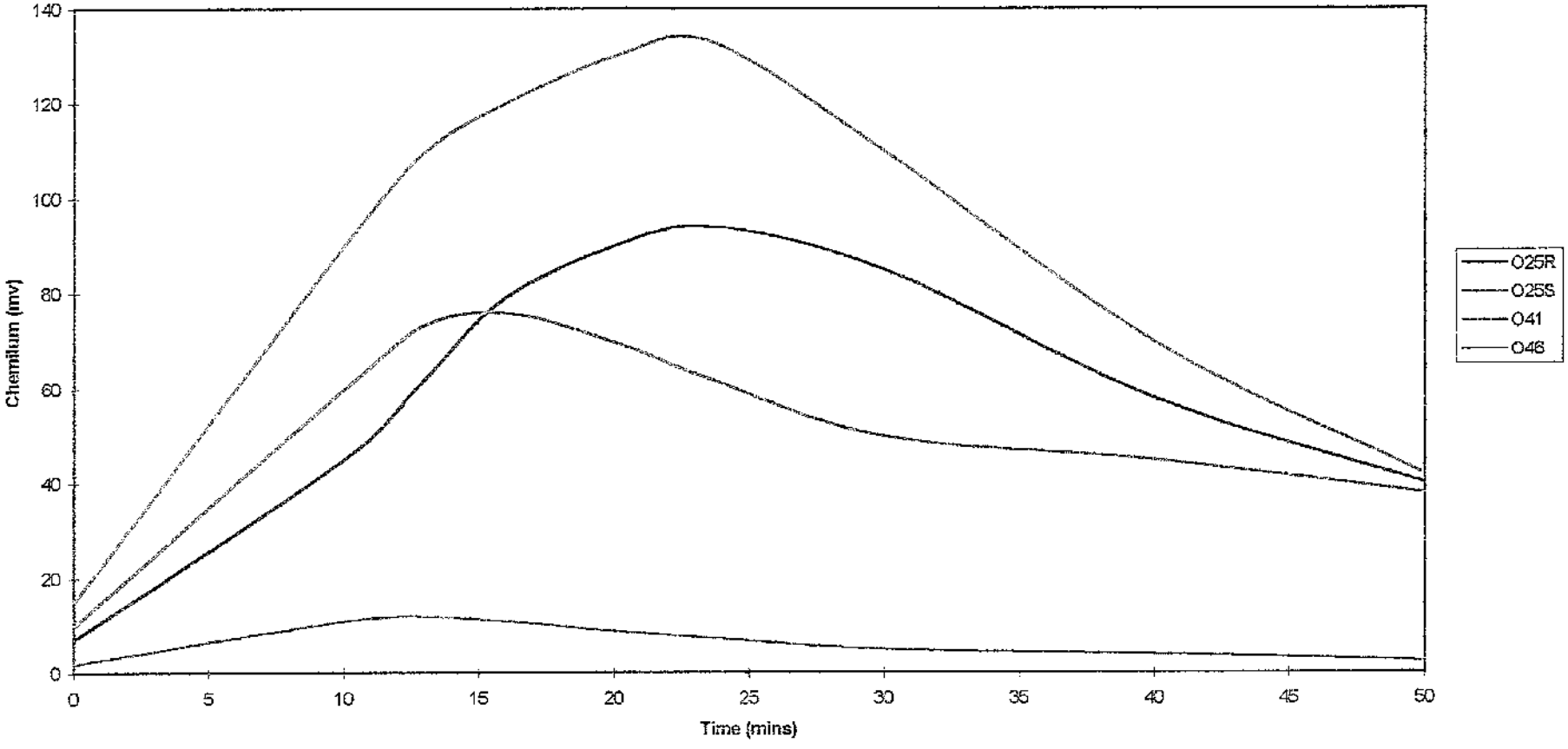


Figure 5.4 (a) Chemiluminescent Response of PMNL to Clinical Isolates of SMG. Traces represent a typical result obtained in a single run.

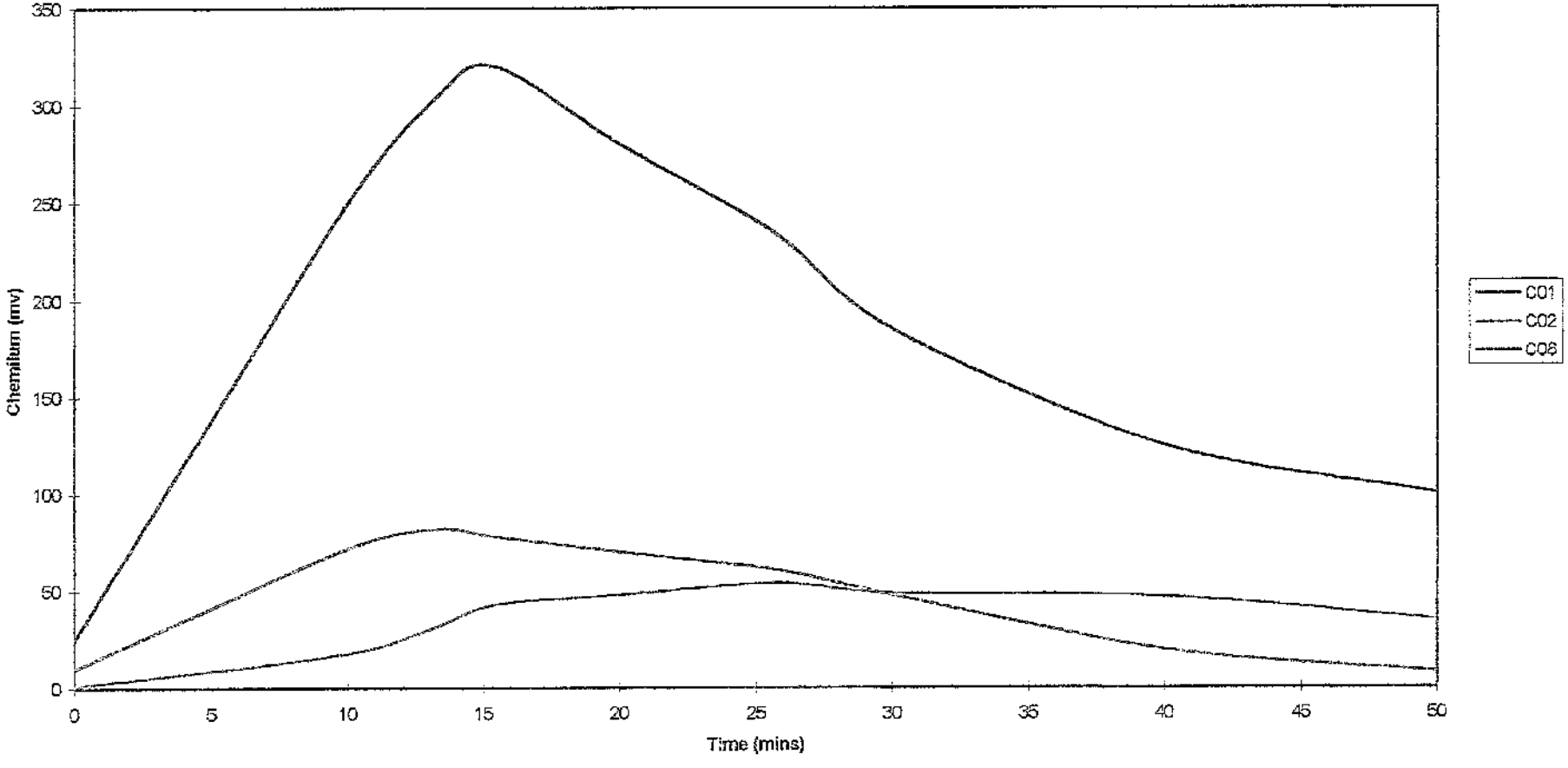


Figure 5.4 (b) Chemiluminscent Response of PMNL to Clinical Isolates of SMG. Traces represent a typical result obtained in a single run.

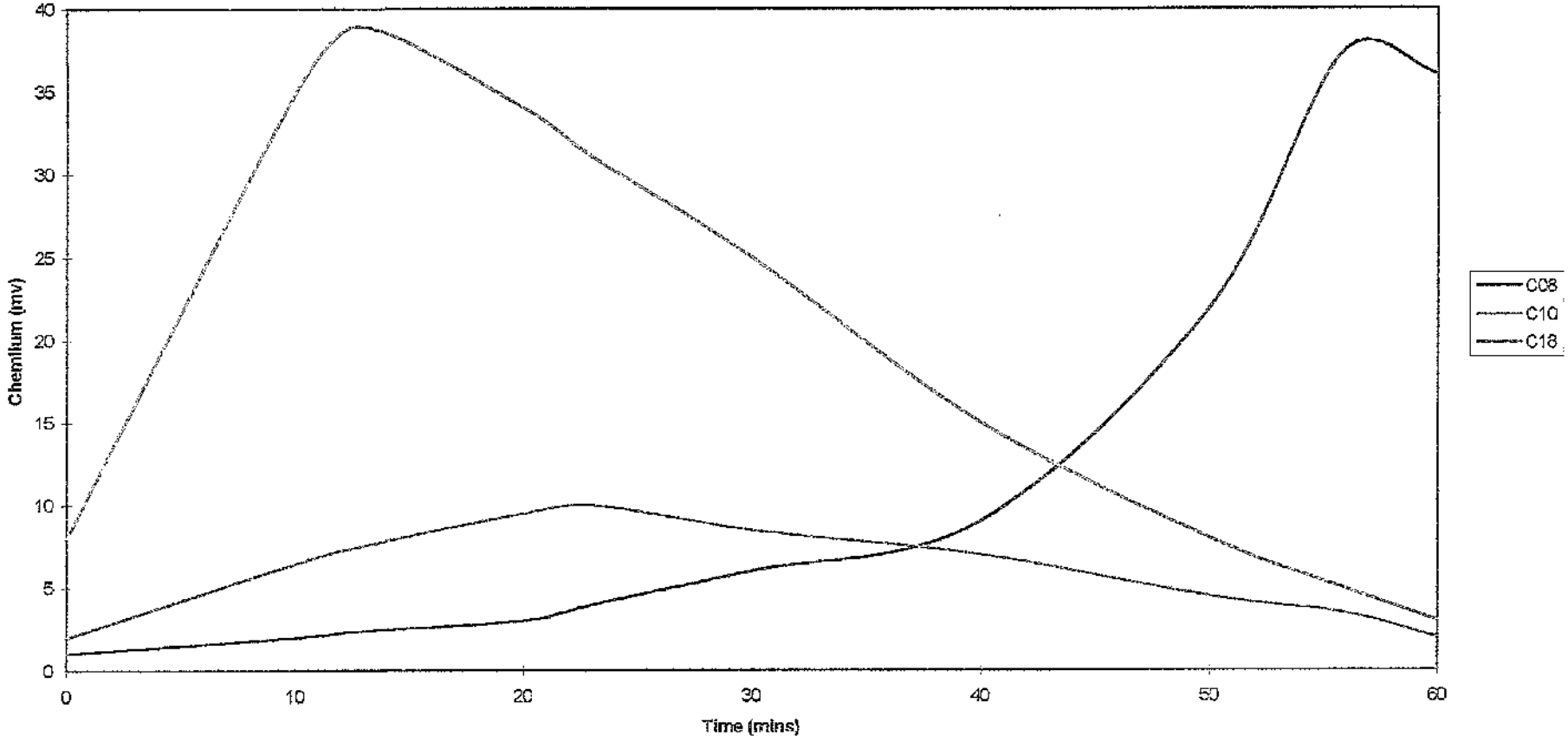


Figure 5.5 (a) Chemiluminescent Response of PMNL to Paque Isolates of SMG. Traces represent a typical result obtained in a single run.

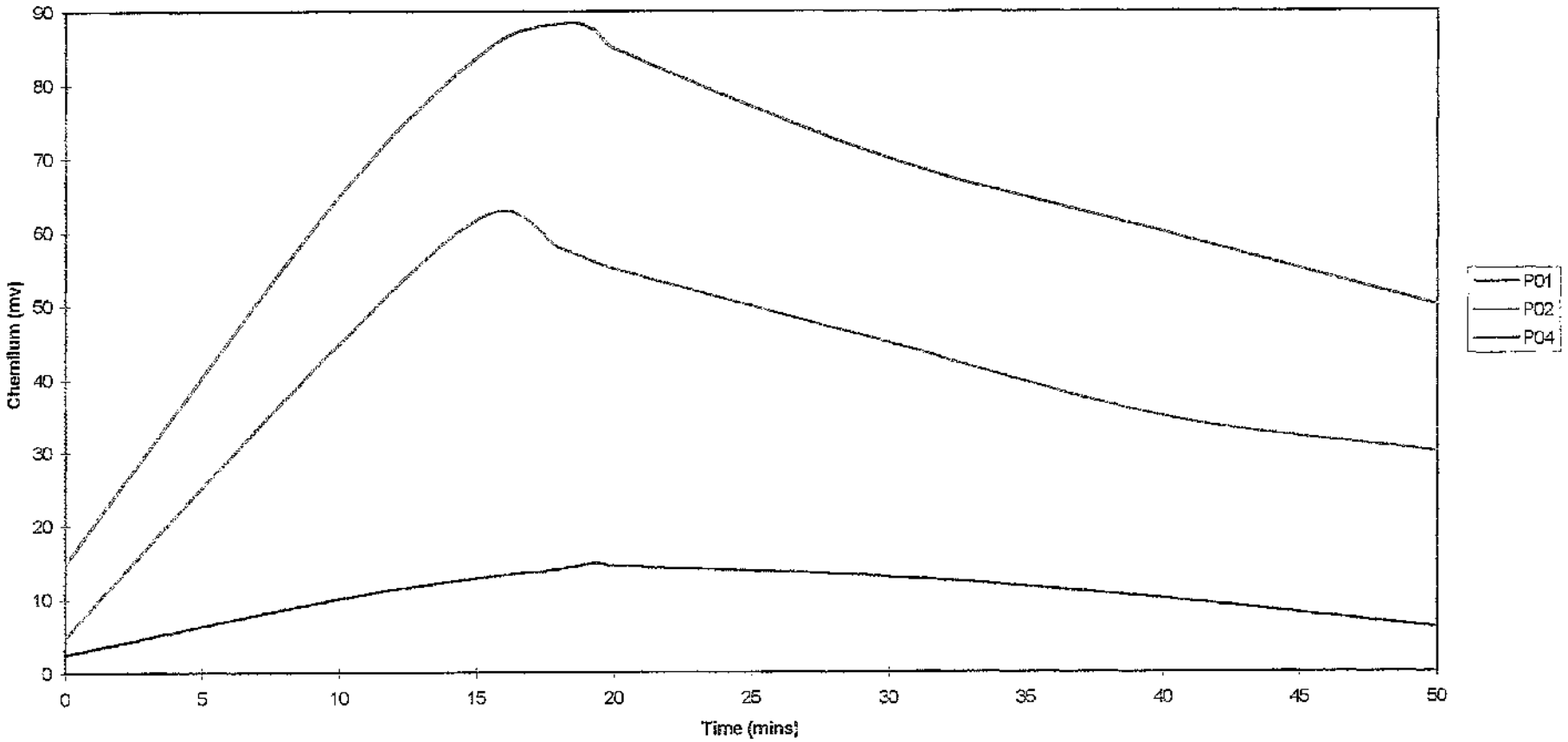
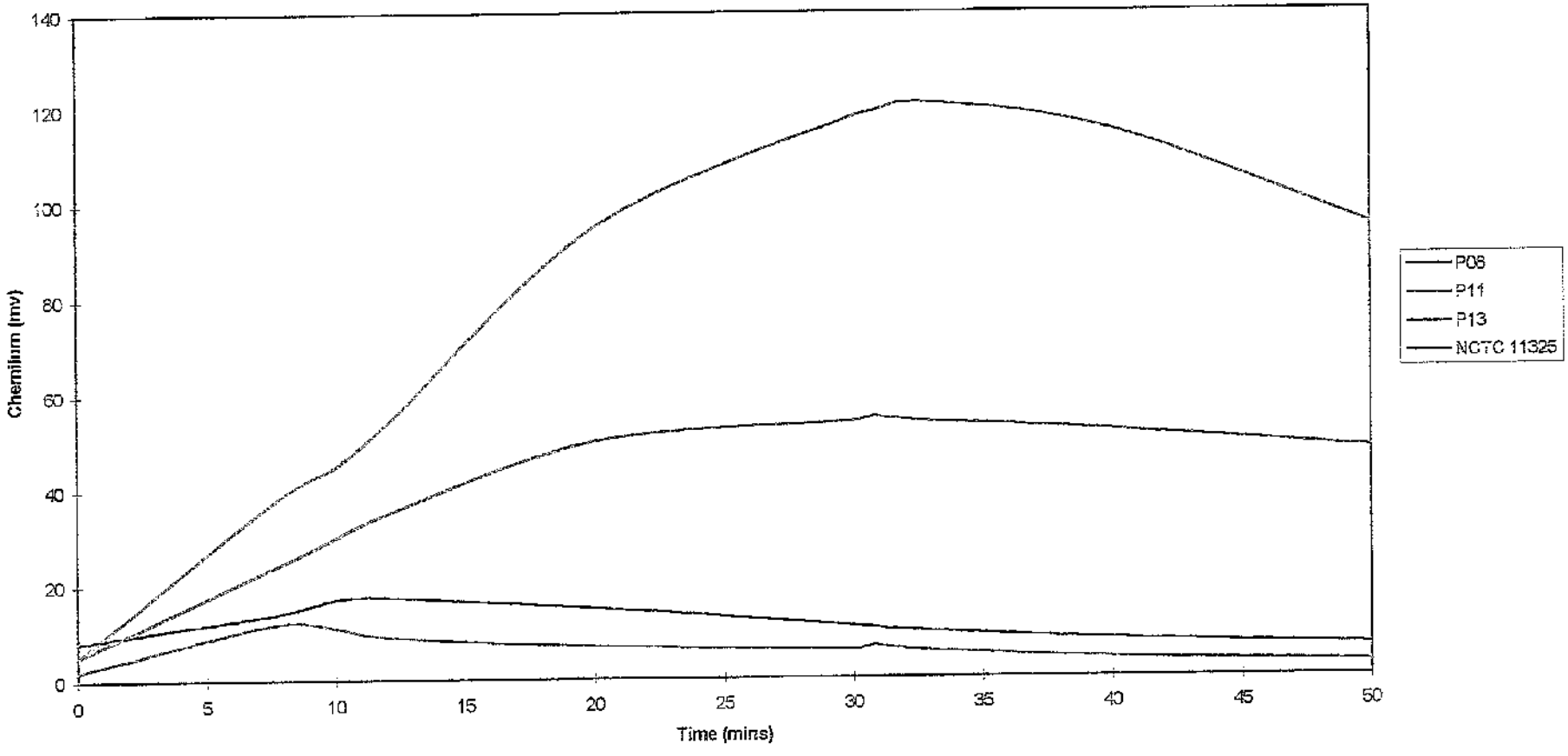


Figure 5.5 (b) Chemiluminescent Response of PMNL to Plaque Isolates of SMG. Traces represent a typical result obtained in a single run.



(MRC) calculated as a function of CL (mV) and time (min) i.e. [mV/min] during the highest acceleration of the response of PMNL, rather than peak values. This was calculated and a comparison of peak chemiluminescence against maximal rate constant (MRC) is illustrated in table 5.5. In 11 out of 19 cases the peak CL value and MRC concurred.

5.3.3 SIGNIFICANCE OF ISOLATE TYPE

There was no statistically significant difference in opsonic requirement or chemiluminescence produced by the isolates from different sites. Thus, rather than considering isolates from plaque, clinical infections and dental abscesses separately, the SMG as a whole can be said to have a common opsonic requirement and to elicit similar chemiluminescent responses.

5.3.4 SPECIES SIGNIFICANCE

There was no statistically significant difference between serum opsonization and chemiluminescent response of *S.anginosus*, *S.constellatus* or *S.intermedius*. Although strains of *S.constellatus* tended to give a higher response, the range of variation within the group resulted in this being statistically insignificant (Figure 5.6).

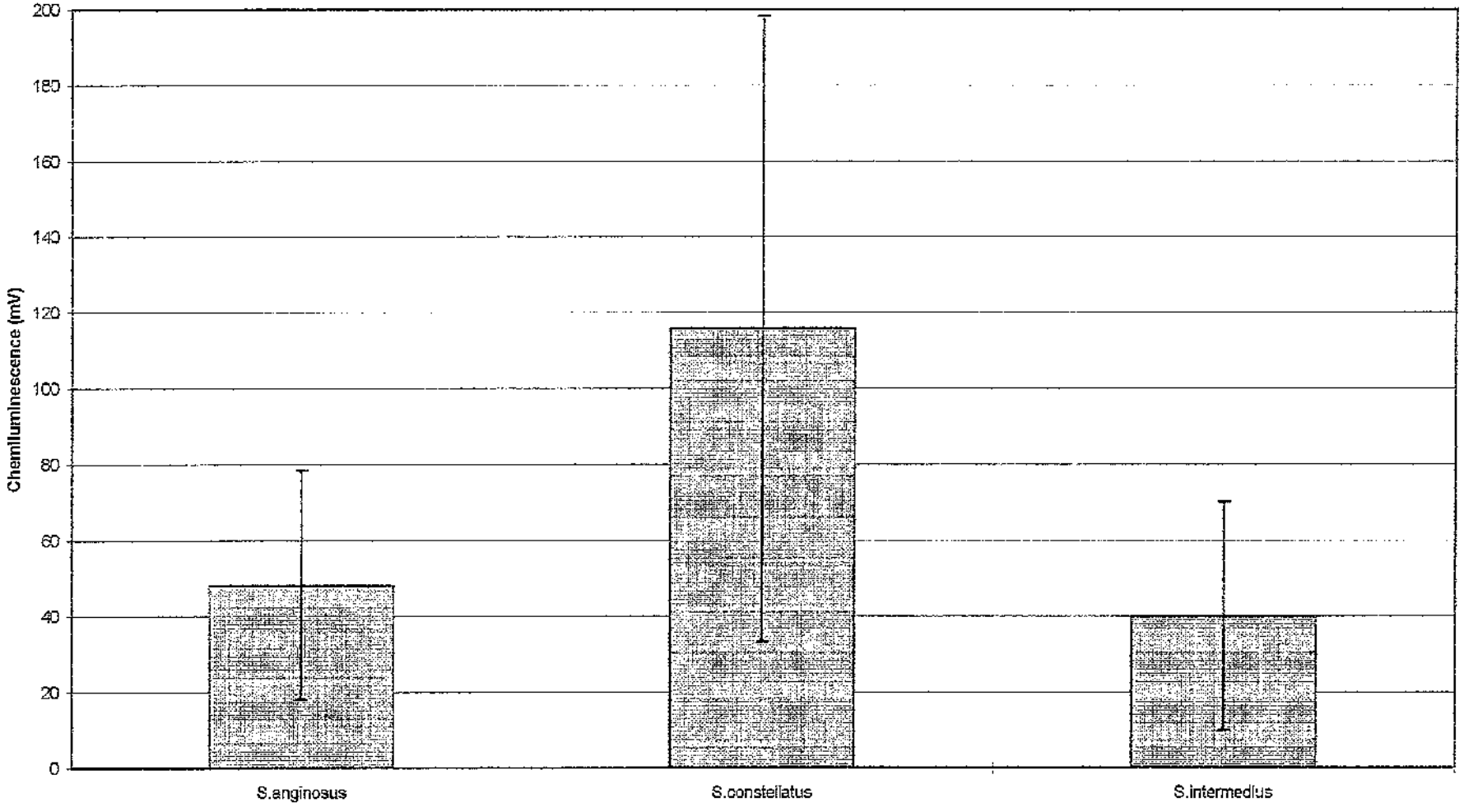
TABLE 5.5 PEAK CHEMILUMINESCENCE (CL) COMPARED TO MAXIMAL RATE CONSTANT (MRC) AS A MEASURE OF THE CHEMILUMINESCENT RESPONSE OF PMNL TO SMG

STRAIN	MRC[1] VALUE (mV/min) 1	2	3	PEAK CL[2] VALUE (mV) 1	2	3
O08	5.5(M)	9.5(M)	6.5(M)	69 (M)	93 (M)	79 (M)
O09	7 (M)	2 (M)	4.8(M)	101(M)	43 (M)	73 (M)
O16	6.5(M)	16 (M)	15 (M)	100(M)	196(M)	182(M)
O25R	0.5(L)	4.5(L)	6.8(M)	22 (L)	94 (M)	109(M)
O25S	5 (L)	7 (M)	8 (M)	121(H)	145(H)	133(H)
O46	0.8(L)	0.9(L)	1.6(L)	10 (L)	11 (L)	20 (L)
C01	12 (H)	22 (H)	27 (H)	168(H)	320(H)	336(H)
C02	2.9(L)	1.9(L)	3.3(L)	72 (M)	53 (M)	57 (M)
C06	3.9(L)	3.9(L)	6.2(M)	60 (M)	63 (M)	82 (M)
C08	2.3(L)	0.4(L)	5.9(M)	66 (M)	14 (L)	37 (L)
C10	0.5(L)	1.3(L)	0.2(L)	9 (L)	21 (L)	3 (L)
C18	0.2(L)	2.7(L)	8 (M)	4.9(L)	39 (L)	87 (M)
NCTC 11325	5 (L)	8 (M)	5.5(M)	121(H)	132(H)	80 (M)
P01	0.5(L)	0.5(L)	0.8(L)	7.2(L)	19 (L)	15 (L)
P02	6 (M)	4 (L)	1.7(L)	85 (M)	62 (M)	59 (M)
P04	6 (M)	3.3(L)	5 (L)	97 (M)	67 (M)	88 (M)
P08	1 (L)	0.9(L)	0.1(L)	21 (L)	17 (L)	2.2(L)
P11	0.4(L)	1.3(L)	1.3(L)	5 (L)	12 (L)	13 (L)
P13	1.5(L)	1.8(L)	2.5(L)	23 (L)	57 (M)	55 (M)

1. MRC = MAXIMAL RATE CONSTANT (mV/min)
Defined as :(L)-Low (0-5 mV/min),(M)-Medium(5.1-10 mV/min),(H)-High (10.1 upwards)

2. CL = Peak chemiluminescence (mV)
Defined as :(L)-Low (0-50 mV), (M)-Medium (51-100 mV), (H)-High (101 upwards)

Figure 5.6 Species Distribution with Respect to Induction of Chemiluminescent Response in PMNL. Chart shows mean chemiluminescence values according to species. Bars indicate +/- one standard deviation



5.3.5 ROLE OF CAPSULE IN BACTERIAL SUSCEPTIBILITY TO Opsonization AND INDUCTION OF CHEMILUMINESCENCE

Treatment with hyaluronidase had an unusual effect of both raising and lowering chemiluminescence for some strains (Table 5.6). However, the differences between treated and untreated values were not statistically significant and so fluctuation in chemiluminescence, either up or down, was presumably due to experimental variation. Therefore capsule appears to play no part in serum opsonization or chemiluminescence.

5.3.6 EFFECT OF HYDROPHOBICITY OF BACTERIAL CELL SURFACE ON CHEMILUMINESCENCE

There was no relationship between the hydrophobic nature of the organism and its ability to elicit a chemiluminescent response in PMNL as shown in table 5.7. The peak chemiluminescent response was categorised as low (0-50 mV), medium (51-100 mV) or high (101 mV upwards), whilst the MRC was categorised as 0-5 mV/min low, 5.1-10 mV/min medium and 10.1 mV/min upwards high. This was compared with cell surface hydrophobicity, categorised as moderate or very hydrophobic/ hydrophilic. As it is generally considered that the more hydrophobic a cell, the more easily it is phagocytosed, it would be expected that strains classed as moderate/very hydrophobic would induce a higher chemiluminescent response. Of 14

TABLE 5.6 EFFECT OF HYALURONIDASE TREATMENT OF SMG ON THEIR ABILITY TO STIMULATE A CHEMILUMINESCENCENT RESPONSE BY HUMAN PMNL

STRAIN	RUN 1	RUN 2	RUN 3	RUN 4	MEAN
O09	258	50.7	17.0	56.4	95.525
O09 T [p]	236	58.2	17.5	47.4	89.775
O25R	217	32.1	42.0	90.3	95.35
O25R T[n]	159	30.4	27.0	76.7	73.27
O25S	144	48.4	11.0	33.8	59.3
O25S T[p]	95.5	25.5	7.5	26.7	38.8
NCTC	465	2.34	5.5	9.42	120.5
NCTC T[f]	504	1.56	3.5	8.05	129.27
C01	167	94.8	132	112	126.45
C01 T [f]	198	115	116	110	134.75
C08	18.7	45	32.4	54.3	37.6
C08 T [f]	18.7	55.0	18.7	46.5	34.75
P02	52.0	13.1	10.2	17.7	23.25
P02 T [f]	35.5	12.9	10.9	11.7	17.75
P11	11.8	3.94	3.27	11.1	7.5275
P11 T [p]	16.4	3.72	7.49	9.77	9.345

Chemiluminescence values in mV.

T denotes hyaluronidase treated strains with capsule removed.

[p] denotes partial capsule removal after treatment with hyaluronidase

[f] denotes full removal of capsule after treatment with hyaluronidase

[n] denoted no change after treatment with hyaluronidase

TABLE 5.7 RELATIONSHIP BETWEEN BACTERIAL CELL HYDROPHOBICITY AND THE CHEMILUMINESCENT RESPONSE OF HUMAN PMNL.

STRAIN	HYDROPHOBICITY	CL[1]	MRC[2]
008	HYDROPHOBIC (M)	MEDIUM	MEDIUM
009	HYDROPHILIC (V)	MEDIUM	LOW
016	HYDROPHOBIC (M)	HIGH	HIGH
025R	HYDROPHOBIC (M)	MEDIUM	LOW
025S	HYDROPHILIC (V)	HIGH	MEDIUM
046	HYDROPHOBIC (M)	LOW	LOW
C01	HYDROPHILIC (M)	HIGH	HIGH
C02	HYDROPHILIC (M)	MEDIUM	LOW
C06	HYDROPHILIC (V)	MEDIUM	LOW
C08	HYDROPHILIC (V)	LOW	LOW
C10	HYDROPHILIC (V)	LOW	LOW
C18	HYDROPHOBIC (M)	LOW	LOW
NCTC	HYDROPHILIC (M)	MEDIUM	MEDIUM
P01	HYDROPHILIC (M)	LOW	LOW
P02	HYDROPHILIC (V)	MEDIUM	LOW
P04	HYDROPHILIC (V)	MEDIUM	LOW
P08	HYDROPHILIC (M)	LOW	LOW
P11	HYDROPHILIC (M)	LOW	LOW
P13	HYDROPHILIC (V)	LOW	LOW

HYDROPHOBICITY : Categorised as stated in Table 3.6

1. CL = Peak Chemiluminescence (mV):
Defined as : 0 - 50 LOW
51 - 100 MEDIUM
101 upwards HIGH

2. MRC = Maximal Rate Constant (mV/min) :
Defined as : 0 - 5 LOW
5.1 - 10 MEDIUM
10.1 upwards HIGH

hydrophilic strains, 6 exhibited low chemiluminescence and 8 high, while of 5 hydrophobic strains 2 had low and 3 had a medium/high respiratory burst. MRC values compared to hydrophobicity revealed that of the 14 hydrophilic strains 11 exhibited low, 2 showed medium and 1 had a high rate of ingestion. Hydrophobic strains had ingestion rates of 2 low, 2 medium and 1 high.

5.4 DISCUSSION

5.4.1 SERUM OPSONIZATION

As shown in figures 5.2 - 5.4, increasing the percentage serum concentration used for opsonization increased the mean peak chemiluminescent response. As the concentration of serum increases, the deposition of C3b and IgG on the bacterial surface increases, so allowing for increased interaction between the cell and the receptors of the PMNL. At a certain concentration the cell surface becomes saturated with opsonins, as there are no more receptors, therefore increasing the serum concentration does not further enhance chemiluminescence. This concentration is 15% for strains O16, P08, P13 and NCTC 11325, and 20% or greater for those remaining.

As shown by the three strains O25R, O25S and NCTC 11325 the SMG also induce a chemiluminescent response when unopsonized. In this instance the PMNL must recognise

and adhere to another aspect of the cell surface other than C3b and IgG. As this has not been studied in the SMG we can only look at this situation in other organisms and suggest a similar role in SMG. *E.coli* is phagocytosed in the absence of opsonins due to the presence of adhesins on its surface which mediate its direct adherence to PMNL (Sobel and Kaye 1990). One of these adhesins is mannose sensitive, and is recognised by mannose-binding receptors on the PMNL allowing subsequent phagocytosis. As mannose is a component of the cell surface of SMG, receptors on the PMNL may be able to recognise it and, in the absence of opsonins, allow ingestion.

The plasma glycoprotein fibronectin may facilitate phagocytosis of SMG in the body in the absence of specific opsonins. Both SMG (Willcox, Geyelin & Knox 1992) and PMNL (Procter, Prendergast & Mosher 1984) have been shown to bind fibronectin, and so the molecule may act as a bridge between the two. Fibronectin could also enhance the opsonic and protective activities of antibodies and complement as it does in relation to Group B streptococci (Hill et al 1984).

Although capsule tends to be antiphagocytic, in the absence of opsonins it can contribute to adherence of the PMNL. The gelatinous nature of the capsule may allow the PMNL to 'stick' to the bacterial cell in the absence of

specific adhesive reactions (Patrick & Larkin 1995). Indeed, results for O25R and O25S, unencapsulated and capsulated varieties of the same strain, show peak chemiluminescence values when unopsonized of 42.4 and 50.9 respectively, which may indicate that capsule enhances ingestion.

As whole pooled serum was used, it cannot be said whether complement or antibody play a larger role in opsonization of the SMG. Complement activity can be destroyed by heating serum to 56°C for 30 minutes, while further treatment can block either the alternative or classical pathways. Specific antibody can be removed by absorption. Use of these altered sera for opsonization of strains for use in the chemiluminescence assay would allow greater understanding of the specific mechanism of opsonization.

5.4.2 RATE OF CHEMILUMINESCENCE

The curve produced of the chemiluminescent response over time corresponds to the events of phagocytosis. The early phase of around 0 to 8 minutes, when the response is steadily rising, corresponds to the initial adherence of the bacteria to the white cell. As engulfment occurs and the killing of the bacteria proceeds at between 10 to 20 minutes, peak response occurs as release of superoxide anion, hydrogen peroxide, singlet oxygen and hydroxyl

radicals are at a maximum in order to kill the bacteria. The reaction then begins tailing off as the bacteria have been digested and PMNL activity returns to its resting rate. Most of the SMG reach their peak between 8 - 20 minutes. Those which peak at a later time, for example 025S and P13, may be more resistant to ingestion due to a factor such as capsule. The rate and magnitude of chemiluminescence should correlate with the radioactive ingestion assay, which is examined in the following chapter.

5.4.3 SPECIES AND ISOLATE TYPE SIGNIFICANCE

As some members of the SMG may remain part of the normal commensal flora and never cause infection, whilst others proceed to cause purulent infection at numerous body sites, it may be suggested that those which cause disease are perhaps aided by resistance to phagocytosis. Indeed, it has also been shown that certain species of SMG preferentially associate with specific infections, again suggesting some distinguishing virulence factor. However, there are no significant differences in chemiluminescence of the three species or in relation to the site of their isolation. Individual strains did show relatively high (C01 (274.6)) or low (P11 (9.94)) values, but these specific strains are likely to have other contributory factors in relation to pathogenicity.

5.4.4 ROLE OF CAPSULE

As has been discussed previously (Chapters 1 and 3), the capsule of the SMG has been implicated as a virulence factor. A high number of strains isolated from purulent infections possess capsular material, and those possessing capsule being able to induce subcutaneous abscesses in mice as the single infecting organism (Lewis et al 1988). In this work we have already seen that capsule hinders the adherence of SMG to BEC (Chapter 4) and suggested that the negative role it plays here must be counteracted by contributing a positive element to virulence, hypothesising resistance to phagocytosis. This supposition was made after considering the anti-phagocytic role of capsule in other organisms.

Capsules associated with bacterial pathogens protect against ingestion by PMNL through interference with opsonization (Finlay and Falkow 1989). They can prevent complement deposition on the bacterial cell surface, with those of pathogens more effective than the capsules of non-virulent strains. Capsule is only weakly immunogenic and masks the more immunogenic bacterial cell surface structures which would directly activate complement.

In pneumococci, C3b is bound to the unencapsulated cell wall via an amide link, while a thiolester-reactive site binds it to the capsule. The amide-linked molecules are

far more potent activators of phagocytosis than the thiolester-binding ones (Gordon, Johnson & Hostetter 1986), providing capsule with another virulence mechanism.

In *S.aureus* the mechanism by which type 1 capsule (mucoid) is antiphagocytic has been elucidated by Verbrugh et al (1982). They showed that the capsule shields C3b molecules deposited on the cell surface making them inaccessible to receptors on the membrane of PMNLs. In contrast Xu et al (1992) found microencapsulated strains of *S.aureus* (capsule types 5 and 8) did not resist opsonophagocytic killing in vitro by normal serum. Dale et al (1996) suggested a similar function of the hyaluronate capsule of type 18 Group A streptococci, that is acting as a physical barrier preventing C3b bound to the cell surface from interacting with the phagocyte receptor. This was suggested as the amount and pattern of C3 deposition on the surface of encapsulated and unencapsulated type 18 streptococci were not significantly different, but the unencapsulated mutant was unable to resist phagocytosis. The authors however found this was not the case for type 24 streptococci, as this strain was more susceptible to phagocytosis and killing despite similar encapsulation to the type 18 strain. They concluded that different mechanisms operate in preventing the opsonophagocytosis of Group A streptococci.

As detailed in the results section (5.3.5), treatment to remove capsule both raised and lowered chemiluminescent response, with no statistically significant difference between treated and untreated. This unusual dual effect may be due to the treatment process which, as detailed in Chapter 3, resulted in a number of outcomes. It may be that when the chemiluminescence increased after treatment this corresponded to the complete removal of surface material, whereas incomplete removal may lower chemiluminescence, perhaps by reducing the 'stickiness' of the bacterium for the PMNL. Chemiluminescence indicates the activation of the PMNL myeloperoxidase system and reflects the functional state of the phagocytic cell, but is not a direct measure of ingestion (McKinley et al 1993). Therefore, bacterial adherence to the surface of the PMNL can induce chemiluminescence rather than specifically ingestion, and this adherence may be aided by capsule although the organism is not internalised.

Capsule of the SMG was found by Lewis et al (1993b) to have no effect on phagocytic ingestion. Although the method used by these investigators did directly measure ingestion it involved counting of ingested organisms in PMNL by microscopic examination of cytospin slides, and therefore may involve human error in distinguishing and counting organisms within only a specified number of PMNL (100). Thus a radiometric ingestion assay was employed

(Chapter 6) to investigate the issues raised in the preliminary work described in the present chapter. This method has the advantage over chemiluminescence measurement of directly quantifying ingestion and is not affected by external factors such as endo/exo toxins (which can induce chemiluminescence). Also, unlike any microscopic examination, it provides a count of bacteria ingested by all the PMNL present in the assay system. This work will be described in the next chapter.

5.4.5 ROLE OF HYDROPHOBICITY

In the absence of opsonin, bacteria with greater surface hydrophobicity are more readily phagocytosed than those with hydrophilic characteristics. When opsonization is considered, the more hydrophobic a bacterial cell surface the more it absorbs the opsonin IgG (Absolom 1988). Indeed, opsonization with IgG is often accompanied by an increase in surface hydrophobicity and gives rise to increased phagocytic ingestion in vitro (Magnusson et al 1985).

Therefore it might have been expected that hydrophobic strains of SMG would induce a higher chemiluminescent response. In fact three of five hydrophobic strains had medium to high chemiluminescence, but there are insufficient data to conclude whether the hydrophobic nature enhanced opsonization and therefore ingestion. It

may also be argued that the difference between moderately hydrophobic and hydrophilic strains is very slight and may be altered by environmental factors. Galdiero et al (1993) found that *S.pyogenes* surface hydrophobicity decreased under prolonged starvation and this resulted in increased chemiluminescence, phagocytic ingestion and killing. A number of hydrophilic strains also showed high chemiluminescence, which suggests that other specific factors are more important in influencing the efficiency of opsonization, rather than the passive absorption of opsonins which is enhanced by hydrophobicity. Also, when considering surface interactions the role played by the surface electrical charge is more determinant than that played by hydrophobic domains.

CHAPTER 6 INGESTION OF THE SMG BY HUMAN PMNL

6.1 INTRODUCTION

As outlined in Chapter 5, resistance to phagocytosis may be an important aspect in the pathogenicity of the SMG. The host's first line of defence against bacterial invasion is phagocytosis and in order to cause disease a bacterium must be able to avoid ingestion and killing by the phagocytic cells. The ability to resist phagocytosis, or to exist within a phagocyte, is an important aspect of the pathogenicity of a number of bacteria, with capsule playing a major role in this resistance. Lewis et al (1993b) investigated the phagocytosis of SMG from dentoalveolar abscesses and the role of capsule in this process. The capsule of the SMG has been suggested as a pathogenicity factor of the group, as a contributory factor in both abscess formation and resistance to phagocytosis (detailed in Chapters 1 and 3), and was found in the previous chapter to have no effect on chemiluminescence. Some investigators believe that chemiluminescence is produced only by oxidation of the ingested organism (Allen 1977), while others state that luminol enhanced chemiluminescence reflects both extra- and intra-cellular generation of oxygen radicals (Fredlund 1993). It can also be induced by other factors such as endotoxin and certain chemicals (McKinley et al 1993). Therefore, as chemiluminescence may not be

measuring only internalised SMG, other methods must be employed. Lewis et al (1993) examined phagocytosis by microscopical examination of cytospin preparations and counted the number of organisms ingested by 100 PMNL. Using this method they found no difference in the degree of uptake between capsulate and non-capsulate SMG strains. A radiometric assay was used in this chapter to investigate the possible effects of isolate source or species, presence of capsule and relative cell surface hydrophobicity on ingestion of SMG strains by human PMNL. This assay allows the examination of the kinetics of ingestion as well as the percentage uptake of bacteria by measuring the PMNL-associated radioactivity of labelled bacteria released by lysis of the phagocytic cells.

6.2 MATERIALS AND METHODS

6.2.1 ISOLATION OF POLYMORPHONUCLEAR LEUKOCYTES

Separation of PMNL from blood was carried out by a slightly different method from that described in Chapter 5. The separating medium was changed to one which gave more efficient separation with whole blood rather than the plasma layer. Normal human blood was collected by venepuncture and added to 10 units per ml of heparin (Multiporin) to prevent clotting. 5 ml of Polymorphprep (Nycomed) was added to conical tubes and 5 ml of whole blood was layered on top, maintaining two tiers. The

tubes were centrifuged at 350 g for 30 minutes, resulting in the formation of 3 layers from which the PMNL layer was removed and washed with gel Hanks (500 g /10 mins). The resultant pellet was resuspended in gel Hanks, counted in a haemocytometer and diluted to a concentration of 1 x 10^7 PMNL/ml.

6.2.2 PREPARATION OF BACTERIA

Bacteria were grown overnight in BHIB + 0.3% YE in the presence of ^3H adenine 2 µCi/ml (Amersham, Buckinghamshire, England). They were harvested and washed three times with saline (1 000g / 15 mins), then adjusted to an OD 620 nm of 0.025 (1 x 10^7 cells/ml).

6.2.3 OPSONIZATION

Bacteria were opsonized with 10% pooled normal human serum as described previously (5.2.5).

6.2.4 ASSAY PROCEDURE

A modification of the method of Peterson et al (1977) was used.

For each test 4 scintillation vials were required to which were added :

TUBE	1	2	3	4
BACTERIA	0.1 ml	0.1 ml	0.1 ml	0.1 ml
PMNL	0.1 ml	0.1 ml	-	-
GEL HANKS	-	-	0.1 ml	0.1 ml

These were incubated in an orbital incubator (Gallenkamp, Glasgow) at 100 rpm for 10, 20 and 30 minutes after which they were processed as follows :

Tubes 1 & 3 :

Ice cold PBS (3 ml) was added and the tubes shaken, followed by centrifugation (110 g/ 5 mins). This procedure was repeated 2 times, followed by the addition of 3 ml of scintillation fluid.

Tubes 2 & 4 :

3 ml of scintillation fluid was added.

Ingestion of SMG by PMNL was calculated from radioactive counts using the following formula :

$$\frac{{}^{3}H \text{ DPM PMNL associated bacteria (Tube 1)} - {}^{3}H \text{ DPM non specific bacteria (Tube 3)}}{{}^{3}H \text{ DPM total bacteria and PMNL (Tube 2)} - {}^{3}H \text{ DPM total bacteria (Tube 4)}} \times 100\%$$

6.2.5 ROLE OF CAPSULE IN DETERMINING BACTERIAL SUSCEPTIBILITY TO INGESTION

Capsule was removed by treatment with hyaluronidase as described previously (3.2.3). For treated samples hyaluronidase (100 µl) was added to 1ml of bacteria at OD 620 nm of 0.025, while control samples of untreated test strains had 100 µl of PBS added. The time of incubation was increased from 20 to 30 minutes in an attempt to produce more effective capsule removal (see Chapter 3 for previous results). After incubation the samples were used in the assay.

6.2.6 MICROSCOPIC EVALUATION OF PHAGOCYTOSIS

Cytospin preparations were made of a selection of bacteria-PMNL mixtures for examination under the microscope. To permit this a second replicate of tube 1 was included in the assay. After the washing stages, the contents of this tube were resuspended in 1 ml of PBS and spun onto silane-coated slides using a Cytotek centrifuge (Miles Scientific, Ames Division, Indiana, USA) at 500 rpm for 5 minutes. After drying for 10 minutes the slides were Gram-stained and viewed.

Photographs were taken using a Nikon FX-35 A camera connected to a Nikon optiphot microscope and attachments.

6.2.7 STATISTICAL ANALYSIS

The Mann-Whitney U test was used to compare data, with a value of $p < 0.05$ being considered significant.

6.3 RESULTS

6.3.1 INGESTION OF THE SMG BY PMNL

The mean % ingestion of strains of SMG after incubation times for up to 30 minutes are illustrated in Table 6.1. The kinetics of ingestion reveals that the rate rose rapidly from 0 to 10 minutes, continued to rise somewhat more slowly from 10 to 20 minutes and finally levelled off by 30 minutes, as shown in Figure 6.1. Strain O25R which is ingested well shows this pattern of ingestion, whilst strain P02, which is poorly ingested, shows a different profile.

Again, as found in the chemiluminescence experiments reported in the previous chapter, there was often a large inter-experimental variation resulting in large standard deviations. This was due to inherent differences in the activity of PMNL from different donors, with some having highly active PMNLs which ingested bacteria well, whilst others had relatively inactive PMNL which were inefficient at ingestion.

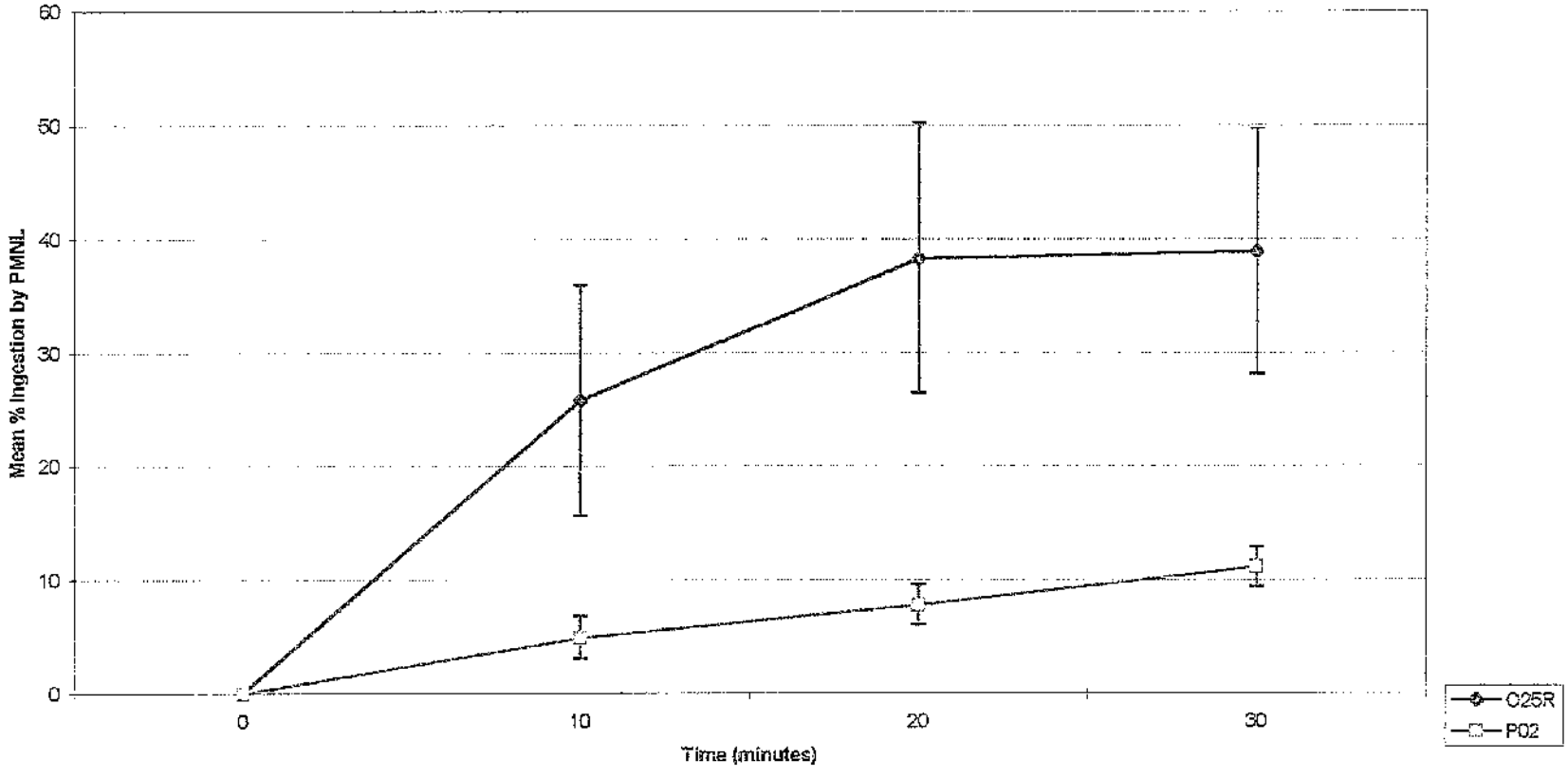
TABLE 6.1 INGESTION OF SMG ISOLATES BY PMNL

	MEAN % INGESTION AFTER INCUBATION TIME		
STRAIN	10 MIN	20 MIN	30 MIN
O08	8.84 (4.4)	13.18 (7.7)	18.16 (6.9)
O09	16.54 (3.1)	15.28 (4.9)	19.6 (13.75)
O16	26.0 (9.6)	33.2 (12.6)	43.7 (3.99)
O25R	25.8 (10.2)	38.3 (11.8)	38.9 (12.5)
O25S	7.2 (2.07)	10.5 (3.5)	9.9 (4.2)
O41	8.78 (5.95)	14.96 (4.2)	20.32 (6.4)
O46	5.98 (4.1)	11.32 (10.3)	14.52 (9.8)
C01	27.6 (21.7)	40.5 (14.1)	37.7 (9.7)
C02	4.5 (3.6)	11.9 (3.05)	19.6 (3.1)
C06	10.8 (7.45)	14.5 (7.9)	24.4 (11.2)
C08	1.8 (1.3)	3.55 (1.06)	5.7 (2.2)
C10	21.7 (13.98)	29.7 (13.9)	36.8 (9.5)
C18	8.2 (4.8)	15.3 (4.2)	26.0 (3.6)
P01	5.84 (3.5)	10.8 (7.3)	16.41 (6.7)
P02	4.9 (1.9)	7.8 (1.8)	11.1 (1.7)
P04	10.64 (7.75)	19.79 (9.6)	28.42 (4.2)
P08	19.86 (13.7)	32.05 (14.7)	48.26 (8.9)
P11	19.66 (15.9)	27.08 (15.5)	42.94 (10.6)
P13	14.6 (9.5)	20.7 (7.3)	37.02 (5.4)
NCTC 11325	18.0 (8.9)	28.9 (6.8)	43.36 (8.9)

Figures in brackets represent the standard deviation.

Mean % ingestion is average of 5 experiments performed with 5 different PMNL donors.

Figure 6.1 KINETICS OF INGESTION . Graph shows mean percentage ingestion of two strains (P02 & O25R) of SMG after incubation with human PMNL for 10, 20 & 30 minutes. Mean is average of 5 runs with 5 different PMNL donors. Bars represent +/- 1 S.D.



6.3.2 SIGNIFICANCE OF SPECIES AND ISOLATE TYPE ON SUSCEPTIBILITY TO PHAGOCYTOSIS

As shown in Figure 6.2 (a & b) there were no significant differences between the ingestion of either the three species types or the different isolate types.

6.3.3 EFFECT OF CAPSULE ON BACTERIAL SUSCEPTIBILITY TO PHAGOCYTOSIS

When the data for mean % ingestion of strains were separated into two groups according to capsule type + or +(s), as defined in Chapter 3, there was a significant difference ($p=0.0304$) between the two groups. Those possessing a larger capsule were ingested to a lesser extent than those with the smaller (or no) capsule, as illustrated in Figure 6.3.

In addition, when capsulate strains were treated with hyaluronidase, the results illustrated in Table 6.2 showed a significant difference ($p=0.0313$) between the mean of untreated and treated cell suspensions. Figure 6.4 shows poorly-ingested strain P02 before and after hyaluronidase treatment, while Figure 6.5 shows well-ingested strain O25R under the same conditions. In both cases a significant increase in the percentage of ingested bacteria can be seen.

FIGURE 6.2 (a). Susceptibility of Different Species to Opsono-phagocytosis. Bar chart shows the mean % ingestion of SMG strains according to species. Bars represent +/- 1 standard deviation.

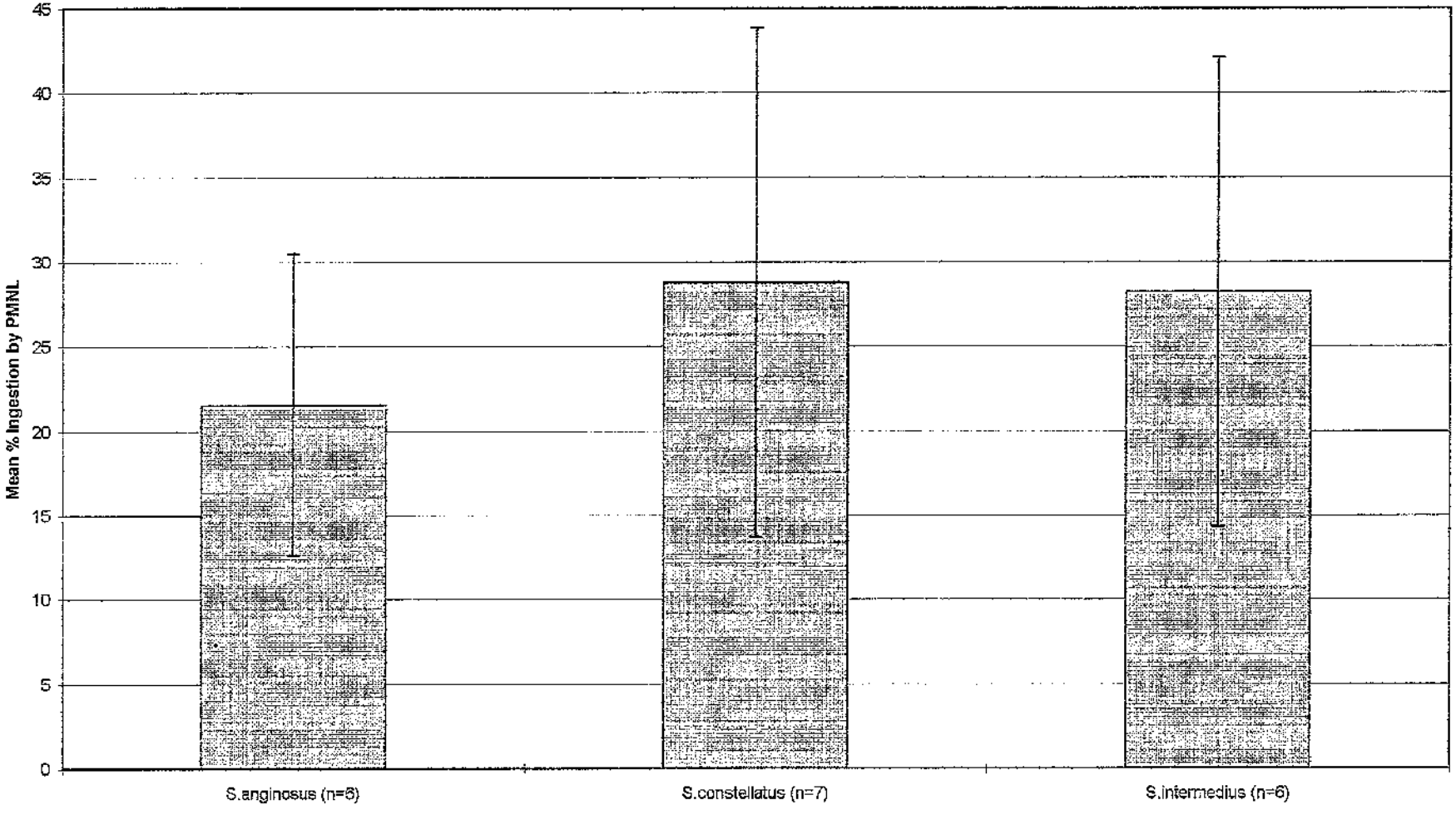


Figure 6.2 (b) Susceptibility of Different Isolate Types to Opsono-phagocytosis. Bar chart shows the mean % ingestion of SMG strains according to isolate type. Bars represent +/- 1 standard deviation.

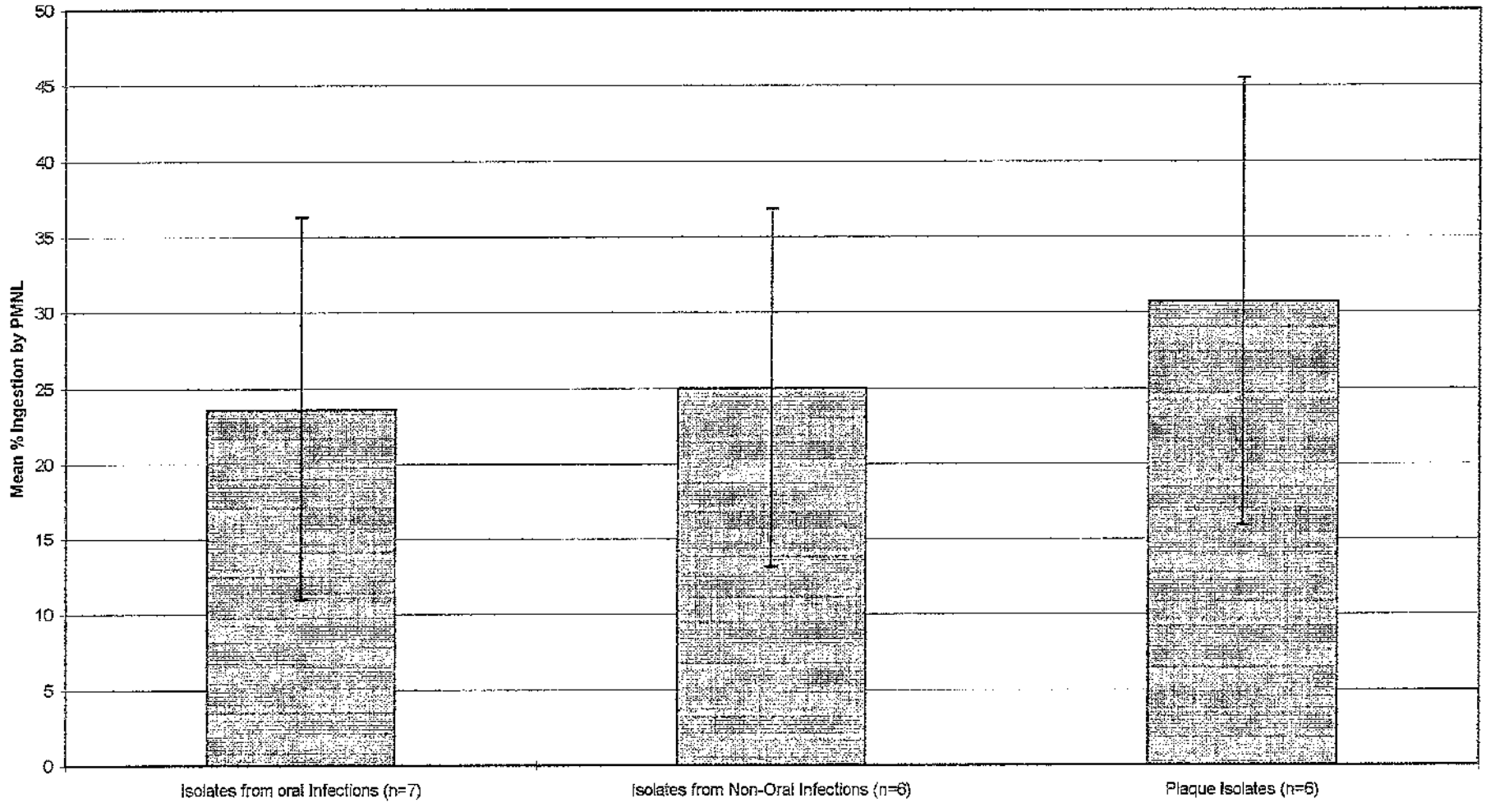


FIGURE 6.3 Effect of Capsule on Bacterial Susceptibility to Phagocytosis. Bar chart shows the mean % ingestion of SMG strains according to their possession of capsular material of either large type (+) or smaller type (+(s)/-). Bars represent +/- 1 S.D.

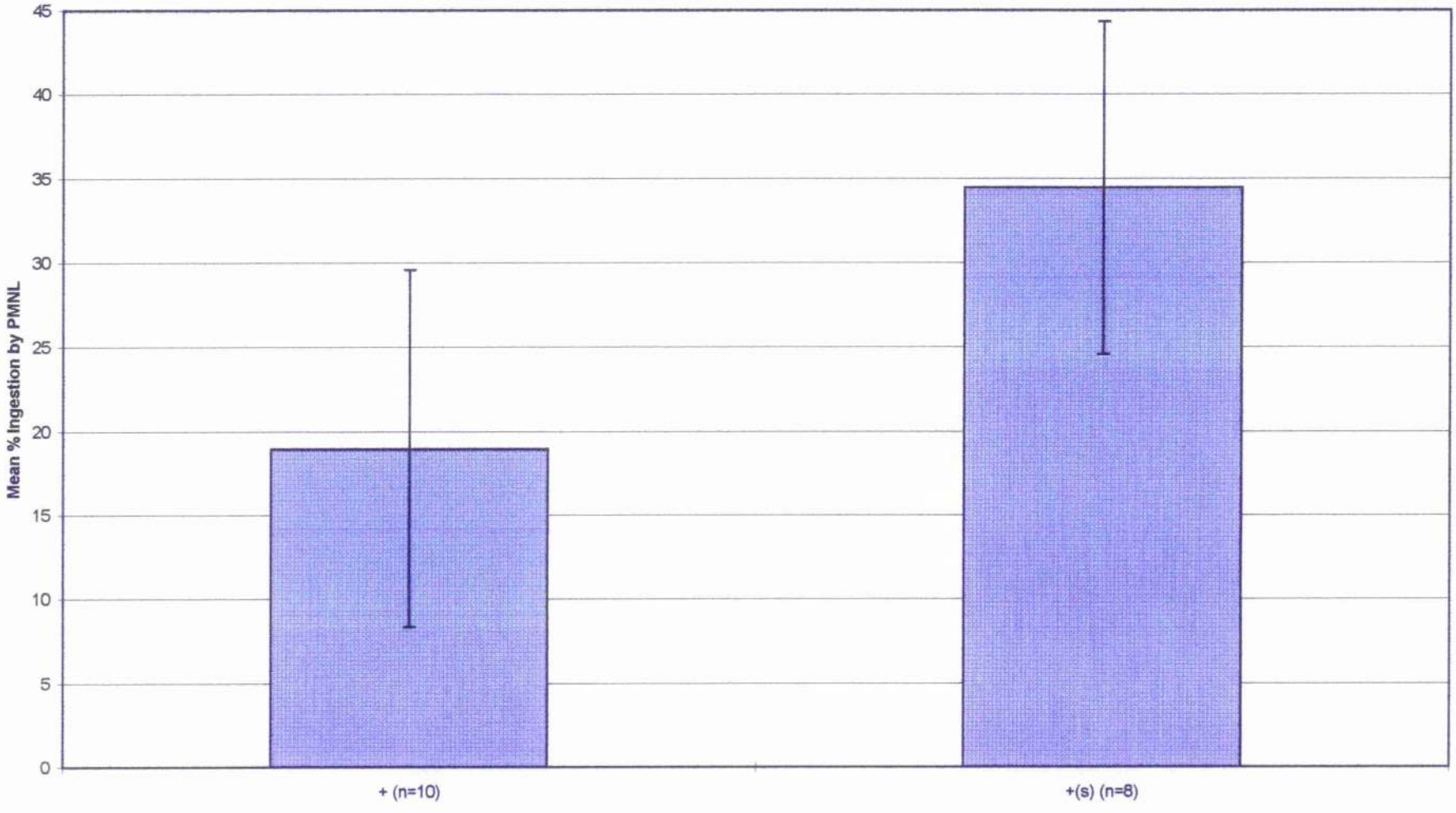
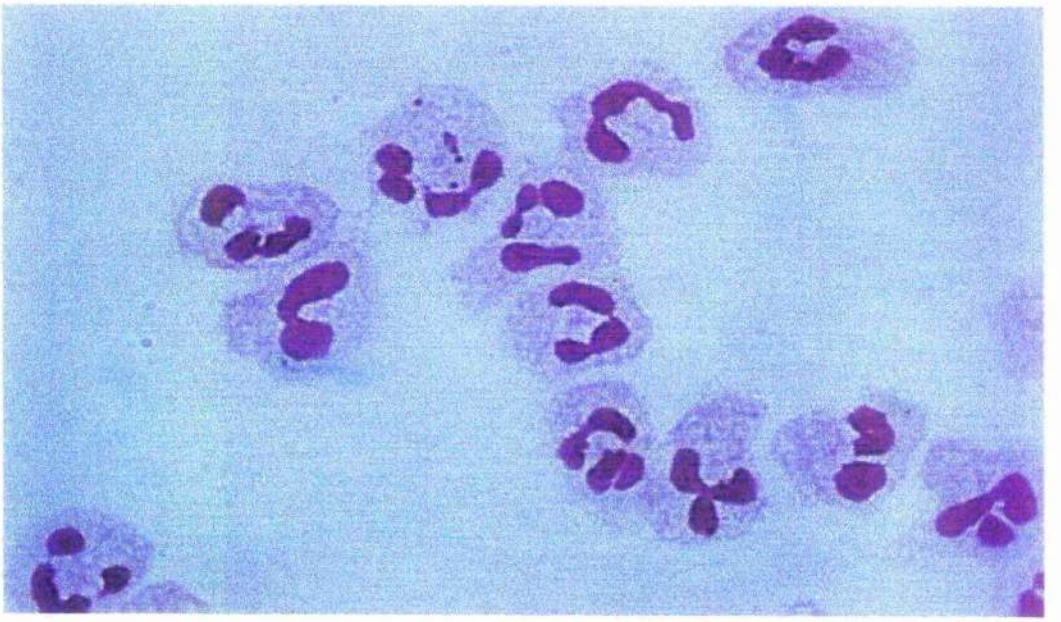


TABLE 6.2 INGESTION OF SMG ISOLATES BEFORE AND AFTER TREATMENT WITH HYALURONIDASE

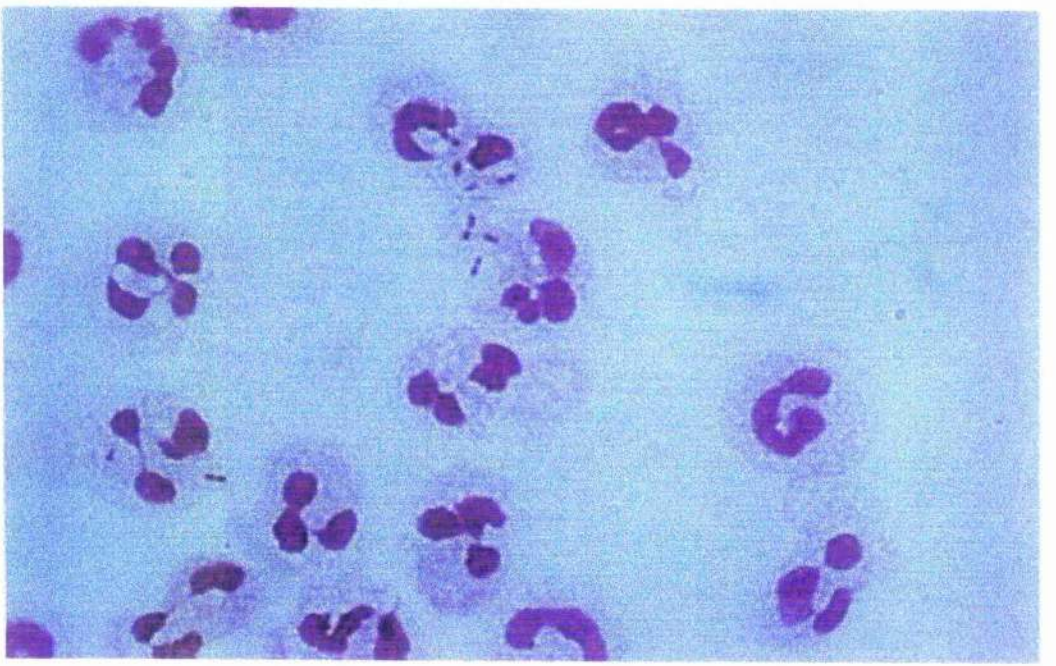
STRAIN	MEAN % INGESTION OF SMG WITHOUT HYALURONIDASE TREATMENT (S.D.)	MEAN % INGESTION OF SMG WITH HYALURONIDASE TREATMENT (S.D.)
O09	16.526 (11.7)	25.07 (10.9)
O25R	11.46 (7.4)	19.1 (4.2)
O25S	20.56 (10.3)	32.7 (16.2)
C01	20.8 (9.1)	24.9 (10.5)
C18	14.3 (6.25)	26.7 (4.7)
P02	1.9 (3.2)	2.9 (2.9)
P11	12.4 (6.1)	21.7 (8.6)
NCTC 11325	12.7 (7.9)	20.1 (7.2)

Mean % ingestion is average of 5 runs performed with 5 different PMNL donors.

FIGURE 6.4 PHAGOCYTOSIS OF POORLY INGESTED STRAIN P02 BEFORE (A) AND AFTER (B) TREATMENT WITH HYALURONIDASE.

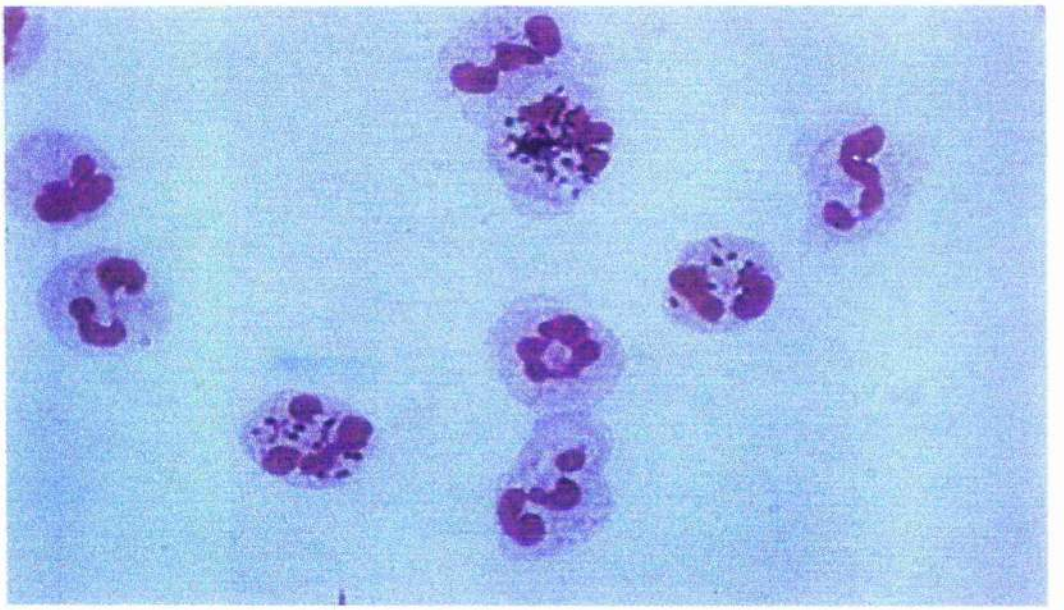


(A) UNTREATED (Mag. x 500)

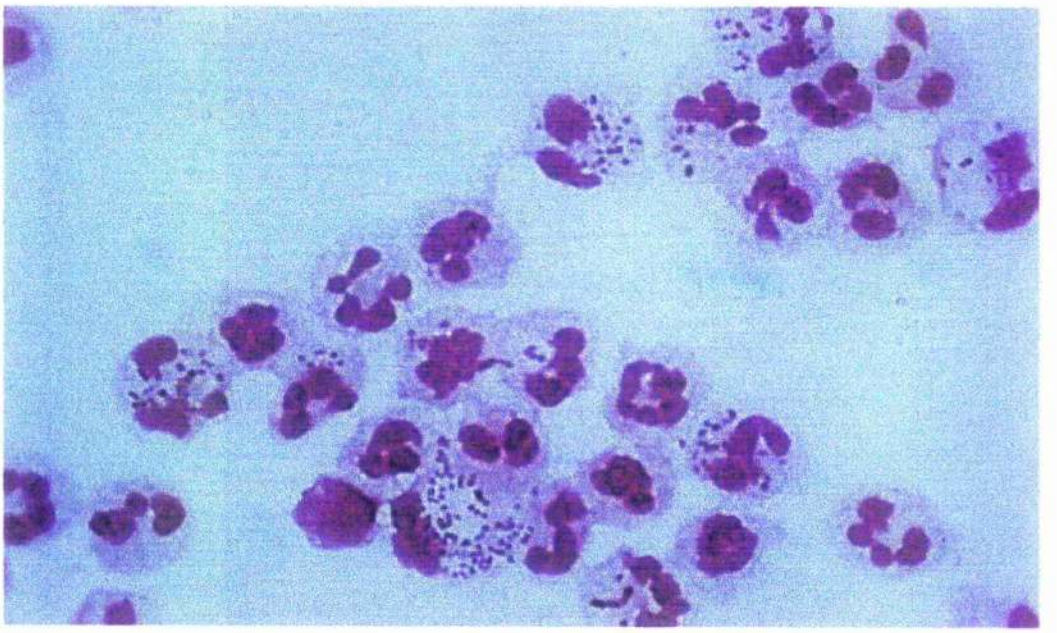


(B) TREATED (Mag. x 500)

FIGURE 6.5 PHAGOCYTOSIS OF WELL INGESTED STRAIN 025R BEFORE (A) AND AFTER (B) TREATMENT WITH HYALURONIDASE.



(A) UNTREATED (Mag. x 500)



(B) TREATED (Mag. x 500)

These observations suggest that the capsule of the SMG has antiphagocytic properties.

6.3.4 COMPARISON BETWEEN INDUCTION OF CHEMILUMINESCENCE AND RADIOMETRIC INGESTION ASSAYS

Table 6.3 shows chemiluminescence and mean % ingestion values for test strains. There was an association between the characterisation of phagocytosis as low, medium or high, as found by chemiluminescence and radiometric assay, in 8/20 strains. As both tests measure similar properties it would be expected that a high chemiluminescence would subsequently give a high % ingestion. However, these tests were often carried out on different days with different PMNL donors, and so variation may have occured due to high and low activity of individual PMNL.

6.3.5 EFFECT OF HYDROPHOBICITY ON SUSCEPTIBILITY TO INGESTION BY PMNL

Table 6.3 also shows the relationship between % ingestion and bacterial cell surface hydrophobicity. As was stated in the previous chapter a hydrophobic nature tends to render a bacterium more prone to phagocytic ingestion than those with a hydrophilic cell surface. Of four hydrophobic strains 3 were moderately to well ingested,

TABLE 6.3 RELATIONSHIP BETWEEN INGESTION, CHEMILUMINESCENCE AND HYDROPHOBICITY

STRAIN	CHEMILUM.	% INGESTION	HYDROPHOBICITY
O08	50.7 (HIGH)	18.16 (MED.)	HYDROPHOBIC (M)
O09	55.2 (MED.)	19.6 (MED.)	HYDROPHILIC (V)
O16	139 (HIGH)	43.7 (HIGH)	HYDROPHOBIC (M)
O25R	65.6 (MED.)	38.9 (HIGH)	HYDROPHOBIC (M)
O25S	123 (HIGH)	9.9 (LOW)	HYDROPHILIC (V)
O41	49.2 (LOW)	20.32 (MED.)	N.T.
O46	11.2 (LOW)	14.52 (LOW)	HYDROPHOBIC (M)
C01	236 (HIGH)	37.7 (HIGH)	HYDROPHILIC (M)
C02	43.2 (LOW)	19.6 (MED.)	HYDROPHILIC (M)
C06	44.6 (LOW)	24.4 (MED.)	HYDROPHILIC (V)
C08	34.5 (LOW)	5.7 (LOW)	HYDROPHILIC (V)
C10	5.9 (LOW)	36.8 (HIGH)	HYDROPHILIC (V)
C18	39.1 (LOW)	26 (MED.)	HYDROPHOBIC (M)
P01	6.7 (LOW)	16.41 (MED.)	HYDROPHILIC (M)
P02	62.4 (MED.)	11.1 (LOW)	HYDROPHILIC (V)
P04	66.8 (MED.)	28.42 (MED.)	HYDROPHILIC (V)
P08	12.1 (LOW)	48.26 (HIGH)	HYDROPHILIC (M)
P11	7.7 (LOW)	42.94 (HIGH)	HYDROPHILIC (M)
P13	36 (LOW)	37.02 (HIGH)	HYDROPHILIC (V)
NCTC 11325	105.1 (HIGH)	43.36 (HIGH)	HYDROPHILIC (M)

Chemiluminescence : 0 - 50 (low) 51 - 100 (medium) 101 upwards (high).

% Ingestion : 0 - 15% (low) 16 - 30% (medium) 31% upwards (high).

Hydrophobicity as determined by Hydrophobic interaction chromatography and categorised as indicated in Table 3.6.

N.T. indicates not tested.

while hydrophilic strains showed varying levels of ingestion (2/8 high, 3/8 medium and 3/8 low). These results were the same as reported in the previous chapter, namely that no correlation could be found between cell surface hydrophobicity and susceptibility to ingestion.

6.4 DISCUSSION

6.4.1 ANTIPHAGOCYTIC EFFECT OF CAPSULAR MATERIAL

The antiphagocytic effect of the hyaluronic acid capsule of the SMG was illustrated in two ways. First, there was a significant difference between ingestion by PMNL of well- and poorly-encapsulated strains. Secondly, removal of capsule by treatment with hyaluronidase caused increased ingestion in all cases. This was in contrast to the findings of Lewis et al (1993b) who found that ingestion of capsulate and non-capsulate SMG organisms did not differ. However, the method employed in the study of Lewis et al (1993b) involved microscopical counting of the number of ingested organisms in 100 PMNL, while the one used in this study measured ingestion of radiolabelled bacteria by all PMNL present in the assay, which may account for the difference in findings. Also no attempt was made by the previous investigators to remove capsule and assess the effect of this structure on ingestion of capsulate SMG. The use here of

hyaluronidase to remove capsule of the SMG follows the basic methods of a number of investigators examining the effects of capsule of Group A and C streptococci. They similarly found that treatment with hyaluronidase removed capsular material and gave a resultant increase in phagocytic ingestion (Kass and Seastone 1943). These findings led to much more detailed investigation of these organisms, arriving at a point today where the capsule and surface structures of Group A and C streptococci are well documented (Ofek and Doyle 1994). It is hoped that the initial work reported in this thesis will prompt the same investigation of the SMG.

Removal of capsule did not significantly affect the results of the chemiluminescence experiments reported in Chapter 5. This may have been due to a number of factors. First, as suggested in Chapter 5, chemiluminescence may be triggered by both organisms adherent to the phagocytic cell as well as those ingested, whereas the assay used here measures only ingestion. Secondly, as was determined in Chapter 3, treatment with hyaluronidase for 20 minutes still left some organisms with capsular material present. With the increase in treatment time to 30 minutes it is possible that more capsule was removed and this was reflected in the results. Finally, it may simply be due to the number of replicates performed in each assay. Any trend towards antiphagocytic effect of capsule may not have proved

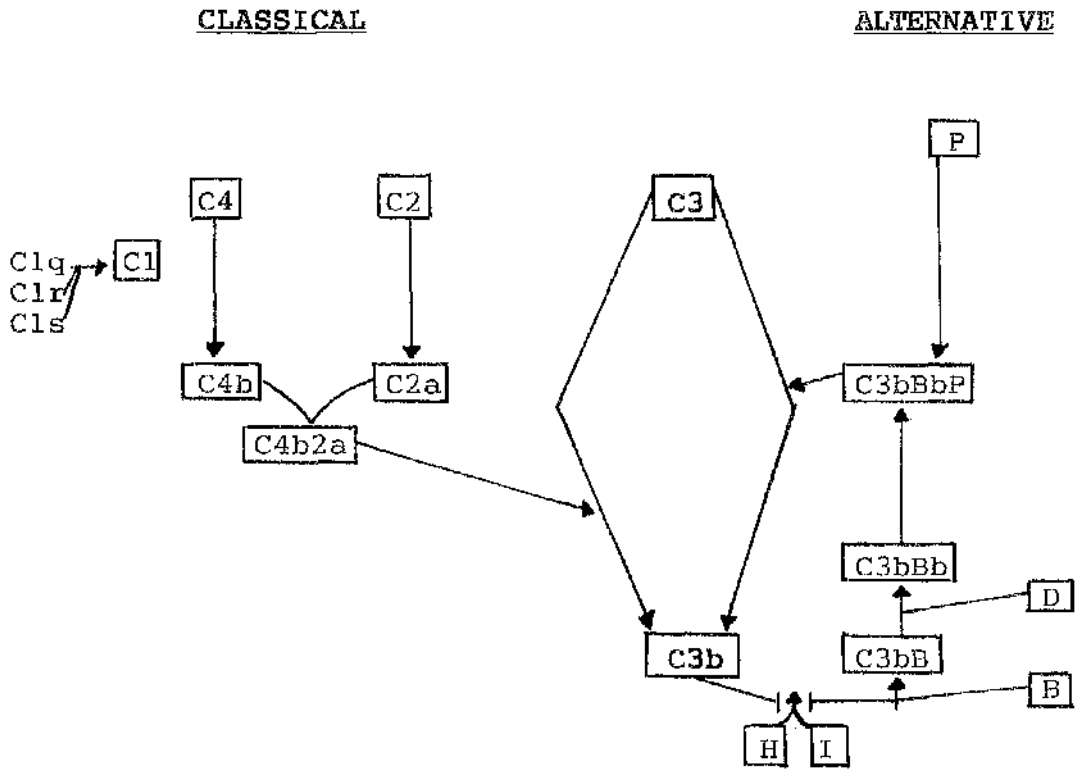
statistically significant with only three replicates in the chemiluminescence assay, but was definitely significant when observed in five runs of radiometric uptake.

Using knowledge gained from the study of other encapsulated organisms, a role of the capsule in SMG resistance to phagocytosis can be suggested. First, polysaccharide capsules have a high negative charge and are hydrophilic (Van Oss & Gillman 1972). These factors are involved in the physico-chemical aspects of non-specific interaction between cells as detailed in Chapter 3, where repulsion between like surfaces occurs. This would make it difficult for the first stage of phagocytosis, recognition, to occur, since close contact would be inhibited. However, in the presence of opsonins these effects will be over-ridden and specific adhesin-receptor interactions will occur between the bacterium and phagocyte. Also, with regard to recognition, the capsule may provide a protective coating, masking surface structures of the cell, thus preventing the PMNL from attaching to receptors on the bacterial cell wall (Fox 1974). Whitnack, Bisno and Beachey (1981) found that the hyaluronate capsule of Group A streptococci prevented attachment of the organism to macrophages, and treatment with hyaluronidase to remove the capsule increased adherence. As mentioned in Chapter 5, *S.aureus* capsule shields C3b molecules, deposited on the cell surface

during opsonization, from receptors on the PMNL surface (Verbrugh et al 1982). Similarly the hyaluronate capsule of Group A streptococci can mask bound C3b on the organism, preventing interaction with the phagocyte receptors (Dale et al 1996). Therefore SMG capsule may contribute to pathogenicity either with or without opsonization by masking the surface of the organism, with capsule removal resulting in better interaction between phagocyte and bacterium.

It has been suggested by a number of investigators (Peterson et al 1978, Edwards et al 1980, Pluschke et al 1983) that the antiphagocytic properties of bacterial capsule revolve around interference with the opsonization process, especially that of deposition of complement on the bacterial surface. Encapsulated organisms can interfere with opsonization via the classical and alternative pathways of complement activation. The activation of complement by both pathways is illustrated in Figure 6.6. The classical pathway is activated by immune complexes, with C1q binding to complexes which activates C1r and C1s, resulting in formation of active C1. This cleaves C4 and C2 giving two proteins, C4b and C2a, which combine to form the classic pathway convertase C4b2a, which converts C3 to C3b. There is a steady turnover of C3 to C3b via the alternative pathway independent of immune complex. However, this is of a low frequency and does not allow for sufficient deposition of

FIGURE 6.6 SCHEMATIC REPRESENTATION OF COMPLEMENT ACTIVATION VIA THE ALTERNATIVE AND CLASSICAL PATHWAYS



C3b on the surface of the bacterium to permit effective opsonization. Amplification of this conversion takes place in serum by means of a feedback loop which, due to deposition of small amounts of C3b on the bacterial surface, causes more factors B, D and P to be recruited, resulting in formation of the alternative pathway convertase C3bBbP, thereby amplifying conversion. However another two serum proteins, H and I, compete with factors B and D for C3b deposited on particle surfaces and therefore can inhibit opsonization.

Kasper (1986) has suggested that capsular material affords resistance to phagocytosis by interfering with the deposition of complement on the bacterial cell surface. Organisms without capsule are able to activate the complement cascade independently of antibody, resulting in direct deposition of C3 which is then converted to C3b on the cell surface. Encapsulated organisms, however, are resistant to this direct complement activation mechanism and require immune complex formation on their surfaces to activate complement. This results in the encapsulated organism having insufficient C3b deposited on its surface, and therefore it is more difficult for the PMNL to adhere.

Capsular polysaccharide can activate the amplification loop in two ways. Edwards et al (1980) showed that sialic acid contained in the capsular polysaccharide of

type III Group B streptococci has a high affinity for factor H. This interaction between the two results in formation of H-C3b complexes, rather than C3bBbP, blocking the amplification loop and giving resistance to opsono-phagocytosis in the absence of antibody. In the presence of antibody the classical pathway would be employed and over-ride the blocking mechanism. Brown et al (1983) illustrated a similar mechanism in type 7 and 12 pneumococcal capsules. These had decreased affinity for factor B compared to cell wall, which has a similar effect as increased affinity for factor H or I, that is, formation of an H-C3b complex which inactivates the amplification loop.

Kasper (1986) also suggested that bacterial capsular polysaccharides mimic oligosaccharides found on glycoproteins throughout the body in order to avoid opsonization. As the complement system has a protective mechanism to prevent activating itself against the body's carbohydrates, encapsulated organisms exhibiting these can escape opsonization, being unrecognised as foreign.

Like the SMG, *Bacteroides fragilis* strains have been found to cause abscesses in mice when encapsulated organisms were used as the inoculum, but not with unencapsulated strains (Onderdonk et al 1977). Simon et al (1982) found that an increase in the size of capsule of *Bacteroides fragilis* gave increased resistance to phagocytosis and opsonophagocytic

killing by neutrophils. Therefore, the capsule of *Bacteroides* species has antiphagocytic properties similar to the SMG, allowing the organism to persist in the host and cause abscesses. It has been suggested that *Bacteroides* species may provide protection against phagocytosis for other organisms present in polymicrobial infections. This and other possible protective mechanisms operating within mixed infections will be discussed in the following section.

6.4.2 ASSOCIATION OF SPECIES AND SOURCE OF ISOLATE WITH RESISTANCE TO PHAGOCYTOSIS

There were no significant differences in the relative efficacy of phagocytic ingestion of the three species of SMG or in relation to the site of their isolation. Therefore the enhanced ability of certain species to cause specific diseases is due presumably to factors other than antiphagocytic properties.

As organisms of the SMG can be both the only isolate from an infection (Kambal 1987) or one of a number of species present (Van der Auwera 1985), it is likely that the overall ability of the group to cause disease involves a combination of virulence factors of the SMG and interaction with other species of the polymicrobial infection. Toyoda, Kusano and Saito (1995) found that *S.constellatus* produced lactic and acetic acids, but these

did not diminish phagocytic killing as they have been shown to do in other organisms (see this section). However, they did detect reduced phagocytic killing of a virulent strain of *S.constellatus* compared to an avirulent strain, suggesting it was mediated by a structural component of the organism. They suggested that this delayed bacterial clearance may be a virulence factor and may explain why the SMG can be the sole organism isolated.

The SMG are often found in mixed infections, especially with anaerobes, where a synergistic relationship has been suggested. Anaerobes may contribute to a polymicrobial infection in a number of ways: 1. by impairing host defences, 2. by providing nutrients for other species, 3. by altering the environment which then promotes survival of other species and 4. by transferring virulence factors between organisms, with several of these occurring at initial phases of infection (Rotstein, Pruett and Simmons 1985).

Bacteroides species have been extensively studied as a group of anaerobes which may produce these effects in mixed infections. They compete with aerobes for opsonins, thereby interfering with the phagocytic process (Ingham et al 1981). Incubation of *Bacteroides* species with serum depletes both heat-stable and heat-labile opsonins which results in insufficient opsonization of

aerobes (Wade, Kasper and Mandell 1983). The location of the components responsible for this is subject to conflicting reports, with Ingham et al (1981) suggesting the intact cell, Connolly et al (1984) the capsule, Wade et al (1983) the whole cell but not the capsule and Jones and Gemmell (1986) lipopolysaccharide. Bacteroides culture supernatants have also been shown to inhibit neutrophil migration, generation of chemiluminescence and killing by phagocytes (Namavar et al 1983). This was shown to be due to production of short chain fatty acids (SCFAs), especially succinic acid. Rotstein et al (1989) found the culture filtrate of *B.fragilis* 9032 to inhibit neutrophil migration and phagocytic killing of *E.coli*, while Eftimiadi et al (1990) found SCFAs to inhibit phagocytosis of *S.aureus*. The mechanism of activity is suggested to be that SCFAs cross the plasma membrane of the neutrophil, lowering the intracellular pH, resulting in impaired neutrophil function (Rotstein et al 1987). Indeed Finlay-Jones et al (1991) found that abscess-derived neutrophils had impaired killing mechanisms against *E.coli* and *P.mirabilis*. Therefore, in these infections there appears to be a synergistic effect between aerobes and anaerobes which could contribute to the pathogenicity of the SMG. This indeed was reported by Shinzato and Saito (1994) who found that *S.constellatus* multiplied at a greater rate in culture mixed with *P.intermedia* (formerly bacteroides) or its culture filtrate

than when cultured alone in broth. Furthermore, the culture filtrate of *P.intermedia* inhibited the bactericidal activity of neutrophils. Therefore, within an abscess the SMG may be protected at the initial stages of infection by their antiphagocytic capsule until the stationary phase is reached, when production of high concentrations of SCFAs by anaerobes lowers the pH and inhibits phagocytic function.

6.4.3 EFFECT OF BACTERIAL HYDROPHOBICITY ON SUSCEPTIBILITY TO INGESTION BY PMNL

As was found previously using the chemiluminescence assay, cell surface hydrophobicity had no effect on the percentage of ingested bacteria. The more hydrophobic a cell surface the more it absorbs IgG, which therefore results in increased phagocytic ingestion. In turn, opsonization with IgG increases cell surface hydrophobicity and therefore increases phagocytosis. However, complement is the major opsonin when whole serum is used and this effect will be minimal. It is likely that cell surface hydrophobicity does not play a large role in the adherence between bacterium and polymorph when opsonization has taken place. This involves specific adhesin-receptor interactions which override any physico-chemical attractive/repulsive forces operating. Therefore, factors which can either enhance or inhibit complement deposition as described in section 6.4.1 are

likely to be more significant in affecting phagocytic ingestion.

CHAPTER 7 FINAL DISCUSSION AND CONCLUSIONS

7.1 INTRODUCTION

As was stated at the beginning of this thesis, the virulence factors of the SMG contributing to their pathogenicity had been little studied, and the aim of this work was to examine some of the factors which may explain their ability to cause purulent infections. This chapter will review the findings of this thesis and suggest possible further work.

7.2 ISOLATION, IDENTIFICATION AND CHARACTERISATION

A group of strains were isolated from dental abscesses, non-oral clinical infections and dental plaque. These were identified as one of the three species *S.anginosus*, *S.constellatus* or *S.intermedius* using both the fluorogenic scheme of Whiley et al (1990) and the commercially available Rapid ID 32 Strep system (API Laboratory Products). Both systems were used as single tests, but occasionally provided doubtful identification and combination of the two provided further characterisation. *S.intermedius* was found to be the most common isolate from plaque, with *S.anginosus* and *S.constellatus* at similar levels in abscess and clinical isolates. Insufficient numbers were studied in these cases to concur with Whiley et al (1992), who found *S.constellatus* to be most common of dental

abscess isolates. Lancefield groups A, C and F were found, with group F most numerous where a grouping was found, but with non-groupable strains predominating. This was in agreement with a number of investigators, who also found Lancefield group G amongst test strains, but again insufficient total numbers may account for its absence here. Hyaluronidase activity was found in *S.constellatus* and *S.intermedius* strains at titres ranging from ½ to 1/128. One strain of *S.anginosus* showed hyaluronidase activity, which contradicts the findings of other investigators who found it confined to the other two species, and who have used this as an identification factor (Homer et al 1993). Streptolysin O and S activity was also found in a range of isolates at titres from ½ to 1/64. DNAse activity was found in 71% of abscess isolates, 60% of clinical isolates and 38% of plaque isolates. Collectively these activities and others not screened for by others, such as possession of chondroitin sulphate depolymerase, may contribute to the pathogenicity of the SMG. It can therefore be seen that the SMG represent a heterogenous group exhibiting a wide range of toxin and enzyme activities, although some could be attributed to either the source of the isolate or the species.

7.3 SURFACE PROPERTIES OF THE SMG

As one of the first areas of interaction between the host

and invading organism, the surface of the SMG was examined. India ink staining of capsule revealed strains had two possible thicknesses of capsule, + or +(s), with one strain having no capsule at all. Plaque and clinical isolates had smaller capsules, with abscess isolates having larger ones. Electron microscopy also revealed + type capsule, which was a thick, densely stained mass, while +(s) capsule appeared wispier and less heavily stained. Treatment with hyaluronidase removed capsule to varying extents, and indicated a hyaluronic acid component. Strains showed hydrophobic and hydrophilic surface characteristics, demonstrated using two test systems which correlated well with each other. There was no correlation between possession of capsule and cell surface hydrophobicity.

7.4 ADHERENCE OF THE SMG TO BEC

Dental abscess isolates were found to adhere to BEC to a greater extent than both clinical and plaque isolates, in agreement with the findings of Willcox and Knox (1990). Capsule hindered adherence of test strains to BEC, and treatment with hyaluronidase to remove capsule resulted in correspondingly increased adherence. It was suggested that the capsule masked specific adhesins on the SMG surface which allowed adherence to BEC. Most poorly adherent strains were hydrophilic, but strongly adherent strains showed both hydrophilic and hydrophobic

characteristics, therefore no significance could be attributed to cell surface hydrophobicity in adherence of the SMG.

7.5 OPSONIC REQUIREMENTS OF THE SMG AND CHEMILUMINESCENCE OF PMNL

Opsonization with normal human pooled serum was found to increase chemiluminescence, with serum concentrations of 15% - 20% or over proving optimum. Neither capsule or cell surface hydrophobicity had any effect on chemiluminescence. No significance could be attributed to either species or isolate type in the ability to elicit a chemiluminescent response.

7.6 PHAGOCYTIC INGESTION OF THE SMG

The capsule of the SMG was found to be antiphagocytic, and strains with small capsules were more readily ingested than those with large capsules. Removal of capsule increased the ingestion of most strains. Cell surface hydrophobicity had no effect on ingestion, and no significance could be placed on species or isolate type and ingestion.

7.7 CONCLUSIONS

At the beginning of this work the aim was to investigate some of the virulence factors of the SMG which helped them to cause pyogenic disease. The group of organisms was found to be highly heterogeneous, with strains possessing a variety of toxin and enzyme activities and various cell surface characteristics. As had been suggested by previous investigators (Brook and Walker 1985; Lewis et al 1988), capsule was found to contribute to the pathogenicity of the SMG. First, it had a negative effect by inhibiting the adherence of strains to BEC and secondly, the positive effect of resistance to phagocytosis. Other virulence factors of SMG strains found by other investigators include the ability of Lancefield Group C SMG to bind albumin which may be related to their ability to aggregate human platelets (Willcox et al 1994). Willcox et al (1990) also found that isolates from clinical infections bound fibronectin to a greater extent than other strains, which may aid pathogenicity. Homer et al (1994) suggested that the ability of *S.intermedius* strains to produce glycosaminoglycan-depolymerising activity may help to explain its association with deep-seated abscess formation, especially in the brain. Lima et al (1992) also found *S.intermedius* to produce an immunosuppressive protein which may aid survival in the host.

Therefore, although the SMG can be a part of the normal flora and cause no harm to the host there are specific factors attributable to either specific species or isolate types which can be considered as virulence factors. However, these are unlikely to fully explain the ability of the SMG to cause abscesses and endocarditis amongst other infections, and the effect of other organisms present in these infections must be considered. The findings of this work have only discovered a small section of the overall pathogenicity factors of the SMG and much further work is needed to gain a full understanding of this clinically significant group.

7.8 FURTHER WORK

The preliminary findings of the experiments performed in this investigation have raised a number of questions to be addressed in further work. First, do the pathogenicity factors demonstrated *in vitro*, such as hyaluronidase and haemolysin activity and capsule, elicit an immune response *in vivo*. This may be investigated by collecting blood and pus samples from patients with SMG infections such as dental abscesses. The organism can then be isolated, cultured to elaborate various exoenzymes and the serum used in immunoblotting in order to determine if antibodies have been produced against these factors.

Secondly, the capsule of the SMG has been confirmed as having a role in pathogenicity, and it is therefore important to understand the structure of the capsule. Hyaluronic acid was shown to be a component, due to digestion with hyaluronidase, but it is not known whether there are other elements. Also useful in further defining the role of capsule would be the creation of a capsule-free mutant strain. Wessels et al (1991) confirmed a hyaluronic acid capsule as a virulence factor for mucoid group A streptococci by deriving an acapsular mutant through transposon mutagenesis. This showed that the acapsular strain was sensitive to phagocytic killing and had 100-fold reduction in virulence in mice. The same method could be employed to confirm its role in the SMG.

Thirdly, further investigation of the phagocytosis of the SMG would be valuable. In addition to resistance to ingestion, organisms can resist intracellular killing, persisting within the phagocyte, thus helping the organism to cause disease. This can be measured using a killing assay, where after incubation of the bacteria/PMNL mixture a sample is added to distilled water to lyse the PMNL, 10 fold dilutions are made and plated out and counts taken, after which a killing index can be calculated (Rotstein et al 1985). This method has been used to study the SMG. Shinzato and Saito(1994) found a culture filtrate of *Prevotella intermedia* inhibited

phagocytic killing of a strain of *S.constellatus*, while Toyoda et al (1995) found a culture filtrate of *S.constellatus* from a pulmonary infection inhibited phagocytic killing. Both these studies were performed on a few strains of *S.constellatus*, and it would be interesting to perform similar investigation on the range of isolates collected for this work. This would enable the determination of relevance of species or isolate. Another area concerning phagocytosis of the SMG which could be investigated is that of the activity of abscess-derived neutrophils. Finlay-Jones et al (1991) suggested that these neutrophils have depleted intracellular killing mechanisms, thus allowing bacteria such as *B.fragilis* and *P.mirabilis* to persist in abscesses whilst surrounded by neutrophils. This may also be the case for SMG strains and experiments may be carried out with abscess derived neutrophils and compared with those results found here with normal human PMNL.

Another area of investigation by other researchers which would be interesting to carry out on the collection of strains in this work is the ability of the SMG to utilise sialic acid (Byers et al 1996) and chondroitin sulphate (Shain et al 1996a). Both groups suggest ability of the SMG to utilise these substrates allows them to persist at an otherwise nutritionally compromised site of infection. Shain et al (1996b) purified a novel glycosaminoglycan depolymerase from *S.intermedius* UNS 35 which showed activity

of a hyaluronidase rather than a chondroitin sulphate depolymerase as originally thought. Screening of the strain collection in this work would prove interesting in comparison, but may also clear up the anomaly of the *S.anginosus* strain detailed in section 2.4.6 which appeared to possess the ability to degrade hyaluronic acid, despite this property being unreported in the species. It may be producing this novel substance.

Finally, all these observations have been factors of the SMG themselves, which are important as they have been the sole organism isolated from infections, but equally important is the interaction of the group with other organisms in mixed infection. Whether it is production by the SMG of substances which promote their survival (Toyoda et al 1995) or substances produced by other organisms aiding the growth and survival of the SMG (Shinzato and Saito 1994), it is clear that the mixed infection is a complex ecosystem which requires further work to understand the role of the SMG .

APPENDICES

Appendix i Solutions and Growth Media

Saline (Oxoid) : 0.85% saline (normal saline) was prepared by adding 1 tablet to 500 ml of distilled water and autoclaving at 121°C for 15 minutes.

PBS (Oxoid) : 1 phosphate buffered saline tablet per 100 ml of distilled water. Autoclaved at 121°C/15 min.

Gel Hanks : 10 ml of x 10 Hanks Balanced Salts solution (Flow lab.) + 10 ml of 1% gelatine + 80 ml sterile distilled water. 100-200 ul 0.1 M NaOH to adjust pH to 7.1.

Luminol (Sigma) : 1.77 mg of luminol (5-Amino-2,3-dihydro-1,4 phthalazinedione) dissolved in 1 ml of Dimethyl sulphoxide to give a concentration of 10^{-2}M. Working solution diluted to 10^{-5} M with PBS.

Polymorphprep (Nycomed) : Sodium metrizoate 13.8% w/v + Dextran 500 8.0% w/v. Density 1.113 g/ml Osmolality 460 mOsm.

8-^{3}H Adenine	: Radioactive concentration 37.0 MBq/ml	
(Amersham)	1.0 mCi/ml.	

Seven Salts	: NaCl	23.48 g/l
Solution	Na_2SO_4	1.96 g/l
	$NaHCO_3$	0.1 g/l
	KCl	0.33 g/l
	$MgCl_2$	2.49 g/l
	$CaCl_2$	0.55 g/l
	H_3BO_3	0.01 g/l

ANAEROBIC BLOOD:	Peptone mixture	15.0	g/l
BROTH	Yeast Extract	10.0	g/l
	Sodium Thioglycollate	0.5	g/l
	Sodium Chloride	2.5	g/l
	Agar no 1	0.75	g/l
	L-Cystein HCl	0.5	g/l
	Resazurin	0.001	g/l
	Sodium Bicarbonate	0.4	g/l
	Haemin	0.005	g/l
	Vitamin K	0.0005	g/l

BHI Broth	: 37 g/l Autoclaved 121°C / 15 minutes.	
(Oxoid)	Calf brain infusion solids	12.5g
	Beef heart infusion solids	5.0g
	Proteose peptone	10.0g
	Sodium chloride	5.0g
	Dextrose	2.0g

	Disodium phosphate	2.5g

Mueller Hinton Broth (Oxoid) : 21 g/l Autoclaved 121°C / 15 minutes.

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

Blood Agar : Colombia agar (Gibco)

5% vol/vol defibrinated horse blood

Appendix ii Electron Microscopy Solutions

Solution A	:	M sodium cacodylate	20 ml
		Distilled water	70 ml
		Adjust to pH 7.4	
		Distilled water	to 100 ml
Solution B	:	25 % glutaraldehyde	2.4 ml
		Distilled water	14.2 ml
Solution C	:	Ruthenium red	0.15 g
		Distilled water	100 ml
Solution D	:	4% Osmium tetroxide	

Primary fixative	:	Soln. A	10 ml (0.07M)
		Soln. B	10 ml (1.2%)
		Soln. C	10 ml (0.075%)

Buffer wash : Equal parts of Soln. A + C

Post fixative (Prepared just before use)

	:	Soln. A	1 ml
		Soln. B	1 ml
		Soln. C	1 ml

Appendix iii Media, Chemical and Equipment Suppliers

Amersham	Buckinghamshire, England.
API Laboratory Products	Hampshire, England.
Bio Orbit	Turku, Finland
Clinicon	Life Sciences UK Ltd., Basingstoke, England.
Difco	Detroit, Michigan.
Fisons	Leicestershire, England.
Gallenkamp	East Kilbride, Scotland.
Heat Systems	New York, USA.
Mickle Engineering	Gomshall, Surrey.
Miles Scientific	Ames Division, Indianna, USA.
National Diagnostics	Atlanta, Georgia, USA.
Nucleopore	Chicago, USA.
Nycomed	Birmingham, England.
Oxoid	Basingstoke, England.
Pharmacia	Uppsala, Sweden.
Sigma	Poole, Dorset, England.
Tech Gen	London, England.
Technical Service Consulatants	Bury, England.
Titertek	Flow Laboratories Ltd., Irvine, Scotland.
Wallac	Turku, Finland.
Weber Scientific	Sussex, England.
Wellcome	Dartford, England.

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