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EFFECT ON ORAL STREPTOCOCCI OF EXPOSURE TO PENICILLIN AND ITS RELEVANCE IN INFECTIVE ENDOCARDITIS PROPHYLAXIS

William Peter John M^{c}Clure

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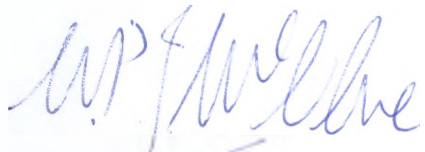
A dissertation submitted to the Faculty of Medicine, The University of the Witwatersrand, Johannesburg, for the degree Master of Science in Medicine.

Johannesburg, 1996

DECLARATION

This is to certify that, except for certain laboratory procedures undertaken on streptococcal isolates at the South African Institute for Medical Research, Johannesburg, this dissertation "Effect on oral streptococci of exposure to penicillin and its relevance in infective endocarditis prophylaxis", submitted for the degree of Master of Science in Medicine at The University of the Witwatersrand, Johannesburg, is my own work and has not been presented at any other University.

Approval to perform this study was obtained from the Committee for Research on Human Subjects, The University of the Witwatersrand, Johannesburg (certificate no. 19/4/90) and the Research and Ethics Committee of the Faculty of Dentistry, Medunsa (project no. D6/90).



William Peter John McClure
March 1996

Parts of this research have been presented at the following congresses:

Congress of the International Association for Dental Research (SA Division), September, 1990: "Cardiology patients and healthy volunteers: penicillin resistance in oral flora". WPJ McClure*, CHJ Hauman.

Congress of the International Association for Dental Research (SA Division), August, 1992: "Effect of oral penicillin on colonisation with viridans streptococcal species". WPJ McClure*, L Liebowitz, M Carmichael.

Pathology Congress, Wild Coast Sun, 1992. "Influence of antimicrobial therapy on susceptibility patterns of isolates of viridans streptococci". L Liebowitz*, WPJ McClure, M Carmichael, J Saunders.

Congress of the International Association of Dental Research (SA Division), September, 1993: "Roxithromycin and erythromycin activity against isolates from cardiology patients". WPJ McClure*, L Liebowitz, M Carmichael, J Saunders.

Congress of the International Association for Dental Research (SA Division), September, 1994: "Anomalous survival of viridans streptococci to the action of penicillin". WPJ McClure*, HJ Koornhof.

The following abstracts of work undertaken in this dissertation have been published:

Cardiology patients and healthy volunteers: penicillin resistance in oral flora. WPJ McClure, CHJ Hauman. J Dent Res 4;70:843 1991.

Effect of oral penicillin on colonisation with viridans streptococcal species. WPJ McClure L Liebowitz, M Carmichael. J Dent Res 72;4:827 1993.

Roxithromycin and erythromycin activity against isolates from cardiology patients. WPJ McClure, L Liebowitz, M Carmichael, J Saunders. J Dent Res 73;4:993 1994.

Anomalous survival of viridans streptococci to the action of penicillin. WPJ McClure, HJ Koornhof. J Dent Res 74;3:1022 1995.

ABSTRACT

The survival of bacteraemic isolates of viridans streptococci, after the administration to subjects of prophylactic antibiotic cover, has been widely reported in the literature. This study demonstrated that the cause of bacteraemia was as a result of phenotypic tolerance amongst populations of bacteria, and both proposed a mechanism responsible for the appearance of this form of tolerance and the design of a model to evaluate candidate drugs for infective endocarditis (IE) prophylaxis.

Dental plaque material from 67 subjects not previously exposed to ß-lactam antibiotics (UE-group) and from 50 individuals who received either rheumatic fever prophylaxis (RF-subgroup) (41) or were given high therapeutic doses of ampicillin (A-subgroup) (9), was inoculated into Todd-Hewitt broth containing 0.125 mg/ml penicillin G, 1.0 mg/ml penicillin G and a combination of 0.125 mg/ml penicillin G plus 5 mg/ml gentamicin. After overnight incubation, the selection broth cultures were subinoculated onto blood agar plates. Alpha-haemolytic colonies were selected for identification according to taxonomic principals of Kilian with a minor modification proposed by Coykendal to accommodate **Streptococcus vestibularis**. The isolates were further investigated by determination of minimum inhibitory (MICs) and bactericidal concentrations (MBCs) to penicillin V, amoxycillin, erythromycin, roxithromycin, clindamycin and vancomycin. This approach permitted the demonstration of genotypic tolerance and the presence of persisters, while survival of streptococci with MBCs less than the equivalent selection concentration of penicillin G allowed for the demonstration of phenotypic tolerance

Results

The **ex vivo** model clearly demonstrated selection of ß-lactam resistant and phenotypically tolerant isolates. **S. oralis**, **S. mitis**

biovar 1 and **S. mitis** biovar 2 were most commonly isolated from both the antibiotic exposed group (E-group) and the group previously unexposed to ß-lactam agents (UE-group). **S. oralis** was significantly more common in the E-group than in the UE-group while **S. mitis** biovar 2 was relatively more plentiful in the UE-group compared with the E-group. Considering the combined findings of the three selection groups, 66 out of 140 isolates (47%) were phenotypically tolerant to penicillin V and 68 (63%) to amoxycillin, ie. survived despite equivalent MBCs being lower than the selection concentrations in broth. The rest of the surviving isolates, following **in vitro** selection, were obviously resistant with MBCs in excess of the selection concentrations. Significantly more isolates had MBCs for either penicillin V or amoxycillin of $\geq$1.0 mg/l antibiotic (highest selection concentration of penicillin G) in the group previously exposed to ß-lactam antibiotics than in the UE-group (p values <0.0001). When the isolates from patients in the rheumatic fever and high-dose ampicillin treatment subgroups were examined for ß-lactam resistance and compared with the isolates from the UE-group, significantly more strains from the exposure groups demonstrated low-level resistance to both penicillin V and amoxycillin. When selection in penicillin G-only (without gentamicin) was analysed, only resistance at a higher level ($\geq$1.0 mg/l) showed a significant difference between the E- and UE-groups in the case of penicillin V (p=0.02) and approached statistical significance in the case of amoxycillin (p=0.06)

Except for vancomycin to which all isolates were consistently susceptible, there were notable differences in the upper limits of susceptibility between the two penicillin exposure groups. Neither the MICs nor the MBCs exceeded 4 mg/l in the UE-group while in the E-group, both the MICs and MBCs for the macrolides and clindamycin

were greater than 64 mg/l antibiotic, and were greater than 8 mg/l for penicillin V and amoxycillin. Exposure to high doses of ampicillin and prolonged exposure to penicillin V resulted in viridans bacteria which were not only more resistant to ß-lactam agents, but also to the macrolides and clindamycin. Erythromycin and clindamycin showed the greater activity with the lowest MICs 50 in both the UE- and E-groups (both 0.007 and 0.015 mg/l, respectively). Roxithromycin was only marginally less active than the latter agents (MICs 50 of 0.015 and 0.06 mg/l, respectively). MIC and MBC ranges were consistently higher in the ampicillin exposure E-subgroup (A-subgroup) than the rheumatic fever E-subgroup (RF-subgroup). Upper limit MIC and MBC concentrations of isolates were often greater than 64 mg/l antibiotic in the A-subgroup, especially against the macrolides and clindamycin, but isolates remained fully susceptible to vancomycin. MICs 50 and MBCs 50 were, for most agents, approximately twice to four times higher in the A-subgroup compared with the RF-subgroup

Only four **S. oralis** isolates, all in the E-group, were genotypically tolerant with MBC:MIC ratios of >10. Of these, three were also tolerant to erythromycin and one to clindamycin (ratios >16:1). MBC:MIC ratios were clearly higher in the E-group than in the UE-group, including genotypic tolerance to erythromycin in the E-group. There was no tolerance to vancomycin

Persisters were most commonly found in the UE-group amongst **S. mitis** biovar 2 (33.3%) strains and least commonly amongst **S. oralis** (17.9%). In the E-group, **S. mitis** biovar 2 again produced proportionately the greatest number of persisters (27.8%) but, in contrast to the above, **S. mitis** biovar 1 had the least (13.3%). Of all species in the study, **S. oralis** (which was also the most commonly isolated species) produced the greatest number of persisters overall in the E-group (50 of a possible 444; 14%) compared with 7% in the UE-group

Results of susceptibility tests of viridans streptococci to penicillin V and amoxycillin after selection in penicillin G-containing broth can be summarised as follows:

i) **S. oralis** and **S. mitis** biovars 1 and 2 were the most common survivors in this model

ii) Amoxycillin was more active than penicillin V amongst viridans streptococci in general, with the differences between the activities of these antibiotics being most marked against **S. mitis** biovar 1 and 2 strains

iii) There was a larger proportion of susceptible strains of **S. oralis** and **S. mitis** biovar 1 in the UE-group surviving **in vitro** than in the E-group but this trend was less marked in the strains surviving the penicillin G-only selection procedure

iv) Peak MICs and MBCs were highest in the E-group isolates and were most clearly observed in **S. oralis** and **S. mitis** biovar 2 strains

Further important aspects related to the susceptibility of viridans isolates were also studied. The observed antibiotic insensitivity of certain bacterial subpopulations within dental plaque deposits (microbial biofilms) was discussed. It was noted that current experimental animal models of IE prophylaxis are inadequate in as much as they only test candidate agents against bacteria while in their usually most susceptible exponential growth phase. Bacterial fractions disregarded by such tests are the phenotypically tolerant dormant or near-dormant bacteria found normally within mature (or stable) dental plaque deposits and other biofilms on mucosae in the body (bacteria exhibiting this form of tolerance would also be found within infected heart valve nodules)

Of note was the susceptibility behaviour exhibited by certain isolates to the macrolide antibiotics. This appeared remarkably similar to phenotypic tolerance recognised traditionally only amongst bacterial exposed to effect of ß-lactam antibiotic agents

Conclusions

The **ex vivo** selection broth model used in the present study clearly demonstrated the phenotypic tolerance phenomenon which also features in bacteraemias following blood-letting dental procedures performed under protection of ß-lactam antibiotic prophylaxis. A mechanism is described which may be responsible not only for aforementioned bacteraemia but also for the presence of strains which subsequently test susceptible to the agent of prophylaxis intended to control such occurrences.

The model was useful in showing the likely effect of previous antibiotic exposure on the resistance profiles of bacteraemic streptococci based on the **in vitro** selection procedure.

These positive aspects auger well for the usefulness of the model in evaluating candidate animicrobial agents for IE prophylaxis, including phenotypically tolerant subpopulations

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I was heartened to learn that the oft felt senses of desperation, panic, discouragement and feelings of disappointment balanced somewhat with those of joy, enlightenment and achievement were not unique to me and are common to many others who wish to take similar unsteady but serious steps into the academic world. After completion of the dissertation though, the results of my efforts have, it appears, been of somewhat less value than the heady sense of exhilaration I occasionally felt led me to believe. What made an especially great impression on me, however, and one which I am sure I will always remember, is the selfless support so modestly offered to me by individuals at the University of the Witwatersrand who themselves have achieved so much in their own right over the years both here in South Africa and abroad. Also of note, and something which may often be taken for granted, are the simple kindnesses and courtesies routinely given me, a complete stranger, by members of the administrative and support staff of the University of the Witwatersrand and at the South African Institute of Medical Research (SAIMR). For this I will always be especially grateful.

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TABLE OF CONTENTS

Chapter 1 Introduction

1.1 Some historical landmarks in the understanding of infective endocarditis -------- 2

1.2 Prophylaxis against infective endocarditis -------- 2
1.2.1 The link between oral streptococci and infective endocarditis -------- 2
1.2.2 Emergence of ß-lactam resistance in viridans streptococci -------- 3

1.3 The present study -------- 4

Chapter 2 Overview of infective endocarditis

2.1 Definition -------- 6

2.2 Epidemiology of infective endocarditis -------- 7

2.3 Aetiology and pathogenesis of infective endocarditis -------- 9
2.3.1 Vegetation development and structure -------- 9
2.3.2 Sequence of events in pathogenesis in infective endocarditis ------- 10
2.3.3 A possible immunological link in the development of infective endocarditis ------- 21
2.3.4 Summary ------- 21

Chapter 3 Sources, routes of infection and bacteraemia in relation to infective endocarditis development

3.1 Indigenous oral flora ------- 23
3.1.1 Bacteria of the oral cavity ------- 23
3.1.2 Environmental niches within the oral cavity ------- 24
3.1.3 Bacterial interactions in the oral cavity ------- 28

3.2 Involvement in bacteraemias of oral streptococci ------- 29
3.2.1 Transient bacteraemias ------- 29
3.2.2 Variation in bacteraemia frequency ------- 32
3.2.3 Inflammation of gingiva ------- 33

3.2.4 Viridans streptococci and orofacial infection ------- 35

3.3 A dermal source and route of infection ------- 36

3.4 Gastro-intestinal source and route of infection ------- 38
3.4.1 Organisms and some predisposing factors ------- 38
3.4.2 Gastro-intestinal malignancy and bacteraemia ------- 39

Chapter 4 Overview of prophylaxis of infective endocarditis

4.1 Introduction ------- 41

4.2 Regimens of antibiotic prophylaxis ------- 41
4.2.1 Oral route ------- 43
4.2.2 Parenteral route ------- 45
4.2.3 Further techniques to reduce the risk of bacteraemia ------- 47

4.3 Experimental models in animals ------- 47
4.3.1 Animal trials ------- 47
4.3.2 Clinical relevance of animal trials ------- 49

4.4 Summary ------- 50

Chapter 5 Antimicrobial candidates for infective endocarditis prophylaxis

5.1 Introduction ------- 52

5.2 ß-Lactams: penicillin G, penicillin V, ampicillin and amoxycillin ------- 54
5.2.1 Introduction ------- 54
5.2.2 Serum levels ------- 57
5.2.3 Resistance to penicillin ------- 58

5.3 Macrolides and related antibiotics: erythromycin, roxithromycin and clindamycin ------- 67
5.3.1 General properties of macrolides ------- 68
5.3.2 Erythromycin ------- 68
5.3.2.1 Pharmacokinetics and activity spectrum of erythromycin ------- 69
5.3.2.2 Erythromycin resistance ------- 70
5.3.3 Roxithromycin ------- 79
5.3.3.1 Improved pharmacokinetic activity ------- 79
5.3.3.1.1 Serum levels, half-life and saliva concentration ------- 79
5.3.3.1.2 Macrophage activity ------- 81

5.3.3.2 Spectrum of activity ------- 82
5.3.3.2.1 Effect of roxithromycin on normal flora ------- 83
5.3.3.3 Resistance to roxithromycin ------- 84
5.3.4 Clindamycin ------- 85
5.3.4.1 General properties ------- 85
5.3.4.2 Mode of action ------- 85
5.3.4.3 Effect of clindamycin on normal flora and its pharmacokinetics ------- 86

5.4 Vancomycin ------- 86
5.4.1 General properties ------- 86
5.4.2 Spectrum and resistance ------- 87
5.4.3 Side-effects ------- 88

Chapter 6 Tolerance to ß-lactam antibiotics

6.1 Description of the phenomenon ------- 90
6.1.1 Genetically determined (genotypic) tolerance ------- 90
6.1.2 Phenotypic tolerance ------- 93
6.1.2.1 Phenotypic tolerance **in vitro** ------- 94
6.1.2.2 Phenotypic tolerance **in vivo** ------- 95
6.1.2.2.1 Experimental **in vivo** phenotypic tolerance ------- 95
6.1.2.2.2 **In vivo** phenotypic tolerance associated with bacteraemia ------- 95

6.2 Tolerance amongst viridans streptococci ------- 96
6.2.1 Tolerance to ß-lactam antibiotics ------- 97
6.2.2 Tolerance to vancomycin ------- 99
6.2.3 "Tolerance" to antibiotics not acting directly upon bacterial cell wall formation ------ 100

6.3 Clinical implications of tolerance in viridans streptococci ------ 101

Chapter 7 Classification of viridans streptococci

7.1 Problems associated with the classification of viridans streptococci ------ 102

7.2 Different systems of classification ------ 104

7.3 Classification of Kilian et al (1989) ------ 104

Chapter 8 Experimental design and methodology

8.1 Introduction ------ 106

8.2 Informed consent ------ 106

8.3 Subjects ------ 106
8.3.1 Subjects exposed to ß-lactam antibiotics ------ 106
8.3.2 Subjects not exposed to ß-lactam antibiotics ------ 107

8.4 Specimen collection ------ 107

8.5 **In vitro** selection in broth: a model ------ 107

8.6 Species identification ------ 108

8.7 Susceptibility testing ------ 109
8.7.1 MIC and MBC determination ------ 109
8.7.2 Definitions relating to survivors of the **in vitro** selection ------ 110
8.7.2.1 Phenotypic tolerance ------ 110
8.7.2.2 Resistance in the context of the selection process ------ 111
8.7.3 Recording of tolerant strains ------ 111
8.7.4 Definition and recording of persisters ------ 111

8.8 Statistical analysis ------ 112

Diagrammatic representation of laboratory procedures used for the identification of viridans streptococci following the methods of Kilian et al (1989) and Coykendall (1989) ..... between text pages 109 and 110

Chapter 9 Identity and susceptibility of oral streptococci to candidate antibiotics

9.1 Introduction ------ 113

9.2 Methodology and definitions ------ 114

9.3 Results ------ 114
9.3.1 Identity of viridans streptococci after **in vitro** selection in penicillin G ------ 114
9.3.2 Susceptibility patterns in the different beta-lactam antibiotic exposure groups ------ 115
9.3.2.1 Susceptibility to candidate antibiotics within UE-group and E-groups as a whole ------ 115
9.3.2.2 Susceptibility within E-subgroups ------ 117

9.3.2.3 Antimicrobial susceptibility of individual species according to exposure group ------ 122

9.4 Discussion ------ 129

Tables (Table Index located at end of Table of Contents)

Chapter 10 Genotypic tolerance and persisters in viridans streptococci

10.1 Introduction ------ 133

10.2 Methods and definitions ------ 134
10.2.1 Definition of genotypic tolerance ------ 134
10.2.2 Persisters ------ 134
10.2.2.1 Refractoriness of persisters to killing ------ 135
10.2.3 Endurance indices ------ 135
10.2.3.1 Endurance indices in relation to tolerant strains ------ 135
10.2.3.2 Endurance indices in relation to persisters ------ 136

10.3 Results ------ 136
10.3.1 Phenotypically tolerant strains and MBC/MIC ratios ------ 136
10.3.2 Endurant strains ------ 140
10.3.3 Persisters ------ 140
10.3.3.1 Persisters at and above MBC levels in different species ------ 140
10.3.3.2 Refractoriness of persisters to killing ------ 145

10.4 Discussion ------ 162
10.4.1 Genotypic tolerance and persisters in UE- and E-groups ------ 162
10.4.2 ß-Lactam candidates for IE prophylaxis based upon susceptibility, tolerance and the frequency of persisters ------ 164
10.4.3 Activity of candidate non-ß-lactam agents of IE prophylaxis based upon thepresence of persisters ------ 165
10.4.3.1 Relative candidature of roxithromycin and vancomycin as agents of IE prophylaxis ------ 167

Tables (Table Index located at end of Table of Contents)

Chapter 11 Effect of penicillin G selection on oral streptococci from antibiotic exposed and non-exposed individuals in an in vitro system

11.1 Introduction ------ 168

11.2 Design of an **in vitro** model of IE prophylaxis with ß-lactam antibiotics ------ 169

11.3 Results ------ 170
11.3.1 Patterns observed following different penicillin G selection procedures ------ 170
11.3.2 Effect of gentamicin in penicillin G selection broth ------ 171
11.3.3 Comparison of MIC and MBC activities of penicillin V and amoxycillin ------ 173
11.3.4 Exposure to two penicillin G concentrations in broth ------ 174
11.3.5 Selection in penicillin G broth of strains with high MBC levels: comparison between E-group and UE-group isolates ------ 174
11.3.5.1 Comparison between E-group and UE-group according to individual selection procedures ------ 175
11.3.5.2 Comparison between E-group and UE-group according to two penicillin G-only selection procedures ------ 175
11.3.5.3 Comparison according to all selection procedures combined ------ 176

11.4 Effect of previous ß-lactam antibiotic exposure on streptococcal species ------ 176
11.4.1 Distribution of streptococcal species according to previous antibiotic exposure ------ 176
11.4.2 Effect of previous exposure to ß-lactam antibiotics on levels of resistance in viridans streptococci as a group ------ 177
11.4.2.1 Differences related to antibiotic exposure following selection procedures in penicillin G and in penicillin G plus gentamicin -------177
11.4.2.2 Differences related to antibiotic exposure following selection procedures in penicillin G alone ------ 177
11.4.2.3 Relative susceptibility to the ß-lactam antibiotics of viridans streptococci in E-subgroups and UE-group based upon MIC 50/MBC 50 and MIC 90/MBC 90 levels ------ 178
11.4.2.4 Relative susceptibility of viridans streptococci to the ß-lactam antibiotics of E-subgroup and UE-group strains based upon resistance breakpoints ------ 178
11.4.3 Effect of exposure to ß-lactam antibiotics on levels of resistance in streptococcal species ------ 180

11.5 Discussion ------ 181
11.5.1 MICs vs MBCs ------ 182
11.5.2 Comparison between the activities of penicillin V and amoxycillin ------ 182

11.5.3 Effect of selection in penicillin G plus gentamicin broth on the susceptibility to penicillin V and amoxycillin according to previous ß-lactam exposure ------ 183
11.5.4 Pooling of findings obtained from different **in vitro** selection concentrations ------ 186
11.5.5 Effect of penicillin G-only selection on exposure group susceptibility to penicillin V and amoxycillin ------ 187
11.5.6 Summary of main findings of **in vitro** selection affecting plaque streptococci in general ------ 188
11.5.7 Effect on species of broth selection ------ 188
11.5.7.1 Relative activities of penicillin V and amoxycillin amongst streptococcal species ------ 189
11.5.7.2 Effect of selection procedures on streptococcal species in relation to previous antibiotic exposure ------ 189
11.5.7.3 Summary of main species-related findings in the **in vitro** selection model ------ 191
11.5.8 Comparative effects of exposure to high ß-lactam concentrations (A-subgroup), prolonged low ß-lactam concentrations (RF-subgroup) and absence of prior ß-lactam exposure (UE-group) ------ 191
11.5.9 Summary of and concluding comments on the most salient features of the **in vitro** selection model in relation to amoxycillin and penicillin V prophylaxis ------ 192
11.5.9.1 Validity of model ------ 192
11.5.9.2 Demonstration of phenotypic tolerance ------ 195
11.5.9.3 Selection of resistant strains ------ 196

Tables (Table Index located at end of Table of Contents)

Chapter 12 General discussion and conclusions

12.1 Rationale and candidate antibiotic agents for prophylaxis ------ 198

12.2 Spectrum of streptococcal species from the oral cavity ------ 199

12.3 Phenotypic tolerance ------ 201
12.3.1 Definition and origin of term ------ 201
12.3.2 Biolayers and biofilms ------ 201
12.3.3 Antibiotic insensitivity of bacteria within plaque (biofilm) deposits ------ 204
12.3.4 Use of animal models ------ 205

12.3.5 The role of adhesion in the prevention of IE by antibiotics ------ 209

12.4 Some conclusions arising from the present **in vitro** selection model ------ 210
12.4.1 Phenotypic tolerance ------ 210
12.4.2 Effect of previous antibiotic exposure on genotypic resistance ------ 210
12.4.3 Relative merits of candidate drugs for IE prophylaxis ------ 210

12.5 Concluding remarks ------ 211

References ------ 213

TABLE INDEX

Table 9.1 Number and identity of viridans streptococcus isolates speciated according to Kilian et al (1989) in different ß-lactam exposure groups following selection in penicillin G-only and penicillin G plus gentamicin ..... after page 114

Table 9.2 Susceptibility of UE- and E-groups to the panel of antibiotics ------ 116

Table 9.3 MIC and MBC profiles of 12 isolates of the A-subgroup ------ 118

Table 9.4 MIC and MBC profiles of 62 isolates in RF-subgroup ------ 119

Table 9.5 Comparison of MIC and MBC ranges between isolates from the different antibiotic exposure groups ------ 120

Table 9.6 Comparison of MICs 50 and MBCs 50 between isolates from the different antibiotic exposure groups ------ 121

Table 9.7 Comparison of MICs 50 and MBCs 50 of ***S. oralis*** ------ 123

Table 9.8 Comparison of MICs 50 and MBCs 50 of ***S. mitis*** biovar I ------ 125

Table 9.9 Comparison of MICs 50 and MBCs 50 of ***S. mitis*** biovar 2 ------ 127

Table 10.1 MBC/MIC ratios of ≥4 amongst streptococcal species ------ 138

Table 10.2 Genotypic tolerance: comparative MBC/MIC ratios in the E- and UE-groups ------ 139

Table 10.3 Strains exhibiting persisters and their respective MBCs ------ 141

Table 10.4 Persister refractory and endurance indices in streptococcal species according to principal ß-lactam exposure groups ------ 145

Table 10.5 Prevalence of persisters and refractory persisters (with PRIs ≥10) by species in the UE- and E-groups ------ 154

Table 10.6 Refractoriness of persisters with PIs >10 exhibited against different antibiotics ------ 157

Table 10.7 Persisters and persister refractory indices (PRIs) related to antibiotics ------ 159

Table 10.8 Prevalence of persisters and refractory persisters (with PRIs ≥10) in UE- and E-groups against 6 antibiotics ------ 160

Table 10.9 Contribution of different ***Streptococcus***

Tables in **Chapter 11** follow consecutively at the end of the chapter between text pages 197 and 198:

Table 11.1 Effect of **in vitro** antibiotic selection on susceptibility of streptococcal isolates from ß-lactam exposed and ß-lactam unexposed individuals

Table 11.2(a) Number and identity of viridans streptococcus isolates speciated according to Kilian et al (1989) in different ß-lactam exposure groups following selection in penicillin G only and penicillin G plus gentamicin

Table 11.2(b) Number and identity of viridans streptococcus isolates speciated according to Kilian et al (1989) in different ß-lactam exposure groups following selection in penicillin G-only broth

Table 11.3(a) ß-lactam susceptibility ranges of streptococcal species, selected in penicillin G-only broth and in combination with gentamicin, from plaque of participants within antibiotic exposed and unexposed groups

Table 11.3(b) ß-lactam susceptibility ranges of streptococcal species, selected in penicillin G-only broth, from plaque of participants in antibiotic exposed and unexposed groups

Table 11.3(c) ß-lactam susceptibility ranges of streptococcal species, selected in penicillin G plus gentamicin only broth from plaque of participants within antibiotic exposed and unexposed groups

Table 11.4(a) Susceptibility in MICs to penicillin V and amoxycillin of isolates selected in penicillin G-only and penicillin plus gentamicin broth from dental plaque of subjects exposed or unexposed to ß-lactam antibiotics

Table 11.4(b) Susceptibility in MICs to penicillin V and amoxycillin of streptococcal species selected in penicillin G only broth from dental plaque of subjects exposed or unexposed to ß-lactam antibiotics

Table 11.4(c) Susceptibility to penicillin V and amoxycillin of isolates selected in penicillin G plus gentamicin-only broth from dental plaque of subjects exposed or unexposed to ß-lactam antibiotics

Table 11.5(a) Susceptibility patterns of viridans streptococci selected in penicillin G and penicillin G plus gentamicin broth from subjects (a) on high-dose ß-lactam treatment, (b) on penicillin V rheumatic fever prophylaxis and (c) unexposed to ß-lactam antibiotics

Table 11.5(b) Susceptibility patterns of viridans streptococci selected in penicillin G only broth from subjects (a) on high-dose ampicillin treatment, (b) on penicillin V rheumatic fever prophylaxis and (c) unexposed to ß-lactam antibiotics

Table 11.6(a) Susceptibility and numbers within susceptibility categories of isolates selected in penicillin G and penicillin G plus gentamicin broth from patients on (a) high ampicillin doses and (b) on penicillin V rheumatic fever prophylaxis

Table 11.6(b) Susceptibility of streptococcal species from dental plaque of patients selected in penicillin G-only broth on (a) high ampicillin doses and (b) on penicillin V rheumatic fever prophylaxis

Table 11.7(a) Significance of **in vivo** exposure to ß-lactam antibiotics on susceptibility of dental plaque streptococci after selection in penicillin G broth, including the procedure involving penicillin G plus gentamicin

Table 11.7(b) Significance of **in vivo** exposure to ß-lactam antibiotics on susceptibility of dental plaque streptococci after selection in penicillin G broth (without gentamicin)

EFFECT ON ORAL STREPTOCOCCI OF EXPOSURE TO PENICILLIN AND ITS RELEVANCE IN INFECTIVE ENDOCARDITIS PROPHYLAXIS

Chapter 1

INTRODUCTION

1.1 Some historical landmarks in the understanding of infective endocarditis

A detailed description of infective endocarditis (IE) appeared near the turn of the century in the classical writings of William Osler who linked viridans streptococci to subacute or "chronic" endocarditis. In his famous textbook "Principals and Practice of Medicine" published early this century (1912), Osler suggested that Streptococcus mitior was the most common cause of this disease. Rushton (1930) reported a direct link between dental extraction (and tonsillectomy) and the development of IE while, five years later in 1935, Okell and Elliot were the first to show clearly that bacteria from the oral cavity may cause this infection

1.2 Prophylaxis against infective endocarditis

1.2.1 The link between oral streptococci and infective endocarditis

Viridans streptococci are micro-organisms indigenous to the oral cavity and oropharynx and are important aetiological agents of IE (Roberts et al, 1979; American Heart Association Report (AHA), 1981; Cotran et al, 1989; Hall and Heimdahl, 1989)

1.2.2 Emergence of ß-lactam resistance in viridans streptococci

Oral streptococci have traditionally been regarded as being susceptible to penicillin (Hall and Heimdahl, 1989) but resistant strains have been isolated from the oral cavity (Longman et al, 1991) and blood (Hess et al, 1983b) of IE-susceptible patients. However, antibiotic use is known to select for bacterial resistance in oral streptococci (Southall et al, 1983; Harrison, Stross et al, 1985; Herbert et al, 1988; Smith et al, 1989; Maskell et al, 1990)

Additionally, exposure has been shown to select for resistance soon after the initial contact between the streptococci and an antibacterial agent and resistant strains emerge readily in individuals with either recent or frequent exposure to such agents Southall et al, 1983; Harrison, Rubin et al, 1985; Woodman et al, 1985). Leviner et al, (1987) detected resistant strains after six hours in 9 (31%) of 29 volunteers following ingestion of the first of three doses of phenoxymethylpenicillin (penicillin V) administered within a 10-hour period. Acquisition of resistant strains may, however, occur without direct antibiotic exposure, especially in hospital settings (Leviner et al, 1984). Persons with no previous exposure to antibacterial agents are known to carry small numbers of antibiotic-resistant bacteria as part of their normal oral flora (Woodman et al, 1985; Longman et al, 1991). It has been shown that bacteraemia with ß-lactam (penicillin)-resistant oral streptococci and IE may follow prophylaxis in animals, and may possibly constitute a threat of prophylaxis failure in humans susceptible to this infection (Longman et al, 1992). As early as 1947, Krumwiede recorded the failure of prophylaxis and the development of IE in a young patient in whom a penicillin-resistant streptococcal strain was isolated from the oral cavity

1.3 The present study

A major feature of the present study is an **in vitro** model designed to mimic IE prophylaxis. In this model, the identity of strains of viridans streptococci within dental plaque (see Section 8.5 for description) and their susceptibility to candidate agents for IE prophylaxis were determined following <u>in vitro</u> selection in benzylpenicillin (penicillin G) and results discussed. Plaque specimens were collected from volunteers who were at the time receiving ß-lactam antibiotics or were without antibiotic exposure for a minimum period of three months prior to sampling. Isolates were speciated and tested for antimicrobial susceptibility at concentrations of antibiotics appropriate to the treatment of IE (breakpoint concentrations). All strains originated from plaque specimens which were subjected to **in vitro** exposure and selection in penicillin G-containing broth

Antibiotic susceptibility levels in viridans streptococci from dental plaque following **in vitro** exposure to penicillin G, especially with regard to ß-lactams but also other candidates for IE prophylaxis, including erythromycin, roxithromycin clindamycin and vancomycin, will be described and explanations for the observed deviations from expected patterns proposed. The anomaly of streptococci surviving in the presence of ß-lactam concentrations expected to have a bactericidal effect, which has been described as phenotypic tolerance (Tuomanen, 1986), feature prominently in this dissertation

In the introductory chapters, background information relevant to the dissertation, and which has a bearing on IE prophylaxis, are discussed. Aspects to be included relate to the pathogenesis of IE (including risk factors), the reputed importance of dental

procedures as a major cause of IE, as well as the role of procedures unrelated to dentistry which affect bacterial niches other than those in the oral cavity. Finally, based on the findings of this study, the use of **in vitro** models to more accurately evaluate candidate antimicrobial agents for IE prophylaxis are discussed

Chapter 2

OVERVIEW OF INFECTIVE ENDOCARDITIS

2.1 Definition

Infective endocarditis (IE) is an inflammatory disease of the endocardium and "one of the most serious of all infections" (Back and Svanbom, 1980; Cotran et al, 1989). The disease is characterised by the colonisation or invasion of heart valves or mural endocardium by microbiological agents leading to the formation of friable vegetations laden with organisms

Prior to the antibiotic era, IE invariably exhibited an extremely high mortality - in the region of 100% (Hayward, 1972a); Tanner and Durack, 1990). However, even with the availabilty of modern therapeutic compounds, it remains a life-threatening condition (Anonymous, 1981; Cotran et al, 1989; Hayward, 1973a). The incidence of new cases of IE appears not to have decreased (Hayward, 1973a) but has actually risen markedly with a longer survival rate and an increase in median age of onset and number of recurrent cases (Tanner and Durack, 1990) (see below)

The infection has been classified on clinical grounds into acute and subacute IE types. The former more severe form is caused by highly virulent organisms which, characteristically, tend to attack the previously normal heart. Necrotising, ulcerative and invasive lesions of heart valves are produced. This process results in the death of patients, often within days. Subacute or chronic IE shows a longer clinical course, is caused by commensal organisms which tend to be less virulent and which affect pre-

viously damaged or scarred endocardium (Cotran et al, 1989) or those with either chronic valvular disease or congenital malformations of the heart (Hayward, 1973a)

2.2 Epidemiology of infective endocarditis

The incidence of IE is difficult to determine accurately since differing diagnostic and reporting criteria exist and only a relatively small number of clinically diagnosed cases, using strict definitions, are proven IE (von Reym et al, 1981)

In a post-mortem investigation, Hayward (1973a) found no definitive reduction in the number of IE cases in the UK from the 1930s through to the early 1970s. He suggested that an alteration in the general clinical picture had occurred and noted that IE still carried a high (30%) mortality rate. He suggested that traditional diagnostic criteria were inadequate - an aspect handled in much greater detail by other authors (von Reyn and Arbeit, 1994) (see later)

Durack and Petersdorf (1977) indicated that the average age of onset had risen from 34 years of age in 1944 to 50 years of age in 1977. They postulated that possible reasons for this include an increased prevalence of arteriovascular and cardiovascular disease in the elderly. This latter proposal was supported by Friedlander and Yoshikawa (1990) who also noted that lesions in the elderly which predispose patients to IE do tend to be age-related degenerative or atherosclerotic valvular defects. In addition, chronic rheumatic heart disease led to the development of IE in this age group - with a few notable exceptions (Veasy et al, 1987). An explanation for the observed trend may be a general decreased incidence of rheumatic fever (Morris, 1985) especially amongst the youth in Western countries. An escalation in the use

of intravenous devices on hospital in-patients could be another contributing factor to the observed later onset of IE

Von Reyn and Arbeit (1994) believe that accurate IE case definition and clinical patterns in this disease should emphasize four fundamental features, ie. (i) predisposing heart disease, (ii) distinctive persistent bacteraemia, (iii) vascular phenomena (cutaneous and visceral) and (iv) evidence of "active intracardiac pathology" ie. active endocardial processes. This diagnostic approach, they contend, may better equip clinicians to compare the results of previous treatments and lead to more accurate diagnoses than was the case in the past. In a multicentre study to determine (amongst others), IE incidence, an optimal therapeutic approach and duration of therapy, Fang et al (1993) divided episodes of IE into "definitive", "presumptive" or "suspicious". Each form of IE was strictly defined

New proposals contained in the Duke schema have been examined critically and are claimed to be an improvement over proposals used currently for the diagnosis of IE (Bayer et al, 1994). The Duke schema involves three major factors for IE diagnosis (not noted here) and, following these, a diagnosis is either "definite", "possible" or a diagnosis is "rejected". A "definite" case of IE, for example, is one which has to satisfy specific pathological and clinical criteria. Each of the two major pathological and clinical criteria is defined as are factors which constitute a definitive or positive IE diagnosis

Hayward (1973b) stated that although often quoted figures on IE mortality appear to indicate a particularly poor prognosis, it is not necessarily the case as the figures include both acute IE and surgically induced cases. A clearer pattern is said to emerge were only bacterially positive cases to be considered. In the author's 300-case series, survival increased as mortality

decreased from 27% prior to 1956 to 14% in the period 1966-72. With the inclusion of all acute and surgical IE cases into the sample, there was a much greater overall mortality figure of 31% for the same period

It appears certain however, that the development of IE tends to occur amongst those with pre-existing cardiac abnormalities (70-75%), some of which may be unknown to those at risk (Durack, 1995). Bayliss et al, (1983) highlighted the incidence of IE amongst the elderly with supposedly normal hearts

2.3 Aetiology and pathogenesis of infective endocarditis

2.3.1 Vegetation development and structure

The accepted (traditional) manner of vegetation development is described by Hayward (1973a) as follows: "minute platelet thrombi settle on valves roughened by congenital or acquired defects or (on) areas of endocardium damaged by jet effects, and that bacteria of low virulence enter from the mouth or genitourinary tract become established and grow in the platelet thrombi and produce the (typical) proliferative and fragile vegetations..."

IE vegetations are single or multiple, vary in size from a few millimetres to several centimetres in diameter and are located along (but not restricted to) the line of valve closure (Lode, 1982. Although this author stressed that lesions found in acute and chronic IE were more similar than dissimilar, Cotran et al (1989) detailed differences in size, site and complications (consequent to their presence) of these structures between the two clinical entities. In the acute form of IE, vegetations are located on previously healthy valves, tend to be larger ("bulkier") and perforate or erode underlying leaflets. Abscesses

may develop within underlying endocardial tissue. Chronic IE on the other hand exhibits smaller vegetations which less commonly produce leaflet erosions or damage but which tend to extend onto adjacent endocardial walls. Such vegetations are commonly associated with the presence of congenital heart defects (Cotran et al, 1989). On prostheses, vegetative lesions tend to occur on the margin of the suture ring

The typical microscopic structure of the endocarditis vegetation consists of an amorphous fibrin mass in which platelets, red blood cells and large numbers of bacteria are imbedded, the latter being centrally placed (Hayward, 1973b). These nodules may "heal" and undergo progressive sterilisation, reorganisation, fibrosis and calcification (Cotran et al, 1989)

As bacteria are present in the valvular lesions of IE, bacteraemia and the resultant seeding of bacteria (into small vessels of the spleen, kidneys and skin leading to vascular changes) are a fundamental requirement for the development of IE (Back and Svanbom, 1980)

2.3.2 Sequence of events in the pathogenesis of infective endocarditis

For subacute (chronic) IE to develop, a number of predisposing (and often unrelated) factors need to be present. Those which play a role include:

* pre-existing congenital or acquired heart defects
* eddy currents and "jet streams" in the flow of blood
* formation of sterile fibrin-platelet deposits
* infective bacteraemic elements
* bacterial adhesion

* infection of sterile vegetations

i) Pre-existing congenital or acquired heart defects

A number of acquired or congenital cardiac abnormalities tend to predispose patients to IE (Back and Svanbom, 1980, and particularly in the young (Cotran et al, 1989). Rheumatic heart disease tended, in the past, to be the most important predisposing factor which lessened considerably in importance in the 1960's and 1970's (Cotran et al, 1989; Jaspers and Little, 1984; Special Report, 1984) but recent reports have indicated an uncharacteristic resurgence of rheumatic fever in the USA (Veasy et al, 1987), occurring in the classical 5-15 year age group

Particular congenital and acquired abnormalities of the heart strongly predispose patients to IE (Cotran et al, 1989) and various categories are tabulated below. Important among these are small shunts formed as a result of limited interventricular defects and which produce jet streams in the blood flow (see below). Others include forms of valvular disease (stenoses), patent ductus arteriosus, tetralogy of Fallot, mitral valve prolapse, degenerative calcific valvular stenoses and bicuspid aortic valve, as well as cardiac valve prostheses and indwelling pulmonary artery catheters. With the observed general decline in rheumatic fever and an increase in the elderly population, Morris (1985) noted the appearance of new groups of high-risk patients with prosthetic heart valve replacements. Cardiac scar tissue at sites of previous injury (damage) or degenerative or inflammatory changes may play a contributory role (Cotran et al, 1989)

Underlying conditions predisposing to infective endocarditis

Congenital:
Ductus arteriosus, ventricular septal defects, co-arctation of the aorta, bicuspid aortic valve, tetralogy of Fallot and pulmonary artery stenosis

Rheumatic:
Mitral valve (85%) and aortic valve stenosis

Degenerative:
Calcified mitral annulus, arteriosclerotic heart disease including calcified nodular stenosis and post-myocardial infarction - mainly in elderly patients

Nosocomial:
Haemodyalsis shunts, pace-maker wires, intracardiac prostheses

Others:
Syphilitic aortitis, idiopathic hypertrophic subaortic stenosis (IHSS), mitral valve prolapse

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Non-cardiovascular predisposing factors include neutropaenia, immunodeficiency and -suppression, and IV drug abuse. The latter two are particularly important at the present time (Cotran, 1989)

ii) Haemodynamic factors

Cardiac abnormalities tend to affect the smooth and even flow of blood through the heart chambers and vasculature. Jet

streams and eddy currents generated as a result of valvular or small septal constrictions initiate the early nonbacterial thrombic stage in the formation of vegetations on the endothelial lining of the heart wall

Interestingly, patients with cardiac lesions with less severe haemodynamic problems are especially at risk of IE while those with severe heart disease (failure) are rarely affected (Hayward, 1973a). Hearts with markedly deficient blood pumping abilities would produce smaller pressure gradients in the blood and would, consequently, be unable to form jet streams etc. severe enough to precipitate thrombus formation and thus initiate vegetation formation

iii) Sterile platelet and fibrin deposits

Platelets and fibrin are deposited onto areas of pre-existing endothelial damage, or where haemodynamic or other injury has occurred (Hayward 1973a; Cotran et al, 1989). This traditional approach was, however, questioned by Hayward in 1973 (1973a) who stated that the exact mechanism of why vegetations form under these conditions is unclear (see later). Cotran et al (1989) appears to lay rather more stress on the haemodynamic changes which occur in heart disease than the presence of endocardial scar tissue and the effect, if any, which this tissue would have on vegetation formation and subsequent infection of the endocardium

Apart from the inductive ability of damaged endothelium, the presence of growing bacteria in the thrombi overlying endothelial lesions acts to further stimulate deposition of fibrin and platelets - albeit deposition under non-sterile conditions. Removal of bacteria from the circulation during therapy removes the catalyst for further such deposition (Hayward, 1973b)

iv) Infective bacteraemic elements

The immediate and essential source of bacteria able to infect the endocardium are those present in the blood. Entry of organisms into the vasculature may occur by way of two mechanisms (Cotran et al, 1989). Firstly, directly as a result of mechanical manipulation of tissues (dental or surgical treatment, the IV route as in drug addiction) or infective processes (sepsis) elsewhere in the body. Secondly, covertly by means of transient bacteraemias after minor injuries of the gut, oral cavity, skin or other areas in the body. These sources tend to seed the blood with organisms of low virulence

Almost any blood-borne microbe has the potential to produce IE. However, like viridans streptococci, some appear have a greater propensity than others. Aetiological agents may be bacterial, fungal, rickettsial, chlamydial and (possibly) viral (Cotran et al, 1989), Hayward, 1973a in origin

A noticable change has also occurred in the type of infective organism responsible for the disease over the years (Hayward, 1973a). In the pre-penicillin era, 95% of cases were caused by viridans streptococci while in their 1969 investigation, Shinebourne et al (Hayward is a co-author) noted that only 44% were caused by these organisms. In a more recent study, Sussman et al (1986) reported that 65% of all cases to be caused by streptococci including the viridans group, as well as S. bovis, Enterococcus faecalis and others

Although oral streptococci may be less common aetiological agents of IE today than in the past, this bacterial group is still the most common associated with diseased heart valves. The severity of viridans endocarditis is illustrated by the findings of Bayliss and his co-workers (1983). In their study of 74 patients who died from IE, at least 15 (20%) were infected with viridans

streptococci. Five of these patients had some form of cardiac abnormality while the rest (67%) experienced infection of a previously "healthy" endocardium. Sussman et al (1986) noted that the clinical course of a number of patients in their 1986 study of viridans endocarditis was comparable to that associated with organisms such as Staphylococcus aureus which tended to be considerably more virulent. They postulated that oral streptococci with enhanced virulence may also have infected healthy hearts in some patients in the study of Bayliss et al (1983) and mimiced staphylococcal IE of previously normal (healthy) endocardium

A recent publication (Douglas et al, 1993) attempted to clarify the confusion relating to different classification schemes and named viridans streptococcal species causing endocarditis in Britain. This was performed according to the most recent taxonomic concepts. Employing a scheme by Beighton et al (1991), of 47 strains collected from 42 confirmed cases of this disease, S. sanguis was found by Douglas et al to be the most common infecting viridans Streptococcus at 32%, S. oralis followed with 30% and S. gordonii with 13%. Other species such as S. mitis were less commonly isolated

v) Bacterial adhesion

Adhesion of bacteria to substrates plays an important physiological role in normal microbe-host interactions, and a critical one in the initiation and progression of disease processes involving these organisms

a) Physiological adherence. A natural biological interaction exists between micro-organisms themselves as well as between micro-organisms and exposed inanimate objects introduced into the vascular system, or between micro-organisms and various anatomical structures in animals and humans. Colonisation of specific areas of the human body involves the inherent attachment

properties of micro-organisms and host tissues (Gibbons and van Houte, 1975). In the mouth, species of oral streptococci are specific in their ability to colonise particular areas. Their particular mechanism of tissue attachment is selective and influenced by a number of factors which includes compatibility with other microbes, host age, saliva composition and diet (Ellen, 1982). Typical colonisatory behaviour which begins immediately post-partum, is observed in the new-born during pioneer colonisation of commensal flora of the skin and mucosae (Hardie, 1983)

b) Adhesion in IE. Adhesion plays a pivotal role in the initial stages of IE. Any property which increases the ease with which organisms attach to target substrates (tissues) prior to colonisation (infection) would serve as a virulence factor under conditions such as those prevailing in the health-compromised cardiac patient. The ability of specific bacterial species to adhere to fibrin and platelet elements present during endocarditis and nonbacterial thrombic endocarditis (NBTE) is important in the pathogenesis of IE (Scheld et al, 1981). Adhesive ability is exhibited by bacteria which colonise damaged or defective endocardiums of patients with congenital or acquired heart disease (van der Bijl, 1992)

Viridans streptococci adhere more strongly to platelet and fibrin thrombi than, for example, **E. coli** (Cotran et al, 1989). Oral streptococci are also known, under certain conditions, to produce substances which increase their adherence capabilities eg: long, extra-cellular polysaccharide polymers called glucans (Hardie, 1983). Polysaccharides synthesised in the presence of sucrose by S. mutans enhance initial bacterial attachment onto certain surfaces (dental) (Socransky and Morganiello, 1971). This feature may, however, not be relevant once strains with the ability to synthesise polysaccharides have reproduced themselves in the bloodstream where nutrients such as sucrose are no longer

present. For attachment of the original bacteraemic bacteria which still carry presynthesised exopolysaccharides, this property may be highly beneficial initially (see below)

Beachey (1981) investigated specificity of bacterial adherence to mucosal surfaces. He noted that bacterial species preferencially populate different tissues - "tissue tropism" - and described the normal habitats of two oral streptococcal species. S. mutans was abundant in "dental plaque" and sparse elsewhere in the oral cavity while S. salivarius was found on tongue epithelium but uncommon elsewhere

Attachment of bacteria to mucosal surfaces is the initial event in the pathogenesis of most infectious diseases in animals and humans (Beachey, 1981) and organisms with an increased ability to do so would exhibit a greater virulence under specific conditions. Lipoteichoic acid (LTA), a cell-membrane constituent in most streptococci, acts as an "adhesin" or receptor for bacterial attachment to target substrates (Beachey, 1981; Courtney et al, 1983). Adhesins, a term first coined by Duguid in 1959, are "adhesive molecules on the surfaces of bacteria". Streptococcal binding sites have been identified on serum albumin, blood platelets, lymphocytes, whole erythrocytes and oral epithelium (Beachey, 1981). LTA has been implicated in a number of inflammatory reactions and other processes (Courtney et al, 1983)

Fibronectin, a glycoprotein, is a normal soluble constituent of blood plasma and other fluids and an insoluble component of extracellular matrix of various cells (buccal mucosal cells) and tissues. It binds to a number of bodily materials and includes collagen, heparin, actin, plasma membranes and certain microorganisms (Staphylococcus aureus) (Akiyama et al, 1981). It plays a role in bacterial attachment processes in the oral cavity and elsewhere. It is present in saliva and gingival fluid and is in-

corporated into the acquired dental pellicle during its formation. The acquired pellicle is the first structure (or coating) to form on teeth and onto which oral bacteria can adhere after all bacterial and other deposits have been cleaned mechanically from the teeth. It is composed of salivary proteins and glycoproteins (Marsh, 1992). The pellicle has been described (Gahnberg et al, 1982; Malamud et al, 1981 and Rosan et al, 1982) as consisting of a variety of salivary products of host and bacterial origin, for example: "high molecular weight glycoproteins (salivary mucins), lysozyme, salivary agglutinins, IgA and bacterial extracellular polymeric substances" including fibronectin. Fibronectin contains fatty acid-binding sites for LTA and may act as a receptor for bacteria with LTA-containing cell-membranes to permit attachment to mucosal cells (Courtney et al, 1983)

The role of LTA was illustrated when it was found to inhibit, to some degree, binding of viridans streptococci to fibronectin-coated hydroxyapatite beads (Hogg and Manning, 1988). In this experiment, a S. sanguis-derived LTA-containing solution was able to attach to and block (or partially block) fibronectin-adherence receptors on the beads before the membrane-bound LTA receptors of bacteria used in the experiment were able to interact. Binding between LTA and fibronectin appears to be of an hydrophobic nature. Hogg and Manning suggest that as both LTA and bacterial cells competed for the same or neighbouring sites on the coated beads, this provided further evidence of the involvement of LTA in viridans streptococcal adhesion. Species were tested for adhesion and no significant difference was apparent between species. However, in this experiment the percentage of adhesion varied in four strains of S. mutans between 66.6-83.2% with one strain <50%, while in the case of S. salivarius 4 isolates varied between 50-90.7% bound, one <50%. Six strains of S. sanguis I were 75.5-92.5% bound. Binding of six strains of S. sanguis II varied

from 69.3–79.3% with one strain <50% and in S. mitis three strains showed binding of 61.4–93.9%. The appearance of a strain each of S. mutans and S. salivarius with LTA-mediated binding percentages of below 50% and lower values is evidence, the authors claimed, for the the involvement of other fibronectin mechanisms. The effect of LTA may be less specific as S. sanguis II and S. mitis were inhibited by LTA although the species do not synthesise LTA

Recent research, employing molecular genetics techniques, has discovered that important virulence factors exist amongst gram-positive bacteria producing experimental endocarditis. Mutant S. sanguis were developed with a reduced ability to bind with immobilised fibronectin and were found to be markedly less virulent in a rat model than the strain with intact fibronectin-binding characteristics. The virulence of the former was infective dose-dependent while the latter was unaffected by inoculum size. Similar tests were performed on S. aureus strains. From data in these studies, it appears that adherence of Gram-positive bacteria to fibronectin is "pivotal" in the pathogenesis of experimental endocarditis (Baddour, 1994)

The ability to synthesise extra-cellular (exo)polysaccharides in S. mutans, for example, and its possible contribution to virulence this species may display has been studied. Through manipulation of its genome, strains with a deficient abilty to produce this substance were markedly less able to produce endocardial disease. What played a definitive role in infectivity was the prior exposure of "normal" test strains to sucrose. Without such exposure, the latter strain exhibited the same (very reduced) degree of infectivity as the mutant strain with such exposure (Baddour, 1994)

In Baddour's 1994 review of virulence factors in IE, he noted

that when S. gordonii was subjected to the same mutagenic manipulation as S. mutans as regards its production of exopolysaccharides and its effect on cariogenicity, it showed no difference in infectivity in the rat endocarditis model. Researchers were unable to explain this phenomenon but it may be that the two processes are unrelated. One is relevant to adherence of strains to hard, dental tissue (and attached protein-rich dental pellicle, bacteria and their products and food debris - see elsewhere) and an ability to produce tooth-dissolving acids, while the other applies to attachment to tissues and substructures of the endocardium. Adherence in cariogenicity and its role in cardiac infectivity are not necessarily comparable

Manning et al (1994) recognised the role which adherence to fibronectin (plentiful at sites of endothelial injury and repair, and also on surfaces of thrombic lesions on platelets) and subsequent platelet aggregation by S. sanguis-group bacteria play in the development of endocarditis

Certain unrelated factors are, however, known to discourage bacterial substrate adherence. Examples include the phenomenon of species specificity - where certain bacteria tend only to infect specific host species (N. gonorrhoeae infects only humans), genetic specificity (susceptibility to certain infections is a genetic trait), the presence of non-specific protective mechanisms on host mucosae (antibodies, protective enzymes, cleansing/flushing action of secretions etc.) and receptor specificity (of bacterial cells for host receptor)

vi) Infection of sterile vegetations

Fibrin-platelet aggregates are initially sterile. Before infection is possible, adhesion to and colonisation of vegetations by micro-organisms circulating in the blood-stream has to occur (Cotran et al, 1989) to produce the typical "infective

vegetations" found in IE

2.3.3 A possible immunological link in the development of infective endocarditis

A strong immunological link in the pathogenesis of IE has been suggested by Hayward (1973a). Leucocytes are absent from vegetations and organisms tend to be centrally placed within the vegetations. He suggested that factors like the (stimulatory) immunological response of the host to the introduction into the blood-stream of organisms of low virulence, could play a role in the development and the complications of IE. In most IE-patients, high levels of circulating antibody to the infecting organism are present and high antibody titre levels may play a major initiatory role in the formation of vegetations, for example. It was found experimentally that chronic bacterial endocarditis is inducible in animals used for the commercial production of antisera after re-introduction of bacteria into the blood-stream during the phase of elevated antibody titres in the immune response. The presence of circulating agglutinating antibodies enables the spread of immune complexes to other areas of the body where they become attached, amongst other sites, to the endothelium of small blood vessels (Hayward, 1973a. Fragile infected vegetations are known to fragment and to spread to other parts of the body in a similar fashion (Hayward 1973a; Cotran et al, 1989)

2.3.4 Summary

Cotran et al (1989) divided the pathogenesis of IE into distinct events. In the presence of bacteraemia, three are important: (i) the formation of sterile platelet-fibrin deposits or non-

bacterial thrombic endocarditis (NBTE), (ii) the seeding of sterile vegetations by clumps of agglutinating antibodies and bacteria, and (iii) the important role which bacterial adhesion plays. Development of the acute form of IE is more difficult to describe except in cases with obvious and frequent bacterial contamination of the blood such as is found in IV drug abusers, or in those with indwelling vascular catheters

An observation by Hayward in 1973 (Hayward, 1973a) during a lecture before the Royal College of Surgeons is particularly apt. When dealing with the confusion and uncertainties of IE, its pathogenesis, why it should develop in the first instance and the reputed involvement of dental procedures, he said the following: "It is difficult to understand why the introduction over a short period of time, such as during a dental extraction, of organisms of low virulence should, in a well patient often with trivial heart trouble, initiate a disease so relentless in its course and destructive in its effects"

It is recognised today that micro-organisms that commonly produce endocarditis in humans, particularly staphylococci, streptococci and enterococci, exploit the wound and wound healing process of the host to produce endocardial infection (see below). Infection would be impossible were there not (i) early colonisation of endothelial wounds by bacteraemic isolates able to survive the hostile conditions present within the bloodstream, and (ii) attachment by bacterial elements to exposed sub-endothelial extracellular matrix proteins within the wounds (Baddour, 1994)

Chapter 3

SOURCES, ROUTES OF INFECTION, AND BACTERAEMIA IN RELATION TO INFECTIVE ENDOCARDITIS

3.1 Indigenous oral flora

3.1.1 Bacteria of the oral cavity

Indigenous populations of micro-organisms inhabiting the skin and mucosal surfaces provide protection against colonisation and/or invasion by pathogenic organisms. However, the organisms need not always serve a protective function and, under certain circumstances, the same microbes may destroy a natural host, either by their own action or together in a mixed infection with exogenous pathogens (Mackowiak, 1982). Opportunistic infections occur commonly in patients with impared immunological defences

The oral cavity in healthy individuals supports one of the "most concentrated and varied of microbial populations of any area of the body" (Hardie, 1983) and numbers of resident organisms in the mouth are extremely great. Gordon et al (1971) state that a "periodontally normal" adult gingival crevice would contain approximately 270 thousand million recoverable micro-organisms per gramme of dental plaque and debris. They determined that aerobic and cultivable anaerobic bacteria made up between 22- and 179 thousand million bacteria per gramme of wet dental plaque depending upon which culture techniques were employed. These figures would include viridans streptococci and other facultatively anaerobic organisms. The sulcus contains a complex, mixed population of organisms the general make-up of which has been found to

be similar in most individuals (Kornman, 1982). The total count in saliva of (mostly tongue derived) organisms is between 430- to 550 million per millilitre with an average sample count of approximately 750 million and 200 000 million cells per gramme for the gingival sulcus (Hardie, 1983)

A difficulty in determining the total viable count is the discrepancy between this figure and the total microscopic count, especially for substances such as dental plaque (used in this study only as a source of streptococci), an aggregate of oral organisms growing within a gelatinous organic matrix (Hardie, 1983). By mechanical disruption of specimens, eg. ultrasonic dispersal, the viable count can be increased significantly (especially of streptococci and actinomyces) or decreased (certain Gram-negative strains)

Microbiological variation amongst oral bacteria is great. Moore et al (1982) found 264 morphologically and biochemically distinct bacterial groups or species amongst 6800 isolates colonising the mouth and dental sites of volunteers with varying degrees of periodontal disease. From a small control sample of four people with healthy periodontia, 316 distinct isolates were cultured. The indigenous flora of the healthy mouth is populated by over 40 named species (Hardie, 1983)

3.1.2 Environmental niches within the oral cavity

Microflora-host relationships are both important and complex and not fully understood (Heimdahl and Nord, 1979). Mechanisms exist which play an important role in the selection of sites where particular organisms tend to be more plentiful. The specific locality of a bacterium type in the host is dependent initially upon the physical ability of that organism to attach or "anchor"

itself to a particular tissue (Gibbons and van Houte, 1975) and for its retention there thereafter (Hardie, 1983). Under favourable conditions, the bacterium will remain at that site and multiply

Different but anatomically related micro-environments or habitats exist in the mouth. Each is chararcterised by, for example, specific pHs, oxygen tensions and nutrient concentrations (Kornman, 1982; Hardie, 1983). Bacteria are more plentiful in areas of the mouth able to provide ideal conditions of growth for that specific strain. The composition of the microflora, therefore, is an indication of the nature of the oral environment from which bacteria are sampled (Hardie, 1983)

Oral niches include the salivary environment, gingival crevice, tongue, dental surfaces and the buccal mucosa (Chow et al, 1978) and colonisation of such sites by oral streptococci is influenced by factors which affect their selective ability to attachment to dental and mucosal surfaces (Ellen, 1982)

S. salivarius is found principally on the tongue and in suspension in saliva where it accounts for approximately 50% of all bacteria. It forms less than 1% of the species identified in either plaque or the gingival crevice (Hardie, 1983). Its presence in saliva in high numbers is likely to be as a result of mechanical dislodgement from its site of attachment (Socransky and Manganiello, 1971). S. salivarius is not dependent upon the presence of teeth for its survival in the mouth (Socransky and Manganiello, 1971). Saliva serves simply as a carrier for this organism

Three strains, S. oralis, S. mitis biovar 1 and S. salivarius were the most abundant species in a study of pioneer oral streptococci undertaken on neonates (Pearce et al, 1995). Samples from

specified sites in the mouth were taken from babies at 1-3 days, 2 weeks and 1 month post-partum. The former species, the most plentiful at the first sampling (41%), was the least common of the three species after a month (20%) while the latter strain rose from an initial 10% to a final 28% after four weeks. There was little change to S. mitis biovar 1 numbers. Other identified species included S. mitis biovar 2, S. sanguis, S. gordonii, and S. anginosis

In the first months of life, microbe populations are relatively unstable (Socransky and Manganiello, 1971). A constant introduction of different species to the baby from other sources (mother) occurs but few will remain and proliferate if environmental conditions are unsuitable. With the eruption of teeth after approximately six months, new micro-environments develop and other aerobic and new anaerobic species are able to colonise the gingival groove and various structures in and on dental hard tissue. The presence of dental caries in the child and adolescent has a marked effect on micobe populations and their composition (Socransky and Manganiello, 1971)

Composition of bacterial populations in the mouth (dental plaque) alters as a result of changes to the oral environment and the integrity of host tissues. This is noticable in the presence of gingival inflammation or during carious processes of teeth. Ellen (1982) examined populations in patients with periodontal disease. The author noted that although Gram-positive bacteria are numerically important oral organisms, they tended to play a lesser role in those with advanced periodontitis and form only a minor (but important) proportion of subgingival microflora at affected sites. Gram-negative anaerobic organisms become more plentiful as the disease process progresses while Gram-positive bacteria tend to increase in number as tissues heal during treatment. The latter are however, like Gram-negative species, also capable of in-

ducing inflammation of the gingiva as they proliferate in dental plaque (Actinomyces species). Even in the "healthy" mouth, signs of gingival inflammation (gingivitis) are evident in concealed locations

The comparative composition of streptococcal populations in caries-active and caries-inactive individuals was examined by Nyvad and Kilian (1990). Seven hundred viridans strains in 4-hour old plaque from 14 adolescent children (median age 14 years, range 12-23 years) were identified. All isolates were taken from identical pieces of human enamel (of identical size etc.) carried in the mouths of volunteers. Streptococci dominated the microflora in 61 and 78% of the viable counts in caries-active and -inactive individuals respectively. Of 700 streptococcal isolates, the predominant isolates consisted of Streptococcus oralis (31.8%), S. mitis biovar I (29.3%) and S. sanguis (21.3%) with S. mitis biovar 2, S. salivarius, S. gordonii and S. mutans making up 5% or less of the total streptococcal count. The first three species accounted for 95% of all streptococci and 56% of the total number of cultivated oral bacteria. The authors found a significantly higher proportion of S. sanguis amongst caries-inactive than caries-active children. Two species, S. oralis and S. gordonii, predominate in health (Marsh, 1992). (Viridans speciation in this study followed the proposals of Kilian et al (1989)

As in the neonate investigation (Pearce et al, 1995), S. oralis, and S. mitis biovar I were plentiful but S. salivarius numbers were substantially reduced. The two to three day difference in sampling times of the two projects may account for this disparity, or the presence of and the attachment to dental hard-tissue by certain other species in the older volunteers, may have played a role

As an adult ages, further floral changes take place. One of the most striking occurs after the loss of teeth (Socransky and Manganiello, 1971)

Proportions of various viridans species may differ in plaque specimens of older (but unspecified) ages. MacFarlane et al (1983) cultured 24 viridans isolates from the plaque of 15 patients about to undergo a molar extraction. Identified organisms (method not noted) consisted of 10 S. mitior, 6 S. sanguis, 5 S. mutans, and 3 S. salivarius isolates. S. mitior (under which S. mitis is also classified) and S. sanguis together formed the majority of isolates in this study of (probably) older plaque but a difference in classification makes comparison difficult

3.1.3 Bacterial interactions in the oral cavity

Certain bacteria may inhance or retard the growth of other species in close proximity (Kuramitsu and Paul, 1980). The growth behaviour of Actinomyces viscosus (isolate T14V) and viridans strains was investigated in an **in vitro** system designed to mimic bacterial compatibility and attachment to dental surfaces. Hydroxy-apatite beads were coated with one strain and the activity of both organisms was observed when a second was permitted to attempt attachment. Two S. mutans strains, in the presence or absensce of sucrose, were found not inhibit A. viscosus attachment. S sanguis (2 strains) tended to inhibit A. viscosus attachment to a marked degree. A. viscosus noticeably increased attachment of one S. sanguis strain and weakly inhibited attachment of the other. The authors postulated that S. sanguis and A. viscosus competed for similar hydroxyapatite attachment sites. S. salivarius and a lactobacillus species exerted little if any effect on A. viscosus while S. mitis possessed activity which was a weakly inhibitory

In another **in vitro** study, behaviour of an antagonistic nature was apparent amongst certain plaque bacteria (Hardie, 1983). S. sanguis displayed a broad inhibitory effect on all other streptococci, filamentous organisms and rods. S. mutans produced bacteriocins which endowed it with a competitive advantage against neighbouring species in the mouth

3.2 Involvement in bacteraemias of oral streptococci

3.2.1 Transient bacteraemias

Since Okell and Elliot's reported link between dental treatment, transitory bacteraemias, the bacteraemic source and the development of IE (1935), much clinical and microbiological research has been undertaken. The transient bacteraemia which follows dental extractions permits oral bacteria to colonise pre-existing cardiac endothelial lesions and is likely to place patients with underlying valvular heart disease at risk of developing IE (Littner et al, 1986; Durack et al, 1983; (British Society for Antimicrobial Chemotherapy Working Party, 1990)

The phenomenon of bacteraemia development after operative procedures within the mouth has received a great deal of attention in the scientific literature. But the proportion of streptococcal bacteria in the blood identified in the literature as being viridans strains varies widely - from less than 40% to well over 60%. In the British Heart Journal, Bayliss et al (1983) analysed the cases of 544 episodes of IE diagnosed in 541 patients. They found the most commonly isolated organisms to be this group of organisms (261 of a 548 isolate total). Sixty-three percent or 164 of 261 streptococcal isolates were viridans streptococci. Of the 544 episodes of I.E., 122 patients (22.65%) had undergone

treatment of a dentally-related nature or had had dental sepsis in the three months prior to the onset of illness. However, 19% of the total number of I.E. cases were considered to be of a "probable" dental origin; ie. 103 of 544 cases or 84% of 122 of patients who presented with a history of either dental treatment (with or without antibiotic cover) or dental infection. Two hundred and thirty patients (43%) either had normal hearts or an unrecognised cardiac abnormality prior to onset of IE

Following a different approach, Durack et al, (1983) found different dental manipulations to be implicated in 48 of 52 (92%) endocarditis prophylaxis failures reported to the National Registry of the American Heart Association. Aetiological agents in almost two-thirds of cases (63.5%) were viridans streptococci

Shanson et al (1984), while investigating a method to better detect viridans bacteraemia after dental extraction, reported the involvement of viridans streptococci in bacteraemias in at least 28 of 58 (48.2%) healthy individuals without a known history of prior antibiotic use. A 30 ml volume of blood was suggested as being of optimal size for detection. Fifteen of 58 persons (41%) yielded viridans streptococci with a lesser 15 ml of blood. Later in 1987, Shanson et al detected this group of bacteria from 12 ml of blood in 32.5% (13 of 40) of volunteers in a comparative study to investigate the effectivity of amoxycillin and teicoplanin as agents of prophylaxis

Coulter et al (1990) reported much higher isolation rates in 58 healthy children following dental extraction using 5 ml of blood. Sixty-three percent (20 of 32) who were not on any form of antibiotic prophylaxis showed post-extraction bacteraemias. Half of 88 organisms identified were either aerobic or facultatively anaerobic and predominantly streptococcal. Thirty one streptococci isolates, identified chiefly as viridans streptococal

species S. mitior and S. sanguis, were found in 20 of 29 children. Other organisms isolated included Actinomyces, Bacteroides (certain of the latter are now classified as Porphyromonas) and Veillonella species. A third of blood cultures in which streptococci were isolated, were found as pure culture. Roberts et al (1987), using an complex culture technique, reported a lower incidence of post-extraction bacteraemia. An incidence of 38.3% in a group of 47 patients was noted. Of the bacteria isolated, 46.6% were viridans streptococci, a percentage almost two thirds that of the 63% of Coulter's et al (1990)

In a trial to assess the efficacy of the prophylactic regimen proposed by the American Heart Associaton (AHA, 1977), Hess et al (1983a) cultured blood specimens drawn from 82 "at risk" children after the extraction of either permanent or primary teeth. Post-extraction bacteraemia was present in 21% (17 children). Of these, 13.4% (10 of 82 or 58.8% of the 17 positive blood specimens) yielded viridans isolates in spite of the volunteers having received pre-operative penicillin G

A link between the size of a bacteraemia and the extraction of certain teeth has been sought. Phillips et al (1976) found no obvious difference in the incidence of bacteraemia in patients undergoing full dental clearance or extraction of wisdom teeth alone. Peterson and Peacock (1976) were, however, able to find a statistically significant difference ($p=<0.001$) in the incidence of bacteraemia relative to the extraction of teeth as compared to non-extraction (restorative) procedures. They were also unable to detect differences between the types of dentition (permanent or deciduous) or dental health status (the gingival health status was not considered)

In an earlier 1968 study, Elliott and Dunbar ran an investigation similar to the later 1976 study of Peterson and Peacock. The

former wished to determine differences, if any, between the occurrence of bacteraemias within younger and older age groups amongst 100 healthy children between the ages of 2 and 13. Blood cultures were performed after extractions. Whether extractions were of diseased or undiseased teeth was not specified. Thirty-six percent yielded blood cultures positive for alpha-haemolytic streptococci - none of which were speciated. Deciduous teeth (only) were removed in 79 children and both permanent and deciduous in 10. In 32% (25 children) of the former and 4 of the latter, viridans streptococci were cultured. In those from whom only permanent teeth were removed (11 children), 63.6% (7 of 11) had viridans streptococci in their blood post-operatively. When ages and positive blood counts were analysed, there was a higher incidence of bacteraemia (52%) amongst the older (8 to 13-year olds) than the younger group (31%, 2 to 7-year olds). Groups were small and no form of statistical analysis was employed

3.2.2 Variation in bacteraemia frequency

The wide variation in the frequency of surgery-linked bacteraemias has been noted above and specifically commented upon in the literature. Frequencies of bacteraemia range between 15-85% (Baltch et al, 1982). Differences in patient selection, times at which blood was drawn after the surgical event, types of surgery performed (Hall and Heimdahl, 1989) and other factors may account for these disparities

Conflicting opinions exist, for example, on what role, if any, the oral hygiene and periodontal health status of a patient or the number of extracted teeth would play in the appearance or magnitude of bacteraemia (Bender et al, 1963). The oral hygiene status of participants is often omitted from investigations of this nature. The contentiousness surrounding the issue is high-

lighted by Coulter et al (1990) who found no relationship between either the incidence of the bacteraemia and the amount of dental plaque, and periodontal (gingival health) status of a patient, or between the magnitude and incidence of a bacteraemia and the number of teeth extracted. However, Wahl (1994) appeared convinced that an indifferent oral hygiene status plays a substantive role in the development of IE. Bartzokas et al (1994) were able to trace to the mouths of patients with extensive periodontal disease, identical strains of **S. sanguis** cultured from infected material taken from total joint replacement prostheses after their failure and subsequent surgical removal

Although the source, locality and portal of entry of oral streptococci into the blood stream from the mouth may be obvious and easily identifiable, it is extremely difficult in retrospect to establish with any degree of certainty how and when offending bacteria entered the vascular system if or when no direct cause for the bacteraemia is detectable

3.2.3 Inflammation of the gingiva

Dental plaque, a soft, non-calcified material composed of bacteria, their products, food debris and other substances, is closely associated with tooth surfaces and the gingival sulcus (Hardy, 1983). Proximity to the gingiva of young (two to three day-old) plaque deposits on the teeth induces mild inflammatory processes in the gingiva after stimulation of the host's immune system (see also later) with accompanied vasodilatation, oedema and vascular fragility. Gingivitis develops initially and is restricted to the soft tissues of the gingival apparatus. Later, with prolonged exposure of the gingiva to bacterial metabolic by-products and toxins produced within older plaque deposits (where species of Gram negative bacteria tend to predominate over the

Gram positive cocci and other species found in gingivitis), tissue breakdown occurs and periodontitis develops (Slots, 1979; Slots and Genco, 1984). Characteristically, this latter condition is not restricted to the gingival apparatus but also affects the alveolar bone and other supportive tissues of the periodontium. Inflammation of periodontal tissues, therefore, eases entry into the blood stream of resident bacteria during forms of physical manipulation. An excellent description of the pathogenesis of periodontal disease is given by Williams et al (1992)

Normal physiological activity of a mechanical nature (chewing, tooth brushing) exerted upon affected gingival tissues may have a similar effect and also produce bacteraemias. Silver et al (1977) were more specific on the contribution which periodontal inflammation played in the development of bacteraemia. Aerobic and anaerobic species of bacteria were cultured in their study in all four volunteer groups who had increasing degrees of defined gingival inflammation. After completion of standard tooth- and gingival brushing procedures, the authors reported a significantly higher incidence of positive blood cultures amongst volunteers with worsening (increased) severities of gingival inflammation: 16%, 33%, 56% and 68% positivity in groups 1-4 respectively. However, like Sconyers et al (1973), any significance between the amount of accumulated dental plaque and the number of positive blood cultures remained undetected. But they were able to correlate an increased number of commensal oral bacterial species occurring in the bloodstream with degrees of specified gingival inflammation and worsening plaque indices. Of note in Silver's investigation was the fact that amongst those volunteers in the group with the best possible standard of oral hygiene (and therefore presumably the least amount of gingival inflammation), bacteraemia was still detected in 16%. None-the -less, early research has shown that the elective removal of teeth does not appear to be warranted to reduce the likelihood of viridans IE

(Croxson et al, 1971; Simon and Goodwin, 1971)

Bacteraemia is not a consequence only of traumatic surgical elective procedures - simple, commonly performed, routine exercises are known to lead to the development of bacteraemia. This was observed after tooth-brushing (as noted above), manipulation during detailed dental examination and periodontal probing, dental scaling, root-canal treatments and after other non-invasive procedures (Robinson et al, 1950; Bender et al, 1963; Conner et al, 1967; Speck et al, 1971; Sconyers et al, 1973; Silver et al, 1977)

In a review of the accepted importance of dental procedures as causes of endocarditis, Guntheroth (1984) found few cases to be related specifically to dental extractions only (47 of 1322 cases or 3.55%). Earlier (and as commented upon above), Hayward (1973b) noted that in many IE cases (two-thirds) neither a precipitating cause for the infection nor the time when the bacteraemia occurred could be satisfactorily determined

A complicating factor in the detection of bacteraemia is the fact that microbiological methods of investigation may differ and dedicated isolation techniques are often required to culture certain bacteria. For example, the identification of "nutritionally-variant" streptococci is only possible if specially supplemented growth media are utilised (Roberts et al, 1979; Feder et al, 1980)

3.2.4 Viridans streptococci and orofacial infection

Viridans and other streptococci need not originate only from dentally-associated foci of infection to precipitate IE and other serious infections. Strains of these bacteria are associated with

episodes of septicaemia and purulent infections of both the oral cavity (Beighton et al, 1991) and peri-oral structures of the human head and neck. Chow et al (1978), noted that (pyogenic) orofacial infections are most commonly odontogenic in origin. They would tend, therefore, to involve organisms (both aerobic and anaerobic) associated with teeth. In exudate cultures from oro-facial soft tissue infections collected during surgical treatment, 36% were found to be viridans streptococci in pure culture (Hunt et al, 1978)

3.3 A dermal source and route of infection

Staphylococci are carried naturally on the skin and on the mucous membranes of the naso-pharynx. These areas of the body serve as important reservoirs, in carriers, for virulent strains of Staphylococcus aureus (von Lichtenstein, 1989). This species is "among the hardiest of nonspore-forming bacteria" (Waldevogel, 1985). It can be cultured from dried clinical material, is able to tolerate high salt levels and is relatively heat resistant. S. aureus causes inflammatory reactions throughout the body and can "invade" the lymphatic and blood systems to give "rise to severe septicaemia or endocarditis"

S. aureus is involved in 20-30% of cases of IE (less than the 35-65% with viridans streptococci, refer above). Characteristically, it is the principal agent in the acute form of endocarditis where previously healthy heart valves are infected. It is widely involved in endocarditis of IV-drug users and is the major cause of IE in patients with valvular prostheses (Cotran et al, 1989). Waldvogel (1985) reports a lower incidence of 10% or less of IE in patients with S. aureus septicaemia while early figures were 60% or higher

Although favouring the adhesion of S. aureus, mucous membranes and the skin form very effective mechanical barriers to tissue invasion (Waldvogel, 1985). Were this barrier to be breached as a result of trauma or surgery (Waldvogel, 1985), large numbers of bacteria would be required for staphylococci to become infective. However, once established and no treatment is initiated, the bacteria tend to become invasive and the infection progressive (von Lichtenstein, 1989). Characteristically, abscess formation is indicative of staphylococcal infection (von Lichtenstein, 1989). If located near the body surface, these lesions heal after natural or surgical drainage. Deeper lesions tend to rupture into serous cavities and suppurative peritonitis or pericarditis may develop. Another clinical condition, cellulitis, becomes apparent when, instead of forming typical localised, well circumscribed collections of purulent material, highly virulent staphylococcal strains spread into and infect loose connective tissue spaces. Staphylococci may disperse throughout the body via the bloodstream and metastatic abscesses or endocarditis may develop if treatment is not instituted promptly. Regional lymphadenitis may develop after S. aureus infection and, with involvement of the venous blood vessels, may lead to the complications typical of staphylococcal cellulitis: the formation and spread of septic emboli which may implant onto heart valves or into other organs (brain, kidney, lung) (von Lichtenstein, 1989)

Another staphylococcusal skin inhabitant, S. epidermidis, which tends to be less virulent, infects surgical or traumatic wounds or prostheses and may be life-threatening in compromised or debilitated patients (von Lichtenstein, 1989). In the multicentre study of Fang et al (1993), this staphylococcal species was the most commonly identified bacterium in all positive blood cultures in the investigation (43 of 171, or 25%). Seventy-four patients of 171 (43%) were classified as having definite prosthetic valve endocarditis and of these, in 30 of 74 patients the organism

responsible for IE was S. epidermidis. The portal of entry most commonly identified in Fangs' study to lead to the development of new cases (18) of prosthetic heart valve IE with nosocomial bacteria, were intravascular catheters and wound and skin infections. In six and five patients, the causative organisms were S. epidermidis and S. aureus, respectively

S. aureus sepsis shows a high mortality in certain clinical settings in spite of the availability of antibiotic chemotherapy. Sepsis with S. epidermidis, on the other hand, is inclined to show a more chronic course and be less of a medical emergency (von Lichtenstein, 1989)

3.4 Gastro-intestinal source and route of infection

3.4.1 Organisms and some predisposing factors

The entry into the blood-stream of bacteria may be overt and can be related directly to some specific and identifiable mechanism, or it may occur covertly as a result of causes difficult to identify (see earlier). Covert transient bacteraemias originate frequently in the gut (and oral cavity) (Cotran et al, 1989). Seeding of the gut blood supply with bacteria of low virulence from the gastro-intestinal tract may occur during normal physiological processes such as intestinal peristalsis or mastication. In two-thirds of patients with IE, no identifiable cause of the initial bacteraemia is known (Hayward, 1973b) - perhaps the latter mechanism may be precipitating factors of greater importance than is currently acknowledged?

Surgical procedures which breach the physical integrity of bacterially-laden mucosal membranes of the (lower) gastro-intestinal tract, are likely to precipitate bacteraemia (Ashby et

al, 1978). Wound infection is common after colon or gastric resection or hysterectomy (Gorbach, 1982) - all surgical procedures involving bacteria-laden mucosae

E. coli, together with Enterobacter and Proteus organisms, are common causes of suppurative infections of the abdominal cavity (von Lichtenstein, 1989). Gram-negative bacteraemia is an extremely serious consequence of infections by these bacteria (acute appendicitis, cholecystitis etc.). Patients die as a result of endotoxaemic reactions, metastatic dissemination of organisms, DIC and shock

Von Lichtenstein makes the point that supports those made by Cotran et al (1989) and Hayward (1973b) on unidentifiable sources of bacteraemic organisms when he wrote that the presence of (Gram-negative) bacteraemia is not necessarily the consequence of suppurative disease. He continued: "...transient contamination of the blood is commonplace in daily life" and is of no consequence in the normal individual but only in those with "lowered resistance..". Bacteroides species, like E. coli, are capable of entering the blood stream after sepsis of the gut (B. fragilis), oral cavity (B. melanogenicus) and associated structures (von Lichtenstein, 1989). Debilitated patients commonly become septicaemic when affected by these species and have a high mortality

3.4.2 Gastro-intestinal malignancy and bacteraemia

Endocarditis caused by certain bacteria present in the colon has been associated with lesions of the large bowel, including colorectal carcinoma. Enterococcus spp. and Streptococcus bovis are examples of organisms which have been shown to be associated with malignancy. Such neoplasms are responsible for entry of these bacteria into the bloodstream with subsequent develoment of en-

docarditis. An association between disease of the gastro-intestinal tract (GIT) and S. bovis bacteraemia was sought by Murray and Roberts (1978). Thirty-six adults with S. bovis endocarditis plus 10 with bacteraemia alone were examined in an attempt to determine possible portals of entry. Of these, 25 had lesions of the GIT or had recently undergone manipulation of this organ system. Twenty-two patients (14 with endocarditis, 8 with bacteraemia) had identifiable lesions in the gut. The authors divided these lesions into "probable" sources of bacteraemia (for example, colon carcinoma, ulcerations, adenomas, peritonitis) or "possible" sources (benign colonic polyps, bleeding haemorrhoids). Two symptomless patients (one each with IE or bacteraemia) after exploration of the GIT, were later found to have previously unidentified villous adenomas

Klein et al (1977) noted a possible link between carriage of S. bovis and the presence of carcinoma of the colon. They speculated on whether S. bovis may increase in number as a result of altered environmental factors in the bowel produced by the malignancy or visa versa

Chapter 4

OVERVIEW OF PROPHYLAXIS OF INFECTIVE ENDOCARDITIS

4.1 Introduction

"Efforts to prevent the disease (IE) have failed, regrettably...." (Morris, 1985)

An important relationship between dental extraction and other blood-letting procedures in dentistry and IE has been recognised for years and it has become accepted (obligatory) to attempt to prevent bacterial endocarditis in patients considered to be at risk

4.2 Regimens of antibiotic prophylaxis

Chemoprophylaxis has as its principal aim the protection of compromised patients from infection by micro-organisms resident in the mouth, nasopharynx and upper respiratory tract. As oral (viridans) streptococci are most commonly implicated in I.E. (Roberts et al, 1979; Bayliss et al, 1983; Durack et al, 1983; Hall and Heimdhal, 1989), safe, practicable methods of protection are directed against these bacteria when they appear in the blood stream

The exact nature of factors predisposing patients to IE development, the disease itself and its prevention may be confusing (Jaspers and Little, 1984) but the known involvement of viridans streptococci in the aetiology of dentally-associated infective

endocarditis (Bourgault et al, 1979) is irrefutable. It is acknowledged that dental treatment is potentially hazardous for those with cardiac disease and that the development of IE in these patients is possible (Durack et al, 1983; Morris, 1985). Following current concepts, the administration of prophylactic antibiotics to reduce the likelihood of susceptible bacteria infecting existing cardiac lesions in susceptable patients is highly advisable. The elective administration of protective antibiotic cover is, however, controversial (Petersdorf, 1978; Morris, 1985; Durack et al, 1983). IE prevention is complex and involves many "diverse issues" (Durack, 1995). Bayer et al (1990) take the view that the administration of antibiotic prophylaxis has simply become a "community standard practice" in the highly compromised patient to prevent prosthetic valve endocarditis in "two major settings": (i) peri-operatively to circumvent operative development of prosthesis colonisation, and (ii) during bacteraemia-inducing oral, urogenital, and other medical procedures

Numbers of European and American IE prophylaxis regimen proposals have been published over the years. The British Society for Antimicrobial Chemotherapy (BSAC) has released several and include the Report of a Working Party in 1982, 1986 and 1990. Those of the American Heart Association (AHA) include releases in the AHA Committee Report (1981), Special Report (1984) and Special Statement (1985). Recommendations and summaries from other parties are available (Van der Bijl, 1992; Medical Letter, 1989; Federation Dentaire Internationale, 1987; Editorial, 1985; Jaspers and Little, 1984)

The detail of many regimens differed markedly in earlier versions and caused considerable confusion amongst dentists and medical practitioners, especially regarding patient-risk categories and antibiotic regimen selection (van der Bijl, 1992).

Attempts have been made to both reduce subsequent confusion and ease patient compliance. Emphasis earlier on parental administration made prophylaxis far less acceptable to both recipients and dentists (Petersdorf, 1978) and in 1982, in an effort to improve compliance, the BSAC proposed a simpler single or double, high-dose, peri-operative oral amoxycillin regimen to cover the critical post-operative risk period

The 1982 BSAC IE-prophylaxis proposal and the AHA (1984) equivalent were the two most commonly used regimens but still, unfortunately, differed sufficiently to prolong confusion. The AHA, however, fell in step with the British (BSAC, 1982; BSAC 1984; BSAC 1986; BSAC 1990) with the publication in 1990 of their updated and amended recommendations (AHA, 1990)

4.2.1 Oral route

At present, the difference between the BSAC and AHA proposals is relatively minor. Both recommend oral erythromycin, clindamycin and large doses of amoxycillin with variations in form and dosage size. The former group prefer larger, single pre-operative doses of antibiotic (except erythromycin, see below) while the AHA tend have an initial dose either identical or smaller in size to the BSAC, with a second administration 6 hours later. Neither the BSAC nor the AHA any longer include penicillin V in their protocols and both recommend amoxycillin 3 g per os 1-hour pre-operatively in non-sensitive adults, while the latter also use a second 1.5 g amoxycillin dose 6 hours later to ensure high post-operative serum amoxycillin concentrations

For penicillin-allergic patients, both oral protocols contain a second erythromycin dose 6 hours after the first but they differ marginally in other areas: the British recommend only the

stearate salt in a 1.5 g stat dose a shorter 1-2 hours prior to the procedure with a 500 mg 6-hour dose later while the Americans prefer a smaller initial 800 mg erythromycin ethylsuccinate or 1 g erythromycin stearate 2-hours pre-operatively plus a second dose of half the first the same period of 6-hours afterwards

Each regimen has some merit. The 50% larger BSAC 1.5 g -stearate dose size ensures higher and more adequate blood levels over a longer period (BSAC, 1986; Shanson, 1985 but may induce a higher incidence of nausea (BSAC, 1986) while the AHA erythromycin-succinate alternative is better tolerated by the patient

Roxithromycin may be a useful alternative to erythromycin (Smith et al, 1989). It has excellent pharmacokinetics and an antibacterial spectrum which is similar to that of erythromycin. It is known to induce less nausea and fewer gastro-intestinal upsets (Blanc et al, 1987) than erythromycin ethylsuccinate and has been shown to produce higher blood levels (than enteric coated erythromycin) from smaller quantities taken by mouth (Nilsen, 1987). Encapsulated enteric-coated erythromycin base produces higher serum levels than the -stearate formulation (Josefsson, Bergan et al, 1982)

Clindamycin is suitable for those who cannot tolerate either penicillin or erythromycin. The total amount of clindamycin administered to IE-susceptible patients in the AHA regimen is less than that in the BSAC. The former has a smaller loading dose of 300 mg clindamycin 1 hour prior to surgery plus 150 mg after 6 hours where the British recommend a single, very much larger 600 mg pre-operative dose only, also given the same time (1 hour) before the blood-letting procedure - without apparent side-effects (BSAC, 1990), apart from the "small risk" of pseudo-membranous colitis (BSAC, 1986)

4.2.2 Parenteral route

The BSAC has only one recommendation for parenteral form of IE prophylaxis for non-allergic adults: a smaller 1.0 g quantity of amoxycillin IM suspended in local anaesthetic plus 120 mg gentamicin is administered initially plus 500 mg amoxycillin orally 6 hours later. This prophylaxis regimen is used to prevent enterococcal endocarditis following gastro-intestinal tract manipulation. The Americans (AHA, 1990) use ampicillin instead of parenteral amoxycillin and use amoxycillin only in its oral form: ampicillin 2.0 g IM or IV 30 minutes before the procedure plus 1.0 g IM or IV or 1.5 g amoxycillin per os 6 hours later. One of three AHA parenteral routes is very similar to that of the BSAC, except that a larger 2.0 g ampicillin IV dose is used (instead of 1.0 g amoxycillin IV) and also with gentamicin up to a maximum of 80 mg. The third adult AHA IV or IM method (non-high-risk patients) uses clindamycin in the lower dose (300 mg) IV plus 150 mg either IV or orally 6 hours later

Both the BSAC and AHA use vancomycin in high-risk, penicillin allergic patients who require treatment under general anaesthesia or have had penicillin more than once the previous month (see below)

In spite of the pain experienced during injection of IV amoxycillin, it is used instead of ampicillin because of its greater and more rapid bactericidal activity (Comber et al, 1975)

For high-risk patients, 1.0 g vancomycin by slow IV infusion 1 hour before surgery is common to the regimens. The BSAC, however, also recommends the additional use of gentamicin (120 mg) IV prior to induction or 15 minutes before the procedure

Paediatric prophylaxis regimens of the BSAC and AHA are adaptions of those of the adult

Recommendations of the BSAC and AHA have been summarised with explanatory comments by van der Bijl (1992). His major differention is between the oral and parental regimens and between penicillin-taking and penicillin-allergic patients. Standard- or high-risk patients (those who recently took penicillin and those who receive treatment under anaesthesia) are included

Since 1982, the BSAC has recommended the use of oral prophylactic cover for almost all high-risk patients (including those with prosthetic heart valves) and the AHA now concurs, although the selection of a particular AHA parenteral prophylaxis regimen is still left to the clinician to a large degree. The single BSAC parenteral route is recommended for special risk cases such as those who have had previous episodes of endocarditis. Included in AHA guidelines are recommendations for patients suffering hypertrophic cardiomyopathy and mitral valve proplapse

While the AHA recommends chemoprophylaxis during blood-letting procedures involving the oral mucosa or gingiva, the BSAC does not unless patients are at special risk

The development of resistance amongst viridans strains initially prompted the BSAC to recommend a minimum interval for amoxycillin of 4 weeks between the last dose and the prophylaxis but later shortened this period to 2 weeks (BSAC, 1986) after further research was published indicating that this was not common (Harrison, Rubin et al, 1985; Woodman et al, 1985. Erythromycin was found to select significantly for resistance but no changes were recommended for this antibiotic (Harrison, Stross et al, 1985

4.2.3 Further techniques to reduce risk of bacteraemia

Prevention of IE may involve techniques which do not require only the administration of chemical agents in those susceptible to its development. Guntheroth (1984) suggested that one of the most effective methods to complement the use of protective chemotherapy would be the practice of good oral and dental hygiene and an aggressive dental management programme. In contrast, certain authors appear to cast doubt on accepted philosophies in this regard (Coulter et al, 1990)

The AHA recognises the risk of poor oral hygiene and periapical and peridontal infections on the development of bacteraemias in at-risk patients and an optimal standard of oral health is encouraged. Suitable oral rinses are recommended as an adjunct (van der Bijl, 1992)

4.3 Experimental models in animals

4.3.1 Animal trials

Investigators have used animal experimentation to support the advisability of antibiotic chemoprophylaxis use. Bacterial species employed in trials are those against which patients would most likely require protection. Studies on the prevention of streptococcal IE have been performed in rat and rabbit models (Bernard et al, 1981; Glauser et al, 1983; Malinverni et al, 1983; Francioli et al, 1985; Moreillon et al, 1986; Pujudas et al, 1986; Berney and Francioli, 1990)

Valvular lesions are produced mechanically by intention (Drake and Sande, 1986) with the insertion of cardiac catheters into the

heart chambers one or more days before initiation of trials. Defined amounts of antibiotics are then administered to the injured animals at predetermined times (usually 30 minutes) prior to the introduction by injection of bacteria into the bloodstream, the inoculum size of which can vary between researchers. Efficacy of specific antibiotics are tested when administered as single agents or in combination, as single or multiple doses and at varying time intervals. Success of prophylaxis is determined by animal sacrifice 24-72 hours after antibiotic administration (Drake and Sande, 1986). To determine the proportion of infected animals, valvular vegetations are excised and cultured

Whatever animal model is employed, all share common pathophysiological features in the (i) production of endothelial cell denudation, (ii) exposure of underlying extracellular matrix, and (iii) local deposition of platelets and fibrin (Baddour, 1994)

Francioli et al, (1985) showed that amoxycillin is equally effective against a small bacterial inoculum when used alone or in combination with gentamicin. In another experiment on rats, a single dose of amoxycillin was used to prevent IE after bacterial challenge with organisms that were amoxycillin-tolerant (Berney and Francioli, 1990). Antibiotic prophylaxis was successful, however, only when the the size of experimental bacterial dose was smaller than the infective dose. Malinverni et al (1987) tested multiple-dose regimens of amoxycillin alone and in combination with gentamicin against two viridans species (S. sanguis and S. intermedius) and two Enterococcus faecalis isolates in experimental streptococcal endocarditis. Amoxycillin as a single agent was effective only against the oral streptococci but not against the E. faecalis strains where only the combination proved successful

When bacteria are sensitive and rapidly killed by an antibiotic,

Glauser and Francioli (1982) found single-dose prophylaxis successful irrespective of the number of organisms used to induce endocarditis

In contrast, tolerant viridans streptococci are only controlled effectively by beta-lactams and other cell-wall active and bacteriostatic antibiotic agents (erythromycin and clindamycin) when bacterial numbers are less than 90% of the infective dose (Glauser et al, 1983; Francioli et al, 1985; Moreillon et al, 1986; Berney and Francioli, 1990)

Further properties of administered antibiotics, other than their bactericidal ability, may, however, play a role. In 1981, Bernard et al, found vancomycin prophylaxis in IE-susceptible rats effective against an injected vancomycin-tolerant S. sanguis strain and ascribed this phenomenon to a reduction of tissue adherence properties of the bacteria

4.3.2 Clinical relevance of animal trials

The relevance of data derived from the experimental development of IE in animal models and extrapolated to IE in humans has been queried (Malinverni et al, 1987). For example, the large quantity of bacteria injected into laboratory animals to produce IE far exceeds that detected in the blood-stream after dental and other procedures in humans. Animal experimentation and clinical situations are incomparable because of vastly unequal number of "seeding bacteria" introduced into the blood-stream (Petersdorf, 1978)

It ought to be borne in mind, however, that the injection of bacteria into the animals is, of necessity, a crude attempt at simulating the biological processes found in nature. Bernard and

his co-workers (1981) indicate, in apparent defence of the laboratory method that, when circulating bacterial levels are determined in the animals shortly after IV injection, numbers soon decrease from the high levels present initially. In the rabbit model, Petersdorf noted that this animal provides a more stingent prophylaxis model than anticipated because of factors which appear to disadvantage the animal more than they apparently would humans: the disproportionally large number of bacteria necessary to induce endocarditis (see above); secondly, the use of indwelling cardiac catheters tends to reduce antibiotic effectivity (see below) and thirdly, the inherent very rapid rate of antibiotic excretion which the rabbit displays

Importantly, results of antibiotic trials for the therapy of IE in the animal models are consistent but experimental design and other variables require critical examination (Drake and Sande, 1986). This latter comment is relevant when observations and conclusions are drawn from data. The authors mention specifically factors such as the effect which the presence or absence of intra-cardiac catheters may exert on antimicrobial agent activity (an aspect discussed by Petersdorf, 1978), the time of antibiotic administration relative to the time of bacterial infection, dose sizes and interdose intervals, and the species of infective bacterial agents used in the model

4.4 **Summary**

From the interpretation of experimental antmicrobial prophylaxis research data, results of its use in the clinical situation is often confusing and inconclusive. Effectiveness of IE prohylaxis regimens is not absolute (Friedlander and Yoshikawa, 1990) and medico-legal aspects generally dictate its employment whenever uncertainty arises on the issue (van der Bijl, 1992)

Despite great strides made in the clinical and scientific fields of medicine, streptococcal IE is still frequently encountered in both the apparently healthy and diseased heart (Bayliss et al, 1983). Prophylactic antibiotic regimens, although based on available clinical knowledge and experimental data, are still empirical in nature (MacFarlane et al, 1983) and no prophylactic regimen has been proven, beyond reasonable doubt, to be effective against the development of endocarditis after invasive procedures of a surgical nature. Only indirect evidence is available on the efficacy of prophylaxis used in humans because of very obvious ethical problems involved with the experimental testing of such regimens on human subjects. What evidence there may be is often, of necessity, anecdotal (Bayliss et al, 1983; Durack et al, 1983). Because of the seriousness of IE development in a patient, routine measures are employed which are hoped would reduce the likelihood of post-operative complications occurring. The serious level of debate is illustrated by Bayer et al in 1990 for instance, who found no controlled studies which, in their view, were able to confirm satisfactorily the benefits of prophylaxis in preventing endocarditis of native valves

A contentious issue which has been raised on many occasions in the medical and scientific literature involves the role which dental procedures may play in the causation of IE. Its importance has been questioned by some researchers (Guntheroth, 1984), while others believe there is a direct dental link (Durack et al, 1983)

Chapter 5

ANTIMICROBIAL CANDIDATES FOR INFECTIVE ENDOCARDITIS PROPHYLAXIS

5.1 Introduction

Recommendations for the use of prophylactic chemotherapy are based mainly on susceptibility patterns of organisms most likely to produce IE in given clinical situations as well as the pharmacokinetic properties of the candidate antibiotics. There is general agreement that the effect of the prophylactic antimicrobial agents on the target organisms should ideally be bactericidal in nature (Coulter et al, 1990)

In an effort to determine possible clinical efficacy in humans, **in vitro** studies into the antimicrobial action of antibiotics against specific bacterial pathogens are performed initially in the laboratory and then tested on animal models. Animal testing is regarded as essential as laboratory models simulating given prophylactic regimens are seldom able to overcome the problems of control of the variable factors present in **in vivo** systems. Variables requiring attention in **in vitro** systems include inoculum size, incubation conditions (duration, temperature, humidity, and oxygen and carbon dioxide concentrations), nature of the culture-medium (solid, semi-solid, broth), pH of the medium and the effect on organisms of the presence of blood or serum. This may account for often widely varying sensitivity values in the literature for specific species against the same antibiotic

Neu and Labthavikul (1983) were able to show an apparent difference in antibiotic sensitivity by varying only one independent

factor. They determined what effect human serum would have on the action of teichoplanin on bacterial isolates. By supplementing broth with the serum to a concentration of 50%, MBC values increased by an average 8- to 16-times (the MBC remained unchanged in one case). MIC's, however, were reduced by a quarter or one half for 5 isolates and doubled in the other

Antibiotic effectivity may be influenced by factors not normally recognised to be important. In an **in vivo** study, Oikarinen and Malmstrom (1972) showed that in spite of high systemic blood concentrations of penicillin V, levels of this antibiotic in oral tissues were found to be markedly affected by vasoconstrictors incorporated into local anaesthetics used to infiltrate areas in the oral cavity. Fifty-six out-patients received one million units (600mg) of penicilllin-V by mouth. Concentrations of this antibiotic in cubital vein blood and in extraction sockets in the mandible and maxilla, with and without vasoconstrictor use respectively, were determined. Penicillin V blood concentrations were markedly higher in those maxillary extraction patients in whom local anaesthetic without vasoconstrictor was used than in those where the local anaesthetic contained vaso-active compounds. In the former, extraction socket levels of penicillin V approached those found in venous blood of the cubital vein. When regional nerve blocks were administered in the mandible prior to extraction, there was no meaningful difference in socket and cubital vein blood penicillin V levels

Toxicity of an antibiotic preparation, if sufficiently severe, may preclude its use in the clinical situation however suitable it may prove to be **in vitro**. However, because of the short duration of IE prophylaxis required even a relatively toxic agent such as vancomicin may be a legitimate candidate for this purpose

To develop suitable antimicrobial prophylaxis regimes, suscep-

tibility patterns of viridans streptococci are required. Oral streptococci are sensitive to virtually all the commonly prescribed beta-lactam antibiotics including penicillins-V and penicillin G, ampicillin and amoxycillin as well as several macrolides (erythromycin, roxithromycin, clarithromycin and azithromycin) and others (eg. clindamycin, vancomycin)

5.2 ß-lactam antibiotics: penicillin G, penicillin V, ampicillin and amoxycillin

5.2.1 Introduction

"ß-Lactam antibiotics are bactericidal because they inhibit bacterial cell-wall synthesis" (Donowitz and Mandell, 1988a)

Penicillin G is the second oldest of the antibacterial agents in clinical use today after the sulphonamides (O'Brien et al, 1987). Observations published in the Lancet in 1940 noted **in vitro** antibacterial action on a number of organisms, including an isolate of a "**Str. viridans** strain from a tooth", and the therapeutic effects on normal animals of a solution of an impure, brown, water-soluble penicillin preparation in powder form (Chain et al) (1940)

As early as 1942, before the use of penicillin G on a world-wide scale, Hobby et al showed this antibiotic to be highly bactericidal on rapidly replicating bacteria but was only bacteriostatic on non-dividing bacterial cells

Penicillin G, penicillin V, ampicillin and amoxycillin are beta-lactam antibiotics in common use for the prevention and treatment of infection by viridans streptococci susceptible to penicillin (Sprunt et al, 1968; Basker et al, 1977; Bougault et

al, 1979; Dowson et al, 1990)

Sensitivity of Gram-positive bacteria to natural and semisynthetic and synthetic penicillins differs. Strains inhibited by natural penicillins tend to be less susceptible to extended-spectrum compounds such as ampicillin and amoxycillin (Moenning et al, 1989). However, viridans streptococci are generally susceptible to penicillin with an average MIC of 0.2 mg/l (Hall and Heimdahl, 1989)

Bourgault et al (1979) found viridans streptococci to be sensitive to penicillin G **in vitro**. Susceptibility ranged from a very sensitive MIC of 0.06-4 mg/l and an MBC of 0.06->8 mg/l penicillin G. Of 63 viridans strains examined, $\leq$1 mg/l penicillin G killed 51 isolates. **S. mitis** and **S. sanguis** II were reported to be the most resistant strains in the study: five of the former and two of the latter were killed only by 2->8 mg/l antibiotic. Ampicillin exhibited activity similar to penicillin G

Coulter et al (1990) found bacteraemic viridans isolates to possess an MIC range of <0.01-2.0 mg/l and MBCs of between 0.01 and 2 mg/l penicillin G. Using broader spectrum amoxycillin, the same isolates had ranges of <0.01-4 mg/l and 0.01-4.0 mg/l for the MIC and MBC's respectively. Penicillin G was seen to be marginally more effective **in vitro** than amoxycillin

In 1978, Shanson et al compared penicillin V and amoxycillin activity in both **in vivo** and **in vitro** tests. They showed preoperative prophylactic antibacterial action against viridans bacteria of 2 g penicillin V to be as efficient in clinical trials as 2 g of amoxycillin. When tested against 43 bacteraemic isolates, all were sensitive to both antibiotics with MICs of $\leq$0.12 mg/l. MICs of both penicillin V and amoxycillin ranged between 0.002 and 0.05 mg/l. For 7 viridans organisms, MBCs were equal to

the MIC and $\leq$0.12 mg/l antibiotic. However, in spite of apparent antibiotic sensitivity of organisms, the authors were able to isolate bacteraemic streptococci from 12% (5 of 40) of the extraction patients on penicillin V and 5% (2/40) on amoxycillin - percentages significantly lower than the 40% (16/40) obtained in the control group. In an earlier study of Phillips et al (1976), most bacteraemic viridans isolates were very sensitive to penicillin V with MICs similar to figures determined by Shanson et al (1978): 0.12 mg/l. Sixteen percent (49/301) were more resistant with an MIC of $\geq$1.0 mg/l penicillin V. Because of the better and more consistent absorption of amoxycillin and its greater and more rapid bactericidal activity, this antibiotic is preferred to penicillin V

Amoxycillin is synthesised by modifying the structure of (semi-synthetic) ampicillin to produce a compound with improved pharmokinetic and other characteristics. Notwithstanding a very similar molecular structure, amoxycillin possesses intrinsic **in vitro** and **in vivo** antibacterial activity which differs marginally from that of ampicillin (Bodey and Nance, 1972; Neu, 1974; Brogden et al, 1975). As noted in Chapter 3, amoxycillin is more rapidly bactericidal than ampicillin although they are equally inhibitory (Comber et al, 1975)

Sutherland et al (1972) found the antibacterial spectrum of amoxycillin to be almost identical to that of ampicillin and to show good activity against viridans streptococci. Similarly, in clinical trials Shanson et al (1978) found spectra of amoxycillin and penicillin V against viridans streptococci to be very similar when used to control post-extraction bacteriaemia in 120 healthy adults. In 1974, Neu reported viridans streptococci to be inhibited by 0.012 mg/l amoxycillin and that it possessed an antibacterial spectrum similar to that of ampicillin. After being taken by mouth, blood (and urine) levels were achieved that were

almost twice those of ampicillin

5.2.2 Serum levels

The clinical advantage of amoxycillin over ampicillin is an improved absorption from the gasto-intestinal tract after oral intake and high blood levels of the former (Sutherland et al 1972). Satisfactory blood levels are evident from experimental data. Cannon et al (1984) determined serum levels of oral amoxycillin after extractions under general anaesthesia in two groups of patients - one older than 12 years of age (24 volunteers) who took 3 g and the other younger group (17) who took half the dose of amoxycillin. The mean duration between amoxycillin administration and the induction of anaesthesia was 4 hours 25 minutes and blood was drawn immediately after completion of the extraction procedures. The average serum level for the older group was 9.08 (+/- 5.59) mg/l amoxycillin and younger 8.21 (+/- 4.7) mg/l. The average for both groups was 8.49 (+/- 5.07) mg/l. In another trial (Shanson et al, 1984), serum levels of amoxycillin reached an average of 16.1 mg/l in 10 healthy adult males two hours after the oral administration of 3 g of the antibiotic in reconstituted powder (syrup) form. The difference in serum levels between the two trials is explained by the difference in time at which the bloods were drawn after administration; more than 4 hours for the first and two hours for the second (Shanson) trial

In an early trial conducted by Neu (1974), amoxycillin serum levels reached an average peak of 7.6 mg/l (range: 4.5-14 mg/l) in eight fasting volunteers. Oral ampicillin in the same trial reached only 3.8 mg/l, range 2.75-8 mg/l. Non-fasting amoxycillin serum levels were found to reach 98% those in fasting subjects. The highest serum levels were reached after 2 hours and after 250, 500, 1500 and 3000 mg doses, levels of 3.2, 7.7, 13.2 and

25.5 mg/l were found

5.2.3 Resistance to penicillin

It is generally accepted that antimicrobial agents select for resistant bacterial mutants. The appearance of resistance in populations of bacteria of clinical importance affects directly the choice of suitable candidate agents

Bacterial resistance to penicillin was recognised soon after it was taken into clinical use. Krumwiede (1949) reported the development in "rheumatic children" on daily doses of oral penicillin, as a "means to protect them from streptococcal pharyngitis", of resistant "alpha-haemolytic streptococcus" (which "was later successfully treated by massive doses of penicillin"). She reported that in strains isolated before and during its administration, penicillin susceptibilities from both the penicillin-treated and control (those not receiving antibiotic) groups of children were comparable (and sensitive), with the exception of four isolates from the controls which were notably more resistant. One child on 50 000 units of penicillin twice daily developed acute bacterial endocarditis (and the offending alpha-haemolytic streptococcus was "not highly resistant"!). Krumwiede recognised that this antibiotic eliminated susceptible strains and selected for resistant clones in the population

Resistance develops as a result of a number of mechanisms. Three genetically controlled forms of penicillin-resistance are known: (i) production of beta-lactamase enzymes by bacteria which inactivate certain members of this antibiotic group; (ii) changes in bacterial cell permeability to beta-lactams (only a problem in gram-negative bacteria) and (iii) structural alteration of

membrane-bound target sites or receptors of beta-lactams, the penicillin-binding proteins (PBPs). Neu (1985) included a tolerance mechanism operating in Staphylococcus aureus and Streptococcus pneumoniae which is dependent upon inhibition of autolytic enzymes required for cell wall formation. Viridans streptococci are not known to produce beta-lactamse enzymes

In 1983, Farber et al found that resistance to penicillin in viridans streptococci occurred with the alteration of PBPs to produce receptors with a lowered affinity for this antibiotic. Dowson et al (1990) investigated this phenomenon in clinical isolates of resistant viridans strains in the United Kingdom and found that the transfer of genetic material which encoded for penicillin resistance via PBPs may occur between resistant S. pneumoniae and previously sensitive S. oralis isolates. They discovered that the PBP2B-gene variant in penicillin-resistant strains of S. oralis was almost identical to that in the pneumococcus

The precise mechanism of killing of sensitive bacteria by penicillin is unknown. Tomasz, in 1986, wrote that "the quantitative relationship between the MIC of a ß-lactam antibiotic and its reactivity with certain PBPs is not well understood" and "also poorly understood is the mechanism by which inhibition of PBP function causes triggering of suicidal autolytic activity" by certain of these agents. Osmotic lysis of bacteria whose cell walls have been damaged by ß-lactams has been postulated to play a role in the final killing of bacteria by these antibiotics but ß-lactams such as imipenem are bactericidal but do not possess any lytic activity

Several studies have been performed to analyse the effect of antimicrobial agents on the sensitivity patterns of oral streptococci. Since the publication of Krumwiede's 1949 article, the

emergence of penicillin-resistance amongst bacteria has been commonly reported. Drucker and Jolly (1971), using antibiotic disks, isolated organisms with differing antibiotic sensitivities from different areas of the mouths of patients with no known prior exposure to specific antimicrobial agents under examination. The investigators found that a large percentage of patients (29%) in the experimental groups carried resistant oral streptococci. The gingival crevice and free gingival margin together contained the highest number of penicillin-resistant oral streptococci (38%). Amongst streptococci in the gingival crevice resistance ranged between 12 and 22% and on the gingival margin between 18 and 30%. The authors did not identify isolates beyond the Streptococcus genus level and were unable to observe that certain species of viridans bacteria are more common in some areas of the mouth than others. As an explanation of the high levels of antibiotic resistance without evidence of administration, the authors noted that some volunteers were medical and dental personnel who may have acquired antibiotic-resistant bacteria from their work environment. The importance of the gingival margin as a source of bacteraemic streptococci in dental procedures, however, was stressed by Drucker and co-worker

Josefsson and Nord (1982) employed a modified version of American Heart Association (AHA) IE-prophylaxis recommendations to determine the susceptibility of oral bacteria after prolonged exposure. Eight volunteers received a stat 2 g penicillin V oral loading dose in tablet form plus a single 800 mg dose one hour later and then every 8 hours thereafter for a total of eight doses. The authors sampled dental plaque after a week and again after eight days and identified viable aerobic and anaerobic organisms. A significant decrease in, amongst other bacteria, S. salivarius and S. sanguis numbers was found and indicated an apparent sensitivity of these oral organisms to penicillin V in their study. However, the opposite was noted later by MacGregor

and Hart (1986). Investigating amoxycillin resistance amongst viridans streptococci in volunteers after an oral 3g dose, they observed that subsequent to a noticable drop in all oral streptococci directly afterwards, numbers of S. salivarius isolates increased to 41.8% of all viridans from an initial 29.3% - a relative increase of 12.5%. S. mutans numbers also increased slightly from 4.6% of all cultures to 7.3% after the amoxycillin was taken. There did not appear to be a marked increase in amoxycillin MICs but MacGregor and Hart did not comment on the possible (clinical) significance of their findings

Leviner and colleagues (1987) identified resistant oral viridans streptococci in volunteers after the taking of penicillin V at doses based on the recommendation of the AHA (Special Report, 1984). Twenty-nine healthy volunteers took 2 g penicillin V, another 1 g 5-hours after the first plus an extra 1 g 10-hours again after the start of the investigation (total 4 g in that period). No further antibiotic was administered. Leviner et al detected resistant oral isolates six hours after the initial penicillin dose in a third (9 or 31%) of the subjects and still found resistant organisms in the mouths of volunteers at least 8 days after the withdrawal of the penicillin. Unlike MacGregor and Hart (1986) (and BSAC, 1986) who saw no reason to limit penicillin use again soon after its employment for prophylactic purposes (see later), Leviner suggested that caution should be exercised for at least 8-days thereafter as resistant strains may still be present in a patient's mouth

Even without evidence of prior antibiotic exposure, marked antibiotic resistance is known to appear. Longman et al (1991) reported viridans amoxycillin-resistance to concentrations of $\geq$6 mg/l (one quarter of the maximum blood-levels after 3g amoxycillin) in 7% of the 54 endocarditis-susceptable patients and 5% of the 65 healthy volunteers. S. sanguis biotypes I and

II, S. mitis and S. salivarius (and one S. milleri) were isolated. All were resistant to either amoxycillin or erythromycin. Neither patients nor volunteers had taken antibiotics in the three months prior to the investigation

In 1983, Southall et al performed a similar trial on volunteers after multiple oral doses of 3g amoxycillin as described by Shanson et al, (1980). All participants, free of penicillin-resistant isolates prior to the start, developed resistant viridans streptococci on completion of the study. Two 3 g amoxycillin doses, administered 8 hours apart, were taken by each of the 11 volunteers once weekly until the first resistant viridans streptococci were found. Specimens for resistance-screening were selected from saliva specimens and gingival sulcus material. By the fifth week, all volunteers had acquired streptococci resistant to 0.5 or 1.0 mg/l amoxycillin. The average time before the emergence of resistance was 3.6 weeks although in one volunteer this occurred after the first of the two 3 g doses. All resistant isolates (17) were identified as S. sanguis and MICs of amoxycillin varied from 1.0 to 16.0 mg/l and MBCs from 2.0-16.0 mg/l. Resistance of viridans isolates to erythromycin varied, but isolates retained susceptibility to clindamycin and vancomycin. MICs against these antibiotics were not stated. After discontinuation of the antimicrobial therapy, resistant streptococci disappeared from the mouths of 8 of 9 volunteers after 7 weeks, and in all 9 volunteers finally after 13 weeks

To examine the development of beta-lactam resistance amongst oral streptococci, Woodman et al (1985) conducted a study using the prophylactic amoxycillin regimen proposed by the BSAC (1982). Amoxycillin was administered to two 10-member volunteer groups. One group (group "A") received a single 3g amoxycillin dose while the second (group "B") received three doses of the same size at weekly intervals. A third 4-member group ("P") was selected from

volunteers of group A to take three doses of placebo, also at 3-weekly intervals, starting 3 months after their single 3g amoxycillin dose. To serve as a microbiological baseline control, specimens were collected from all volunteers in an attempt to partially identify and quantify resident salivary populations of amoxycillin-sensitive and -resistant bacteria before the investigation was initiated. All group "A" and "B" subjects harboured bacteria resistant to 2 mg/l amoxycillin when tested before the culture medium was supplemented with the antibiotic, although not all strains from the same subjects (2) were always resistant. Specimens from 9 (of 20) subjects grew bacteria resistant to 40 mg/l amoxycillin. The authors calculated that the volunteers carried less than 1% of resistant oral flora in total - still a very large pool of resistant strains if one accepts that 1 g of wet plaque contains 22-179 thousand million bacteria (Gordon et al, 1971)

After the orally administered dose of amoxycillin, the streptococcal count in group "A" (including those resistant to 2 mg/l antibiotic) returned to baseline counts after 3 weeks. However, streptococci resistant to 40 mg/l amoxycillin fell in number to levels below those which existed prior to antibiotic administration. Total streptococcal (and anaerobic) counts in group "B" soon returned to baseline levels after the three doses but there was a statistically significant rise in amoxycillin resistance after the second and third doses. High counts of resistant streptococci lasted on average for 28 and 21 days for the organisms resistant to 2 mg/l and 40 mg/l repectively. In two subjects, amoxycillin resistance returned to baseline levels only after 7 weeks and it took 11 weeks for resistance to 40 mg/l to disappear from the saliva of all subjects. Placebo group members showed no statistically significant change in anaerobe or streptococcal numbers although there were noticeable fluctuations in anaerobe numbers

The time interval between induction of penicillin resistance and oral clearance of mutant penicillin-resistant organisms would have important clinical implications were compromised patients to require further antibiotic treatment within this period. Woodman et al (1985) suggested that either a single or double dose 3 g amoxycillin regimen (with a 7 day interval in the latter instance) would be acceptable for at-risk IE patients. If spaced, multiple dental procedures are required in a patient, either a suitable alternative antibacterial agent should be used or an absolute minimum time interval of 4 weeks between treatments should be observed were the original antibiotic to be re-used. Woodman et al are supported by Southall et al (1983); others not subscribing to this view are discussed below

Penicillin-resistant micro-organisms may be part of normal oral flora and writers state that it is possible to isolate resistant organisms in 7% or less of the general population and up to 100% in special groups (Drucker and Jolly, 1971; Sukchotiratana et al, 1975; Phillips et al, 1976; Elliot and Dunbar, 1977; Leviner et al, 1983; Leviner et al; 1984. Sukchotiratana et al (1975) suggested that the emergence of resistant streptococci so soon after taking the antibiotic (24 h in their study of dental crevicular flora) was as a result of resistant isolates already present in undectable numbers before the trial was initiated - a point supported later by Woodman et al (1985). Bacteria resistant to gingival fluid or salivary concentrations of penicillin V quickly increased in number after the elimination of sensitive elements by the antibiotic, and persisted for 8-weeks after its discontinuation

Several reports are contrary to often quoted studies on the appearance and development of penicillin-resistant strains. MacGregor and Hart (1986) found no significant increase in resis-

tance amongst viridans streptococci to amoxycillin when taken as a single "prophylactic" dose. Further, they found there to be no contra-indication for the repeat administration, during the three weeks following the first dose, of another dose were this required. This is a position on prophylaxis use similar to that taken by the BSAC in the same year (BSAC, 1986) who recommended a shorter 2-week minimum period before a repeat dose. MacGregor and Hart used ten suitable participants who acted as their own controls. They had no prior antibiotic exposure for four weeks prior to their inclusion in the study. Saliva specimens were collected and cultured for viridans streptococci at specified times beginning 3 weeks before and ending three after the oral administration of 3g amoxycillin. Viridans numbers dropped off markedly immediately (1 hr after swallowing this antibiotic) but, after 7-days, numbers had returned to levels which existed prior to the administration of the amoxycillin. During the 21-day control (pre-administration) phase of the investigation, one particpant had two salivary isolates (both **S. mitis**) with MICs of 1.0 mg/l amoxycillin but most other cultures were found to have lower MICs between <0.03 and 0.25 mg/l. After the amoxycillin, 3 isolates had MICs of 1.0 mg/l; two were **S. mitis** species and a single **S. salivarius** strain. The fact that participants in this study only received a single dose of antibiotic may account for the disappearance of resistant mutants so soon after the administration of the stat dose

Penicillin V, proposed in older AHA prophylaxis regimens, was found to leave normal salivary, throat and faecal flora unaffected in 10 subjects screened for 29-days after the start of a week's oral administration of 800 mg 12-hourly in capsule form (Heimdahl and Nord, 1979). Although serum penicillin V levels in blood drawn 1 hour after the first daily dose increased gradually from an initial 3.8 to 5.2 mg/l during the trial, no antibiotic was detected in any salivary (or faecal) specimens. A small

decrease in unspeciated salivary streptococcal numbers was noted and no resistant strains were isolated. The lack of any apparent disturbance in oral flora is ascribed by the authors to the use of the antibiotic in capsular and not tablet form. Clindamycin was tested in a similar manner (discussed later)

In a 1976 publication, Phillips et al speculated on whether the presence of resistant strains carried by an individual was as the result of (i) the acquisition from patients, with prior antibiotic exposure, of such strains of oral streptococci, (ii) the overgrowth of certain intrinsically resistant species or (iii) the selection of resistant mutants from previously sensitive commensal streptococci. The researchers isolated a greater number of resistant streptococci from patients with known prior penicillin exposure than from those without. Leviner et al (1984) found that hospital staff in contact with patients "who were consumers of high doses of penicillin" had greater numbers of resident penicillin-resistant oral viridans streptococci (48.08%) than "low-contact" staff groups (7.89%). Phillips et al (1976), Leviner et al (1984) and Drucker and Lolly (1971) shared the same views on the concept of carriers or reservoirs of resistant strains

Antibiotic resistance in oral streptococci may, theoretically, be used to advantage through their ability to compete for colonisation by more difficult-to-treat organisms such as gram-negative bacilli but this potentially beneficial effect would be difficult to apply in clinical situations on a routine basis. Studies were undertaken by Sprunt et al (1971) on the exertion of such an interference phenomenon by certain antibiotic-resistant bacteria on others in compromised patients. They investigated whether overgrowth by pathogenic gram-negative bacteria in the oro-pharynx might be prevented by some bacteriological means in patients after cardiac surgery. A total of 29 patients who received IV

penicillin plus 1g of streptomycin for 5-7 days post-operatively, were studied. Thirteen of 29 patients showed an overgrowth of enteric bacilli and all but one demonstrated a loss of alpha-haemolytic streptococci in the pharynx during the period of high antibiotic administration. However, during this period, in 15 of the 16 patients in whom no pharyngeal gram-negative overgrowth could be demonstrated, alpha-haemolytic streptococci were demonstrated in their pharynges instead. Alpha-haemolytic streptococci were found to be susceptibile to <0.3-0.6 mg/l. Five patients, in whom resistant alpha-haemolytic strains were selected after receiving oral penicillin for 3-5 weeks prior to surgery, were all found to be free of enteric Gram-negative bacilli. The hypotheses which Sprunt et al postulated viz. that (i) the persistence of viridans streptococci after large doses of penicillin would result in the absence of Gram-negative enteric bacilli from the patients' pharynges and that (ii) the presence of interfering bacteria would be as a result of penicillin resistance which would develop in the streptocooci, appear to be supported by their investigation. The resistant viridans streptococci apparently served a useful, protective (bacillus repellent) role which may conceivably have contributed positively towards post-operative recuperation of patients in the study. The potentially beneficial result of microbial incompatibility (Ellen, 1982) may occur between resident penicillin-resistant alpha-haemolytic streptococci and enteric bacilli competing for colonisation of parts of the oro-pharynx of compromised patients in an area of the body not normally associated with their presence in the healthy host

5.3 Macrolides and related antibiotics: erythromycin, roxithromycin and clindamycin

5.3.1 General properties of macrolides

Macrolides are a broadly homogenous group of antibiotics with four notable characteristics: they possess similar (i) macrocyclic lactone rings, (ii) antibacterial spectra, (ii) mechanisms of action and bacterial resistance and (iii) pharmacokinetics. Additionally, they possess relatively high lipid solubility, a low degree of ionisation and wide tissue distribution. Macrolides exert their antibacterial activity by binding bacterial 50s-ribosomal subunits and interfering with normal protein synthesis by competitively inhibiting access of t-RNA-amino acid complexes to the m-RNA (Wilson and Cockerill, 1983), at the level of DNA translation (Molinari, 1983)

Macrolides in clinical use at present are erythromycin, roxithromycin, clarithromycin, azithromycin and dirythromycin. The latter four compounds share similar activities and spectra to erythromycin but have markedly improved pharmacokinetics. Only roxithromycin of the newer macrolides was included in the experimental design of this study and will therefore be discussed in some detail even though other recently released agents of this group of antibiotics (which were not yet available in South Africa when this investigation was initiated) may be similarly useful for IE prohylaxis

5.3.2 Erythromycin

Erythromycin, one of the so-called 14-carbon lactonic ring class, was isolated originally from Streptomyces erythreus and is currently one of the most important of the macrolide antibiotics. Although serious adverse reactions are rare, gastro-intestinal (GI) intolerance is more common (Mandell, 1985) but is usually the consequence of large dose size (Maskell et al, 1988). Apart

from GI intolerance, mild drug fever and hypersensitivity to one of the erythromycin forms may occur. It is regarded as being one of the safest antibiotics in common clinical use (Neu, 1988)

Erythromycin is available in erythromycin-estolate, -stearate and base (enteric-coated) preparations. The latter two are safer to use in patients with liver disease than the first which may also induce sensitivity reactions

5.3.2.1 Pharmacokinetics and activity spectrum of erythromycin

Unpredictable absorption of erythromycin is a prominent clinical failing of this agent (Maskell et al, 1988). Film-coated preparations produce significantly higher maximum peak serum levels than either the estolates or stearates. In a study by Josefsson, Bergan et al (1982), a 500 mg enteric-coated erythromycin-base dose produced a significantly higher serum level (a 3.5 mg/l average) than the equivalent stearate form (2.5 mg/l). The maximum individual serum concentrations for the 250, 500 and 100 mg coated base doses were 1.9, 3.8 and 6.5 mg/l respectively, and for the 500 mg stearate equivalent, 2.9 mg/l. Absorption was erratic as indicated by late or bi-phasic serum peaks after oral intake

Erythromycin has a broad spectrum of activity against both Gram-positive and Gram-negative bacteria and is often used as an alternative to penicillin - to which it has a similar spectrum (Molinari, 1983), but is regarded as having fewer primary therapeutic indications (Steigbigel, 1985)

Erythromycin is highly active against viridans streptococci. When tested against unspeciated groups of viridans bacteria, Jones (1985) determined an MIC range of 0.1-0.8 mg/l erythromycin for these bacteria

A dose administered orally one hour prior to dental extraction reduced the incidence of post-operative streptococcal bacteraemia markedly. In a study by Shanson et al in 1985, viridans streptococci were isolated from blood cultures in only 15% of patients (6 of 40) given 1.5 g oral erythromycin pre-operatively compared to three times that number (43%) of patient controls (18/42) who had not received pre-operative erythromycin. Species isolated from the six members of the group given erythromycin consisted of three isolates each of S. sanguis and S. mitis. Minimum inhibitory (and minimum bactericidal) concentrations were 0.03, 0.03 and 0.01 (0.06, 0.12, 0.12) for the former and 0.06, 0.01 and 0.01 (0.12, 0.06, 0.06) for the latter strain, respectively. Similar MICs were obtained for species isolated from patients who had not received prophylaxis (range 0.01-0.06 mg/l, mean 0.02 mg/l). Their MBCs reached higher concentrations (0.06-1.0 mg/l, mean 0.19 mg/l). Shanson's team also found that amongst patients who had received erythromycin, those who were culture-negative had higher serum erythromycin levels (3.7 mg/l erythromycin) than the small number (6) from whom blood-borne viridans streptococci were cultured (1.8 mg/l). Harrison, Stross et al (1985) measured serum concentrations which ranged from undetectable levels to 8.3 mg/l erythromycin 2 -3 hours after oral administration of a smaller 1 g dose

5.3.2.2 Erythromycin resistance

Resistant strains possessing biochemically altered erythromycin binding sites on ribosomal 50S sub-units (O'Brien et al, 1987) are selected after prolonged erythromycin exposure. Molinari (1983) stated that resistance to this antibiotic (as well as to clindamycin and chloramphenicol) develops as a result of bacteria becoming impermeable to the drug

Researchers appear not to agree on the effect which prolonged erythromycin exposure to this antibiotic had on bacterial flora. Some detected only minor changes to resistance patterns while others found these to be marked and/or prolonged. Heimdahl and Nord (1982) studied what effect erythromycin exposure may exert on the resident oral, throat and faecal flora. They were able to detect what they termed "minor" changes after the ingestion of 500 mg erythromycin (in stearate form) twice daily for 7-days. Ten volunteers, none of whom had received any antibacterial agent for 6 months prior to the start of the study, had saliva, throat and faecal specimens cultured for aerobic and anaerobic bacteria for 16 days after the first dose. Antibiotic levels were also determined in these specimens. Serum concentrations determined in finger-prick blood taken 1.5 hours after the first daily 500 mg erythromycin dose ranged from approximately 1.5-3.25 mg/l over the test period. The authors were unable to detect erythromycin in certain blood specimens of two patients. Salivary levels ranged from 0-1.8 mg/l and 4 of 10 volunteers showed pronounced drop in S. salivarius numbers. Changes in populations of other species was also noted (3 of 10 showed a drop in staphylococci). They saw that aerobe numbers in the saliva and throat appeared to be less affected, and were minor, although there was an increase in colonisation by enterobacteria and enterococci with MICs to erythromycin of >16 mg/l. Changes which occurred in the composition of bacterial populations after the long-term use of this macrolide were significant enough to affect aerobic and anaerobic faecal flora

Also in 1982, Josefsson and Nord detected a significant decrease in numbers of certain oral streptococci after erythromycin. They used an initial loading dose in eight volunteers of 500 mg erythromycin (base) followed 2 hours later with a further 500 mg plus 500 mg every 8 hours up to a total of 6 doses. A significant

decrease in S. salivarius numbers (like Heimdahl and Nord 1982) and micrococci was detected although these authors also considered it to be "minor" (sensitivity changes which developed did so, they postulated, as a result of the appearance of erythromycin in the saliva). They found neither an increase in resistance to erythromycin (or to penicillin V) amongst the bacteria tested nor colonisation of oral areas by enterobacteria or enterococci

In 1983, Southall et al planned an experiment to detect and identify amoxycillin-resistant oral streptococci, if any, after a repeated two-dose regimen of this antibiotic and to test the resultant (resistant) isolates against erythromycin and two other antibiotics (clindamycin and vancomycin). They administered 3 g amoxycillin by mouth plus another 3 g eight hours thereafter to 12 healthy volunteers (9 of whom were finally to complete the study) once weekly until oral streptococci resistant to 0.5 or 1.0 mg/l amoxycillin appeared. Oral secretions were sampled on the day following the intake of the antibiotic. All subjects were screened prior to the study and found to be free of resistant organisms but all carried strains after the fifth weekly "double dose". One subject developed resistant viridans after the first weekly doses and another after the second week. Subjects showed a marked decrease in numbers of sensitive species within 24-hours of the first doses and numbers of resistant organisms returned to undetectable levels within 13 weeks after the last dose in all 9 volunteers. Seventeen resistant isolates were identified as S. sanguis (non-dextran-producing) by two methods of identification. The cultures showed an MIC range of 1.0-16.0 mg/l amoxycillin and MBCs of 2.0-16.0 mg/l. However, when tested against erythromycin, 3 of 11 volunteers carried amoxycillin-resistant strains sensitive to this antibiotic (MIC $\leq$0.25 mg/l) and the other 8 had MICs which were resistant or moderately resistant to it (1-2 mg/l erythromycin). By disc testing, the 17 S. sanguis strains were

sensitive to clindamycin and vancomycin

Other authors reported that long-term exposure of oral streptococci to erythromycin induces noticeable bacterial resistance. Harrison, Stross, et al (1985) found that the repeated administration to volunteers of erythromycin stearate selected for erythromycin resistance amongst oral streptococci after the second of 2 identical regimens after a 7-day interval. Each consisted of 1 g erythromycin per os initially plus 500 mg 6 and 12 hours thereafter. Whether the antibiotic was in a capsular form or not was not specified. Prior to the study, none of the 10 volunteers carried streptococci with erythromycin MICs $\geq$0.25 mg/l. After the first three-dose regimen, the total number of plaque streptococci dropped off in all participants but had returned to normal levels after the second course of antibiotics when erythromycin resistance was detected. On withdrawal of the antibiotic, although erythromycin resistance showed a general decline, eight volunteers still harboured these strains after 23 weeks and in at least 4 of them at 43 weeks. Two species (S. sanguis and S. mitior) isolated from four volunteers were highly resistant to erythromycin having MICs from 16->256 and MBCs between 128->256 mg/l. The rest (six) yielded strains with MICs of between 1-4 mg/l but of these isolates, a number had MBCs >256 mg/l - so-called "tolerant" strains. More than one species was isolated from some participants and, in total, 9 highly resistant cultures were identified; five S. mitior and four S. sanguis species. Seven showed MICs >256 mg/l and all had MBCs of this magnitude. Different isolates of these two species were found to show moderate resistance to erythromycin. All nine highly resistant isolates were very sensitive to vancomycin and benzylpenicillin. Harrison found no correlation between concentrations of erythromycin in saliva and the occurrence of highly resistant viridans streptococci, unlike Josefsson and Nord (1982)

Multiple doses of erythromycin were also found by others to induce resistance to this antibiotic. Herbert et al, (1988) screened plaque specimens isolated from the two volunteers in his study which, where possible, followed closely that of Harrison, Stross et al (1985). He used the erythromycin regimen proposed by the BSAC (1982) where erythromycin 1.5 g was administered to volunteers and followed by 500 mg 6 hours later. Herbert administered the antibiotic regimen three times with 7-day intervals and found after the second two-dose set that both subjects carried a significant proportion of the total streptococcal count resistant to erythromycin. After the third 1.5 g dose, erythromycin resistance was found to consitute between 30-100% of all isolates. Minimum inhibitory concentrations of resistant isolates were, however, less than 8 mg/l erythromycin while MBCs were $\geq$32 mg/l in the very small two subject sample. From volunteer 2, Herbert et al identified 18 of 22 resistant isolates as S. mitior, three as S. sanguis and the remaining strain as a S. salivarius specimen. Volunteer 1 produced mostly S. mitior isolates with fewer S. sanguis and others also being cultured. A significant proportion of resistant streptococci appeared 3 hours after the second administration in both volunteers and maintained this high level until the third. Persistence of resistance was not measured

In a 7-day study, Heimdahl et al (1984) showed that, unlike penicillin V and doxycycline, erythromycin and clindamycin each suppressed S. salivarius numbers in the oral cavity to a degree that its continued use could result, the authors claimed, in an overgrowth of other oral bacteria such as Group A streptococci. This situation could, they speculated, compromise yet further the health of any patients already medically compromised when the normal inhibitory effect of bacteriocins produced by commensial S. salivarius populations against other (potentially harmful) oral bacteria was either reduced or absent. Contrary to the study of Heimdahl et al, Herbert et al, (1988) found an isolate of S.

salivarius which was resistant to erythromycin. In 1982, Josefsson and Nord identified the suppression of S. salivarius numbers by both erythromycin and penicillin V

Maskell et al (1990) conducted an investigation to determine macrolide resistance amongst salivary viridans streptococci in volunteers after and/or before the oral intake of two different macrolides. Either erythromycin (1.5 g stat plus a single 500 mg dose 6 hours thereafter) or another macrolide (josamycin) was administered. Maskell et al showed substantially more resistant streptococci in the volunteers after macrolide therapy than before the time, when a small proportion was identified. Volunteers who took part in the experiment were 45 hospital staff members who only supplied saliva specimens, and 2 groups of 8 dental students who each supplied both pre- and post-antibiotic specimens of saliva. Of 45 staff members, 44 (97.8%), 44 (97.8%), 38 (84.4%) and 38 (84.4%) harboured streptococci with MICs of 0.25 (an MIC indicating sensitivity), while resistant strains had MICs of 1, 4 and 64 mg/l erythromycin respectively. Before taking the antibiotic, streptococci resistant to 1 mg/l erythromycin were cultured from all 16 students (and 15 to 1 mg/l josamycin). All were resistant to erythromycin at a concentration of 1 mg/l; 87% (14 of 16 volunteers) to 4 mg/l; and 50 (8 of 16) to 64 mg/l. Additionally, the authors found that 13 of the 16 students (81%) harboured streptococci that were also resistant to 1 mg/l amoxycillin. After administration of erythromycin, 23%, 17% and 6% of the total oral streptococci were found to be resistant 48 hours after the 1.5 g dose to 1, 4 and 64 mg/l of the antibiotic, respectively. Five days later only 7% of isolates showed low-level resistance (1 mg/l) and 0.5-1.5% were moderately ($\geq$4 mg/l) to highly resistant ($\geq$8 mg/l). One month thereafter erythromycin resistance amongst the bacteria declined to 2% and even lower after three months. Average erythromycin concentrations in saliva were 3.88 and 1.1 mg/l at 90 minutes and 6-hours respectively

However, in terms of volunteer numbers, highly resistant viridans streptococci ($\geq$64 mg/l) were isolated from 7 of the 8 students who took erythromycin. Three individuals carried the organisms prior to dosing and these were still present in the mouth after 90 days in at least two of the students (a third was not re-tested). Three streptococcal species, S. sanguis, S. mitis and S. salivarius, were isolated from the three prior to dosing and S. sanguis (2 isolates) and S. mitis (4) were found in 7 students thereafter. In total, 17 highly resistant strains were identified in this study: S. mitis (9), S. sanguis (7) and S. salivarius (1). All isolates were sensitive to amoxycillin at MICs which varied from between 0.015-0.12 mg/l but the majority were resistant to 256 mg/l of roxithromycin, clindamycin (and azithromycin, clarithromycin) but susceptible to pristinamycin (0.25 mg/l)

A degree of bias was evident in the trial as volunteers who made up both experimental groups were not randomly selected from an "unaffected" population. Volunteers commenced the study with erythromycin-resistant viridans streptococci already present in their mouths and this could possibly have skewed results and conclusions. To clarify the situation, the authors state that as none of the participants had taken antibiotics in the three months prior to the study, the resistant strains appeared to have originated from the hospital environment or from foodstuffs. However, in its favour, this form of exposure to resistant strains may simulate in some measure, the selection which occurs amongst those taking daily penicillin V prophylaxis

Leviner et al (1984) identified hospital-borne antibiotic-resistant viridans isolates in hospital staff volunteers in a similar study

Harrison, Stross et al (1985) supported the findings of Maskell et al (1990) on the general behaviour of oral streptococci with regard to erythromycin resistance. Both research teams found that resistant organisms persisted for long periods after exposure to erythromycin: 43 weeks in Harrison's and at least three months in Maskell's study (and resistance to >64 mg/l erythromycin was still identifiable in 4 of 6 volunteers). However, the latter authors did not speculate on whether any of the resistant isolates identified in the staff and student groups during the screening period, subsequent to completion of the antibiotic course, had had a possible direct environmental origin or not

Furthermore, Maskel et al did not appear to recognise that saliva carries organisms flushed from populations of bacteria attached to the various exposed oral (especially the tongue; Hardie, 1983) and dental structures. Bacteria which find their way into the blood-stream after trauma to bone and soft tissue of the jaws tend to be those attached, characteristically, to specific oral structures (Chow et al, 1978). When these structures are located directly within a surgical field, for example, bacteraemia arises from recognised populations of organisms specific to those areas and not necessarily from those suspended in saliva which, naturally, is sterile prior to its emptying into the mouth

In 1978, Hunt et al attempted culture of 74 clinical exudate specimens from soft tissue oral infections and 68 yielded bacteria for antibiotic susceptibility testing (six specimens failed to yield growth on culture). Of interest was the finding that streptococci were found in pure culture in 57% of cases in this study. A total of 31 viridans isolates were cultured and 20 were in pure culture. It is conceivable that surgery of the infected sites could have produced a viridans bacteraemia. Only 5 tested sensitive to erythromycin and 26 were resistant (no MICs were supplied). However, all 31 isolates were sensitive to ampicillin

and 26 to penicillin V. Isolates were not identified to species level beyond the general viridans grouping. Important selection forces were absent in this investigation as none of the patients received prolonged antibiotic therapy that may have lead to the development and retention of antibiotic-resistant viridans strains

The serious consequences of erythromycin resistance amongst oral streptococci was demonstrated by Eng et al (1982) in a patient after maxillo-facial surgery. IE developed in a penicillin-allergic, 44 year-old man after undergoing maxillary sinus surgery in spite of his having received 4 g intravenous erythromycin lactobionate pre-operatively. After completion of the surgical procedure, he received oral preparations of erythromycin stearate (2 g) daily for 4 days and again for 3 days after day 10 when he complained of feeling unwell. Seven weeks after surgery he was diagnosed as having IE. All blood cultures were positive and yielded S. sanguis biotype I. MICs and MBCs were determined for penicillin (MIC:0.007; MBC:0.007 mg/l, respectively), vancomycin (0.5; 2.0 mg/l) and erythromycin (40; $\geq$320 mg/l). The patient recovered uneventfully on vancomycin therapy

The inappropriateness of erythromycin use as IE treatment was illustrated in an experimental rabbit model in 1975. Pelletier et al found that erythromycin at 15 mg/kg, in multiple doses, failed to protect heart catheter-damaged animals with IE against the causative organism, an erythromycin-sensitive S. sanguis II (MIC 0.08 mg/l, MBC 0.15 mg/l erythromycin). An S. sanguis II inoculum of 100 million cfu's was utilised to infect the animals and its large size may have rendered the treatment ineffective

5.3.3 Roxithromycin

Roxithromycin is chemically related to erythromycin and has improved pharmakinetic activity (Nilsen, 1987) in addition to useful properties common to other macrolide-type antibiotics. These include lipid solubility, low degree of ionisation and wide tissue and body fluid distribution

Roxithromycin is well tolerated by patients after oral intake (Smith et al, 1989) and displays an excellent safety profile in both adults and children where a small number of between 3.1 and 4.1% of approximately 3000 patients taking 150 mg roxithromycin twice daily experienced side-effects of nausea, gastro-intestinal pain or diarrhoea in equal proportions (Blanc et al, 1987; Neu, 1988). In another study on 211 subjects (Sasaki, 1987), 2.4% (5) had gastro-intestinal upsets and minor changes in liver function tests in 3 of 92 subjects (3.3%). Neu (1988) noted that there was no significant haematological or renal toxicity and less than 1% of patients had changes in liver function tests in his overview

5.3.3.1 Improved pharmacokinetic activity

5.3.3.1.1 Serum levels, half-life and saliva concentration

Roxithromycin has been shown to produce markedly higher serum concentrations than erythromycin at similar or higher oral doses. Dose-related serum concentrations were detected after ingestion of single 150 and 300 mg dose sizes. After the smaller dose, plasma concentrations were in the range of 6.61-7.9 mg/l and for the larger 300 mg roxithromycin, 9.1-10.82 mg/l (Nilsen, 1987; Puri and Lassman, 1987; Kees et al, 1988; Tremblay et al, 1988)

Six male subjects produced a mean peak serum level of 3.0 mg/l

(range: 0.3-7.3 mg/l) one hour after receiving 300mg roxithromycin on three occasions after 7-day intervals in a trial conducted by Smith et al (1989). Saliva and gingival fluid were found to be free of roxithromycin at concentrations $\geq$0.2 mg/l

In the clinical trial of Kess et al (1988), roxithromycin 150 mg was found to have an elimination half-life of approximately 15.5 hours, a time which was 9-times longer than that of 500 mg erythromycin (1.7 hours). Investigators reported the half-life to range from between 10 to 16 hours with an average in the region of 12 hours (Neu, 1988). The half-life was not prolonged when the single-dose size was increased from 150mg to 300mg or 450mg (Tremblay et al, 1988). The values in the trial of Tremblay et al (1987), using the three dose sizes, ranged from 10.5 hours for 150mg of roxithromycin to 11.91 and 13.84 hours for the higher 300 and 450 mg doses respectively. Puri and Lassman (1987) determined the half-life for this antibiotic and their data did not differ substantially from those of either Kess et al (1988) or Tremblay et al (1988). The half-life values of Puri and Lassman were 10.5 and 10.3 hours for the 300 and 450 mg doses but 8.4 hours for 150 mg - an half-life marginally less than the other authors using the same dose. The roxithromycin half-life for the 150 mg and 300 mg doses for all the above authors, therefore, ranged from 8.4 to 15.5 hours. Nilsen (1987), too, reported the mean half-life for roxithromycin at different doses to be between 8 and 14 hours and for erythromycin to be substantially shorter, ranging from 1.5 to 3 hours

In saliva, roxithromycin levels after a 300 mg loading dose followed by six 150 mg doses at 12-hourly intervals were either undetected (Puri and Lassman, 1987) or showed very low concentrations of $<$0.05 mg/l saliva (Acar et al, 1988)

5.3.3.1.2 Macrophage activity

Polymorphonuclear neutrophils and other phagocytic cells form an important part of the body's immunological defence system against invading pathogens (Sohnle et al, 1991) and these antibiotic compounds are actively concentrated in the cells. Roxithromycin was found to accumulate in polymorphonuclear neutrophils to levels which were 14-times the extracellular concentration (erythromycin, 8-times) and in alveolar macrophages from smokers to 190-times the concentration (non-smoker macrophages, 61-times). For erythromycin, levels in smoker and non-smoker macrophages were 46- and 38-times the extracellular concentration respectively (Carlier et al, 1987)

Anderson et al (1987) investigated further the intracellular activity of roxithromycin and erythromycin. His results showed that neutrophils concentrated both antibacterial agents (roxithromycin 30- and erythromycin 10-fold) but, additionally, that intracellular activity of the compounds was dependent upon normal, intact oxygen-dependent intrinsic phagocytic cellular antimicrobial systems

By employing human phagocytes **in vitro**, Yokota and Bhattacharyya (1988) were able to show that cells incubated with roxithromycin ingested more bacteria per cell (in this instance **S. aureus** in numbers larger than 30 bacteria per cell) than in the presence of either erythromycin or josamycin. Roxithromycin was bactericidal with lower MICs and lysozymal concentrations than either of the other two antibiotics. This phenomenon was noted by the authors to indicate synergy between this antibiotic and lysozyme activity. The serum bactericidal activity of this antibiotic in whole blood was found to be no different to that of erythromycin

White blood cells were used in a similar investigation. In a

preliminary **in vitro** study, Prieto-Prieto et al (1988) compared the effects which roxithromycin and three other macrolide antibacterial agents exerted on certain polymorphonuclear neutrophil (PMN) functions at concentrations of 0.5, 5.0, and 50 mg/l antibiotic. PMN adhesion, spontaneous cell mobility, chemotaxis and phagocytosis were observed. Although the results were very similar, roxithromycin enhanced polymorphonuclear adherence and chemotactic activity to a slightly greater degree than the others. No agent appeared to affect the phagocytic function of the white blood cells in this study. Spontaneous PMN mobility, however, was reduced by all compounds

5.3.3.2 **Spectrum of activity**

Roxithromycin, like erythromycin and other macrolides, produces its antibacterial effect by the intracellular inhibition of protein synthesis

The **in vitro** antibacterial spectrum of roxithromycin is similar to that of erythromycin (Barlem and Neu, 1984; Young et al, 1989). Streptococcal strains involved in endocarditis such as S. mutans, S. sanguis, S. mitior and other viridans streptococci, are inhibited by ≤1 mg/l roxithromycin (Neu, 1988)

Barlam and Neu (1984) compared the activity of roxithromycin (RU 28965) with that of erythromycin. Several different aerobic and anaerobic species were tested. Thirteen unspeciated viridans streptococci were equally sensitive with identical ranges (<0.1->100 mg/l) and MIC 50 and MIC 90 values (both 0.8 mg/l). Against four S. mutans, roxithromycin was markedly more effective with the range of the former ≤0.1-0.4 while the latter was ≤0.1->200; MIC 50s were identical at 0.1 mg/l but MIC 90s were 0.4 and >200 mg/l antibiotic, respectively. Against eight S. mitis strains,

MIC 50 (0.1) and MIC 90 values (0.4 mg/l) were identical but only the MIC upper range limits differed marginally: roxithromycin range was $\leq$0.1->200 mg/l and erythromycin's, $\leq$0.1->300 mg/l

This antibiotic was tested by Sasaki (1987) against bacteria isolated from oro-facial infections (pericoronal and periodontal infections and osteitis). Of 144 mixed aerobic (60) and anaerobic (84) clinical isolates from 73 of 193 patients, 85.4% were sensitive to roxithromycin (MIC $\leq$3.13 mg/l). A 144 isolate total included 36 unidentified alpha-haemolytic streptococci where 12 isolates were inhibited by 0.20 mg/l, 9 by 0.39 mg/l and 1 by 0.78 mg/l of this antibiotic. However, 14 were not inhibited by concentrations >100 mg/l roxithromycin. The latter highly resistant viridans isolates comprised 66.6% (14 of 21) of all clinical strains recovered from patients in the trial

5.3.3.2.1 Effect of roxithromycin on normal flora

The consumption over 10 days of either roxithromycin or erythromycin in drinking water produced only minor changes in the faecal flora of experimental animals (mice) in the trial of Koopman et al (1988). The authors postulated that gut flora would remain generally unaffected after single doses or very short prophylaxis regimens

In human trials, no effect on the colonisation resistance of the oropharynx to bacteria resident in the area could be demonstrated after oral roxithromycin (or erythromycin) during a shorter 7-day trial (Vollaard et al (1987). Gut flora was also examined and, contrary to Koopman's et al (1988) work on mice, the authors noted a detectable effect on the composition of normal bowel flora. Secondary colonisation of the bowel by Enterobacteriaceae after both macrolides and amoxycillin occurred. However, roxith-

romycin and erythromycin, unlike amoxycillin, did not appear to affect gut counts of enterobacteria unduly - numbers were not raised to levels which were higher than the original pre-dose counts. With less disturbance of normal flora, roxithromycin may produce fewer microbe-related gastro-intestinal side-effects than amoxycillin

5.3.3.3 Resistance to roxithromycin

Following the administration of roxithromycin to two volunteers, Smith et al (1989) observed an increase in resistance to roxithromycin amongst streptococci isolated from smooth surface dental plaque. Three resistant (but unidentified) isolates from the 2 volunteers were detected prior to its start: two from one volunteer which were resistant to 2 and 8 mg/l, respectively, and a third resistant to 2 mg/l roxithromycin from the other. After completion of the study - after the third weekly single 300 mg oral stat dose - all isolates had become resistant to roxithromycin at the same two concentrations. Five of 7 distinguishable strains, identified as four S. mitior and a single S. sanguis strain, were resistant to >64 mg/l roxithrmycin, erythromycin and clindamycin but susceptible to penicillin G. From the results of this study, prolonged administration of roxithromycin to patients would appear to induce the development of resistance to macrolides and the prophylactic use of erythromycin or clindamycin in IE-susceptible patients shortly after roxithromycin therapy would appear to be unwise. Roxithromycin in concentrations greater than 0.2 mg/l was not detected in the saliva or gingival fluid

5.3.4 Clindamycin

5.3.4.1 General properties

Clindamycin is a chlorine derivative of lincomycin isolated from Streptomyces lincolnensis and belongs to the lincosamide class of antibiotics. They have a completely different chemical composition compared to macrolides but its mode of action and spectrum is very similar to that of the macrolides

The drug is well absorbed from the GIT where food has little effect on absorption and penetrates most body tissues (Wilson and Cockerill, 1983). It attains levels in bone which are very favourable when compared to those in serum: expressed as ratio of bone/serum concentration (serum concentrations in brackets), it produced values in rats of 1.11 (5.8); 0.83 (4.8); 0.90 (2.5); 0.78 (2.3) and 1.30 (1.3) respectively when measured at 30 minutes and 1, 2, 3 and 4 hours after administration of 15 mg/kg IV clindamycin (Summersgill at al, 1982)

5.3.4.2 Mode of action

The lincosamides and macrolides, two otherwise unrelated groups of antibiotics, exert their anti-bacterial effect by the biochemical modification of the 50s subunit in bacterial ribosomes (O'Brien et al, 1987). Clindamycin and macrolide antibiotics show cross-resistance amongst viridans streptococci. Sprunt (1970) reported erythromycin-resistance amongst viridans streptococci resistant to lincomycin. Similar cross-resistance between clindamycin and erythromycin is found in pneumococci and staphylococci (Barber and Waterworth, 1964)

5.3.4.3 Effect of clindamycin on normal flora and pharmacokinetics

During an investigation into the effects of penicillin V on normal resident viridans streptococci, Heimdahl and Nord (1979) also ran a test on the effects which clindamycin might have on the flora in saliva, the throat and faeces of 10 other subjects. One hundred and fifty milligrammes of clindamycin was taken six-hourly in capsule form by mouth. Serum and salivary concentrations varied and showed values of 1.8-2.3 mg/l in the former and 0-1.7mg/l clindamycin in the latter during the administration period. Like penicillin V, only a slight change in salivary streptococcal numbers was observed. However, a great reduction in anaerobe numbers was detected in the mouth, throat and gut. Clindamycin-resistant flora spread into and proliferated in these areas after sensitive populations of bacteria were eliminated after the multiple-dose, week-long antibiotic regimen. The use of clindamycin, as is the case with several other antibiotics has been linked to antibiotic-associated colitis caused by Clostridium difficile (George et al, 1980)

5.4 Vancomycin

5.4.1 General properties

Vancomycin, identified originally in Amycolatopsis orientalis (previously Streptomyces orientalis) (Nagarajan, 1991), contains a central hepta-peptide core common to all members of the glycopeptide group of antibacterial agents. It is poorly absorbed from the intestinal tract. The antibiotic inhibits the cell-wall peptidoglycan synthesis process in sensitive organisms and its action is largely bactericidal (Hermans and Wilhelm, 1987). In addition to inhibition of peptidoglycan polymerase by steric

hindrance, binding of glycopeptides to the D-alanine terminus of the subunit effectively blocks the vital transpeptidation reaction that links the growing peptidoglycan chain to the mature cell wall (Reynolds, 1989). Vancomycin may also alter the permeability of the cell membrane and selective inhibition of RNA synthesis is possible (Watanakunakorn, 1984). The antimicrobial spectrum is narrow and is used traditionally to treat serious infections caused by Gram-positive organisms (S. aureus and others) resistant to alternative therapeutic compounds. This agent is indicated only when penicillins and other more conventional compounds cannot be used (penicillin allergies) (Molinari, 1983)

5.4.2 Spectrum and resistance

Leuconostoc species are intrinsically resistant (Dutka-Malen et al (1990) and Gram-negative bacteria are resistant to vancomycin (Nagarajan, 1991; Hermans and Wilhelm, 1987). Almost all Gram-positive bacteria are susceptible to this antibiotic (Dutka-Malen et al, 1990; Watanakunakorn, 1984). Southall et al (1983) found all 17 S. sanguis strains with varying degrees of penicillin-resistance in their study, to be sensitive to vancomycin. Shanson et al (1987) found oral streptococci, isolated after extraction from patients who received prophylactic pre-extraction doses of different antibiotics, to be sensitive to this antibiotic at MIC ranges of between 1-2 mg/l (mean 1.2 mg/l vancomycin)

The vast majority of viridans streptococci are susceptible. Bayer et al, 1990 considered viridans streptococci to be "universally" susceptible, but already in 1984 Watanakunakorn listed resistant strains which had been reported in the literature

Vancomycin is used as a reserve antibiotic for IE-prophylaxis in

high-risk patients (Hermans and Wilhelm, 1987). It was wholly effective as treatment of an IE patient who carried an erythromycin-resistant strain of S. sanguis biotype I with MIC and MBC values of 40 and ≥320mg/l, respectively (Eng et al, 1982). Vancomycin MIC and MBC values for the isolate were 0.5 and 2.0 mg/l, respectively

High resistance to vancomycin by a viridans strain was reported by Shlaes et al (1984) after complications arose in a patient after resection of a malignant tumour of the mandible. S. sanguis II was isolated with a MIC and MBCs >128 mg/l vancomycin. Additionally, it was found to be tolerant to penicillin (MIC=0.5; MBC >16 mg/l) and to cephalothin (MIC=2; MBC >16) and cefoxitin (MIC=256; MBC >256), and a third generation cephalosporin, cefotaxime (MIC=2; MBC >64)

Tolerance of bacteria to vancomycin is known to occur (Shanson and Tadayon, 1986), and is common amongst enterococci

Tuomanen (1986) found vancomycin (and other compounds such as imipenem) to be effective in killing non-growing, amino acid deprived E. coli and S. pneumoniae bacteria

5.4.3 Side-effects

Systemic toxic reactions, like the so-called "red neck" or "red man" syndrome, have been described in patients receiving vancomycin who exhibit pruritis, a maculopapular rash of the face, neck and upper torso and possibly also hypotension. This reaction has been ascribed to a massive release of histamine and mast cell degranulation after the rapid intravenous infusion of vancomycin (Hermans and Wilhelm, 1987). It has, however, been noted that this syndrome may also develop rarely after a slow infusion

(Bayer et al, 1990). Harmful side-effects include thrombophlebitis, oto- and nephrotoxicity and allergenic reactions (Molinari, 1984) 226]. Ototoxicity may develop when serum levels exceed 30 mg/l vancomycin (Hermans and Wilhelm, 1987)

Chapter 6

TOLERANCE TO ß-LACTAM ANTIBIOTICS

6.1 Description of the phenomenon

Certain bacteria are known to exhibit unusual growth behaviour in the presence of particular antibiotic agents

6.1.1 Genetically determined (genotypic) tolerance

Shockman et al (1979) and Tomasz (1979) noticed in their studies that some strains of susceptible organisms appeared only to undergo growth inhibition rather than succoumb to the normally bactericidal action of penicillins when exposed to these agents. This unusual reaction was observed when penicillin G and other beta-lactams inhibited synthesis of the bacterial cell wall and did not to lead, eventually, to cell rupture and death. These ß-lactams normally disrupt critical stages in the biosynthesis or assembly of the bacterial cell wall which results in lysis and death of the cells (Tomasz et al, 1970)

Strains which survived in the presence of antibiotics traditionally active against the cell-wall biosynthesis, were subsequently shown to be deficient in certain essential, naturally-occurring enzymes necessary for penicillin and other beta-lactams to exert a lytic effect on micro-organisms. These mediators of lysis-associated bacterial killing, present after exposure of the organisms to beta-lactam agents, were discovered to be endogenous peptidoglycan hydrolases or autolysins (Handwerger and Tomasz,

1985)

The function of autolysins in the bacterial cell is to produce enzymatic breaks in the mesh-work of cell-wall polymers to permit the insertion, in specific areas, of newly synthesised wall material required during cell growth. Thus, any process which only inhibits the production of new cell wall material without also inhibiting the action of cellular autolysins, would lead to disintergration of the cell wall (Tomasz et al, 1970)

Toumanen, Durack and Tomasz (1986) note that antibiotic tolerance differs fundamentally from all other resistance mechanisms in that a change in the drug MIC is not involved - bacteria evade only the killing action of the antibiotic (as reflected by the MBC values)

The phenomenon where bacterial growth only was inhibited at low concentrations and the cells not lysed or killed by the normally bactericidal beta-lactam antibiotics at higher concentrations of the antibiotic (a bacteriostatic effect), became known as antibiotic "tolerance" - a term originally proposed by Tomasz et al in 1970

Certain bacterial substances appear able to reduce the antibacterial effect of antibiotics. Raynor et al (1979) suggested that tolerance could result from the enhanced secretion by certain organisms (S. aureus in his study) of an autolysin inhibitor such as lipoteichoic acid (LTA). Shockman et al (1979) supported this finding when they found that in the presence of LTA, normally lethal concentrations of penicillin G produced only sub-lethal effects in a wide variety of Gram-positive bacteria able to generate and release this substance

Subsequently, four mechanisms of improved survival amongst clini-

cal isolates have been described and collectively referred to as "survivor mutations" by Toumanen et al (1986). These authors noted that all isolates exhibited susceptible levels of drug susceptibility in terms of MICs but possessed some secondary mechanisms which involved changes in cidal and lytic effects. All four mechinisms contributed to what the authors termed "superior" survival during exposure to cell wall inhibitory antibiotics: (i) this is a strain-specific phenomenon (which also affects viridans streptococci) and appears independent of antibiotic concentration (Goessens et al, 1984). Survivors appear to die at a slower rate than other cells in the culture which have lower degrees of survival. This mechanism is found amongst so-called **persister mutants** and may be dependent upon the sensitivity to antibiotics of the organism during particular stages of the cell cycle (Moyed and Bertrand, 1983). (ii) The **paradoxical effect** is exhibited by several bacterial pathogens (including viridans stretococci) in which bactericidal activity fails to occur above certain concentrations of antibiotic (Handwerger and Tomasz, 1985). (iii) A third form is drug specific and resembles that which occurs amongst **LYT(+) TOL(+)** laboratory mutant pneumococci not deficient in autolytic activity (Williamson and Tomasz, 1980). (iv) The final mechanism described by Tuomanen et al (1989) is exhibited by several bacterial species which have autolytic defects similar to those of **lysis-defective** (tolerant) laboratory mutuants of pneumonococci

In studies by Voorn et al (1994), non-tolerant variants of S. aureus were induced by repeated subculture of a tolerant clinical isolate on an antibiotic-free medium. The parent strain exhibited a paradoxical response to cloxacillin both **in vivo** and **in vitro**. The authors showed that continuous administration of this antibiotic in the treatment of endocarditis in rats caused by the tolerant strain failed to kill the organisms because of sustained high concentrations of cloxacillin in the blood. The same authors

showed that after repeated cycles of exposure to 25 mg/l cloxacillin or 10 mg/l vancomycin alternating with growth in antibiotic-free medium, they could induce tolerance in non-tolerant S. aureus strains. Conversely, tolerant strains lost their tolerance after repeated subculture in antibiotic-free medium at 37 degrees Celcius for 50 days (Voorn et al, 1994). In similar studies, Moore et al (1994) generated an isogenic pair of ß-lactam tolerant and non-tolerant strains of S. aureus by selecting tolerant variants from a non-tolerant strain. They suggested that there may well be a relationship between tolerance and the paradoxical effect as both phenomena can be induced in the same strain (Voorn et al, 1994). Voorn et al (1994) speculated on whether (uncommon) altered responses to the bactericidal activity of ß-lactam agents exhibited by strains may be elicited as a direct result of exposure to those agents; ie. sampled specimens already exposed to the agents during therapy of the infection

6.1.2 Phenotypic tolerance

Tuomanen (1986) distinguished between genotypic tolerance to beta-lactam antibiotics - where a mutant, non-functional autolytic enzyme is produced by an organism - and the phenomenon which non-growing (and for that reason beta-lactam insensitive) bacteria exhibit in the presence of most of these antibiotics. She suggested that the latter be referred to as phenotypic tolerance. This term will be used in this dissertation to distinguish the phenotypically tolerant or refractory state exhibited when susceptible bacteria with low MICs and MBCs **in vitro** survive the bactericidal activity of antibiotics, from the traditional use of the term tolerance, based on the difference between MICs and MBCs of bacteria

Evidence of phenotypic tolerance has been observed to occur both **in vitro** and **in vivo**

6.1.2.1 Phenotypic tolerance in vitro

Chain suggested in 1945 that it was not bacterial cell division which was essential for effective killing by penicillin, but rather active bacterial metabolism. Jawetz et al in 1951 expanded upon the studies of Chain and concluded that active synthesis of specific proteins within the cell (and which can be inhibited by chloramphenicol) was critical for penicillin activity. Derangement of synthesis was noted to occur with the deprivation of a wide variety of essential nutritional requirements which included usable carbon and nitrogen sources, vitamins and minerals. With the experimental removal of these substances from the bacterial environment, arrest of the logarithmetic increase in cell numbers occurred within minutes and there was a marked and coordinated reduction in all macromolecule synthesis rates (Goodell and Tomasz, 1980). All changes which ensure the survival of an organism and which occur in situations of deprivation are said to be under the control of the **rel A** genetic locus - the so-called "stringent response" (Ramey and Ishiguro, 1978) - a mechanism not well understood (Tuomanen, 1986)

The use of the chemostat, however, has shown that there is a correlation between killing and cell lysis amongst bacteria grown in the absence of essential nutrients and is a direct function of the generation time of the bacterium (Tuomanen et al, 1986)

Slow rates of growth or actual cessation of growth are commonplace at numerous sites of bacterial infection **in vivo** (Tuomanen, 1986)

6.1.2.2 Phenotypic tolerance *in vivo*

6.1.2.2.1 Experimental *in vivo* phenotypic tolerance

Toumanen et al (1985) used a rabbit model of experimental pneumococcal meningitis to examine bacterial growth in the subarachnoid space. Two important observations relevant to the tolerance phenomenon were made: (i) a long lag period was evident between inoculation of test organisms into the cerebrospinal fluid and the initiation of growth and (ii) maximal growth rate of bacteria in the space was significantly lower than levels achieved **in vitro**. Retarded growth characteristics in the space may be linked to lower concentrations of buffer (a third of optimum level) and concentrations $\geq$100-fold deficient in leucine, isoleucine, cysteine, glycine, valine, serine and the minerals magnesium and manganese in cerebrospinal fluid (CSF) compared with a minimal chemically defined medium. This type of growth behaviour was also noted **in vitro** in bacteria in nutrient-deficient growth media

Phenotypic tolerance was further investigated by these authors in an **ex vivo** animal experiment using heat-treated serum or leukocyte-free CSF removed from healthy rabbits. Pneumococci inoculated into these fluids behaved in a manner observed **in vivo** - they displayed retarded and slower growth patterns similar to those strains inoculated into the subarachnoid space of rabbits discussed above

6.1.2.2.2 *In vivo* phenotypic tolerance associated with bacteraemia

Hall et al (1993) unknowingly observed the phenomenon of

phenotypic tolerance in humans **in vivo.** It was noted that viridans streptococci survived in the blood of individuals who, following dental extraction procedures and the administration of penicillin V or amoxycillin, survived killing despite the fact that isolates were subsequently shown to have MICs and MBCs well below the respective ß-lactam blood concentrations

As already noted, the precise nature of events which produces phenotypic tolerance to antimicrobial agents remains unclear but the response is observed to occur under a number of different conditions: (i) amongst bacteria which exhibit reduced growth rates in altered micro-environments (for example, where there is an increased concentration of various enzymes and metabolic products), (ii) after nutrient deprivation or depletion of essential substances and substrates such as carbon and nitrogen sources, dissolved gases, vitamins and minerals (iron) or (iii) in bacteria buried in adherent microbial biofilms through which either nutrients and antibiotic compounds have difficulty in passing (Holmes and Evans, 1989; Cozens et al, 1986; Brown et al, 1988). Bacteriological conditions similar to any one or more of the above exist within bacterial films coating urinary catheters (Nickel et al, 1985) and may exist within endocarditis vegetations, on vascular or similar prostheses and other devices, as well as in dental plaque

6.2 Tolerance amongst viridans streptococci

In this study the term "tolerance", when not specifically qualified, will refer to genotypic tolerance and the adjective "phenotypic" will precede this term when the phenomenon of phenotypic tolerance is referred to

6.2.1 Tolerance to ß-lactam antibiotics

Species of viridans streptococci are reported to exhibit tolerance to penicillin G (Holloway et al, 1980; Dankert and Hess, 1982). S. sanguis was the first such oral streptococcus that was studied in detail by Horne and Tomasz in 1977. Penicillin G halted bacterial growth of this strain at an extremely low MIC of 0.013 mg/l, and produced reversible, non-lethal morphological changes at 10x MIC where 63% of cells survived. It also displayed tolerance to oxacillin, cephalexin and cephaloridine. Against the latter antibiotics the strain had a survival rate of 67%, 100% and 79%, respectively. The tolerant response of this strain to penicillin and other cell-wall inhibitors mimicked typical features of "autolysin-defective bacteria" (Horne and Tomasz, 1977)

Penicillin tolerance is not limited only to S. sanguis (19%), however, and was noted by Holloway et al, (1980) to occur in all viridans species tested except S. salivarius. The phenomenon was common in strains of S. mutans (27%), S. mitior (20%) and S. sanguis (19%). Two hundred and fourteen gingival sulcus, dental and bacteraemic isolates were screened for penicillin sensitivity in their study and 151 (71%) had MICs <0.8 U/ml (equivalent to 0.48 mg/l) penicillin. Eighty-three of 151 (55%) had MBCs lower than this value and 68 (45%) higher. Tolerance (MBC = ≥10x MIC) was noted in 29 (19%) of the 151 "sensitive" strains (MIC <0.48 mg/l) and in 14 (22%) "resistant" strains (MIC ≥0.48 mg/l). A high average MBC/MIC ratio of 153 was found amongst the 43 tolerant strains and for two isolates it was approximately 1000-fold. The authors suggested that the former, where the MIC's are in the normal range but MBCs are high, be termed "endurant" streptococci to distinguish them from those which also exhibited high (resistant) MICs

Viridans streptococci were screened for tolerance by Powley et al in 1989. Sputum specimens (of no particular clinical significance) were collected and viridans cultures identified. Of these, 13 of 40 strains (32.5%) were found to be tolerant to penicillin G: 3 of 5 S.sanguis I biovar 2 isolates (60%), 6 of 20 S. sanguis II (30%) and 4 of 9 S. mitis (44%) isolates. No isolates of either S. sanguis biovar 1 or S. mitis exhibited tolerance to this antibiotic in the small sample tested

Hess et al (1983) stated that the the presence of tolerant viridans species **in vivo** may increase the risk of IE development in spite of prophylaxis with penicillin G alone after experimental development of this infection in the rabbit model. Using strains of S. sanguis II isolated from the gingival sulci of children (three strains) and one from a patient with endocarditis, the IE infection rate in compromised rabbits who received penicillin G prophylaxis ranged from 9–80% (average 47%) depending upon which isolate was introduced to produce the infection

In a case report (Denning et al, 1984), an indigent patient died after the extraction of 24 teeth in spite of his receiving 3 g amoxycillin stat 2 hours pre-operatively. S. milleri was isolated from the blood and found to be tolerant to amoxycillin with an MIC of 0.025 mg/l and MBC >10 mg/l

In a publication in which the involvement in IE of S. mutans was reputedly first identified (Harder et al, 1974), manipulation of penicillin G susceptibility data showed that in all cases (9 in total) MBC:MIC ratios fulfilled the lower requirements for tolerance (see Section 6.2.2) and were ≥10. Of these, seven strains had ratios ≥125, two were ≥625 and one had an extremely high MBC:MIC ratio of ≥1000. Four and two of the six isolates tested against vancomycin had MICs of 5 and 1 mg/l antibiotic, respectively

6.2.2 Tolerance to vancomycin

Amongst 13 penicillin-tolerant viridans cultures (penicillin MBC:MIC ratio ≥20) from patients with endocarditis - all collected prior to their receving antibiotic, vancomycin MICs ranged between 2-4 mg/l (Shanson and Tadayon, 1986). Sixteen mg/l vancomycin failed to produce a 99.9% viable count reduction after overnight culture. MBC values were variable. Four strains possessed MBCs of ≥32 mg/l vancomycin and for 3 strains it was ≥16 mg/l. Of the organisms with MBCs ≥32 mg/l, three were **S. mitior** and one a **S. sanguis** I. Two isolates with MBCs greater ≥16 mg/l were both **S. mitior**

Bourgault et al (1979) tested more than 63 clinical viridans isolates against 12 different antibiotics. At a concentration of 1 mg/l, vancomycin was bactericidal to only 25.7% of strains (19 of total of 73). Seventy-three percent (54 of 73) were only inhibited by this concentration. Eleven isolates, including four **S. mitis** and three **S. sanguis** II species, required concentrations of >8 mg/l vancomycin for killing. Seven strains required vancomycin MBCs of >8mg/l. In 1984, Watanakunakorn reported vancomycin MIC values for viridans streptococci to range between <0.312 and 8.0 mg/l

Tolerance was common amongst clinical isolates from IE patients in a study by Meylan et al (1986) where susceptibility of 25 oral streptococci against amoxycillin and vancomycin was determined. Ten **S. mitis**, 7 **S. sanguis** and 2 **S. salivarius** strains were identified. Five were unspeciated. All had amoxycillin MICs of ≤0.25 mg/l and MBCs which were either low (<0.5 mg/l) in 6 non-tolerant strains (25%) or high (>128 mg/l) in 18 (75%) tolerant strains. The MIC of vancomycin against all 24 strains was ≤1.0 mg/l while

the MBC was <1mg/l in 3 (12.5%) non-tolerant isolates and >128 mg/l in 21 (87.5%) displaying tolerance

In another study, two unspeciated vitamin B-6 dependent IE viridans isolates were found to be tolerant to vancomycin with MBCs of ≥32 mg/l and individual vancomycin MICs of 0.05 mg/l and 1 mg/l (Feder et al, 1980). Several other examples of nutritionally variant streptococci that are tolerant have been reported in the literature (Gephart and Washington, 1982; Holloway and Dankert, 1982)

There is a noticable variation in the literature as to what MBC:MIC ratio constitutes tolerance to antibiotics. Ratios of ≥10 (Holloway et al, 1980), ≥20 (Shanson and Tadayon, 1986) and ≥32 (Dankert, Holloway et al, 1982; Powley et al, 1989) have been employed by various researchers

6.2.3 "Tolerance" to antibiotics not acting directly on bacterial cell wall formation

A number of viridans strains collected in the study of Harrison, Stross, et al (1985) exhibited MICs in the range 1-4 mg/l of erythromycin but had MBCs >256 mg/l. These concentrations clearly fit the parameters of tolerance described previously. However, the term tolerance is normally reserved for agents acting upon cell walls of Gram-positive bacteria. Several antibiotics such as macrolides and tetracyclines which act on protein synthesis are described as being mainly bacteriostatic. However, at high concentrations, which are unachievable in tissues, they may be bactericidal. This situation is not normally described as tolerance. For the purposes of this study, it is proposed, however, to apply this term to the macrolides and clindamycin in the case of streptococci where these antibiotics are normally bactericidal at

relatively low concentrations which are achievable at sites of infection

6.3 Clinical implications of tolerance in viridans streptococci

Clinical implications of bacterial antibiotic tolerance to penicillin, for example, are uncertain, but were it of any significance, "it is likely to be in those conditions in which the use of bactericidal agents is essential for therapeutic success" (Powley et al, 1988). This is clearly the case in the treatment of IE

Serum bactericidal activity against the causative organisms in IE is often used to predict the outcome of therapy. A serum bactericidal level required for efficient use against tolerant pathogens may be very useful but unachievable in some cases in clinical situations, eg endocarditis chemotherapy. Such levels are especially important for prognostic purposes when drug combinations are used for the treatment of IE

Serum bactericidal levels believed to be essential for effective therapy against particular pathogens may be unachievable in clinical situations (endocarditis chemotherapy). Metlan et al (1986), using data from the separate exposure of isolates to vancomycin and amoxycillin, proposed that for (all) tolerant streptococci, the "reduction in viability" (time-kill kinetics) was a more accurate indication than the MBC alone of successful clinical outcome which depends on bactericidal antibiotic activity. It has also been suggested that the ability of antibiotics to kill bacteria affects the efficacy of prophylaxis (Glauser, Bernard et al, 1983)

Chapter 7

CLASSIFICATION OF VIRIDANS STREPTOCOCCI

7.1 Problems associated with the classification of viridans streptococci

The classification of oral streptococci has proved to be particularly difficult: "viridans-type streptococci encountered in oral cavities and pharynges have been curiously refractory to satisfactory classification" (Kilian et al, 1989). Their definition is surprisingly poor in spite of their "dominant role in early dental plaque and caries" (Nyvad and Kilian, 1990) and other diseases

Considerable problems have been encountered by researchers studying post-operative streptococcal bacteraemias and these have been compounded by inconsistencies relating to the taxonomy of oral streptococci. The diversity of this group of bacteria severely hampered efforts to determine the identity, distribution and numbers of micro-organisms which colonise and inhabit the oral cavity. Difficulties were encountered during sampling, culture and isolation of such organisms (Hardie, 1983)

The most abundant streptococci in the mouth are undoubtedly the viridans group of streptococci. These bacteria are gram-positive, spherical or ovoid, facultatively anaerobic, non-spore forming, non-motile organisms which most commonly produce alpha-haemolysis when cultured on blood agar. Streptococci occur singly, in pairs, in short or longer chains of 8 to 10 cocci when grown on suitable media (Hardie, 1983; Schuster, 1983)

Non-haemolytic streptococci are those whose colonies are neither alpha-haemolytic nor do they produce clear haemolytic zones on blood agar after the complete destruction of erythrocytes (beta-haemolysis) incorporated into the medium. In contrast, alpha-haemolysis, the haemolysis form exhibited by most viridans streptococci, is characterised by a greenish zone of discolouration which develops around colonies after partial digestion of the haemoglobin (Coykendall, 1989)

Several methods of classification have been proposed but agreement on this matter has yet to be obtained internationally. The inabilty of investigators to reach consensus on the choice of phenotypic traits of viridans streptococci which would distribute these bacteria into clusters that could serve as easily identifyable species has contributed to the confusion. Organisms giving identical biochemical reactions have often been allocated different identities in different schemes

Viridans streptococci belong to the genus **Streptococcus**. The taxonomic position of the genus as well as several other Gram-positive cocci with similar physiological properties has not been finalised (Bergey's Manual, 1986). Characteristics of chain or coccal pair formation, the lack of cytochromes and catalase negativity typify the **Streptococcus** genome (Facklam and Smith, 1976). Sherman (1937) distinguishes viridans streptococci from enterococci by the former's inability to tolerate extreme temperatures, lack of growth in the presence of bile, high salt concentrations and alkali, and by their weak reducing powers

Major differences are apparent in British and American taxonomic schemes (Facklam, 1984; Hardie, 1983). In Facklam's editorial (Facklam, 1984) mention is made of the more convenient and informative Lancefield antigen grouping system used for identifying

beta-haemolytic streptococci. But it is unsuitable for use on "non-beta-haemolytic" streptococci which often fail to react to Lancefield grouping sera

7.2 Different systems of classification

A plethora of schemes for the identification of the oral viridans streptococci have been proposed in the last two to three decades and include those of Carlsson (1968), Colman and Williams (1972), Colman (1976), Cowan and Steel (1974), Deibel and Seeley (1974), Hardie and Bowden (1976), Parker and Ball (1976), Facklam (1977), Jones (1978) and Parker (1983). Later schemes include Colman and Ball (1984), French et al 1989, Coykendall (1989) and Kilian et al (1989) and others

7.3 Classification of Kilian et al (1989)

The method of Kilian et al (1989) was used in this investigation and was complemented in part by proposals of Coykendal (1989) to identify **S. vestibularis** colonies. An extensive list of biochemical and physiological characteristics is used by Kilian to differentiate between the various viridans species and include differences in biochemical activity, antigenic cell-wall components, fimbriation, coaggregation properties, affinity for salivary glycoproteins and others. In his study (Kilian et al, 1989), 151 isolates were subjected to categorisation based on nine colony morphological types, five inhibition tests, 28 biochemical tests, 26 enzymatic tests and 16 antiserum reactions. The authors noted that many cultures were not stable, and even after at least five serial subcultures continued to show a mixture of colony types

A total of 7 species and a number of biovariants were recognised,

namely: S. sanguis (4 biovars), S. gordonii (3 biovars), S. oralis, S. mitis (2 biovars), S. salivarius, S. anginosus and S. mutans

Usefully, most strains gathered for the Kilian et al study had previously been subjected to genetic analyses. This information proved especially valuable where "difficult" decisions on taxonomy had to be made

The classification proposed by Kilian et al correlates reasonably well with Facklam's. Although full recognition has not been awarded to all species proposed by Killian et al (1989), their classification has received considerable support and identification schemes based on this classification are freely employed (Douglas et al, 1993; Whiley and Beighton, 1991)

Although, by way of DNA hybridisation and cellular polypeptide electrophoresis, Coykendall (1989) found S. vestibularis to be related most closely to S. salivarius, he considered it sufficiently distinct to warrant assignment to a separate species. This proposal was incorporated into the present study

Chapter 8

EXPERIMENTAL DESIGN AND METHODOLOGY

8.1 Introduction

The *in vitro* experiments described in this dissertation were designed to assess the potential efficacy of antimicrobial agents which are candidates for the prophylaxis of infective endocarditis (IE) following dental procedures. The effect of *in vitro* exposure of dental plaque streptococci from different groups of individuals to a representative ß-lactam agent (penicillin G) formed an essential part of the studies and the susceptibility of survivors to candidate prophylactic agents was used to compare the potential usefulness of candidate prophylactic agents

8.2 Informed consent

Informed consent was obtained from all participants prior to their inclusion in the study. The project was approved by the Research and Ethics Committee of the Medical University of Southern Africa, Medunsa, and The University of the Witwatersrand, Johannesburg

8.3 Subjects

8.3.1 Subjects exposed to ß-lactam antibiotics

Two subgroups of patients who received prior antibiotics were

used. The first consisted of 41 volunteers who received daily doses of penicillin V for rheumatic fever prophylaxis (the RF subgroup) and a second smaller group of nine patients who received high therapeutic doses of ampicillin at Ga-Rankuwa Hospital for at least one week and not longer than 8 weeks (A-subgroup). The two subgroups were considered together as ß-lactam "exposed" individuals (E-group) or, where appropriate, independently

8.3.2 Subjects not exposed to ß-lactam antibiotics

Sixty-seven volunteers about to undergo dental extraction formed a separate group of individuals who have not recently been exposed to antibiotics. None of these volunteers had received antibiotics for a minimum period of three months prior to plaque sampling. They were labelled the "unexposed" group (UE-group)

8.4 Specimen collection

Dental plaque was removed from adjacent interproximal supra- and subgingival surfaces of the first and second upper molars using sterile Gracy currettes (Ash Instruments). In persons without maxillary molars, plaque specimens were removed from comparable anatomical sites on mandibular molars

8.5 *In vitro* selection in broth: a model

Dental plaque material was inoculated into 10 ml Todd-Hewitt broth (Oxoid, Basingstoke, UK) containing 0.125 mg/l penicillin G (Hoechst Pharmaceuticals), 0.125 mg/l penicillin G plus 5% gentamicin (Eli Lilly) and 1.0 mg/l penicillin G. The gentamicin-

containing broth cultures were used not only to simulate regimens recommended for IE therapy due to viridans streptococci with MICs $\geq$0.5 mg/l and enterococci (Tanner and Durack, 1990), assuming that the combination may deal more effectively with the streptococci than penicillin G alone: but also, and more importantly, because a combination of penicillin plus an aminoglycoside has been suggested for IE prophylaxis following studies demonstrating survival of viridans streptococci in the blood - despite parental penicillin G prophylaxis used singly (Hess et al, 1983. Blood agar plates inoculated with gentamicin-containing broth cultures showed better separation of streptococcal colonies from those of non-streptococcal species compared with plates inoculated with broth cultures containing penicillin G alone

Following overnight incubation at 37 degrees Celcius, a loop-full from each of the three suspensions was transferred onto one antibiotic-free blood-agar plate (Oxoid, Columbia agar plus 5% horse blood) and incubated for a further 24 hours. Alpha-haemolytic colonies with differing morphological characteristics were selected for speciation and susceptibility testing from one or more of the plates

Cultures of isolates were labelled according to their sources and selection procedure and stored at -20 degrees Celcius until further studied

8.6 Species identification

Strain identification was performed according to the taxonomic approach of Kilian, Mikkelsen and Hendrichsen (1989). Mannitol, melibiose and maltose fermentation tests were performed, as were tests based upon growth in the presence of 4% and 6.5% NaCl and in aesulin-bile, hydolysis of aesulin, arginine and urea, suscep-

tibility to sulphamethoxazole and vancomycin (using disc diffusion) and production of dextran, laevan and alkaline phosphatase. In addition, the Vogues-Proskauer reaction (VP) of isolates was determined, and indicator plates which allowed for the selection of strains producing hydrogen peroxide (Muller, 1984) were included in the series of biochemical tests. Coykendall's method (1989) was used to classify **S. vestibularis**, while the identification of **S. anginosus**, which includes **S. intermedius** and **S. constellatus** as well as **S. milleri**, was based on recommendations of Coykendall (1989) and Kilian et al (1989). The identification of the various **Streptococcus** species and biovars was based on the following flow chart

8.7 Susceptibility testing

8.7.1 MIC and MBC determinations

MICs and MBCs were determined by a micro-titre broth dilution technique performed according to NCCLS recommendations (NCCLS Document, 1990). Antibiotic concentrations were prepared within the ranges of 0.003-256 mg/l for penicillin V (Sigma, Missouri), 0.003-4 mg/l for amoxycillin (Beecham Pharmaceuticals), erythromycin (Abbott Laboratories), roxithromycin (Roussel Laboratories) and clindamycin (Upjohn), and 0.03-32 mg/l for vancomycin (Eli Lilly). The inoculum was prepared according to NCCLS methodology to give a final concentration of 100-500 thousand CFU/ml in each well. **Staphylococcus aureus** ATCC 29213 served as control

The MIC was defined as the lowest concentration of the antibiotic that inhibited growth as assessed by the naked eye, while the MBC denoted the lowest concentration that produced a $\geq$99.9% kill as demonstrated on blood agar plates (5% horse blood) where $<$0.11%

LABORATORY PROCEDURES FOR THE IDENTIFICATION OF VIRIDANS STREPTOCOCCI FOLLOWING THE METHODS OF KILIAN et al AND COYKENDALL

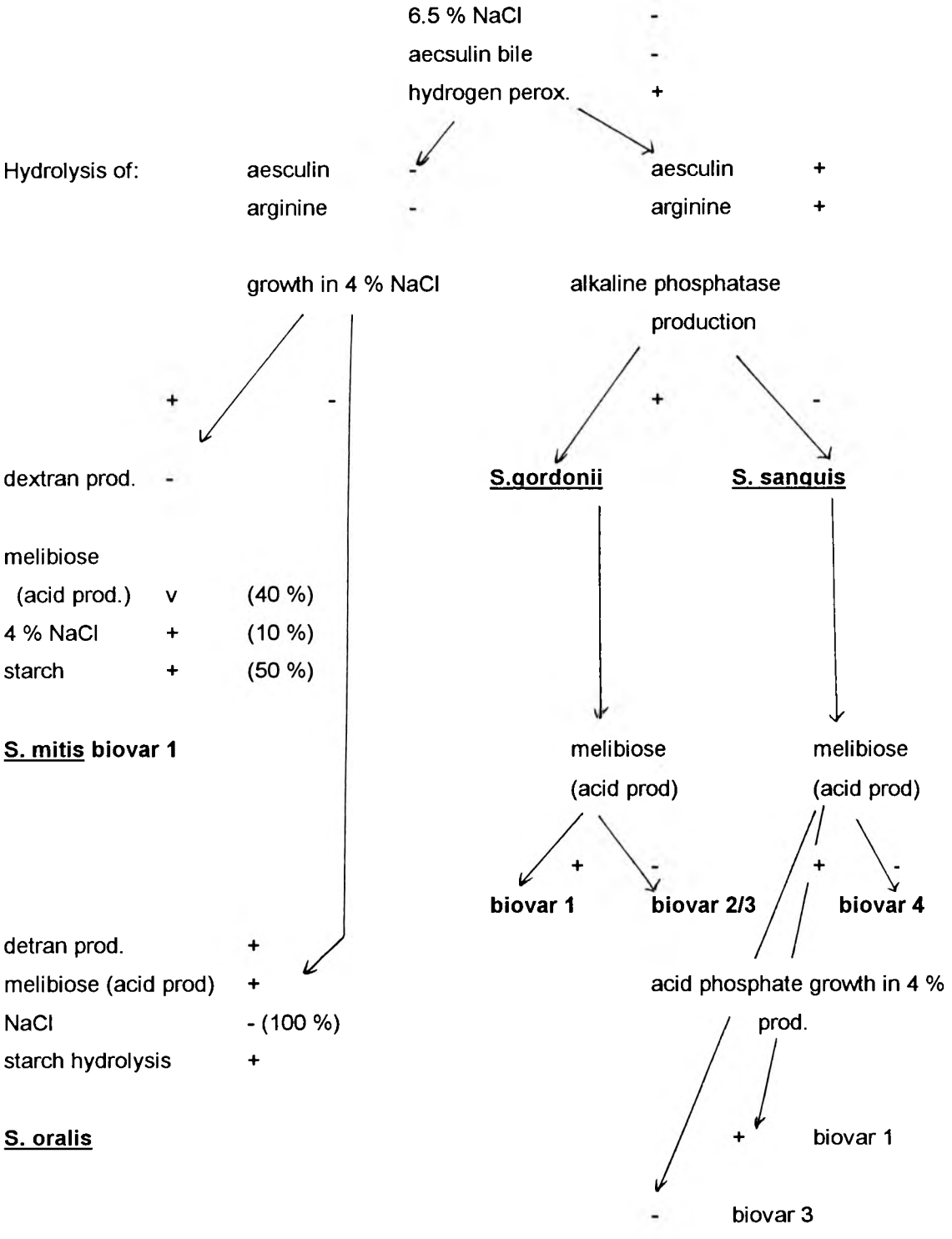
Part I: Preliminary steps

cell morphology	cocci	+
	cocco-bacillary	+
	rods	+
cell arrangement	chains	+
	pairs	+
	clusters	-
gas production in presence of glucose		-
vancomycin susceptibility		yes
streptococcal typing		variable (v)
growth in aesculin bile		v
pyrase		v
growth:	in 6.5 % NaCl	-
	at 45 Celcius	v
	at 10 Celcius	-

Part II: Species identification

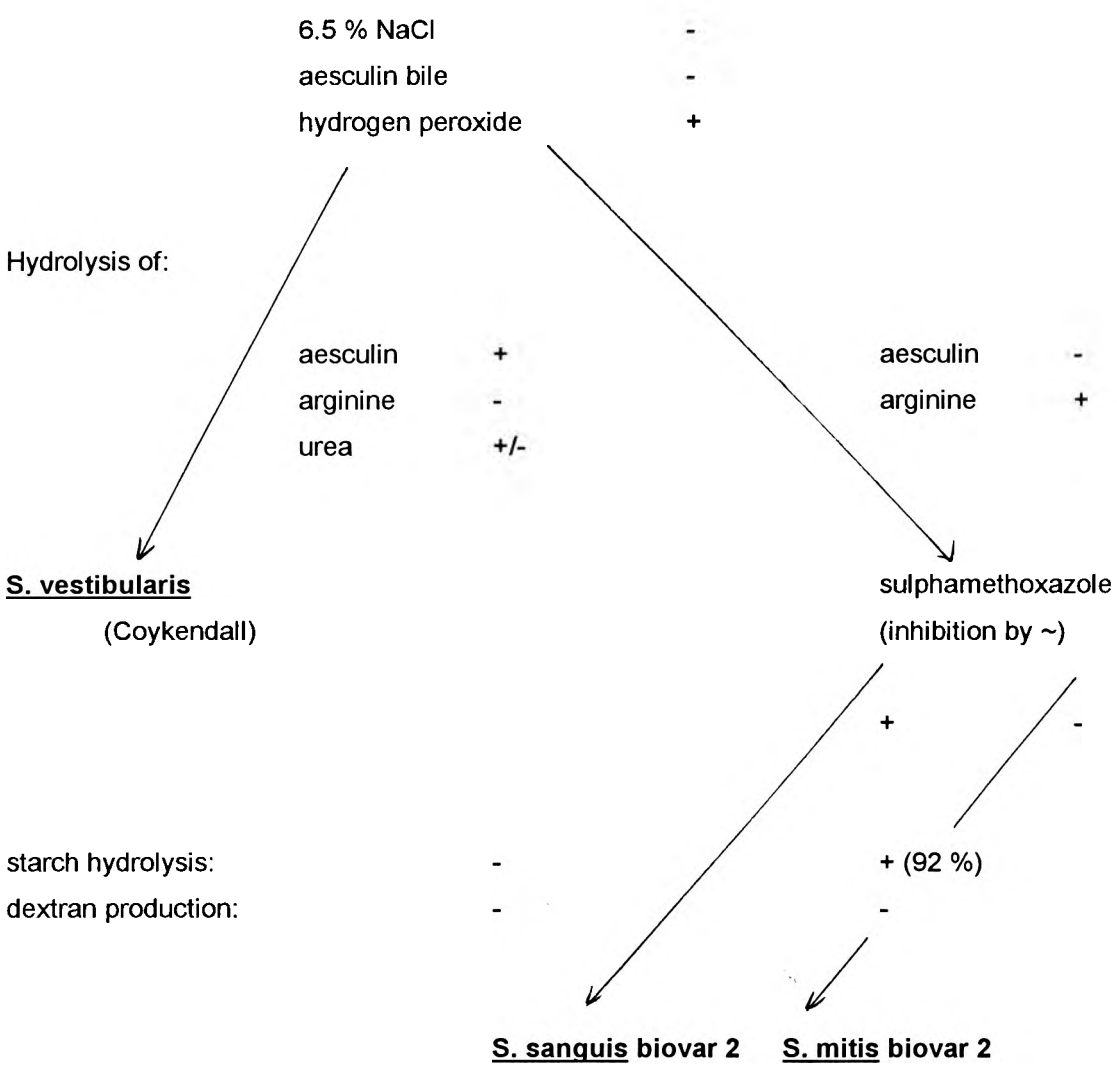
Step 1.

Growth in the presence of:



Step 2.

Growth in the presence of:



Step 3.

arginine hydrolysis -
aesculin hydrolysis +
mannitol (acid prod.) +/-

S. mutans

VP reachtion +
alk. phos'tase prod. -

aesulin hydrolysis +
arginine hydrolysis -
mannitol (acid prod) -
laevan +

S. salivarius

VP reaction +
urea hydrolysis + (71 %)

Growth in the presence of:

6.5 % NaCl -
aesculin bile -
hydrogen peroxide -

aesculin hydrolysis +/-
arginine hydrolysis +/-
mannitol (acid prod) -
alkaline phos. prod +/-

S. anginosus

(Coykendall)

of the original inoculum for MIC testing survived as CFUs from wells showing no growth after overnight incubation during the first step (MIC determination) of the MBC technique

For penicillin V and amoxycillin, isolates were regarded as susceptible when their MICs were $\leq$0.12 mg/l, intermediately resistant when their MICs ranged from 0.25-2 mg/l and resistant when MICs were $\geq$4 mg/l. For comparative purposes, it should be noted that penicillin G is more active against streptococci than penicillin V, the former being roughly up to two-fold more active (Garrod, 1960a; Garrod, 1960b). Penicillin G is also slightly more active against non-enterococcal streptococci than amoxycillin (Coulter et al, 1990). MIC levels of penicillin V and amoxycillin are for many strains the same as for penicillin G but a minority of strains have MICs twice those of penicillin G (Garrod, 1960a; Garrod, 1960b; Coulter et al, 1990)

8.7.2 Definitions relating to survivors of <u>in vitro</u> selection process

8.7.2.1 Phenotypic tolerance

Isolates of viridans streptococci which had MBCs of penicillin V and amoxycillin less than the selection concentrations of penicillin G (0.125 mg/l and 1.0 mg/l) were regarded as being phenotypically tolerant. This assumption was based upon the premise that penicillin V and amoxycillin are equivalent to or slightly less active against viridans streptococci than penicillin G. Strains with low MBCs of penicillin V and amoxycillin should therefore, under favourable conditions, be killed by an equivalent concentration of penicillin G

8.7.2.2 Resistance in the context of the selection process

Based on the same reasoning as above, survivors with penicillin V and amoxycillin MBCs greater than the equivalent penicillin G selection concentrations, were regarded as intrinsically resistant at the relevant concentrations. It should be noted that the levels of resistance in the context of the *in vitro* selection model are not exactly the same as those based on internationally regarded breakpoint concentrations denoting resistance or degrees of resistance

8.7.3 Recording of tolerant strains

Degrees of tolerance (genotypic) were recorded as MBC:MIC ratios. Although it is accepted that most authorities regard ratios of $\geq$32:1 to denote tolerance by definition, differences in excess of 2:1 ratios between MBCs and MICs were also noted

8.7.4 Definition and recording of persisters

The presence of persisters was based on the survival of up to 0.1% of bacterial cells in the original inoculum at the MBC, demonstrated as CFUs in the second step of the MBC technique (see 8.7.1) by subculture on blood agar plates. When persisters were present at concentrations higher than the MBC (but with $\leq$0.1% survivors of the original inoculum), these concentrations were also recorded

8.8 Statistical analysis:

The design of the study did not allow for the accurate standardisation of the amount of dental plaque material removed from the teeth for investigation. Also, the number of colonies selected per broth selection procedure varied as described earlier. These factors may have affected, to some extent, the accuracy or validity of our statistical analyses which were, none-the-less, applied when considered appropriate. For analysis of categorical variables, the Fisher exact and Chi-squared tests were used and the U-test of Mann-Whitney for continous variables

Chapter 9

IDENTITY AND SUSCEPTIBILITY OF ORAL STREPTOCOCCI TO CANDIDATE ANTIBIOTICS

9.1 Introduction

Extensive **in vitro** studies have been conducted to evaluate candidate drugs used in the prophylaxis of infective endocarditis (IE). Most of these studies based their assessment on the effect of these antibiotics on the drug susceptibility of oral flora, including their potential to select for resistant viridans strains, and in a few studies, their propensity to give rise to colonisation by other bacteria. Further investigations dealt with susceptibility profiles of oral streptococcal species within the oral cavity, and in others, the effect of prophylactic ß-lactam antibiotics on bacteraemic isolates. These latter studies demonstrated that both resistant and susceptible organisms may survive in the blood despite serum concentrations higher than the MBCs of the isolates (Hall et al, 1993)

In this chapter, streptococci from dental plaque which survived exposure to penicillin G alone and penicillin G plus gentamicin, were subjected to MIC and MBC determinations against six candidate antibiotics and the possible effect of previous exposure to ß-lactam antibiotics on the susceptibility of these streptococci to a battery of antibiotics analysed

9.2 Methodology and definitions

The methods employed in this chapter of the study have been recorded in detail in Chapter 8. Briefly, Streptococcus species were identified according to the classification of Kilian et al (1989) with a number of modifications, as described in that chapter. MICs and MBCs were performed in microtitre trays as specified in the NCCLS guidelines (NCCLS Document, 1990)

9.3 Results

9.3.1 Identity of viridans streptococci after in vitro selection in penicillin G

The identity of viridans streptococci following selection in penicillin G is given in Table 9.1 and is recorded into groups according to their previous exposure to ß-lactam antibiotics (E-group) or lack of recent exposure (UE-group)

Seventy-nine viridans streptococcal isolates collected from the plaque of 67 UE-group volunteers were classified into eight species according to Kilian et al (1989) and Coykendall (1989) (Table 9.1): S. oralis (31 isolates), S. mitis biovar 1 (22), S. mitis biovar 2 (18), S. sanguis biovar 4 (2), S. vestibularis (2), S. sanguis biovar 1 or 3 (1), S. gordonii biovar 2 or 3 (2) and S. sanguis biovar 2 (1). Seventy-four isolates from the 50 member E-group comprised 49 S. oralis, 5 S. mitis biovar 1, 12 S. mitis biovar 2, 2 S. sanguis biovar 4, 2 S. vestibularis and 4 S. gordonii biovar 2 or 3. With the exception of S. sanguis biovar 1 or 3 and S. sanguis biovar 2 which were not recovered from the E-group, all the abovementioned species were isolated from both UE- and E-groups. Coykendall's classification was utilised to identify S. vestibularis strains not recognised by

Table 9.1 Number and identity of viridans streptococcus isolates speciated according to Kilian et al (1989) in different β-lactam exposure groups following selection in penicillin G only and penicillin G plus gentamicin

	Species according to antibiotic exposure			
	Unexposed, speciation only (n = 67)*	Unexposed, also susceptibility tested (n = 54)	Exposed to rheumatic fever prophylaxis (n = 41)	Exposed to ampicillin treatment (n = 9)
Streptococcus oralis	31	26	40	9
S. mitis biovar 1	22	15	5	0
S. mitis biovar 2	18	18	9	3
S. sanguis biovar 4	2	2	2	0
S. vestibularis (Coykendall)	2	2	2	0
S. sanguis biovar 1 or 3 †	1	1	0	0
S. gordonii biovar 2 or 3 †	2	1	4	0
S. sanguis biovar 2	1	1	0	0
Total	79	66	62 ‡	12 ‡

* n = number of subjects in each group

† Range of identification tests used did not allow for differentiation between biovars 1 and 3 of S. sanguis or biovars 2 and 3 of S. gordonii respectively

‡ The respective subgroups together form the E-group: 62 + 12 = 74 isolates

NOTE: This table is identical to Table 11.2(a)

Kilian et al (1989)

S. oralis was significantly more plentiful (49 out of 74; 66%) in the E-group than in the UE-group (31 of 79; 39%) (chi squared test, Yates modification; $p<0.001$) while S. mitis biovar 1 was more frequently found in the UE-group than the other group (Fisher exact test; $p<0.001$). S. mitis biovar 2 (12/74; 16%) was, like S. oralis, common in the ß-lactam-exposed group but more so in those not exposed to these antibiotics: 18 out of 79 (23%) (Table 9.1)

S. salivarius attaches preferentially to the tongue (Hardie, 1983). None was isolated from the plaque samples in this study. No other important oral streptococcal species such as S. mutans (Hall and Heimdahl, 1989; Franklin, 1992; Bayliss et al, 1983; Baddour, 1994) or S. milleri (Sussman et al, 1986) were recovered

9.3.2 Susceptibility patterns in the different ß-lactam antibiotic exposure groups

All 74 identified viridans isolates from E-group volunteers were tested for susceptibility to a panel of 6 antibiotics but 13 isolates from 13 UE-group subjects were not available for further investigation and only underwent speciation. The remainder (66 of 79) were tested (Table 9.1)

9.3.2.1 Susceptibility to candidate antibiotics within the UE-group and the E-groups as a whole

Susceptibility profiles of isolates from the two exposure groups are presented in Table 9.2 for comparative purposes. The MICs 50 denote concentrations at which 50% of strains, ranked according

to their MICs, are inhibited

Table 9.2 Minimum inhibitory concentrations (in mg/l antibiotic)

The following abbreviations are used in this table and elsewhere: pen V = penicillin V; amoxy = amoxycillin; ery = erythromycin; rox = roxithromycin; clinda = clindamycin; vanco = vancomycin

a) UE-group

	MIC range	MIC 50	MBC 50	MBC range
pen V	0.015–2	0.12	0.25	0.015–2
amoxy	≤0.007–2	0.03	0.03	≤0.007–2
ery	≤0.007–0.25	≤0.007	0.007	≤0.007–1
rox	≤0.007–4	0.015	0.03	≤0.007–4
clin	≤0.007–0.06	0.007	0.015	≤0.007–4
vanco	0.25 –1	1	1	0.25–2

b) E-group

	MIC range	MIC 50	MBC 50	MBC range
pen V	0.003–8	0.25	0.5	0.003–16
amoxy	0.007–16	0.25	0.25	0.007–32
ery	≤0.003–>64	0.015	0.03	≤0.007–>64
rox	≤0.007–>64	0.06	0.12	≤0.007–>64
clinda	≤0.007–>64	0.015	0.03	≤0.007–>64
vanco	0.25 –1	0.5	0.5	0.25–2

There are notable differences between the UE-group and E-group when the upper limits of both the MIC and MBC ranges for all antibiotics in the study are considered, except for vancomycin to which organisms were consistently susceptible. The differences between the two exposure groups were especially pronounced for the macrolides and clindamycin. Similar but less marked differences were observed when MIC 50 values in the two antibiotic exposure groups were compared. It is obvious from these observations that exposure to high doses of ampicillin and prolonged exposure to penicillin V may result in viridans bacteria that are not only more resistant to ß-lactam agents, but also to macrolides and to clindamycin

With regard to comparative susceptibilities between the various antimicrobial agents, erythromycin and clindamycin showed the greater activity with the lowest MICs in both the UE- and E-groups. Roxithromycin was only slightly less active

9.3.2.2 **Susceptibility within E-subgroups**

As indicated in Chapter 8, the E-group consisted of two subgroups: one (subgroup A) receiving high doses of therapeutic ampicillin (the 9 member, 12 isolate so-called "high-exposure" subgroup) and the other (RF-subgroup) who were on rheumatic fever prophylaxis (62 isolates from 41 individuals). Susceptibility ranges and MICs 50 and MBCs 50, as well as MICs 90 and MBCs 90, were calculated for these isolates:

Table 9.3 MIC and MBC profiles of 12 isolates of the A-(ampicillin) subgroup (in mg/l antibiotic)

	MIC range	MIC 50	MIC 90
pen V	0.12–4	1	4
amoxy	0.06–16	0.5	8
ery	≤0.007–>64	0.03	4
rox	0.015–>64	0.12	8
clinda	0.007–>64	0.03	4
vanco	0.5–1	0.5	1

	MBC range	MBC 50	MBC 90
pen V	0.12–4	2	4
amoxy	0.6–32	1	16
ery	≤0.007–>64	0.06	4
rox	0.015–>64	0.12	16
clinda	0.007–>64	0.03	>64
vanco	0.5–1	0.5	1

Table 9.4 MIC and MBC profiles of 62 isolates in RF- (rheumatic fever) subgroup (in mg/l antibiotic)

	MIC range	MIC 50	MIC 90
pen V	0.003–8	0.5	4
amoxy	0.007–8	0.25	4
ery	0.003–8	0.015	2
rox	0.007–>64	0.06	4
clinda	0.003–>64	0.015	0.06
vanco	0.25–1	0.5	1

	MBC range	MBC 50	MBC 90
pen V	0.003–16	0.5	4
amoxy	0.007–16	0.25	4
ery	0.003–16	0.015	4
rox	0.007–32	0.06	8
clinda	0.007–>64	0.03	0.06
vanco	0.25–2	0.5	1

Listed below in Table 9.5 are, for comparative purposes, susceptibility ranges of candidate antibiotics using MIC values and then MBC values, for the **E-group** as a whole, the **A-** and **RF-subgroups** as well as the UE-group

Table 9.5 Comparison of MIC and MBC ranges (in mg/l antibiotic) between isolates from the different antibiotic exposure groups

MIC ranges:

	E-group	A-subgroup	RF-subgroup	UE-group
pen V	0.003-8	0.12-4	0.003-8	0.015-2
amoxy	0.007-16	0.06-16	0.007-8	≤0.007-2
ery	≤0.003->64	≤0.007->64	0.003-8	≤0.007-0.25
rox	≤0.007->64	0.015->64	0.007->64	≤0.007-4
clinda	≤0.007->64	0.007->64	0.006->64	≤0.007-0.06
vanco	0.25-1	0.5-1	0.25-1	0.25-1

MBC ranges:

	E-group	A-subgroup	RF-subgroup	UE-group
pen V	0.003-16	0.12-4	0.003-16	0.003-2
amoxy	0.007-32	0.6-32	0.007-16	0.007-2
ery	0.003->64	≤0.007->64	0.003-16	≤0.007-1
rox	≤0.007->64	0.015->64	0.007-32	≤0.007-4
clinda	≤0.007->64	0.007->64	0.007->64	≤0.007-4
vanco	0.25-2	0.5-1	0.25-2	0.25-2

In order to determine the possible effects of previous ß-lactam antibiotic exposure, the following lists of MICs 50 and MBCs 50 for the **E-group**, **A-subgroup**, **RF-subgroup** and the UE-group are provided

Table 9.6 Comparison of MICs 50 and MBCs 50 between isolates from the different antibiotic exposure groups (in mg/l)

	E-group		A-subgroup		RF-subgroup		UE-group
	MIC 50	(R)*	MIC 50	(R)	MIC 50	(R)	
pen V	0.5	(4.2)	1	(8.3)	0.5	(4.2)	0.12
amoxy	0.25	(8.2)	0.5	(16.7)	0.25	(8.3)	0.03
ery	0.015	(≥2)	0.03	(≥4.3)	0.015	(≥2)	≤0.007
rox	0.06	(4)	0.12	(8)	0.06	(4)	0.015
clinda	0.015	(≥2)	0.03	(≥4.3)	0.015	(≥2)	≤0.007
vanco	0.5	(0.5)	0.5	(0.5)	0.5	(0.5)	1

	E-group		A-subgroup		RF-subgroup		UE-group
	MBC 50	(R)	MBC 50	(R)	MBC 50	(R)	
pen V	0.5	(2)	2	(8)	0.5	(2)	0.25
amoxy	0.25	(8.3)	1	(33.3)	0.25	(8.3)	0.03
ery	0.03	(≥4.2)	0.06	(≥8.6)	0.015	(≥2)	≤0.007
rox	0.12	(4)	0.12	(4)	0.06	(2)	0.03
clinda	0.03	(2)	0.03	(2)	0.03	(2)	0.015
vanco	0.5	(0.5)	0.5	(0.5)	0.5	(0.5)	1

* (R) is ratio between the respective exposure group and the UE-group

When the MBCs 50 and MBCs 50 of the two group E-subgroups are compared with those of the UE-group (where differences are expressed in ratios for convenience) it is clear that the MICs and MBCs of both the A-subgroup and the RF-subgroup are consistently higher than in the UE-group. This applies to all an-

timicrobial agents except for vancomycin. Furthermore, the MICs 50 and MBCs 50 were, for most agents, approximately twice to four times higher in the A-subgroup compared with the RF-subgroup. Noticably, there appears only to be marginal differences between erythromycin, roxithromycin and clindamycin activity. MIC values and ratios for these agents are either almost identical or differ only by a factor of approximately two

9.3.2.3 Antimicrobial susceptibility of individual species according to exposure group

Only S. oralis, S. mitis biovar 1, and S. mitis biovar 2 strains were present in sufficiently large numbers to permit the meaningful comparison of susceptibility data relating to E- and UE-groups (shown hereunder). The following lists indicate MIC and MBC data (in mg/l antibiotic) per streptococcal species in the form of ranges, MICs 50 and MBCs 50 as well as respective MIC 50 ratios and MBC 50 ratios of E-groups to UE-groups

Table 9.7 Comparison of MICs 50 and MBCs 50 of *S. oralis*

(a) Minimum inhibitory concentrations:

A) UE-group (26 isolates)

	MIC range	MIC 50	E-/UE-group
pen V	0.03–1	0.06	
amoxy	≤0.007–0.25	0.03	
ery	≤0.007–0.015	0.007	(see below)
rox	≤0.007–0.06	0.015	
clinda	≤0.007–0.06	0.007	
vanco	0.25–1	1	

B) E-group (49 isolates):

pen V	0.003–8	0.5	8.3
amoxy	0.007–16	0.25	8.3
ery	≤0.007–>64	0.015	2
rox	≤0.007–>64	0.06	4
clinda	≤0.007–>64	0.015	2
vanco	0.5–2	0.5	0.5

(b) Minimum bactericidal concentrations

A) UE-group (26):

	MBC range	MBC 50	E/UE-group MBC Ratios
pen V	0.03–1	0.12	
amoxy	≤0.007–0.5	0.03	
ery	≤0.007–0.06	≤0.007	(see below)
rox	≤0.007–0.06	0.015	
clinda	≤0.007–0.06	0.015	
vanco	0.25–1	1	

B) E-group (49):

pen V	0.003–16	1	8
amoxy	≤0.007–32	0.5	16.7
ery	≤0.007–>64	0.03	4.3
rox	≤0.007–>64	0.12	8
clinda	≤0.007–>64	0.03	2
vanco	0.5–2	1	1

Table 9.8 Comparison of MICs 50 and MBCs 50 of *S. mitis* biovar 1 (in mg/l antibiotic)

(a) Minimum inhibitory concentrations

A) UE-group (15):

	MIC range	MIC 50	E/UE MIC 50 ratios
pen V	0.03–2	0.12	
amoxy	≤0.007–2	0.03	
ery	≤0.007–0.25	≤0.007	(see below)
rox	≤0.007–2	0.03	
clinda	≤0.007–0.06	≤0.007	
vanco	0.25–1	0.5	

B) E-group (5):

pen V	0.5–1	1	8.3
amoxy	0.25–2	1	33.3
ery	0.007–2	0.03	>4.3
rox	0.03–4	0.12	4
clina	0.015–0.6	0.015	>2
vanco	0.5–1	0.5	1

(b) Minimum bactericidal concentrations

A) UE-group (15):

	MBC range	MBC 50	Ratios
pen V	0.06–2	0.25	
amoxy	0.015–2	0.03	
ery	≤0.007–0.25	≤0.007	(see below)
rox	≤0.007–2	0.03	
clinda	≤0.007–0.25	0.015	
vanco	0.25–2	0.5	

B) E-group (5):

pen V	1–2	1	4
amoxy	0.25–2	1	33.3
ery	0.007–4	0.03	4.3
rox	0.03–8	0.25	8.3
clinda	0.015–0.06	0.03	2
vanco	0.5–1	0.5	1

Table 9.9 Comparison of MICs 50 and MBCs 50 of S. mitis biovar 2

a) Minimum inhibitory concentrations

A) UE-group (18):

	MIC range	MIC 50	E/UE MIC 50 ratios
pen V	0.015–0.5	0.12	
amoxy	≤0.007–0.5	0.06	
ery	≤0.007–0.12	≤0.007	(see below)
rox	≤0.007–4	0.015	
clinda	≤0.007–0.06	≤0.007	
vanco	0.5–1	0.5	

B) E-group (12):

pen V	0.003–8	0.12	1
amoxy	0.03–4	0.12	2
ery	≤0.003–2	0.03	>4.3
rox	0.015–4	0.06	4
clinda	≤0.007–0.03	0.015	>2
vanco	0.25–1	0.5	1

b) Minimum bactericidal concentrations

A) UE-group (18):

	MBC range	MBC 50	Ratios
pen V	0.015–1	0.25	
amoxy	0.015–1	0.12	
ery	≤0.007–1	≤0.007	(see below)
rox	≤0.007–4	0.03	
clinda	≤0.007–4	0.015	
vanco	0.5–1	1	

B) E-group (12):

pen V	0.003–16	0.12	2
amoxy	0.03–4	0.25	2
ery	≤0.007–2	0.03	4.3
rox	0.015–8	0.06	2
clinda	≤0.007–0.06	0.015	1
vanco	0.25–1	0.5	2

Of all strains in this study, **S. oralis** was the only species to to exhibit MICs and MBCs in excess of 64 mg/l for any particular antibiotic. Isolate number C5 was especially resistant to erythromycin, roxithromycin and clindamycin: all MICs and MBCs were >64 mg/l

S. mitis biovar 1 displayed the greatest differences in susceptibility to the various antibiotics when E- and UE-groups were compared. This was particularly noticable in the case of the ß-

lactam agents where a 33.3x difference in MIC 50 values occurred with amoxycillin. Of the two agents, the S. mitis biovar 2 species showed the smallest difference in sensitivity to penicillin V between the two exposure groups. MIC 50 values of this antibiotic for S. mitis biovar 2 were the same at 0.12 mg/l in both E- and UE-groups. MIC ratios were $\geq$4 for all streptococcal species against roxithromycin and, with the exception of S. oralis (a ratio of 2), against erythromycin. All isolates remained susceptible to vancomycin

9.4 Discussion

Of all species in this study, S. oralis was significantly more common in the E-group (49 out of 74) compared with the UE-group (31 out of 79; p=0.001) and S. mitis biovar 1 in the UE-group (22 out of 79; p=0.001) than the E-group (5 of 74; p=0.0001). S. mitis biovar 2 was common in the UE-group (18 of 79 isolates) but somewhat less so in the E-group (12 of 74 isolates)

Except for vancomycin, MICs 50 and MBCs 50 were consistently higher in the E-group than the UE-group. Similarly, MICs/MBCs 50 MICs/MBCs 90 figures for all antibiotics, except vancomycin, were slightly (but consistently with one or two exceptions) higher in the A-subgroup than the RF-group. MIC or MBC indices were identical in the case of penicillin V (MICs 90 and MBCs 90) and clindamycin (MBC 50). Compared with the UE-group, the A-subgroup had 4.3 to 16.7-fold higher MICs 50 and a 2 to 33.3-times higher MBCs 50. MICs 50 and MBCs 50 were also somewhat higher in the RF-subgroup compared with the UE-group: both MICs 50 and MBCs were from 0.5 to 8.3-fold higher in the former subgroup

The reasons for the selection of resistance to antibiotics other than ß-lactams in dental plaque for which no record of prior ex-

posure to the non-ß-lactam antibiotics are available, are not clear. It is possible that some of the patients in the A- and RF-subgroups were previously exposed to non-ß-lactam antibiotics. It also possible that the **in vivo** and **in vitro** selection pressures of the model used in the present studies may have selected for genetic material coding for multiple drug resistance such as plasmids or transposons. Examples of transposons (Tn 917 and Tn 1545) carrying the erythromycin resistance gene erm AM in streptococci, including **S. sanguis**, which also code for clindamycin resistance, are well described in the literature and have been recently reviewed by LeClercq and Courvalin (1991)

In this study, strains with increased MICs and MBCs to the macrolides and clindamycin tended also to be intermediately or fully resistant to penicillin and amoxycillin

Penicillin susceptibility findings in this study approximate those in the literature. Isolates in the study of Neu (1974) were sensitive to 0.012 mg/l amoxycillin. The MICs of isolates against both penicillin V and amoxycillin ranged from 0.002-0.05 mg/l with MICs 50 $\leq$0.12 mg/l (Shanson et al, 1976) while Phillips et al, in the same year, found MICs of approximately 0.12 mg/l and MBCs $\geq$1.0 mg/l penicillin V. Against amoxycillin, MacGregor and Hart (1986) determined a MIC range of <0.003-0.25 mg/l for their viridans isolates and Hall and Heimdahl (1989) found isolates to be susceptible to 0.2 mg/l penicillin. Coulter et al (1990) tested isolates against both penicillin V and amoxycillin. MIC and MBC ranges for penicillin V were <0.01-2 mg/l and 0.01-2 mg/l, respectively, and for amoxycillin, <0.01-4 mg/l and 0.01-4 mg/l. Against penicillin G, oral streptococcal MIC ranges were 0.06-4 mg/l and MBC ranges 0.06-8 mg/l (Bourgault et al, 1990). The latter authors found that isolates of two species, **S. mitis** and **S. sanguis** II were killed only by levels >2-8 mg/l antibiotic

Other researchers identified strains of oral streptococci with far higher levels of resistance to ß-lactam agents. Isolates in the study of Southall et al (1983) showed MICs of 1-16 mg/l and MBCs of 2-16 mg/l to amoxycillin. Yet higher levels of resistance resistance to amoxycillin were identified in the study of Woodman et al (1985) who illustrated what effect prior exposure to an antibiotic may have on the development of resistance. These authors screened salivary oral flora for susceptibility to this agent (Group A: after a single dose or, Group B; after three doses of 3 g amoxycillin at weekly intervals) and found that all subjects in their study carried streptococcal strains resistant to 2 mg/l antibiotic (median count of 5700 cfu/ml saliva) prior to any receiving the agent, while 9 of 20 had isolates resistant to 40 mg/l (116 cfu/ml saliva). In those who received 3 g amoxycillin in multiple doses, the number of streptococci resistant to 2 mg/l and 40 mg/l of this antibiotic agent increased markedly after the second and third doses ($p<0.001$) and remained at elevated levels for 28 and 21 days respectively. But, to put their findings in perspective, the researchers calculated that resistance occurred in substantially less than 1% of the total count - still a substantial proportion of all isolates, however. Their results support the trend of increased antibiotic resistance which was identified in this study amongst isolates from the E-group (Tables 9.2-9.9)

As noted earlier in this chapter (Section 9.3.2.2), activity of the two macrolides and clindamycin were broadly similar against the UE-group isolates and against the E-group isolates

The significance of the susceptibility of the viridans streptococcal isolates of the present study to the macrolides erythromycin and roxithromycin and clindamycin, particularly in relation to their suitability as candidates for IE prophylaxis, is discussed in Chapter 10. This will permit the inclusion of con-

siderations of tolerance and the persister phenomenon

Chapter 10

GENOTYPIC TOLERANCE AND PERSISTERS IN VIRIDANS STREPTOCOCCI FOLLOWING SELECTION IN AN IN VITRO MODEL

10.1 Introduction

Examples of mechanisms of genotypic tolerance have been given in Chapter 8. Tolerance based upon MBC/MIC ratios, as is the practice in clinical laboratories, is likely to have a genetic basis provided the test media are nutritionally adequate and the inocula used in the first step of the experiments (MIC determination) are in the logarithmic growth phase. The term "genotypic tolerance" will be used to describe this type of tolerance in order to distinguish it from phenotypic tolerance which is used in this dissertation to denote susceptible survivors of selection in penicillin G and penicillin G plus gentamicin broth cultures

The genetic basis and mechanism of persisters are not fully understood but are probably similar to those operating in genotypic tolerance. Persisters represent a small proportion of the inoculum ($\leq$0.1%) that survive longer than the 24 h exposure period used in MIC determinations and are therefore probably examples of genotypic tolerance based upon a slow rate of killing of the strain concerned. In this chapter, the presence of genotypic tolerance and persisters (as defined above) amongst the survivors of plaque streptococci following **in vitro** selection in penicillin G containing broth will be recorded

10.2 Methods and definitions

10.2.1 Definition of genotypic tolerance

Definitions of genotypic tolerance to an antibiotic have been variously reported as ratios of the MBC to MIC greater than 10:1, 20:1 or 32:1 (see earlier). A ratio of 32:1 is now generally accepted as the preferred definition by clinical microbiologists. Tolerance ratios were determined in this study for all the antibiotics evaluated, including the macrolides and clindamycin, even though tolerance to the latter agents (which do not act directly on the bacterial cell wall and which are not generally regarded as primarily bactericidal) is not usually recorded in the literature

10.2.2 Persisters

A small proportion of bacterial cells still produce colony forming units at MBCs of some isolates. This was demonstrated when a percentage of the original inoculum in the broth wells grew as colonies on the blood agar subcultures. The MBC endpoint by definition was achieved when $\geq$99.9% of the bacteria in the original inoculum were killed. The remainder of viable bacteria ($\leq$0.1% of the original inoculum) produced 1 to 50 colonies from the 10 microlitre volumes of broth transferred to the blood agar plates. These surviving colonies were recorded as persisters. Concentrations at which persisters were identified are listed hereunder per **Streptococcus** strain and exposure group

Persister colonies, as described above, were recorded at concentrations greater than MBCs, including the highest concentrations at which they occurred

10.2.2.1 Refractoriness of persisters to killing

The figures obtained when the lowest concentrations of antibiotics at which persisters were completely eliminated are divided by the MBCs of the strains involved, relate to the relative refractoriness of the persisters to killing. The ratios obtained in this manner are expressed as persister refractory indices (PRIs)

10.2.3 Endurance indices

10.2.3.1 Endurance indices in relation to tolerant strains

The term endurance in relation to viridans streptococci has been proposed by Holloway et al (1980) to denote streptococci with penicillin G MICs less than the standard "breakpoints for resistance" but with MBCs appreciably greater than the MICs (see later). The term, as proposed, defined the penicillin G breakpoint required for a bactericidal action in IE cases as 0.8 units/ml (equivalent to 0.5 mg/l) and included strains with MBC/MIC ratios of <10. In this dissertation, the British (British Society for Antimicrobial Chemotherapy's: A guide to sensitivity testing, J Antimicrob Chemother 1991, **27** Suppl D:1-47) and NCCLS breakpoint of $\geq$0.25 mg/l for both penicillin V and amoxycillin was used to measure endurance, and the term endurance index (EI) is proposed for the purpose in this study. This index was determined by dividing the MBCs of the relevant strains by their MBC breakpoint of 0.25 mg/l in the case of both penicillin V and amoxycillin. Endurance to vancomycin using a breakpoint concentration of 8 mg/l did not occur and endurance indices for the macrolides and clindamycin (MBC breakpoints have not been proposed in the literature) were not calculated

10.2.3.2 Endurance indices in relation to persisters

When the minimal antimicrobial concentrations required to eliminate persisters are divided by the resistance breakpoints of the antibiotics concerned (according to NCCLS criteria but based upon MBCs), the figure obtained is expressed as a persister endurance index (PEI). This index relates to concentrations that are achievable in the blood and indices of >1 would suggest that such persister strains may be difficult to eradicate by the antibiotic concerned

10.3 Results

10.3.1 Genotypically tolerant strains and MBC:MIC ratios

The identity of isolates with MBC/MIC ratios of 4 or greater, their actual MICs and MBCs, adjusted ratios and the antibiotic agent against which the specific isolate was tested are shown in Table 10.1. Endurance indices in the case of cell wall-active antibiotics are also recorded. Table 10.2 summarises the genotypic tolerance data and MBC/MIC ratios in the UE- and E-groups

Amongst isolates from UE-group volunteers, all had MBC/MIC ratios of 1 or 2 (data not shown) except for nine isolates including an isolate (isolate H61 I) with a marginally elevated MBC/MIC ratio of 4 against vancomycin (but with the MBC still within achievable blood levels) (see Table 10.1). The majority of isolates in the E-group, like those in the UE-group, displayed no tolerance as defined above. Many strains strains had marginally elevated ratios of 4 and higher; some against more than one antibiotic (four isolates). Amongst **S. oralis**, **S. mitis** biovar 1 and **S.**

mitis biovar 2, four strains had ratios >10 (see Table 10.1). Two isolates in the E-group approached the 10:1 ratio against amoxycillin (8:1) and penicillin V (8.3:1). Another two isolates exhibited ratios of 10:1 against erythromycin (16.6:1) and clindamycin (16.6:1), while a further two exceeded the higher 32:1 requirement for tolerance with ratios of 66.6:1 and 33.3:1 - both against erythromycin. All four of the latter isolates were strains of **S. oralis** and belonged to the E-group. One of the isolates (C4) which was by definition potentially tolerant to clindamycin with a ratio >16, originated from the high-exposure ß-lactam A-subgroup

MBC/MIC ratios were clearly higher in the E-group compared with the UE-group, including classical tolerance to erythromycin as mentioned earlier (see Table 10.1 and Table 10.2)

Table 10.1 MBC/MIC ratios of ≥4 amongst streptococcal species

Isolate number	Species	MIC	MBC	Adjusted MIC/MBC ratio *	Endurance index (EI)	Anti-biotic
UE-group (9 out of 66 isolates, 13.6%)						
H1	S. oralis	0.03	0.12	4:1	**0.5**	pen V
H7 I	S. mitis 2	0.25	1	4	na **	rox
H12	S. mitis 2	≤0.007	0.03	≥4.3	na	ery
	S. mitis 2	0.03	0.25	8.3	na	clinda
H13	S. mitis 2	0.12	1	8.3	na	ery
H53 III	S. sanguis 1/3	≤0.007	0.03	≥4.3	na	clinda
H53 IV	S. oralis	≤0.007	0.06	≥8.6	na	ery
H55 II	S. mitis 2	0.03	0.12	4	na	ery
H61 I	S. oralis	0.25	1	4	**0.125**	vanco
E-group (20 out of 74 isolates, 27.0%)						
C1 #	S. oralis	0.015	0.06	4	na	clinda
C5	S. mitis 2	0.25	1	4	na	ery
C4 #	S. oralis	4	16	4	na	rox
	S. oralis	4	≥64	>16	na	clinda
C9	S. mitis 2	0.06	0.5	8.3	**2**	pen V
	S. mitis 2	<0.003	0.015	>5	na	ery
C10 I	S. oralis	4	32	8	na	rox
C10 II	S. mitis 2	4	16	4	na	ery
C19 I	S. oralis	0.03	0.12	4	na	rox
C19 II	S. oralis	0.015	0.06	4	na	clinda
C21 II	S. oralis	1	4	4	na	ery
C24 I	S. oralis	2	16	8	**64**	amoxy
	S. oralis	0.03	2	66.6	na	ery
C24 II	S. gordonii 2/3	4	16	4	na	rox
C31 I	S. oralis	0.015	0.25	16.6	na	ery

C32 #	S. oralis	0.03	0.12	4	na	clinda
C16 II	S. oralis	0.03	0.12	4	0.5	amoxy
	S. oralis	0.03	1	33.3	na	ery
C39	S. oralis	0.06	0.25	4.2	na	ery
	S. oralis	0.06	0.25	4.2	na	clinda

* = MIC:MBC ratio adjusted to unity (ie. x:1)

** = not applicable (na) to non-ß-lactam agents

\# = isolates which originated from the high exposure ampicillin A-subgroup of the E-group

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Table 10.2 Genotypic tolerance: comparative MBC/MIC ratios in the E- and UE-groups

	UE-group			E-group		
	MBC/MIC ratios			MBC/MIC ratios		
	≥4	≥10	≥32	≥4	≥10	≥32
Pen V	1	0	0	1	0	0
Amoxy	0	0	0	2	0	0
Ery	4	0	0	8	3	2
Rox	1	0	0	4	0	0
Clinda	2	0	0	5	1	0
Vanco	1	0	0	0	0	0
Total	9	0	0	20	4	2

10.3.2 Endurant strains

Only four isolates exhibited endurance with tolerance in the face of MICs below the proposed breakpoints to penicillin V and amoxycillin (Table 10.1). Of these four strains, two had endurance indices (EI) of >1 (C9, S. mitis 2 with EI = 2 and C24, S. oralis 2 with EI = 64). Both of these isolates had MBC:MIC ratios of approximately 8. (Endurance indices = strain MBC divided by MBC breakpoint, see Section 10.2.3.1)

10.3.3 Persisters

10.3.3.1 Persisters at and above MBC levels in different species

MBCs of strains exhibiting persisters on plates are listed in Table 10.3. When strains showing persisters at a given MBC were also refractory to concentrations higher than their MBCs, the highest concentrations at which persisters were still present are given and are underlined to distinguish them from persisters at isolate MBCs only. The numbers of strains showing persisters against the different antibiotics are given in parentheses in the right hand column

Table 10.3 Strains exhibiting persisters and their respective MBCs

a) UE-group

		Nos of strains with persisters
S. oralis (26 isolates tested):		
pen V	0.25	(1)
amoxy	1; 0.06; 0.03; 0.03; 0.03; 0.03; 0.015	(7)
ery	0.12; 0.03; 0.03; 0.03; 0.03; 0.015	(6)
rox	0.03; 0.12; 0.12; 0.03; 0.03; 0.06; 0.06	(7)
clinda	0.06; 0.06; 0.015; 0.03; 0.03; 0.015; 0.015	(7)
vanco		(0)
S. mitis biovar 1 (15):		
pen V		(0)
amoxy	0.5; 0.06	(2)
ery	2; 0.25; 0.12; 0.03; 0.03; 0.03; 0.015	(7)
rox	4; 0.5; 0.25; 0.12; 0.06; 0.03;	(6)
clinda	4; 0.25; 0.12; 0.015	(4)
vanco		(0)
S. mitis biovar 2 (18):		
pen V	4; 4	(2)
amoxy	4; 0.5; 1; 1	(4)
ery	8; 2; 1; 0.06; 0.03; 0.03; 0.015	(7)
rox	8; 8; 0.12; 0.06; 0.06; 0.06; 0.06; 0.03; 0.03; 0.03; 0.015	(11)
clinda	8; 0.06; 0.06; 0.06; 0.03; 0.03; 0.03 0.03; 0.03; 0.03; 0.015; 0.015; 0.015	(13)
vanco		(0)

S. sanguis biovar 4 (2):

pen V	1; 0.25	(2)
amoxy	0.5	(1)
ery		(0)
rox	0.06	(1)
clinda	0.06; 0.06	(2)
vanco		(0)

S. sanguis biovar 1 or 3 (1):

pen V	1	(1)
amoxy		(0)
ery		(0)
rox		(0)
clinda		(0)
vanco		(0)

S. vestibularis (2):

pen V		(0)
amoxy	0.015	(1)
ery	0.03	(1)
rox	0.06;	(1)
clinda	0.06; 0.03	(1)
vanco		(0)

NB: No persisters were recorded in one isolate each of S. sanguis biovar 2 and S. gordonii

b) E-group

		Nos of strains with persisters
S. oralis (49):		
pen V	16; 8; 16; 4 (C8 III); 8; 8; 8; 8; 4; 4; 2; 1; 0.12; 0.12; 0.12; 0.12;	(16)
amoxy	2; 2; 2; 1;	(4)
ery	4; 4; 8; 8; 2; 0.12; 0.12; 0.06; 0.06; 0.06; 0.06; 0.03; 0.03; 0.015; 0.015	(15)
rox	8; 8; 2; 0.5; 0.25; 0.25; 0.25; 0.12; 0.12; 0.06; 0.03	(11)
clinda	4; 2; 1; 0.5; 0.25; 0.25; 0.12; 0.12; 0.12; 0.12; 0.06; 0.06; 0.06	(13)
vanco		(0)
S. mitis biovar 1 (5):		
pen V		(0)
amoxy	0.5	(1)
ery	0.015	(1)
rox	0.06	(1)
clinda	0.06	(1)
vanco		(0)
S. mitis biovar 2 (12)		
pen V	8; 4; 0.5; 0.12	(4)
amoxy	1; 0.25; 0.25	(3)
ery	0.25; 0.12; 0.12; 0.06	(4)
rox	2; 0.5; 0.25 0.12; 0.12	(5)
clinda	1; 0.25; 0.25	(3)
vanco	4	(1)

S. sanguis biovar 4 (2):

pen V	2	(1)
amoxy		(0)
ery	0.06	(1)
rox	0.25	(1)
clinda	0.25	(1)
vanco		(0)

S. gordonii biovar 2 or 3 (4):

pen V	256; 16; 8	(3)
amoxy	16	(1)
ery	8; 0.12	(2)
rox	1	(1)
clinda	0.25	(1)
vanco		(0)

S. vestibularis (2):

pen V	2; 0.5	(2)
amoxy	2; 1	(2)
ery	0.06	(1)
rox	0.12	(1)
clinda		(0)
vanco	2 (isolate no. C49 I)	(1)

10.3.3.2 **Refractoriness of persisters to killing**

Refractoriness to killing amongst persisters and persister endurance indices are shown in Tables 10.4–10.9 (according to species and antibiotic) in both the UE- and E-groups

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Table 10.4 Persister refractory (PRI) and endurance indices (PEI) in streptococcal species according to principal ß-lactam exposure groups (the PEI is based upon resistance breakpoint, RBP, concentrations)

a) **UE-group**

Isolate	**Species**	Persister MBC/strain MBC ratio	Persister refractory index (PRI)	Persister endurance index (PEI) *
Penicillin V: persisters in 6 strains, RBP ≥0.25 mg/l				
H7 II	S sanguis 4	1:0.25	4	
H18	S. mitis 2	4:0.5	8	
H22	S. mitis 2	4:2	2	
H52 II	S. sanguis 4	0.25:0.12	2	
H64 II	S. oralis	0.25:0.06	4	
H53 III	S. sanguis 1/3	1:0.5	2	
Amoxycillin: persisters in 15 strains, RBP ≥0.25 mg/l				
H4	S. mitis 1	0.5:0.12	4	
H7 I	S. mitis 2	1:0.06	17	4
H13	S. mitis 2	1:0.25	4	
H19	S. mitis 2	1:0.5	2	
H20	S. oralis	2:0.03	67	8
H43	S. mitis 1	0.06:0.03	2	

H48	S. oralis	0.03;0.015	2
H52 II	S. sanguis 4	0.5:0.25	2
H53 II	S. oralis	0.03:0.015	2
H55 I	S. oralis	0.03:0.015	2
H55 II	S. mitis 2	4:0.5	8
H59	S. vestib.	0.015:0.007	2
H60	S. oralis	0.06:0.03	2
H63 II	S. oralis	0.015:≤0.007	>2
H64 II	S. oralis	0.03:0.007	4

Erythromycin: persisters in 21 strains, RBP ≥1 mg/l

H6	S. mitis 2	0.03:≤0.007	>4
H7 I	S. mitis 2	1:0.12	8
H12	S. mitis 1	0.25:0.003	8
H13	S. mitis 2	2:1	2
H20	S. mitis 1	4:≤0.007	>571 4
H21	S. mitis 1	0.03:≤0.007	>4
H26	S. mitis 1	0.03:≤0.007	>4
H40	S. mitis 1	0.03:≤0.007	>4
H43	S. mitis 1	0.015:≤0.007	>2
H44 I	S. oralis	0.12:0.015	8
H46	S. mitis 1	0.12:0.015	8
H49	S. oralis	0.03:≤0.007	>4
H50 I	S. mitis 2	0.06:≤0.007	>9
H51 I	S. mitis 2	0.03:≤0.007	>4
H52 I	S. mitis 2	0.015:≤0.007	>2
H55 II	S. mitis 2	8:0.12	7
H58 II	S. oralis	0.03:0.015	2
H59	S. vestib.	0.03:≤0.007	>4
H61 I	S. oralis	0.015:0.007	2
H61 II	S. oralis	0.03:≤0.007	>4
H64 II	S. oralis	0.03:0.015	2

Roxithromycin: persisters in 26 strains, RBP ≥2 mg/l

H1	S. mitis 1	1:0.015	67	0.5
H4	S. mitis 2	0.015:0.007	2	
H6	S. mitis 2	0.06:0.015	4	
H9	S. oralis	0.03:≤0.007	>4	
H12	S. mitis 1	0.03:0.015	2	
H13	S. mitis 2	8:4	2	
H16	S. mitis 2	0.03:0.015	2	
H20	S. mitis 1	8:0.06	133	4
H26	S. mitis 1	0.12:0.03	4	
H43	S. mitis 1	0.06:0.03	2	
H46	S. mitis 1	0.5:0.03	17	0.25
H48	S. oralis	0.12:0.03	4	
H50 I	S. mitis 2	0.06:0.03	2	
H50 II	S. mitis 2	0.03:0.015	2	
H51 I	S. mitis 2	0.06:≤0.007	>9	
H52 I	S. mitis 2	0.12:0.03	4	
H52 II	S. sanguis 4	0.06:0.015	4	
H53 I	S. mitis 2	0.03:0.015	2	
H53 IV	S. oralis	0.06:0.015	4	
H54 II	S. oralis	0.12:0.015	8	
H55 I	S. oralis	0.03:0.015	2	
H55 II	S. mitis 2	16:0.12	133	8
H56 I	S. vestib.	0.06:0.03	2	
H58 I	S. oralis	0.03:0.015	2	
H63 II	S. oralis	0.06:0.015	4	
H67 I	S. mitis 2	0.06:0.015	4	

Clindamycin: persisters in 28 strains, RBP ≥1 mg/l

H1	S. mitis 1	0.5:0.007	71	0.5
H6	S. mitis 2	0.03:≤0.007	>4	
H7 II	S. sanguis 4	0.6:0.015	4	
H9	S. oralis	0.015:≤0.007	>2	
H13	S. mitis 2	0.03:≤0.007	>2	

H15		S. mitis 2	0.015:≤0.007	>2	
H16		S. mitis 2	0.03:0.015	2	
H18		S. mitis 2	0.03:0.015	2	
H19		S. mitis 2	0.015:≤0.007	>2	
H20		S. mitis 1	8:0.03	266	8
H26		S. mitis 1	0.015:≤0.007	>2	
H40		S. mitis 2	0.03:0.015	2	
H44	II	S. mitis 2	0.015:≤0.007	>2	
H46		S. mitis 1	0.12:0.015	8	
H48		S. oralis	0.06:0.015	8	
H51	I	S. mitis 2	0.06:0.015	4	
H52	I	S. mitis 2	0.12:≤0.007	>17	0.12
H52	II	S. sanguis 4	0.12:≤0.007	>17	0.12
H53	IV	S. oralis	0.015:≤0.007	2	
H55	I	S. oralis	0.015:0.007	2	
H55	II	S. mitis 2	16:0.6	267	16
H56	I	S. vestib.	0.03:0.015	2	
H57	I	S. oralis	0.03:0.015	2	
H58	I	S. oralis	0.06:0.03	2	
H58	III	S. oralis	0.03:0.015	2	
H59		S. vestib.	0.06:0.015	2	
H64	I	S. mitis 2	0.03:0.015	2	
H67	I	S. mitis 2	0.06:0.03	2	

* PEIs are only recorded when respective PRIs are ≥10

b) **E-group**

Isolate	Species	Persister MBC/strain MBC ratio	Persister refractory index (PRI)	Persister endurance index (PEI) *
Penicillin V: persisters in 26 strains, RBP ≥0.25 mg/l				
C7	S. mitis 2	0.5:0.25	2	
C8 I	S. mitis 2	0.12:0.03	4	
C8 III	S. oralis	8:0.007	2667	32
C11 II	S. oralis	32:0.5	64	128
C12 II	S. mitis 2	16:0.03	533	64
C15 II	S. gord. 2/3	≥256:2	≥128	>1024
C17 I	S. oralis	0.12:0.3	4	
C18 I	S. gord. 2/3	16:0.12	133	64
C21 I	S. oralis	16:2	8	
C21 II	S. oralis	8:0.5	16	32
C23	S. oralis	8:4	2	
C24 II	S. gord. 2/3	32:0.5	64	128
C25 II	S. oralis	16:8	2	
C27 I	S. mitis 2	4:2	2	
C30	S. oralis	4:2	2	
C31 I	S. oralis	0.12:0.06	2	
C32	S. oralis	8:4	2	
C34	S. oralis	8:2	4	
C35	S. oralis	0.12:0.06	2	
C43	S. oralis	1:0.5	2	
C45	S. oralis	0.12:0.06	2	
C46 I	S. sanguis 4	2:1	2	
C47 I	S. oralis	4:2	2	
C49 I	S. vestib.	2:0.4	4	
C49 II	S. vestib.	0.5:0.25	2	
C50	S. oralis	2:1	2	

Amoxycillin: persisters in 11 strains, RBP ≥0.25 mg/l

C3	S. oralis	2:1	2	
C7	S. mitis 2	1:0.5	2	
C15 I	S. mitis 2	0.5:0.03	17	2
C18 I	S. gord. 2/3	≥16:0.12	≥133	64
C21 I	S. oralis	2:1	2	
C26	S. mitis 1	0.5:0.25	2	
C27 II	S. mitis 2	0.25:0.12	2	
C43	S. oralis	2:1	2	
C47 I	S. oralis	1:0.5	2	
C49 I	S. vestib.	2:0.5	4	
C49 II	S. vestib.	1:0.12	8	

Erythromycin: persisters in 24 strains, RBP ≥1 mg/l

C7	S. mitis 2	0.25:0.03	8	
C8 I	S. mitis 2	0.12:0.015	8	
C8 III	S. oralis	0.12:0.03	4	
C9	S. mitis 2	0.06:0.015	4	
C10 I	S. oralis	8:4	2	
C11 II	S. sanguis 4	0.12:0.003	40	0.12
C12 III	S. oralis	0.06:0.03	2	
C13 I	S. mitis 1	0.015:0.007	2	
C16 II	S. oralis	4:≤0.007	≥571	4
C17 I	S. oralis	0.12:0.03	4	
C18 I	S. gord. 2/3	0.25:0.007	36	0.25
C19 I	S. oralis	0.03:0.015	2	
C19 II	S. oralis	8:0.03	267	8
C20	S. mitis 2	0.12:0.03	4	
C21 I	S. oralis	8:4	2	
C24 II	S. gord. 2/3	8:4	2	
C32	S. oralis	≥8:2	≥4	
C29 I	S. oralis	0.06:≤0.007	≥9	
C41	S. oralis	0.015:≤0.007	≥2	
C44	S. oralis	0.03:≤0.007	4	

C47 I	S. oralis	0.015:≤0.007	2	
C48 II	S. oralis	0.06:≤0.007	≥9	
C49 I	S. vestib.	0.12:0.007	17	0.12
C50	S. oralis	0.06:≤0.007	≥9	

Roxithromycin: persisters in 20 strains, RBP ≥2 mg/l

C2	S. mitis 2	2:0.03	67	1
C7	S. mitis 2	0.5:0.06	8	
C8 I	S. mitis 2	0.12:0.015	8	
C8 III	S. oralis	0.25:0.06	4	
C9	S. mitis 2	0.12:0.015	8	
C11 I	S. sanguis 4	0.25:0.03	8	
C13 I	S. mitis 1	0.06:0.03	2	
C16 II	S. oralis	4:0.015	266	2
C17 II	S. oralis	0.5:0.06	8	
C18 I	S. gord. 2/3	1:0.12	8	
C19 II	S. oralis	≥8:0.12	≥67	4
C20	S. mitis 2	0.25:0.06	4	
C21 I	S. oralis	8:4	2	
C29 I	S. oralis	0.12:0.015	8	
C31 II	S. oralis	0.25:0.12	2	
C38	S. oralis	0.03:0.015	2	
C44	S. oralis	0.12:0.015	8	
C47 I	S. oralis	0.06:0.03	2	
C49 II	S. vestib.	0.12:0.03	4	
C50	S. oralis	0.25:0.03	8	

Clindamycin: persisters in 19 strains, RBP ≥1 mg/l

C6	S. oralis	2:0.03	67	2
C7	S. mitis 2	2:0.03	67	2
C8 I	S. mitis 2	0.25:0.03	8	
C8 II	S. oralis	0.25:0.06	4	
C8 III	S. oralis	1:0.03	33	1
C9	S. mitis 2	0.5:0.015	33	0.5

C11	I	S. sanguis 4	0.5:0.03	17	0.5
C11	II	S. oralis	0.25:0.06	4	
C14		S. mitis 1	0.06:0.015	4	
C16	II	S. oralis	4:≤0.007	≥571	4
C17	II	S. oralis	0.12:0.03	4	
C18	I	S. gord. 2/3	0.25:0.06	4	
C18	II	S. oralis	0.12:0.06	2	
C19	II	S. oralis	8:0.06	133	8
C23		S. oralis	0.12:0.03	4	
C29	I	S. oralis	0.06:0.007	9	
C41		S. oralis	0.06:0.03	2	
C44		S. oralis	0.06:0.03	2	
C50		S. oralis	0.12:0.015	8	
Vancomycin: persisters in 2 strains, RBP ≥8 mg/l					
C7		S. mitis 2	4:0.5	8	
C49	I	S. vestib.	2:1	2	

* PEIs are only recorded when PRIs are ≥10

[Reminder: PRI and PEI ratios:-
PRI = lowest concentration at which persisters are eliminated divided by strain MBC (a measure of refractoriness, see Section 10.2.2.1)
PEI = persister MBC divided by NCCLS resistance breakpoints (relates to achievable antibiotic blood levels, see Section 10.2.3.2)]

Refractoriness was common amongst persisters and the majority fell in the range of PRI values of 2-9 in both the UE-group and E-group (see above). In some strains, PRIs were found to be very high. Levels in the region of 500-times the MBCs of 4 strains

were required to eliminate persisters completely within the prescribed period of 24 h (one of which originated from isolate H20, the only one in the UE-group) and in one organism (isolate C8 III), a concentration of more than 2500 times its MBC was required. In this particular strain, and most others, the original MBCs were low and the PEIs were therefore also considerably lower. This trend applied to all persisters in the UE-group where values of corresponding PEIs (which ranged from 0.12-16) dropped substantially from their PRI values of 17-267 (Table 10.3a). However, this did not apply to all persisters in the E-group (Table 10.3b). Examples of high PRIs and PEIs are strains C8 III and C15 II which had PRIs and PEI values of 2337 (PRI) and 32 (PEI), and $\geq$1·28 (PRI) and >1024 (PEI), respectively, against penicillin V (see Table 10.4 above)

Of the total number of times strains exhibiting persisters to one or more of the six antibiotics tested (198), refractoriness was observed 36 times (18.2%; see Table 10.7 and Table 10.8). The frequency of refractoriness was 24 out of 103; 23.5% in the E-group and 12 out of 96; 12.5% in the UE-group (Table 10.8 and Table 10.9)

Table 10.5 Prevalence of persisters and refractory persisters (with PRIs ≥4 and PRIs ≥10) by species in the UE- and E-groups

	UE-group		E-group	
	No.	%	No.	%
S. oralis	26		49	
Potential maximum no. persisters	156		294	
Persisters	28	17.9%	59	20%
Refractory persisters				
a) PRI ≥4	12	7.7% (42.8%)	31	10.5% (52.5%)
b) PRI ≥10	1	0.6% (3.6%)	11	3.7% (18.6%)
S. mitis 1	15		5	
Potential maximum no. persisters	90		30	
Persisters	19	21.1%	4	13.3%
Refractory persisters				
a) PRI ≥4	14	15.5% (73.7%)	1	3.3% (25%)
b) PRI ≥10	6	6.7% (31.6%)	1	3.3% (25%)

	UE-group			E-group		
	No.	%		No.	%	
S. mitis 2	18			12		
Potential maximum no. persisters	108			72		
Persisters	37	34.3%		20	27.8%	
Refractory persisters						
a) PRI ≥4	17	15.7%	(45.9%)	16	22.2%	(80%)
b) PRI ≥10	4	3.7%	(10.8%)	4	5.6%	(20%)
S. sanguis 4	2			2		
Potential maximum no. persisters	12			12		
Persisters	6	50%		4	33.3%	
Refractory persisters						
a) PRI ≥4	4	33.3%	(66.7%)	3	25%	(75%)
b) PRI ≥10	1	8.3%	(16.7%)	2	16.7%	(50%)
S. vestibularis	2			2		
Potential maximum no. persisters	12			12		
Persisters	5	41.7%		7	58.3%	
Refractory persisters						
a) PRI ≥4	1	8.3%	(20%)	4	33.3%	(57.1%)
b) PRI ≥10	0	0	0	1	8.3%	(14.3%)

	UE-group		E-group		
	No.	%	No.	%	
S. sanguis 1/3	1		0		
Potential maximum no. persisters	6		0		
Persisters	1	16.7%	0	0%	
Refractory persisters					
a) PRI ≥4	0		0		
b) PRI ≥10	0		0		
S. gordonii 2/3	1		4		
Potential maximum no. persisters	6		24		
Persisters	0		8	33.3%	
Refractory persisters					
a) PRI ≥4	0		6	25%	(75%)
b) PRI ≥10	0		5	20.8%	(62.8%)

S. mitis biovar 2 had the highest number of persisters in both UE- and E-groups (Table 10.5). With UE- and E-groups combined, the highest proportion of persisters were refractory amongst *S. mitis* biovar 1 strains (15 out of 23; 65%). *S. mitis* biovar 2 and *S. oralis* followed with 59% (33 of 56) and 49% (43 of 87) respectively

Table 10.6 Refractoriness of persisters with PRIs >10 exhibited against different antibiotics

Species	Pen V		Amoxy		Ery		Rox		Clinda		Total PRIs >10		Total	
	UE	E	UE	E	UE	E	UE	E	UE	E	UE	E	pers's	(%*)
So #	0	3	1	0	0	2	0	2	0	4	1	11	87	(14)
Sm 1	0	0	0	1	1	0	3	0	2	0	6	1	23	(30)
Sm 2	0	1	1	0	0	0	1	1	2	2	4	4	57	(14)
Ss 4	0	0	0	0	0	1	0	0	1	1	1	2	10	(30)
Sv	0	0	0	0	0	1	0	0	0	0	0	1	12	(8.3)
Ss 1/3	1	0	0	0	0	0	0	0	0	0	1	0	1	(0)
Sg 2/3	0	3	0	1	0	1	0	0	0	0	0	5	8	(63)
	0	7	2	2	1	5	4	3	5	7	12	24	198	

* Total no. of persisters (pers's) with PRIs >10 as % of no. of pers's per species (eg. for So: [1 + 11] divided by 87 = 14%)

\# So = S. oralis, Sm 1 = S. mitis biovar 1, Sm 2 = S. mitis biovar 2, Ss 4 = S. sanguis biovar 4, Sv = S. vestibularis, Ss 1/3 = S. sanguis biovar 1 or 3 and Sg 2/3 = S. gordonii biovar 2 or 3

Although numbers were small, a high proportion of S. gordonii strains (5 of 8; 63%) showed refractory persisters while figures for the more common species varied from 14-30%. The only persisters to show a persister endurance index (PEI) greater than the PRI value (Table 10.4) were two S. gordonii biovar 2 or 3 strains (isolates C 15 II and C 24 II) and a single S oralis isolate (C 11 II). All three showed higher PEIs than PRIs to penicillin V and could be more difficult to control (kill) with usual blood concentrations of antibiotic (see explanation of terms after Table 10.4)

High index refractoriness was demonstrated 24 times against the six antibiotics tested in the E-group - double the number from the UE-group (Tables 10.6, 10.7 and 10.8). The high frequency of PRIs $\geq$10 in the E group was particularly pronounced in S. oralis and S. gordonii while a reverse trend was seen in S. mitis I strains (Table 10.9). Of agents to which Streptococcus species showed this phenomenon in the former group, fewest were refractory to amoxycillin (2) and roxithromycin (3) while five were refracory to erythromycin and seven each to clindamycin and penicillin V. The latter antibiotic also elicited the highest refractoriness (PRI of 2667) amongst the oral streptococci tested and was of a persister in S. oralis in the E-group. After clindamycin (observed 5 times), roxithromycin had the second highest refractoriness develop against it in the UE-group (4 times) (Table 10.6 and Table 10.7). Amoxycillin, the second lowest after vancomycin, had two refractory persisters (PRIs >10) and erythromycin only one in contrast to the E-group, where erythromycin was second highest (5 times) only to penicillin V (7) and clindamycin (7). As noted elsewhere above, certain persisters of isolates exhibited refractoriness to more than one agent

Sixty-six strains have the potential of producing the persister phenomenon in a total of 396 instances against the six antibiotics in the UE-group. In this group, persisters were found in 12 isolate-antibiotic interactions giving a percentage value of 3.0. The corresponding figures in the E-group were: 74 strains yielding 24 out of a potential 444 positive tests to give a percentage of 5.4 (Table 10.8)

Table 10.7 Persisters and persister refractory indices (PRIs) related to antibiotics

	UE-group (66 strains)		**E-group** (74 strains)	
	Persisters	PRI ≥10 (%)	Persisters	PRI ≥10 (%)
pen V	6 (9%)	0 (0%)	26 (35%)	7 (9%)
amoxy	15 (23%)	2 (3%)	11 (15%)	2 (3%)
ery	21 (32%)	1 (2%)	25 (34%)	5 (7%)
rox	26 (39%)	4 (6%)	20 (27%)	3 (4%)
clinda	28 (42%)	5 (8%)	19 (26%)	7 (9%)
vanco	0 (0%)	0 (0%)	1 (1%)	0 (0%)

Table 10.8 Prevalence of persisters and refractory persisters (with PRIs ≥10) in UE- and E-groups against 6 antibiotics

	UE-group		E-group	
	No.	(%)	No.	(%)
Strains	66		74	
Potential maximum number of persisters	396		444	
Persisters	96	(24.2)	102	(22.9)
Refractory persisters (PRIs >10)	12	(3.0)* (12.5)#	24	(5.4)* (23.5)#

* Percentage of the potential maximum number of persisters in 66 and 74 strains in the UE- and E-groups respectively which show refractory persisters

\# Percentage of persisters to show refractoriness

Table 10.9 Contribution of different *Streptococcus* species to produce persisters

	UE-group		E-group	
Species	No.	% *	No.	% *
S. oralis	28	7.1	59	13.3
S. mitis 1	19	4.8	4	0.9
S. mitis 2	37	9.3	20	4.5
S. sanguis 4	6	1.5	4	0.9
S. vestibularis	5	1.3	7	1.6
S. sanguis 1/3	1	0.2	0	0
S. gordonii 2/3	0	0	8	1.8
S. sanguis 2	0	0	0	0
Totals	96	24.2%	102	23.0%
Potential maximum no. of persisters	396		444	

* Percentage of antibiotic-isolate interactions that produced persisters out of the potential maximum number of persisters

S. oralis produced the greatest proportion of persisters in the E-group (59 of 444; 13.3%) compared with 7% in the UE-group. The two *S. mitis* biovars produced the greatest proportion in the UE-group with 4.8% and 9.1%, respectively

10.4 Discussion

10.4.1 Genotypic tolerance and persisters in UE- and E-groups

Genotypic tolerance, according to one of the less stringent definitions (MBC/MIC ratios >10), was not identified amongst isolates from the UE-group. This applied equally to all isolates in the E-group, except for four S. oralis strains whose tolerance ratios ranged from >16-66.6 (Table 10.1)

S. oralis was also the commonest species amongst which persisters developed (87 out of 226; 38%) (Tables 10.4-6 and 10.9) and in which persisters were refractory (43 out of 87; 49.4%) (Table 10.5). However for this species, the highly refractory (PRI >10) proportion of all persisters (12 of 87; 14%) was smaller than those of S. mitis biovars 1 and 2 (both 30%), as well and S. gordonii biovar 2 or 3 (63%) (Table 10.4 and Table 10.6)

The occasions when viridans streptococci in the UE-group produced persisters to the six antibiotics tested were very similar in frequency (96 out of 396; 24.2%) to those in the E-group (102 of 444; 22.9%). However, refractoriness with PRI >10 in persisters in the UE-group (12 of 96; 12.5%) was statistically lower than in the E-group (24 of 102; 23.5%) ($p<0.05$, chi-squared test with Yates modification). These findings suggest that prior exposure to ß-lactam antibiotics may select for refractoriness in persisters with PRIs >10. Why previous administration of ß-lactam antibiotic should select for persister refractoriness against non-ß-lactam antibiotics is, however, not clear. Not surprisingly, PEIs show very similar trends to those of PRIs >10

As noted, there are isolates in the study with a high degree of tolerance to antibiotics. The ability of antibiotic agents to alone routinely contain, control and cure IE in a patient without

the participation of the host's own intact defence system, would appear unlikely. But this particular property to kill target bacteria is still regarded as being essential (Glauser et al, 1983; Coulter et al, 1990) and critical for therapeutic success (Powley et al, 1989). However, whether the need to kill persisters is required in the clinical situation has not been addressed in the literature and few routine diagnostic laboratories report on the presence of persisters, even in patients with IE

Some isolates which display tolerance appear to be what Holloway et al (1980) termed "endurant" (ie. with MICs in the susceptible and not resistant ranges). In this dissertation, the term endurance index (EI) was coined to describe endurant strains with MBCs of penicillin V and amoxycillin $\geq$2.5 mg/l. Strains with EIs appreciably greater than 1 would theoretically be more difficult to eradicate during therapy than non-tolerant strains or endurant strains with MBCs below the MBC breakpoint of 2.5 mg/l. Only two isolates had EIs to penicillin V or amoxycillin of >1 (Table 10.1). Both these isolates were in the E-group and had EIs of 2 (S. mitis 2) and 64 (S. oralis) while one of the two isolates with EIs of 0.5 was in the UE-group and the other in the E-group (both were S. oralis strains). The clinical relevance of EIs is, at present, not known

Endurance amongst persisters with PEIs of >1 in strains with PRIs of >10 (Table 10.4) occurred 7 out of a potential maximum of 396 antibiotic-strain interactions (from 12 strains) in the UE-group (1.8%) as opposed to 16 out of 444 antibiotic-strain interactions (3.6%) from 21 strains in the E-group. The PEIs in the E-group were considerably higher with a range of 0.12->1024 and a mean of 74.9, compared with a range of 0.12-16 and a mean of 4.5 in the UE-group

10.4.2 ß-Lactam candidates for IE prophylaxis based upon susceptibility, tolerance and the frequency of persisters

With regard to the choice of agents for IE prohylaxis following dental procedures, it appears that when the presence of persisters is considered as a criterion on its own, penicillin V would be more appropriate than amoxycillin in individuals not exposed to antibiotics during the previous three months, while amoxycillin would be the preferred agent in individuals exposed to ß-lactam agents (Table 10.7). No clear trend emerged, however, when genotypic tolerance was considered as a criterion for chosing between penicillin V and amoxycillin as candidates for prophylaxis (Table 10.1)

Genotypic tolerance was uncommmon in the present study and was less frequently found than in some other studies (Harder et al, 1974; Pulliam et al, 1979 and Holloway et al, 1980). Many examples of tolerance in strains causing endocarditis involve S. mutans isolates (Harder et al, 1974), or nutritionally defective viridans streptococcal isolates which do not feature in the present study. It should also be noted that in some of these studies (Roberts et al, 1979) a definition of tolerance involved a 10:1 MBC:MIC ratio rather than a 32:1 ratio (Pulliam et al, 1979; Holloway, Dankert and Hess, 1980). Tolerant strains have also been isolated from the blood of patients following dental procedures involving the gingival sulcus (Pulliam et al, 1979)

It is noteworthy that there was poor correlation between strains which were phenotypically tolerant during the selection process and those that were subsequently found to be genotypically tolerant (data not shown)

10.4.3 Activity of candidate non-ß-lactam agents of IE prophylaxis based on genotypic tolerance and the presence of persisters

It is interesting to examine the comparative activities of the various agents using MIC 50 and MBC 50 data independently (ß-lactam and vancomycin data are included here for comparative purposes). The ranking order of MIC 50 values of UE-group volunteers from most to least active using dosage size (mg/l) had clindamycin and erythromycin as the most active candidates, followed by roxithromycin, amoxycillin, penicillin V and vancomycin. For E-group isolates, the most active were erythromycin, clindamycin, roxithromycin, penicillin V together with amoxycillin, and lastly vancomycin. However, the picture altered slightly using UE-group MBC 50 values: here erythromycin headed the list followed by clindamycin, roxithromycin paired with amoxycillin, then penicillin V and finally vancomycin. Clindamycin and erythromycin tie as the apparently most effective according to MBC 50s for the E-group, then follow roxithromycin, amoxycillin and penicillin V and vancomycin to take up the lowest rankings yet again

In Table 10.2, numbers of strains with MBC/MIC ratios $\geq$4, $\geq$10 or $\geq$32 (degrees of genotypic tolerance) are presented for each candidate agent. The UE-group produced the smallest number and all were of the lowest magnitude (MBC/MIC ratio $\geq$4): most were genotypically tolerant to erythromycin (4 strains), none to amoxycillin and only one each to roxithromycin, penicillin V and vancomycin. Genotypic tolerance amongst E-group strains was markedly more pronounced and occurred at all three possible MBC/MIC ratios. Again, genotypic tolerance against erythromycin was most common (8 of 26 strains, 30.7%) but none was detected in the study against vancomycin. Of the macrolide and clindamycin trio, genetic tolerance was least common against roxithromycin (4

of a 26 strain total, 15.4%) and all were at the lower MBC/MIC genetic tolerance ratio

As noted previously, all strains were exposed to each antibiotic agent and the appearance of persisters was noted and their MBCs determined (Tables 10.3 and 10.4). The non-ß-lactam agent to produce the greatest number of persisters amongst UE-group strains was clindamycin (a total of 28 of all strains and 37% of 75 persisters) and the least erythromycin (21, ie. 28%). (No persisters developed against vancomycin). Ranks were almost reversed in the E-group. Clindamycin had the least (19, 29%) and erythromycin the most (24, 37%) persisters. (It is noteworthy that vancomycin had 2 persisters, or 3%, develop against it from E-group strains)

In all four of the initial rankings above, the macrolides and clindamycin nominally appear to give the best results. On the other hand, with regards to persisters, the two ß-lactam agents and vancomycin would appear to be superior (the exception being penicillin V with 26 persisters amongst E-group strains)

Although there appeared to be a variation between the ability of erythromycin, roxithromycin and clindamycin to produce persisters when the exposure groups were dealt with separately (a total of 75 occurrences in the UE-group and 65 in the E-group), there was little difference between these agents when data from both exposure groups was combined. The frequency of persister development for erythromycin was 32.1% (45 of 140), roxithromycin 32.8% (46 of 140) and clindamycin 34% (47 of 140)

10.4.3.1 Relative candidatures of roxithromycin and vancomycin as agents of IE prophylaxis

As noted elsewhere, roxithromycin has superior pharmacokinetics, comparable antibacterial activity to erythromycin, the smallest number of strains to show genetic tolerance and a similar general overall propensity to erythromycin and clindamycin to produce persisters. It appears to be marginally better than erythromycin in the latter regard in those with prior ß-lactam exposure. It deserves further attention as a candidate for routine IE prophylaxis

When all available criteria are assessed, vancomycin emerges as a very strong (the best) IE prophylaxis candidate because of the relatively few persisters which develop and the markedly rare appearance of tolerance to this agent. Although the MBCs of vancomycin are higher than is the case with other antibiotics, blood levels are easily obtainable

More information is required on vancomycin concentrations obtainable within the oral and plaque environments, and whether "free" salivary vancomycin would increase the development of resistance amongst oral streptococci - as it appeared to do in the case of unencapsulated penicillin V (dosage form) in a study of Heimdahl and Nord (1979) - is open to speculation. The use of this agent simply for prophylaxis purposes is not justified in the light of its possible toxic side-effects and its status as a "reserve" antibiotic in chemotherapy

Chapter 11

EFFECT OF PENICILLIN G SELECTION ON ORAL STREPTOCOCCI FROM ANTIBIOTIC EXPOSED AND NON-EXPOSED INDIVIDUALS IN AN *IN VITRO* MODEL

11.1 Introduction

As discussed in earlier chapters, viridans streptococci are indigenous to the oral cavity and important aetiological agents of infective endocarditis (IE) (Roberts et al, 1979; AHA Report, 1981; Cotran et al, 1989; Hall and Heimdahl, 1989). It was also indicated in Chapter 5 that oral streptococci are generally regarded as being susceptible to penicillin (Hall and Heimdahl, 1989), but resistant strains have been isolated from both the oral cavity (Longman et al, 1991) and blood (Hess et al, 1983) of IE-susceptible patients

Antibiotic use is known to select for bacterial resistance in oral streptococci (Southall et al, 1983; Harrison, Stross et al, 1985; Herbert et al, 1988; Smith et al, 1989; Maskell et al, 1990). Antibiotic-resistant strains emerge readily in individuals with either recent or frequent exposure (Southall et al, 1983; Harrison, Rubin et al, 1985; Woodman et al, 1985). As discussed in Chapter 5, resistance may be detected in populations of oral bacteria shortly after administration of antibiotics to patients previously unexposed to such compounds (Leviner et al, 1987). Acquisition of resistant strains may, however, occur without direct antibiotic exposure especially in hospital settings (Leviner et al, 1984), and persons without a history of exposure to antibiotic are known to carry small numbers fo antibiotic-resistant

bacteria as part of their normal oral flora (Longman et al, 1991; Woodman et al, 1985). It has also been shown that bacteraemia with penicillin-resistant oral streptococci - and IE - may follow amoxycillin prophylaxis in a rabbit model (Longman et al, 1992)

Recently Hall et al (1993) demonstrated that penicillin V- and amoxycillin-sensitive viridans streptococci and anaerobic bacteria survive in the blood circulation following prophylaxis with those antibiotics. The present study was designed to determine the identity and penicillin V and amoxycillin susceptibility, as well as susceptibility to other candidate antibiotics, of viridans streptococci from dental plaque which survive the presence of penicillin at levels which would exert ß-lactam activity equivalent to those likely to be obtained in blood during IE-prophylaxis. As described in Chapter 8, the source of streptococci in this **in vitro** model was dental plaque from individuals who had had or without prior antibiotic exposure. Penicillin G was used for the **in vitro** selection process not only as a compromise candidate to simulate the effect of penicillin V and amoxycillin prophylaxis, but also as this antibiotic is most often the single agent of choice in IE therapy. In an extention of the study, gentamicin which is known to act synergistically with ß-lactam agents, was used to determine whether this combination will modify the effect of penicillin G alone on the survival of oral streptococci

11.2 Design of an <u>in vitro</u> model of IE prophylaxis with ß-lactam antibiotics

The **in vitro** model described in this dissertation was designed to mimic IE prophylaxis using ß-lactam antibiotics and the selection pressure prophylaxis may exert on oral streptococci. Because of cost considerations, penicillin G was used as a surrogate ß-

lactam agent to obviate a study using both amoxycillin and penicillin V (see last paragraph above)

This model was described in full in Chapter 8 but, briefly, it entails the sampling of plaque material from the teeth of two groups of individuals. One group represented persons recently exposed to ß-lactam antibiotics and the second group comprised individuals who had not received such agents during the preceeding three months. Plaque specimens were inoculated into broth media containing 0.1 and 1.0 mg/l penicillin G, as well as into broth containing 0.125 mg/l penicillin G plus 5 mg/l gentamicin. After overnight incubation, these selection broth cultures were subcultured onto blood agar plates for single colonies. Representative colonies on the plates were selected for susceptibility evaluation against penicillin V and amoxycillin and the four other antibiotics covered in Chapter 9. Susceptibility assessment included MIC and MBC determinations and the demonstration of tolerant strains and strains yielding persisters

11.3 Results

11.3.1 Patterns observed following different penicillin G selection procedures

The distribution of viridans streptococci isolated from dental plaque of individuals who were either receiving ß-lactam antibiotics (E-group) or who were not recently exposed to these agents (UE-group), and their penicillin V and amoxycillin MICs and MBCs following **in vitro** selection by penicillin G or penicillin G plus gentamicin, are shown in Table 11.1. It is clear that **in vitro** exposure to the two concentrations of penicillin not only permitted the survival of strains with penicillin V and amoxycillin MBCs greater than the equivalent concentrations of

penicillin G in broth as expected (phenotypic tolerance) (66 of 144; 47% and 88 of 140; 63% respectively), but also failed to destroy plaque bacteria that were refractory to killing at penicillin concentrations appreciably higher than their MICs and MBCs (genetically-based resistance)

11.3.2 Effect of gentamicin in penicillin G selection broth

Survival of strains of viridans streptococci with penicillin V and amoxycillin MICs and MBCs both higher and lower than the equivalent selection concentration of penicillin G also occurred in the presence of gentamicin (Table 11.1)

The addition of gentamicin to 0.125 mg/l penicillin G did not appear to affect, to any great extent, the selection of isolates with MICs greater than the selection concentration of penicillin G in the case of penicillin V, ie. the selection of strains resistant to this antibiotic with MICs $\geq$0.125 mg/l (12 out of 19; 63% for selection in penicillin G only, as opposed to 29 of 54; 54% with p=0.66, chi-squared test with Yates modification). However, in the case of amoxycillin, the addition of gentamicin appeared to have made a significant difference in the survival of resistant streptococci (MICs $\geq$0.25 mg/l) (11 of 19; 58% vs. 10 of 54; 19%, p=0.003; chi-squared test, Yates modification)

The corresponding figures with regard to MBCs are 14 of 19 (74%) and 36 of 54 (67%) for penicillin V at the penicillin G-only 0.125 mg/l selection concentration, and at the same selection concentration plus gentamicin, respectively. The difference is not statistically significant. With regard to amoxycillin, however, the proportions of resistant to susceptibile (phenotypically tolerant) isolates according to MBCs at the 0.125 mg/l penicillin G-only selection concentrations are 12 of 19

(63%) vs 10 of 54 (19%) ($p<0.001$; chi-squared test, Yates modification)

An interesting observation is that the effect of penicillin G at the concentration 0.125 mg/l plus gentamicin compared with that of penicillin G only at 1.0 mg/l on amoxycillin MBC patterns was very similar in both the E-group and UE-group. The proportion of resistant to phenotypically tolerant strains in the UE-group was 6 out of 45 (13.3%) vs 2 out of 12 (16.7%), and 4 of 9 (44.4%) vs 23 of 55 (41.8%) respectively in the E-group. The differences were clearly not significant. However, as mentioned previously, there were statistically significant differences between both the amoxycillin MICs and MBCs when selection by the antibiotic combination was compared with penicillin G alone at the 0.125 mg/l level. These differences were, however, comfined to the UE-group

With regard to penicillin V, the patterns after selection at penicillin G 0.125 mg/l plus gentamicin and with penicillin G alone (0.125 mg/l) are similar, as indicated earlier. It is interesting that in the E-group the penicillin V patterns were similar when selection at 1.0 mg/l penicillin G was compared with selection in penicillin 0.125 mg/l plus gentamicin. This was, however, not the case when the corresponding penicillin V patterns in the UE-group were compared ($p=0.003$; chi-squared, Yates modification)

In summary, these comparisons suggest a modifying effect on survivors of penicillin G plus gentamicin selection with regard to amoxycillin susceptibility which was only convincingly shown in the UE-group. But a similar effect based upon penicillin V susceptibility was not demonstrated, not even in the UE-group (MICs: 24 out of 45; 53% vs 5 out of 9; 56% and MBCs: 31 out of 45; 69% vs 7 of 9; 78%)

11.3.3 Comparison of MIC and MBC activities of penicillin V and amoxycillin

When the MIC and MBC findings in Table 11.1 are studied it is clear that amoxycillin exhibited greater activity against viridans streptococci than penicillin V. For example, at the MIC level of <0.125 mg/l, 73 out of 140 strains (52%) were inhibited by amoxycillin compared with 46 of 140 (33%) by penicillin V ($p<0.002$, chi-squared test, Yates modification) while the corresponding figures for MBC values were 70 of 140 (50%) for amoxycillin as opposed to 34 of 140 (24%) for penicillin V ($p<0.00002$). Furthermore, when the E- and UE-groups were conpared, a larger number of isolates had MBCs $\geq$0.125 mg/l in the E-group compared with the UE-group (58 of 74; 78% and 48 of 66; 73%, respectively, in the case of penicillin V while the amoxycillin figures were 51 of 74; 69% vs 19 of 66; 29%). It is, none-the-less, clear that the difference in the bactericidal activity between penicillin V and amoxycillin was much more evident in the UE-group (48 of 66; 73% vs 19 of 66; 29% and $p=0.000001$; chi-squared, Yates correction). As expected, very similar trends were shown when breakpoints were used (see Tables 11.4a and 11.4c). Interestingly, the difference in bacteriostatic activity between penicillin V and amoxycillin following selection in penicillin G only (Table 11.4b) was not as marked as was the case when gentamicin was added to penicillin G in the selection procedure (Table 11.4c)

Amoxycillin also appeared to be more active than penicillin V, especially in the UE-group, when the individual species (*S. oralis*, *S. mitis* I, and *S. mitis* II) were compared (Tables 11.3a, 11.3c, 11.4a, 11.4c). An interesting observation is demonstrated in Tables 11.5a and 11.5b where amoxycillin was shown to be more active than penicillin V at the MIC 50/MBC 50 levels but the

trend was reversed at the MIC 90/MBC 90 levels

Not only was amoxycillin more active than penicillin V overall, but when the penicillin V and amoxycillin MICs of individual strains were compared, a good correlation was obtained (correlation co-efficient, 0.87). The few deviations from this excellent correlation occurred mainly in the strains with higher MICs/MBCs (see Tables 11.5a and 11.5b)

11.3.4 Exposure to two penicillin G concentrations in broth

After the 24 hour exposure at 37 degrees Celcius to the higher concentration of penicillin G in broth, 27 strains out of 67 (40%) had penicillin V MBCs $\geq$1.0 mg/l while the remaining 40 strains (60%) survived selection despite having MBCs of $\leq$1.0 mg/l penicillin V (Table 11.1). The amoxycillin figures were 25 out of 67 (37%) and 42 of 67 (63%), respectively. Following selection in 0.125 mg/l penicillin G broth cultures (excluding the survival of isolates exposed to the addition of 5 mg/l gentamicin), 14 out of 19 surviving strains (74%) had penicillin V MBCs $\geq$0.125 mg/l (corresponding figures for amoxycillin were 12 out of 19; 63%). Selection at 0.125 mg/l penicillin G yielded fewer isolates (6 of 19; 32%) with penicillin V MBCs $\geq$1.0 mg/l compared with 27 out of 67 (40%) at the 1.0 mg/l penicillin G selection level. The corresponding figures for amoxycillin were 4 of 19 (21%) and 25 of 67 (37%), respectively, at the low and high selection concentrations

11.3.5 Selection in penicillin G broth of strains with high MBC levels: comparison between E-group and UE-group isolates

11.3.5.1 Comparison between E-group and UE-group according to individual selection procedures

A clear trend showing a higher proportion of isolates with penicillin V and amoxycillin MBCs $\geq$1.0 mg/l in the E-group compared with the UE-group, was evident following the various selection procedures (Table 11.1). This finding applies to the higher selection concentration (25 of 55; 45%) in the E-group vs 2 of 12 (17%) in the UE-group (p=0.03) for penicillin V and 23 of 55 vs 2 of 12 in the case of amoxycillin (not significant). However, at the lower penicillin selection concentration, 5 of 10 (50%) in the E-group had MBCs >1.0 mg/l as opposed to the 1 of 9 (11%) in the UE-group. The respective amoxycillin figures were 3 out of 10 (30%) and 1 of 9 (11%). The numbers in this comparison are too low for statistical analysis. When the corresponding figures in the penicillin G (0.125 mg/l) plus gentamicin selection broth were compared, the difference between the E- and UE-groups according to previous antibiotic exposure was significant (3/9 and 33% vs 3/45 and 7% giving a p-value of 0.05 (Fisher Exact test). THe corresponding figures for amoxycillin were 2 of 9 (22%) as opposed to 1 of 45 (2.2%)

11.3.5.2 Comparison between E-group and UE-group according to the two penicillin G-only selection procedures

Comparison of resistance at MBCs $\geq$1.0 mg/l according to the two penicillin G only selection procedures (Table 11.1) showed that more strains in the E-group had penicillin V MBCs $\geq$1.0 mg/l than in the UE-group (30/65 vs 3/21; p<0.02, chi-squared test with Yates modification). For amoxycillin, statistical analysis indicates a difference which approaches significance; the corresponding figures being 26/65 vs 3/21; p=0.06 (chi-squared test, Yates modification)

11.3.5.3 Comparison according to all selection procedures combined

When the UE- and E-groups are compared (Table 11.1), considering all the selection procedures, significantly more strains had MBCs $\geq$1.0 mg/l antibiotic for both penicillin V and amoxycillin in the E-group compared with the other (33/74 vs 6/66; $p<0.00001$ and 28/74 vs 4/66; $p<0.00002$)

11.4 Effect of previous ß-lactam antibiotic exposure on streptococcal species

11.4.1 Distribution of streptococcal species according to previous antibiotic exposure

S. oralis, S. mitis biovar 1 and S. mitis biovar 2 were the predominent species found in both the UE-group and E-groups, and S. oralis and S. mitis biovar 2 were the only species isolated from the ampicillin-exposed (A-) subgroup. S. mitis biovar 1 was common in the UE-group but only four strains were recovered from ß-lactam exposed individuals, all receiving rheumatic fever prophylaxis. Less common isolates belonged to S. sanguis biovar 4, S. vestibularis and biovars 2 or 3 of S. gordonii species (the latter two biovars were not distinguished from one another), while other S. sanguis strains were distinctly uncommon (Table 11.2a)

Very similar trends were observed when selection in the penicillin G-only concentrations were analysed (see Table 11.2b)

11.4.2 Effect of previous exposure to ß-lactam antibiotics on levels of resistance in viridans streptococci as a group

11.4.2.1 Differences related to antibiotic exposure following selection procedures in penicillin G and in penicillin G plus gentamicin

MICs and MBCs of both penicillin V and amoxycillin were clearly higher in the E-group of 74 isolates compared with 66 strains in the UE-group as reflected in the MIC 50 and the peak MIC and MBC levels (Table 11.3a). This was also the case when individual species were considered (Table 11.3a), except for S. mitis biovar 2 MIC 50 levels of penicillin V which were the same as in the two exposure groups. Similar trends could be observed when the findings were presented according to susceptible, intermediately resistant and resistant categories (Table 11.4a)

11.4.2.2 Differences related to antibiotic exposure following selection procedures in penicillin G alone

The findings when the selection process involved penicillin G-only and penicillin G plus gentamicin are given in Tables 11.3b and 11.4b, and Tables 11.3c and 11.4c, respectively. They show similar trends as the results of the selection procedures described under Section 11.4.2.1. However, the differences of the proportion of susceptible strains in the UE-group compared with the E-group were less pronounced in the isolates from the penicillin G-only selection process

11.4.2.3 Relative susceptibility to the ß-lactam antibiotics of viridans streptococci in E-subgroups and UE-group based upon MIC 50/MBC 50 and MIC 90/MBC 90 levels

The MIC 50 and MBC 50 levels of penicillin V and amoxycillin against isolates from the two E-subgroups consisting of hospitalised patients exposed to high doses of ampicillin over relatively short periods (ampicillin (A)-subgroup; 9 patients, 12 isolates) and rheumatic fever patients with prolonged low-level exposure to penicillin V (rheumatic fever (RF)-subgroup; 41 individuals, 62 isolates) are given in Table 11.5a (including isolates processed in penicillin G plus gentamicin selection broths) and Table 5b (penicillin G only selection broth). MIC 50/MIC 90 and MBC 50/MBC 90 levels of both penicillin V and amoxycillin were, with the exception of the three S. mitis biovar 2 strains, higher in the A-subgroup than the RF subgroup which, in turn, were higher than those in the UE-group, especially as indicated by the MIC 90 and MBC 90 figures of the latter. These findings applied to both the penicillin G plus gentamicin selection broth procedures and the penicillin G only procedure (see Table 11.5a and Table 11.5b)

11.4.2.4 Relative susceptibility of viridans streptococci to the ß-lactam antibiotics of E-subgroup and UE-group strains based on resistance breakpoints

Numbers of isolates in the different susceptibility categories are shown in Table 11.6a and Table 11.6b and an analysis of the statistical significance of the effect of **in vivo** exposure to ß-lactam antibiotics on resistance to penicillin V and amoxycillin is presented in Table 11.7a and Table 11.7b. A significantly greater number of isolates resistant to either penicillin V ($p=0.012$) or amoxycillin ($p=<0.001$) was encountered in the E-

group compared with the UE-group (Table 11.7a). Both short-term ampicillin therapy and long-term penicillin V prophylaxis also resulted in significantly larger proportions of penicillin V-resistant and amoxycillin-resistant isolates compared with those susceptible to these agents. P-values ranged from <0.0001 for the A-subgroup vs UE-group in the case of amoxycillin, to p=0.012 for A-subgroup vs UE-group against penicillin V (Table 11.7a). In Table 11.7b, the equivalent figures are shown after selection in the absence of gentamicin and no significant difference was evident between any of the respective groups. No significant difference was detected between the two E-subgroups irrespective of whether gentamicin was present in the penicillin G broths or not

However, when isolates obtained from the penicillin plus gentamicin selection procedure in the UE-group were compared with those of the E-group, 32 of 45 (71%) of isolates were susceptible to penicillin V in the UE-group compared with 5 of 9 in the E-group. The difference was not significant but the respective figures for amoxycillin (44 of 45 vs 6 of 9) yielded a p-value of 0.03 (Fisher Exact test). Furthermore, when, as shown in Tables 11.4b and 11.6b, high level resistance ($\geq$4 mg/l) strains were compared with less resistant and sensitive strains in the three antibiotic exposure groups (2 and 3 out of 12, respectively, for penicillin V and amoxycillin in the A-subgroup and 6 and 5 of 53 in the RF-subgroup, respectively, were highly resistant while no highly resistant strains were encountered in the UE-group). When the A-subgroup was compared with the UE-group, p-values (using the Fisher Exact test), were 0.12 and 0.04 for penicillin V and amoxycillin, respectively, and 0.17 and 0.3 when the RF-subgroup was compared with the UE-group. Thus, only in the case of amoxycillin in the A-subgroup could a significant difference between prior antibiotic exposure and non-exposure be demonstrated - and even then, small numbers of isolates involved render statistical analysis uncertain

11.4.3 Effect of exposure to ß-lactam antibiotics on levels of resistance in streptococcal species

S. oralis and S. mitis biovar 2 exhibited the highest MIC and MBC levels to the two ß-lactam antibiotics in the E-group, ranging from 4-32 mg/l. The corresponding peak MICs and MBCs in the UE-group were much lower and ranged from 0.25-1 mg/l antibiotic (Table 3a). MIC 50 figures of S. oralis and S. mitis biovar 1 were approximately 8-fold higher in the E-group compared with the UE-group but no difference in MIC 50 figures of S. mitis biovar 2 figures were observed. Similar trends, based on amoxycillin MIC 50 figures, were demonstrated (Table 11.3a), as were those apparent after selection in in penicillin G alone (Table 11.3b), albeit with fewer available UE-isolates

Comparison of MICs 50/MBCs 50, MICs 90/MBCs 90 and peak MICs/MBCs of strains from the A- and RF-subgroups and the UE-group (Table 11.5a) clearly shows that the resistance concentrations to both penicillin V and amoxycillin were consistently highest in the A-subgroup followed by the RF-subgroup. This trend was more clearly shown in S. oralis strains than in S. mitis biovar 2 strains (Table 11.5a)

With all isolates distributed into susceptible, intermediately resistant and resistant categories (Table 11.4a and Table 11.6a), only strains of S. oralis and S. mitis biovar 2 were found to be resistant to the ß-lactams (MICs $\geq$4.0 mg/l). All highly resistant strains were limited to specimens which originated in the dental plaque of E-group volunteers: 16.7% of 12 isolates and 9.7% of 62 strains which were resistant to penicillin V came from the A-subgroup and RF-subgroup respectively, while the corresponding percentages for amoxycillin were 25 and 6.5 in the two respective

subgroups

When the relative activities of penicillin V and amoxycillin were compared in the various streptococcal species, the latter antibiotic was found to be generally more active than penicillin V. This was also apparent when the UE- and E-groups were considered. With the exception of relatively small numbers of S. mitis biovar 2 strains in the E-group (12 isolates) and unnamed species in the UE-group, the amoxycillin MIC 50 levels were approximately 4-fold lower than the corresponding MICs 50 of penicillin V in the species and biovars listed according to ß-lactam exposure groups in Table 11.3a, with very similar trends in Tables 11.3b and 11.3c. Table 11.4a also shows that more isolates were susceptible to amoxycillin than penicillin V amongst S. oralis and S. mitis (both biovars) isolates as well as amongst the total number of strains in the UE-group. A similar but less pronounced trend was seen amongst strains in the E-group. The same trends were observed in Tables 11.4b and 11.4c concerning penicillin G-only and the combination selection procedures. However, more S. oralis strains in the high ampicillin exposure group showed higher MBC 90 and peak MBC values of amoxycillin compared with penicillin V. This was also an overall finding in the 12 isolates in the A-subgroup as well as in the 62 isolates of the RF-subgroup (Tables 11.5a and 11.5b)

11.5 Discussion

Although the findings in the **in vitro** penicillin G selection model exclude the **de novo** susceptibility status of streptococci in dental plaque and does not indicate how many of the originally susceptible streptococci in the plaque were killed in the process of selection, it does provide the relative numbers of phenotypically tolerant streptococci with MBCs below the equivalent

penicillin G selection concentrations, as well as resistant streptococci surviving the **in vitro** selection procedure

The most important aspect of this model is however that it should give an indication of the phenotypically tolerant or resistant status of dislodged streptococci from dental plaque which may enter the blood stream during ß-lactam prophylaxis

11.5.1 MICs vs MBCs

MICs and MBCs for the E-group and UE-group isolates are recorded in Table 11.1. The MIC findings are interesting as they express the conventional assessment of susceptibility of organisms to antibiotics. However, in the context of the present study, MBC findings are more relevant as they more appropriately reflect the potential for survival of the streptococci to the two ß-lactam antibiotics tested. This is obviously important in the prophylaxis of IE where killing (MBCs) of organisms is of paramount importance as opposed to mere inhibition (as reflected by the MICs) where surviving bacteria may still be able to colonise the endocardium after the antibiotic effect has waned

11.5.2 Comparison between the activities of penicillin V and amoxycillin

Comparatively few studies have been conducted in which the relative activities of penicillin G, amoxycillin and penicillin V have been compared (Garrod, 1960a; Garrod 1960b; Coulter et al, 1990). The present study clearly shows that amoxycillin is somewhat more active against viridans streptococci than penicillin V, regarding both their relative bacteriostatic and bactericidal potential against these bacteria. These findings are in accord-

ance with findings of the quoted authors

11.5.3 The effect of selection in penicillin G plus gentamicin broth on the susceptibility to penicillin V and amoxycillin according to previous ß-lactam exposure

The addition of gentamicin to 0.125 mg/l penicillin G in broth resulted in ratios of phenotypic tolerance to resistance (T/R) ratios of surviving strains that resemble selection at 1.0 mg/l concentrations more closely than the 0.125 mg/l penicillin-only selection process - but only in the case of amoxycillin. For penicillin V the T/R ratios resemble more closely the selection that occurred at 0.125 mg/l penicillin G only

The gentamicin plus penicillin G selection process in the case of UE-group isolates resulted in a much higher amoxycillin T/R ratio of 39/45 (87%) compared with 3/9 (33%) when the selection broth contained only penicillin G ($p=0.015$, Fisher exact test). No meaningful difference was, however, observed when the penicillin V T/R ratios of penicillin G plus gentamicin (14/45; 31%) were compared with those of penicillin alone (2/9; 22%) ($p=0.71$, Fisher Exact test). Thus while exposure of isolates to selection in the penicillin plus gentamicin broth resulted in little difference between isolate sensitivity to penicillin V compared to selection in penicillin G only (0.125 mg/l), this was not the case for amoxycillin where this combination resulted in the selection of appreciably fewer resistant isolates with MBCs greater than the selection concentration of penicillin G at 0.125 mg/l in the combination. These findings suggest that the addition of gentamicin to penicillin G in the selection process in the case of UE-group isolates rendered the organisms more phenotypically tolerant to amoxycillin compared with penicillin V. A high proportion of phenotypically tolerant isolates was also seen in

the UE-group when selection took place at the high penicillin G concentration of 1.0 mg/l without gentamicin: 10 out of 12 (83%) were phenotypically tolerant to amoxycillin and 2 out of 12 (17%) had MBCs >10 mg/l (Table 11.1). However, at this concentration the proportion of strains phenotypically tolerant to penicillin V was similar to that of amoxycillin. The trend of a preponderance of amoxycillin-tolerant (phenotypic) strains relative to penicillin V was also seen, with this one exception, in the other selection exercises in the UE- and E-groups, but much less so and certainly not statistically important (Table 11.1). In the case of selection at 10 mg/l penicillin G-only in the UE-group, however, there was no difference between penicillin V and amoxycillin T/R ratios (Table 11.1)

The high proportion of isolates phenotypically tolerant to amoxycillin in the penicillin plus gentamicin selection process may be partly due to the greater activity of amoxycillin compared with penicillin V under the conditions pertaining to MIC/MBC determinations which are designed to prevent the expression of phenotypic tolerance (inocula for MIC/MBC determinations contain cells from fresh isolates in the very active logarithmic growth phase - see later). Under these laboratory conditions the same cohort of streptococcal isolates will show proportionally more strains to be susceptible to amoxycillin, by virtue of its greater activity, than to penicillin V

It is possible that the higher concentrations of penicillin G, and the addition of gentamicin to the lower penicillin G concentration, may enhance the expression of phenotypic tolerance over and above the role growth conditions may play in dental plaque - which probably also favour phenotypic tolerance (see later). In the case of a possible gentamicin effect, it is conceivable, but highly speculative, that its action on protein synthesis may result in defective enzymes which could slow down or

halt metabolic processes reversably, thus facilitating phenotypic tolerance. Furthermore, one could speculate that previous exposure to ß-lactam antibiotics (E-group subjects) may have eliminated some strains susceptible to such effects of gentamicin or high levels of penicillin G which may operate during the selection process. Alternatively, previous antibiotic exposure may have selected mutants not affected in this manner by these selection procedures. A more plausable explanation, however, is that previous antibiotic exposure may have affected the physical formation of the dental plaque or changed its integrity and composition, resulting in modified bacterial films which are more likely to promote phenotypic tolerance than undisturbed films

The similarity between the T/R ratios amongst the survivors of the high penicillin G and the low penicillin G plus gentamicin selection concentrations, respectively, could at least in part, be explained by synergy between gentamicin and penicillin G. The premise that synergy may play a role is based upon the following assumptions: a) Susceptible cells that are not phenotypically tolerant will be killed by both penicillin G alone and by the antibiotic combination but the number of cells killed are not known; b) Phenotypically tolerant cells will be refractory to killing; c) More genetically-based resistance cells will be killed if there is synergy between the drugs in the combination compared with the bactericidal effect of penicillin G alone and d) The T/R ratio will therefore tend to be greater if synergy is present (see Table 11.1)

This argument is corroborated by the presence of a high percentage of susceptible strains with MICs $\leq$0.12 mg/l (and therefore phenotypically tolerant) following selection by this combination amongst the UE-group individuals: 71% against penicillin V and 98% against amoxycillin amongst 45 isolates, as opposed to 52% and 57% respectively amongst 21 isolates selected by the equiv-

alent penicillin G concentration only. A similar but less pronounnced trend was evident in the E-group: 56% and 67% for penicillin V and amoxycillin out of the small number of 9 isolates from the penicillin G plus gentamicin broth, compared with 35% and 42% out of 65 isolates from the penicillin G-only broths (see Tables 11.4b and 11.4c)

Similar trends were noticed when the MICs 50 of strains surviving the various selection procedures were examined. Thus, in the case of penicillin V, no change occurred in the MIC 50 values of the viridans streptococci in the UE-group (0.12 mg/l) when selection in penicillin G-only broths (Table 11.3b) was compared with the penicillin G plus gentamicin selection procedure (Table 11.3c). There was, however, a decrease in the MICs 50 of E-group strains from 0.5 mg/l in the penicillin G-only broths to 0.12 mg/l in the penicillin G plus gentamicin strains (Tables 11.3b and 11.3c). For amoxycillin, there was a similar 4-fold decrease in MICs 50 in both the E- and UE-groups (Table 11.3b and 11.3c)

11.5.4 Pooling of findings obtained from different <u>in vitro</u> selection concentrations

As the selection procedures could all reflect what may happen **in vivo** in patients exposed to prophylactic ß-lactam antibiotics (also keeping in mind that selection patterns of 0.125 mg/l penicillin G plus gentamicin resemble those of selection by the higher concentration of 1.0 mg/l penicillin G), the findings of resistance to both ß-lactams of all three selection procedures were combined for statistical analysis of the exposure groups even though the differences described above appear to be real. However, although there may have been justifiable grounds for the combination of data, it was decided to also analyse the findings separately for the three selection procedures

11.5.5 The effect of penicillin G-only selection on exposure group susceptibility to penicillin V and amoxycillin

More isolates were found to be phenotypically tolerant at 1.0 mg/l concentrations in the UE-group (10/12: 83%, both agents, see Table 11.1) as opposed to the E-group where the amoxycillin figures were 32/55; 58% and those of penicillin V 30/55; 55% (see Table 11.1). Possible reasons for this finding were discussed in Section 11.5.9.2. In the lower 0.125 mg/l selection concentration of penicillin G-only very little difference can be observed between the two exposure groups but numbers were too small for any valid conclusions to be drawn

Examination of the penicillin G-only selection model (without the addition of gentamicin) shows very little difference between the phenotypic tolerance to resistance (T/R) ratios of penicillin V and amoxycillin in both groups as reflected in the MBC values: UE-group, 10/12 for both antibiotics at the 1.0 mg/l penicillin G selection concentration and 2/9 and 3/9 for the two respective agents at the lower 0.125 mg/l selection. For the E-group, the equivalent T/R ratios for penicillin V and amoxycillin at 1.0 mg/l are 30/55 and 32/55 (1.0 mg/l), and 3/10 and 4/10 for the same two at 0.125 mg/l antibiotic. It is worth noting again that the higher T/R ratios at the 1.0 mg/l selection concentration are demonstrated in both the E-group and UE-group but this trend in T/R ratios is reversed in these two groups at the lower 0.125 mg/l selection concentration. The proportionately fewer resistant survivors at the high selection concentration can be partly explained on the assumption that of the metabolically active streptococci from the dental plaque exposed to the selection concentration, a smaller proportion would be resistant and survive at he higher compared with the lower selection concentrations.

Unfortunately, the relative proportions of resistant cells with higher MBCs that were metabolically inactive in the plaque samples, were not known. Such phenotypically tolerant cells would, of course, have survived the selection process irrespective of the level of the selection concentration or the strain MBC. It is, however, reasonable to assume that the mix of susceptible and resistant cells in the plaque samples submitted to the various selection concentrations were similar in each of the UE- and E-groups groups

11.5.6 Summary of main findings of <u>in vitro</u> selection affecting plaque streptococci in general

Selection in penicillin G broth results in the survival of both resistant strains and phenotypically tolerant forms. The addition of gentamicin in the selection process failed to abolish either phenotypically tolerant or resistant strains and may result in the development of proportionally more phenotypically tolerant bacteria, but not to penicillin V. It would also appear that the state of phenotypic tolerance is not due to traditional (genetically-based) tolerance phenomena and finally, and not unexpectedly, that previous ß-lactam exposure results in the selection of proportionally more resistant forms

11.5.7 Effect on species of broth selection procedures

The overall effects of the three selection procedures on the various streptococcal species are given in Tables 11.3a and 11.4a while the effects of penicillin G-only selection and that of penicillin G plus gentamicin are given in Tables 11.3b and 11.4b and 11.3c and 11.4c, respectively

11.5.7.1 Relative activites of penicillin V and amoxycillin amongst streptococcal species

As pointed out earlier (Section 11.3.3), amoxycillin showed greater activity amongst the more common S. oralis, S. mitis biovar 1 and 2 strains. In the UE-group proportionately more S. oralis strains were susceptible to penicillin V (81% overall; Table 11.4a) than S. mitis biovar 1 (53%) and biovar 2 (56%). This was not demonstrated in the E-group (41%, 0% and 47%, respectively), nor in the case of amoxycillin in either of the exposure groups. Similar trends were seen in the penicillin G plus gentamicin selection group (Table 11.4c) but not in the penicillin G-only selection groups (table 11.4b) where the difference in susceptibility to penicillin V between S. oralis and S. mitis biovar 2 was reversed (43% vs 55%) compared with the data in the other two selection groups. The numbers of isolates belonging to these two species in the penicillin G-only selection groups were, however, too small for valid comparisons to be made

11.5.7.2 Effect of selection procedures on streptococcal species in relation to previous antibiotic exposure

Except for S. mitis biovar 2, there was a clear trend amongst S. oralis and S. mitis biovar 1 isolates of larger numbers of susceptible strains in the UE- as opposed to the E-group when selection groups were combined (Table 11.4a), as well as in the penicillin G plus gentamicin selection group. This pattern was, however, not as pronounced in the penicillin G-only selection groups where the total number of isolates in the UE-group was only 21 with 5, 4 and 9 strains respectively in the three most common species/biovars, respectively (S. oralis, S. mitis biovar 1, S. mitis biovar 2). In the E-group, there was a total of 57

isolates of these species/biovars with a respective species distribution of 41, 5 and 11 isolates (Table 11.4b)

With regard to MICs 50 (see Tables 11.3a, 11.3b and 11.3c), the values (with exception of S. mitis biovar 2) were generally higher in the E-group than in the UE-group amongst the species. This was exemplified in S. oralis and S. mitis biovar 1 strains, the latter biovar showing somewhat higher MICs 50 (but not peak MIC or MBC levels) compared with the other species/biovars (penicillin V MICs 50 of the three combined selection procedures of 0.12 and 1.0 mg/l in the UE- and E-groups, respectively, in contrast to 0.06 and 0.5 mg/l for S. oralis strains). When the MICs 50 of both penicillin V and amoxycillin in the penicillin G plus gentamicin selection group were compared, they were generally the same or one serial concentration lower than in the penicillin G-only selection group

More striking were the differences between the peak MIC and MBC levels of penicillin V and amoxycillin which were, with the exception of S. mitis biovar 2 strains, appreciably lower in the penicillin G plus gentamicin selection category than was the case with the penicillin G-only selection procedures (see Tables 11.3a, 11.3b and 11.3c). This finding may, however, be fortuitous as 45 isolates from the penicillin G plus gentamicin selection process came from the UE-group (from which lower peak levels would be expected) while only 9 isolates from this combination selection procedure came from the E-group and only 2 of these had MICs or MBCs higher than 1.0 mg/l (see Table 11.1). On the other hand, 65 isolates from the E-group (55 from the high penicillin G concentration selection procedure) compared with 21 from the UE-group (12 of these in the high penicillin G concentration procedure) came from the two penicillin G-only selection process, thus increasing the likelihood of selecting strains with high MIC/MBC values

11.5.7.3 Summary of main species-related findings in the *in vitro* selection model

a) *S. oralis* and *S. mitis* biovars 1 and 2 were the most common survivors in this model

b) Amoxycillin was more active than penicillin V in viridans streptococci in general and the differences between the activities of these antibiotics was most marked in *S. mitis* biovar 1 and *S. mitis* biovar 2 strains

c) There was a larger proportion of susceptible strains of *S. oralis* and *S. mitis* biovar 1 in the UE-group than the E-group (Tables 11.4a, 11.4b and 11.4c) but this trend was less marked in strains surviving the penicillin G-only selection broth procedure (Table 11.4b)

d) Peak MICs/MBCs were highest in the E-group isolates and were most clearly observed in *S. oralis* and *S. mitis* biovar 2 strains

11.5.8 Comparative effects of exposure to high ß-lactam concentrations (A-subgroup), prolonged low ß-lactam concentrations (RF-subgroup) and absence of prior ß-lactam exposure (UE-group)

In vivo exposure to high concentrations of ampicillin (A-subgroup) resulted in the selection of viridans streptococci with higher penicillin V and amoxycillin MICs and MBCs compared with streptococci selected from those exposed to prolonged administration of penicillin V in rheumatic prophylaxis individuals. The latter, in turn, had higher MICs/MBCs than oral streptococci

selected from the UE-group (Tables 11.5a, 11.5b, 11.6a and 11.6b). This pattern was most clearly shown in S. oralis strains

These findings not only show that prior antibiotic exposure does affect the susceptibility of viridans streptococci in plaque, but also demonstrates that exposure to high concentrations of ß-lactam antibacterial agents would tend to select for strains with higher levels of resistance. They also suggest that selective pressures affect some species more than others and that S. oralis selected in the process had higher MICs/MBCs than S. mitis biovar 2 strains

It is interesting to note (but difficult to explain) that prior exposure to ß-lactam antibiotics also led to the selection of strains that were resistant to antibiotics other than ß-lactams (see Chapter 9). It is possible that transposons coding for resistance eg. to erythromycin and clindamycin by Tn 917 and Tn 1545 (Leclerq and Courvalin, 1991) may play a role. But, these latter transposons do not, however, code for resistance to ß-lactam agents

11.5.9 Summary of, and concluding comments on, the most salient features of the in vitro selection model in relation to amoxycillin and penicillin V prophylaxis

11.5.9.1 Validity of model

The experimental model used in this study has a number of potential shortcomings viz:

a) the original composition of the streptococcal species in plaque transferred to the penicillin G selection cultures and their susceptibility patterns were not determined

b) dental plaque specimens could not, for practical reasons, be standardised with regard to size. However, the same site was used to collect dental plaque whenever possible (upper first or second molars, see Section 8.4)

c) the antibiotic chosen for selection was penicillin G (supplemented with gentamicin in one selection process) rather than amoxycillin which is used in practice for IE prophylaxis (and penicillin V in the past). The author feels that the case of penicillin G is justified by the fact that it is an agent of choice - "the mainstay of therapy" (Johnson and Tunkel, 1995) - either as a single agent or in combination with an aminoglycoside, in the treatment of cases of IE caused by viridans streptococci

The choice of the low concentration of penicillin G in the **in vitro** model is a compromise but can be justified on the grounds that the end result (death of the bacteria) after the duration of exposure is likely to be the same at 0.125 mg/l or 1.0 mg/l as it is with much higher concentrations such as 20 mg/l, as these are all in excess of the MBCs of susceptible viridans streptococci. Very high serum levels of 20 mg/l and greater are achievable in practice following amoxycillin prophylaxis at high (3g) doses (Shanson et al, 1984). Furthermore, the long-term consequences of selection of resistant strains relate to the resistant streptococci in dental plaque where the antibiotic concentrations are much lower, probably approximately 10% of the blood levels, ie. in the range of selection concentrations used in the present model (Sukchotiratana and Linton, 1975)

d) The decision to expose the plaque-derived bacteria to an antibiotic selection period of 18 hours (overnight incubation) may also be a matter for debate. The British Society for An-

timicrobial Chemotherapy (BSAC) recommended 3g amoxycillin 1 hour before the dental procedure giving an exposure time of a few hours only while the American Heart Association (AHA) recommended an additional 1.5g 6 hours later resulting in an adequate exposure time of approximately 12 hours. This study, therefore, had an exposure time of bacteria to antibiotic activity which was somewhat longer than that achievable by the AHA schedule. Workers who studied prophylaxis in **in vivo** experiments (Phillips et al, 1976; Shanson et al, 1978; Hess et al, 1983) have suggested that the failure to show killing of susceptible bacteraemic organisms by prophylactic antibiotics in their studies may be as a result of the short exposure of the bacteria to the antimicrobial agents at the time of taking blood samples (approximately 10 minutes) and diluting out of the antibiotic during the blood culture process. These problems have been eliminated in the present model

Deficiencies and debatable aspects of the model described in the dissertation would have been costly to rectify. They do not, however, appear to have markedly compromised the validity of the findings presented here. The model finally employed in this study attempted to mimic IE prophylaxis in persons at risk who are about to undergo dental procedures. It includes important features such as removal of plaque material containing biofilms of bacteria which are likely to be prone to phenotypic tolerance and to harbour amongst them, resistant strains selected by previous antibiotic usage. Furthermore, streptococci from dental plaque are subjected to antibiotic pressures likely to obtain at the dental plaque sites as well as the blood of persons following administration of a prophylactic ß-lactam antibiotic (even though concentrations in the blood are greater than in the model) following administration of a prophylactic ß-lactam antibiotic. Surviving bacteria would therefore tend to include those which may cause IE should prophylaxis fail. The model could also be of value as a screening procedure in experiments to predict the

potential efficacy of antibiotics that are candidates for IE prophylaxis, also in persons previously exposed to antibiotics

The rationale for the use of penicillin G plus gentamicin in the selection model is based upon the known synergistic action of this combination on viridans streptococci and further studies using such combinations were recommended by Hess et al (1983) following failure of penicillin G to destroy bacteraemic streptococci in their **in vivo** investigations. However, the selection of strains in the presence of penicillin G plus gentamicin introduced a confounding aspect to the model. When all three selection processes (ie. high and low penicillin G levels and penicillin G plus gentamicin) were considered together statistically, significant differences in resistance frequencies to penicillin V and amoxycillin in the antibiotic exposure groups, compared with unexposed individuals, were demonstrated (Table 11.7a). Such differences could, unfortunately, not be shown when the penicillin G-only selection procedures were considered (Table 11.7b) except when an MBC level of $\geq$1.0 mg/l was taken as the breakpoint for resistance (see footnote Table 11.7b). Were similar models to be designed in future, it may be wise to use an agent or combination of agents which is/are actual candidates for prophylaxis rather than a surrogate compound such as penicillin G

11.5.9.2 **Demonstration of phenotypic tolerance**

Phenotypic tolerance was clearly demonstrated in the **in vitro** model and this is entirely in keeping with those reported in early **in vivo** studies (Phillips et al, 1976; Shanson et al, 1978; Hess et al, 1983; Hall et al, 1993), the latter of whom showed that bacteraemic viridans streptococci survive amoxycillin and penicillin V prophylaxis despite bactericidal antibiotic levels obtained in the blood of subjects in the study

The model described here suggests that phenotypic tolerance is more likely to be operative with amoxycillin prophylaxis, especially in individuals not previously exposed to ß-lactam antibiotics. This form of tolerance may be associated with prophylaxis failure (although not necessarily so as tolerant strains subjected to ß-lactam antibiotics may possibly fail to adhere to platelet vegetations at the initiation of IE - see Section 2.3.2). On the other hand, phenotypically tolerant strains demonstrated **in vivo** and in this model, would probably become susceptible to this type of antibiotic agent in the IE setting. Here metabolically active non-tolerant bacteria susceptible to the bactericidal action of the antibiotic are likely to prevail - at least during stages of the disease when vegetations are developing or maintained as "active" entities. Degrees and/or phases of phenotypic tolerance may indeed be responsible for the prolonged therapy that is required for cure of the disease

Results of the study recorded here also showed that previous administration of antibiotics to individuals affected their plaque streptococci in such a way that when these organisms were exposed to high penicillin G-only concentrations or low penicillin G plus gentamicin they were less likely to exhibit phenotypic tolerance to amoxycillin than in persons not recently given antibiotic. This may be as a result of altered biofilm caused by prior exposure to antibiotics and this phenomenon may well apply to IE prophylaxis with ß-lactam agents

11.5.9.3 **Selection of resistant strains**

The model strongly suggests that ß-lactam-resistant strains may be selected during IE prophylaxis with penicillin V or amoxycillin. Furthermore, such strains would more likely be present in

individuals previously exposed to ß-lactam agents (see Table 11.7a) while the highest levels of resistance can be expected when high-dose ß-lactam therapy had been given recently to hospitalised patients (Tables 11.3a and 11.5a). The results are in accordance with previous publications relating to the effect of antibiotic exposure on viridans streptococci in the oral cavity (see Section 1.2.2 and Section 5.2.3)

This study shows that **S. mitis** biovar 1 strains are more likely than other viridans streptococci to be resistant to penicillin V and amoxycillin based upon MIC 50/MBC 50 values (Table 11.3a). S. oralis strains, on the other hand, exhibited the highest resistance levels to amoxycillin (peak MBCs; 32 mg/l) while S. oralis and **S. mitis** biovar 2 strains displayed increased levels of resistance to both amoxycillin and penicillin V (peak MBCs; 16 mg/l; see Table 11.3a). Thus, high-level resistance with MBCs >4 mg/l were only encountered in **S. oralis** and **S. mitis** biovar 2 isolates (Tables 11.3a and 11.5a)

Table 11.1 Effect of in vitro antibiotic selection on susceptibility of streptococcal isolates from β-lactam-exposed (E-group) and β-lactam-unexposed individuals (UE-group)

Susceptibility ranges (mg/l) of surviving streptococci	Selection broths (mg/l penicillin G)											
	0.125				1.0				0.125 + 5 mg/l gentamicin			
a) E-group isolates (74)	(10 isolates)				(55 isolates)				(9 isolates)			
	P†		A		P		A		P		A	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
< 0.125	3*	3	4	4	11	9	16	14	4	4	5	5
≥ 0.125-0.9	3	2	3	3	20	21	18	18	2	2	2	2
≥ 1.0	4	5	3	3	24	25	21	23	3	3	2	2
b) UE-group isolates (66)												
	(9 isolates)				(12 isolates)				(45 isolates)			
< 0.125	4	2	4	3	3	2	5	5	21	14	39	39
≥ 0.125-0.9	5	6	5	5	7	8	5	5	24	28	6	5
≥ 1.0	0	1	0	1	2	2	2	2	0	3	0	1

* Underlined figures denote number of isolates with MIC's lower than the selection concentrations

† P = penicillin V & A = amoxycillin

Table 11.2(a) Number and identity of viridans streptococcus isolates speciated according to Kilian et al (1989) in different b-lactam exposure groups following selection in penicillin G only and penicillin G plus gentamicin

	Species according to antibiotic exposure			
	Unexposed, speciation only (n = 67)*	Unexposed, also susceptibility tested (n = 54)	Exposed to rheumatic fever prophylaxis (n = 41)	Exposed to ampicillin treatment (n = 9)
Streptococcus oralis	31	26	40	9
S. mitis biovar 1	22	15	5	0
S. mitis biovar 2	18	18	9	3
S. sanguis biovar 4	2	2	2	0
S. vestibularis (Coykendall)	2	2	2	0
S. sanguis biovar 1 or 3 †	1	1	0	0
S. gordonii biovar 2 or 3 †	2	1	4	0
S. sanguis biovar 2	1	1	0	0
Total	79	66	62 ‡	12 ‡

* n = number of subjects in each group

† Range of identification tests used did not allow for differentiation between biovars 1 and 3 of S. sanguis or biovars 2 and 3 of S. gordonii respectively

‡ The respective subgroups together form the E-group: 62 + 12 = 74 isolates

NOTE: This table is identical to Table 9.1

Table 11.2(b) Number and identity of viridans streptococcus isolates speciated according to Kilian et al (1989) in different b-lactam exposure groups following selection in penicillin G only broth

	Number of isolates within species according to antibiotic exposure			
	Unexposed, speciation only (n = 67)*	Unexposed, also susceptibility tested (n = 18)	Exposed to rheumatic fever prophylaxis (n = 33)	Exposed to ampicillin treatment (n = 9)
Streptococcus oralis	31	5 (21) †	32 (8)	9 (0)
S. mitis biovar 1	22	4 (11)	5 (0)	0 (0)
S. mitis biovar 2	18	9 (9)	8 (1)	3 (0)
S. sanguis biovar 4	2	1 (1)	2 (0)	0 (0)
S. vestibularis ||	2	0 (2)	2 (0)	0 (0)
S. sanguis biovar 1 or 3 ‡	1	1 (0)	0 (0)	0 (0)
S. gordonii biovar 2 or 3 ‡	2	0 (1)	4 (0)	0 (0)
S. sanguis biovar 2	1	1 (0)	0 (0)	0 (0)
		21 (45)	53 (9)	12 (0)
Total number of isolates	79	66 §	62§	

* n = number of subjects in each group

† Numbers in parenthesis denote the total number of isolates recovered from penicillin plus gentamicin selection broth

‡ Range of identification tests used did not allow for differentiation between biovars 1 and 3 of S. sanguis or biovars 2 and 3 of S. gordonii res

§ Total number of isolates identified in UE-group = 21 + 45 = 66; E-group = 53 + 9 + 12 = 74

|| Coykendall (1989) classification

Table 11.3(a) β-lactam susceptibility ranges of streptococcal species, selected in penicillin G broth only and in combination with gentamicin, from plaque of participants within antibiotic exposed (E) and unexposed (UE) groups

Classification of Kilian et al (1989)				Penicillin V (mg/l)			Amoxycillin (mg/l)		
Species	Group	No.	(%)	MIC-range	MIC-50	MBC-range	MIC-range	MIC-50	MBC-range
S. oralis	UE	26	(39.4)	0.03-1	0.06	0.03-1	≤0.007-0.25	0.03	≤0.007-0.5
	E	49	(66.2)	0.003-8	0.5	0.003-16	0.007-16	0.25	0.007-32
S. mitis b/v 1	UE	15	(22.7)	0.03-2	0.12	0.06-2	≤0.007-2	0.03	0.015-2
	E	5	(6.8)	0.5-1	1	1-2	0.25-2	1	0.25-2
S. mitis b/v 2	UE	18	(27.2)	0.015-0.5	0.12	0.015-1	≤0.007-0.5	0.06	0.015-1
	E	12	(16.2)	0.003-8	0.12	0.003-16	0.03-4	0.12	0.03-4
Other species*	UE	7	(10.6)	0.03-0.5	0.12	0.03-1	0.007-0.5	0.12	0.007-1
	E	8	(10.8)	0.06-1	0.25	0.06-2	0.03-1	0.12	0.06-2
All species	UE	66	(100)	0.015-2	0.12	0.015-2	≤0.007-2	0.03	≤0.007-2
	E	74	(100)	0.003-8	0.25	0.003-16	0.007-16	0.25	0.007-32

* S. sanguis biovar 2 (1 isolate in UE-group, 0 in E-group); S. sanguis biovars 1 or 3 (1,0); S. sanguis biovar 4 (2,2); S. vestibularis (2,2) S. gordonii biovars 2 or 3 (1,4)

Table 11.3(b) β-lactam susceptibility ranges of streptococcal species, selected in penicillin G only broth, from plaque participants in antibiotic exposed (E) and unexposed (UE) groups

Classification of Kilian et al (1989)				Penicillin V (mg/l)			Amoxycillin (mg/l)		
Species	Group	No.	(%)	MIC-range	MIC-50	MBC-range	MIC-range	MIC-50	MBC-range
S. oralis	UE	5	(23.8)	0.06-1	0.06	0.06-1	0.015-0.25	0.06	0.015-0.5
	E	41	(63.1)	0.015-8	0.5	0.015-8	0.007-16	0.5	0.007-32
S. mitis biovar 1	UE	4	(19.0)	0.12-2	0.25	0.25-2	0.015-2	0.12	0.015-2
	E	5	(7.7)	0.5-2	1	1-2	0.25-4	1	0.25-4
S. mitis biovar 2	UE	9	(42.9)	0.03-0.5	0.12	0.03-1	≤0.007-0.5	0.06	0.015-0.5
	E	11	(16.9)	0.003-8	0.12	0.003-16	0.03-4	0.12	0.03-4
Other species*	UE	3	(14.3)	0.06-0.5	0.12	0.12-0.5	0.12-0.5	0.25	0.12-1
	E	8	(12.3)	0.06-1	0.25	0.06-2	≤0.007-2	0.12	≤0.007-2
All species	UE	21	(100)	0.03-2	0.12	0.03-2	≤0.007-2	0.12	0.015-2
	E	65	(100)	0.003-8	0.5	0.003-16	0.007-16	0.25	0.007-32

* S. sanguis biovar 2 (1 isolate in UE-group, 0 in E-group); S. sanguis biovars 1 or 3 (1,0); S. sanguis biovar 4 (1,2); S. vestibularis (0,2) and/or S. gordonii biovars 2 or 3 (0,4)

Table 11.3(c) β-lactam susceptibility ranges of streptococcal species, selected in penicillin G plus gentamicin only broth from plaque participants within exposed (E) and unexposed (UE) groups

Classification of Kilian et al (1989)				Penicillin V (mg/l)			Amoxycillin (mg/l)		
Species	Group	No.	(%)	MIC-range	MIC-50	MBC-range	MIC-range	MIC-50	MBC-range
S. oralis	UE	21	(47)	0.03-0.25	0.06	0.03-1	≤0.007-0.12	0.015	≤0.007-0.12
	E	8	(89)	0.003-4	0.25	0.003-4	≤0.007-2	0.06	≤0.007-2
S. mitis b/v 1	UE	11	(24)	0.03-0.5	0.12	0.06-0.5	≤0.007-0.06	0.03	0.015-2
	E	0	(0)	-	-	-	-	-	-
S. mitis b/v 2	UE	9	(20)	0.015-0.5	0.12	0.015-1	0.015-0.12	0.06	0.015-1
	E	1	(11)	(0.06)†	-	(0.06)	(0.06)	-	(0.06)
Other species*	UE	4	(9)	0.03-0.25	0.25	0.03-0.25	0.007-0.25	0.06	0.007-0.25
	E	0	(0)	-	-	-	-	-	-
All species	UE	45	(100)	0.015-0.5	0.12	0.015-1	≤0.007-0.25	0.03	≤0.007-1
	E	9	(100)	0.003-4	0.12	0.003-4	≤0.007-2	0.06	≤0.007-2

* S. sanguis biovar 2 (0 isolate in UE-group, 0 in E-group); S. sanguis biovars 1 or 3 (0,0); S. sanguis biovar 4 (1,0); S. vestibularis (2,0) and/or S. gordonii biovars 2 or 3 (1,0)

† Respective MIC or MBC value

Table 11.4(a) Susceptibility in MICs to penicillin V and amoxycillin of isolates selected in penicillin G only and penicillin plus gentamicin broth from dental plaque of subjects exposed or unexposed to β-lactam antibiotics

Species	Unexposed to β-lactams (n = 66 isolates)					Exposed to β-lactams (n = 74 isolates)					
Kilian et al (1989) classification	susceptible *		intermediately resistant †		resistant ‡	susceptible *		intermediately resistant †		resistant ‡	
	P §	A	P	A		P	A	P	A	P	A
Streptococcus oralis	21 (81%)	25 (96)	5	1	0	18 (37)	22 (45)	23	21	8	6
S. mitis biovar 1	8 (53)	13 (87)	7	2	0	0	0	5	4	0	1
S. mitis biovar 2	10 (56)	14 (78)	8	4	0	7 (58)	7 (58)	4	4	1	1
S. sanguis biovar 4	1	0	1	2	0	1	0	1	2	0	0
S. vestibularis ||	2	2	0	0	0	0	1	2	1	0	0
S. sanguis biovar 1 or 3	0	1	1	0	0	0	0	0	0	0	0
S. gordonii biovar 2 or 3	0	1	1	0	0	2	2	2	2	0	0
S. sanguis biovar 2	1	0	0	1	0	0	0	0	0	0	0
Total	43	56	23	10	0	28	32	37	34	9	8
(%)	(65.1)	(84.8)	(34.8)	(15.2)		(37.8)	(43.2)	(50)	(45.9)	(12.2)	(10.8)

* MIC ≤ 0.12 mg/l for both penicillin V and amoxycillin

† MICs; 0.25-2 mg/l for both penicillin V and amoxycillin

‡ MIC ≥ 4.0 mg/l for both penicillin V and amoxycillin

§ P = penicillin V, A = amoxycillin

|| Coykendall (1989) classification

Table 11.4(b) Susceptibility in MICs to penicillin V and amoxycillin of streptococcal species selected in penicillin G only broth from dental plaque of subjects exposed or unexposed to β-lactam antibiotics

Species	Unexposed to β-lactams (n = 21 isolates)					Exposed to β-lactams (n = 65 isolates)					
Kilian et al (1989) classification	susceptible *		intermediately resistant †		resistant ‡	susceptible *		intermediately resistant †		resistant ‡	
	P §	A	P	A		P	A	P	A	P	A
Streptococcus oralis	3 (60%)	4 (80)	2	1	0	13 (32)	17 (41)	21	18	7	6
S. mitis biovar 1	1 (25)	2 (50)	3	2	0	0	0	5	4	0	1
S. mitis biovar 2	5 (56)	5 (56)	4	4	0	7 (64)	6 (55)	3	4	1	1
S. sanguis biovar 4	1	0	0	1	0	1	1	1	1	0	0
S. vestibularis ||	0	0	0	0	0	0	1	2	1	0	0
S. sanguis biovar 1 or 3	0	1	1	0	0	0	0	0	0	0	0
S. gordonii biovar 2 or 3	0	0	0	0	0	2	2	2	2	0	0
S. sanguis biovar 2	1	0	0	1	0	0	0	0	0	0	0
Total	11	12	10	9	0	23	27	34	30	8	8
(%)	(52.4)	(57.1)	(47.6)	(42.9)		(35.4)	(41.5)	(52.3)	(46.2)	(12.3)	(12.3)

* MIC ≤ 0.12 mg/l for both penicillin V and amoxycillin

† MICs; 0.25-2 mg/l for both penicillin V and amoxycillin

‡ MIC ≥ 4.0 mg/l for both penicillin V and amoxycillin

§ P = penicillin V, A = amoxycillin

|| Coykendall (1989) classification

Table 11.4(c) Susceptibility to penicillin V and amoxycillin of isolates selected in penicillin G plus gentamicin only broth from dental plaque of subjects exposed or unexposed to β-lactam antibiotics

Species	Unexposed to β-lactams (n = 45 isolates)					Exposed to β-lactams (n = 9 isolates)					
Kilian et al (1989) classification	susceptible *		intermediately resistant †		resistant ‡	susceptible *		intermediately resistant †		resistant ‡	
	P §	A	P	A		P	A	P	A	P	A
Streptococcus oralis	18 (86%)	21 (100)	3	0	0	5 (63)	5 (63)	2	3	1	0
S. mitis biovar 1	7 (64)	11 (100)	4	0	0						
S. mitis biovar 2	5 (56)	9 (100)	4	0	0	0	1	1	0	0	0
S. sanguis biovar 4	0	0	1	1	0						
S. vestibularis ‖	2	2	0	0	0						
S. gordonii biovar 2 or 3	0	1	1	0	0						
Total	32	44	13	1	0	5	6	3	3	1	0
(%)	(71)	(98)	(29)	(2)		(55.6)	(66.7)	(33.3)	(33.3)	(11.1)	(0)

* MIC ≤ 0.12 mg/l for both penicillin V and amoxycillin

† MICs; 0.25-2 mg/l for both penicillin V and amoxycillin

‡ MIC ≥ 4.0 mg/l for both penicillin V and amoxycillin

§ P = penicillin V, A = amoxycillin

‖ Coykendall (1989) classification

Table 11.5(a) Susceptibility patterns of viridans streptococci selected in penicillin G and penicillin G plus gentamicin broth from subjects (a) on high-dose β-lactam treatment, (b) on penicillin V rheumatic fever prophylaxis and (c) unexposed to β-lactam antibiotic

Isolates from β-lactam exposed groups	MIC50/MBC50 (mg/l)		MIC90/MBC90 (mg/l)		MIC/MBC range (mg/l)	
	Penicillin V	Amoxycillin	Penicillin V	Amoxycillin	Penicillin V	Amoxycillin
a) High β-lactam exposure group 12 isolates (9 subjects)	1/2	0.5/1	4/4	8/16	0.12/0.12-4/4	0.06/0.06-16/32
S. oralis I subgroup 9 isolates	2/2	1/1	4/4	8/16	0.12/0.12-4/4	0.06/0.06-16/32
S.mitis II subgroup 3 isolates	0.12/0.12	0.25/0.25	1/2	2/2	0.12/0.12-1/2	0.12/0.12-2/2
b) Rheumatic fever prophylaxis group 62 isolates (41 subjects)	0.25/0.5	0.25/0.25	4/4	4/4	0.003/0.003-8/16	0.007/0.007-8/16
c) Unexposed group 66 isolates (54 subjects)	0.12/0.12	0.03/0.03	0.5/0.5	0.25/0.25	0.015/0.015-2/2	≤0.007/≤0.007-2/2

Table 11.5(b) Susceptibility patterns of viridans streptococci selected in penicillin G only broth from subjects (a) on high-dose ampicillin treatment, (b) on penicillin V rheumatic fever prophylaxis and (c) unexposed to β-lactam antibiotics

Isolates from β-lactam exposed groups	MIC50/MBC50 (mg/l)		MIC90/MBC90 (mg/l)		MIC/MBC range (mg/l)	
	Penicillin V	Amoxycillin	Penicillin V	Amoxycillin	Penicillin V	Amoxycillin
a) High β-lactam exposure group 12 isolates (9 subjects)	1/2	0.5/1	4/4	8/16	0.12/0.12-4/4	0.06/0.06-16/32
S. oralis 9 isolates	2/2	1/1	4/4	8/16	0.12/0.12-4/4	0.06/0.06-16/32
S.mitis biovar 2 3 isolates	0.12/0.12	0.25/0.25	1/2	2/2	0.12/0.12-1/2	0.12/0.12-2/2
b) Rheumatic fever prophylasis group 53 isolates (33 subjects)	0.25/0.5	0.25/0.25	4/4	2/4	0.003/0.003-8/16	0.007/0.007-8/16
c) Unexposed group 21 isolates (18 subjects)	0.12/0.25	0.12/0.12	0.5/0.5	0.5/1	0.03/0.03-2/2	≤0.007/0.015-2/2

Table 11.6(a) Susceptibility (based on MICs) and numbers within susceptibility categories of streptococcal species from dental plaque of patients selected in penicillin G and penicillin G plus gentamicin broth from patients on (a) high ampicillin doses and (b) on penicillin V rheumatic fever prophylaxis

Species	(a) Short-term high-dose ampicillin recipients (n = 12 isolates)						(b) Prolonged penicillin V prophylaxis recipients (n = 62 isolates)					
	susceptible (≤0.12 mg/l)		intermediate resistance (0.25-2 mg/l)		resistant (≥4 mg/l)		susceptible (≤0.12 mg/l)		intermediate resistance (0.25-2 mg/l)		resistant (≥4 mg/l)	
	P	A	P	A	P	A	P	A	P	A	P	A
Streptococcus oralis	1	2	6	4	2	3	17	20	17	17	6	3
S. mitis biovar 1							0	0	5	4	0	1
S. mitis biovar 2	2	1	1	2	0	0	5	6	3	2	1	1
S. sanguis biovar 4							1	1	1	1	0	0
S. vestibularis (Coykendall)							0	1	2	1	0	0
S. sanguis biovar 1 or 3							0	0	0	0	0	0
S. gordonii biovar 1 or 3							2	2	2	2	0	0
S. sanguis biovar 2							0	0	0	0	0	0
Total	3	3	7	6	2	3	25	30	30	27	7	5
(%)	(25)	(25)	(58)	(50)	(17)	(25)	(40.3)	(48.4)	(58.4)	(43.5)	(11.3)	(8)

Table 11.6(b) Susceptibility (based on MICs) and numbers within susceptibility categories of streptococcal species from dental plaque of patients selected in penicillin G only broth on (a) high ampicillin doses and (b) on penicillin V rheumatic fever prophylaxis

Species	(a) Short-term high-dose ampicillin recipients (n = 12 isolates)						(b) Prolonged penicillin V prophylaxis recipients (n = 53 isolates)					
	susceptible (≤0.12 mg/l)		intermediate resistance (0.25-2 mg/l)		resistant (≥4 mg/l)		susceptible (≤0.12 mg/l)		intermediate resistance (0.25-2 mg/l)		resistant (≥4 mg/l)	
	P	A	P	A	P	A	P	A	P	A	P	A
Streptococcus oralis	1	2	6	4	2	3	12	15	15	14	5	3
S. mitis biovar 1							0	0	5	4	0	1
S. mitis biovar 2	2	1	1	2	0	0	5	5	2	2	1	1
S. sanguis biovar 4							1	1	1	1	0	0
S. vestibularis (Coykendall)							0	1	2	1	0	0
S. sanguis biovar 1 or 3							0	0	0	0	0	0
S. gordonii biovar 1 or 3							2	2	2	2	0	0
S. sanguis biovar 2							0	0	0	0	0	0
Total	3	3	7	6	2	3	20	24	27	24	6	5
(%)	(25)	(25)	(58)	(50)	(17)	(25)	(37.7)	(45.3)	(50.9)	(45.3)	(11.3)	(9.4)

Table 11.7(a) Significance of in vivo exposure to β-lactam antibiotics on susceptibility of dental plaque streptococci after selection in penicillin broth including the procedure involving penicillin G plus gentamicin

β–lactam exposure	Penicillin V				Amoxycillin			
	S *	R *	(%)	Significance (p-value) †	S	R	(%)	Significance (p-value)
Short-term ampicillin (Subgroup A)	3	9	(75)	A vs UE (0.012)	3	9	(75)	A vs UE (<0.0001)
Rheumatic fever prophylaxis (Subgroup RF)	25	37	(60)	A vs RF (NS ‡)	30	32	(52)	A vs RF (NS)
β-lactam exposure (Group E)	28	46	(62)	RF vs UE (0.008)	32	42	(57)	RF vs UE (<0.0001)
Not exposed (Group UE)	43	23	(35)	E vs UE (0.001)	56	10	(15)	E vs UE (0.001)

* S = susceptible and R = resistant, including intermediately resistant

† p-values are based on either Fisher Exact tests or Yates' correction of Chi-squared

‡ NS = not significant

Table 11.7(b) Significance of in vivo exposure to β-lactam antibiotics on susceptibility of dental plaque streptococci after selection in penicillin broth (without gentamicin)

β–lactam exposure	Penicillin V				Amoxycillin			
	S *	R *	(%)	Significance (p-value) †	S	R	(%)	Significance (p-value)
Short-term ampicillin (Subgroup A)	3	9	(75)	A vs UE (NS ‡; 0.24)	3	9	(75)	A vs UE (NS; 0.16)
Rheumatic fever prophylaxis (Subgroup RF)	20	33	(62)	A vs RF (NS)	24	29	(55)	A vs RF (NS)
β-lactam exposure (Group E)	23	42	(65)	RF vs UE (NS; 0.37)	27	38	(58)	RF vs UE (NS; 0.51)
Not exposed (Group UE)	11	10	(48)	E vs UE (NS; 0.26) §	12	9	(43)	E vs UE (NS; 0.32)

* S = susceptible and R = resistant, including intermediately resistant

† p-values are based on either Fisher Exact tests or Yates' correction of Chi-squared

‡ NS = not significant

§ Comparison of E-group vs US-group using MBCs >1.0 mg/l as a criterion of resistance yielded a statistically significant p-value of 0.02 in the case of penicillin V and approached significance in the case of amoxycillin (p = 0.06) as given in Section 11.3.5.2

Chapter 12

GENERAL DISCUSSION AND CONCLUSIONS

This chapter will be devoted to the discussion of selected topics relating to the present field and will include a substantial section on phenotypic tolerance with respect to biofilms in dental plaque and its relation to IE prophylaxis. It will conclude with a summary of the main findings produced by the **in vitro** selection model and suggest further studies emanating from the findings recorded in this dissertation

12.1 Rationale and candidate antibiotic agents for prophylaxis

"... Guidelines are not based on any controlled studies: there have never been a placebo-controlled human study on antibiotic prophylaxis for the prevention of IE" (Wahl, 1994)

None-the-less, guidelines have been proposed for the administration of prophylactic antibiotic cover prior to clinical procedures which may produce bacteraemia in persons regarded as being susceptible to the development of IE (BSAC, 1982; Special Report, 1984; BSAC, 1986; BSAC, 1990; Van der Bijl, 1992; AHA, 1990). Both parenteral and oral preparations of penicillin are widely used as IE-prophylaxis. Amoxycillin is the most commonly recommended ß-lactam agent and is administered orally in large doses pre-operatively (3 g for adults) with or without an additional post-operative administeration (Van der Bijl, 1992. Penicillin V

was in vogue in the 1970's and early 1980's but is no longer recommended as it offers no advantage over the better absorbed and more active amoxycillin. Before the inclusion of clindamycin in IE-prophylaxis recommendations, erythromycin was the only suitable alternative oral agent in patients allergic to penicillin. Recent efforts by the British Society for Antimicrobial Chemotherapy, the American Heart Association and others have simplified and standardised prophylaxis regimens (Van der Bijl, 1992; Wahl, 1994). In this study, the susceptibility to penicillin V, amoxycillin, erythromycin, roxithromycin, clindamycin and vancomycin of dental plaque-derived streptococci which survived selection in overnight broth culture, was determined and the isolates speciated

Various **in vivo** studies have shown that after the administration of most of the candidate antibiotics for purposes of prophylaxis, not only does bacteraemia still occur (Phillips et al, 1976; Shanson et al, 1978; Hess et al, 1983a), but that bacteria are viable (Phillips et al, 1976) and still sensitive to the administered antibacterial agent (Hall et al, 1993). The latter authors noted that administered prophylactic penicillins (penicillin V and amoxycillin) do not prevent the occurrence of post-extraction bacteraemia by highly susceptible organisms despite the fact that more than adequate blood levels were obtained. The present **in vitro** model, the validity of which was discussed in Chapter 11.5.9, clearly demonstrated the phenomenon of phenotypic tolerance. Furthermore, it offers a means for the evaluation of candidate antibiotics for IE prophylaxis

12.2 Spectrum of streptococcal species from the oral cavity

The streptococcal species isolated in this study have also been recovered from similar sites by other researchers (Longman et

al, 1991). S. oralis was found to be the predominant species in plaque that survived **in vitro** exposure to penicillin G (Tables 11.2a and 11.2b). S. sanguis and S. oralis are commonly found on (smooth surface enamel of) caries-inactive teeth (Marsh, 1992). Of note was the failure to demonstrate in plaque specimens the important oral streptococci S. mutans and S. milleri which are recognised causes of IE (Bayliss et al, 1983; Young, 1987; Franklin, 1992). The absence of hydrogen peroxide-negative colonies on the hydrogen peroxide indicator plates (Muller, 1984) lends credence to reports that at least one of these species, S. mutans, does not commonly colonise smooth dental surface sites sampled in this study but rather inhabits retentive (occlusal) surfaces (Ellen, 1982). An increase in numbers of this species has, however, been reported to be associated with dental caries (Marsh, 1992). Additionally, the presence of S. mutans is known to be rare in those with a reduced dietary intake of sucrose (Hardie, 1983) - a situation which may apply to many subjects who participated in this study. While our laboratory approach allowed for the identification of alpha-haemolytic S. milleri strains, none was found. However, any ß-haemolytic forms of this species-complex which includes strains of S. anginosus, S. intermedius and S. constellatus (Kilian et al, 1989; Facklam, 1984; French et al, 1989) would have been missed in the present study. S. milleri species were identified in another study (Longman et al, 1991) from plaque specimens gathered from similar areas, although their method of collection differed markedly from that employed in this study. The latter researchers were unable to culture S. mutans from gingival sulcus material. As its typical habitat was not sampled, the failure to isolate S. salivarius was not unexpected. Although **in vitro** exposure to penicillin G and this antibiotic plus gentamicin may have affected the isolation of this streptococcal species in the present study, it is likely that phenotypically- or genotypically tolerant bacterial cells from these species would have survived the selection procedure

12.3 Phenotypic tolerance

12.3.1 Definition and origin of term

Explanations of the survival phenomenon of susceptible strains in the presence of ß-lactam and other antibiotics have been offered by a number of authors (Hobby et al, 1942; Bigger, 1943; Greenwood, 1972; Tuomanen, 1986; Gilbert et al, 1990; Shockman et al, 1979; Brown et al, 1988). The response of bacterial clones to antibiotic pressure may be related to the growth status of the original (parent) streptococci within the plaque inoculum. Hobby et al noted in 1942 that the antibacterial action of penicillin only appeared to occur when "active multiplication" occurred. Gilbert et al (1990), in a minireview, postulated that asynchronous populations of bacteria contain balanced proportions of organisms in the various stages of growth associated with the normal cell cycle. As penicillin G and other ß-lactam antibiotics presumably affect only growing cultures (Tuomanen, 1986; Shockman et al, 1979), dormant or near-dormant bacteria in mitotic interphase would remain largely unaffected when subjected to these antibacterial agents, while those which were growing actively would either have survived their effects and multiplied if resistant, or have been destroyed if susceptible. Toumanen (1986) used the term "phenotypic tolerance" to describe this form of bacterial behaviour which allows what appears to be "susceptible" bacteria survive in the presence of antibiotic agents

12.3.2 Biolayers and biofilms

In a leading article, Brown, Allison and Gilbert (1988) suggested that slowly growing organisms within what was termed "biolayers"

may exhibit phenotypic tolerance. Evans and Holmes (1987) described these as being "microbial biofilms" consisting of "adherent, dense populations of micro-organisms and their associated exopolysaccharides...". Gilbert et al, 1990, who widened this concept to include chronic infections, called them "functional bacterial concortia which grow as adherent biofilms within extended polysaccharide glycocalices" and which are able to "condition their environment" within this "glycocalyx barrier". Although unintended, Gilbert's description of chronic infection would serve usefully as one of mature ("chronic") bacterial accumulations on dental hard tissue. Marsh (1992) termed dental plaque simply as "film(s) of micro-organisms found on the tooth surface embedded in a matrix of polymers of salivary and bacterial origin" while Hardie (1983) called this material a "soft, non-calcified accumulation of bacteria and their products" associated closely with the tooth surface and the gingival sulcus

Extracellular glycocalyx barriers synthesised by micro-organisms may protect susceptible bacteria by means of two mechanisms. Directly, by restricting or preventing the penetration into plaque of antibiotic agents (Gilbert et al, 1990; Nickel et al, 1985; Dall et al, 1987; Dall et al, 1990), or indirectly, by slowing cell growth-rate to reduce bacterial sensitivity to particular agents (Brown et al, 1988). Growth-rate and nutrient limitation or deprivation are "fundamental modulators of drug activity" (Brown et al, 1988). Glycocalyces may also shield bacteria from host defence mechanisms **in vivo** (Ashby et al, 1994). Similar conditions within residual material carried over in plaque specimens, existed in the artificial broth-culture selection system employed in this study ("captive" bacteria enclosed within bacterial glycocalyx polymers and adherent salivary proteins etc.)

However, Nickel et al (1985) expressed certain reservations about

the protection supposedly granted to biofilm bacteria as a result of cellular dormancy. They stated that an increased degree of "metabolic inactivity" (and antibiotic resistance) need not exist in bacteria within biofilms when compared to their planktonic (free-floating) counterparts. They also propose that (the bacteria within) biofilms may actually be more active biochemically than planktonic forms but support other workers by emphasising that decreased antibiotic susceptibility of biofilms (and in their experiment there appeared to be strong evidence for this) may be far more as a result of protection granted by the intercelluar exopolysaccharide matrix than that as a result of the role played by cellular metabolic activity. If this were to be the case in this **in vitro ex vivo** study, one would have to postulate that exopolysaccharide matrix protected bacterial cells in the **in vitro** situation where these bacteria were exposed to an active ß-lactam agent in the test tube. It would be interesting to explore whether mechanical shearing of matrix may reduce phenotypic tolerance in viridans streptococci

Supporting the concept of the importance of dormancy, it has been demonstrated in chemostat experiments that killing of certain bacteria by ß-lactam antibiotics may vary and that the availability of specific nutrients any affect growth rate (Cozens et al, 1986. Although the latter authors found that the elimination of **E. coli** by amoxycillin was not markedly affected in such a system compared with a number of other ß-lacatm antibiotics, the effect of this antibiotic on nutrient-deprived viridans populations eg. within (mature) "plaque-biofilm" environments, is undocumented

12.3.3 Antibiotic insensitivity of bacteria within plaque (biofilm) deposits

As bacteria which enter the blood-stream during surgical and other dental procedures originate from dislodged colonies of plaque-embedded viridans and other bacteria, the initial metabolic status of the organisms would presumeably reflect both the (mitotic) asynchronicity and variable antibiotic sensitivity to the administered antibiotics of the original plaque populations themselves. Consequently, a ß-lactam antibacterial agent may be incapable of destroying simultaneously all plaque-derived organisms in the blood-stream as dormant or near-dormant subpopulations of phenotypically tolerant organisms would survive its action - the presence also of protective extra-cellular substances (noted above) notwithstanding. This concept, which is corroborated by our findings (Tables 1, 3-6), questions the postulated efficacy of prophylactic antibiotic regimens used at present, irrespective of whether or not the organisms are labelled susceptible according to conventional **in vitro** testing criteria

Differing isolate susceptibilities and the selection which occurred within specific groups of isolates described previously would tend to support reservations about the effectivity of prophylaxis. Hall et al (1993) reported on the apparent inability of either penicillin V or amoxycillin to eliminate post-extraction streptococcal bacteraemia. In their **ex vivo** study, they (as in this investigation), found susceptible strains which survived the effects of the two ß-lactams. More than 90% of their 126 surviving strains had MICs $\leq$0.125 mg/l antibiotic

But IE prophylaxis does appear, somehow, to bestow significant clinical protection on experimental animals (Bernard et al, 1981; Glauser et al, 1983; Pujadas et al, 1986; Moreillon et al, 1986).

Mechanisms other than bactericidal efficacy are necessary to assist in the protective role (Hall et al, 1993; Glauser et al, 1982; Berney and Francioli, 1990) and are reported to include the sensitisation by antibiotics (penicillin) of bacteria to the action of certain protective host factors (Horne and Tomasz, 1980), bacterial growth inhibition (Moreillon et al, 1986; Brown et al, 1988) and the ability of certain antibiotics to interfere with bacterial adhesion to tissues (Scheld et al, 1981; Lowy et al, 1983). Other factors include antibiotic dose and frequency (Pujadas et al, 1987; Berney and Francioli, 1990), and bacterial inoculum size (Pujadas et al, 1986; Berney and Francioli, 1990; Pujadas et al, 1987)

Durack (1995) summarised the rationale for prophylaxis against endocarditis simply (? simplistically) as follows: "endocarditis usually follows bacteraemia; bacteraemia with organisms which may cause endocarditis follows certain health care procedures; these bacteria are usually sensitive to antibiotics; therefore, these agents should be given to those susceptible to the infection"

Hall et al (1993), in an effort to explain the unexpected survival of bacteria after the antibiotic prophylaxis, wrote that "the protective effect of prophylactically administered penicillins must be due to interference with crucial steps in the development of endocarditis - other than the transient bacteraemia that occurs"

12.3.4 **Use of animal models**

It is worth noting that inocula of fresh, actively growing bacteria (only) are administered in animal models used to assess the potential efficacy of agents employed in prophylaxis regimens (Dall et al, 1987; Dall et al, 1990; Bernard et al, 1981; Glauser

et al, 1983; Pujadas et al, 1986; Moreillon et al, 1986). Animal trials using bacteria in the exponential growth phase of their cell-cycle would be unable to adequately mimic clinical conditions which might prevail during dental procedures in humans. Dental biofilm is likely to contain the metabolically mixed, dormant and asynchronously mutiplying bacterial populations discussed above

Elements of bacterial populations which originate elsewhere in the body and find their way into the blood-stream would be similarly constituted (see below)

Microbiological properties of importance during the sterilisation of cardiac vegetations in IE-patients under treatment (intact, sessile bacterial colonies within enveloping matrices; Gilbert et al, 1990) differ materially and physically from those which prevail during prophylaxis in susceptible patients of bacteraemia-producing events (Brown et al, 1988) - although metabolically dormant or inactive bacteria would occur within both vegetations (Cremieux and Carbon, 1992) and suspended bacteraemic plaque fragments. To improve success of treatment of valvular infection, infected prostheses and other devices involving vasculature, some understanding of factors affecting bacterial constituents associated with conditions above may be useful (Ashby et al, 1994)

Incongruities of the sort raised by Hall et al (1993) remain unresolved in the case of IE prophylaxis. Fang et al (1993), in a prospective multicentre study, found that lengthened duration of antimicrobial therapy failed to prevent the development of endocarditis in bacteraemic patients with prosthetic valvular implants. Perhaps dormancy of existing subpopulations within established colonies of bacteria (biofilms) adherent to valve prostheses - and resistant to the onslaught of normally effective an-

tibiotics as a result of phenotypic tolerance - played a pivotal role in the progression to IE amongst their patients?. And how would these dormant subpopulations be able to actively infect these patients over a prolonged period while still in the presence of antibiotic?. Possibly enveloping serum-protein deposits on bacterial colonies may have slowed or hampered attempts at chemotherapeutic sterilisation of IE cardiac vegetations in a manner which was additional to and separate from that exerted by other (unrelated) protective films of an extracellular bacterial nature. Or factors such as the paradoxical effect (Handwerger and Tomasz, 1985) may have played a meaningful role. It is interesting that Tuomanen et al (1986) noted that the most abundant sources in the body of tolerant bacteria are those infection sites not easily accessible to the immune system. They give the deep seated infections, valvular endocarditis and meningitis as examples - all infections of sites in the body where some form of nutrient restriction to infecting organisms occurs (see below)

Generally, however, the desired result in compromised patients of antibiotic prophylaxis against bacteraemic elements involving a growing population of bacteria from any site in the body ought, by the very nature of the pathogenic mechanism involved, to be similar. Fragments in the blood of suspended asynchronous (mature) populations originating from mucosal surfaces of the gastro-intestinal, respiratory or female genital tracts (Wehrmacher, 1994), as examples, would be subject to the same fundamental objectives and principals of prophylaxis as those from within the oral cavity itself. On the basis of current concepts and practice, the origin within the body of culprit bacteria ought to be of little consequence when appropriate antibacterial agents and prophylaxis protocols are employed

The metabolic state of blood-borne or invasive bacteria, and

their reaction to antibacterial agents, is difficult to simulate in artificial (**in vitro**) systems as noted previously. In a mini-review, Gilbert et al (1990) highlighted possible behavioural properties of bacterial populations involved in chronic infections (as noted above, dental plaque accumulations fit the description of chronic infections admirably - especially long-standing ones). These cells often exhibit phenomema associated with starvation and dormancy under certain biological/ physiological conditions. Bacteria in infected valvular vegetations, for example, - and which exhibit stationary phase cultures (Brown et al, 1988), could react unpredictably to antibiotic activity and may also be tolerant (Tuomanen et al, 1986) in any event (see above)

Besides the effect which cellular metabolic activity may have on efficiency of antibiotic activity, the precise mechanism(s) of protection exerted by prophylactic agents themselves in the development of IE, and discussed in earlier chapters, are confusing and often contradictory. Tuomanen et al (1986) commented on the fact that although only bactericidal antibiotic agents are acceptible for the successful treatment of endocarditis, studies into the prevention of this disease have revealed that the prophylaxis process appeared to be far more complex than was originally supposed - especially when nonbactericidal concentrations of antibiotic were shown to have the desired protective effect as well. However, genetic tolerance to certain agents was also found to markedly lessen the effectivity of these agents when this factor was taken into account. Hess et al (1983), in their study on rabbits, found failure of penicillin to protect the animals to be closely related to the MBC of penicillin for each isolate. But, in 1973, Durack and Petersdorf were the first to show that bactericidal action appeared not to be essential in prophylaxis - subinhibitory concentrations of streptomycin successfully prevented the development of experimental IE. Later

in 1981, Bernard et al (1981) published similarly favourable results using vancomycin against a tolerant viridans strain. The latter authors were able to identify inhibition of bacterial adherence by vancomycin as a mechanism of protection rather than its notable killing action

12.3.5 The role of adhesion in the prevention of IE by antibiotics

Much of the confusion, some of which is highlighted above, was resolved to some degree in the **in vitro** and **in vitro** plus **in vivo** experiments of Scheld et al (1981) and Lowy et al (1983), respectively. The former worked on adherence of bacteria to fibrin-platelet complexes in the presence of vancomycin, penicillin and three other antibacterial agents, whilst the latter studied adherence behaviour to scarred heart tissue of tolerant S. sanguis pretreated with penicillin (with the resultant loss of lipoteichoic acid). Mechanisms explained by the latter two publications were noted by Tuomanen et al (1986) to apply to bacteria irrespective of their tolerance status, noting further that loss of adhesins occurs at concentrations above MICs and that these adhesin-related concentrations remain unchanged after the development of tolerance

Findings in this dissertation suggest that certain strains "primed" for antibiotic resistance in the E-group as a result of previous ß-lactam exposure, may flourish in the presence of additional antibiotic pressure at the time of IE prophylaxis. It is unlikely that such genotypically resistant strains will lose their adherence potential when exposed to antibiotics to which they are truly resistant

12.4 Some conclusions arising from the present in vitro selection model

12.4.1 Phenotypic tolerance

ß-Lactam antibiotics exert an irregular and unpredictable killing effect on metabolically non-uniform or mixed populations of normally sensitive bacteria. Only dividing cells are killed in such populations while nondividing or dormant cells would survive exposure to these agents to exhibit normal sensitivity patterns when re-exposed later to the same agents. This appears to be the case in the present study. The **in vitro** results here are in accordance with the presence of fully susceptible bacteraemic oral viridans streptococci in the **ex vivo** study conducted by Hall et al (1993)

12.4.2 Effect of previous antibiotic exposure on genotypic resistance

However, the study was able to go further. Bacterial selection in ß-lactam broths or broths with a ß-lactam plus gentamicin produced susceptibility behaviour which differed dependant upon the history of the original isolates with regard to prior contact with antibiotic agents. This was observed in the UE and E-group isolates recovered from penicillin G and gentamicin broth which exhibited greater resistance penicillin V and amoxycillin in the E-subgroups

12.4.3 Relative merits of candidate drugs for IE prophylaxis

The two macrolides, erythromycin and roxithromycin, plus clindamycin appeared to be the most active purely on MBCs alone, fol-

lowed by the two ß-lactam agents tested, and vancomycin. However, and this is especially important when the bactericidal action of agents is explored, when the emergence of persisters and the development of tolerance is taken into account, the rankings are reversed. On **in vitro** data alone, vancomycin would appear to be an ideal candidate. Its killing of non-growing Streptococcus pneumoniae (and therefore probably other alpha-haemolytic streptococci) may prove a great advantage (Tuomanen, 1986) and needs to be verified

Based on **in vitro** data, there appears to be little to choose between the macrolides and clindamycin for IE prophylaxis. The superior pharmacokinetic properties of roxithromycin would be very useful in the clinical situation where its far higher serum levels, increased activity in macrophages and fewer side-effects at therapeutic dosages are apparent

Other apparently useful information emerged from the study. Interestingly, with regard to the history of prior ß-lactam antibiotic exposure of a patient at risk of IE, penicillin V appeared to be a better candidate for IE prophylaxis use than amoxycillin in patients who had not had ß-lactams in the previous three months because of a lesser chance of persisters developing. On the other hand, in someone who had had antibiotics during this time, amoxycillin would appear to be the wiser choice for the same reason

12.5 **Concluding remarks**

The **in vitro** model used in the present studies demonstrated selection of both phenotypically tolerant and genotypically resistant strains of viridans streptococci. The findings generated by the model are likely to reflect the status of bac-

teraemic streptococci following procedures which would disturb dental plaque and lead to plaque-derived bacteria entering the blood circulation where they are exposed to candidate ß-lactam (or other) prophylactic agents. Some modifications to the model which are mooted in Chapter 11 (Section 11.5.9) may be considered in future studies

Because of their bactericidal activity against streptococci, the macrolides - especially roxithromycin based on its improved pharmacokinetics - and clindamycin would be good candidates for study in the model. Newer macrolides such as clarithromycin and azithromycin, with their superior pharmacokinetic properties, could also be studied, while vancomycin is perhaps the most deserving candidate for further investigation. Evidence reported by Tuomanen (1986) that this agent may be active against metabolically inactive streptococci can be tested in this model which hopefully will show reduced phenotypic tolerance against vancomycin

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