REVIEW

Australian Dental Journal 2005;50:(1):2-5

Why be down in the mouth? Three decades of research in oral microbiology

AH Rogers*

Abstract

This paper describes some of the work done in the author's laboratory over the past 35 years. The research covers the following areas: the physiology of oral streptococci and their interactions; the physiology of some Gram-negative anaerobes and their interactions in relation to periodontal diseases; preventing the major dental diseases; and the future of oral microbiology.

Key words: Oral microbiology, dental plaque, dental caries, periodontal diseases.

Abbreviations and acronyms: HSP = heat shock proteins; MS = mutans streptococci.

(Accepted for publication 23 December 2004.)

INTRODUCTION

From the beginning of the last century it has been accepted that bacteria play a pivotal role in the aetiology of dental caries. However, this was not proven until the early 1960s and oral microbiology, as a discipline, did not gain momentum until the mid- to late-1960s. At this time, attention was focused on *Streptococcus mutans*, a group of organisms now referred to as mutans streptococci (MS). These organisms are not only acidogenic but also aciduric and, together with lactobacilli, are the dominant bacteria found in most carious lesions.

The physiology of oral streptococci and their interactions

Early studies in our laboratory concentrated on the distribution of MS in carious lesions and in plaque from sound tooth surfaces.[1] We also began to examine some of the organism's physiological characteristics, among which was the production of bacteriocins. These are proteinaceous antibacterial substances produced by many different bacteria and are usually active only against closely-related strains. Utilizing the production of, and susceptibility to, MS bacteriocins, we devised a bacteriocin typing ("fingerprinting") scheme for various MS strains – especially those isolated from the plaque of individual members of family groups.[2] We were subsequently able to demonstrate that most children harboured MS transmitted by their mother and were thus among the first to show that caries is an infectious transmissible disease.[3] In a series of experiments with colleagues at the University of Nijmegen, The Netherlands, it was shown that bacteriocin activity in MS appears to confer on the organisms an ecological advantage in developing dental plaque. Briefly, a specific pathogen-free plaque ecosystem was established in the mouths of rats. In the early stage of plaque development, invading MS quickly became dominant. However, with time, as the ecosystem stabilized into a so-called "climax community", it became much more resistant to the invasion and establishment of MS. Nevertheless, bacteriocin-producing (bac^+) strains generally established at a higher level than bac^- strains.[4] This theme will be re-visited a little later in relation to the concept of "replacement therapy" as a caries-preventive measure.

The next phase of our studies stemmed from two now widely-accepted concepts. Firstly, that oral infectious diseases are often polymicrobial in nature and result from disturbances in the microbial ecology of the mouth; and secondly, that understanding this ecology – both in terms of the metabolic capabilities of the resident bacteria as well as their interactions – is crucial in the prevention and treatment of such diseases.

In studying the metabolic activities of oral streptococci and their subsequent interactions with one another, we elected to use continuous culture rather than the traditional batch culture technique. The latter is unnatural in the sense that it is a closed system in which a bacterium is cultured in a nutrient-replete environment where it can grow at rates approaching its maximum until lack of nutrient(s), biological space and the build-up of toxic metabolic end-products eventually cause growth to cease; the death-rate then begins to exceed the growth-rate and viable cell numbers decline. When growing *in vivo* (e.g. the mouth), the limitation

*Microbiology Laboratory, Dental School, The University of Adelaide, South Australia.

of one or more nutrients, usually fermentable carbohydrate, and the rate at which they are supplied reduces bacterial growth rates. The technique of continuous culture is one in which the culture medium, deficient in one or more nutrients, is continuously supplied at a rate which, in turn, dictates the bacterial growth rate. This can be adjusted to that which is thought to occur *in vivo*. Not only can the continuous culture system be used to mimic the *in vivo* situation but, by controlling environmental parameters such as pH, temperature and gaseous atmosphere, the effect of these individual variables on microbial behaviour can be investigated.

The co-existence of bacteria in natural environments can often be explained in terms of competition for growth-limiting substrate(s). Since the outcome of such competition depends upon relevant growth parameters, such as substrate affinity and biomass yield, in collaboration with colleagues in The Netherlands, we determined the growth parameters for some oral bacteria. Perhaps the most striking finding was that some of the non-MS plaque streptococci, such as *Streptococcus sanguis*, completely consumed arginine (Arg).[5] The utilization of this amino acid, as an alternative source of carbon and energy, releases ammonia – thus favouring a pH rise and would therefore counteract the harmful effect of low pH on the tooth surface. Indeed, we demonstrated, by both *in vitro* and *in vivo* experiments that such organisms can dominate over MS when sufficiently high levels of Arg and low levels of fermentable carbohydrate are present.[6,7] In a further series of experiments we demonstrated that, because of their peptidase activities, a number of common plaque organisms, such as *S. sanguis*, can obtain the metabolically important Arg from a variety of peptides and also from whole proteins, such as casein.[8,9] Studies such as these indicate that the microbial homeostasis maintained in most sites throughout the human body can be disturbed by a number of environmental factors, such as nutrient availability. One of the best-documented examples of this is the outgrowth of the cariogenic MS when diets are high in fermentable carbohydrate, especially sucrose. The relationship between plaque bacteria and the host in health and disease has been well-delineated in Philip Marsh's "Ecological Plaque Hypothesis".[10] Implicit in this hypothesis is the concept that disease can be prevented, not only by inhibiting potential pathogens but also by interfering with those environmental factors modulating the selection and enrichment of such bacteria.

While continuous culture techniques, in which bacteria growing in the planktonic state have provided much useful information on the interactions of microbial communities in dental plaque, the notion that such communities in nature actually exist as biofilms on surfaces has recently been re-born. This biofilm mode of growth is advantageous for bacteria in a number of ways. For example, when organized in biofilms, they are less susceptible to anti-microbials and more resistant to immune defence mechanisms. Indeed, the concentration of an agent that kills planktonic bacteria may have to be increased by 10-1000 times to have the same effect as those grown as a biofilm.[11] However, this does not negate studies on planktonic bacteria since it has been shown that the metabolic behaviour of dental plaque communities grown as biofilms is essentially the same as that of their planktonic counterparts.[12]

Gram-negative anaerobes – periodontal microbiology

During the 1990s we focused our attention on some of the Gram-negative anaerobic bacteria thought to be involved in the aetiology of periodontal diseases. In particular, we investigated various aspects of the growth and metabolism of *Fusobacterium nucleatum*, a member of various bacterial consortia associated with these diseases. Moreover, in the development of dental plaque, and as this community becomes more complex, it is clear that *F. nucleatum* plays an important role as a "microbial bridge", facilitating co-aggregation between 'early' and 'late' colonizers.[13] We found that, when grown in chemically-defined media, the organism was able to ferment either simple sugars or amino acids to obtain energy. Much higher biomass levels were obtained from glucose, reflecting the fact that mixed acid fermentation of this sugar yields ca. 3ATP/mol of substrate while only 1ATP/mol is obtained from amino acid fermentation.[14] Also stemming from these studies was the observation that butyric acid, produced by the fermentation of both sugars and amino acids, is a potent inhibitor of human gingival fibroblast proliferation.[15] The metabolic versatility of *F. nucleatum* may, in part, explain its occurrence in widely-different oral niches. Further studies demonstrated that the organism could attack small peptides, releasing key energy-yielding amino acids.[16] One of the enzymes responsible for peptide cleavage was subsequently purified and found to be a cobalt-activated metallo-aminopeptidase. In addition, the importance of this enzyme in the nutrition of *F. nucleatum* was shown by the finding that specific enzyme inhibitors prevented growth in a complex medium but not in one which was chemically-defined; that is, containing free amino acids.[17] The organism appeared unable to attack proteins such as albumin – in plentiful supply in the (diseased) subgingival environment – but the endopeptidase activity necessary to provide small peptides would be provided by organisms such as *Porphyromonas gingivalis* with which it is often associated in diseased sites. This organism, strongly implicated in the aetiology of periodontal diseases because of its manifold virulence factors, is asaccharolytic and so obtains energy solely from amino acid fermentation. Working with colleagues at The University of Melbourne School of Dental Science, we found that the key amino acids appear to be serine (Ser), threonine (Thr) and Arg in

free or peptide form. We also characterized the Ser/Thr uptake system.[18]

Due to its extensive array of peptidases, *P. gingivalis* can grow on whole proteins such as haemoglobin and albumin. Thus, the efficiency with which peptides are utilized might be a key to the microbial ecology of Gram-negative anaerobes in the gingival sulcus.

Apart from its direct implication in periodontal diseases, we decided to investigate the possibility that the presence of *F. nucleatum* is essential for the establishment and survival of other periodontopathic bacteria in subgingival plaque, possibly protecting them from oxidative damage. It was found that the organism could grow and maintain a relatively low redox potential in the presence of 40 per cent oxygen in air – due mainly it seemed, to increased NADH oxidase activity.[19] Moreover, *F. nucleatum* was able to support the growth of *P. gingivalis* in aerated and carbon dioxide-depleted environments in which *P. gingivalis*, as a monoculture, was unable to survive.[20] However, the organism does possess some basic mechanisms enabling it to cope with moderate or transient oxidative stress. For example, we found that haemin, produced by the proteolysis of haemoglobin, can provide anti-oxidant protection.[21] Nevertheless, it relies on organisms such as *F. nucleatum* for its survival and replication in highly oxygenated environments. Thus, from an ecological viewpoint, control of species such as *F nucleatum*, which support the growth of pathogens such as *P. gingivalis*, might radically alter the pathogenic ecosystem in favour of the host.

Apart from oxidative stress, bacteria growing in or on the human body are, as previously mentioned, subjected to other stresses imposed by changes in environmental conditions. Such changes lead to the synthesis of proteins known as heat shock proteins (HSP). Two of these, GroEL (HSP60) and DnaK (HSP70), act as molecular chaperones in the assembly and folding of proteins and are considered to be immunodominant antigens in many human pathogens growing *in vivo*. Because they share common epitopes, they cross-react with HSP from human cells in which they also play a role in protecting from damage in response to stress stimuli. The presence of these common epitopes has led to the notion that chronic inflammatory reactions, such as periodontal diseases, may be due to defects in the body's ability to regulate anti-self compatibility. It has also been suggested that cross-reactive HSP responses may contribute to cardiovascular disease progression.[22] Since the complete gene sequence of *F. nucleatum* is known, we can define patterns of expression of gene products (proteins). This is the emerging science of "Proteomics", the biophysical technologies of which we have employed to show that when *F. nucleatum* is grown at elevated pH, such as occurs in the diseased periodontal pocket, both GroEL and DnaK are up-regulated. Further studies along these lines may reveal up- or down-regulation, according to environmental conditions, of other gene products potentially involved in virulence.

Preventing caries and periodontal diseases

In relation to caries prevention, public health measures are clearly the most effective since they avoid the need to rely on patient compliance. At present, water fluoridation remains the only effective choice. Of many other approaches that have been suggested, most are based on the thesis that MS are the major group of cariogenic bacteria. In this context, the reduction of MS numbers in first-time mothers, by strict adherence to oral hygiene, together with dietary advice, has been shown to delay or prevent transmission of these organisms to their off-spring – with a concomitant and significant reduction in caries.[23]

Methods for specifically reducing MS levels include the use of the alcohol sugar (substitute) xylitol. However, for a number of reasons, including cost, its widespread use is not practicable. Much the same seems to apply to a number of other sugar substitutes and food additives. In relation to the chemical control of plaque, chlorhexidine, one of the most widely recommended, has limited effectiveness. In addition, it does not have a narrow anti-microbial spectrum. Thus, to minimize the risk of interfering with the overall microbial ecology of the mouth, the identification, in pathogens, of genes which are vital for their survival *in vivo* may facilitate the design of specific inhibitors of the gene product(s) based on the deduced properties of the protein(s). The incorporation of such compounds in toothpastes could be a useful public health measure – at least in developed countries.

A novel approach to reducing caries is that of "replacement therapy". Briefly, the idea is to colonize the mouth with an MS strain, genetically engineered to be defective in lactic acid production and to express elevated levels of mutacin, an anti-MS bacteriocin. In experimental animals, such a strain aggressively displaces indigenous MS and is of very low cariogenicity.[24] This probiotic concept has, of course, been applied to the gastro-intestinal tract where ingested lactobacilli and bifidobacteria, found in large numbers in commercial yoghurt, have been shown to control pathogens such as *Salmonella, Shigella* and *E. coli*.[25]

Some anti-caries vaccines appear to be effective in animal models[26] but have not yet successfully been tested in humans. Apart from the choice of the appropriate immunogen(s), one of the problems with such vaccines lies in optimizing the timing and route of administration. In relation to periodontal diseases, it is doubtful whether a vaccine targeted to a specific pathogen, such as *P. gingivalis* would have a marked impact since consortia of organisms, possessing various virulence factors, have been implicated.

The future

Oral microbiology, as a discipline, has made significant scientific contributions, particularly in the areas of microbial attachment to surfaces and to the subject of mucosal immunology. In a more general way,

attention has been focused primarily on characterizing dental plaque organisms and their potential roles in the major dental diseases. While the more recent application of molecular genetic techniques has greatly increased our knowledge of many of these bacteria, laboratory-accumulated information has not yet been translated into dental practice in terms of having a significant impact on these diseases. However, using such techniques it is probable in the future that many previously-unidentified but potentially pathogenic oral bacteria will be detected. As mentioned above, the science of proteomics may help to identify specific virulence determinants that may only be expressed *in vivo*. Even so, in order to understand disease aetiology, we need to know how pathogens interact with other indigenous, but non-harmful, bacteria: the "ecological approach".

In a broader context, periodontal microbiology and immunology may well assume greater significance in the future, since periodontitis has been linked to diseases such as atherosclerosis, pre-term birth abnormalities and arthritis.

ACKNOWLEDGEMENTS

I am indebted to John Thonard, who, in late 1967, took me on as Post-doctoral Fellow and continued to support and encourage me over the years. Laboratory colleagues whose help and friendship have been invaluable include Peter Zilm, Neville Gully and Andrea Pfennig, together with a number of post-graduate students. Interstate researchers whose collaboration has been most stimulating include Eric Reynolds and Stuart Dashper from The University of Melbourne School of Dental Science. International collaborators whose expertise and friendship are highly valued include Philip Marsh and John Smalley from the UK and Hans van der Hoeven and Frans Mikx from The Netherlands. Financial support from local and national granting bodies has been much appreciated; in particular the continued support of the Australian Dental Research Foundation Inc.

REFERENCES

1. Rogers AH. The proportional distribution and characteristics of streptococci in human dental plaque. Caries Res 1969;3:238-248.
2. Rogers AH. The bacteriocin patterns of strains belonging to various serotypes of Streptococcus mutans. Arch Oral Biol 1976;21:243-249.
3. Davey AL, Rogers AH. Multiple types of the bacterium Streptococcus mutans in the human mouth and their intra-family transmission. Arch Oral Biol 1984;29:453-460.
4. Rogers AH, van der Hoeven JS. Stability of the resident microflora and the bacteriocinogeny of Streptococcus mutans as factors affecting its establishment in specific pathogen-free rats. Infect Immun 1979;23:206-213.
5. Rogers AH, van der Hoeven JS, Zilm PS, de Jong MH. Estimation of growth parameters for some oral bacteria grown in continuous culture under glucose-limiting conditions. Infect Immun 1986;52:897-901.
6. Rogers AH, Zilm PS, Gully NJ. The utilisation of arginine by oral streptococci grown glucose-limited in a chemostat. FEMS Microbiol Letts 1986;37:9-13.
7. Rogers AH, Zilm PS, Gully NJ. The influence of arginine on the co-existence of Streptococcus mutans and Streptococcus milleri in glucose-limited mixed continuous culture. Microb Ecol 1987;14:193-202.
8. Rogers AH, Pfenning AL, Gully NJ, Zilm PS. Factors affecting peptide catabolism by oral streptococci. Oral Microbiol Immunol 1991;6:72-75.
9. Rogers AH, Reynolds EC. The utilization of casein and amino acids by Streptococcus sanguis P_4A_7 in continuous culture. J Gen Microbiol 1990;136:2545-2550.
10. Marsh PD. Sugar, fluoride, pH and microbial homeostasis in dental plaque. Proc Finn Dent Soc 1991;87:515-525.
11. Lewis K. Riddle of biofilm resistance. Antimicrob Agents Chemother 2001;45:999-1007.
12. Bradshaw DJ, Marsh PD, Watson GK, Allison C. Role of Fusobacterium nucleatum and coaggregation in anaerobic survival in planktonic and biofilm oral microbial communities during aeration. Infect Immun 1998;66:4729-4732.
13. Kolenbrander PE, London J. Adhere today, here tomorrow; oral bacterial adherence. J Bacteriol 1993;175:3247-3252.
14. Rogers AH, Chen J, Zilm PS, Gully NJ. The behaviour of Fusobacterium nucleatum chemostat-grown in glucose and amino acid-based chemically defined media. Anaerobe 1998;4:111-115.
15. Bartold PM, Gully NJ, Zilm PS, Rogers AH. Identification of components in Fusobacterium nucleatum chemostat-culture supernatants that are potent inhibitors of human gingival fibroblast proliferation. J Periodontal Res 1991;26:314-322.
16. Rogers AH, Gully NJ, Pfennig AL, Zilm PS. The breakdown and utilization of peptides by strains of Fusobacterium nucleatum. Oral Microbiol Immunol 1992;7:299-303.
17. Rogers AH, Gunadi A, Gully NJ, Zilm PS. An aminopeptidase nutritionally important to Fusobacterium nucleatum. Microbiology 1998;144:1807-1813.
18. Dashper SG, Brownfield L, Slakeski N, Zilm PS, Rogers AH, Reynolds EC. Sodium ion-driven serine/threonine transport in Porphyromonas gingivalis. J Bacteriol 2001;183:4142-4148.
19. Diaz PI, Zilm PS, Rogers AH. The response to oxidative stress of Fusobacterium nucleatum grown in continuous culture. FEMS Microbiol Letts 2000;187:31-34.
20. Diaz PI, Zilm PS, Rogers AH. Fusobacterium nucleatum supports the growth of Porphyromonas gingivalis in oxygenated and carbon-dioxide-depleted environments. Microbiology 2002;148: 467-472.
21. Diaz PI, Zilm PS, Wasinger V, Corthals GL, Rogers AH. Studies on NADH oxidase and alkyl hydroperoxide reductase produced by Porphyromonas gingivalis. Oral Microbiol Immunol 2004;19:137-143.
22. Okuda K, Kato T, Ishihara K. Involvement of periodontopathic biofilm in vascular diseases. Oral Dis 2004;10:5-12.
23. Kohler B, Andreen I. Influence of caries-preventive measures in mothers on cariogenic bacteria and caries experience in their children. Arch Oral Biol 1994;39:907-911.
24. Hillman JD. Genetically modified Streptococcus mutans for prevention of dental caries. Antonie Van Leeuwenhoek 2002;82:361-366.
25. Ouwehand AC, Salminen S, Isolauri E. Probiotics: an overview of beneficial effects. Antonie Van Leeuwenhoek 2002;82:279-289.
26. Smith DJ, Taubman MA. Potential for glycosyltransferase-based synthetic peptides in a dental caries vaccine. Adv Exp Med Biol 1995;371:1157-1159.

Address for correspondence/reprints:
AH Rogers
Visiting Research Fellow
Dental School
The University of Adelaide
North Terrace
Adelaide, South Australia, 5005
Email: tony.rogers@adelaide.edu.au