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ADRF Research Grant Abstracts

Maternal antioxidant supplementation does not reduce the incidence of phenytoin-induced cleft lip and related malformation in rats

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There is considerable evidence that phenytoin-induced birth defects in the rat are a consequence of a period of bradycardia and hypoxia in the embryos. Experiments were designed to test the hypothesis that phenytoin-induced birth defects result from free-radical damage to the embryos during the reoxygenation period post-hypoxia. Female rats (>9 per group) were fed either a control diet or a diet high in antioxidants (vitamins C and E and coenzyme Q10) both before and during pregnancy and were then given a teratogenic dose of phenytoin (180mg/kg) on gestational day 11 (GD11). The rats were killed on GD20 and the fetuses examined for malformations.

The initial results showed that the antioxidant diet had a significant protective effect, with far fewer antioxidant-group fetuses showing cleft lip or maxillary hypoplasia compared with the control group. However,

this result was confounded by reduced food intake by the rats fed the antioxidant diet and a significantly lower maternal body weight at the time of phenytoin administration. Since the phenytoin was administered by intraperitoneal injection the control rats received higher absolute doses of phenytoin and it is speculated that this results in higher fetal exposure. A second experiment, in which the rats were pair-fed, failed to demonstrate any protective effect of the high antioxidant diet. These results do not support the reoxygenation hypothesis for phenytoin teratogenesis. An alternative explanation would be hypoxia-induced transcription-related changes resulting in cell cycle arrest and apoptosis.

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The spatial distribution of lead in teeth as a biomarker of prenatal and neonatal lead exposure

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Lead is amongst the most hazardous toxins in our environment and continues to pose a significant health risk. Environmental exposure to lead has been implicated in a number of health disorders including neuropsychological impairment, hypertension, anaemia and dental caries. While it is known that susceptibility to the toxic effects of lead is greatest during early development, no suitable retrospective biomarker has been developed to determine lead exposure experienced during the prenatal and neonatal periods. In the present study, it was hypothesized that the spatial distribution of lead in enamel and coronal dentine of human primary teeth would provide temporal information regarding the pattern and intensity of lead exposure experienced during the prenatal and neonatal periods of development.

A Wistar rat model was firstly used to study the spatial distribution of lead in teeth at a fixed level of lead exposure. The high-resolution map of lead in teeth, developed in this experiment, showed four distinct zones of lead distribution. High concentrations of lead (>88µg/g) were found in the outer layers of enamel up to a depth of approximately 40µm from the surface. The interior of enamel had substantially lower concentrations of lead (<39µg/g). In dentine, high concentrations of lead (>138µg/g) were observed in the region, approximately 70-100µm in width, immediately adjacent to the pulp. This map of lead distribution in enamel and coronal dentine served as the basis for a larger animal model where female Wistar rats were exposed to varying doses of lead in drinking water during the periods of gestation and lactation. Lead

concentrations in the prenatally and postnatally formed enamel and dentine of the rat pups were measured using a nuclear microprobe and were compared to maternal intake of lead during the corresponding time periods. The spatial distribution of lead in coronal dentine adjacent to the dentinoenamel junction showed a strong positive correlation with lead intake ($r>0.68$). Lead levels in enamel, however, did not mirror the pattern of maternal lead consumption ($r<0.42$). In addition to mapping the spatial distribution of lead in enamel and coronal dentine, lead concentrations were also determined in the whole first molar teeth, blood, bone and key organs (liver, kidney and brain) using inductively coupled plasma-mass spectrometry. Tooth-lead levels consistently showed a positive correlation with the organ-lead levels ($r>0.83$), which was greater than the correlation between blood- and organ-lead levels ($r<0.73$).

This methodology was subsequently applied in a pilot human study. Primary teeth, blood-lead and environmental data were collected from 10 children living in the Broken Hill region of New South Wales, Australia. The lead concentrations in prenatally and

postnatally formed enamel and dentine, measured using laser-ablation inductively coupled plasma-mass spectrometry, were compared to blood-lead levels taken at birth and around the age of one year. Lead concentrations in dentine reflected the blood-lead concentrations. Enamel-lead levels, however, did not agree with the lead concentrations in blood or coronal dentine. Furthermore, when participants were categorized according to their blood-lead levels, dentine-lead concentrations of those participants with high blood-lead levels ($>10\mu\text{g/dL}$) were significantly greater than dentine-lead concentrations of participants with low blood-lead levels ($<10\mu\text{g/dL}$) ($p<0.05$). Enamel-lead concentrations did not show a statistically significant difference between the two groups ($p<0.13$). The results of the animal experiments and pilot human study support the applicability of dentine-lead levels as a biomarker for prenatal and neonatal lead exposure.

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The role of antigen-presenting cells in regulation of immune responses against *Candida albicans* infection

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We demonstrated substantial differences between mouse strains in the cytokine profiles produced after infection with *Candida albicans in vivo*, and after stimulation of separated lymphocyte subsets *in vitro*. IL-10 was produced early in CBA/CaH mice, and both IL-10 and IFN- γ were present later in the course of infection. In BALB/c mice, IFN- γ was dominant early,

and IL-10 only appeared when the infection was being cleared. Cells derived from cervical and submandibular lymph nodes after oral infection showed little antigen-specific cytokine production. In CBA/CaH mice, IL-10 was shown to be produced by a population of CD4⁺, CD25^{hi} lymphocytes, as well as by CD11b⁺ cells, most probably macrophages. T cell lines were successfully established from both spleen and lymph nodes, and showed similar patterns of cytokine production to those of cells assayed *ex vivo*.

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Genetic regulation of *Porphyromonas gingivalis* infection

RB Ashman, GJ Seymour*

Although BALB/c mice are susceptible to infection with *Porphyromonas gingivalis*, crosses between susceptible BALB/c and resistant C57Bl/6 mice produced susceptible offspring, whereas crosses between BALB/c and resistant A/J mice gave resistant progeny. These results suggested that A/J mice possessed dominant resistance alleles, whereas relevant resistance alleles in C57Bl/6 mice were recessive. We explored this further by backcrossing [BALB/c x C57Bl/6] F1 hybrid mice to BALB/c mice carrying the dominant (susceptible) allele of the C57Bl/6 resistance gene, and resistant [BALB/c x A/J] F1 hybrids to BALB/c mice that also carry the recessive allele of the A/J resistance gene. We expected that this breeding

strategy would produce equal numbers of susceptible and resistant progeny in each experimental group.

Of the 53 [BALB/c x A/J] x BALB/c backcross mice, 16 developed lesions averaging $2.5\text{mm} \pm 0.3\text{SE}$ in size after subcutaneous challenge with *P. gingivalis*. There were 59 [BALB/c x C57Bl/6] x BALB/c backcross mice, of which 13 developed lesions averaging $4.6\text{mm} \pm 0.4\text{SE}$. The lesion sizes were significantly different, indicating that the resistance gene carried by the C57Bl/6 strain was weaker in its effect than that carried by the A/J strain. Contrary to prediction, only about one quarter of the mice in each group demonstrated the susceptible phenotype – a result consistent with the actions of two independent genes. This may indicate heterozygosity of the alleles carried by the parental strains, but further breeding experiments would be required to clarify the situation.

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Periodontal development and inflammation in syndecan-1 knock-out mice

PM Bartold*

Syndecan-1 is a molecule located on the surface of many cells. It plays a significant role during the development of many tissues and is also an important regulator of inflammatory cell function.

In this project mice which lack the gene to express syndecan-1 to study its role in the development of periodontal tissues and in periodontal inflammation were used.

All wild-type and knock-out specimens were obtained from our collaborator in Germany. The heads were subjected to micro-CT analysis and morphometric analyses. The samples were decalcified and are currently being processed for histological analyses.

Micro CT scanning and morphometric assessments indicate that the development of the orofacial and

dental tissues in the knock-out animals do not differ significantly from the wild-type specimens. This is encouraging as it means that any changes observed during induction of experimental inflammation can be attributed to the molecular defect rather than morphological changes.

The model for induction of infectious periodontitis in the mouse has been established and, pending suitable breeding, we hope to commence studies investigating the effect of the absence of syndecan-1 in periodontal disease in the next few months.

If syndecan-1 acts as a negative regulator of developmental processes or leukocyte-mediated inflammatory responses it could have use as a target for the manipulation of cells during tissue regeneration or inflammation. These findings have significance to periodontal development and inflammation and also to inflammation in general.

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Isolation and characterization of stem cells from the periodontal ligament

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This project investigated the presence of mesenchymal stem cells in the periodontal ligament and progressed in three fronts: (i) isolation and cell culture of clonogenic ovine periodontal ligament stem cells (PDLSC); (ii) small animal xenogeneic experimentation to determine the ability of ovine PDLSC to produce components of the periodontium such as periodontal ligament and cementum; and (iii) commencement of larger animal studies in preparation for cell transplantation into periodontal defects.

We were able to successfully isolate, culture and expand cells from the sheep periodontal ligament. Early characterization using an array of stem cell associated antibodies indicates that the PDLSC possess the likely characteristics of mesenchymal stem cells.

These cells have been incorporated into hydroxyapatite/tricalcium phosphate scaffolds and implanted subcutaneously into the backs of NOD/SCID mice. Histological assessment after eight weeks has shown that the implanted cells have the capacity for significant amounts of mineralized tissue with the

morphological feature of both bone and cementum. Currently immunohistochemical analyses are underway to determine the molecular composition of these tissues. We were able to obtain from a collaborator in the USA two antibodies which recognize cementum specific proteins which will significantly strengthen our hypothesis that periodontal ligament stem cells have the potential to produce cementum.

We have now commenced large animal studies using sheep whereby periodontal defects are created around the mandibular second molars. Pilot studies were performed to perfect the technically difficult surgery in order to establish a protocol which results in minimal trauma to the animals as well as creating standardized periodontal defects. Surgical implantation of potential scaffold materials in the form of teflon membranes has been carried out and we now await histological assessment of this phase of the study.

Published: Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY, Shi S. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 2004;364:149-155.

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Molecular mediators of bone loss in periodontitis

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Periodontal disease is the most common bone resorption pathology in humans. Successful performance of this project will not only lead to better understanding of the molecular mechanisms of this poorly understood disease, but will also identify potential treatments.

This study tested the hypothesis that elevated levels of receptor activator of NF κ B ligand (RANKL) are associated with reduced osteoprotegerin (OPG) in inflamed periodontal tissues associated with periodontitis. Accordingly the aims of this study were to identify the presence of both RANKL and OPG in normal and inflamed periodontal tissues and to

determine how the levels of these might change with inflammation associated with periodontitis.

Immunohistochemical staining of normal and inflamed human periodontal tissues has been completed for osteoprotegerin, RANKL and TRAIL. We have demonstrated that OPG and RANKL are expressed in biopsies of inflamed periodontitis lesions. In addition we have also found that another ligand for OPG, TRAIL is expressed in both types of tissue (although not from the same patient). In these studies we have noted that OPG decreases with inflammation, RANKL increases with inflammation and TRAIL increases with inflammation.

These findings may be of considerable significance in light of OPG's ability to block the activity of TRAIL (and vice versa) and TRAIL's anti-inflammatory properties.

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Modulation of the immune response to *Porphyromonas gingivalis* in a mouse model

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Periodontal disease results from the inflammatory response to bacteria present in dental plaque. This results in an inflammatory cell infiltrate into the periodontal lesion which consists of lymphocytes and macrophages. We have hypothesized that T-lymphocytes predominate in a stable lesion while B lymphocytes and plasma cells increase in number in a progressive lesion. One suggestion is that the T lymphocytes with a Th1 cytokine profile may be the predominant T cell in early/stable lesions. While the dominance of B cells and plasma cells in progressive lesions may suggest a role for Th2 cells, B lymphocytes produce specific antibodies that the plasma cells secrete. The antibodies have a protective function which includes prevention of bacterial adherence, inactivation of bacterial toxins and opsonins for phagocytosis by neutrophils and macrophages. Conflicting results have been reported that show higher levels of specific antibodies to *P. gingivalis* in patients with disease when compared to gingival health. So, whether elevated levels of antibody to specific bacteria can eliminate the pathogen is still questionable. Antibodies may not eliminate pathogens because of poor antigenicity or poor antibacterial properties. Therefore the aim of this study was to use a mouse model to investigate the modulatory effects on the specific IgG1 (Th2) and IgG2a (Th1) subclass IgG antibody responses and neutrophil phagocytosis of opsonized bacteria of serum from mice successively immunized with *P. gingivalis* and *Fusobacterium nucleatum*.

The project was approved by the institutional animal ethics review committee. BALB/c female mice were divided into five groups (six per group). Group 1 mice received 1×10^8 viable *F. nucleatum* intraperitoneally (ip) in saline weekly for four weeks; Group 2 mice, 1×10^8 viable *P. gingivalis*; Group 3 mice were injected with 1×10^8 viable *P. gingivalis* weekly for two weeks followed by 1×10^8 viable *F. nucleatum* weekly for two weeks; Group 4 mice were injected with 1×10^8 viable *F. nucleatum* weekly for two weeks followed by 1×10^8 viable *P. gingivalis* weekly for two weeks. Group 5 mice were sham immunized with weekly injects for the four weeks. One week after the final immunizations mice were killed and blood samples collected. The serum was used to determine specific IgG subclass antibody levels and for opsonization of *F. nucleatum* and *P. gingivalis* in a chemiluminescence assay. The results were analysed by multivariate analysis of variance using the general linear model used to test the

differences in specific levels of anti-IgG1 and IgG2a *F. nucleatum* and *P. gingivalis* antibodies and neutrophil chemiluminescence within and between each of the five groups. Selected pairs of groups were then tested for significance using the Students *t* test.

The results of this study showed that while IgG1 and IgG2a subclasses were induced in all immunized groups, there was a tendency towards an IgG1 response in mice immunized with *P. gingivalis* alone. Mice immunized with *F. nucleatum* followed by *P. gingivalis* induced significantly higher anti-*P. gingivalis* IgG2a levels rather than IgG1. The results also showed that the peak chemiluminescence of neutrophils phagocytosis of *F. nucleatum* opsonized with immune serum occurred at 10 minutes, whereas the peak activity of *F. nucleatum* opsonized with non-immune serum or of *F. nucleatum* that was not opsonized was delayed until 40 minutes. Therefore the immune serum from *F. nucleatum* had an enhanced opsonization property. When *F. nucleatum* was opsonized with serum from Group 4 mice immunized with both *F. nucleatum* and *P. gingivalis* this showed the lowest phagocytic effect. In contrast to *F. nucleatum* the phagocytosis of *P. gingivalis* was reduced and occurred at 40 minutes, while the response using non-immune serum showed the least inhibition. The results also demonstrated a much higher level of chemiluminescence during phagocytosis of *F. nucleatum* compared to *P. gingivalis*. This reduction may be due to the inhibitory effect of *P. gingivalis*. Clinical studies in humans have shown deficiencies in phagocytic activity of neutrophils in periodontitis cases when compared to health. Also, anti-*P. gingivalis* protease antibodies that occur late in periodontitis have been demonstrated to block the anti-opsonizing activities of C3 and IgG may be responsible for the reduced chemiluminescence.

In conclusion, the results of the study have demonstrated a Th1/Th2 response in mice after immunization with *F. nucleatum* and *P. gingivalis* with a trend towards a Th2 response in *P. gingivalis*-immunized mice. When mice were immunized with *F. nucleatum* prior to *P. gingivalis* there was a significantly increased IgG2a (Th1) response to *P. gingivalis*. Further, the inhibition of neutrophils phagocytosis of immune serum-opsonized *P. gingivalis* was modulated by the presence of anti-*F. nucleatum* antibodies, while anti-*P. gingivalis* antibodies induced an inhibitory effect on the phagocytic response to *F. nucleatum*. Further experiments involving the modulation of *P. gingivalis* by other periodontopathic bacteria are required.

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Development of effective communication skills in dental students

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Oral health care providers and consumers depend on effective communication to generate, access and exchange relevant oral health information. Recognizing this, oral health educators are placing an increasing emphasis on the development of communication skills within oral health curriculum. The purpose of this study was to investigate the effects of a new curriculum introduced in a School of Dentistry in an Australian university which offers courses on effective communication skills. The current research examined how dental students' subjective norms, attitudes and self-efficacy towards effective communication impact on their intention to communicate effectively in their future roles as oral health care providers and their perceptions of their communicative behaviour in a real interaction with a patient.

We used a theoretical framework which integrates the Theory of Reasoned Action (TRA)^{1,2} and the Theory of Planned Behavior (TPB).³ In the model, we proposed that if a dental student's *subjective norms about becoming a dentist with good communication skills* (asking questions, expressing concerns, being assertive and providing and inviting speech narratives) are positive, his or her *attitude about becoming a dentist with good communication skills* is positive and *perceived behavioural control about becoming a dentist with good communication skills* is high, the *intention to engage in participative communication during encounters with patients and clinical staff* will be strong, which in turn will increase the chance that the student will actually use effective communication skills in academic and clinical settings.

A total of 148 dental students (79 males and 69 females – 12 participants did not report their sex), enrolled in the curriculum in the School of Dentistry completed the survey. The median age was 20 (range 18-42). Approximately 49 students were enrolled in their second year of university, 50 were in their third

year and 35 were in their fourth year of the program. The rest of the students ($n=4$) were enrolled in first year.

A confidential self-report survey was distributed to second, third and fourth year students from the School of Dentistry at a major Australian university during class. An information sheet was given to students, stating that the data they provided the research team would be kept completely confidential and anonymous. It also described the aim of the project.

Students were told that the survey was designed to measure their perceptions of: (1) communication subjective norms within dentistry; (2) their attitudes towards communication skills components in their course of study; (3) whether or not they believe it will be easy or difficult to communicate effectively after being taught communication skills; (4) their intention to incorporate the communication skills in the future; and (5) their perceptions of their communicative behaviour in a real interaction with a patient.

When students' perceived subjective norms towards communication were positive, they were more likely to indicate that they intended to communicate effectively in the future and that they had communicated effectively in a recent interaction with a patient. Other factors that were related to more positive communication intentions and communicative behaviour were students' attitudes towards communication and their self-efficacy (or perceived behavioural control).

To improve the effectiveness of dental programs, oral health educators can focus on the social norms supporting effective communication, students' perceived behavioural control and their attitudes towards communication. Collectively, it is hoped that these steps will ultimately improve the communication skills of oral health graduates.

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Structural studies of biomimetic peptides of bovine dentine phosphophoryn

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Bovine dentin phosphophoryn (BDP), a protein rich in aspartyl (Asp) and O-phosphoseryl (Ser(P)) residues, is synthesized by odontoblasts and believed to be involved in matrix-mediated biomineralization of dentin.¹ When immobilized on a stable support and incubated in physiological solutions of calcium and phosphate, phosphophoryn can induce the formation of hydroxyapatite HA.² At high phosphophoryn concentrations, when phosphophoryn is free in solution, phosphophoryn tends to inhibit the crystallization of HA. This dual ability to initiate or inhibit HA formation has led to the currently accepted view that phosphophoryn plays an important role in the mineralization process. At low levels when attached to the gap regions of collagen fibrils, phosphophoryn induces the formation of initial apatite crystals while at higher levels it controls the size, shape and orientation during crystal growth. Characterization of this protein has proved difficult due to the high degree of phosphorylation, extreme negative charge (pI 1.1)³ and redundant amino acid composition (80-90 per cent Asp and Ser residues).¹

Using ¹H NMR spectroscopy, it has been recently demonstrated that phosphophoryn is a uniformly flexible molecule which is consistent with the relatively featureless sequences.⁴ The significance of these results is that phosphophoryn, due to its uniform nature, has now the potential to be replaced by biomimetic peptide analogues that together with amorphous calcium phosphate may lead to the development of novel, dental restorative materials and calcium phosphate delivery vehicles. Since phosphophoryn is highly resistant to proteolysis,⁵ the design and synthesis of multiphosphorylated biomimetic peptides was investigated. The aim of this study was to examine the solution structure of the nonphosphorylated peptide Asp-Ser-Ser-Asp-Ser-Ser-Asp-Ser-Ser-Asp in the presence of calcium ions.

The synthesis of the nonphosphorylated Asp-Ser-Ser-Asp-Ser-Ser-Asp-Ser-Ser-Asp was carried out using standard Fmoc protocols. Following cleavage from the resin support, the crude peptide was purified using reversed-phase HPLC. MALDI-TOF mass spectrometry and N-terminal sequence analysis confirmed the identity of the peptide.

All experiments on the DSSDSSDSSD peptide were performed on a UNITY^{NOVA} spectrometer operating at a proton frequency of 600MHz. The following spectra of the peptide in the presence of 90mM calcium were accumulated in the phase sensitive mode using the States-TPPI method,⁶ NOESY^{7,8} and TOCSY (HOHAHA).^{9,10} The resonances were assigned using the standard sequential assignment procedure.^{11,12} The chemical shifts were compared to the random coil

values determined by Wishart *et al.*¹³ and Merutka.¹⁴ The Asp and Ser amide and H α chemical shifts were significantly dispersed indicating non-random coil values.

Molecular modeling was performed using the software package SYBYL 6.8 (Tripos Associates, St. Louis, MO, USA) on a SGI Octane workstation. The Kollman all atom forcefield was utilized without the electrostatic term with no cutoff for nonbonded interactions. Procheck was used to check angles, bond lengths, chirality and omega angles. The *in silico* constructed peptide was subjected to a simulated 20ps of dynamics at 300°C followed by 500 cycles of minimization both *in vacuo* and while solvated in a 8Å radius shell of water. The modeling results show that the peptide prefers to adopt a non-random coil conformation. This was tested further by allowing the peptide constructed in an α -helical conformation and solvated within a 8Å radius shell of water to relax through minimization and dynamics cycles. The α -helical conformation was not stable and hence unraveled. In conclusion, our NMR and modelling results are similar to those of the highly acidic casein phosphopeptides that also adopt non-random coil structures consisting of loops and turns.¹⁵⁻¹⁸

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Investigation of the anticariogenic casein phosphopeptide α_{s1} -casein(59-79) interacting with the mineral ions in amorphous calcium phosphate, using NMR spectroscopy

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The repair of early enamel lesions has been recently demonstrated by tryptic phosphopeptides derived from milk caseins that associate with amorphous calcium phosphate (ACP), forming stable complexes. These casein phosphopeptides (CPP), containing the phosphorylated cluster motif -Pse-Pse-Pse-Glu-Glu-, form calcium phosphate delivery vehicles that retard enamel demineralization and promote remineralization.

The CPP consist predominantly of four multi-phosphorylated peptides whose sequences are shown below with the cluster sequence underlined. Pse represents a phosphoserine residue.

[1] Gln⁵⁹-Met-Glu-Ala-Glu-Pse-Ile-Pse-Pse-Pse-Glu-Glu-Ile-Val-Pro-Asn-Pse-Val-Glu-Gln-Lys⁷⁹.

α_{s1} -casein(59-79)

[2] Arg¹-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Pse-Leu-Pse-Pse-Pse-Glu-Glu-Ser-Ile-Thr-Arg²⁵.

β -casein(1-25).

[3] Asn⁴⁶-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-Pse-Pse-Pse-Glu-Glu-Pse-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys⁷⁰.

α_{s2} -casein(46-70)

[4] Lys¹-Asn-Thr-Met-Glu-His-Val-Pse-Pse-Pse-Glu-Glu-Ser-Ile-Ile-Pse-Gln-Glu-Thr-Tyr-Lys²¹.

α_{s2} -casein(1-21)

In an approach to understand the ultrastructure of the casein phosphopeptide-amorphous calcium phosphate complexes CPP-ACP complex, we have been studying the solution structures of the predominant peptides bound to calcium ions, and complexed with amorphous calcium phosphate using Nuclear Magnetic Resonance (NMR) Spectroscopy. We have previously reported the structural features, derived from proton NMR spectroscopic studies of the individual peptides α_{s1} -CN(59-79), β -CN(1-25) and α_{s2} -CN(2-20) in the presence of calcium ions.¹⁻³ The casein phosphopeptide α_{s2} -CN(59-79) was selectively precipitated from a tryptic digest of casein using Ca²⁺ and ethanol and further purified by anion exchange FPLC and reverse

phase HPLC.^{3,4} The purity of the peptide was checked by amino acid sequencing and mass spectrometry as previously described.^{5,4} The phosphopeptide α_{s1} -CN(59-79) was dissolved at 1.67g/L in Milli Q water. To the peptide solution, 50 μ M CaCl₂ and 21.3mM Na₂HPO₄ was added stepwise slowly. The pH was held at 9.0 by titration of 50mM NaOH. The final α_{s1} -CN(1-25)-ACP solution was then dialysed. For the NMR experiments, the pH of the peptide complex was adjusted to 6.1 and dialysed overnight prior to lyophilisation. The peptide complex was dissolved in 90% H₂O 10% D₂O.

All spectra of the α_{s1} -CN(59-79)-ACP complex were recorded with spectral widths of 6000.6Hz in F₁ and F₂ with 1024 complex data points. Phase-sensitive spectra were collected using the States-TPPI method.⁶ The WET⁷ pulse sequence was used for solvent suppression in the NOESY^{8,9} and TOCSY^{10,11} spectra. Pre-saturation of the solvent peak was used when acquiring the DQ-COSY^{12,13} spectra. Mixing times of 250 minutes and 300 minutes were used in acquiring the NOESY spectra and a mixing time of 80 minutes was used for the TOCSY spectrum. A total of 100 t₁ increments of 16 transients were collected for the TOCSY spectrum. The 250 minutes NOESY had 128 t₁ increments of 128 transients while the 300 minutes NOESY had 200 t₁ increments of 64 transients. The DQ-COSY spectra had 200 t₁ increments of four transients.

The amino acid spin systems of α_{s1} -CN(59-79) were identified by examining the TOCSY and NOESY recorded in H₂O at pH 6.00 at a temperature of 25°C. The single histidine, valine, asparagine and serine, two isoleucines, and three phosphoserines were recognized by their chemical shifts and the connectivity patterns in the TOCSY spectrum.¹⁴ The β H₂ to NH nOes of the five O-phosphoserines were observed in the fingerprint region of the TOCSY and NOESY permitting their sequence specific assignments. The nOe crosspeaks

connecting the side-chain amide resonances of N⁷⁴ and Q⁷⁸ were observed and sequentially assigned.

The secondary H α and NH proton chemical shifts of the phosphoserine residues were calculated using the 'random coil' chemical shifts reported by Bienkiewicz and Lumb.¹⁵ The secondary H α and NH proton chemical shifts of the other residues were calculated using the 'random coil' chemical shifts reported by Merutka *et al.*,¹⁶ with the sequence-dependent corrections recently reported by Schwarzingler *et al.*¹⁷

The secondary structure of the peptide α_{s1} -CN(59-79) was characterized by sequential (i, i+1), medium-range (i, i+2,3) nOes and C α H chemical shifts. The spectral data was compared with that of the peptide α_{s1} -CN(59-79) bound to calcium ions,¹ revealing that the significant secondary amide and alpha chemical shifts are similar. The majority of medium-range nOes detected in the α_{s1} -CN(59-79)-ACP complex consist of sidechain to sidechain noes indicating interactions with the mineral ions.

In conclusion the α_{s1} -CN(59-79) peptide backbone adopts a non-random-coil conformation consisting of loops and turns. In addition, the α_{s1} -CN(59-79) peptide side chains adopt orientations to interact with the mineral, with the corresponding accommodation of the backbone.

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Role of macrophage migration inhibitory factor (MIF) in oropharyngeal candidiasis

CS Farah, RB Ashman*

We have developed various animal models of oropharyngeal candidiasis in our laboratory. An oral *Candida albicans* infection can be established in inbred, immunodeficient, and head and neck irradiated mice, all of which show a cytokine profile weighted towards a pro-inflammatory Th1-type response in recovering animals.^{1,2} In irradiation-induced oropharyngeal candidiasis, one particular cytokine, macrophage migration inhibitory factor (MIF), was detected by ribonuclease protection assay (RPA) in the oral mucosa of both irradiated and non-irradiated mice recovering from an oral *C. albicans* challenge at different time-points during recovery.² In addition, MIF was detected in the early stages of recovery in T cell deficient mice following reconstitution with functional splenic

lymphocytes.¹ Furthermore, we have shown that clearance of the yeast from the oral cavity is dependent on T cell augmentation of phagocytic action by macrophages and neutrophils.³

MIF is known to promote pro-inflammatory cytokine release,⁴ and to suppress the anti-inflammatory effects of glucocorticoids.⁵ MIF is primarily produced by T cells and monocytes/macrophages, and mediates pro-inflammatory effects by stimulating macrophages to produce TNF- α and nitric oxide.^{6,4} We have recently shown however, that mice that lack TNF- α develop an acute oral infection, whereas mice that lack iNOS (the inducible form of nitric oxide synthase) do not develop an infection of increased severity compared to the wildtype control mice (Farah, in submission).

Furthermore, IL-12p40 knockout mice develop a chronic oropharyngeal infection, whereas IFN- γ knockout mice do not (Farah, in submission).

It is not clear what role, if any, MIF plays in recovery or susceptibility to oropharyngeal candida infection. From our previous studies, MIF was observed in oral tissues of mice recovering from an oral infection, and it is postulated that MIF plays a role in regulating the inflammatory process in the oral tissues, and contributes to the eradication of the yeast. MIF has been shown to play a role in mediating T cell activation and antigen-specific immunity,⁷ promoting TNF- α ,⁴ and phagocytosis by macrophages.⁸

In this experiment, we set out to examine the presence of MIF, and its correlation to the presence of pro-inflammatory cytokines (IL-12, TNF- α and IFN- γ), in the oral tissues and lymph nodes of IL-12p40, TNF- α and IFN- γ cytokine knockout mice.

Specific pathogen-free female IL-12p40, TNF- α , or IFN- γ -deficient knockout mice and their respective controls (C57BL/6), six to eight weeks of age, were infected orally with 10^8 live *C. albicans* yeasts in 20 μ l PBS. The infection was monitored by swabbing the oropharyngeal cavity of days 1, 4, 8, 14 and 21 with sterile cotton swabs buffered in sterile PBS, and plating on Sabouraud's agar plates. Colony forming units were counted and assigned into five groups correlating with the level of recoverable yeast from the oropharyngeal cavity. This method provided a semi-quantitative measure of the levels of floridity of the infection. The scoring system used was as follows: 0=No detectable yeasts, 1=some (1-10) CFU detected, 2=significant numbers (11-100) of CFU detected, 3=greater levels (101-1000) of CFU detected, 4=florid levels (1000+) of CFU detected.

Mice were sacrificed during the course of the experiment for histopathological examination of oral tissues, and frontal skull sections were stained with haematoxylin and eosin, and according to the periodic acid-Schiff technique.

Total RNA was isolated from oral tissues of knockout and control mice using Ultraspec RNA reagent, and the concentration and purity was determined by spectrophotometry at 260 and 280nm. cDNA was prepared by reverse transcription of 1 μ g of each RNA, using an oligo d(T)¹⁵ primer and AMV reverse transcriptase. cDNA was amplified by polymerase chain reaction in an amplification mix consisting of Taq DNA polymerase, the primer, and 1 μ l cDNA in a total volume of 25 μ l. Negative controls (without cDNA) were included in each run. A housekeeping gene GAPDH was also used. Following amplification, the product was analysed by electrophoresis through 2.5 per cent (w/v) agarose gels stained with ethidium bromide and the bands visualized using an ultraviolet transilluminator.

Table 1. Cytokine gene expression in oral tissues and lymph nodes in cytokine knockout mice

Oral Tissues	Primer			
	IL-12	TNF- α	IFN- γ	MIF
IL-12 KO	-	+	+	-
TNF- α KO	-	+	+	-
IFN- γ KO	-	+	+	-
C57/BL6	-	+	+	-
Lymph Nodes				
IL-12 KO	-	+	+	-
TNF- α KO	-	+	+	-
IFN- γ KO	-	+	+	-
C57/BL6	-	+	+	-

IL-12 KO mice develop a very severe chronic oropharyngeal infection, while TNF- α mice develop only an acute exacerbation early in the course of the infection. IFN- γ mice do not show any increased susceptibility compared to control C57/BL6 mice. The histopathology shows extensive hyphal elements penetrating the epithelium only in IL-12 KO mice.

As can be seen in Table 1, MIF was not detected in the oral tissues or lymph nodes of any of the mouse groups tested. Furthermore, IL-12 was not detected in any mouse strain. TNF- α and IFN- γ were detected in both oral tissues and lymph nodes in all mouse strains tested. A correlation does not seem to exist between the level of infectivity of the oral tissues, the presence of pro-inflammatory cytokines, and the presence of MIF. It is perhaps not surprising that IL-12p40 KO mice did not show any MIF, since IL-12 is a key dominant regulator of pro-inflammatory cytokines, and these cytokines would not be present in its absence. On the other hand, it was expected that the C57/BL6 control mice, and perhaps even IFN- γ KO mice, might express MIF in the tissues since these mice clear the infection within 21 days, and do not show any signs of acute exacerbation as seen in TNF- α mice, or chronic colonization as seen in IL-12p40 mice.

From these results, it would appear that MIF does not play a significant role in inducing pro-inflammatory cytokine release, regulating the inflammatory process in the oral tissues, or contributing to the eradication of the yeast.

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Active and passive immunization against oropharyngeal candidiasis

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Mucosal infections caused by *Candida albicans* are commonly encountered in patients with an impairment of cell-mediated immune responses. HIV+/AIDS patients, in particular, demonstrate a reduction in CD4⁺ T cells,^{1,2} so it can be inferred that protective responses are dependent on the presence and functionality of CD4⁺ T lymphocytes. There has been little clinical evidence for a role for humoral immunity in oral candidiasis;³⁻⁵ however, Matthews *et al.*,⁶ have speculated that in AIDS patients, spread of the yeast from the oral cavity and dissemination throughout the body may be inhibited by the presence of an antibody to a 47kDa protein of *C. albicans*.⁶ The role of humoral immunity in oropharyngeal candidiasis remains unclear, and most of what is known about protective antibodies to candida has been derived from the systemic model.⁷⁻⁹ The purpose of this study was to examine the role of active and passive immunization against an oral *C. albicans* infection in a murine model.

Specific pathogen-free BALB/c and CBA/CaH euthymic female mice, six to eight weeks of age, were primed either systemically by intravenous injection of 3×10^5 *C. albicans* in 200 μ l PBS or orally with 10^8 viable yeast cells in 20 μ l PBS, and rested for four to six weeks. Primed mice were subsequently challenged orally with 10^8 candida yeasts. Naïve inbred BALB/c and CBA/CaH mice were intravenously injected with either 3×10^7 lymphocytes or 250 μ l of serum from either orally or systemically immunized isogenic animals. After injection, the mice were inoculated orally with 10^8 yeasts. Serum samples were tested for the presence of candida-specific IgG1, IgG2a and IgM, while saliva samples were tested for IgA, by ELISA. Antigens of *C. albicans* were detected by Western Blot on probing with serum following immunization.

Active oral immunization was protective in BALB/c and CBA/CaH mice, reducing both fungal burden and duration of infection after secondary challenge ($P < 0.01$), whereas systemic immunization failed to protect against subsequent oral challenge. Candida-specific IgM was the predominant antibody detected in serum, by ELISA, following both primary and secondary oral challenge; however, candida-specific salivary IgA was not detectable. Immunization by

passive transfer of either lymphocytes or immune serum did not confer any significant protection against either oral infection in either susceptible (CBA/CaH) or resistant (BALB/c) mouse strains.

Antibodies produced after oral immunization tended to show a more restricted range, and a lower intensity of recognition than those elicited by systemic immunization as determined by Western Blot analysis. CBA/CaH mice produced antibody of both IgG1 and IgG2a subclasses, to a wider range of antigenic determinants than did BALB/c mice, which produced predominantly IgG1. No IgM reactivity was detected in serum from either CBA/CaH or BALB/c mice. Provisional correlation with protein antigens of the yeast ranged from 30-51kDa in CBA/CaH mice and 35-38kDa in BALB/c mice. Some of the antigens identified include triose phosphate isomerase (TPIS), and glyceraldehyde-3-phosphate dehydrogenase (G3P).

The data demonstrate a role for mucosa-associated immunity following immunization by the oral route, most likely exerted by local T lymphocytes resident in the oral mucosa, but there was no evidence to support a role for humoral immunity in protection against oral candidiasis.

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The role of cell surface epithelial mucin (MUC-1) in oropharyngeal candidiasis

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Cell surface mucins are glycoproteins thought to play a fundamental primary role in host defence at mucosal surfaces, particularly in the gastro-intestinal tract (GIT).¹ Mucosal epithelial cells undertake absorption and secretion whilst providing a critical barrier between the luminal environment and the internal organs.²

Epithelial mucins are large, complex mucosal glycoproteins that can be divided into two distinct sub-families: (a) secreted gel-forming mucins; and (b) cell surface mucins (cs-mucins). While much is known about gel-forming mucins, the functions of cs-mucins remain to be established. Emerging data suggest cs-mucins are involved in intracellular signaling and are likely to have both a barrier and reporting function on the apical surface of all mucosal epithelial cells. At least seven human epithelial cs-mucin genes (MUC-1, 3A, 3B, 4, 11, 12) have been identified to date.^{3,4} All seven cs-mucins are expressed in the colon.

The cs-mucins act as decoys preventing adhesion of bacteria to other molecules in the glycocalyx. Interactions with microbial adhesins may trigger release of the cs-mucin α -subunit thereby coating the micro-organism and preventing access to the cell surface via the cs-mucin. Bacterial binding then stimulates cs-mucin signalling resulting in defensive responses by the epithelial cell, including recruitment of neutrophils. Neutrophils bind directly to the apical surface of epithelial cells during infections, and the molecular events underlying neutrophil-epithelial cell adhesion are partially understood. Neutrophils have been shown to exert candidacidal activity, and represent the first line of defence against *Candida albicans* yeast.⁵ Indeed, neutrophil infiltration is the hallmark of candidal lesions,^{6,7} often forming micro-abscesses in the oral epithelium of infected tissue.^{8,9}

Preliminary data show that cs-mucins limit bacterial infections in the GIT, and that the deficit in MUC1 leads to increased susceptibility to mucosal bacterial infection.^{10,11} In this study we set out to explore the potential for cs-mucin to limit fungal infections *in vivo* by using MUC1^{-/-} mice. Our aim was to provide *in vivo* evidence that cs-mucins protect oral mucosal surfaces from infection, as well as limiting systemic spread via disruptions to the GIT mucosa.

Specific pathogen-free female MUC1 knockout (*Muc1*^{-/-}) mice and their respective wild type controls (*Muc1*^{+/+}), six to eight weeks of age, were used. Animal experiments were approved by the Animal Experimentation Ethics Committee of the University of Queensland, and carried out in accordance with the National Health and Medical Research Council's Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 1997.

C. albicans isolate 3630 was grown in Sabouraud's broth for 48 hours at room temperature. Mice were inoculated orally with 10⁸ live *C. albicans* yeasts in 20 μ l PBS. The infection was monitored by swabbing the oral cavity on days 1, 4, 8, 14 and 21. Colony forming units (cfu) were counted and assigned into five groups correlating with the level of recoverable yeast from the oral cavity as described previously.^{8,9,12}

Mice were examined for overt signs of systemic infection (e.g. diarrhoea). Mice were sacrificed and had various organs including the stomach, colon, small intestine, brain and kidneys removed, weighed, and transverse slices homogenized in 1ml of PBS. Samples were diluted appropriately (1:10, 1:100, 1:1000), and 100 μ l aliquots plated on Sabouraud's agar containing chloramphenicol as described previously.¹³ The plates were incubated at 37°C for two days, and the colonies counted. Each determination was carried out in duplicate. Frontal sections of the skull were taken at approximately 3mm intervals, with consecutive sections stained with haematoxylin and eosin, and according to the periodic acid-Schiff technique. Sections were examined by light microscopy.^{8,9} Systemic organs were also stained accordingly.

A monoclonal anti-PMN antibody (RB6-8C5) was used to deplete neutrophils. This rat IgG2b monoclonal antibody reacts with the Gr-1 surface antigen expressed on murine granulocytes but not with monocytes or lymphocytes.^{14,15} The antibody was produced and purified at the Institute of Medical and Veterinary Science, Adelaide, South Australia, and pre-tested to determine the optimal dose for depletion. Mice were depleted of neutrophils by i.p. administration of the antibody preparation on day -2, then at 48 hour intervals until day 8, as described previously.⁸ Complete depletion of neutrophils was confirmed by blood smears. Quantitative data were analysed using the statistical features of GraphPad Prism Version 2.01 (GraphPad Inc, San Diego, CA, USA). Student's *t* test and one-way ANOVA were used with *P*<0.05.

There was no systemic dissemination of the yeast, or any gut involvement in both knockouts and controls, either before or after PMN depletion. There was also no evidence of hyphal penetration in the oral tissues in both mouse strains. Although PMN depletion did increase the severity of the infection on day 1, this did not result in an increase in the duration of the infection, or in hyphal penetration of the oral mucosa. MUC1 does not appear to contribute to protection against *C. albicans* at the oral mucosal surface, and a role for a neutrophil-mucin interaction in the defense against an oral *C. albicans* infection seems unlikely. In conclusion, it does not appear that cs-mucins modulate adhesion, invasion or infection of oral mucosal epithelial cells by *C. albicans*.

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) enhances macrophage killing of *Candida albicans*

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Phagocytic cells are the first line of defense against candida infections.¹⁻⁵ They are primarily responsible for the elimination of fungi from infected tissues and organs,⁶ and quantitative or qualitative differences in their function may be responsible, at least in part, for the variations in susceptibility or resistance that have been documented in inbred strains of mice.

Mutations that affect the phagocytic or killing capacity of neutrophils have been shown to increase susceptibility to candida infection. Beige mice show an increased susceptibility to intravenous challenge,⁷ and neutrophils from these mice demonstrate a significantly decreased capacity to kill *Candida albicans* hyphae when compared with BALB/c mice.⁸ Depletion of neutrophils *in vivo* markedly increases susceptibility to both systemic⁹ and oral¹⁰ candidiasis, and in the latter case, revealed differences between mouse strains in their contribution to host resistance. Macrophages also have been shown to be important in host recovery, although their spontaneous effector activity varies depending on the tissue or organ from which they are isolated,¹¹ and their state of activation.

It is possible that T cell augmentation of neutrophil and macrophage activity takes place either by supporting recruitment of phagocytic cells from bone marrow, or by enhancing the functional activity of these cells. The purpose of this study was to compare the phagocytosis and killing of *C. albicans* by

neutrophils and macrophages from BALB/c and CBA/CaH mice, and to evaluate the effect of GM-CSF on these responses.

Long-term bone marrow cultures for the production of neutrophils were established as previously described.¹² Bone marrow cells were flushed from femurs and tibias in bone marrow culture medium, and incubated for three weeks, replacing half of the medium with fresh medium every week. On week four, the culture medium was removed and an equal volume of freshly isolated bone marrow cells added into the flasks, after which they were cultured for a further week. The total supernatant was collected on week five for isolation of neutrophils on a discontinuous Percoll density gradient.

Mouse bone marrow cells were obtained, as described above, and incubated in macrophage culture medium (MCM) for seven days. On days four and six, the medium was replaced with an equal volume of MCM. The macrophages were harvested using a cell scraper, washed and resuspended in RPMI-1640 at the appropriate concentration for use.

For the phagocytosis assay, yeasts were fixed in 75 per cent ethanol, labeled with FITC and incubated with functional neutrophils or macrophages. After incubation, EDTA was added to stop phagocytosis, and ethidium bromide was added before analysis by flow cytometry (FACS). For the killing assay, live *C. albicans*

were labeled with FITC and incubated with phagocytic cells. After incubation, cells were lysed by hypotonic shock and extracellular DNA removed. Propidium iodide was added before FACS analysis.

In order to examine the effects of cytokines on killing, GM-CSF (800U/ml) IFN- γ (2000U/ml), and TNF- α (200U/ml) were used. Before incubation with live yeasts, phagocytic cells were activated by each cytokine at 37°C, in an atmosphere of 5% CO₂ and 95 per cent humidity for one hour, and the killing assay was performed in the presence of the same dose of cytokine.

Killing of yeast by macrophages and neutrophils tended to follow the established pattern of susceptibility and resistance of these two strains of mice. BALB/c macrophages were significantly more efficient than CBA/CaH macrophages at killing *C. albicans*, but they were less efficient at phagocytosing the yeast. Likewise, neutrophils from BALB/c mice were slightly more effective at killing candida than neutrophils from CBA/CaH mice, and were less capable of candida phagocytosis compared to CBA/CaH neutrophils.

Of note was the fact that macrophages from both mouse strains were able to kill the yeast more effectively than neutrophils from these same mice, while phagocytosis displayed an opposite pattern. The addition of recombinant GM-CSF remarkably enhanced killing of the yeast by macrophages from CBA/CaH mice, but there was no enhancement of killing by macrophages from the BALB/c strain. Enhancement of macrophage killing was also seen with activation by IFN- γ and TNF- α , but to a lesser degree compared to GM-CSF.

BALB/c macrophages are not unresponsive to cytokines, as they can be activated to increase resistance against *Paracoccidioides brasiliensis*,¹³ so the defect appears to be candida-specific. It is possible that candida infection in BALB/c mice induces cytokines that act on macrophages in an autocrine fashion to increase their level of activation, whereas this innate cytokine response does not occur in CBA/CaH mice.

Such a mechanism might explain the inherent tissue resistance of BALB/c, as compared to CBA/CaH mice.

In conclusion, BALB/c macrophages are more effective than CBA/CaH macrophages at killing *C. albicans*, but the killing efficiency of the latter can be markedly enhanced by GM-CSF.

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The immune response to *Porphyromonas gingivalis* in the periodontal tissues of cytokine-specific knockout mice

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It is now well known that periodontal disease results from the inflammatory response of the host to bacteria in dental plaque. The nature of this immunoinflammatory response determines the outcome of the disease process, and the tissue destruction that follows. With increased bacterial challenge, neutrophils under the influence of pro-inflammatory cytokines and chemokines migrate to the gingival tissues and exert a protective effect.¹ However, with the continued presence of bacteria in these tissues, a cell-mediated inflammatory infiltrate dominated by lymphocytes and macrophages takes form in the connective tissues. Failure of the adaptive response to contain the bacterial challenge leads to a shift in the inflammatory response, mostly B cells and plasma cells, which ultimately results in the production of either protective antibodies and control of the infection, or non-protective antibodies and subsequent connective tissue destruction and bone loss mediated by IL-1 and TNF- α .¹

In recent years, great attention has been paid to the role of T lymphocytes and Th1/Th2 cytokines in periodontal disease progression. The Th1/Th2 concept is generally accepted, but the results from human studies are equivocal. Studies on cytokines have led to the formulation of several hypotheses as to which T cell subsets are associated with periodontitis.^{2,4}

The aim of this study was to determine the response of alveolar bone to *P. gingivalis* in an experimental murine model, using genetically-modified cytokine-specific knockout mice.

Specific pathogen-free female IL-4, IL-10, IL-12p40, TNF- α and IFN- γ knockout mice and their respective controls, six to eight weeks of age, were used in this study. *P. gingivalis* isolate W50 was grown anaerobically, as described previously.⁵ *P. gingivalis* infection was established.⁶ Mice were given sulfamethoxazole-trimethoprim (Bactrim) *ad libitum* in de-ionized water for 10 days prior to infection, followed by a four day antibiotic-free period. Mice were inoculated orally with 10⁹ CFU of live *P. gingivalis* in 100 μ l PBS by gavage three times at two day intervals. Controls included sham-infected mice which received the antibiotic water pre-treatment, and the PBS gavage without *P. gingivalis*. Seventy days after the last gavage, mice were euthanized and samples collected.

After the animals were sacrificed, the skulls were dissected and jaws defleshed after treatment in 6% Triton-100 at 85°C for three hours. Subsequently, the samples were immersed overnight in 3% H₂O₂, followed by a short treatment with 1% NaOCl, air-dried and stained with 0.5% eosin for five minutes followed by 1% methylene blue for one minute in order to delineate the CEJ more clearly. Alveolar bone loss

was measured morphometrically according to the method of Tatakis and Guglielmoni with minor modifications.⁷ The area of bone loss (mm²) was calculated as the sum of the exposed molar root surface on all three molars, in both the maxilla (buccal and palatal) and mandible (lingual). Blinded measurements were performed using a dissecting microscope and a computer-assisted image analysis system (Axiovision, CARL ZEISS VISION, Germany) by a sole blinded operator. Quantitative data were analysed using the statistical features of GraphPad Prism Version 2.01 (GraphPad Inc, San Diego, CA, USA). Student's *t* test and one-way ANOVA were used with P<0.05. Serum was screened for IL-4, IL-10, IL-12, TNF- α and IFN- γ by ELISA using Mouse OptEIA Cytokine Sets.

On average, *P. gingivalis* infected IL-12, IFN- γ and TNF- α KO mice experienced less bone loss than their uninfected counterparts, while infected IL-4 and IL-10 KO mice experienced more alveolar bone loss compared to uninfected mice. This effect was most evident in IL-12 KO mice. It would appear from these results that Th1 cytokines are less protective compared with Th2 cytokines in terms of alveolar bone loss in this *P. gingivalis* animal model.

High levels of IL-12 (840-2727 pg/ml) and low levels of IL-10 (36-128 pg/ml) were detected in the serum of *P. gingivalis* infected mice. No TNF- α , IFN- γ or IL-4 was detected. These results show that challenge with *P. gingivalis* induces IL-12 in the serum, but it would appear that this is not associated with increased levels of IFN- γ . The actual mechanism of alveolar bone loss is unknown and may reflect a more systemic response. The absence of IFN- γ and IL-4 may in fact suggest non-immune mechanisms.

Although the present study can not clarify the actual mechanisms of bone loss it does appear that in the presence of *P. gingivalis*, IL-12 seems to play a central role in regulating this response in this model. As this regulation seems not to involve an increase in either Th1 or Th2 cytokines, further analysis of these findings is warranted.

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***Porphyromonas gingivalis* specific T-cells in cardiovascular disease**

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Atherosclerotic cardiovascular disease is a leading cause of death worldwide and the importance of the role of infection and inflammation in atherosclerosis is now widely accepted. Chronic inflammatory periodontal disease is a significant oral health problem with *Porphyromonas gingivalis* being one of the major causative organisms for disease progression. Individuals with severe periodontitis have been reported to have a significantly increased risk of developing cardiovascular diseases including atherosclerosis, myocardial infarction and stroke. Infection may initiate and facilitate the progression of atherosclerosis as a result of the immune response to bacterial heat shock proteins (HSPs). All cells, both prokaryotic and eukaryotic, express HSPs on exposure to various forms of stress including temperature, oxidative injury and infection. Factors such as bacterial lipopolysaccharide, cytokines and mechanical stress can induce the expression of host protective (h) HSP60 on endothelial cells. Due to the homologous nature of HSPs among species, cross-reactivity of the immune response to bacterial HSP (GroEL) with hHSP60 on endothelial cells may result in endothelial dysfunction and the subsequent development of atherosclerosis. Chronic infections such as those caused by periodontopathic bacteria such as *P. gingivalis* have been associated with atherosclerosis possibly due to cross-reactivity of the immune response to bacterial GroEL with hHSP60. The aim of this study therefore was to test this hypothesis.

Carotid endarterectomy specimens were obtained from 29 patients for immunohistology. Generally, the atherosclerotic lesion consisted mainly of CD14+ macrophages, mostly as foam cells. An inflammatory cell infiltrate was observed at the periphery of the lesion. These were almost entirely CD3+ T cells, with many more CD4+ T cells than CD8+ T cells. Very few CD19+ B cells were detected. HSP60+ cells included the endothelium and some smooth muscle cells. *P. gingivalis* organisms were demonstrated in four of the arteries using immunohistology.

GroEL-specific T cell lines from peripheral blood and GroEL-, hHSP60- and *P. gingivalis*-specific T cell lines from atherosclerotic plaques were established and

characterized in terms of their cross-reactive proliferative responses, and cytokine and chemokine profiles and T cell receptor (TCR) V β expression by flow cytometry. Cross-reactivity of several lines was demonstrated. The cytokine profiles of the artery T cell lines specific for GroEL, hHSP60 and *P. gingivalis* demonstrated Th2 phenotype predominance in the CD4 subset and Tc0 phenotype predominance in the CD8 subset. A higher proportion of CD4 cells were IP-10+ and RANTES+ with low percentages of MCP-1+ and MIP-alpha+ cells while a high percentage of CD8 cells expressed all four chemokines. Finally, there was overexpression of the T cell receptor V β 5.2 family in all lines. These cytokine, chemokine and V β profiles are similar to those demonstrated previously for *P. gingivalis*-specific lines established from periodontal disease patients.

We examined cross-reactivity of anti-GroEL and anti-*P. gingivalis* antibodies with hHSP60 in atherosclerosis patients and quantified a panel of six pathogens in atherosclerotic lesions. After absorption of plasma samples with hHSP60, there were variable reductions in levels of anti-GroEL and anti-*P. gingivalis* antibodies, suggesting that these antibodies cross-reacted with hHSP60. All of the artery specimens were positive for *P. gingivalis*. *Fusobacterium nucleatum*, *Tannerella forsythia*, *Chlamydia pneumoniae*, *Helicobacter pylori* and *Haemophilus influenzae* were found in 84 per cent, 48 per cent, 28 per cent, 4 per cent and 4 per cent of arteries respectively. The prevalence of the three periodontopathic microorganisms, *P. gingivalis*, *F. nucleatum* and *T. forsythia*, was significantly higher than that of the remaining three microorganisms.

These results further support the hypothesis of molecular mimicry of GroEL and hHSP60 and the importance of *P. gingivalis* infection and therefore of periodontitis, in atherosclerosis. This study has provided further evidence for the role of infection in atherosclerosis disease progression, suggesting the importance of avoiding and treating infections, including periodontitis, especially for those patients with other cardiovascular disease risk factors.

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Investigation of two genetically distinct populations within the species *Fusobacterium nucleatum*, with different clinical niches

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Fusobacterium nucleatum is one of a number of species implicated in periodontal diseases. A previous study in this laboratory¹ revealed the presence of at least two major groups of isolates (distinct genetic populations) within the species. One of these was found only in the oral cavity while members of the second group were isolated from either that site or an extra-oral site. The aim of the present project was to investigate further the existence of these two groups within *F. nucleatum* isolates, both oral and extra-oral, using partial 16S rRNA gene sequence comparisons. The 16S rRNA gene was targeted because it is highly conserved and is often used for phylogenetic investigations of bacterial species.

Thirty isolates used in the previous electrophoretic study, and chosen to represent the range of genetic diversity observed within the two populations, were presently studied. A 490-bp section of the 16S rRNA gene was sequenced. The PCR primers were designed from alignments of all available *Fusobacterium* species GeneBank sequences. The sequences obtained were aligned with GeneBank sequences using Clustal X

(1.64b) and phylogenetically analysed using the Phylip version 3-6 (alpha 2) package (J. Felsenstein). Supporting their inclusion into a single species, all *F. nucleatum* sequences formed a cluster distinct from other *Fusobacterium* species. Most of the relationships between isolates were in agreement with our previous electrophoretic study. Within *F. nucleatum*, a number of distinct clusters were identified, some associated with particular infection sites. Again, in agreement with the electrophoretic study, these clusters did not support the proposed sub-speciation of *F. nucleatum*.

It is concluded that, although the 16S rRNA gene is highly conserved within *F. nucleatum*, phylogenetic analysis of clinical isolates, using a variable region of the gene, revealed a number of distinct lineages within the species. While these may constitute sub-species, they conflict with those previously described. In a broader context, physiological studies of members of the 'sub-species' may reveal characteristics responsible for niche selection, perhaps leading to treatment regimes interfering with the oral and extra-oral colonization of the organism.

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Prevalence and carriage of *Bacteroides forsythus* in subjects with clinical attachment loss and association with *prtH* genotype

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Cross-sectional studies have demonstrated a high prevalence of *Bacteroides forsythus* in rapidly progressive periodontitis. However, the nature of these studies i.e. the presence or absence of *B. forsythus* in already diseased subjects, precludes any determination as to whether *B. forsythus* is a risk factor for the future development of disease. Longitudinal studies where subjects with little or no initial periodontal disease were followed for periods of up to five years, have indeed demonstrated that *B. forsythus* presence characterized the conversion of periodontally healthy sites into diseased sites as indicated by loss of attachment, loss of alveolar crest height and tooth mortality.

Although fewer studies have examined the role of *B. forsythus* in periodontal health, this organism may be present in as many as 45 to 55 per cent of healthy subjects. Clearly the presence of *B. forsythus* in

subgingival plaque alone may be insufficient for disease initiation. *B. forsythus* has been shown to produce a variety of virulence factors that may initiate disease. The *prtH* gene from *B. forsythus* encodes a cysteine protease that has both proteolytic and haemolytic activity. Tan *et al.*,¹ have subsequently demonstrated among subjects with adult periodontitis, of those infected with *B. forsythus*, 85 per cent carried the *prtH* genotype. In contrast, of the *B. forsythus* infected healthy subjects they examined, only 10 per cent possessed the *prtH* genotype.

In this retrospective study we aimed to first compare the prevalence and carriage rate of *B. forsythus* in a population identified as having lost attachment over a five-year period with matched subjects who did not lose attachment. Secondly, by correlating the prevalence of the *prtH* genotype in *B. forsythus* infected subjects with

corresponding disease progression, we may help elucidate the role of *B. forsythus* in the aetiology of periodontal disease.

Clinical investigations were performed in the Colgate Oral Care Research Unit within the University of Queensland. Subjects were drawn from a prospective five-year longitudinal study on the progression of periodontal disease in an Australian population. Subgingival plaque samples collected at baseline and then yearly for five years were examined from 35 subjects with attachment loss (≥ 2 mm in at least two sites over five years) (LOA+) and compared with 36 age and sex matched subjects with no attachment loss during this period (LOA-). A Real-Time polymerase chain reaction (RT-PCR) assay using the ABI Prism[®] 7700 sequence detection system was used for both the enumeration of *B. forsythus* and detection of the *prfH* genotype in subgingival plaque. The amplification plot generated by the ABI Prism[®] 7700 sequence detection system is a plot of the fluorescence signal versus the cycle number. For each sample, the threshold cycle (Ct) is the cycle number at which the midpoint of the exponential phase of amplification (as indicated by the fluorescent signal) is reached. The higher the initial amount of *prfH*-containing DNA present, the sooner the amplified product is detected in the RT-PCR process, and hence the lower the Ct value. The non-parametric Mann-Whitney U test was used to determine whether there were any significant differences in the levels of the *prfH* genotype between the LOA+ and the LOA- groups.

As few as 10 *B. forsythus* were detected in 98 per cent and 97 per cent of LOA- and LOA+ subjects

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Dental employer and graduate survey

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Over the past three years there have been significant changes to the dental curriculum in the School of Dentistry at the University of Western Australia (UWA). Most recently a fifth year, called the Pre-Graduation Intern year, has been added to the course. It primarily involves the students participating in clinical attachments. Students attend various clinical placements both within the Oral Health Centre of Western Australia, as well as in clinical settings within the community. In order to evaluate the effectiveness of these changes, a study was conducted to elicit the perceptions of both students who graduated prior to these changes, as well as employers of these graduates. The primary focus was on the graduates' preparedness for employment.

respectively using the RT-PCR method. When the numbers of *B. forsythus* detected were grouped into four ranges ($<10^3$, $10^3 - 10^4$, $10^4 - 10^5$ and $>10^5$), at baseline, no difference in prevalence between either LOA- or LOA+ subjects was seen. Subsequently however, the percentage of subjects with $>10^3$ *B. forsythus* was significantly higher at each examination in the LOA+ subjects ($p < 0.05$).

With the exception of one subject from the LOA- group, all *B. forsythus* infected subjects demonstrated detectable levels of *prfH* at some point over the five years. From baseline to year 1, 22 subjects in the LOA+ group experienced attachment loss. These subjects demonstrated significantly higher levels of *prfH* ($p = 0.018$) compared to the 36 LOA- subjects. Similarly, between baseline and year 2, 27 subjects in the LOA+ group experienced attachment loss. Again a significant difference was observed ($p = 0.022$) when their baseline Ct values were compared to the baseline Ct value of the LOA- group, with the LOA+ group demonstrating higher levels of *prfH*. This significant difference was also observed over the baseline to year 4 and baseline to year 5 time periods. Changes in the levels of the *prfH* genotype during the above time periods were also examined. In general, the level of *prfH* genotype was not maintained over the period of time during which the loss of attachment occurred.

These results suggest that while high numbers of *B. forsythus* may be associated with loss of attachment, the baseline level of *prfH* genotype may be indicative of future loss of attachment.

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A total of 107 dental graduates from the years 2000 and 2001 were offered a survey. Of the 107 surveys offered, 36 were completed and returned (38.5 per cent response rate). A total of 45 dental clinicians who may have employed graduates from the 1999, 2000, 2001 or 2002 UWA graduating years were also offered a survey. Of the 45 invitations, 23 employers responded (51 per cent response rate). The 60 survey items were based on the Graduate Outcomes of the Dental School. Both the dental graduate and dental employer surveys were based on a six point Likert scale indicating the level of preparedness that the dental course provided, five points being 'very well' and one point being 'very poorly'. The midpoint was 'neutral' and the additional point was 'don't know'. Participants were also invited to provide written comments.

Overall, the graduates' perceptions of their preparedness to practice scored very highly for approximately 95 per cent of the curriculum. In general, graduates felt well-prepared. However, there were some specific areas of concern. Graduates felt that they were not adequately prepared to: use dental research methods; evaluate the impact of social factors on illness; and perform simple oral surgery and surgical extractions.

Overall, the employers were very complimentary of the graduates' preparedness to practice. The most frequent comments in terms of suggested improvements noted specific clinical skills, especially simple oral surgery, extractions, dentures and occlusion-related problems, as requiring a greater level of competence. Employers repeatedly commented positively on the improved difference between pre- and post-2001 UWA graduates.

Criticism such as the 'approach to the work environment', 'lack of confidence' and other aspects of professionalism, was mostly related to students graduating in 1999 and 2000. There also appeared to be a difference in expectation by former UWA graduates who were now employing UWA graduates, and those employers who were not UWA graduates themselves, with the former having a higher expectation of employees.

Out of the 44 survey items a total of 26 items scored as 'highly positive'. Several items had a high 'don't

know' or non-response rate. These included: gold in/onlays, single crowns, bridgework, tooth-coloured restorations and orthodontic treatment. The most likely explanation for this is that the employed graduates may not have had the opportunity to implement these skills, or may have had insufficient opportunities for the employer to warrant comment.

It is important to note that the employers (where they did attempt to 'rank' the skills as requested) gave equal weighting to the importance of all the individual clinical skills. They also noted where students initially lacked confidence upon commencement within the practice, this was gained very quickly within the first year of experience. The changing trends in dental practice were also indicated by the survey results. For example, a high rate of non-response in reference to 'gold inlays/onlays' indicates this is very limited in its application in current dental practice.

While the overall survey response rate was relatively low (graduates n=36, employers n=23), the survey data does show repetition in similar areas between both populations surveyed and in the written feedback from both graduates and employers. Accordingly, the main area of improvement relates to specific clinical skills, as noted, that have now been addressed by the UWA School of Dentistry.

The survey found that the current dental curriculum is preparing graduates for employment to the satisfaction of the employers surveyed, who stated that 'preparation of graduates was excellent', with a 'huge difference in the level of confidence and the standard of treatment' in the current graduates. As one employer stated, 'the Faculty can be very proud of itself'.

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Genetic programs involved in periodontal regeneration

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The ultimate goal of periodontal therapy is complete regeneration of the tissues lost as a result of periodontitis. However, most regenerative clinical procedures are unpredictable, largely because there is a lack of understanding of how the various tissues comprising the periodontium (gingival epithelium, gingival connective tissue, periodontal ligament, alveolar bone and cementum) interact during the regenerative process. A greater understanding of the cellular and molecular events involved in periodontal regeneration would result in the development of novel, predictable clinical techniques.

In order to study the cell and molecular events associated with periodontal regeneration, we have previously isolated and characterized primary cell cultures derived from human regenerating defects and compared them with patient-matched gingival

fibroblasts (GF) and periodontal ligament cells (PLC).^{1,2} These regenerating tissue-derived cells (RTC), which were obtained using the surgical technique of 'guided tissue regeneration', were found to have specific properties in terms of cell proliferation, matrix synthesis and bone-marker gene expression, suggesting that they had superior regenerative properties.^{1,2} This study aimed to identify the gene expression profiles that are responsible for the distinct nature of RTC when compared to GF and PLC.

A microarray analysis using the Hu133A Affymetrix arrays containing probes for over 22 000 well characterized gene transcripts was used to identify the genes and pathways that were characteristic of the three different tissues – regenerating tissue, gingival and periodontal ligament. Changes in gene expression were analysed as previously described³ and allowed the

creation of gene lists which represented increases and decreases of gene expression with a minimal fold change of 1.5. This methodology generated a list of 185 genes (160 increases and 25 decreases) that were consistently changed between RTC and PLC, irrespective of inter-patient heterogeneity. A similar comparison between RTC and PLC identified relatively few differentially expressed genes with 19 genes being identified (10 increases and nine decreases). Selected marker genes were validated by real time PCR. The mRNA expression of *IGFBP3*, *EGR1*, elastin, osteoprotegrin and *CD14* was quantified and there was a good correlation between the real time PCR analysis and microarray results, thus validating the array results.

Hierarchical clustering was used in order to determine which variables (patient source or cell type) had a dominant effect on gene expression. When the entire dataset was used, we found that the three cell types had unique expression profiles, although there was some similarity between the RTC and GF. However, clustering analysis using the differentially expressed genes that were either up-regulated or down-regulated in RTC compared to PLC and GF showed distinct clustering according to cell type, indicating that these genes can be considered characteristic of RTC.

Functional classification of the differentially regulated genes in RTC was carried out using the gene ontology (GO) annotation tools, NetAffx GO Browser and EASE.⁴ EASE analysis identified 26 GO groups that were up-regulated and nine that were down-regulated in RTC. The categories most significantly increased in the RTC were cytoplasmic components, such as cytoskeletal structural proteins (vimentin, adducin 1 and 3, β - and γ -actin), and proteins associated with skeletal development (e.g., exostoses 2) and calcium binding (e.g., calreticulin, matrix metalloproteinase 3), which are of particular functional relevance for hard tissue formation. A number of transcriptional co-repressor genes, including calreticulin and *YY1* were up-regulated in RTC, potentially identifying the transcriptional mechanisms that define the distinct phenotypic properties of RTC. Among the GO categories most significantly down-regulated were cell growth and regulation of cell growth (insulin-like growth factor binding proteins-2, -3 and -7). There was

also regulation of gene expression of extracellular matrix molecules (lumican, fibulin 1 and elastin).

The RTC had a greater similarity in gene expression with GF than with PLC. Both GF and RTC share a higher proliferation rate compared to PLC,² suggesting a common, relatively undifferentiated phenotype. In contrast, the PLC cells were derived from the periodontal ligament, which is a more specialized and mature tissue, containing a greater proportion of committed cells. This difference in tissue maturity appears to have a major impact on the gene expression profile, a finding which is consistent with another microarray analysis of GF and PLC which attributed differential gene expression between the two cell types to the higher proliferative and metabolic rate of GF.⁵

The stringent analytical microarray methodology used in this study allowed us to identify a set of functionally relevant genes, most of which are involved in wound healing and mineralized tissue formation, that can be used as markers for RTC cells. The majority of the markers identified in this study relate to differences between RTC and PLC. Since the RTC are derived from a PLC sub-population, these changes are important for our understanding of the mechanisms involved in progenitor cell activity during regeneration. In particular, the up-regulation of genes encoding proteins involved in skeletal development and calcium binding support the notion that the RTC contain progenitors with osteogenic/cementogenic potential. In conclusion, this study has demonstrated that RTC can be characterized by a consistent and robust set of differentially expressed genes. Some of these genes have known functional implications for periodontal regeneration and may present potential therapeutic targets.

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The role of iNOS in *Porphyromonas gingivalis* induced alveolar bone loss

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Nitric oxide (NO) is a short-lived free radical involved in the regulation of diverse physiological and pathological mechanisms and can also act as a cytotoxic agent in inflammatory disorders.¹ The biosynthesis of NO occurs through the catalysis of L-arginine by a family of isoenzymes, globally known as NO synthases (NOS). Inducible NOS (iNOS) is expressed only after its induction by inflammatory stimuli such as IL-1, TNF- α , IFN- γ and LPS in macrophages and polymorphonuclear cells.² This inducible process yields high amounts of NO for extended periods.

The inflammatory mechanisms involved in the pathogenesis of periodontitis results from a complex interaction between a microbial biofilm (dental plaque), and the host. Although bacteria are the cause of the disease, the presence of plaque alone is insufficient to produce periodontitis in the non-susceptible host. The nature of the host inflammatory response to the plaque determines the extent of the destruction. There is evidence implicating NO involvement in the pathogenesis of periodontitis, although its role remains ambiguous.³ Numerous studies have indicated an increased local production of NO and increased expression of iNOS in gingivitis and periodontitis, relative to healthy controls.³ Furthermore, elevated NO production has been observed in murine macrophages stimulated with LPS from *Porphyromonas gingivalis* and *A. actinomycetem comitans*.⁴ However, it has also been reported that NO retards the growth and survival of periodontopathic bacteria⁵ and can prevent excessive bone resorption.⁶ Additionally, the mere increase of nitric oxide in the local environment does not necessarily correlate with periodontal destruction.³ Therefore, it remains unknown whether NO aids in the elimination of periodontopathic bacteria or contributes to tissue destruction.³ The aim of this study was to investigate *P. gingivalis*-induced alveolar bone loss in iNOS knockout mice in order to gain insight into its possible role in bone loss during periodontitis.

The *P. gingivalis* W50 strain used in this study was cultured as previously described.⁷ The bacteria were suspended in reduced normal saline at a concentration of 10¹⁰/ml. *P. gingivalis* infection was established in the gingival tissues as previously described.⁸ Briefly, mice were given sulfamethoxazole-trimethoprim (Bactrim) *ad libitum* in de-ionized water for 10 days prior to infection, followed by a four-day antibiotic-free period. Mice were inoculated orally with 10⁹ cfu of live *P. gingivalis* in 100 μ l PBS by gavage three times at two day intervals. Controls included sham-infected mice which received a PBS gavage without *P. gingivalis*. Seventy days after the last gavage, the mice were

ethanased and samples collected. A separate group of iNOS+/+ and iNOS-/- mice were aged naturally and euthanased at 30 weeks of age.

After the animals were sacrificed, the skulls were dissected and jaws defleshed after treatment in 6% Triton-100 at 85°C for three hours. Subsequently, the samples were immersed overnight in 3% H₂O₂, followed by a short treatment with 1% NaOCl, air-dried and stained with 0.5% eosin followed by 1% methylene blue in order to clearly delineate the CEJ. Alveolar bone loss was measured morphometrically according to established methodology.⁹ Briefly, the area of bone loss (mm²) was calculated as the sum of the exposed molar root surface on all three molars, in both the maxilla (buccal and palatal) and mandible (lingual). Measurements were performed using a dissecting microscope and a computer assisted image analysis system (Axiovision, CARL ZEISS VISION, Germany) by a sole blinded operator. Quantitative data were analysed using Student's *t* test and one-way ANOVA statistical features (GraphPad Prism Version 2.01, GraphPad Inc, San Diego, CA, USA).

In naturally aged, uninfected mice, there was significantly more alveolar bone loss at 30 weeks of age compared to mice aged six or 16 weeks in both the iNOS+/+ and iNOS-/- groups. However, when mice were colonized with *P. gingivalis*, a significantly greater amount of alveolar bone loss ($P < 0.0001$) occurred in the infected iNOS-/- mice, compared to the infected iNOS+/+ mice at day 70 after inoculation. Therefore, although both iNOS knockout mice and their controls lost bone over time in the absence of externally introduced bacterial challenge, there was no significant difference in the amount of bone lost. In contrast, once *P. gingivalis* was introduced into the oral environment, there was an exaggerated amount of bone loss seen in the iNOS-/- mice, but not the iNOS+/+ mice.

Bone remodelling is a highly regulated and ordered process, characterized by osteoblast-mediated bone formation coupled with osteoclast mediated resorptive episodes. Osteoblasts and osteoclasts both produce and respond to NO.^{10,11} Studies examining the role of NO in bone cell activity have reported both stimulatory and inhibitory effects on bone remodelling, depending on the levels of NO and the model used.⁶ *In vitro* studies have shown a bi-phasic effect of NO on both osteoblastic and osteoclastic activity. Low levels of NO promote osteoblastic growth and differentiation in both an autocrine¹² and paracrine fashion,¹³ and also enhance IL-1 mediated resorption.⁶ iNOS has been shown to be essential for IL-1 mediated resorption by sustaining NF κ B in osteoclastic precursors.¹⁴ Conversely, high levels of NO have been shown to inhibit osteoblastic growth and differentiation,^{10,13}

possibly due to its pro-apoptotic effects on osteoblasts.¹⁵ These high levels have also been shown to inhibit osteoclastic formation and activity, together with promoting apoptosis of osteoclastic progenitors, when stimulated by IFN- γ in combination with IL-1 or TNF- α .^{6,11}

It appears that *P. gingivalis*-induced alveolar bone loss is increased in the absence of iNOS and NO. This would suggest that NO is protective against alveolar bone loss in the presence of an oral infection with *P. gingivalis*. This may be explained by higher levels of NO in wildtype mice resulting in inhibition of osteoclastic activity in the presence of a local inflammatory tissue response mediated by TNF- α or IFN- γ .

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A bioinformatics study of salivary proteins

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Saliva is a glandular secretion that is vital in the maintenance of healthy oral tissues. Recently, several proteomic studies have confirmed the identification of over 100 proteins and protein fragments by immunoblotting, 2D PAGE and HPLC combined with mass spectrometry. The aim of this study was to collate the reported proteins in saliva and investigate the protein source, attributed function, structure and disease association as well as gene locations.

A variety of molecular databases were searched including Online Mendelian Inheritance in Man (OMIM), LocusLink (now superseded by Entrez Gene), Swiss-Prot, Protein Data Bank (PDB), GeneCards® and CATH. The currently known total numbers of genes associated with craniofacial, oral, dental and salivary proteins and disorders were initially extracted from the human genome and OMIM databases and grouped according to chromosome number. A total of 1082 genes, encoding for proteins relating to the craniofacial, dental, salivary and oral environment, were found in the OMIM database. Of these only 704 genes were

matched to a specific chromosome. A further search for genes encoding proteins relating to craniofacial, dental, salivary and oral disorders was performed and 261 genes with known chromosomal loci were listed. Subsequently, analysis of the genes coding for the salivary proteins demonstrated a non-uniform chromosomal distribution, with chromosomes 1, 4, 11, 14 and 20 having the largest number of genes encoding for salivary proteins. Furthermore, chromosome 4 has the largest proportion of genes encoding for proteins derived from the salivary glands.

Analysis of the functions showed that two-thirds of the proteins include enzymes, proteinase inhibitors, inhibitors of crystal growth, immunoglobulins and proteins that are involved in immune activities. Since several diseases are associated with salivary disorders including Sjögren's syndrome, diabetes, periodontitis, Prader-Willi syndrome, rheumatoid arthritis, cystic fibrosis, caries and stress-related disorders, the reported roles of salivary proteins in various human disorders were collated. This literature search revealed a list of possible salivary biomarkers including defensins, histatins, calgranulin A, B and basic proline rich lacrimal protein.

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Narrowing of the inferior dental canal in relation to the lower third molars

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Removal of impacted lower third molars is a common event in dentistry and associated complications are well documented. Traditionally, periapical radiographs and rotational panoramic radiographs (panoramic radiographs) have been used to assess the relationship between the inferior dental canal and the adjacent third molar roots before surgery. Both conventional imaging modalities have their limitations, but perhaps the greatest limitation is the lack of three-dimensional information provided. Over the past few years, with the increased use of CT to assess the inferior dental canal in cases of impacted third molars, one of the current investigators noted that, in a relatively large number of cases, the inferior dental canal appears to be compressed between the roots of the adjacent third molar and the lingual or buccal cortex.

The objective of this study was to assess narrowing of the inferior dental canal in the lower third molar regions using computed tomography and to determine the value of radiographic markers on rotational panoramic radiographs in assessing the true relationships of the inferior dental canal.

Patients referred for computed tomography assessment of impacted lower third molars were used in this study. The lower third molars were assessed using computed tomography to determine the position and morphology of the inferior dental canal relative to the roots and the cortical plates. The radiographic markers on rotational panoramic radiographs were correlated

with the computed tomography findings when rotational panoramic radiographs were available.

The patients referred had 202 lower third molars. Inferior positioning of the inferior dental canal was the most common location on computed tomography. Narrowing of the inferior dental canal was found using computed tomography in relation to the lower third molars in 66.8 per cent of cases. The chance of narrowing of the inferior dental canal, as shown using computed tomography, increased when at least one of the radiographic markers – superimposition, narrowing, deviation or reduction in density – were present on the rotational panoramic radiograph. Deviation of the inferior dental canal on rotational panoramic radiographs was found to be the most significant predictor of narrowing of the inferior dental canal and a close relationship to the roots, as shown in computed tomography.

Narrowing of the inferior dental canal is a common finding when impacted lower third molars are assessed using computed tomography. Our current study demonstrates that rotational panoramic radiography can be of value in predicting when there is narrowing of the canal. Deviation of the canal on a panoramic radiograph is the most reliable predictor. When there is superimposition of the canal over the third molar roots or deviation of the canal in relation to the third molar roots, a close relationship between the canal and the third molar is very likely.

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Distribution of *Tannerella forsythensis* in an adolescent and an adult population

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Tannerella forsythensis (formerly called *Bacteroides forsythus*) is a fusiform, fastidious, anaerobic, Gram-negative organism found in sites of periodontal breakdown in higher numbers than in sites of gingivitis or health. *T. forsythensis* has also been detected more frequently and in higher numbers in active periodontal lesions than in inactive lesions. Also, it has been reported that subjects who harboured *T. forsythensis* were at greater risk of attachment than subjects in whom *T. forsythensis* were not detected. In the World Workshop in Periodontology *T. forsythensis* was

identified as a key periodontopathogen. While previous studies have shown that *T. forsythensis* can be detected in the plaque of adolescents – in 24 per cent of adult subjects with periodontal health and up to 53 per cent of adult patients with advanced periodontal disease – the distribution of *T. forsythensis* within the normal population is unknown. Therefore we have used a 16S rDNA based PCR to detect *T. forsythensis* in the dental plaque of an adolescent population and in an adult population. Subgingival plaque was collected from 228 subjects representing an adolescent population (aged

11-13 years) from Greater Manchester, UK and 498 subjects representing a normal adult population from the University of Queensland, aged 18-65 years. Samples were collected from the deepest and shallowest sites in each sextant of the dentition (up to 12 samples per subject) and placed in vials containing phosphate buffered saline and 0.01% Thimerosal then stored at -80°C. The samples from each subject were pooled and the DNA was extracted by boiling. The supernatant containing DNA was assayed using a standard PCR assay. The product (641bp) from the PCR was visualized using agarose gel electrophoresis. Restriction enzyme analysis of the *T. forsythensis* bands using DraI and ClaI, confirmed the specificity of the PCR result with expected 37, 102 and 287bp products. As controls, *T. forsythensis* type strain ATCC 43037 and a conserved 16S rDNA ubiquitous primer were included in all sample assays.

We have also used two specific primers which we have shown under our assay conditions to have different 'sensitivities' (10^3 and 10^7 cells) which allowed a semi-quantification of numbers of *T. forsythensis* organisms to be made. In the adolescent population, 25 per cent were found to carry *T. forsythensis*, albeit in relatively low numbers. In the adult population a total of 36.9 per cent were found to carry the organism, 7.5 per cent with high numbers ($>10^7$ cells). Although the incidence of *T. forsythensis* increased with age, there was no statistically significant association between age and level of *T. forsythensis* observed.

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A total of 100 *T. forsythensis* positive adult subjects were selected and IgG and IgG subclass antibodies in the serum samples were determined using a direct ELISA to detect the presence of anti-*T. forsythensis* antibodies. Standards of purified human IgG (15 to 250 ng/ml), negative and positive controls were included in each assay. A horse-radish peroxidase conjugated goat anti-human IgG was used to detect the specific *T. forsythensis* antibodies. A standard curve was then generated and used to determine the unknown values. The mean serum anti-IgG *T. forsythensis* antibody concentration was determined to be 1.8µg/ml, with a maximum concentration of IgG antibody being 11µg/ml. The medium IgG concentration was within the range of 0-3µg/ml. The predominant IgG subclass in this population of 100 *T. forsythensis* positive subjects was IgG1 with 68 per cent of the subjects exhibiting specific IgG₁ antibodies to *T. forsythensis*. This was followed by IgG4 with 53 per cent of the subjects, IgG3 with 48 per cent and IgG2 with 19 per cent. It was found that there was no statistical association of IgG antibody levels and periodontal disease. The overall low IgG levels and the presence of *T. forsythensis* in the plaque of these subjects may indicate that *T. forsythensis* antigens are non-antigenic with only low levels of antibodies elicited in some subjects and in some no antibodies were detected. Whether the serum IgG and subclass antibody responses are to protein or carbohydrate antigens of *T. forsythensis* will need further work.

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Synthesis of multiphosphorylated peptides of bovine dentine phosphophoryn for the development of novel dental restorative material

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Bovine dentin phosphophoryn (BDP), a protein rich in aspartyl (Asp) and O-phosphoseryl (Ser(P)) residues is synthesized by odontoblasts and believed to be involved in matrix-mediated biomineralization of dentin.¹ When immobilized on a stable support and incubated in physiological solutions of calcium and phosphate, phosphophoryn can induce the formation of hydroxyapatite (HA).² At high phosphophoryn concentrations, when phosphophoryn is free in solution, phosphophoryn tends to inhibit the crystallization of HA. This dual ability to initiate or inhibit HA formation has led to the currently accepted view that phosphophoryn plays an important role in the mineralization process. At low levels when attached

to the gap regions of collagen fibrils, phosphophoryn induces the formation of initial apatite crystals while at higher levels it controls the size, shape and orientation during crystal growth. Characterization of this protein has proved difficult due to the high degree of phosphorylation, extreme negative charge (pI 1.1)³ and redundant amino acid composition (80-90 per cent Asp and Ser residues).¹

Using ¹H NMR spectroscopy, it has been recently demonstrated that phosphophoryn is a uniformly flexible molecule which is consistent with the relatively featureless sequences.⁴ The significance of these results is that phosphophoryn, due to its uniform nature, has now the potential to be replaced by biomimetic peptide

analogues that together with amorphous calcium phosphate may lead to the development of novel, dental restorative materials and calcium phosphate delivery vehicles. Since phosphophoryn is highly resistant to proteolysis,⁵ the design and synthesis of multiphosphorylated biomimetic peptides was investigated. The aim of this study was to synthesize the nonphosphorylated peptide Asp-Ser-Ser-Asp-Ser-Ser-Asp-Ser-Ser-Asp and multiphosphorylated Asp-Ser(P)-Ser(P)-Asp-Ser(P)-Ser(P)-Asp-Ser(P)-Ser(P)-.

The synthesis of the unphosphorylated Asp-Ser-Ser-Asp-Ser-Ser-Asp was carried out using standard Fmoc protocols. Following cleavage from the resin support, the crude peptide was purified using reversed-phase HPLC. MALDI-TOF mass spectrometry and N-terminal sequence analysis confirmed the identity of the peptide. The purified peptide was analysed by 1D NMR spectroscopy. The β resonances of the aspartyl groups (β D) were observed at approximately 3ppm. These resonances were assigned using the random coil ¹H chemical shifts reported by Wüthrich⁶ and are also consistent with the Asp β shift assignments of bovine dentine phosphophoryn observed at 3.3ppm.⁴ The β resonances of the seryl groups are observed at approximately 3.9ppm and the α resonances of the seryl groups are observed at 4.5ppm. The amide resonances of aspartyl and seryl groups were observed between 8 and 9ppm. The J coupling of amides to alphas (6Hz) is consistent with this peptide being a random-coil.⁶ The amino acid side chains are expected to be in a flexible, extended confirmation.

The synthesis of the phosphorylated peptide analogue of phosphophoryn Asp- Ser(P)- Ser(P)-Asp-Ser(P)- Ser(P)-Asp- Ser(P)- Ser(P)- proved to be more challenging. Standard solid-phase peptide synthesis protocols for Fmoc chemistry were used.⁷ Synthesized Fmoc-amino acids were obtained from Auspep (Melbourne, Australia) and Fmoc-Ser(PO(OBzl)OH)-OH were obtained from Calbiochem-Novabiochem Pty Ltd (New South Wales, Australia). Peptides were assembled as the carboxamide form using PAL-Peg-PS resin (PerSeptive Biosystems Inc., Framingham, MA).

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The Fmoc group was removed with a continuous flow of 2% v/v DBU in NMP containing 2% v/v piperidine. Cleavage of the peptide from the resin support was performed using TFA:thioanisole:TIPS: water (90:5:2.5:2.5) cleavage cocktail for six hours. A number of synthesis strategies were explored in order to optimize the procedure. The first attempt using HBTU activation resulted in a low yield of the multiphosphorylated peptide. This was likely to be due to the difficulties of coupling and steric hindrance during assembly of multiphosphorylated residues. HATU has been reported to give superior results in coupling efficiencies, due to the presence of a tertiary amine, which has a greater catalytic effect.⁸ Synthesis of the multiphosphorylated peptide was subsequently optimized using HATU and a double coupling regime that resulted in a higher yield of product.

A variety of separation methods including reverse phase HPLC, anion exchange chromatography and size exclusion chromatography were employed to purify the short and highly acidic peptides. N-terminal sequence analysis confirmed the position of the residues. MALDI-TOF mass spectrometry confirmed the mass of the peptide. NMR spectroscopy showed that the chemical shifts of phosphoserine and aspartate groups to be in close agreement with those of phosphophoryn and confirmed the expected 3:2 ratio of phosphoseryl to aspartyl groups.

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Detection and quantitation of *Porphyromonas gingivalis* using peptide nucleic acid probes

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Chronic periodontitis is an inflammatory disease of the supporting tissues of the teeth leading to resorption of alveolar bone and eventual tooth loss. The disease is

a major public health problem in all societies and is estimated to affect up to 15 per cent of the adult population with severe forms affecting 5-6 per cent.

The development and progression of chronic periodontitis has been associated with specific Gram-negative bacteria in subgingival plaque. The presence of three Gram-negative bacterial species *Bacteroides forsythus*, *Treponema denticola* and in particular *Porphyromonas gingivalis* in subgingival plaque has been strongly associated with disease. The persistence of *P. gingivalis* in subgingival plaque from periodontitis patients after treatment (scaling and root planing) has been reported to be significantly associated with progressive alveolar bone loss. Furthermore, an increase in *P. gingivalis* cell numbers in subgingival plaque has been shown to correlate with disease severity as measured by attachment loss, periodontal pocket depth and bleeding on probing, and in animal models of disease oral inoculation with *P. gingivalis* leads to alveolar bone loss. A number of methods have been employed to detect *P. gingivalis*, including the use of DNA hybridization probes. Peptide nucleic acids (PNA) are synthetic pseudo-peptide DNA/RNA mimics, consisting of a synthetic backbone made up of N-(2-amino-ethyl)-glycine. The side-chain of each backbone unit is made up of the same nucleobases of DNA; guanine, adenine, thymine and cytosine and within the PNA oligomer the nucleobases are in the same confirmation and spacing that occurs naturally in DNA. Unlike DNA, PNA oligomers are uncharged, acyclic, achiral, biologically stable and are protease resistant. These unique properties of PNA oligomers allow faster and more effective hybridization and higher affinities of binding to DNA compared to DNA oligomers.

The aim of this project was to design a *P. gingivalis* specific and a *P. gingivalis* strain specific DNA and peptide nucleic acid probes and compare the level of detection using the dot-blot chemiluminescence assay.

Oligonucleotide probes for *P. gingivalis* 16sRNA (*P. gingivalis* specific) and ISPg4 (*P. gingivalis* W50 strain specific) genes were designed using NetPrimer (available online at www.PremierBiosoft.com). Biotinylated 16sRNA and ISPg4 DNA probes with the sequences GGTTCACCATCCGTCATCTACA (T_m; 58.54°C) and GCTTTTAGGACACTAATGGTTCG (T_m; 58.23°C), respectively, were synthesized by GeneWorks Pty Ltd (Adelaide, Australia). Biotinylated 16sRNA and ISPg4 PNA probes with the sequences CACCATCCGTCATC (T_m; 37.4°C) and ACAC-TAATGGTTCG (T_m; 30.55°C), respectively, were synthesized using standard solid-phase peptide synthesis protocols for Fmoc chemistry. The PNAs were assembled as the carboxamide form using Fmoc-XAL-Pal resin. Double coupling was achieved with HATU

activation using 5 equiv of Fmoc-PNA monomer in NMP and 7.5 equiv of DIPEA/lutidine solution in NMP for 20 minutes, followed by a capping step (acetic anhydride, two minutes) and then Fmoc removal with 20 per cent v/v piperidine in DMF for 2.5 minutes. Once synthesis was complete the PNA was removed from the solid support using TFA:m-Cresole:Triisopropylsilane (92.5:5:2.5) cleavage cocktail and purified using a Zorbax 300SB-C18 9.4 x 250mm 5µm column (Aligent, USA) installed in a Waters Delta 600 HPLC system, using 0.1 per cent v/v TFA in water and 0.1 per cent v/v TFA in 90 per cent aqueous acetonitrile as the limit buffer.

Serial dilutions of *E. coli* (JM109), *P. gingivalis* W50 and *P. gingivalis* ATCC 33277 DNA were hybridized with the biotinylated DNA and biotinylated PNA probes using a dot-blot hybridization assay. CDP-Star™ (Sigma, New South Wales, Australia) was used to detect the bound biotinylated probes, following manufacturer's instructions for streptavidin-alkaline phosphatase detection system and chemiluminescence was detected using a 1450 MicroBeta TriLux Liquid Scintillation and Luminescence Counter (Applied-Biosystems, New South Wales, Australia). Data was analysed using an independent samples one-way ANOVA with a Tukey's post Hoc test.

P. gingivalis species-specific and strain-specific biotinylated probes were manually synthesized using standard solid phase Fmoc chemistry. Reverse phase HPLC analysis of the biotinylated probes showed a single major product for each synthesized probe and MALDI-TOF mass spectrometric analysis gave observed masses of 4347.5Da and 4767.6Da for the 16s rRNA and ISPg4 PNA probes, respectively, which for both probes was 113Da more than the calculated mass which is typical of a TFA salt adduct. Dot-blot hybridization showed that DNA and PNA *P. gingivalis* 16s rRNA probes were specific for *P. gingivalis* and hybridized to DNA from both W50 and ATCC 33277 strains but not *E. coli* DNA. Furthermore, biotinylated PNA probes specific for the ISPg4 sequence had strong hybridization to *P. gingivalis* W50 DNA, with a detection limit of 6ng of DNA, but did not hybridize to *P. gingivalis* ATCC 33277 DNA or to *E. coli* DNA. Although the biotinylated synthetic DNA oligonucleotide probe specific for ISPg4 did not hybridize with *E. coli* DNA it hybridized at similar intensities to both *P. gingivalis* W50 and ATCC 33277 DNA. These results imply that the biotinylated PNA ISPg4 probe was more specific than the biotinylated synthetic DNA oligonucleotide ISPg4 probe. The data of this project suggest that PNAs could be employed as a hybridization probe for periodontopathic organisms such as *P. gingivalis* in a highly sensitive assay to aid in the diagnosis, treatment and management of periodontitis.

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Characterization of the role of the RgpA-Kgp proteinase-adhesin complex of *Porphyromonas gingivalis* W50 in the murine periodontitis model

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Periodontal diseases are bacterial-associated inflammatory diseases of the supporting tissues of the teeth and range from mild forms of gingivitis to more aggressive forms such as chronic periodontitis, which are characterized by the destruction of the tooth's supporting structures. Chronic periodontitis is associated with the subgingival colonization of a consortium of anaerobic, Gram-negative bacteria. Among them, *Porphyromonas gingivalis* has attracted considerable attention as it has been more closely associated with disease. Among a number of virulence factors associated with the pathogenicity of *P. gingivalis*, the trypsin-like proteinases have received considerable attention for their ability to degrade a broad range of host proteins including structural proteins and others involved in defence. The trypsin-like proteinase activity of *P. gingivalis* has been attributed to two Arg-specific proteinases designated RgpA and RgpB, which are encoded by the genes *rgpA* and *rgpB* respectively, and the Lys-specific proteinase Kgp, encoded by the *kgp* gene. Using the mouse lesion model, we have shown these proteinases contribute to virulence in the order $Kgp \gg RgpB \geq RgpA$.

The aim of this project was to determine the virulence and alveolar bone loss induced in the mouse periodontitis model when *rgpA*⁻, *rgpB*⁻, *kgp*⁻, *rgpA/rgpB*⁻ and *rgpA/rgpB/kgp*⁻ proteinase isogenic mutants of *P. gingivalis* strain W50 are used to orally infect mice compared with the wild type *P. gingivalis* W50 strain.

The *P. gingivalis* W50 wild type strain and the *rgpA*⁻, *rgpB*⁻, *kgp*⁻, *rgpA/rgpB*⁻ and *rgpA/rgpB/kgp*⁻ isogenic mutants were obtained from the culture collection of the Cooperative Research Centre for Oral Health Science, School of Dental Science, The University of Melbourne, Australia.

The mouse periodontitis experiments were approved by The University of Melbourne Ethics Committee for Animal Experimentation. Six to eight week old BALB/c mice (10 mice per group) received kanamycin (Sigma, New South Wales, Australia) at 1mg/mL in deionised water for seven days. Three days after the antibiotic treatment mice were orally inoculated four times, two days apart with 1×10^{10} viable *P. gingivalis* W50 cells (25µL of wild type or isogenic mutants) in PG buffer containing 2% wt/vol carboxymethylcellulose (CMC, Sigma, New South Wales, Australia). A control group was sham-infected with PG buffer containing 2% wt/vol CMC alone. Two weeks later mice received another four doses (two days apart) of 1×10^{10} viable *P. gingivalis* W50 cells (25µL of wild type or isogenic mutants) in PG buffer containing 2% wt/vol CMC. Four weeks after the second oral challenge, mice were

bled from the retrobulbar plexus and three days later, mice were killed and the maxillae removed.

Maxillae were boiled for one minute in deionised water, mechanically defleshed and immersed in 2% wt/vol potassium hydroxide (16 hours, 25°C). The maxillae were then washed (2 x deionised water) and immersed in 3% wt/vol hydrogen peroxide (6 hours, 25°C). After washing (2 x deionised water) the maxillae were stained with 0.1% wt/vol aqueous methylene blue and a digital image of the buccal side was captured with an Olympus DP12 digital camera mounted on a dissecting microscope using OLYSIA BioReport software version 3.2 (Olympus Australia Pty Ltd, New South Wales, Australia) to assess horizontal bone loss. Horizontal bone loss is loss occurring in a horizontal plane, perpendicular to the alveolar bone crest that results in a reduction of the crest height. Maxillae were aligned so that the buccal and lingual molar cusps were superimposed. A micrometer scale in plane with the maxillae was digitally imaged at the same time so that measurements could be standardized for each image. The area from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) for each tooth was measured using OLYSIA BioReport software version 3.2 imaging software. Bone loss measurements were determined twice in a random and blinded protocol by two standardized examiners.

To evaluate the virulence of the isogenic mutants, BALB/c mice were orally inoculated with eight doses (1×10^{10} /viable cells per dose) of the proteinase mutants and wild type *P. gingivalis* W50 and the resulting periodontal bone loss measured. Mice inoculated with the *rgpA*⁻ mutant developed similar levels of alveolar bone loss compared to the wild type. Furthermore, although there was a reduction in the mean alveolar bone loss in mice inoculated with *rgpB*⁻ and *rgpA/rgpB/kgp*⁻ the level of bone loss was not significantly different from wild type. However, mice inoculated with either the *kgp*⁻ or the *rgpA/rgpB*⁻ double mutant did have significantly less ($p < 0.05$) alveolar bone loss compared with the wild type *P. gingivalis* W50 strain. Mice inoculated with *P. gingivalis* wild type and mutant strains were bled and serum IgG, IgG1, IgG2a, IgG2b, IgG3, IgA and IgM titers were determined by ELISA. The predominant *P. gingivalis*-specific antibody response induced was IgG3. No IgG1, IgG2b, IgG2a, IgM or IgA response was detected. Mice challenged with the proteinase isogenic mutants also had the same *P. gingivalis*-specific antibody response as induced by the wild type strain.

In this study the role of RgpA, RgpB and Kgp in the virulence of *P. gingivalis* was studied in the mouse periodontitis model using proteinase isogenic mutants

of *P. gingivalis*. Inactivation of either the *kgp* gene or both the *rgpA* and *rgpB* genes (*rgpA*⁻/*rgpB*⁻ double mutant) resulted in a significant reduction in the pathogenicity of *P. gingivalis* W50, however, inactivation of the *rgpA* gene alone had no significant

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Anxiety control in dental practice: use of lavender

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Dental odours, especially eugenol, are regularly identified as conditioning factors contributing to dental anxiety due to their ability to provoke unpleasant memories. Many dentists use perfumes to mask the dental odour in their reception areas and surgeries. Some of these perfumes are derived from lavender oil, which in aromatherapy has a long history of application as a sedative and in treating anxiety and depression.¹ The effectiveness of lavender has been tested in delivery rooms,² intensive care units³ and hemodialysis units⁴ with indications that it may reduce stress. It has also been tested as a masking odour in hospital wards with positive results.⁵

This study was a controlled clinical trial of the use of lavender as an anxiety-reducing agent for dental patients in private practice. Four dentists were recruited to undertake the trial in their private practices on general patients, eliminating those with appointments for excessively fear-provoking treatment or with respiratory conditions. Four odours were compared using a standardized protocol for delivery of the odour. The four odours were water (neutral), vanilla (pleasant odour with no known therapeutic applications), eugenol (unpleasant odour with negative dental responses) and lavender (test odour). An equal number of patients in each surgery were exposed to each odour. Each patient completed two questionnaires based on Kleinknecht's Dental Fear Scale⁶ – one prior to the appointment and one in the reception area immediately following the appointment. The dentist also completed a questionnaire following the appointment. While the patients consented to filling in the questionnaires they were not told of the use of odour in the surgery. Only one patient mentioned any of the odours (lavender).

Valid responses for all three questionnaires were obtained from 115 respondents (10 patients had one or more forms missing). The patients were all adults, 60 per cent were female and 46 per cent were in the 30-50 year age bracket. Fifty-five per cent were regular dental attendees. Using a 1-10 scale, 32 per cent of patients were not afraid prior to treatment commencing

effect. These results suggest that the Lys- and Arg-specific proteinase activity may have a significant role in the virulence directly or indirectly by processing of virulence factors of *P. gingivalis*. In conclusion, by characterizing the virulence of isogenic proteinase mutants, we have shown that both Lys- and Arg-specific proteolytic activity contribute to the virulence of *P. gingivalis* in the mouse periodontal model.

and 27 per cent expressed fear levels greater than five. There was no difference in the levels between regular and irregular attendees. Irregular attendees reported delaying making appointments ($p>0.001$) and failing to attend arranged appointments ($p>0.05$) more frequently than regular attendees, however this was unrelated to self-reported fear levels. Overall, males were less fearful than females both before treatment and following treatment ($p>0.05$) but the changes in fear levels before and after treatment were similar for both sexes.

No significant differences were recorded in the use of lavender as a means of anxiety reduction as compared with any of the other odours. Lavender was, however, the only odour that did not record an increase in self-reported anxiety levels post-treatment. The dental staff were positive in their acceptance of lavender as a base for perfume in the dental surgery environment. However, results of this study do not support the use of lavender as an anxiety-reducing therapy.

A second component of the study was the patient reaction to completing an anxiety questionnaire before and after treatment. Sixty-two per cent of patients wanted their anxiety results forwarded to their dentist with no difference in attendance patterns or fear levels. In the comments section, both patients and staff indicated that undertaking a self-reported anxiety questionnaire was a valuable tool in treatment and wanted to continue its use.

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Effect of prostaglandin E₂ on the synthesis of RANKL, OPG, ALP and PGEs in the periodontium: an *in vivo* and *in vitro* study

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The osteoblast differentiates and matures from progenitor cells and during this process it produces regulatory cytokines which modulate the bone remodelling cycle. The aims of this study were to determine *in vitro* how exogenous PGE₂ affects the expression of the genes for nuclear factor κβ-ligand (RANKL), osteoprotegerin (OPG), alkaline phosphatase (ALP) and prostaglandin synthase (PGEs) in cultured primary osteoblasts. Osteoblastic cells obtained from the long bones and jaws of three Wistar rats were cultured and stimulated with 10⁻³M, 10⁻⁵M or 10⁻⁷M PGE₂, ascorbic acid plus β-glycerophosphate (positive controls), or cultured in Dulbecco's modified media (negative controls) for 5, 10, 15 and 20 days. Relative quantitation PCR was used to determine the relative expression of the target genes. In addition, the ratio for RANKL and OPG genes expression was calculated. The results were analysed by one-way ANOVA. RANKL gene expression was significantly higher (p<0.001) in osteoblasts treated with PGE₂ at the various concentrations for five days. Higher RANKL gene expression was maintained in cells

treated with 10⁻³M PGE₂ over the other experimental periods, but not in those treated with 10⁻⁵ and 10⁻⁷M PGE₂, where this gene expression was reduced. Furthermore, the RANKL/OPG ratio showed that higher levels of OPG were produced by osteoblasts at the various experimental periods when the cultured osteoblasts were stimulated with the two lower doses of the eicosanoid. PGEs gene expression was significantly reduced in cells treated with the two lower doses of the eicosanoid after culturing for 15 days (P<0.01) and 20 days (p<0.05). Conversely, ALP gene expression increased in the same PGE₂-treated groups for the same experimental periods. Osteoblasts treated with the two lower concentrations of PGE₂ showed a similar pattern of expression of the target genes to that observed in the positive controls, whereas osteoblasts treated with the higher concentration of the eicosanoid followed a pattern similar to that observed for the negative controls. It is concluded that *in vitro* PGE₂ affects osteoblast biology in a dose dependent manner, whereas at concentrations of 10⁻⁵ and 10⁻⁷M, the eicosanoid stimulates the expression of genes favouring osteoblasts maturation and bone mineralization, but a higher concentration appears to produce the opposite effect.

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Investigation of the role of a novel C-terminal protein motif in anchoring proteins to the *Porphyromonas gingivalis* outer membrane

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Porphyromonas gingivalis is a major aetiological agent in adult periodontitis. Establishment and persistence of the organism in the periodontal pocket involves molecules both secreted into the extracellular environment and upon the surface of the cell, such as fimbriae and proteinases, particularly RgpA, RgpB and Kgp. RgpA and RgpB are arginine-X specific enzymes and Kgp is a lysine-X specific enzyme that are encoded by *rgpA*, *rgpB* and *kgp* respectively. RgpA and Kgp associate with various adhesin molecules to form a noncovalently associated complex at the cell surface. RgpB also occurs on the cell surface, but does not appear to be associated with the RgpA/Kgp/adhesin complex. The means by which RgpB and the RgpA/Kgp/adhesin complex, and other proteins, attach to the outer membrane of the bacterium is unknown.

RgpB is recognized by an antibody (mAb 1B5) raised against *P. gingivalis* lipopolysaccharide (LPS) that has been shown to recognize a carbohydrate epitope. We

have demonstrated that other *P. gingivalis* outer membrane proteins (OMPs) RgpA27, P27 and P59 are also recognized by mAb 1B5 yet have no sequence similarity to RgpB other than in the very 70-80 C-terminal amino acids, suggesting that the carbohydrate modifications may be in the C-termini. Alignment of these and other proteins identified from translation of the *P. gingivalis* W83 genome sequence have revealed that numerous other (novel) *P. gingivalis* proteins that are predicted to be extracellular have similar C-terminal sequences. Several amino acids, two tyrosines, an aspartic acid and two glycines appear to be conserved between most of these proteins which indicates that there may be a common secretory and or attachment process used for *P. gingivalis* OMPs. Further, it is possible that the tyrosines are targets for O-linked glycosylation and so would be important for the post-translation modifications seen with *P. gingivalis* OMPs.

The aims of this study were to begin investigation of this novel C-terminal motif found within *P. gingivalis* W50 OMPs, using RgpB as a model. More specifically the aims were to use site-directed mutagenesis to alter the RgpB C-terminal residue tyrosine(718) to phenylalanine and ascertain the effect of this mutation on the outer membrane localization and activity of RgpB.

Site-directed mutagenesis of Tyr(718) followed the protocol of the Stratagene QuickChange™ Site-Directed Mutagenesis Kit and employed the complementary pair of oligonucleotides (CACAAAACGGCGTGTGGCCGTTTCGCATCGC) and (GCGATGCGAACGGCAAACACGCCGTTTTGTG). The mutagenesis was performed using pBH1.1 as template, a plasmid previously generated in our laboratory that has a PCR-derived *P. gingivalis* W50 *rgpB* gene ligated into pGEM-Teasy. The mutated plasmid was transformed into *E. coli* and the DNA sequence verified. The unmodified (positive control) and mutated genes were then ligated into the *E. coli/P. gingivalis* shuttle plasmid pYH411 and used to transform *E. coli*. Successful recombinant plasmids pLC1 (pYH411::*rgpB*, positive control), *prgpBPhe*(718) and unmodified pYH411 (negative control) were used to transform *P. gingivalis* strain YH522AB. YH522AB has *rgpA* and *rgpB* inactivated by gene insertions (*rgpA*::*cat*,*rgpB*::*tetQ*) and has no endogenous Arg-X hydrolytic activity. Selection of pYH411 constructs in *P. gingivalis* was on horse blood agar plates supplemented by 10µg/ml erythromycin.

Recombinant *P. gingivalis* strains ECR7 (YH522AB::pLC1), ECR138 [YH522AB::*prgpBPhe*(718)] and ECR5 (YH522AB::pYH411), were assayed for Arg-X

specific substrate hydrolysis using N α -Benzoyl-L-arginine-*p*-nitroanilide (BAPNA) as substrate. Cultures were grown at 37°C, in batch culture, using brain heart infusion broth supplemented with 10µg ml⁻¹ erythromycin, 5mg ml⁻¹ cysteine and 5µg ml⁻¹ haemin, to a cell density of 2.5x10⁹ cells per ml. Cells were harvested by centrifugation at 6000g for 20 minutes and washed in a buffer composed of 50mM Tris-HCl, 5mM CaCl₂, 150mM NaCl, pH 8.0.

Whole cells of ECR5, ECR7 and ECR138 displayed 0.0, 7.0±0.7 and 4.4±0.6 units of hydrolytic activity per 10¹¹ cells assayed respectively, showing a significant decrease in whole cell-associated Arg-X activity by the mutated RgpB relative to wild-type. Culture supernatants of ECR5, ECR7 and ECR138, which had been subjected to centrifugation at 40,000g for 30 minutes to pellet vesicles, displayed 0.0, 0.3±0.03 and 0.4±0.08 units of hydrolytic activity per 10¹¹ cells at harvest respectively, showing a definite increase in Arg-X activity associated with the mutated RgpB relative to that of wild type. Preliminary western immunoassay showed that RgpB[Tyr(718)Phe] displayed the typical non-specific molecular mass profile observed for the wild-type enzyme and that like wild type RgpB it was also associated with vesicles. Interestingly, the western immunoassays suggested RgpB[Tyr(718)Phe] to be more abundant in the culture supernatant than the wild-type control, which agreed with the activity data. Together the data indicated that although, by itself, the mutation Tyr(718)Phe is not sufficient to prevent transport of the enzyme through the outer membrane, subsequent post-translation modification and outer membrane association, it does influence the distribution of RgpB in a growing culture.

The roles of the other conserved C-terminal residues in the outer membrane localization and activity of RgpB are currently under investigation.

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Construction of *Porphyromonas gingivalis* strains and plasmids for improved gene cloning and expression

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The periodontal pathogen *Porphyromonas gingivalis* produces three major extracellular proteolytic enzymes, the gingipains. The gingipains comprise of two arginine-specific enzymes RgpA and RgpB and a lysine-specific enzyme Kgp that are encoded by *rgpA*, *rgpB* and *kgp* respectively. The gingipains are believed to have a role in the maturation of each other as well as other *P. gingivalis* proteins, such as FimA and the haemagglutinins. However, when purifying *P. gingivalis* proteins the gingipains can be a hindrance because they can degrade the target protein. To prevent this degradation it is necessary to use inhibitory compounds, such as tosyl-L-lysine-chloromethyl ketone (TLCK), which are hazardous and expensive. Therefore

it would be useful to purify *P. gingivalis* proteins from cells in which the gingipain-encoding gene(s) have been inactivated. In some cases however maintaining Rgp or Kgp proteinase activity would be of use in the study of proteins such as FimA which require processing by these enzymes.

The study of *P. gingivalis* genes and proteins using heterologous hosts such as *Escherichia coli* has been limited by poor promoter recognition or production of biologically inactive proteins. The ability to introduce genes into *P. gingivalis* via a plasmid vector would be of use in gene expression and recombinant protein analysis. Therefore the aim of this work was to produce isogenic mutants of *P. gingivalis* lacking RgpA, RgpB

and Kgp activity, individually and in concert, and to construct a plasmid for gene cloning and expression within these mutants.

Mutants were constructed using *P. gingivalis* strains YH522, which is a restriction-modification mutant that is amenable to transformation with plasmids, and strain W50, which is a laboratory strain used in virulence studies. Gingipain-encoding gene inactivations were achieved using electroporation to introduce linear DNAs containing the insertion-inactivated genes into cells with allele replacement occurring via double crossover homologous recombination. All mutations were confirmed by Southern hybridization using the appropriate genes as probes.

rgpA::cat mutants were made as follows. A *cat* gene, which encodes chloramphenicol acetyl transferase was ligated into the *EcoRV* site of plasmid pNS3.8 that contains a *Bam*HI fragment encompassing the 5' end of *rgpA* in pUC18. Successful transformants were selected on horse-blood agar (HBA) containing 10 µg/ml⁻¹ chloramphenicol. *rgpB::tetQ* mutants were made by ligating a 2.65kb *Eco*ICRI fragment from the *E. coli/Bacteroides* shuttle plasmid pNJR12 (that contains *tetQ* and produces resistance to tetracycline in *P. gingivalis*) into the *Eco*72I site of plasmid pBH1.1. pBH1.1 contains a polymerase chain reaction (PCR)-derived insert of the *P. gingivalis* W50 *rgpB* gene, in pGemT-Easy. The recombinant plasmid was used as a template for PCR to amplify *rgpB::tetQ* and this PCR-product used to transform *P. gingivalis*. Transformants were selected on HBA containing 1 µg/ml⁻¹ tetracycline. *rgpA::cat,rgpB::tetQ* double mutants were made by insertion inactivation of the *rgpB* gene in the *rgpA::cat* mutant, using the method to derive the *rgpB::tetQ* single mutant.

The gene *kgp* was disrupted with either *ermF-ermAM* (erythromycin resistance), or the *Bacteroides fragilis* cephalosporinase encoding gene, *cepA*, which results in resistance to β-lactam antibiotics. *ermF-ermAM* was excised from the *E. coli/Porphyromonas* shuttle replicon pYH411 (a gift from Hisashi

Yoshimoto, Kanaga Dental College, Yokosuka, Japan) using a *Sca*I/*Eco*RI double digest, blunt ended and inserted into the *EcoRV* site of *kgp*, which was previously cloned in our laboratory as a PCR fragment into pGemT-Easy. The *kgp::ermF-ermAM* DNA was used to transform *P. gingivalis* and erythromycin resistant transformants selected on HBA containing 10 µg/ml⁻¹ erythromycin. *cepA* was excised from pEC474 (a gift from Professor CJ Smith, East Carolina University, North Carolina, USA) as an *EcoRV/Msc*I fragment and ligated to *Mfe*I in *kgp*, as above. Recombinants were selected using 5 µg/ml ampicillin in HBA plates.

P. gingivalis proteinase deficient mutants we have constructed to date include: YH522A, *rgpA::cat*; YH522B, *rgpB::tetQ*; YH522AB, *rgpA::cat,rgpB::tetQ*; YH522-G2, *kgp::ermF-ermAM*; YH522ABK, *rgpA::cat,rgpB::tetQ,kgp::ermF-ermAM*; YH522KAB, *rgpA::cat,rgpB::tetQ,kgp::cepA*; and W50ABK, *rgpA::cat,rgpB::tetQ,kgp::ermF-ermAM*.

Mutants such as YH522ABK and W50ABK have resistance to both tetracycline and erythromycin, so selection of cells transformed with currently available shuttle plasmids such as pYH411 or pNJR12 would be difficult. Therefore a new *E. coli/Porphyromonas* shuttle plasmid, pCS22 which uses *cepA* as a selection marker was constructed. To construct pCS22 we replaced the *ermF-ermAM* cassette from pYH411 with the pUC18 *lacZ* gene, creating pCS14 and facilitating blue-white recombinant screening in *E. coli*. We then used PCR to amplify *cepA*, incorporating *Aat*II restriction enzyme sites at each end. This DNA was ligated to the *Aat*II site in pCS14 which completed the vector. *P. gingivalis* pCS22 transformants exhibit up to 100 µg/ml ampicillin resistance on HBA. Our successful expression of *cepA* in *P. gingivalis* is the first demonstration of β-lactamase production by this species.

The *P. gingivalis* gingipain-deficient strains and plasmid pCS22 created during this study are expected to facilitate production of proteins for use in virulence studies accelerating progress in our aim of devising an effective vaccine for the prevention of periodontal diseases associated with *P. gingivalis* colonization.

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A C-terminally truncated recombinant Lys-specific proteinase is transcribed but not expressed in *Porphyromonas gingivalis*

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Several putative virulence factors have been implicated in the pathogenicity of *Porphyromonas gingivalis*, a major aetiological agent of chronic periodontitis. Of these, the extracellular Arg- and Lys-specific proteinases (Gingipains) encoded by three

genes (*rgpA*, *rgpB*, *kgp*) have been suggested to play a major role in disease pathogenesis. The proteins encoded by *rgpA* and *kgp* of strain W50 form cell-associated complexes composed of 45kDa Arg-specific proteinase (*RgpA_{cat}*) associated with four sequence-

related adhesins, RgpA_{A1} – RgpA_{A4} all encoded by *rgpA*, and a 48kDa Lys-specific proteinase (KgpAcat) associated with potentially five sequence-related adhesins KgpA₁₋₅ all encoded by *kgp*. Kgp_{cat} and RgpA_{cat} contain an adhesin binding motif (ABM1) that is repeated in several of the adhesin domains and is proposed to be responsible for the non-covalent association of the catalytic and adhesin domains in the complex.

Previous attempts to purify native Kgp_{cat} directly from wild-type *P. gingivalis* culture showed that the Kgp_{cat} could not be purified at the scale or the level of purity required. Further, attempts at producing the *P. gingivalis* RgpA_{cat} and Kgp_{cat} using *E. coli* expression systems in our laboratory and by others, has produced insoluble recombinant proteins with very low specific activity. The objective of the present study was to produce a plasmid-encoded recombinant Lys proteinase (r-Kgp) in *P. gingivalis* Kgp mutant strains in order to allow for efficient large-scale purification of r-Kgp for further investigation.

We have constructed a recombinant modified Δkgp that encodes residues 1-445 of Kgp_{cat} followed by a hexa-His tag, a KKRR proteinase processing site and the 70 C-terminal residues of Kgp_{A5}.

The gene Δkgp was designed so that r-Kgp_{cat} (residues 446-509) would be truncated in the C-terminus to remove ABM1, thus enabling purification of an adhesin-free recombinant proteinase. Codons for the Kgp_{A5} C-terminal extension were incorporated in Δkgp as evidence suggests that this region is essential for the processing and export of the protein through the cell membrane (EC Reynolds, personal communication). Codons for a 'KKRR' proteinase processing site were incorporated in Δkgp to allow proteolytic release of the truncated r-Kgp from the C-terminal extension which remains attached to the cell outer membrane. The Δkgp was constructed to facilitate purification of the r-Kgp_{cat} from the culture supernatant using nickel column chromatography.

Δkgp was constructed using a DNA 'SOEing' procedure as follows. A polymerase chain reaction (PCR) was used to generate a fragment from pUC18::*prtK*¹ plasmid DNA using various oligonucleotides: oligo 1, (5'GCCTGGCCATCTAGAGTTCGACCCGACTCCTTAGTCAAGGATC3') that encodes a buffer followed by a Ball, XbaI, Sall site and 21 nucleotides that are found approximately 350 bases upstream from the promoter of *kgp* and oligo 2, (5'GCGACGCTTCTTATGATGATGATGATGATGAGGAAGATAATTAGAGCGAGTGATAAC3'). Oligo 2 is reverse and complimentary to a sequence that encodes residues 437-445 of Kgp_{cat} followed by six His

residues, two Lys and two Arg residues. A second PCR fragment was generated from *P. gingivalis* W50 genomic DNA using oligo 3 and oligo 4. Oligo 3 (5'CATCATCATCATCATCATAAGAAGCGTCGCGCAGACGTAACGGCTCAGAAG3') encoded six His residues followed by two Lys and two Arg residues and seven residues of the Kgp_{A5} adhesin. Oligo 4 (5'CGGTGGCCAGGTACCGTCGACTTACTTTACAGCGAGTTTCTCTACG3') is reverse and complementary to a sequence downstream of *kgp* followed by a Sall, KpnI, Ball site and a buffer. PCR fragments 1-2 and 3-4 were annealed via their overlapping regions and then subjected to a further PCR using oligos 1 and 4 to generate fragment 1-4. Fragment 1-4 was ligated into pGEMT-Easy (Promega) and transformed into *E. coli* JM109. The insert from pGEMT-Easy::*kgp* was ligated into pNJR12, electroporated into *E. coli* DH5 α and the integrity of the insert confirmed by sequence analysis. Recombinant pNJR12::*kgp* plasmid DNA was transferred into electrocompetent *P. gingivalis* W50KIA (Kgp mutant) and selected on horse blood agar containing 1 μ g/ml tetracycline and 10 μ g/ml erythromycin to generate strain ECR78.

The presence of Δkgp mRNA in ECR78 was analysed by northern blot and a recombinant transcript of appropriate size (2.9kb) was detected. The presence of r-Kgp in ECR78 was tested using enzymatic assay and western blot analysis. No Lys-specific proteolytic activity was present in ECR78 nor could r-Kgp be detected in a western immunoassay. Further, attempts to purify r-Kgp from whole cells and culture fluid by nickel-affinity chromatography were also unsuccessful. In support of these results Sztukowska *et al.*,² have reported a similar phenomenon in a study investigating expression of a series of deletion variants encoding C-terminally truncated Kgp in *P. gingivalis*. Results show that *kgp* constructs truncated to amino acids 374 or 453 of Kgp_{cat} with His-tags on the carboxyl termini, were readily transcribed yet little or no protein product could be detected, whereas longer *kgp* constructs were less affected by the truncation.² Similarly, attempts to produce His-tagged, C-terminally truncated rRgpB in our laboratory have resulted in recombinant mRNA but no detectable protein product. Together these results suggest that the C-terminal region of the Gingipains is important for protein translation and/or stability of the nascent polypeptide.

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The effect of fluoride on rat enamel amelogenins

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Enamel fluorosis is a defect in tooth enamel mineral resulting from toxic doses of fluoride ingested during amelogenesis. Although no definitive mechanism is currently available to explain the formation of this abnormal enamel, it is generally accepted that the fluoride influences the ameloblasts in their production and maturation of the enamel matrix. In an enamel fluorosis model in which rats are treated with sodium fluoride, secretory ameloblasts of incisor tooth germs exhibit disruption of intracellular transport of newly synthesized amelogenins. The toxic influence of fluoride on ameloblast enamel protein secretory metabolism is particularly apparent with increased numbers of lysosomal-like organelles.^{1,2} *In vitro* studies suggest fluoride promotes the intracellular degradation of amelogenins in secretory ameloblasts.³ Lysosomal organelles degrade proteins, through the activities of specific proteases. In previous studies, we have histologically identified lysosomal proteases dipeptidyl peptidase II [E.C. 3.4.14.2] (DPPII) and cathepsin B [3.4.22.1] (CathB) in rat incisor secretory ameloblasts.^{4,5}

The aims of this study were three fold: first, to measure the activities of DPPII and CathB in the enamel organ of the rat incisor; second, to ascertain whether rat secretory enamel proteins (amelogenins) are degraded by these proteases *in vitro*; and third, to ascertain whether the rat incisor enamel organ activities of either DPPII or CathB are influenced by fluoride treatment.

Results for the first two aims were achieved (see *Published*) by harvesting rat incisors and kidneys (positive control tissue) from six female, 10 week old Wistar rats. Whole enamel organs were dissected from rat teeth. Then, DPPII and CathB activities were measured in the enamel organ tissue extracts using specific fluorogenic substrates. Analysis indicated that the rat incisor enamel organs contained specific activities of both DPPII and CathB at levels comparable with those in kidneys which are rich in both these lysosomal proteases. A purified extract of rat incisor enamel, positive for amelogenins (as determined by SDS-PAGE and Western blot), was incubated *in vitro* with purified preparations of both DPPII and CathB. Loss of protein bands in Western blots of SDS-PAGE of enamel incubation samples confirmed that the rat incisor enamel amelogenins were degraded by both DPPII and CathB.

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Results for the third aim were obtained from the *in vivo* fluoride treatment of 12 female, three week old Wistar rats. Randomly divided into three groups, the rats were given two intraperitoneal injections per day of either isotonic saline, NaF, 10mg per kg body weight, or NaF, 20mg per kg body weight over a period of 4.5 days. Then, as previously, DPPII and CathB activities were measured in the incisor enamel organs from each rat. The enamel organs from rats given 0, 10 and 20mg NaF per kg body weight had, respectively, DPPII specific activities of 50.2±6.5, 54.4±5.9, and 59.8±11.7mmol/min/mg protein and CathB specific activities of 58.7±4.7, 64.6±12.3, and 76.5±12.2mmol/min/mg protein (mean ± 2SEM, n=4). Linear regression analysis of both sets of data showed a significant (P<0.001) positive trend between protease activity and fluoride dose.

This study has shown: (a) that rat incisor enamel organ demonstrates potent levels of activity of lysosomal proteases DPPII and CathB; (b) that these two proteases are capable of degrading amelogenins *in vitro*; and (c) that both proteases show increases in the level of their activity with *in vivo* treatment with increasing levels of sodium fluoride.

These results support the premise of an increased protein catabolism occurring in ameloblasts producing fluorotic enamel.

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The effect of fluoride on rat enamel glycoproteins

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If fluoride is ingested, even in relatively small amounts, during the period of amelogenesis of a developing tooth, the resulting mature enamel mineral is abnormal. Although no definitive mechanism is currently available to explain the formation of this defective (fluorosed) enamel, it is generally accepted that the fluoride influences the production and maturation of the enamel matrix by the ameloblasts. Biom mineralization of extracellular matrices is thought to involve not only the presence of particular matrix molecules e.g. proteins, but also their associated sugar moieties.

In enamel fluorosis model rats treated with sodium fluoride, secretory ameloblasts of incisor tooth germs exhibit disruption of intracellular transport of newly synthesized-secretory enamel protein. The influence of fluoride on ameloblast intracellular vesicular transport is particularly apparent in the Golgi apparatus, the site of post-synthetic protein glycosylation.

The aim of this study was to assess the effect of fluorotic levels of sodium fluoride (NaF) in drinking water on the total sugar content of rat incisor secretory enamel matrix. Previous studies have established that, in rats, ingestion of drinking water containing 100ppm fluoride, as NaF, for a few weeks, results in the formation of fluorosed incisors. Forty-eight female, three week old, Wistar rats were randomly divided into three treatment groups: animals with drinking water

which was either de-ionized water, de-ionized water containing 10ppm fluoride, as NaF or de-ionized water containing 100ppm fluoride, as NaF. All rats were fed the drinking water *ad libitum* and maintained on the same rat chow for 49 days. On the last day of treatment all rats were killed and their incisor teeth were removed, snap-frozen and freeze-dried. Secretory enamel samples were dissected and pooled from the upper and lower incisors of four rats in a treatment group. From these enamel samples, protein was obtained by sequential extraction with 0.5M acetic acid (containing protease inhibitors). Acid extracts, for each enamel sample, were pooled, de-salted (by 5kDa cut-off, aqueous dialysis) and then freeze-dried. The enamel protein preparations were analysed for both total sugar content (measured as galactose using 2-aminothio phenol) and total protein content (measured as bovine serum albumin using bicinchoninic acid). The secretory enamel from rats given 0, 10 and 100ppm fluoride had, respectively, total sugar contents of $9.6 \pm 2.4 \mu\text{g}/\text{mg}$, $8.3 \pm 2.5 \mu\text{g}/\text{mg}$, and $13.0 \pm 3.6 \mu\text{g}/\text{mg}$ and total protein contents of $117 \pm 16 \mu\text{g}/\text{mg}$, $112 \pm 17 \mu\text{g}/\text{mg}$, and $107 \pm 6 \mu\text{g}/\text{mg}$, (mean \pm 2SEM, n=4). The sugar content of the fluorosed incisor secretory enamel (100ppm fluoride treatment) was significantly higher ($P < 0.05$, ANOVA) compared with non-fluorosed incisor secretory enamel (0 and 10ppm fluoride treatments).

This study has shown that rat incisor secretory enamel contains measurable levels of total sugar and these levels are significantly altered in fluorotic rat teeth.

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Identification of mouse haplotype H-2b, H-2d and H-2k MHC-restricted immunodominant T-cell epitopes from the RgpA-Kgp proteinase-adhesin complex of *Porphyromonas gingivalis*

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Porphyromonas gingivalis is a Gram-negative bacterium that has been associated with chronic periodontitis, a chronic inflammatory disease of the supporting tissues of the teeth. Previous reports have shown that animals immunized with killed, whole *P. gingivalis* cells were protected against *P. gingivalis*-induced bone loss in the rat periodontitis model and the ligature monkey model, indicating that a vaccine against *P. gingivalis* may protect against chronic periodontitis. A major virulence factor common to both invasive and non-invasive strains of *P. gingivalis* is the RgpA-Kgp proteinase-adhesin complex. The RgpA-

Kgp complex has been shown to induce a protective immune response, with a predominant Th2 cytokine and antibody response in the mouse lesion and periodontitis models. Furthermore, synthetic peptides (from the RgpA-Kgp complex) conjugated to diphtheria toxoid, when used as vaccines induced protective antibodies in mouse lesion and periodontitis models. In order to produce a fully synthetic peptide vaccine, which has considerable advantages over conventional vaccines, both B-cell and T-cell epitopes from the pathogen of interest need to be identified. We have previously identified protective B-cell epitopes

from the RgpA-Kgp complex, however, little work has been conducted on the investigation of T-cell epitopes of the RgpA-Kgp complex. The aim of this research was to identify the immunodominant T-cell epitopes of the RgpA-Kgp complex in three genetically different mouse strains: BALB/c (H-2d), CBA (H-2k) and C57BL/6 (H-2b).

192 overlapping peptides (offset by 10 and overlapping by five residues) representing the RgpA-Kgp complex were synthesized on diketopiperazine pins using standard solid phase peptide synthesis protocols for Fmoc chemistry. Prior to cleavage from the pins, the side-chain protecting groups were removed by submerging the pins in TFA:ethanedithiol:anisole (95:2.5:2.5). Cleavage of the peptides from the pins was achieved by submerging the pins in 0.1M ammonium bicarbonate (pH 8.4) containing 40 per cent v/v acetonitrile. Cleaved peptides were lyophilized and stored at -20°C. RgpA-Kgp complex was extracted and purified from the *P. gingivalis* W50 cells as previously described by O'Brien-Simpson *et al.*¹

BALB/c mice were obtained from the animal facility at the Department of Immunology and Microbiology at The University of Melbourne. RgpA-Kgp complex (50µg/mouse) was emulsified in Complete Freund's Adjuvant and used to immunize mice subcutaneously in the hock of the hind legs. T lymphocytes were isolated from pooled inguinal and popliteal lymph node cell suspensions of mice primed seven days previously with the RgpA-Kgp complex and spleens were isolated from non-immunized mice as a source of syngeneic antigen presenting cells. T-cells were cultured in enriched DMEM at a concentration of 3x10⁵ T-cells/well in a 96 well microtitre plate in the presence of syngeneic γ -radiated (2200R, ⁶⁰Co Source) spleen cells (4.8x10⁵ cells/well) together with antigen (RgpA-Kgp complex or synthetic peptide) in a total volume of 250µL. T-cells were incubated for four days at 37°C in an atmosphere of 5 per cent CO₂ in air. T-cell proliferation was detected using ³H-thymidine incorporation, which was added during the last 18 hours of the assay. Cells were then lysed and the DNA harvested onto glass fibre filter mats. Incorporation of ³H-thymidine was measured using a Beta counter. A positive proliferative response was recorded when there was an S.I of greater than 2 (where S.I is calculated as the test cpm divided by the negative control cpm).

Using PEPSCAN methodology, a number of T-cell epitopes were identified from each mouse strain. Eleven BALB/c (H2d) mouse T-cell epitopes were identified. Two of these epitopes were located within the RgpA

proteinase domain and the remaining epitopes were from the Kgp proteinase domain. Twenty-one CBA (H-2k) mouse T-cell epitopes were identified. Of these, two epitopes were located within the Kgp proteinase domain, five epitopes were located within the RgpA proteinase domain, and the remainder were located in adhesin domains Kgp^{A1}/RgpA^{A1} (9), Kgp^{A2}/RgpA^{A2} (3), Kgp^{A3}/RgpA^{A3} (1) and Kgp^{A5} (1). Thirty-eight C57BL/6 (H-2b) mouse T-cell epitopes were identified. Of these, 13 were located within the Kgp proteinase domain, nine epitopes were located within the RgpA proteinase domain, and the remainder were located in adhesin domains Kgp^{A1}/RgpA^{A1} (6), Kgp^{A2}/RgpA^{A2} (3), Kgp^{A3}/RgpA^{A3} (1), Kgp^{A4}/RgpA^{A4} (3) and Kgp^{A5} (3). Four epitopes (two from the Kgp proteinase domain and two from the RgpA proteinase domain) were common to both the BALB/c and C57BL/6 mouse strains. Five epitopes were common to both CBA and C57BL/6 and were located in the RgpA proteinase domain (1), Kgp proteinase domain (1), and in the adhesin domains Kgp^{A1}/RgpA^{A1} (1) and Kgp^{A2}/RgpA^{A2} (2). No common epitopes to all three mouse strains were found. In order to identify the immunodominant T-cell epitopes, the PEPSCAN-identified epitopes were synthesized and used to immunize mice. The peptide primed T-cells were then stimulated with the RgpA-Kgp complex and the T-cell proliferative responses were compared. The immunodominant epitope for BALB/c mice was found to be ⁴³⁹ANYTAHGSETAWADP⁴⁵³, which is located within the Kgp proteinase domain. The immunodominant T-cell epitopes for CBA and C57BL/6 mice were ¹¹¹GRFSCESKEDLKTQI¹²⁵ and ³⁶¹QINLT-DASVNSVCDY³⁷⁵, respectively and are both located in the RgpA proteinase domain.

A number of T-cell epitopes were identified from each of the mouse haplotypes. All of the identified BALB/c mouse T-cell epitopes were located in the proteinase domains of the RgpA-Kgp complex, indicating that the proteases are more immunogenic than the adhesins in this mouse strain. Epitopes from both the proteinase and adhesin domains were identified for the CBA and C57BL/6 mouse strains, signifying that both the proteases and adhesins are immunogenic in these strains. The immunodominant epitopes for each mouse strain were located within the Kgp or RgpA proteinase domains, indicating that the proteinases contain the most immunogenic regions in the RgpA-Kgp complex for these strains of mice. This work will aid in the development of a vaccine towards *P. gingivalis*.

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Expression of a *Porphyromonas gingivalis* adhesin fragment, Kgp39, in *Escherichia coli* and analysis of its immunogenicity using the murine lesion model

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Porphyromonas gingivalis is an opportunistic pathogen associated with chronic periodontitis, an inflammatory disease of the supporting tissues of the teeth. A major virulence factor of *P. gingivalis* is the RgpA-Kgp proteinase-adhesin complex, which is the product of post-translational cleavage of the RgpA and Kgp polyproteins.^{1,2} Within this proteinase-adhesin complex there are five repeated motifs, designated adhesin binding motifs (ABM), that have been implicated in aggregation and binding to host substrates. When synthesized as peptides and tested in a murine lesion model, three ABM motifs (ABM1, ABM2 and ABM3) were protective against challenge with *P. gingivalis*.³ Further, epitope mapping of the complex adhesin component RgpA27, identified three immunoreactive peptide epitopes (EP1, EP2 and EP3).⁴ Examination of primary amino acid sequence showed that one adhesin sub-unit of the RgpA-Kgp proteinase-adhesin complex, Kgp39, has the immunoreactive ABM and EP epitopes clustered together. Therefore it was proposed that this sub-unit is potentially highly immunogenic.

The aim of this study was to assess the immunogenicity of Kgp39. A full length Kgp39 (aa 738-1099) and a fragment of Kgp39 (aa 759-989) containing the ABM and EP epitope cluster only, designated r-Kgp39 FL and r-Kgp39 HS respectively, were produced as recombinant proteins. Both proteins were produced using an *E. coli* pET28 expression system resulting in the recombinant proteins being produced with a hexahistidine sequence, to aid purification. The recombinant proteins formed inclusion bodies within *E. coli* and were recovered from sonicated cell lysates by treatment with 8M urea. The proteins were purified using nickel affinity chromatography, and the urea removed by buffer exchange.

To assess the immunogenicity of the recombinant proteins they were tested in a murine lesion model. BALB/c mice, six to eight weeks old (10 mice per group), were immunized subcutaneously with either 35µg of each recombinant protein, 2x10⁹ formalin-killed cells of *P. gingivalis* ATCC 33277, as the positive control, or with buffer only, as the negative control. Each antigen was emulsified in Incomplete Freund's Adjuvant (IFA). After 30 days the mice were boosted with antigen and then bled from the retrobulbar plexus to obtain pre-challenge sera. Two days after bleeding, the mice were challenged with 7.5x10⁹ viable cells of *P. gingivalis* ATCC 33277 by subcutaneous injection in the abdomen and lesion size was monitored over 14 days. The maximum lesion size attained for each group was calculated. All of the sham-immunized controls (buffer alone) developed lesions that had a

maximal average lesion size (mm²) of 58.8±26.6. Mice immunized with formalin-killed *P. gingivalis* did not develop lesions. Immunization with r-Kgp39 FL and r-Kgp39 HS resulted in a maximal average lesion size (mm²) of only 3.5±5.6 and 2.9±4.6 respectively.

The antibody isotypes in the antisera induced by r-Kgp39 FL and r-Kgp39 HS immunization were determined by Enzyme-Linked Immunosorbent Assay (ELISA). The RgpA-Kgp proteinase-adhesin complex, r-Kgp39 FL and r-Kgp39 HS were immobilized to wells of a microtitre plate, and probed with protective antisera raised against: (i) formalin-killed whole cells; (ii) r-Kgp39 FL; and (iii) r-Kgp39 HS and the antibody titre and isotype determined. It was found that the predominant antibody subclass for each protective antisera was IgG1 and that the antibody response induced by the recombinant proteins was cross-reactive with native antigen and the RgpA-Kgp proteinase-adhesin complex.

To investigate the potential cross-reactivity of the antibodies induced by r-Kgp39 FL and r-Kgp39 HS to other *P. gingivalis* proteins, the antisera were used to probe purified RgpA-Kgp proteinase-adhesin complex, an outer membrane protein preparation and *P. gingivalis* W50 whole cells in an immunoblot. Mass-spectrometry of the immunoreactive proteins showed that as well as recognizing Kgp39, antibodies raised to r-Kgp39 FL and r-Kgp39 HS were also immunoreactive with other components of the RgpA-Kgp complex which included RgpA44, RgpA27 and RgpA17.

In conclusion, we have shown that r-kgp39 FL and r-Kgp39 HS are able to elicit a protective immune response in the murine lesion model and that the predominant antibody subclass induced is IgG1. Further, antibodies produced in response to r-Kgp39 FL and r-Kgp39 HS antigens were immunoreactive with native Kgp39 and also with other adhesins of the RgpA-Kgp proteinase-adhesin complex. Together, these results indicate that both recombinant proteins r-Kgp39 FL and r-Kgp39 HS have potential as vaccines in the treatment of *P. gingivalis*-associated periodontitis.

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3-D morphometric analysis of craniofacial morphology in children with and without cleft lip (+/-cleft palate)

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Although patient data are available that characterize morphology and growth in various craniofacial anomalies, including cleft lip and palate (CLP), most are based on two-dimensional assessments of cephalometric radiographs. Advances in medical imaging and computer processing of digital images now enable accurate visualization of craniofacial structures in 3-D, providing an opportunity to carry out more detailed analyses.

The aim of this study was to compare the 3-D morphology of selected structures, including the hyoid bone, cervical spine, nasopharynx and cranial base, in Malaysian infants with and without CLP. We hypothesized that there would be morphological differences between the CLP and non-cleft (NC) groups in these different regions, supporting the view that CLP is part of a generalized disturbance in growth and development and not just a localized defect. Prior to this study there were no normative 3-D data available for craniofacial morphology in Malaysian children. Furthermore, as far as we are aware, this is the first 3-D study of craniofacial morphology in unoperated CLP infants.

CT scans were collected from 29 unoperated infants with CLP and from 23 NC infants in the age interval of 0-12 months. The CT scans were obtained from the Department of Radiology, Hospital Universiti Sains Malaysia, then saved on CD-ROM and transferred to the Australian Craniofacial Unit (ACFU) at the Women's and Children's Hospital, Adelaide. The PERSONA software package, developed by the Research Unit at the ACFU, was then used to produce 3-D reconstructions of the images on a Silicon Graphics computer workstation. This package allowed the identification and location in 3-D of a large number of craniofacial landmarks. Double determinations confirmed that the landmarks could be located reliably.

Various linear and angular variables were measured and comparisons made between the CLP and NC samples. Measurements involving the hyoid bone included length and height of the greater horn and body, and the level of the hyoid bone and tip of the epiglottis in relation to the cervical vertebrae. The heights of individual vertebral bodies and intervertebral

spaces were measured and any cervical spine anomalies present in the CLP and NC infants were recorded. Other variables were computed from selected landmarks to quantify nasopharyngeal width, height and depth, and to enable cranial base length and height and cranial base angle to be determined. The width of the sphenoccipital synchondrosis was also computed. Linear modelling approaches were used to compare distances and angles between the CLP and NC samples, as they enabled the effects of age and sex to be taken into account statistically.

Very few of the study variables displayed significant sex differences within either sample but there were several significant differences between the samples. The hyoid bone was positioned at the level of C3 or C4 in the CLP infants, whereas it was positioned at a higher level (C2-C3) in the NC infants. In addition, five patients in the CLP group had significant hyoid bone anomalies, such as non-ossified bodies, whereas none of the NC group was affected. Three cervical vertebral bodies were significantly smaller in height in the CLP sample whereas two intervertebral spaces were significantly larger. Several cervical spine anomalies were noted in the CLP sample, including fusion of adjacent vertebrae and asymmetry of the anterior tubercle of C1. Pharyngeal width was significantly greater in the CLP sample. The cranial base was smaller in the CLP sample and the sphenoccipital synchondrosis was significantly wider.

Our findings indicate that several regions of the cranio-cervical complex are affected in CLP, not just those in the region of the cleft. The phenotypic changes relate to structures derived from the first, second and third branchial arches and may reflect alterations in cartilage growth and/or ossification in CLP infants. The observed morphological differences in the CLP infants may also be associated with common clinical problems. For example, the lower positioning of the hyoid bone may be associated with compromised function of the epiglottis in forming a seal with the larynx, thereby increasing the risk of aspiration pneumonia. The altered dimensions of the nasopharynx may also be associated with disruptions in the dilatatory mechanism of the Eustachian tube thus contributing to recurrent middle ear infections and subsequent hearing loss.

The apparently smaller cranial base in CLP infants could be associated with their observed mid-face hypoplasia and the wider speno-occipital synchondrosis may be related to a defect in ossification of the chondrocranium of the cranial base. The finding of shorter vertebral bodies and larger intervertebral spaces in the cervical spines of CLP infants is also consistent with a generalized alteration in the pattern of ossification in CLP. Altered development of the cervical spine in CLP may influence the ability of the fetus to lift the

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head and could be associated with failure of the palatal shelves to fuse.

Overall, our 3-D analysis has disclosed new information about phenotypic variation in CLP and has also helped to explain possible reasons for the clinical problems faced by affected children, such as aspiration pneumonia, speech problems, otitis media and upper respiratory tract infections. The question of whether the phenotypic findings in cranio-cervical structures are the cause of CLP, reflect a common underlying aetiological problem, or are an effect, cannot be answered definitively at this time. However, the fact that there are several structures affected together suggests that clefting may be one aspect of a more general problem.

A cell and polymer based composite scaffold for bone tissue engineering

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The use of bone substitutes to help repair, regenerate or replace damaged or diseased bone tissues is of particular clinical interest. Tissue engineering is an emerging interdisciplinary field involving principles of the life sciences and engineering and is the science of reconstructing or mimicking natural processes through the use of synthetic polymer scaffolds with the expectation of tissue regeneration. One aspect of tissue engineering is concerned with the formation of three-dimensional tissue substitutes by culturing cells on natural or synthetic polymer scaffolds. After implantation into patients, such engineered devices may help to create or restore the lost tissue or organ function. The three essential ingredients for both morphogenesis and tissue engineering are cellular components, growth and differentiation factors, and a scaffold or three-dimensional matrix. The cellular components must not only be present, but also be able to give rise to new structural tissue.

Bone defects, which result from tumors, diseases, infections, trauma, biochemical disorders and abnormal skeletal development, pose a significant clinical problem with regards to their management. Different types of cells have been used for the reconstruction of bone tissue, including bone marrow stromal cells, periosteal cells, skeletal muscle cells and cells derived directly from bone, as well as cells transduced with bone marrow morphogenetic protein genes. The relatively simple harvesting of bone marrow cells or osteoblasts from an easily accessible bone site, such as the alveolar bone of the jaw, can provide a good source of autologous bone tissue. In the present study the hypothesis was tested that cells from alveolar bone can retain their osteogenic properties in a three-dimensional collagen scaffold and subsequently synthesize a bone matrix, which after implantation, can induce new bone formation in critical-size calvarial bone defects.

Regeneration of osseous defects using a tissue engineering approach provides a novel means of treatment utilizing cell biology, materials science and molecular biology. In this study the concept of tissue engineering was tested using collagen type I matrices seeded with cells with osteogenic potential implanted into sites where osseous damage had occurred. Explant cultures of cells from human alveolar bone and gingiva were established. When seeded into a three-dimensional type I collagen-based scaffold, the bone-derived cells maintained their osteoblastic phenotype as monitored by mRNA and protein levels of the bone-related proteins including bone sialoprotein (BSP), osteocalcin (OCN), osteopontin (OPN), bone morphogenetic protein (BMP) 2&4 and alkaline phosphatase (ALP). These *in vitro*-developed matrices were implanted into critical-size bone defects in skulls of immunodeficient (SCID) mice. Wound healing was followed for up to four weeks. When measured by microdensitometry the bone density within the defects filled with the osteoblast-derived matrix was significantly higher compared with the defects filled with either collagen scaffold alone or collagen scaffold impregnated with gingival fibroblasts. New bone formation was found at all of the sites treated with the osteoblast-derived matrix at 28 days, while no obvious new bone formation was identified at the same time point in the control groups. *In situ* hybridization for the human-specific *Alu* gene sequence indicated that the newly formed bone tissue resulted from both transplanted human osteoblasts and endogenous mesenchymal stem cells. The results indicate that cells derived from human alveolar bone can be incorporated into bioengineered scaffolds and synthesize a matrix, which on implantation can induce new bone formation.

Extracellular matrix materials as scaffolds for the repair and regeneration of tissues are receiving

increased attention. The current study also tested whether extracellular matrices formed by osteoblasts *in vitro* could be used as a scaffold for osteoblast transplantation and induce new bone formation in a critical defect site *in vivo*. Human osteoblasts derived from alveolar bone were cultured in six-well plates until confluent and then in mineralized media for a further culture of three weeks in order to form an osteoblast-mineralized matrix complex. Histologically, at this time point a connective tissue phenotype was formed with cells and enriched extracellular matrix. Type I collagen was the major extracellular component and the structural molecule which determined the

matrix macrostructure. Other bone-related proteins such as ALP, BMP 2&4, BSP, OPN and OCN also accumulated in these matrixes. Osteoblasts embedded in this matrix expressed mRNAs for these bone-related proteins very strongly. Bone nodules were detected in the matrix and there was a correlation between mineralization and the distribution of BSP and OPN. When this matrix was transplanted into a critical-size bone defect in skulls of immunodeficient mice (SCID), new bone formation occurred. Furthermore, the cells inside the matrix survived and proliferated in the recipient sites, and were traceable by the human specific *Alu* gene sequence by *in situ* hybridization. It was found that bone-forming cells differentiated from both transplanted human osteoblasts and activated endogenous mesenchymal stem cells. This study indicates that mineralized bone matrix, formed by human osteoblasts *in vitro*, can be used as a scaffold for osteoblast transplantation and subsequently can induce new bone formation.

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An *in vitro* model to measure the effect of a silver fluoride and potassium iodide treatment on the permeability of demineralized dentine to *Streptococcus mutans*

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Microhardness and Ca:P ratio of carious and Carisolv™ treated caries-affected dentine using an ultra-micro-indentation system and energy dispersive analysis of x-rays – A pilot study

R Sakoolnamarka, MF Burrow, M Swain, MJ Tyas

ADRF Undergraduate Summer Vacation Research Grant Abstracts

Comparison of perceptions of dental clinical teaching by students and by teachers

T McLean, J Fairley; TM Gerzina (Supervisor)*

The objectives of this study were to compare the perceptions of both dental students and clinical teachers about dental clinical teaching and the teaching styles that foster independent clinical practice and to provide recommendations to the faculty for future dental clinical teaching.

Qualitative methodology was the use of student focus groups where participants were asked to discuss and rank order styles of clinical teaching dentistry they considered most aided their preparation for independent clinical practice. Quantitative methodology was the use of a questionnaire for students and teachers that was informed by the focus group data.

Three themes – the student/clinical teacher relationship, aspects of educational theory in dental clinical teaching and the professional skills required for dental clinical practice were explored in the questionnaire. In the theme of the *teacher/student*

relationship no statistically significant differences were seen between teacher and student groups ($P>0.011$). In the theme of *educational theory applied in dental clinical teaching*, a statistically significant difference was seen between teacher and student groups in the value of pre-clinical instruction in senior clinical years ($P<0.0029$) and in the value of a clinical log book ($P<0.0038$). In the theme of *skills required for clinical dental practice*, a statistically significant difference was seen between teacher and student groups in the value of a critical appreciation of evidence-based practice as one of the skills ($P<0.0013$).

Overall, the study indicated that the dental clinical learning environment supports close perceptual conformity between students and clinical teachers in regard to teaching styles that student preparation for dental clinical practice.

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An *in vitro* study of wear prevention in dentine

T Narayana; S Ranjitkar, J Kaidonis, GC Townsend, LC Richards (Supervisors)*

Tooth wear is a significant problem facing clinicians and several approaches are being used to manage this challenge. These include patient education, nightguards, dietary advice, varnishes and remineralizing agents such as fluorides, which show considerable potential. This study aimed to quantitatively and qualitatively test the ability of Tooth Mousse® (GC Corporation), a commonly used remineralizing agent containing casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), to reduce dentine wear under highly controlled conditions.

Twelve freshly extracted third molar teeth were sectioned mesiodistally along their longitudinal axis, and the enamel was removed from the buccal halves of these teeth. The dentine specimens obtained were worn against their enamel antagonists for 75 000 cycles, at a rate of 80 cycles per minute, in a custom-made electro-mechanical machine at a load of 9.95kg. Eight were worn with an acidic lubricant (pH=3), and had Tooth Mousse® applied to them for five minutes at 20 minute intervals. Four had Tooth Mousse® as the sole lubricant, and these had Tooth Mousse® applied to them continuously. Dentine wear was quantified using

profilometry, and assessed qualitatively by examining epoxy resin replicas of the specimens under a scanning electron microscope. These data were then compared with data from two control experiments conducted at the same load, with a lubricant of pH=3 and deionized water, in which Tooth Mousse® was not used.

Dentine wear was minimal in the specimens where Tooth Mousse® was applied continuously. Those that were worn at pH=3 with Tooth Mousse® applied at regular intervals showed less wear than both the control groups. Control specimens worn with deionized water showed greater wear than those worn at pH=3. Wear facets on the specimens to which Tooth Mousse® had been continuously applied were very smooth and shiny. Those from all the other groups showed more striations due to the wear process.

The results show that Tooth Mousse® is capable of reducing dentine wear. Its contribution appears to be greatest when it is used continuously under dry conditions simulating attrition only, but it also shows promise in reducing wear under conditions simulating a combination of attrition and erosion. Further research is planned to clarify the means by which Tooth Mousse® reduces dentine wear, and to ascertain its clinical usefulness in managing tooth wear.

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Dental jurisprudence: an analysis of complaints against Victorian dental care providers 2000-2004

D Sanduja; M Hopcraft (Supervisor)*

There are few data available on the number and type of complaints made against dental care providers in Australia despite anecdotal reports of an increasing trend in health-related complaints and litigation.

Data from this study were obtained from the Dental Practice Board of Victoria on complaints received between July 2000 and December 2004.

There were a total of 651 complaints against all dental care providers in the study period, which equates to a rate of 4.1 complaints per 100 dental care

providers per year. Dentists were responsible for 490 of the complaints, with 66 complaints against dental prosthetists and 43 complaints against dental specialists. There were very few complaints against dental therapists and no complaints against dental hygienists. 47 complaints were made against unregistered people or institutions.

This study found that there was a relatively low rate of complaints made against dental care providers in Victoria, with most occurring against dentists in private practice in Melbourne. Less than 10 per cent of complaints resulted in an adverse finding against the dental care provider.

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Effect of light-accelerated tooth bleaching on tooth surface and intrapulpal temperature rise *in vitro*

EH Wainwright; IA Meyers, LJ Walsh (Supervisors)*

The aim of this study was to investigate a number of bleaching systems and determine the *in vitro* increase in temperature of bleaching gel on the surface of the tooth, and temperature changes within the pulp during various 'light-accelerated' in-office bleaching procedures.

A thermocouple was placed on the dentine surface of a pulpal access cavity in extracted incisor teeth. Intrapulpal temperature rises were recorded while using the light from four output sources, with or without the addition of a bleaching agent to the enamel surface. Surface temperature of the tooth and the bleaching gel was recorded using an infrared thermometer, and dentine-enamel thickness measured.

Mean peak intrapulpal temperature rises recorded during bleaching ranged from 6.2°C with the Zoom light, to 2.6°C using the LED LeDemetron curing unit. The Zoom system also resulted in the greatest surface temperature increases. The thickness of the tooth or the gel used was found to have no significant effect on temperature, with the exception of the KTP laser gel system, which significantly raised gel temperature in comparison to the control.

While temperature increases induced by some bleaching systems were high, the potential for pulpal damage *in vivo* may be reduced by physiological processes not simulated in this study.

To reduce the risk of thermal trauma as a result of in-office bleaching procedures, consideration should be given by dentists to the choice of accelerating light unit and bleaching agent used.

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