PAULA CRISTINA TREVILATTO

SUSCETIBILIDADE GENÉTICA À SEVERIDADE DA DOENÇA PERIODONTAL

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, para a obtenção do título de Doutor em Biologia e Patologia Buco-Dental.

Piracicaba

2002

PAULA CRISTINA TREVILATTO

SUSCETIBILIDADE GENÉTICA À SEVERIDADE DA DOENÇA PERIODONTAL

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, para a obtenção do título de Doutor em Biologia e Patologia Buco-Dental.

Orientador: Prof. Dr. Sergio Roberto Peres Line

Banca Examinadora:

Prof. Dr. Sergio Roberto Peres Line
Prof. Dr. Edgard Graner
Prof. Dr. Antonio Wilson Sallum
Prof. Dr. Vinícius Augusto Tramontina
Profa. Dra. Raquel Fernanda Gerlach

Piracicaba

2002

Ficha Catalográfica

~~T728s~~
T729s

Trevilatto, Paula Cristina.
Suscetibilidade genética à severidade da doença periodontal. / Paula Cristina Trevilatto. -- Piracicaba, SP : [s.n.], 2002.
xx, 179p. : il.

Orientador : Prof. Dr. Sergio Roberto Peres Line.
Tese (Doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Diagnóstico. 2. Odontologia. 3. Biologia molecular. 4. Géis. 5. Prevenção. 6. Patologia bucal. 7. Periodontia. 8. Reação em cadeia da polimerase. 9. Saliva. 10. Polimorfismo (Genética). I. Line, Sergio Roberto Peres. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Ficha catalográfica elaborada pela Bibliotecária Marilene Girello CRB/8–6159, da Biblioteca da Faculdade de Odontologia de Piracicaba - UNICAMP.

UNICAMP

FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, em sessão pública realizada em 10 de Outubro de 2002, considerou a candidata PAULA CRISTINA TREVILATTO aprovada.

1. Prof. Dr. SERGIO ROBERTO PERES LINE _____

2. Profa. Dra. RAQUEL FERNANDA GERLACH _____

3. Prof. Dr. VINÍCIUS AUGUSTO TRAMONTINA _____

4. Prof. Dr. EDGARD GRANER _____

5. Prof. Dr. ANTONIO WILSON SALLUM _____

DEDICATÓRIA

A DEUS, que nos proporciona
tantas oportunidades...

Aos meus pais, NARCISO e DORA, pelos
ensinamentos constantes ao longo de nossa
caminhada evolutiva e por ampararem
toda a minha existência

Aos meus irmãos CLAUDIA e NARCISO Jr.,
pela convivência e por iluminarem meu caminho

Ao ANDRÉ, pelo amor e pelo companheirismo incansável

AGRADECIMENTO ESPECIAL

AO ***PROF. DR. SERGIO ROBERTO PERES LINE***, PELA ORIENTAÇÃO E EXEMPLO DE CARÁTER. PELA CONDUÇÃO COMPETENTE DE TODOS OS PASSOS DE MEU CAMINHO CIENTÍFICO. PELA LIBERDADE ASSISTIDA. UM EXEMPLO DE TRABALHO SÉRIO E VERDADEIRA VOCAÇÃO À INVESTIGAÇÃO CIENTÍFICA.

AGRADECIMENTOS

À Faculdade de Odontologia de Piracicaba (FOP) - UNICAMP, pela utilização de suas instalações desde a minha graduação. Levarei seu nome em meu peito com orgulho.

Ao Prof. Dr. Antonio Wilson Sallum, Diretor da FOP - UNICAMP, pela amizade e pela ótima administração desta casa.

À Profa. Dra. Altair Antoninha Del Cury, Coordenadora Geral da Pós-Graduação da FOP-UNICAMP, pelo exemplo de seriedade, competência e poder de liderança, e pela preocupação em bem encaminhar os formados nesta instituição.

À Profa. Dra. Darcy de Oliveira Tosello, ex-Coordenadora do Curso de Pós-Graduação em Biologia e Patologia Buco-Dental da FOP, pela contínua disponibilidade e simpatia.

À Profa. Dra. Silvana Pereira Barros, Coordenadora do Curso de Pós-Graduação em Biologia e Patologia Buco-Dental da FOP, por todo apoio e pela amizade dentro e fora do âmbito profissional.

Ao Prof. Dr. Pedro Duarte Novaes, Chefe do Departamento de Morfologia da Faculdade de Odontologia de Piracicaba (FOP) - UNICAMP, por ser extremamente acessível aos questionamentos, e por permitir a utilização dos laboratórios durante o desenvolvimento de nossas pesquisas.

À Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP, pela bolsa que me foi conferida e pelo auxílio financeiro nos congressos e de reserva técnica, que permitiram a boa execução desta pesquisa. Muito obrigada.

Aos professores do Departamento de Periodontia, especialmente Prof. Dr. Antonio Wilson Sallum, Prof. Dr. Enilson Antonio Sallum e Prof. Dr. Francisco H. Nociti Jr., que tornaram possível a realização de nossa pesquisa, através da coleta de DNA dos pacientes nas clínicas de graduação e especialização desta instituição.

A todos os professores do Departamento de Morfologia, Laboratório de Histologia, especialmente ao Prof. Dr. José Merzel, o meu muito obrigada pelo constante acesso ao diálogo científico.

Às "meninas" do Depto. de Morfologia, Cidinha, Eliene, Suzete e Ivani, muito obrigada pela paciência e pela convivência no dia-a-dia.

À Érica, da Secretaria da Pós-Gradução, pelos esclarecimentos cabíveis a qualquer tempo.

Às garotas da Biblioteca, especialmente à Heloísa e à Marilene, pela paciência em me atender na elucidação de dúvidas pertinentes à tese.

À minha amiga Lúcia Elvira Alvarez que, ao longo do tempo, apesar de por vezes distante, sempre esteve presente nos momentos importantes de minha vida e por clarear momentos difíceis.

À Ana Paula, por sua constante amizade e pela alegria da convivência.

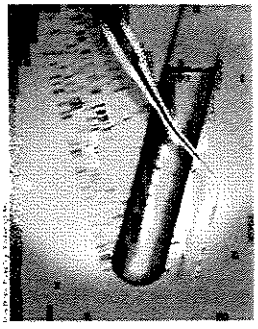
À Raquel Scarel, por sua dedicação e pela sensibilidade nos momentos difíceis.

À Silvana Pasetto, pelo estímulo e pelas palavras amigas.

Ao Rui, pelo auxílio constante e pelo compartilhamento de alegrias e dissabores.

Ao Marco, pelo incentivo e pelos bons momentos.

À Cristina e à Isabela, à motivação do trabalho futuro.



"A felicidade tem base
no dever cumprido"

SUMÁRIO

RESUMO 1

ABSTRACT 3

1. INTRODUÇÃO 5

1.1.1 MEDIADORES INFLAMATÓRIOS DA DP. 6

1.1.2 INTERLEUCINA-1 (IL-1) 6

1.1.3 ANTAGONISTA DO RECEPTOR DA IL-1 (IL-1 RA) 7

1.1.4 INTERLEUCINA-6 (IL-6) 7

1.1.5 FATOR DE NECROSE TUMORAL-α (TNF-α) 8

2. PROPOSIÇÃO 11

3. ARTIGOS 13

ARTIGO 1 15

ARTIGO 2 35

ARTIGO 3 59

ARTIGO 4 75

ARTIGO 5 101

ARTIGO 6 119

ARTIGO 7 143

4. CONCLUSÃO 163

REFERÊNCIAS BIBLIOGRÁFICAS 165

ANEXO 171

ARTIGO 8 173

APÊNDICE 178

RESUMO

Citocinas constituem importantes mediadores inflamatórios que influenciam o início e a progressão de diversas doenças inflamatórias crônicas, incluindo a doença periodontal. Polimorfismos gênicos têm sido relacionados ao aumento da expressão das citocinas correspondentes. O objetivo do presente estudo foi investigar a correlação entre os polimorfismos nos genes da IL-1α, IL-1β, IL-1ra, IL-6 e TNF-α e a severidade da periodontite crônica. Cento e treze indivíduos não-aparentados, não-fumantes, acima dos 25 anos (média: 41,6 anos), foram divididos de acordo com o nível de severidade da doença periodontal crônica: 44 indivíduos saudáveis, 31 com periodontite moderada e 38 com periodontite severa. O DNA genômico foi obtido de células epiteliais da mucosa bucal através de um bochecho com 5 mL de solução de glicose a 3 % e leve raspagem da mucosa jugal. Foram analisados os seguintes polimorfismos: *IL-1A* (-889), *IL-1B* (-511), *IL-1B* (+3953), *IL-1RA* (intron 2), *IL-6* (-174) e *TNFA* (-308), pela técnica PCR-RFLP. A significância das diferenças nas freqüências dos polimorfismos no grupo controle e em grupos com periodontite foi acessada pelo teste qui-quadrado (p<0,05). Diferenças significantes nas freqüências alélicas e genotípicas foram encontradas nos polimorfismos *IL-1B* (-511), *IL-1RA* (intron 2) e *IL-6* (-174). Concluiu-se que os polimorfismos *IL-1A* (-889), *IL-1B* (+3953) e *TNFA* (-308) não se correlacionaram à severidade da periodontite, enquanto que os polimorfismos *IL-1B* (-511), *IL-1RA* (intron 2) e

IL-6 (-174) se relacionaram à susceptibilidade à periodontite crônica na população estudada. Os mesmos polimorfismos foram investigados em uma família com periodontite agressiva, juntamente com parâmetros microbiológicos. Os resultados microbiológicos e genéticos não foram suficientes para predizer a susceptibilidade à periodontite agressiva na família estudada.

ABSTRACT

Inflammatory cytokines represent important pathological mediators in chronic inflammatory diseases, including periodontitis. Polymorphisms in cytokine genes have been associated with enhanced expression of cytokines, which mediate the development of inflammatory diseases. The aim of this study was to investigate the association between polymorphisms in the IL-1α, IL-1β, IL-1ra, IL-6 and TNF-α genes and severity of chronic periodontitis in Brazilians. One hundred and thirteen unrelated non-smoking subjects, over 25 years (mean age 41.6), were divided according to the severity level of periodontal disease: 44 healthy individuals, 31 subjects with moderate and 38 with severe periodontitis. DNA was obtained from epithelial cells through a mouthwash with 5 mL 3% glucose and scraping of oral mucosa. The samples were analyzed for *IL-1A* (-889), *IL-1B* (-511), *IL-1B* (+3953), *IL-1RA* (intron 2), *IL-6* (-174) and *TNFA* (-308) polymorphisms using PCR-RFLP. The significance of the differences in the frequencies of the polymorphisms in the control and groups with periodontitis was assessed by Chi-square test ($p<0.05$). Significant differences in the allele and genotype frequencies were found among the groups regarding *IL-1B* (-511), *IL-1RA* (intron 2) and *IL-6* (-174) polymorphisms. It was concluded that *IL-1A* (-889), *IL-1B* (+3953) and *TNFA* (-308) polymorphisms were not associated with the severity of chronic periodontal disease in the study population whereas polymorphisms *IL-1B* (-511), *IL-1RA* (intron 2) and *IL-6* (-174) correlated to disease

susceptibility. These polymorphisms were also investigated in a family with aggressive periodontitis, together with clinical and microbiological parameters. The results showed that the microbiological and genetic parameters did not predict the susceptibility to periodontitis in the study family.

1. INTRODUÇÃO

A doença periodontal (DP) é constituída por um grupo de afecções inflamatórias crônicas causadas por bactérias específicas, como *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* e *Bacteroides forsythus* (NONNENMACHER *et al.*, 2001; SLOTS & KAMMA, 2001). As bactérias ativam mecanismos inflamatórios que acabam por destruir colágeno e osso dos tecidos de suporte periodontal (POTEMPA *et al.*, 2000). Embora as bactérias sejam essenciais para a iniciação da periodontite, a quantidade e os tipos de bactérias não são variáveis suficientes para explicar diferenças na severidade da DP (KORNMAN *et al.*, 1997). Nas doenças inflamatórias crônicas, há fatores modificadores que não causam a doença, mas amplificam alguns mecanismos que tornam a doença mais severa. O cigarro (HABER, 1994; SCHENKEIN *et al.*, 1995; JOHNSON & SLACH, 2001), o estresse psicossocial (LINDEN *et al.*, 1996), certas doenças sistêmicas (MEALEY, 2000; WACTAWSKI-WENDE, 2001) e determinados polimorfismos genéticos (KORNMAN *et al.*, 1997; TREVILATTO *et al.*, *in press*) são exemplos de fatores que tornam os indivíduos suscetíveis à progressão/gravidade da DP.

Polimorfismos são variações genéticas encontradas na população, nas quais a freqüência do alelo mais raro de um determinado loco é maior que 1% (THOMPSON *et al.*, 1991). Existem polimorfismos que influenciam a atividade de fatores reguladores da resposta inflamatória, estando assim associados ao

aumento significativo do risco à severidade da doença (POCIOT *et al.*, 1992; DANIS *et al.*, 1995; WILSON *et al.*, 1997; SHIRODARIA *et al.*, 2000).

1.1 Mediadores inflamatórios da DP

Bactérias específicas, predominantemente anaeróbias gram-negativas, ativam mecanismos teciduais que produzem uma série de alterações inflamatórias e imunológicas que levam à destruição do tecido conjuntivo e osso alveolar. Em humanos, a resposta imune às bactérias é promovida e regulada em parte por um número de citocinas (GEMMELL & SEYMOUR, 1994). Como ocorre em outras infecções, a inflamação nos tecidos gengivais protege o organismo contra o ataque microbiano local e impede os microrganismos de se disseminarem. No entanto, a resposta inflamatória causa danos às células e estruturas periodontais, incluindo o osso alveolar.

1.1.1 Interleucina-1 (IL-1)

As interleucinas são mediadoras-chave do processo inflamatório, pois modulam componentes da matriz extracelular e osso que compõem os tecidos periodontais (GENCO, 1992). Níveis mais altos de IL-1 foram encontrados na gengiva (HONIG *et al.*, 1989) e em sítios ativos (STASHENKO *et al.*, 1991a,b) de pacientes com periodontite crônica, e diminuídos no fluido gengival após tratamento da DP (MASADA *et al.*, 1990). A IL-1α e a IL1β são produzidas e liberadas localmente na DP, apresentando atividades biológicas similares pró-

inflamatórias e catabólicas, como induzir a desmineralização óssea, aumentar a adesão de leucócitos às células endoteliais, proporcionar a desgranulação de neutrófilos, e estimular a síntese de prostaglandina e a atividade colagenolítica pelas células do tecido conjuntivo. Além disso, promovem a proliferação epitelial e são quimiotáticas para neutrófilos e macrófagos, tornando-os ativos (MASADA *et al.*, 1990).

1.1.2 Antagonista do receptor da IL-1 (IL-1 ra)

O antagonista do receptor da interleucina-1 compete com a IL-1 pela ocupação de receptores de superfície celular, desempenhando papel inibitório no desenvolvimento de doenças mediadas por esta citocina. Constitui importante regulador endógeno do processo inflamatório (TARLOW *et al.*, 1993), estando associado à susceptibilidade a diversas doenças inflamatórias (MANSFIELD *et al.*, 1994; BOIARDI *et al.*, 2000; BUCHS *et al.*, 2000; SHU *et al.*, 2000).

1.1.3 Interleucina-6 (IL-6)

A interleucina-6, assim como a IL-1, está envolvida em mecanismos de reabsorção óssea (GEMMELL & SEYMOUR, 1994) e se encontra presente em concentrações mais elevadas no fluido gengival de pacientes com DP refratária (REINHARDT *et al.*, 1993).

1.1.4 Fator de necrose tumoral-α (TNF-α)

O fator de necrose tumoral-α (TNF-α) é uma citocina pró-inflamatória produzida por macrófagos, que desempenha papel similar às IL-1 e IL-6 (ALEXANDER & DAMOULIS, 1994). Ele induz a secreção de colagenase, prostaglandina E_2 (PGE_2) e IL-6 por fibroblastos e osso em cultura (MEIKLE *et al.*, 1989), além de estimular a reabsorção óssea (BERTOLINI *et al.*, 1986). O fator de necrose tumoral-α está presente no fluido gengival de pacientes com gengivite e periodontite (ROSSOMANDO *et al.*, 1990). No entanto, o TNF-α é cerca de 500 vezes menos potente que a IL-1 na indução de reabsorção óssea (STASHENKO *et al.*, 1991a; ALEXANDER & DAMOULIS, 1994).

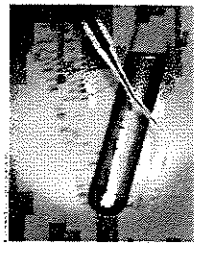
Como se vê por estes exemplos, o número de mediadores da resposta do hospedeiro, que atuam na destruição dos tecidos periodontais, é vasto e com ação interdependente. Estudos em periodontite crônica indicam que grande parte da perda de inserção e da reabsorção do osso alveolar pode ser prevenida pela ação de drogas antiinflamatórias não-esteróides (HOWELL & WILLIAMS, 1993; JEFFCOAT *et al.*, 1993). Portanto, alterações gênicas que resultem em modificação da resposta inflamatória merecem ser estudadas.

Mais que ser o interesse principal focalizar níveis de higiene oral, identificando-se e quantificando-se a microbiota potencialmente patogênica, o principal desafio para o futuro parece ser encontrar meios de identificar grupos de risco para a severidade da doença periodontal. Clinicamente, estes fatores podem

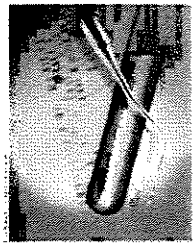
ser usados para a identificação de indivíduos com uma resposta desfavorável ao ataque microbiano, isto é, pessoas que sejam suscetíveis à doença periodontal, para que sejam priorizados programas de prevenção, antes que ocorra a destruição irreversível dos tecidos periodontais, e para que se conheça melhor o curso da doença. Como a placa bacteriana parece explicar, apenas em parte, o desenvolvimento da DP (GENCO, 1992), fatores sistêmicos diretos e indiretos que influenciam a resposta do hospedeiro parecem ser de grande relevância e merecem atenção nesta fase do desenvolvimento científico. A DP compreende um grupo de doenças crônicas multifatoriais complexas cuja etiopatogênese é pobremente compreendida, desta forma, dificultando testes diagnósticos do risco à sua severidade e progressão (KORNMAN & DI GIOVINE, 1998). Mediadores pró-inflamatórios parecem ser o foco de atenção neste momento em que a resposta do hospedeiro se mostra determinante na intensidade da destruição periodontal. Assim, a busca de marcadores genéticos que permitam a detecção de indivíduos com maior probabilidade de desenvolver a doença é fundamental para a prevenção da instalação do processo destrutivo, ou na instauração de terapêutica individualizada e proservação adequada de pacientes, nos quais sinais clínicos da doença já se manifestaram.

2. PROPOSIÇÃO

O presente trabalho, composto por sete artigos, teve como objetivo geral avaliar a associação entre polimorfismos genéticos e susceptibilidade à doença periodontal em uma família com periodontite agressiva e em pacientes com diferentes graus de periodontite crônica. Os polimorfismos *IL-1A* (-889), *IL-1B* (-511), *IL-1B* (+3953), *IL-1RA* (intron 2), *IL-6* (-174) e *TNFA* (-308) foram estudados pela técnica PCR-RFLP.



3. ARTIGOS



DIAGNÓSTICO MOLECULAR DA DOENÇA PERIODONTAL
Revista da Associação Paulista dos Cirurgiões Dentistas (APCD)
v.55, n.2, Mar./Apr. 2002, p.100-103

DIAGNÓSTICO MOLECULAR DA DOENÇA PERIODONTAL

Paula Cristina TREVILATTO, Antonio Wilson SALLUM, Sérgio Roberto Peres LINE

Paula Cristina Trevilatto - Doutoranda em Biologia e Patologia Buco-Dental da Faculdade de Odontologia de Piracicaba – UNICAMP

Antonio Wilson Sallum - Professor Titular do Departamento de Periodontia e Prótese da Faculdade de Odontologia de Piracicaba – UNICAMP

Sérgio Roberto Peres Line - Professor Livre Docente do Departamento de Morfologia da Faculdade de Odontologia de Piracicaba – UNICAMP

Paula Cristina Trevilatto: Faculdade de Odontologia de Piracicaba-UNICAMP

Depto. Morfologia

Av. Limeira, 901

Piracicaba - SP

CEP. 13.414-018

Tel: (0XX19) 430-5214

Fax: (0XX19) 430-5218

e-mail: pctrev@yahoo.com.br

DIAGNÓSTICO MOLECULAR DA DOENÇA PERIODONTAL

SINOPSE

O cigarro, o estresse psicossocial e certos polimorfismos gênicos são exemplos de fatores que aumentam o risco à periodontite, tornando os indivíduos mais suscetíveis à progressão ou ao agravamento da doença. Polimorfismos em genes de mediadores pró-inflamatórios, que caracterizam a resposta do hospedeiro, têm sido alvo de estudos recentes, que objetivam diagnosticar precocemente mecanismos destrutivos da doença. A realização de um simples bochecho com solução açucarada pelo paciente pode permitir a análise de seu perfil de susceptibilidade genética à doença periodontal, o que pode ser de grande valia na prevenção da instalação e progressão da doença periodontal em pacientes de risco.

UNITERMOS: *Periodontite. Polimorfismo genético. Suscetibilidade.*

MOLECULAR DIAGNOSIS OF PERIODONTAL DISEASE

ABSTRACT

Smoking, stress and genetic polymorphisms are examples of factors that increase the risk of periodontitis, making the individuals more susceptible to the progression or aggravation of periodontal disease. Polymorphisms in genes of pro-inflammatory mediators, which characterize the host response, have been investigated with the aim of accomplishing the early diagnosis of the destructive mechanisms of the disease. A simple mouthwash performed by the patient can determine the individual pattern of genetic susceptibility to periodontal disease, which can help to prevent the beginning and progression of the disease in high-risk patients.

DESCRIPTORS: *Periodontitis. Genetic risk. Polymorphisms.*

A periodontite (DP) constitui doença inflamatória crônica em resposta à presença de bactérias específicas, como *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* e *Bacteroides forsythus*. As bactérias ativam mecanismos inflamatórios que acabam por destruir colágeno e osso dos tecidos de suporte periodontal. Embora as bactérias sejam essenciais para a iniciação da periodontite, a quantidade e os tipos de bactérias não são suficientes para explicar diferenças na progressão e severidade da DP. O cigarro e o estresse psicossocial são exemplos de fatores que aumentam o risco à doença, tornando os indivíduos mais suscetíveis à progressão ou ao agravamento da doença periodontal. No entanto, há indivíduos que, mesmo sofrendo exposição a esses fatores ambientais e apresentando pobre higiene oral, e, portanto, possuindo altos índices de placa, não mostram níveis significativos de doença. O contrário também é verdadeiro: pacientes com baixos índices de placa podem desenvolver altos níveis de doença e responder mal ao tratamento periodontal, o que caracteriza a periodontite refratária. Nas doenças inflamatórias crônicas, há fatores modificadores que não causam a doença, mas amplificam alguns mecanismos que tornam a doença mais severa. Esses fatores são geneticamente determinados e constituem a resposta do hospedeiro.

FATORES RELACIONADOS À DOENÇA PERIODONTAL

1. Placa Dental

Diferenças na prevalência e gravidade da DP foram associadas à raça, à área geográfica, ao sexo, à condição sócio-econômica e ao nível educacional. A partir da década de 60, com o emprego do Índice de Higiene Oral (OHI), foi observado que diferenças raciais, geográficas e demográficas aparentes na gravidade da DP desaparecem quando os níveis de doença são relacionados diretamente com índices de higiene oral. A associação entre a DP e a higiene oral foi demonstrada epidemiologicamente (LINDHE[8], 1992). A quantidade de placa e cálculo, juntamente com a idade, poderia explicar até 90 % da experiência da doença.

2. Resposta do Hospedeiro

A reação imunoinflamatória representa a resposta do hospedeiro à microbiota da placa dental e seus produtos. A própria qualidade da placa (quais as bactérias e em que quantidade relativa estão presentes) é determinada pela resposta individual do hospedeiro, que apresenta componentes imunológicos próprios que permitirão ou não o desenvolvimento da microflora em questão. O tipo e a severidade da DP são reflexo da

competência da resposta do hospedeiro mais do que da virulência da microflora patogênica, o que torna difícil determinar se um certo tipo de microflora bacteriana associado a doenças periodontais específicas seria a causa ou condição da doença. Muito embora placa e cálculo estejam indubitavelmente relacionados com a gengivite e a destruição dos tecidos periodontais, há pacientes que, tendo placa e cálculo em abundância e restaurações mal-adaptadas pela vida inteira, não mostram evidências de alterações destrutivas no sistema de inserção. Inversamente, pacientes jovens com higiene oral satisfatória podem revelar destruição avançada de tecidos periodontais (LINDHE[8], 1992). Assim, em cada paciente isoladamente, o grau de DP não é determinado apenas pela presença da placa microbiana. Qualquer alteração patológica em nível periodontal é conseqüência direta da resposta individual do hospedeiro em função da presença da placa. A idade poderia, talvez, refletir a manifestação da queda imunológica e o desequilíbrio de fatores que regulam a atividade colagenolítica. Dessa forma, o argumento de que 90 % dos casos de DP podem ser explicados com base na idade e higiene oral, no mínimo, não é ideal. Embora estudos epidemiológicos revelem que a freqüência e a severidade da DP aumentam com a idade e com a higiene oral inadequada, não basta somente avaliar os tipos de microrganismos presentes

na placa e sua quantidade relativa, juntamente com fatores retentivos. A associação etiológica direta entre a colonização bacteriana sobre a superfície dentária e a destruição da inserção periodontal pela inflamação é clara, mas a taxa de destruição em resposta a esta colonização varia consideravelmente de uma pessoa para outra, dependendo de diferenças na resposta tecidual à irritação bacteriana. Isto explica por que a gengivite, que reflete a presença da colonização bacteriana, não resulta em destruição periodontal em todos os casos. Apesar de as bactérias serem essenciais para a iniciação da periodontite, não há mecanismos seguros que determinem a trajetória clínica da doença em nível individual, diferenciando pacientes que apresentarão uma forma de leve à moderada da doença e responderão bem ao simples cuidado profissional, daqueles que são suscetíveis a desenvolver formas mais severas, que demandem terapia intensiva e resultem na morbidade dental.

MEDIADORES PRÓ-INFLAMATÓRIOS DA DP

A preocupação com aspectos causais é enorme, mas pouco se conhece ainda acerca de sinalizadores/mediadores fundamentais na patogênese e evolução da doença. Diferenças individuais na expressão de determinadas citocinas têm efeito dramático na progressão da doença (KORNMAN et al.[7],

1997). O número de fatores que atuam na destruição dos tecidos periodontais, mediadores da resposta imunoinflamatória do hospedeiro, é enorme e com ação interdependente. Estudos sobre periodontite do adulto indicam que 2/3 da perda de inserção e reabsorção de osso alveolar são passíveis de prevenção pela ação de drogas antiinflamatórias não-esteróides. Aspectos da resposta imunoinflamatória do hospedeiro à infecção bacteriana, que são o elemento-chave do início e da progressão da periodontite, podem ser geneticamente determinados (HART[4], 1994). Portanto, alterações em nível gênico, que resultam em modificação da resposta via mediadores, merecem ser estudadas. Dessa forma, nos últimos anos, atenção tem sido amplamente dirigida para fatores do hospedeiro, que aumentam significativamente o risco à doença, especialmente polimorfismos em genes de mediadores pró-inflamatórios, moduladores ou intensificadores dos mecanismos destrutivos da doença. Polimorfismos são variações genéticas encontradas na população, consideradas dentro da normalidade. Há polimorfismos que influenciam a atividade de fatores que regulam a intensidade da resposta inflamatória, estando assim associados ao aumento significativo do risco à severidade da doença. Desde que o grau de destruição nos tecidos periodontais, que caracteriza a DP severa, seja essencialmente resultado da resposta

inflamatória, faz-se necessário o estudo de polimorfismos genéticos que alteram a produção de fatores relacionados com a intensidade da resposta inflamatória e a severidade da DP em adultos e em indivíduos com doença periodontal de início precoce (EOP).

MARCADORES GENÉTICOS DE SUSCETIBILIDADE À DP

São recentes as primeiras descrições de polimorfismos gênicos associados à DP. Polimorfismos nos genes da interleucina-1 α e β (IL-1 α e β) e fator de necrose tumoral-α (TNF-α) foram os primeiros marcadores genéticos da DP a serem descritos, identificando indivíduos suscetíveis à severidade da doença periodontal (KORNMAN et al.[7], 1997, GORE et al.[3], 1998, DIEHL et al.[1], 1999 e MCGUIRE; NUNN[10], 1999). As interleucinas (IL) são mediadoras-chave do processo inflamatório, pois modulam a síntese e degradação de componentes da matriz extracelular e osso que constituem os tecidos periodontais (GENCO[2], 1992). Níveis mais altos de IL-1 foram encontrados na gengiva (HONIG et al.[5], 1989) e em sítios ativos (STASHENKO et al.[13,14], 1991a e b) de pacientes com periodontite do adulto, e diminuídos no fluido gengival após tratamento da DP (MASADA et al.[9], 1990). Diferenças individuais nos níveis de interleucina relacionados aos diferentes graus de

suscetibilidade à DP são atribuídas a alelos de genes polimórficos da interleucina-1. O alelo 2 do polimorfismo IL-1β^{+3953} está associado ao aumento da produção de IL-1β *in vitro* (POCIOT et al.[11], 1992) e por polimorfonucleares do sangue periférico (GORE et al.[3], 1998). Em adultos, o polimorfismo no gene da IL-1β^{+3953} (alelo 2) sugere fortemente um papel desta citocina na patogênese da DP (KORNMAN et al.[7], 1997; GORE et al.[3], 1998). A freqüência do alelo 2 do polimorfismo IL-1β^{+3953} esteve significativamente aumentada em pacientes com DP avançada quando comparados com aqueles com DP leve à moderada, o que fornece subsídios para a implicação desse polimorfismo na suscetibilidade à severidade da DP em adultos (GORE et al.[3], 1998). Indivíduos heterozigotos para o alelo 2 (polimorfismo IL-1β^{+3953}) produzem 2 vezes mais IL-1β, enquanto a homozigose para esse alelo proporciona o aumento de 4 vezes na produção desta interleucina-1 β (POCIOT et al.[11], 1992). KORNMAN et al.[7] (1997) associaram um genótipo composto por alelos resultantes de polimorfismos no gene da IL-1β^{+3953} (alelo 2) e da IL-1α^{-889} (alelo 2) à severidade do desenvolvimento da DP em adultos e estimaram a freqüência desse genótipo composto na população em cerca de 30 %. Foi demonstrado estatisticamente, num estudo longitudinal de 14 anos, que a presença de genótipos variantes (chamados de genótipos positivos para a

IL-1), resultantes de polimorfismos, aumenta as chances de morbidade dental em 2,7 vezes e, num efeito combinado com o fumo, pode resultar num risco 7,7 vezes maior de perda dental (McGuire; Nunn[10], 1999). Estes autores observaram um alto grau de colinearidade entre a combinação fumo-genótipo positivo para IL-1 e parâmetros clínicos relacionados à perda dental, tais como profundidade de sondagem, envolvimento de furca e mobilidade. Para os autores, é possível prever quais pacientes são passíveis de perder mais dentes, especialmente quando fumantes, o que denota a relevância clínica da informação genética. O valor dos parâmetros clínicos tradicionalmente usados na determinação do prognóstico da DP parece estar pelo menos em parte na dependência de genótipos específicos. A associação entre uma dada doença e um marcador genético conhecido, além de permitir a identificação de indivíduos de maior risco ao desenvolvimento da doença em questão, contribui para decifrar a heterogeneidade etiológica da mesma na elucidação de sua patogênese (Sofaer[12], 1990).

DIAGNÓSTICO MOLECULAR DA DOENÇA PERIODONTAL

Mais do que focalizar níveis de higiene oral, identificando-se e quantificando-se a microbiota potencialmente patogênica, o principal desafio

para o futuro parece ser encontrar meios de identificar grupos de risco para a severidade da doença periodontal. Clinicamente, esses fatores podem ser usados para a identificação de indivíduos com uma resposta desfavorável ao ataque microbiano, isto é, pessoas que sejam suscetíveis à doença periodontal, para que sejam priorizados programas de prevenção, antes que ocorra a destruição irreversível dos tecidos periodontais, e para que se conheça melhor o curso da doença. A DP compreende um grupo de doenças crônicas multifatoriais complexas cuja etiopatogênese é pobremente compreendida, dessa forma dificultando testes diagnósticos do risco de sua severidade e progressão (KORNMAN; DI GIOVINE[6], 1998). Mediadores pró-inflamatórios parecem ser o foco de atenção neste momento em que a resposta do hospedeiro mostra-se determinante na intensidade da destruição periodontal. Assim, a busca de marcadores genéticos que permitam a detecção de indivíduos com maior probabilidade de desenvolver a doença é fundamental para a prevenção da instalação do processo destrutivo, ou para a instauração de terapêutica individualizada e proservação adequada de pacientes, nos quais sinais clínicos da doença já se manifestaram. Sendo a DP uma doença multifatorial complexa, quanto mais genes forem considerados, mais efetivo será o diagnóstico.

A mensuração da perda óssea através de sondagem na dentição mista e em dentes apenas parcialmente erupcionados é considerada difícil. Devido à severidade das periodontites de início precoce (EOP) e à sua tendência a progredir para a morbidade dental em curto espaço de tempo e em indivíduos jovens, a detecção precoce da EOP em crianças e adultos jovens é de suma importância para o diagnóstico de indivíduos suscetíveis a desenvolverem a doença a partir da infecção. Pelo fato de a EOP apresentar tendência à agregação familiar, a investigação genética em indivíduos jovens, principalmente naqueles descendentes de pais que perderam dentes pela DP, poderia garantir acompanhamento mais cuidadoso do profissional quanto ao controle de placa, juntamente com aconselhamento contra hábitos de risco, como o fumo. A descoberta de marcadores genéticos relacionados à EOP pode ser de inestimável valia para a compreensão de sua etiologia e patogênese e, no futuro, para a instalação de análise epidemiológica preventiva.

A análise de DNA humano é amplamente empregada em estudos genéticos de famílias e populações. Na maioria dos casos, é realizada em amostras obtidas de sangue periférico. No entanto, constitui um procedimento invasivo, além de esbarrar em problemas éticos, tais como em casos que envolvem pessoas extremamente doentes, idosos e crianças; também requer

supervisão médica e equipamento específico, o que aumenta os custos. A obtenção de células epiteliais da mucosa bucal como fonte de DNA permite uma análise genética fácil, rápida e sem entraves do ponto de vista ético. Ela consiste na realização de um bochecho com solução de glicose a 3 % pelo paciente por 1 minuto (SCAREL et al.[15], 2000; TREVILATTO; LINE[16]). Essa solução transporte contém as células epiteliais nucleadas, a partir das quais se extrai o DNA, o que permite as análises de suscetibilidade genética à DP. O perfil de suscetibilidade representa a somatória das análises individuais de genes que expressam mediadores/fatores celulares envolvidos na patogênese da doença.

CONCLUSÃO

Num futuro muito próximo, a prática da coleta de um simples bochecho do paciente poderá permitir ao cirurgião-dentista o conhecimento do perfil individual de risco à progressão e severidade da doença periodontal. Essa técnica de coleta é simples, rápida e não-invasiva, de apropriada execução durante uma consulta clínica e indicada, por exemplo, para pacientes cujos progenitores perderam elementos dentários em conseqüência da DP Dessa forma, sugere um acompanhamento mais próximo do paciente pelo CD e

desaconselha hábitos de risco, como o fumo. Ainda, do ponto de vista do cirurgião-dentista, este terá como se proteger judicialmente se de posse de termo de compromisso devidamente assinado pelo paciente, em ciência de sua condição genética.

REFERÊNCIAS BIBLIOGRÁFICAS

1. DIEHL, S. R.; WANG, Y.; BROOKS, C. N.; BURMEISTER, J. A.; CALIFANO, J. V.; WANG, S.; SCHENKEIN, H. A. Linkage disequilibrium of interleukin-1 genetic polymorphisms with early-onset periodontitis. *J Periodontol*, v. 70, n. 4, p. 418-430, April 1999.
2. GENCO, R. J. Host responses in periodontal diseases: current concepts. *J Periodontol,* v. 63, n. 4, p. 338-355, April (*Suppl.*) 1992.
3. GORE, E. A.; SANDERS, J. P.; PANDEY, J. P.; PALESCH, Y.; GALBRAITH, G. M. P. Interleukin-1β^{+3953} allele 2: association with disease status in adult periodontitis. *J Clin Periodontol*, v. 25, p. 781-785, 1998.
4. HART, T. C. Genetic considerations of risk in human periodontal disease. *Curr Opin Periodontol*, p. 3-11, 1994.

5. HONIG, J.; RORDORF-ADAM, C.; SIEGMUND, C.; WIEDEMANN, W.; ERARD, F. Increased interleukin-1 beta (IL-1β) concentration in gingiva tissue from periodontitis patients. *J Periodont Res*, v. 24: 362-367, 1989.

6. KORNMAN, K. S.; DI GIOVINE, F. S. Genetic variations in cytokin expression: a risk factor for severity of adult periodontitis. *Ann Periodontol*, v. 3, n. 1, p. 327-338, July 1998.

7. KORNMAN, K. S.; CRANE, A.; WANG, H-Y.; DI GIOVINE, F. S.; NEWMAN, M. G.; PIRK, F. W.; WILSON JR. T. G.; HIGGINBOTTOM, F. L.; DUFF, G. W. The interleukin-1 genotype as a severity factor in adult periodontal disease. *J Clin Periodontol*,. v. 24, p. 72-77, 1997.

8. LINDHE, J. Tratado de Periodontologia Clínica. Guanabara Koogan S.A., 2ª ed., cap. 5, p. 107-135, 1992.

9. MASADA, M. P.; PERSSON, R.; KENNEY, J. S.; LEE, S. W.; PAGE, R. C.; ALLISON, A. C. Measurement of interleukin-1α and -1β in gingival crevicular fluid: implications for the pathogenesis of periodontal disease. *J Periodont Res*, v. 25, p. 156-163, 1990.

10. MCGUIRE, M. K.; NUNN, M. E. Prognosis versus actual outcome. IV. The effectiveness of clinical parameters and IL-1 genotype in accurately

predicting prognoses and tooth survival. *J Periodontol*, v. 70, n. 1, p. 49-56, Jan. 1999.

11. POCIOT, F.; MOLVIG, J.; WOGENSEN, L.; WORSAAE, H.; NERUP, J. A Taq I polymorphism in the human interleukin-1 beta (IL-1β) gene correlates with secretions in vitro. *Eur J Clin Invest*, v. 22, p. 396-402, 1992.

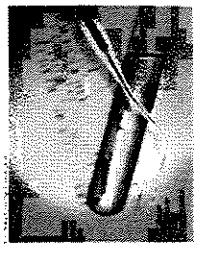
12. SOFAER, J.A. Genetic approaches in the study of periodontal diseases. *J Clin Periodontol*, v. 17, p. 401-408, 1990.

13. STASHENKO, P.; FUJIYOSHI, P.; OBERNESSER, M. S.; PROSTAK, L.; HAFFAJEE, A. D.; SOCRANSKY, S. S. Levels of interleukin 1β in tissue from sites of active periodontal disease. *J Clin Periodontol*, v. 18, p. 548-554, Jul. 1991a.

14. STASHENKO, P.; JANDINSKI, J. J.; FUJIYOSHI, P.; RYNAR, J.; SOCRANSKY, S. S. Tissue levels of bone resorptive cytokines in periodontal disease. *J Periodontol*, v. 62, n. 8, p. 504-509, Aug. 1991b.

15. SCAREL, R. M.; TREVILATTO, P. C.; DI HIPÓLITO JR. O.; CAMARGO L. E. A.; LINE, S. R. P. Absence of mutations in the homeodomain of the *MSX1* gene in patients with hypodontia. *Am J Med Genet*, v. 92, n. 5, p. 346-349, June 2000.

16. TREVILATTO, P. C.; LINE, S. R. P. Use of buccal epithelial cells for PCR amplification of large DNA fragments. *J Forensic Odonto-Stomatol.* Aceito para publicação.



CLINICAL, GENETIC AND MICROBIOLOGICAL FINDINGS IN A BRAZILIAN FAMILY WITH AGGRESSIVE PERIODONTITIS
Journal of Clinical Periodontology
v.29, n.3, Mar. 2002, p.233-239

Clinical, Genetic and Microbiological Findings in a Brazilian Family with Aggressive Periodontitis

Paula C. Trevilatto[1], Vinícius A. Tramontina[2], Maria A. N. Machado[2], Reginaldo B. Gonçalves[3], Antonio W. Sallum[2] and Sergio R. P. Line[1]

Departments of [1]Morphology, [2]Periodontology and [3]Oral Diagnosis, Dental School of Piracicaba, State University of Campinas, Piracicaba, SP, Brazil.

Short title: *Clinical, Genetic, Microbiologic aspects.*

Abstract. In this study, the clinical, genetic and microbiological profile of a 14-individual family with Aggressive Periodontitis was reported. Microbiological samples were obtained from subgingival plaque of affected sites and PCR was utilized to detect pathogenic bacteria. RFLP-PCR was used to determine cytokine genetic polymorphisms. Localized Aggressive Periodontitis was diagnosed for a 18-yr-old systemically healthy non-smoking female (proband), associated with *Actinobacillus actinomycetemcomitans* infection and detectable levels of *Porphyromonas gingivalis*, *Bacteroides forsythus* and *Treponema denticola*. The siblings displayed clinically detectable Aggressive Periodontitis. *Bacteroides forsythus* and *T. denticola* were the most frequent pathogens found. *P. gingivalis* was detected in 50 % of the individuals. Allele 2 of the IL-1α (-889) polymorphism was found in all the individuals as well as allele 1 of the IL-1β (+3953). Alleles 1 and 2 (50 % each) of the IL-1β (-511), allele 1 of the TNF-α (-308) and allele 2 (in homo or heterozygosity) of the gene IL-RN (intron 2) were present. The low prevalence of *A. actinomycetemcomitans* in this family may question the validity of the microbiological analysis to diagnose Localized Aggressive Periodontitis. The results clearly show that microbiological and genetic parameters were irrelevant for the prediction of periodontitis susceptibility in this family.

Key words: Aggressive Periodontitis; cytokine; risk factors; gene polymorphism; periopathogens

Aggressive Periodontitis comprises a group of rare (roughly 1 % of the population), rapidly progressive forms of periodontitis, characterized by severe destruction of periodontal attachment apparatus at an early age. Aggressive Periodontitis seems to be inherited in a Mendelian Fashion, and both X-linked and autosomal modes of transmission have been proposed (Hart et al. 1992, Hodge et al. 2000). Diagnosis of Aggressive Periodontitis requires exclusion of the presence of systemic diseases that may severely impair host defenses and lead to premature tooth loss (Tonetti & Mombelli 1999). The short time of manifestation of clinically detectable levels of disease is generally interpreted as being the expression of either aggressive etiological agents, that implies infection with a highly virulent microbiota, or a high level of subject susceptibility to Periodontal Disease (PD), or a combination of the two (Tonetti & Mombelli 1997). *Actinobacillus actinomycetemcomitans* (*A.a.*) has received particular attention in recent years for being considered a key microorganism in Localized Aggressive Periodontitis. Bacterial lipopolysaccharide (LPS) activates host cells to secrete inflammatory mediators such as prostaglandins, interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α). Subject differences in the interleukin levels are related to the different degrees of susceptibility to periodontal disease and are due to polymorphic alleles of some genes.

Polymorphisms in the IL-1α, IL-1β and TNF-α can identify individuals susceptible to periodontitis (Gore et al. 1998, Diehl et al 1999, McGuire & Nunn 1999). These cytokines are key regulators of the host immuno-inflammatory responses to microbial infection (Kornman et al. 1997). They modulate the synthesis and resorption of extracellular matrix components and bone of the periodontal tissues (Genco 1992). Interleukin-1 (IL-1) has been implicated in disease pathogenesis because it is considered to be a multifunctional pro-

inflammatory cytokine (Birkedal-Hansen 1993). Besides participating in several aspects of the immune response (Tatakis 1993), it enables ingress of inflammatory cells into sites of infection, stimulates release of metalloproteinases that degrade proteins of the extracellular matrix and promotes bone resorption (Birkedal-Hansen 1993, Diehl et al 1999). The allele 2 of IL-1β (+3953) gene was associated with increased production of IL-1β *in vitro* (Pociot et al. 1992) and by peripheral blood leukocytes (Gore et al. 1998). Heterozygous individuals for the IL-1β (+3953) allele 2 can produce twice as IL-1β, while homozygosity promotes a 4-fold increase in the production of IL-1β (Gore et al. 1998). A longitudinal study with a follow up of 14 years (McGuire & Nunn 1999) showed a close relationship between a positive IL-1 genotype (allele 2 of the IL-1β^{+3953}) status and tooth loss. However, alleles 1 of IL-1α (-889) and IL-1β (+3953) polymorphisms were shown to be associated with Aggressive Periodontitis and the variation at the IL-1α (-889) marker does not seem to be closely linked with this disease (Diehl et al. 1999). It was also observed that homozygous individuals for allele 2 of IL-1α (-889) marker were quite rare (only 3 % of the patients with Aggressive Periodontitis).

In spite of its rare occurrence, Aggressive Periodontitis has been the focus of many investigations aimed at understanding its etiology and pathogenesis. In this study we report the clinical and microbiological profile, as well as the analysis of specific inflammatory mediator genotypes of a 14-individual Brazilian family with Aggressive Periodontitis.

Materials and Methods

Clinical procedure

Within a protocol approved by an institutional review board, subjects signed a consent form after being advised of the nature of the study. The two-generation family was formed by 14 individuals: 8 women and 6 men. The average age was 21 years, with a range between 10 and 48 years. Evaluations in each family member consisted of medical and dental history, full-mouth periapical radiographic survey and dental examination. Patients did not smoke and did not have any systemic disease. Clinical examination consisted of visual inspection and photography of the teeth and oral tissues. Measurements of probing depth and of attachment level were made at four sites for each tooth using standard periodontal probe (Hu-Friedy, Chicago, IL). Inflammation was measured using the Gingival Index (GI) and microbial deposits were assessed using the Plaque Index (PI) (Löe 1967). Alveolar bone loss was verified by the use of periapical radiographs (Rx). Any tooth with a clinical attachment loss (CAL) $\geq$ 5 mm was considered to be affected (Flemming 1999, Hedge et al. 2000). Siblings 3 and 4 refused from clinical and radiographic examination. It should be noted that the detection of clinically evident disease is very unlikely in patients less than 13 years of age, unless they are suffering from any variety of systemic conditions that interferes with resistance to bacterial infection in addition to or as part of their Aggressive Periodontitis (Armitage 1999).

Microbiological analysis

Sampling

Microbiological samples were obtained from subgingival plaque of affected sites. Sample sites were isolated and air-dried. Supragingival plaque was removed and paper points were

inserted to the depth of the pockets, held in place for 15 s and placed into a vial containing 0.5 mL sterile water. The samples were boiled for 10 min, centrifuged, and the supernatant was subsequently used for PCR analysis.

Bacterial samples

The following bacterial strains were obtained from American Type Culture Collection and used as positive and/or negative controls: *Porphyromonas gingivalis (P.g.)* (ATCC 33277), *Porphyromonas endodontalis (P.e.)* (ATCC 35406), *Bacteroides forsythus (B.f.)* (ATCC 43037), *Actinobacillus actinomycetemcomitans (A.a.)* (ATCC 29522), *Prevotella intermedia* (*P.i.*) (ATCC 25611), *Prevotella nigrescens* (*P.n.*) (NCTC 9336) and *Treponema denticola* (*T.d.*) (clinical isolate).

Primers

The primers used have been described previously: *P. gingivalis* (Benkirane et al. 1995); *B. forsythus*, *T. denticola* and *A. actinomycetemcomitans* (Slots et al. 1995); *P. endodontalis*; *P. intermedia* and *P. nigrescens* (Guillot et al. 1997). Most of these bacteria are considered to be predictors for the progression of periodontitis (Flemming 1999).

PCR

Amplifications were performed in a reaction mixture containing 5 µL of sample, 1X PCR buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl; 1.5 mM $MgCl_2$), 200 µM of each of the 4 dNTPs, 1.25 U Taq DNA polymerase, 1.0 µM of each primer in a final volume of 50 µL. The PCR temperature profile included an initial denaturation step at 95°C for 2 min, followed by 36 cycles of denaturation at 95°C for 30 s, primer annealing at 65°C for 1 min and extension at 72°C for 1 min, with a final extension step of 72°C for 2 min. PCR

amplification products were analyzed in 1.5 % agarose gel electrophoresis. The gel was stained with ethidium bromide and photographed on a ultraviolet light transilluminator.

Genes of the host inflammatory response

Sampling

Fourteen individuals undertook a mouthwash after 2 min, containing 5 mL 3 % glucose. Following mouthwash, a sterile wood spatula was used to scrape oral mucosa. The tip of the spatula was then shaked into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm for 10 min. The supernatant was discarded and the cell pellet resuspended in 500 µL of extraction buffer [10 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5 % SDS]. The samples were then frozen at -20 °C until used for DNA extraction.

DNA extraction

After defrosted, samples were incubated overnight with 100 ng/mL proteinase K (Sigma Chemical Co., St. Louis, MO, USA) at 37°C with agitation. DNA was then purified by sequential phenol/chloroform extraction and salt/ethanol precipitation (Sambrook et al. 1989). DNA was dissolved in 70 µL TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA]. The concentration was estimated by measurements of OD 260.

Analysis of genetic polymorphisms

Amplification reactions (PCR) were performed with 300 to 700 ng DNA in a volume of 50 µL in reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, deoxyribonucleotides (200 µM each), 2 U *Taq* DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden). Five genetic markers, considered to be predictors of Adult Periodontitis severity by Kornman et al. (1997), were investigated: IL-1α (-889),

IL-1β (-511), IL-1β (+3953), TNF-α (-308) and IL-RN (intron 2) genes. The primers, $MgCl_2$ concentration, cycle parameters and restriction assays (RFLP) were used as described (Kornman et al. 1997). Amplification was carried out in a *Perkin-Elmer GeneAmp 2400* thermal cycler. Restriction products were visualized by electrophoresis on vertical 5 % polyacrylamide gels in 1X TBE (89 mM Tris-Borate, 89 mM boric acid, 2 mM EDTA), followed by silver staining (Bio-Rad Silver Stain Kit).

Results

Case report

Proband features

The proband (subject 10: Fig.1, Table 1) was examined and complained about tooth mobility. The patient was systemically healthy and did not present susceptibility to infections. She suffered from red, swollen and easy-bleeding gingiva, progressive mobility on all first molars and gingival recessions. At the moment of clinical examination, the right maxillary and mandibular first molars and the left maxillary first molar were extremely mobile. The teeth were free from caries and calculus was not found. Radiographs revealed extensive alveolar bone loss on the first molars. Deep pockets were clinically detected by probing. Localized Aggressive Periodontitis was diagnosed for the proband according to the criteria of the *1999 World Workshop for a Classification of Periodontal Diseases and Conditions* (Armitage 1999).

Siblings

Subjects 11, 13, 14, 15 (Fig.1, Table 1) did not show any clinical attachment loss. At their age (10 to 16 years) the disease phenotype may not have developed sufficiently so that could

be reliably differentiated from diseased subjects. The youngest siblings of the family (13, 14 and 15) were in the period of mixed dentition. Six siblings (5, 6, 7, 8, 9 and 12: Fig.1, Table 1) presented signs of Aggressive Periodontitis. Siblings 5, 6, 7 and 8 presented several missing teeth and clinical attachment loss were in the range of 5 to 7 mm; radiographs revealed angular defects affecting the related teeth, especially in sibling 7. Siblings 5 and 6 showed a moderate amount of calculus and plaque. Sibling 7 did show neither calculus nor amounts of plaque. Localized Aggressive Periodontitis was diagnosed for subjects 5 and 8. Incipient LAP was diagnosed for subject 9, which presented only two affected sites, and sibling 12, with affected sites on the first molars (she was in the period of mixed dentition). The large number of missing teeth and loss of attachment ≥ 5mm in other teeth than molars and incisors in patients 6 and 7 suggest a generalization of the process of disease. The clinical condition of the mother (subject 2: Fig. 1) suggests that she used to portrait periodontal disease (Table 1).

Microbiological findings

The specificity of PCR primers was confirmed against the tested species. No cross reaction was observed for all tested species. The most frequent pathogens found in the subgingival microbiota of the analyzed patients were *B. forsythus* and *T. denticola* (detected in 45 % plaque samples). *Porphyromonas gingivalis* was found in about 50 % of the aggressive periodontitis individual plaque samples. The proband (subject 10) was colonized by *A. actinomycetemcomitans*, *B. forsythus*, *T. denticola* and *P. gingivalis* (Table 2).

Genetic analysis

The following alleles of the polymorphisms studied were observed in the family: allele 2 of IL-1α gene (-889), allele 1 for the IL-1β gene (+3953), alleles 1 and 2 (50 % each) for IL-1β

gene (-511), allele 1 for TNF-α gene (-308) and allele 2 (in homo or heterozygosity) for IL-RN gene (intron 2) (Table 3).

Discussion

Gram-negative organisms are believed to constitute approximately two thirds of the inhabitants of aggressive periodontitis pathogenic microbiota. *Actinobacillus actinomycetemcomitans*, a facultative anaerobic, is considered to be closely associated with LAP (Tonetti & Mombelli 1999). In fact, this microorganism was detected in the proband sites of infection. Together with the clinical attachment loss and alveolar bone loss localized in the mesial aspect of the first molars, the presence of this pathogen is considered to be an important factor in the diagnosis of LAP (Lench et al. 1988). Generalized aggressive periodontitis has been frequently associated with *P. gingivalis*, *B. forsythus* and *A. actinomycetemcomitans* (Tonetti & Mombelli 1999). One of the siblings with generalized aggressive periodontitis had *P. gingivalis* and the two presented *B. forsythus*. Sibling 15 was infected with *A.a.*, *P.g.*, *P.e.* and *T.d.*. This patient had a significant microbiota and should be monitored. He did not present signs of disease, which could be due to low age (10 years).

Since bacteria alone will not explain periodontal disease progression, genetic risk factors implicated in the host immune and inflammatory response to bacterial etiologic agents might significantly contribute to the comprehension of the pathogenesis of this group of diseases.

Allele 1 (genotype 1/1) of the IL-1β (+3953) polymorphism was present in all the family members analyzed. If the presence of allele 1 for the IL-1β (+3953) polymorphism determines low levels of IL-1β (and we found it in homozygosity for all the family members), other mediators should be playing a role and having an effect on severity of

periodontal disease. Perhaps, the lower expression of the IL-1β may be compensated by the homozygosity of the IL-1α gene (allele 2). All the individuals studied did present allele 2 (genotype 2/2) of the IL-1α (-889) marker. Although IL-1α is about 15-fold less potent than IL-1β (Stashenko et al. 1991 b), polymorphism in this gene has been associated with juvenile arthritis, a chronic inflammatory disease (McDowell et al. 1995). Otherwise, allele 1 of the IL-1β (+3953) polymorphism associated or not with IL-1α (-889) allele 2 can possibly cause individuals to be more susceptible to aggressive periodontitis. It is possible that the non-affected siblings of this study do not show signs of disease because of low age (principally individuals 14 and 15 who carry pathogenic bacteria). The distinct roles of IL-1α and β in periodontal diseases indicate a possible association between periodontitis severity and IL-1 genotypes, but conclusive data about which allele of both polymorphisms really and undoubtly contributes to the increase of susceptibility to periodontal disease remain to be deciphered.

Regarding the IL-1β (-511) polymorphism, we have found alleles 1 and 2 in homo or heterozygosity, with a similar frequency. The frequency of alleles 1 and 2 has been estimated as about 60 % and 40 % respectively (Di Giovine et al. 1992).

The tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine, produced by macrophages, with a similar role as IL-1 (Alexander & Damoulis 1994). It induces the secretion of collagenase, prostaglandin E_2 (PGE_2) and interleukin-6 (IL-6) by fibroblasts and bone in culture (Meikle et al. 1989), stimulates bone resorption, and has important immunological activities (Bertolini et al. 1986). It is present in the crevicular fluid of patients with gingivitis and periodontitis (Rossomando et al. 1990). However, TNF-α is about 500-fold less potent than IL-1 in inducing bone resorption (Stashenko et al. 1991 a,

Alexander & Damoulis 1994). Polymorphism within TNF-α locus (allele 2) has been associated with autoimmune diseases (Wilson et al. 1993). In our study, we observed only allele 1 (in homozygosity) in all individuals analyzed. In fact, the frequency of this allele was around 84 % when a sample of forty unrelated individuals was screened (Wilson et al. 1992).

The IL-1 receptor antagonist (IL-1ra), expressed by IL-RN gene, is an IL-1 antagonist by blocking IL-1 receptors, inhibiting the development of diseases mediated by IL-1. Out of the four alleles described allele 2 is more frequent in patients with some forms of immuno-inflammatory diseases (Tarlow et al. 1993, Blakemore et al 1994, Clay et al. 1994, Mansfield et al. 1994). Allele 2 was present in all genotypes studied at least in heterozygosity.

We have investigated some already reported markers, but also have to consider the effect of other host modifying factors, acting in antagonistic or synergistic ways with the genes of principal effect, that can modulate the severity of the inflammatory response. Besides, it is not certain yet whether IL-1 is indeed the major factor determining the genetic susceptibility to periodontitis. It is possible that the findings and definitions of factors composing the host response, together with the local microbiota associated, could help clarify the etiology and pathogenesis of this heterogeneous disease, this way providing the best way to prevent and monitor the treatment of this pathology. Analysis of families which portrait forms of aggressive periodontitis may help to add information to clarify the factors that really contribute to progress disease.

It is also worth mentioning that the use of buccal epithelial cells as source of DNA for PCR amplifications has several advantages over blood sampling (Trevilatto & Line 1999, Scarel et al. 2000). The collection of material is fast and inexpensive, buccal samples can be

easily obtained from patients who are reluctant to donate blood and there is no need of medical supervision during sampling (Lench et al. 1988, Meulenbelt et al 1995). Besides, children's consent becomes easier, which is suitable for epidemiological analysis and diagnostic assays involving a large number of individuals.

Acknowledgements

The authors wish to thank Dr. Francisco H. Nociti Jr. for the usage of the Clinic to treat the patients. This work was supported by FAPESP grant 97/06484-5.

References

Alexander, M. B. & Damoulis, P.D. (1994) The role of cytokines in the pathogenesis of periodontal disease. *Current Opinion in Periodontology* 39-53.

Benkirane, R. M., Guillot, E. & Mouton, C. (1995) Immunomagnetic PCR and DNA probe for detection and identification of *Porphyromonas gingivalis. Journal of Clinical Microbiology* **33**, 2908-2912.

Bertolini, D. R., Nedwing, G. E., Bringman, T. S., Smith, D. D. & Mundy, G. R. (1986) Stimulation of bone resorption and inhibition of bone formation *in vitro* by human tumour necrosis factors. *Nature* **319**, 516-518.

Birkedal-Hansen, H. (1993) Role of matrix metalloproteinases in human periodontal diseases. *Journal of Periodontology* (*Suppl*) **64**, 474-484.

Blakemore, A. I. F., Tarlow, J. K., Cork, M. J., Gordon, C. & Emery, P. (1994) Interleukin-1 receptor antagonist gene polymorphism as a severity factor in systemic lupus erythematosus. *Arthritis & Rheumatism* **37**,1380-1385.

Clay, F. E., Cork, M. J., Tarlow, J.K., Blakemore, A. I., Harrington, C. I., Lewis F. & Duff G. W. (1994) Interleukin-1 receptor antagonist gene polymorphism association with lichen sclerosus. *Human Genetics* **94**, 407-410.

Di Giovine, F. S., Takhsh, E. , Blakemore, A. I. F. & Duff, G. W. (1992) Single base polymorphism at –511 in the human interleukin-1 beta gene (IL-1β). *Human Molecular Genetics* **1**(6), 450.

Diehl, S. R., Wang, Y., Brooks, C. N., Burmeister, J. A., Califano, J. V., Wang, S. & Schenkein, H. A. (1999) Linkage disequilibrium of interleukin-1 genetic polymorphisms with early-onset periodontitis. *Journal of Periodontology* **70**, 418-430.

Genco, R. J. (1992) Host responses in periodontal diseases: current concepts. *Journal of Periodontology* **63**, 338-355.

Gore, E. A., Sanders, J. P., Pandey, J. P., Palesch, Y. & Galbraith, G. M. P. (1998) Interleukin-1β^{+3953} allele 2: association with disease status in adult periodontitis. *Journal of Clinical Periodontology* **25**, 781-785.

Guillot, E. & Mouton, C. (1997) PCR-DNA probe assays for identification and detection of *Prevotella intermedia* sensu stricto and *Prevotella nigrescens*. *Journal of Clinical Microbiology* **35**, 1876-1882.

Honig, J., Rordorf-Adam, C., Siegmund, C., Wiedemann, W. & Erard, F. (1989) Increased interleukin-1 beta (IL-1β) concentration in gingiva tissue from periodontitis patients. *Journal of Periodontal Research* **24**, 362-367.

Kornman, K. S., Crane, A., Wang, H-Y., Di Giovine, F. S., Newman, M. G., Pirk, F. W., Wilson Jr. T. G., Higginbottom, F. L. & Duff, G. W. (1997) The interleukin-1 genotype as

a severity factor in adult periodontal disease. *Journal of Clinical Periodontology* **24**, 72-77.

Lench, N., Stanier, P. & Williamson, R. (1988) Simple non-invasive method to obtain DNA for gene analysis. *Lancet* 1356-1358.

Löe, H. (1967) The gingival index, the plaque index and the retention index systems. *Journal of Periodontology* **38**, 610-616.

Mansfield, J. C., Holden, C., Tarlow, H., Di Giovine, F. S., McDowell, T. L., Wilson, A. G., Holdsworth, C. D. & Duff, G. W. (1994) Novel genetic association between ulcerative colitis and the antiinflammatory cytokine interleukin-1 receptor antagonist. *Gastroenterology* **106**, 637-642.

Masada, M. P., Persson, R., Kenney, J. S., Lee, S. W., Page, R. C. & Allison, A. C. (1990) Measurement of interleukin-1α and -1β in gingival crevicular fluid: implications for the pathogenesis of periodontal disease. *Journal of Periodontal Research* **25**, 156-163.

McDowell, T. L., Symons, J. A., Ploski, R., Fárre, O. & Duff, G. W. (1995) A genetic association between juvenile rheumatoid arthritis and a novel interleukin-1 alpha polymorphism. *Arthritis & Rheumatism* **38**, 221-222.

McGuire, M. K. & Nunn, M. E. (1999) Prognosis versus actual outcome. IV. The effectiveness of clinical parameters and IL-1 genotype in accurately predicting prognoses and tooth survival. *Journal of Periodontology* **70**, 49-56.

Meikle, M. C., Atkinson, S. J., Ward, R. V., Murphy, G. & Reynolds, J. J. (1989) Gingival fibroblasts degrade type I collagen films when stimulated with tumor necrosis factor and interleukin 1. *Journal of Periodontal Research*. **24**, 207-213,.

Meulenbelt, I., Droog, S., Trommelen, G. J. M., Boomsma, D. I. & Slagboom, P. E. (1995) High-yield noninvasive human genomic DNA isolation method from genetic studies in geographically dispersed families and populations. *American Journal of Human Genetics* **57**, 1252-1254.

Pociot, F., Molvig, J., Wogensen, L., Worsaae, H. & Nerup, J. (1992) A Taq I polymorphism in the human interleukin-1 beta (IL-1β) gene correlates with secretions in vitro. *European Journal of Clinical Investigation* **22**, 396-402.

Rossomando, E. F., Kennedy, J. E. & Hadjimichael, J. (1990) Tumour necrosis factor alpha in gingival crevicular fluid as a possible indicator of periodontal disease in humans. *Archives of Oral Biology* **35**, 431-434.

Sambrooks, J., Maniatis, T. & Fritsch, E. F. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd Edition, vol. 2. Cold Spring Harbor Laboratory Press.

Slots, J., Ashimoto, A., Flynn, M. J., Li, G. & Chen, C. (1995) Detection of putative periodontal pathogens in subgingival specimens by 16S ribossomal DNA amplification with the polymerase chain reaction. *Clinical & Infectious Disease* **20**, 304-307.

Stashenko, P., Fujiyoshi, P., Obernesser, M. S., Prostak, L., Haffajee, A. D. & Socransky, S. S. (1991a) Levels of interleukin 1β in tissue from sites of active periodontal disease. *Journal of Clinical Periodontology* **18**, 548-554.

Stashenko, P., Jandinski, J. J., Fujiyoshi, P., Rynar, J. & Socransky, S.S. (1991b) Tissue levels of bone resorptive cytokines in periodontal disease. *Journal of Periodontology* **62**, 504-509.

Tarlow, J. K., Blakemore, A. I. F., Lennard, A., Solari, R., Steinkasserer, A. & Duff, G. W. (1993) Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-bp tandem repeat. *Human Genetics* **91**, 403-404.

Tatakis, D. (1993) Interleukin-1 and bone metabolism: a review. *Journal of Periodontology* **64**, 416-431.

Tonetti, M. S. & Mombelli, A. (1997) Early onset periodontitis. In: *Clinical periodontology and implant dentistry.* 3rd edition, eds. Lindhe, J., Karring, T., Lang, N. P., pp. 227-257. Copenhagen: Munksgaard.

Trevilatto, P. C. & Line, S. R. P. (2000) Use of buccal epithelial cells for PCR Amplification of large DNA fragments. *Journal of Forensic Odonto-Stomatology*. **18** (1): 6-9.

Wilson, A. G., de Vries, N., Pociot, F., di Giovine, F. S., van der Putte, L. B. A. & Duff, G. W. (1993) An allelic polymorphism within the human Tumor Necrosis Factor a promoter region is strongly associated with HLA A1, B8, and DR3 alleles. *Journal of Experimental Medicine* 77, 557-560.

Wilson, A. G., di Giovine, F. S., Blakemore, A. L. & Duff, G. W. (1992) Single base polymorphism in the human tumour necrosis factor alpha (TNF alpha) gene detectable by Ncol restriction of PCR product *Human Molecular Genetics* **1,** 353.

Adress:
Sergio Roberto Peres Line
Faculdade de Odontologia de Piracicaba-UNICAMP
Departamento de Morfologia
Av. Limeira 901, CEP. 13414-018
Piracicaba - SP
Brazil
Fax: +55 (019) 430-5218
E-mail: sergline@fop.unicamp.br

Table 1. Clinical features of the individuals within the family with aggressive periodontitis.

Patient	Age	Sex	Missing Teeth	Teeth = CAL (mm) [*tooth face**]	Alveolar Bone Loss (Rx)
2	48	Female	All the maxillary 37/38/46/47/48	absent	31/32/41/42/ 43/44 /45
5	25	Female	16/12/11/21/22/24/26 36/37/32/46	27=7[*m*]	27/33/ 31/41/42
6	24	Male	17/14/12/11/21/22/26/36/ 34/32/33/31/41/42/46	16=5[*m*]; 24=5[*m*]; 37=5[*v*] 43=7[*m*]	16/24/25/26/ 36/43/47
7	23	Female	12/11/21 31/32/41/42	16=5[*m*]; 24=7[*m*]; 46=7[*m*]; 44=7[*v*]; 36=5[*b*]	16/22/24/26/34/ 35/36/ 44/45/46
8	20	Female	36/45/46	16=7[*m/d*]; 21=5[*d*]; 22=5[*m*]; 24=9[*m*]; 26=7[*m*]	absent
9	19	Female	absent	16=7[*m*]; 26=5[*m/d*]	16/26
10 Proband	18	Female	absent	16=7[*m*]; 26=5[*m*]; 36=7[*m*]; 46=8[*d*]	16/36/46
11	16	Female	absent	absent	absent
12	14	Female	absent	16=5[*d*]; 26=5[*d*]; 36=5[*d*]; 46=5[*d*]	absent
13	13	Male	absent	absent	absent
14	11	Male	absent	absent	absent
15	10	Male	absent	absent	absent

* ***m**=mesial; **d**=distal; **v**=vestibular; **b**=buccal*

Table 2. Presence of specific bacteria in crevicular sites of the aggressive periodontitis patients.

Patients	***A.a.***	***B.f.***	***P.e.***	***P.g.***	***P.i.***	***P.n.***	***T.d.***
2	-	-	-	-	-	-	-
3	-	+	-	-	-	-	+
4	-	-	-	-	-	-	-
5[§]							
6	-	+	-	+	-	+	+
7	-	+	+	-	-	-	-
8	-	+	-	+	+	-	+
9	-	+	+	-	-	-	+
10	+	+	-	+	-	-	+
11	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-
14	-	+	-	+	-	-	+
15	+	-	+	+	-	-	+

+ positive PCR reaction; - negative PCR reaction.

[§] This patient was not analyzed because she had already started taking antibiotics.

Table 3. Gene markers analyzed with the respective genotypes of each individual of the Brazilian family with aggressive periodontitis.

Patient	**IL-1α^{-889}** ***(alleles 1/2)***	**IL-1β^{+3953}** ***(alleles 1/2)***	**IL-1β^{-511}** ***(alleles 1/2)***	**TNF-α^{-308}** ***(alleles 1/2)***	**IL-RN** ***(alleles1/2/3/4/5)***
2	(2/2)	(1/1)	(1/2)	(1/1)	(2/2)
3	(2/2)	(1/1)	(2/2)	(1/1)	(2/4)
4	(2/2)	(1/1)	(1/1)	(1/1)	(2/2)
5	(2/2)	(1/1)	(1/2)	(1/1)	(2/2)
6	(2/2)	(1/1)	(1/2)	(1/1)	(2/2)
7	(2/2)	(1/1)	(1/2)	(1/1)	(2/4)
8	(2/2)	(1/1)	(1/2)	(1/1)	(2/2)
9	(2/2)	(1/1)	(1/1)	(1/1)	(2/2)
10	(2/2)	(1/1)	(1/2)	(1/1)	(2/2)
11	(2/2)	(1/1)	(1/2)	(1/1)	(2/2)
12	(2/2)	(1/1)	(1/1)	(1/1)	(2/2)
13	(2/2)	(1/1)	(2/2)	(1/1)	(2/2)
14	(2/2)	(1/1)	(2/2)	(1/1)	(2/4)
15	(2/2)	(1/1)	(1/1)	(1/1)	(2/2)

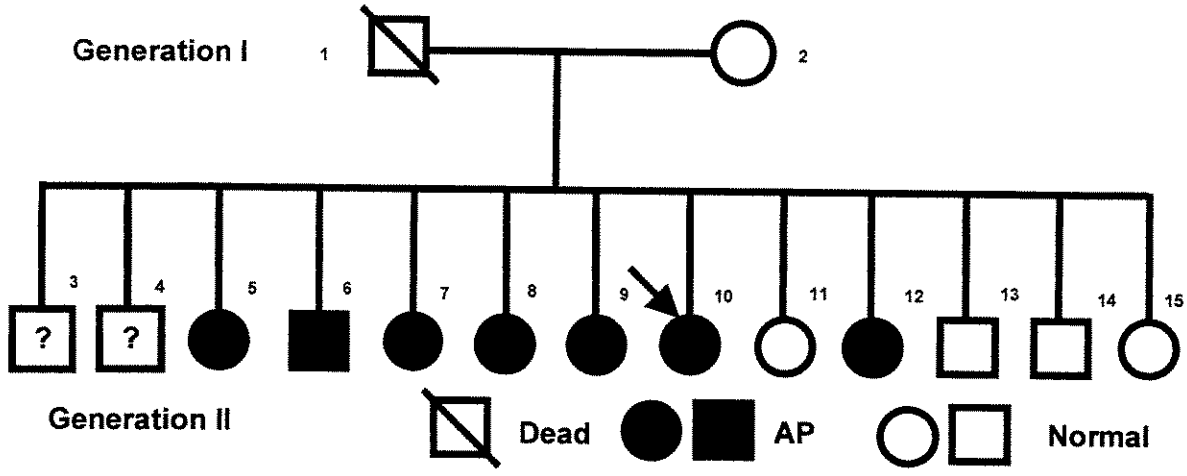
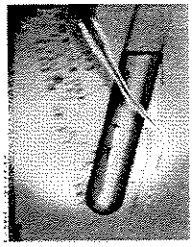


Fig.1. Pedigree of the family. Circles designate female and squares males. The proband is indicated with an arrow.



POLYMORPHISMS IN THE IL-1α AND IL-1β GENES ARE NOT ASSOCIATED WITH SUSCEPTIBILITY TO CHRONIC PERIODONTITIS IN A BRAZILIAN POPULATION

The Journal of Infectious Diseases, submitted

Title: Polymorphisms in the IL-1α and IL-1β Genes Are Not Associated with Susceptibility to Chronic Periodontitis in a Brazilian Population

Paula C. Trevilatto,[1] Raquel M. Scarel-Caminaga,[1] Rui B. de Brito Jr,[1] Ana P. de Souza,[1] Antonio W. Sallum,[2] and Sergio R. P. Line[1]

Departments of [1]Morphology and [2]Periodontology, Dental School of Piracicaba, State University of Campinas, Piracicaba, SP, Brazil

Abstract: 150 words

Text: 1565 words

Presented in part: XVIII Reunião Anual da Sociedade Brasileira de Pesquisas Odontológicas (*SBPqO*) – **Brazilian Oral Research**, 1-5 September 2001, Águas de Lindóia, São Paulo, Brazil, v. **15** *supplement*, (abstract H012).

This study was approved by the Research Ethics Committee of Dental School of Piracicaba - State University of Campinas (UNICAMP), Piracicaba, SP, Brazil (protocol 63/99).

Financial support: FAPESP grants 99/05610-2 and 99/06094-8.

Reprints and correspondence: Dr. Sergio R. P. Line, Departamento de Morfologia, Faculdade de Odontologia de Piracicaba-UNICAMP, Av. Limeira 901, CEP. 13414-018, Piracicaba – SP, Brazil. Fax: +55 (019) 3412-5218. Tel: +55 (019) 3412-5333 (e-mail: [redacted])

Abstract

The aim of this study was to investigate the association between polymorphisms in the IL-1α and IL-1β genes and severity of chronic periodontitis in Brazilians. 113 non-smoking subjects over 25 years (mean age 41.6) were divided according to the severity level of periodontal disease: 44 healthy individuals (control group), 31 subjects with moderate and 38 with severe periodontitis. DNA was obtained from epithelial cells through a mouthwash with 3 % glucose and scraping of oral mucosa. The samples were analyzed for *IL-1A* (-889) and *IL-1B* (+3953) polymorphisms using PCR-RFLP. The significance of the differences in the frequencies of the polymorphisms in the control and groups with periodontitis was assessed by Chi-square test ($p<0.05$). No significant differences in the allele and genotype frequencies were found among the groups. It was concluded that neither *IL-1A* (-889) nor *IL-1B* (+3953) polymorphism was associated with the severity of chronic periodontal disease in the study population.

Periodontal diseases (PD) comprise a group of infectious immuno-inflammatory diseases, characterized by irreversible loss of tissue support around the teeth, which often leads to tooth loss. Bacterial lipopolysaccaharides (LPS) of the dental plaque activate cells to produce cytokines such as interleukin-1 (IL-1), which plays a central role in the pathogenesis of many autoimmune and infectious diseases, including periodontitis [1]. Interleukin-1 is a pro-inflammatory cytokine that has been observed in up to 100 % of the gingival crevicular fluid (GCF) from periodontally diseased sites [2]. It has been postulated that this cytokine could be considered a clinical indicator of the active phase of periodontal disease [3].

Together with age, race and smoking, polymorphisms in immuno-regulatory genes seem to be risk factors for the onset and progression of PD. Within the IL-1β gene, a polymorphism in exon 5 (at position +3953) has been reported to influence the production of IL-1β protein. Genotype 2/2 of *IL-1B* (+3953) polymorphism has been associated with a 4-fold increase in IL-1β production [4]. A composite genotype formed by allele 2 of a bi-allelic polymorphism at position -889 of *IL-1A* plus allele 2 of *IL-1B* (+3953) occurred in 80 % of the individuals severely affected and did correlate to the clinical status of chronic periodontitis [1].

In this study we investigated the association between polymorphisms *IL-1A* (-889) and *IL-1B* (+3953) and the severity of chronic periodontitis in a Brazilian population.

Methods

Subject Selection. A convenience sample of 113 unrelated, non-smoking subjects over 25 years of age (mean age 41.6 years) from the Southeastern region of Brazil were recruited for study from the patient pool at the Dental Clinics of the Faculty of Dentistry of Piracicaba - UNICAMP. The baseline clinical parameters for the subject population are presented in table 1. The criteria of exclusion and diagnosis were described elsewhere [5]. Subjects were divided into the following categories according to PD severity: 44 healthy individuals (control group), with no signs of disease, 31 subjects with moderate periodontitis and 38 patients with severe periodontitis.

Sampling. DNA was obtained from epithelial buccal cells and extraction was performed as described by Trevilatto & Line (2000) [6].

Polymorphism in the IL-1α gene at position -889. The oligonucleotides 5'-AAGCTTGTTCTACCACCTGAACTAGGC-3' and 5'-TTACATATGAGCCTTCCATG-3' were used as primers. Polymerase chain reaction (PCR) was carried out with 500 ng genomic DNA in a total volume of 50 μL, containing 10 mM Tris-HCl

(pH 8.3), 50 mM KCl, 1 μM of each primer, 200 μM each dATP, dCTP, dGTP and dTTP, 2.5 mM $MgCl_2$ and 2.5 units T*aq* DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden). The reaction was incubated for 3 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C and a final extension at 72°C for 5 min. Amplifications were carried out in a Perkin-Elmer GeneAmp 2400 thermal cycler. Restriction fragment length polymorphism (RFLP) was performed with 3 U per 25 μL reaction of N*co*I at 37°C overnight (ON) to detect allele 1 [T] (16 bp + 83 bp) and allele 2 [C] (99 bp).

Polymorphism in the IL-1β gene at position +3953 (5th exon). The region that contains the T*aq*I polymorphic site within exon 5 of IL-1β gene was amplified by polymerase chain reaction (PCR). The oligonucleotides 5'-CTCAGGTGTCCTCGAAGAAATCAAA-3' and 5'-GCTTTTTTGCTGTGAGTCCCG-3' flanking this region were used as primers. Amplification reactions were carried out with 500 ng genomic DNA in a total volume of 50 μL, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 μM of each primer, 200 μM each dATP, dCTP, dGTP and dTTP, 2.5 mM $MgCl_2$, and 2.5 units T*aq* DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden). The solution was incubated for 2 min at 95°C, 1 min at 67.5°C and 1 min at 74°C by 2 cycles followed by 35 cycles of 1 min at 95°C, 1 min at 67.5°C and 1 min at 74°C and 3 cycles of

1 min at 95°C, 1 min at 67.5°C and 5 min at 74°C. The products were digested with 3 U per 25 µL reaction of T*aq*I at 65°C ON to yield allele 1 [C] (85 bp + 97 bp) and allele 2 [T] (182 bp). In both cases a constant 12 bp band was also produced

Statistical Analysis. The allele ratio and genotype distribution of periodontitis patients and healthy control subjects were analyzed with Chi-square test. A p-value <0.05 was considered significant.

Results

There was no significant difference in the genotype distribution between healthy controls and periodontitis patients in the polymorphisms *IL-A*$^{-889}$ (p=0.903) and *IL-1B*$^{+3953}$ (p=0.663), neither was observed statistical variation in the frequency of the alleles in either polymorphisms studied: *IL-A*$^{-889}$ (p=0.647) and *IL-1B*$^{+3953}$ (p=0.351). The distribution of the genotype frequencies and the frequencies of the alleles for both polymorphisms studied are shown in table 2. Allele 2 of the *IL-A* (-889) polymorphism was carried by 51.3% (58/113) of the subjects; of these, only 5.3% (6/113) were homozygous. Allele 2 of the *IL-1B* (+3953) polymorphism was rarer with 31% (35/113) of the study population carrying this allele; of these, 3.5% (4/113) were homozygous. There was no difference in the frequency of the composite genotype, consisting of

IL-A (-889) allele 2 and *IL-1B* (+3953) allele 2, between the healthy group and the groups with periodontitis (p=0.386). Only 33 of the 113 subjects (29.2%) carried the composite genotype.

Discussion

Although the indications that more than half of the increased susceptibility to periodontal disease may depend on genetic factors [7], the genetic factors contributing to periodontitis susceptibility remain poorly understood. Among potential new candidate susceptibility genes, there is a strong evidence to support investigation of the IL-1 gene family on chromosome 2q13, encoding three proteins, IL-1α, IL-1β and the IL-1 receptor antagonist (IL-1ra), which competes with IL-1α and IL-1β for binding to the IL-1 receptors and is a potent inhibitor of IL-1 activity. In this study polymorphisms in the pro-inflammatory *IL-1A* and *IL-1B* were investigated. It was not realized significant association between *IL-1A* and *IL-1B* polymorphisms and severity of periodontal disease. Neither was observed a correlation between the composite genotype (formed by at least one copy of the allele 2 of both polymorphisms) and periodontitis in the study population.

When Caucasians were taken alone, differences in the allele and genotype frequencies among the groups were not observed as well (data not shown).

As can be noted in table 1, the majority (77 %) of the individuals belonging to the population studied is of Caucasian heritage. The high number of white individuals in the sample can be explained by the predominance of European ancestry in South and Southeast of Brazil and is consistent with the data from the Official Brazilian Census. Allele 2 carriage rate for both *IL-1A* (-889) and *IL-1B* (+3953) polymorphisms in the Caucasians was respectively 54% (47/87) of the population, with 5.8% (5/87) of homozygous, and 35.6% (31/87) of the subjects, being 3.5% (3/87) homozygous. Studies on the relationship between IL-1 polymorphisms and periodontitis in Caucasians found the following allele 2 carriage rates: 48% [8], 50.3% [1], 40.6% [9], 52% [10] and 56.4% [11] at the *IL-1A* (-889), and 44.2% [1], 40.6% [9], 32.3% [12] and 49% [11] at the *IL-1B* (+3953) locus. The composite genotype was carried by 33.3% (29/87) of the study Caucasians, similar to that (36.3%) reported by Kornman et al. (1997) [1]. Lower prevalence of 28% was obtained by McDewitt et al (2000) [13] and 29.1% by Duff and di Giovine in Northern Europeans subjects of unknown periodontal status [1], similar to what was obtained in this study for the whole Brazilian population (29.2%).

Recently, a number of single nucleotide polymorphisms (SNPs), which quantitatively affect mRNA synthesis, have been identified in the IL-1 gene cluster, and their frequency is associated with the incidence and severity of

several infectious and inflammatory diseases, such as rheumatoid arthritis [14], meningococcal disease [15] and chronic periodontitis [1, 9, 11, 12].

Our results failed to detect an association between specific alleles of *IL-1A* (-889) and *IL-1B* (+3953) polymorphisms and chronic periodontal disease in a Southeastern Brazilian population. Even though the IL-1 genes do affect the production of the cytokines, we speculate the total influence might be weak and these alleles may not represent a risk factor for Brazilian periodontitis patients. Although the role IL-1 plays in leading to tissue destruction, it is worth mentioning the need for evaluation of other potential candidate genes as contributors to periodontitis, since the existence of a unique gene of principal effect has not yet been confirmed, and chronic periodontitis may possibly represent a polygenic disease. Besides, an individual may also increase susceptibility to severe disease due to non-genetic factors, such as economic status, poor education or lack of professional dental care and bacterial challenge. Probably, in each population an individual risk factor may explain a different proportion of severe periodontitis cases than in other population groups. Polymorphisms in these and/or other genes of the host response, yet to be identified, may bring more information as to which factors could indeed be implicated in the process of disease.

It was concluded that neither *IL-1A* (-889) nor *IL-1B* (+3953) polymorphism was associated with the severity of chronic periodontal disease in the population studied. Although the prevalence of the polymorphisms studied for the Brazilian population was similar to that reported for Europeans, findings from this study bring into question the usefulness of the cited polymorphisms as a method for determining the susceptibility of Brazilian patients to chronic periodontitis.

References

1. Kornman KS, Crane A, Wang H-Y, et al. The interleukin-1 genotype as a severity factor in adult periodontal disease. J Clin Periodontol 1997; 24:72-7.
2. Masada MP, Persson R, Kenney JS, Lee SW, Page RC, Allison AC. Measurement of interleukin-1α and -1β in gingival crevicular fluid: implications for the pathogenesis of periodontal disease. J Periodont Res 1990;25:156-63.
3. Yavuzyilmaz E, Yamalik N, Bulut S, Özen S, Ersoy F, Saatçi Ü. The gingival crevicular fluid interleukin-1β and tumor necrosis factor-α levels in patients with rapidly progressive periodontitis. Aust Dent J 1995;40:46-9.

4. Pociot F, Molvig J, Wogensen L, Worsaae H, Nerup J. A T*aq*I polymorphism in the human interleukin-1 beta (IL-1β) gene correlates with secretions in vitro. Eur J Clin Invest 1992;22:396-402.

5. Scarel-Caminaga RM, Trevilatto PC, Souza AP, Brito Jr. RB, Line SRP. Investigation of an IL-2 polymorphism in patients with different levels of chronic periodontitis. J Clin Periodontol 2002; 07, *in press*

6. Trevilatto PC, Line SRP. Use of buccal epithelial cells for PCR amplification of large DNA fragments. J Forensic Odonto-Stomatol 2000;18:6-9.

7. Michalowics BS, Diehl SR, Gunsolley JC, et al. Evidence of a substantial genetic basis for risk adult periodontitis. J Periodontol 2000;71:1699-707.

8. McDowell TL, Symons JA, Ploski R, Fárre O, Duff GW. A genetic association between juvenile rheumatoid arthritis and a novel interleukin-1 alpha polymorphism. Arthritis & Rheum 1995;38:221-2.

9. Gore EA, Sanders JP, Pandey JP, Palesch Y, Galbraith GMP Interleukin-1β^{+3953} allele 2: association with disease status in adult periodontitis. J Clin Periodontol 1998;**25**:781-5.

10. Shirodaria S, Smith J, McKay IJ, Kennet CN, Hugh FJ Polymorphisms in the IL-1A gene are correlated with levels of interleukin-1 alpha protein

in gingival crevicular fluid of teeth with severe periodontal disease. J Dent Res 2000;79:1864-9.

11. Papapanou PN, Neideud A-M, Sandros J, Dahlén G. Interleukin-1 gene polymorphism and periodontal status. J Clin Periodontol 2001;28: 389-96.

12. Galbraith GMP, Hendley TM, Sanders JJ, Palesch Y, Pandey JP Polymorphic cytokine genotypes as markers of disease severity in adult periodontitis. J Clin Periodontol 1999;26:705-9.

13. McDewitt MJ, Wang H–Y, Knobelman C, et al. Interleukin-1 genetic association with periodontitis in clinical practice. J Periodontol 2000;71:156-63.

14. Buchs N, di Giovine FS, Silvestri T, Vannier E, Duff GW, Miossec P. IL-1B and IL-1Ra gene polymorphisms and disease severity in rheumatoid arthritis: interaction with their plasma levels. Genes Immunol 2001;2:222-8.

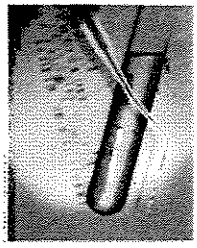
15. Read, R. C., Camp, N. J., di Giovine, F. S., Borrow, R., Kaczmarski, E. B., Chaudhary, A. G., Fox, A. J. & Duff, G.W. (2000) An interleukin-1 genotype is associated with fatal outcome of meningococcal disease. J Infect Dis 2001;182:1557-60.

Table 1. Baseline clinical parameters of the subject population (n=113)

	Healthy (n=44)	**Moderate (n=31)**	**Severe (n=38)**
Age (years)			
Mean (± SD)	43.2 (±14.0)	36.9 (±11.2)	43.6 (±14.4)
Gender %			
Female	68.2	80.6	84.2
Male	31.8	19.4	15.8
Ethnic Group %			
Caucasoid	84.1	77.4	68.4
Afro-American	6.8	16.1	13.2
Mulatto	6.8	6.5	18.4
Japanese	2.3	0.0	0.0

Table 2. Distribution of the *IL-1A* (-889) and *IL-1B* (+3953) alleles (n=226) and genotypes (n=113) in the healthy group and in groups with moderate and severe periodontitis of the whole Brazilian population

	IL-1A (-889)				**IL-1B (+3953)**			
	Genotype- n (%)			*p value*	Genotype - n (%)			*p value*
	1/1	1/2	2/2		1/1	1/2	2/2	
Healthy	20 (45.5)	21 (47.7)	3 (6.8)	0.903	27 (61.5)	15 (34.0)	2 (4.5)	0.663
Moderate	15 (48.5)	14 (45.0)	2 (6.5)		24 (77.5)	6 (19.3)	1 (3.2)	
Severe	20 (52.7)	17 (44.7)	1 (2.6)		27 (71.0)	10 (26.3)	1 (2.7)	
	Allele - n (%)				Allele - n (%)			
	1	2			1	2		
Healthy	61 (69.3)	27 (30.7)		0.715	69 (78.4)	19 (21.6)		0.351
Moderate	44 (71.0)	18 (29.0)			54 (87.0)	8 (13.0)		
Severe	57 (75.0)	19 (25.0)			64 (84.2)	12 (15.8)		



ASSOCIATION BETWEEN IL-1β-511 (C→T) POLYMORPHISM AND CHRONIC PERIODONTITIS IN A BRAZILIAN POPULATION
Scandinavian Journal of Immunology, submitted

Association between IL-1β -511 (C→T) Polymorphism and Chronic Periodontitis in a Brazilian Population

P. C. TREVILATTO,[*] R. M. SCAREL-CAMINAGA,[*] R. B. DE BRITO JR[*], A. P. de SOUZA,[*] A. W. SALLUM[§] & S. R. P. LINE[*]

Departments of [*]Morphology and [§]Periodontology, Dental School of Piracicaba, State University of Campinas, Piracicaba, SP, Brazil

Present address:

Sergio Roberto Peres Line

Faculdade de Odontologia de Piracicaba-UNICAMP

Departamento de Morfologia

Av. Limeira 901, CEP. 13414-018

Piracicaba - SP

Brazil

Tel: +55 (019) 3412-5333

Fax: +55 (019) 3412-5218

E-mail: sergiln@fop.unicamp.br

SHORT TITLE: *IL-1β (-511) polymorphism, periodontitis*

ABSTRACT

Polymorphisms in the interleukin-1 (IL-1) gene cluster have been associated with many types of inflammatory diseases including chronic periodontitis. The aim of this study was to investigate the association between IL-1β (-511) polymorphism and severity of chronic periodontitis in Brazilians. 113 non-smoking subjects over 25 years (mean age 41.6) belonging to different ethnic groups were divided according to the severity level of periodontal disease: 44 healthy individuals, 31 subjects with moderate and 38 with severe periodontitis. DNA was obtained from epithelial cells through a mouthwash with 3% glucose and scraping of oral mucosa. The samples were analyzed for IL-1β (-511) polymorphism using PCR-RFLP. The significance of the differences in the frequencies of the polymorphism in the control and groups with periodontitis was assessed by Chi-square test ($p<0.05$). Significant differences in the allele ($p=0.018$) and genotype ($p=0.051$) frequency were found between control and groups with periodontitis only in the subgroup of Brazilian blacks and mulattos. We concluded that allele 2 of IL-1β (-511) polymorphism was associated with susceptibility to chronic periodontitis in the subpopulation composed of blacks and mulattos, which may suggest a racial component in the determination of the degree of periodontitis severity.

INTRODUCTION

Periodontitis comprises a group of chronic immuno-inflammatory diseases that affect the tissues surrounding teeth and can lead to tooth loss. The etiology of periodontal disease (PD) has been primarily attributed to the interaction of bacterial antigens and inflammatory cells resulting in the production of cytokines [1]. Monocytes are activated by lipopolysaccaharides (LPS) to produce cytokines such as interleukin-1 (IL-1), which may contribute to the destruction of periodontal tissues [2].

Interleukin-1β (IL-1β) is a member of the interleukin-1 family that has a broad spectrum of biological actions. The degradation of type I collagen by fibroblasts when stimulated with IL-1β suggests that connective tissue destruction could be initiated by this cytokine [3]. This molecule also promotes bone resorption by stimulating proliferation, differentiation and activation of osteoclasts [4]. Interleukin-1β has been observed in up to 100% of the gingival crevicular fluid (GCF) from periodontally diseased sites [1, 4-6]. It was postulated that this cytokine could be considered a clinical indicator of the active phase of periodontal disease [1].

Together with age, sex, race, stress and smoking [7-10], genetic polymorphisms seem to be risk factors for the onset and pathogenesis of PD.

Polymorphisms in the IL-1β gene (chromosomal localization: 2q13) have been reported. A single base polymorphism at position -511 (C→T) was described [11]. Allele 2 (T) of the IL-1β -511 gene polymorphism has been associated with certain infectious and immuno-inflammatory diseases, such as asthma [12], meningococcal disease [13], cirrhosis [14], hippocampal sclerosis [15], inflammatory bowel disease [16, 17] and gastric carcinoma [18].

Epidemiological studies indicate that periodontitis is widespread among the Brazilian population [19-23]. Since IL-1β seems to have key roles in the development of chronic periodontitis, in this study we investigated the association between the IL-1β (-511) polymorphism and the severity of chronic periodontitis in a Brazilian population.

MATERIAL AND METHODS

Subject Selection. A convenience sample of 113 unrelated, non-smoking subjects >25 years of age (mean age 41.6 years old), were recruited for study from the patient pool at the Dental Clinics of the Faculty of Dentistry of Piracicaba - UNICAMP (approved by the Ethical Committee in Research at FOP/UNICAMP, protocol 63/99). The patients are from the Southeastern

region of Brazil. The principal ethnic groups that constitute the Southeast Brazilian population [24, 25] were included in this study: 87 Caucasian (white), 13 Afro-American (black), 12 mulatto and 1 Japanese individuals. The baseline clinical parameters for the subject population are presented in table 1. All subjects were in good general health and had at least 20 teeth in the mouth. Subjects did not have any of the following exclusion criteria: diseases of the oral hard or soft tissues except caries and periodontal disease; use of orthodontic appliances; need for pre-medication for dental treatment; chronic usage of anti-inflammatory drugs; a history of diabetes, hepatitis or HIV infection; immunosuppressive chemotherapy; history of any disease known to severely compromise immune function; present acute necrotizing ulcerative gingivitis or current pregnancy or lactation. Subjects completed personal medical and dental history questionnaires, and within a protocol approved by an Institutional Review Board, signed a consent form after being advised of the nature of the study. Diagnosis and classification of disease severity were made on the basis of clinical parameters and consisted of physical examination, medical and dental history, probing depth, assessment of clinical attachment loss (CAL), tooth mobility, gingival recession and observation of bleeding on probing. Measurements of probing depth and

attachment level were recorded at 4 points around each tooth. Subjects were included in clinical categories according to PD severity:

1) *Healthy group*: Subjects found to exhibit no signs of periodontal disease as determined by the absence of clinical attachment loss and no sites with probing depth >3 mm (n = 44),

2) *Moderate Periodontitis*: Patients with tooth sites exhibiting ≥3 mm and <7 mm CAL (n = 31),

3) *Severe Periodontitis*: Patients with teeth exhibiting ≥7 mm CAL (n = 38).

Sampling. The sampling of epithelial buccal cells was performed as described by Trevilatto & Line [26]. Briefly, 113 individuals undertook a mouthwash after 1 min, containing 5 mL 3 % glucose. Following mouthwash, a sterile wood spatula was used to scrape oral mucosa. The tip of the spatula was then shaked into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm for 10 min. The supernatant was discarded and the cell pellet resuspended in 500 μL of extraction buffer [10 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5 % SDS]. The samples were then frozen at -20°C until used for DNA extraction.

DNA Extraction. After defrosted, samples were incubated overnight (ON) with 100 ng/mL proteinase K (Sigma Chemical Co., St. Louis, MO, USA) at 37°C with agitation. DNA was then purified by sequential phenol/chloroform

extraction and salt/ethanol precipitation. DNA was dissolved in 70 μL TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA]. The concentration was estimated by measurements of OD 260.

Polymorphism in the IL-1β gene at position -511 (C→T). The oligonucleotides 5'-TGGCATTGATCTGGTTCATC-3' and 5'-GTTTAGGAATCTTCCCACTT -3' were used as primers. Amplification reactions were performed with 500 ng genomic DNA in a total volume of 50 μL, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 μM of each primer, 200 μM each dATP, dCTP, dGTP and dTTP, 4 mM $MgCl_2$, and 2.5 units T*aq* DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden). Cycling was 2 cycles of 2 min at 95°C, 1 min at 53°C and 1 min at 74°C, followed by 38 cycles of 1 min at 95°C, 1 min at 53°C and 1 min at 74°C. The products were digested with 3 U per 25 μL reaction of A*va*I at 37°C ON to detect allele 1 (C) (114 bp + 190 bp) and allele 2 (T) (304 bp).

Gel electrophoresis. Restriction products were visualized by electrophoresis on vertical 10% non-denaturing polyacrylamide gels in 1X TBE (89 mM Tris-Borate, 89 mM boric acid, 2 mM EDTA), followed by silver staining (Bio-Rad Silver Stain Kit).

Statistical Analysis. The differences in allele and genotype frequency of periodontitis patients and healthy control subjects were analyzed with Chi-

square test. *P*-values less than 0.05 were considered to be statistically significant. The analyses were performed by the program BioEstat [27].

RESULTS

There was no significant difference in the genotype distribution between healthy controls and periodontitis patients on the IL-1β (-511) polymorphism (p=0.433), neither was observed statistical variation in the frequency of the alleles for the polymorphism studied (p=0.522). The distribution of the genotype frequencies for the polymorphism studied in the whole sample is shown in table 2 and the frequencies of the alleles are shown in table 3. Allele 2 was carried by 65.5% (74/113) of the study population, being 14% (16/113) homozygous.

When Caucasians alone were investigated, there were no differences among the groups in the genotype (p=0.716, Table 4) and allele (p=0.955, Table 5) frequencies for the IL-1β (-511) polymorphism. However, there was a significant difference in the genotype (p=0.051, Table 6) and allele (p=0.018, Table 7) frequencies between the healthy group and groups with periodontitis in the subgroup comprising the Afro-Americans (blacks) and mulattos for the IL-1β (-511) locus. For genotypes 1/1 *vs* 1/2 together with 2/2 [odds ratio:

control *vs* moderate/severe disease = 18 (1.37-235.69), p=0.049]. For allele 1 *vs* allele 2 [odds ratio: control *vs* moderate/severe disease = 6.5 (1.48-28.4), p=0.020]. Allele 2 was carried by 84% (21/25) of the whole Afro-American and mulatto individuals, being 32% (8/25) homozygous. The allele 2 carriage rate for the patients with moderate and severe periodontitis was 94.7% (18/19), of whom 42% (8/19) were homozygous.

DISCUSSION

Polymorphisms in certain host-response genes have been related to hyper-secretion of several cytokines upon microbiological challenge [28-30]. Recently, a number of polymorphisms, which quantitatively affect mRNA synthesis, have been identified in the IL-1 gene cluster [11, 28, 31, 32] and their frequency has been associated with several chronic inflammatory diseases [15-17, 32-41]. The allele 2 carriage rate for a polymorphism in the 5^{th} exon of IL-1β gene [28] appears to be related to the incidence and severity of periodontal disease [42-45].

In order to elucidate the genetic background to chronic periodontitis it is useful to investigate associations with genetic polymorphisms of immune response genes, whose products play a role in the inflammatory process. The

aim of this study was to examine IL-1 (-511) polymorphism in unrelated Brazilian patients with several degrees of periodontal disease.

As can be noted in table 1, the majority (77 %) of the individuals belonging to the population studied is of Caucasian heritage. The sample is consistent with the data from the Official Brazilian Census [24, 25], which shows that the population of São Paulo State (Southeast of Brazil) is composed by 71.8% of white subjects. The high number of white individuals can be explained by the predominance of European ancestry in South and Southeast of Brazil [24, 25].

Allele 2 carriage rate for the IL-1β (-511) polymorphism in the white Caucasians was 58% (52/87), being 9% (8/87) of the individuals homozygous. A similar 60% allele 2 carriage rate at IL-1β (-511) locus was found by Kornman *et al.* [42] in Caucasians of north European origin. Studies on other cytokine genes have shown similar allele frequencies in European and Southeastern Brazilian populations, reinforcing a common ancestry [46].

No association was found between chronic periodontitis and IL-1β (-511) polymorphism in the Caucasian subgroup. Kornman *et al.* [42] also failed to detect an association between this polymorphism and chronic periodontitis in Caucasians.

As can be observed in table 1, the percentage of black and mulatto individuals increases in the groups with periodontitis. Besides, the prevalence of black and mulatto subjects is higher in the periodontitis groups than the prevalence of whites (75% *vs* 60%). In the severe disease group the difference is still more evident (50% *vs* 30%). Destructive periodontal diseases and differences in subgingival microbiota and host immune response have been reported disproportionately more prevalent and severe in African-Americans, indicating that risk factors for disease progression may differ for these populations [10]. However, it is not yet clear whether these differences are really genetic or due to confounding variables such as social economic status, poor education or lack of professional dental care [9]. Sometimes genetic and environmental factors can interact to influence aspects of disease. In black subjects, but not in the withes, smoking in patients with chronic periodontitis was associated with IgG1 lower concentration [47].

Allele 2 carriage rate is high in the black and mulatto subgroup (84%) and is still greater if only the diseased patients are taken into account (94.7%). Allele 2 carriage rate was higher in the subgroup of blacks and mulattos than in whites for all status of disease (84% *vs* 60%) and this difference was more expressive in patients with periodontitis (94.7% *vs* 42%). In addition to the higher frequency of allele 2 in the black and mulatto population, this allele

was associated with chronic periodontitis only in this racial group (p=0.018). The present results indicate that blacks and mulattos are at higher risk of chronic periodontitis than their white neighbors when they present allele 2 of IL-1 (-511) polymorphism in their genotypes [OR: 18 (1.37-235.69), p=0.049]. The fact that allele 2 only correlates to disease in the group composed of black and mulatto individuals may suggest a race-related genetic susceptibility to this disease. It could also indicate that additional efforts are needed to identify complex relationships between allele 2 of IL-1 (-511) gene and other inflammatory modifying genes. Our results point at genetic racial components as contributing risk factors for chronic periodontal disease progression. The substantial ethnic variability in the frequency of allele 2 of the IL-1β (-511) gene reinforces the concept of the complexity of the causative mechanisms of chronic periodontitis and the variety of genetic interactions that are likely to determine the diseased phenotype.

It was concluded that allele 2 of the IL-1β (-511) polymorphism was associated with susceptibility to chronic periodontal disease in the Brazilian sub-population of blacks and mulattos studied. This is the first study relating the IL-1β (-511) polymorphism to susceptibility of chronic periodontitis. Although the sample size of blacks and mulattos is small (25 subjects), this

finding may indicate that ethnic and racial differences could interfere with disease-associated polymorphisms.

ACKNOWLEDGEMENTS

This study was supported by FAPESP grants 99/05610-2 and 99/06094-8.

REFERENCES

1 Yavuzyilmaz E, Yamalik N, Bulut S, Özen S, Ersoy F, Saatçi Ü. The gingival crevicular fluid interleukin-1β and tumor necrosis factor-α levels in patients with rapidly progressive periodontitis. Aust Dent J 1995;40:46-9.

2 Havemose-Paulsen A, Holmstrup P. Factors affecting IL-1-mediated collagen metabolism by fibroblasts and the pathogenesis of periodontal disease: a review of the literature. Crit Rev Oral Biol Med 1997;8:217-36.

3 Meikle MC, Atkinson SJ, Ward RV, Murphy G, Reynolds JJ. Gingival fibroblasts degrade type I collagen films when stimulated with tumor necrosis factor and interleukin-1. J Periodont Res 1989;24: 207-13.

4 Wilton JMA, Bampton JLM, Griffiths GS, Curtis MA, Life JS, Johnson NW *et al.* Interleukin-1 beta (IL-1β) levels in gingival crevicular fluid from

adults with previous evidence of destructive periodontitis - a cross sectional study. J Clin Periodontol 1992;19:53-7.

5 Masada MP, Persson R, Kenney JS, Lee SW, Page RC, Allison AC. Measurement of interleukin-1α and -1β in gingival crevicular fluid: implications for the pathogenesis of periodontal disease. J Periodont Res 1990;25:156-63.

6 Gamonal J, Acevedo A, Bascones A, Jorge O, Silva A. Levels of interleukin-1β, -8, and -10 and RANTES in gingival crevicular fluid and cell populations in adult periodontitis patients and the effect of periodontal treatment. J Periodontol 2000;71:1535-45.

7 Haber J. Smoking is a major risk factor for periodontitis. Curr Opin Periodontol Review 1994;12-8.

8 Schenkein HA, Gunsolley JC, Koertge TE, Schenkein J, Tew JG. Smoking and its effects on early-onset periodontitis. J Am Dent Assoc 1995;126:1107-13.

9 Fox CH. New considerations in the prevalence of periodontal disease. Curr Opin Dent 1992;2:5-11.

10 Craig RG, Boylan R, Yip J, Bamgboye P, Koutsoukos J, Mijares D *et al.* Prevalence and risk indicators for destructive periodontal diseases in 3 urban American minority populations. J Clin Periodontol 2001;28:524-35.

11 Di Giovine FS, Takhsh E, Blackmore AL, Duff GW. Single base polymorphism at -511 in the human interleukin-1 beta gene (IL-1β). Hum Mol Genet 1992;1:450.

12 Karjalainen J, Nieminen MM, Aromaa A, Klaukka T, Hurme M. The IL-1beta genotype carries asthma susceptibility only in men. J Allergy Clin Immunol 2002;109:514-6.

13 Read RC, Camp NJ, di Giovine FS, Borrow R, Kaczmarski EB, Chaudhary AG *et al*. An interleukin-1 genotype is associated with fatal outcome of meningococcal disease. J Infect Dis 2000;182:1557-60

14 Takamatsu M, Yamauchi M, Maezawa Y, Saito S, Maeyama S, Uchikoshi T. Genetic polymorphisms of interleukin-1 beta in association with the development of alcoholic liver disease in Japanese patients. Am J Gastroenterol 2000;95:1305-11.

15 Kanemoto K, Kawasaki J, Miyamoto T, Obayashi H, Nishimura M. Interleukin (IL) 1 beta, IL-1 alpha, and IL-1 receptor antagonist gene polymorphisms in patients with temporal lobe epilepsy. Ann Neurol 2000;47:571-4.

16 Nemetz A, Nosti-Escanilla MP, Molnar T, Kope A, Kovacs A, Feher J *et al*. IL1B gene polymorphisms influence the course and severity of inflammatory bowel disease. Immunogenetics 1999;49:527-31.

17 Heresbach D, Alizadeh M, Dabadie A, Le Berre N, Colombel JF, Yaouanq J *et al.* Significance of interleukin-1 beta and interleukin-1 receptor antagonist genetic polymorphism in inflammatory bowel diseases. Am J Gastroenterol 1997;92:1164-9.

18 Machado JC, Pharoah P, Sousa S, Carvalho R, Oliveira C, Figueiredo C *et al.* Interleukin 1B and interleukin 1RN polymorphisms are associated with increased risk of gastric carcinoma. Gastroenterology 2001;121:823-9

19 Gjermo P, Bellini HT, Marcos B. Application of the community periodontal index of treatment needs (CPITN) in a population of young Brazilians. Comm Dent Oral Epidemiol 1983;11:342-6.

20 Gjermo P, Bellini HT, Pereira Santos V, Martins G, Ferracyoli JR. Prevalence of bone loss in a group of Brazilian teenagers assessed on bite-wing radiographs. J Clin Periodontol 1984;11:104-13.

21 Pecora JD, da Cruz Filho AM. Study of the incidence of radicular grooves in maxillary incisors. Braz Dent J 1992;3:11-6.

22 Dini EL, Guimaraes LO. Periodontal conditions and treatment needs (CPITN) in a worker population in Araraquara, SP, Brazil. Int Dent J 1994; 44:309-11.

23 Tinoco EM, Beldi MI, Loureiro CA, Lana M, Campedelli F, Tinoco NM *et al.* Localized juvenile periodontitis and Actinobacillus

actinomycetemcomitans in a Brazilian population. Eur J Oral Sci 1997;105:9-14.

24 IBGE - Instituto Brasileiro de Geografia e Estatística, Brasil: 500 anos de povoamento. IBGE, Rio de Janeiro, 2000.

25 IBGE - Instituto Brasileiro de Geografia e Estatística, *http://www.ibge.gov.br*

26 Trevilatto PC, Line SRP. Use of buccal epithelial cells for PCR amplification of large DNA fragments. J Forensic Odontostomatol 2000;18:6-9.

27 Ayres M, Ayres M Jr. BioEstat: aplicações estatísticas nas áreas das ciências biológicas e médicas. Manaus: Sociedade Civil Mamirauá, 1998.

28 Pociot F, Molvig J, Wogensen L, Worsaae H, Nerup J. A Taq I polymorphism in the human interleukin-1 beta (IL-1β) gene correlates with secretions in vitro. Eur J Clin Invest 1992;22:396-402.

29 Wilson AG, Symons JA, McDowell TL, McDewitt HO, Duff GW. Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. Proc Natl Acad Sci USA 1997;94: 3195-9.

30 Shirodaria S, Smith J, McKay IJ, Kennet CN, Hugh FJ. Polymorphisms in the IL-1A gene are correlated with levels of interleukin-1 alpha protein in gingival crevicular fluid of teeth with severe periodontal disease. J Dent Res 2000;79:1864-9.

31 Tarlow JK, Blakemore AIF, Lennard A, Solari R, Steinkasserer A, Duff GW. Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-bp tandem repeat. Hum Genet 1993;91:403-4.

32 McDowell TL, Symons JA, Ploski R, Fárre O, Duff GW. A genetic association between juvenile rheumatoid arthritis and a novel interleukin-1 alpha polymorphism. Arthritis Rheum 1995;38:221-2.

33 Griffin WS, Nicoll JA, Grimaldi LM, Sheng JG, Mrak RE. The pervasiveness of interleukin-1 in Alzheimer disease pathogenesis: a role for specific polymorphisms in disease risk. Exp Gerontol 2000;35:481-7.

34 Kantarci OH, Atkinson EJ, Hebrink DD, McMurray CT, Weinshenker BJ. Association of two variants in IL-1 beta and receptor antagonist genes with multiple sclerosis. J Neuroimmunol 2000;106:220-7.

35 Rebeck GW. Confirmation of the genetic association of interleukin-1A with early onset sporadic Alzheimer's disease. Neurosci Lett 2000;293:75-7.

36 Shu KH, Lee SH, Cheng CH, Wu MJ, Lian JD. Impact of interleukin-1 receptor antagonist and tumor necrosis factor-alpha gene polymorphism IgA nephropathy. Kidney Int 2000;58:783-9.

37 Buchs N, di Giovine FS, Silvestri T, Vannier E, Duff GW, Miossec P. IL-1B and IL-1Ra gene polymorphisms and disease severity in rheumatoid arthritis: interaction with their plasma levels. Genes Immunol 2001;2:222-8.

38 Donaldson P, Agarwal K, Craggs A, Craig W, James O, Jones O. HLA and interleukin 1 gene polymorphisms in primary biliary cirrhosis: associations with disease progression and disease susceptibility. Gut 2001;48:397-402.

39 Kolsch H, Ptok U, Bagli M, Papassotiropoulos A, Schmitz S, Barkow K, Kockler M, Rao ML, Maier W, Heun R. Gene polymorphims of interleukin-1 alpha influence the course of Alzheimer's disease. Ann Neurol 2001;49:818-9.

40 Nishimura M, Kawakami H, Maruyama H, Izumi Y, Kuno S, Kaji R, Nakamura S. Influence of interleukin-1 beta gene polymorphism on age-at-onset of spinocerebellar ataxia 6 (SCA6) in Japanese patients. Neurosci Lett 2001;307:128-30.

41 Yucesoy B, Vallyathan V, Landsittel DP, Sharp DS, Weston A, Burleson GR *et al.* Association of tumor necrosis factor-alpha and interleukin-1 gene polymorphisms with silicosis. Toxicol Appl Pharm 2001;172:75-82.

42 Kornman KS, Crane A, Wang H-Y, Di Giovine FS, Newman MG, Pirk F W *et al.* The interleukin-1 genotype as a severity factor in adult periodontal disease. J Clin Periodontol 1997;24:72-7.

43 Gore EA, Sanders JP, Pandey JP, Palesch Y, Galbraith GMP. Interleukin-$1\beta^{+3953}$ allele 2: association with disease status in adult periodontitis. J Clin Periodontol 1998;25:781-5.

44 Galbraith GMP, Hendley TM, Sanders JJ, Palesch Y, Pandey JP. Polymorphic cytokine genotypes as markers of disease severity in adult periodontitis. J Clin Periodontol 1999;26:705-9.

45 Papapanou PN, Neideud AM, Sandros J, Dahlén G. Interleukin-1 gene polymorphism and periodontal status. J Clin Periodontol 2001;28:389-96.

46 Scarel-Caminaga RM, Trevilatto PC, Souza AP, Brito Jr RB, Line SRP. Frequencies of the –330 (T→G) *IL2* and –590 (T→C) *IL-4* gene polymorphism in a South-eastern Brazilian population. Eur J Immunogenet 2000, *in press*.

47 Gunsolley JC, Pandey JP, Quinn SM, Tew J, Schenkein HA. The effect of race, smoking and immunoglobulin allotypes on IgG subclass concentrations. J Periodontal Res 1997;32:381-7.

Table 1. Baseline clinical parameters of the subject population (n=113)

	Healthy (n=44)	Moderate (n=31)	Severe (n=38)	Total (n=113)
Age (years)				
Mean (± SD)	43.2 (±14.0)	36.9 (±11.2)	43.6 (±14.4)	41.6 (±12.4)
Gender %				
Female	68.2	80.6	84.2	78
Male	31.8	19.4	15.8	22
Ethnic Group %				
Caucasoid	84.1	77.4	68.4	77.2
Afro-American	6.8	16.1	13.2	11.5
Mulatto	6.8	6.5	18.4	10.5
Japanese	2.3	0.0	0.0	0.8

Table 2. Distribution of the IL-1β (-511) genotypes in the healthy group and in groups with moderate and severe chronic periodontitis in the whole Brazilian population

Genotype	Healthy		Moderate		Severe		*p* value
	n	(%)	n	(%)	n	(%)	
1/1	17	(38.7)	11	(35.5)	11	(29.0)	0.433
1/2	23	(52.3)	13	(42.0)	22	(58.0)	
2/2	4	(9.0)	7	(22.5)	5	(13.0)	

Table 3. Distribution of the IL-1β (-511) alleles in healthy, moderate and severe groups with chronic periodontitis in the whole Brazilian population

Allele	Healthy		Moderate		Severe		*p* value
	n	(%)	n	(%)	n	(%)	
1	57	(64.8)	35	(56.5)	44	(57.9)	0.522
2	31	(35.2)	27	(43.5)	32	(42.1)	

Table 4. Distribution of the IL-1β (-511) genotypes in the healthy group and in groups with moderate and severe chronic periodontitis in the Caucasian Brazilian population

Genotype	Healthy		Moderate		Severe		*p* value
	n	(%)	n	(%)	n	(%)	
1/1	14	(37.8)	11	(46.0)	10	(38.5)	0.7157
1/2	20	(54.0)	10	(41.5)	15	(57.5)	
2/2	3	(8.2)	3	(12.5)	1	(4.0)	

Table 5. Distribution of the IL-1β (-511) alleles in healthy, moderate and severe groups with chronic periodontitis in the Caucasian Brazilian population

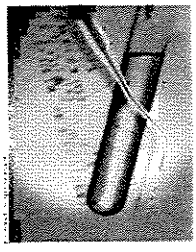
Allele	Healthy		Moderate		Severe		*p* value
	n	(%)	n	(%)	n	(%)	
1	48	(64.8)	32	(66.5)	35	(67.5)	0.9555
2	26	(35.2)	16	(33.5)	17	(32.5)	

Table 6. Distribution of the IL-1β (-511) genotypes in the healthy group and in groups with moderate and severe chronic periodontitis in the subgroup composed of Brazilian black and mulatto individuals

Genotype	Healthy		Moderate		Severe		*p* value
	n	(%)	n	(%)	n	(%)	
1/1	3	(50.0)	0	(0.0)	1	(8.3)	0.0515
1/2	3	(50.0)	3	(43.0)	7	(58.3)	
2/2	0	(0.0)	4	(57.0)	4	(33.4)	

Table 7. Distribution of the IL-1β (-511) alleles in healthy, moderate and severe groups with chronic periodontitis in the Brazilian blacks and mulattos

Allele	Healthy		Moderate		Severe		*p* value
	n	(%)	n	(%)	n	(%)	
1	9	(75.0)	3	(21.5)	9	(37.5)	0.0184
2	3	(25.0)	11	(78.5)	15	(62.5)	



INTERLEUKIN-1 RECEPTOR ANTAGONIST (IL-1RA) GENE POLYMORPHISM IS ASSOCIATED WITH THE SEVERITY OF CHRONIC PERIODONTITIS IN A BRAZILIAN POPULATION

Cytokine, submitted

Interleukin-1 receptor antagonist (IL-1ra) gene polymorphism is associated with the severity of chronic periodontitis in a Brazilian population

Paula C. Trevilatto[1], Rui B. de Brito Jr[1], Raquel M. Scarel-Caminaga[1], Ana P. de Souza[1], Antonio W. Sallum[2], Sergio R. P. Line[1]

From the Departments of [1]Morphology and [2]Periodontology, Dental School of Piracicaba, State University of Campinas, Piracicaba, SP, Brazil

KEY WORDS: chronic periodontitis/IL-1ra/polymorphism

Correspondence to: Sergio Roberto Peres Line

Fax: +55 (019) 3412-5218

Tel: +55 (19) 3412-5333

Faculdade de Odontologia de Piracicaba-UNICAMP

Departamento de Morfologia

Av. Limeira 901, CEP. 13414-018

Piracicaba - SP

Brazil

E-mail: sergiln@fop.unicamp.br

Short title: *IL-1ra and chronic periodontitis*

ABSTRACT

Inflammatory cytokines represent important pathological mediators in chronic inflammatory diseases. Interleukin-1 receptor antagonist (IL-1ra) regulates interleukin-1 (IL-1) activity in inflammatory disorders, including chronic periodontitis. Polymorphism in the IL-1ra gene was associated with enhanced production of IL-1ra protein. This study aimed to investigate the association between IL-1ra polymorphism and severity of chronic periodontitis in Brazilians. 113 non-smoking subjects over 25 years (mean age 41.6) were divided according to the severity level of periodontal disease: 44 healthy individuals, 31 subjects with moderate and 38 with severe periodontitis. Genomic DNA was obtained through a mouthwash with 3 % glucose and scraping of oral mucosa. Polymerase chain reaction (PCR) amplification was used to identify the IL-1 receptor antagonist intron 2 variable number of tandem repeat (VNTR) polymorphism. The significance of the differences in the genotype and allele frequencies of the polymorphism in the healthy, moderate and severe groups with periodontitis was assessed by Chi-square test ($p<0.05$). Significant differences in the genotype frequencies of the polymorphism were found between control/moderate and severe periodontitis groups ($p=0.029$). It was concluded that IL-1ra intron 2 polymorphism was associated with the severity of chronic periodontal disease in the Brazilian population.

Periodontitis is a complex multifactorial condition involving multiple genes and environmental factors. Approximately half of the variance in periodontal disease seems to be attributed to genetic factors.[1] Inflammatory mediators, such as interleukin-1 (IL-1) and IL-1 receptor antagonist (IL-1ra), play a key role in the pathogenesis of several inflammatory disorders, including chronic periodontitis.[2,3] Interleukin-1 receptor antagonist regulates IL-1β activity by inhibiting its competitive binding to the IL-1β receptor, thus suppressing the IL-1β induction of cytokines.[4] Some cytokine gene polymorphisms may contribute to inter-individual variation in cytokine production upon microbial challenge[5-7]. The polymorphism in human IL-1ra gene intron 2 is caused by variable number of an 86-bp tandem repeat.[8] The number of repeats at the polymorphism site may be of functional significance because the repeated sequence contains possible binding sites for transcription factors.[8] Allele 2 was reported to be associated with enhanced production of IL-1ra protein.[9] Recently, the IL-1ra gene allele 2 was reported to be associated with inflammatory bowel disease,[10] rheumatoid arthritis,[11] nephropathy,[12] polymialgia rheumatica,[13] alopecia areata,[14] lichen sclerosus,[15] multiple sclerosis,[16] systemic lupus erythematosus,[17] Sjögren's syndrome[18] and myasthenia gravis.[19]

Since periodontitis is a chronic inflammatory disease, this study aimed to investigate the association between the IL-1ra (intron 2) polymorphism and chronic periodontitis in Southeast Brazilians.

RESULTS

Fifty-four percent of the individuals studied (61/113) had the A1A1 genotype and 33.6% (38/113) had A1A2 type; only 3.5% (4/113) A2A2 type individuals were included; 0.9% (1/113) was of A1A3 type; 0.9% (1/113) of A2A3 genotype; 5.3% (6/113) were of A1A4, and 1.8% (2/113) were of the A2A4 type. There was no significant difference in the genotype distribution between healthy controls and periodontitis patients at the study polymorphism (p=0.1536) (Table 2). But, when the groups comprising control healthy individuals and subjects with moderate periodontitis were analyzed together against the severe periodontitis group, we found a statistically significant association of the genotype A2A2 (genotype frequency 10.5% *vs* 0%, Table 2) of the IL-1ra polymorphism with severe disease (p=0.029). There was no statistical variation in the frequencies of the alleles between controls and groups with periodontal disease (p=0.799) (Table 3). The allele frequencies in the study population were: allele 1, 73.9%; allele 2, 21.7%; allele 3, 0.9%; allele 4, 3.5% and allele 5, 0% (Table 4). The corresponding carriage rates were: 94%, 37%, 1.7%, 7% and 0%, respectively. Allele 2 carriage rate at

IL-1ra polymorphism was not different (37%) when only the diseased patients were taken in account, and when severely impaired patients alone were analyzed (37%).

DISCUSSION

Interleukin-1β and its endogenous antagonist, interleukin-1 receptor antagonist, play important roles in immune responses. interleukin-1 receptor antagonist inhibits IL-1β activity competitive binding to the IL-1β receptor, thus suppressing the IL-1β induction of cytokine.[4] It may therefore play a crucial role in many IL-1 mediated diseases, acting as an important endogenous regulator of inflammation. Polymorphism in the IL-1ra gene may account for variation in the production of IL-1ra and IL-1β proteins.[9,20] Interleukin-1β and IL-1ra may function as immunomodulators of chronic periodontitis, [21] so that allelic variation in those genes might be expected to have an impact on periodontal disease. Allele 2 of IL-1ra polymorphism has been associated with many chronic inflammatory diseases, but studies on its implication in periodontal disease are rare, if they so far exist. The allele frequencies in the study population were very similar to that obtained by Tarlow *et al.* (1993) [8] (Table 4). The allele 2 frequency obtained in the present study was 21.7%. The following similar frequencies for allele 2 were reported: 21.4%[8] and 27%[18]. Allele 2 was carried by 37% of the study population and by 36% of

Caucasian controls of northern European ancestry.[18] A study on the relationship between the IL-1ra polymorphism and periodontitis in Caucasians found an allele 2 carriage rate of 47%,[22] but it can be much rarer in other population groups.[4,12] Studies have demonstrated a significant increase in both the frequency and carriage rate of allele 2 in several diseases.[17,18]

In the study population, the allele 2 (homozygous genotype) was related to risk of severity for chronic periodontitis. It was concluded that IL-1ra intron 2 polymorphism is associated with the severity of, but not with susceptibility to, chronic periodontal disease in the Brazilian population studied. Although IL-1ra represents an anti-inflammatory cytokine, with allele 2 increasing the production of the corresponding protein, these results implicate the potential involvement of both pro- and anti-inflammatory cytokines in the regulation of chronic periodontitis. The paradox of the association of allele 2 with inflammatory diseases, and with increased production of IL-1ra, perhaps resides in the fact that beyond being anti-inflammatory, it stimulates IL-1β production, which, at a time, is a potent pro-inflammatory mediator. Another point of consideration is that A2 effect on local and systemic IL-1ra production may be distinct. That can be illustrated by the fact that lower salivary levels of IL-1ra could be detected in patients with severe Sjögren's Syndrome, in contrast with the increase of this protein in the patients serum, suggesting specific regulation in different compartments.[18,23]

MATERIAL AND METHODS

Subject selection

A convenience sample of 113 unrelated, non-smoking subjects >25 years of age, were recruited for study from the patient pool at the Dental Clinics of the Faculty of Dentistry of Piracicaba – UNICAMP (approved by the Ethical Committee in Research at FOP/UNICAMP, protocol 63/99). The patients are from the Southeastern region of Brazil. The baseline clinical parameters for the subject population are presented in table 1. All subjects were in good general health and had at least 20 teeth in the mouth. Subjects did not have any of the following exclusion criteria: diseases of the oral hard or soft tissues except caries and periodontal disease; use of orthodontic appliances; need for pre-medication for dental treatment; chronic usage of anti-inflammatory drugs; a history of diabetes, hepatitis or HIV infection; immunosuppressive chemotherapy; history of any disease known to severely compromise immune function; present acute necrotizing ulcerative gingivitis or current pregnancy or lactation. Subjects completed personal medical and dental history questionnaires, and within a protocol approved by an Institutional Review Board, signed a consent form after being advised of the nature of the study.

Diagnosis and classification of disease severity were made on the basis of clinical parameters and consisted of physical examination, medical and dental

history, probing depth, assessment of clinical attachment loss (CAL), tooth mobility, gingival recession and observation of bleeding on probing. Measurements of probing depth and attachment level were recorded at 6 points around each tooth. Subjects were included in clinical categories according to periodontal disease severity:

1) *Healthy group*: Subjects found to exhibit no signs of periodontal disease as determined by the absence of clinical attachment loss and no sites with probing depth >3 mm (n = 44),
2) *Moderate Periodontitis*: Patients with tooth sites exhibiting ≥3 mm and <7 mm CAL (n = 31),
3) *Severe Periodontitis*: Patients with teeth exhibiting ≥7 mm CAL (n = 38).

Sampling

The sampling of epithelial buccal cells was performed as described by Trevilatto & Line (2000).[24] Briefly, 113 individuals undertook a mouthwash after 1 min, containing 5 ml 3% glucose. Following mouthwash, a sterile wood spatula was used to scrape oral mucosa. The tip of the spatula was then shaked into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm for 10 min. The supernatant was discarded and the cell pellet resuspended in

500 µl of extraction buffer [10 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5% SDS]. The samples were then frozen at -20°C until used for DNA extraction.

DNA extraction

After defrosted, samples were incubated overnight (ON) with 100 ng/mL proteinase K (Sigma Chemical Co., St. Louis, MO, USA) at 37°C with agitation. DNA was then purified by sequential phenol/chloroform extraction and salt/ethanol precipitation. DNA was dissolved in 70 µL TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA]. The concentration was estimated by measurements of OD 260.

Polymerase chain reaction (PCR) and variable number of tandem repeat (VNTR)

Polymorphism in the IL-1ra (intron 2) gene

The oligonucleotides 5'-CTCAGCAACACTCCTAT-3' and 5'-TCCTGGTCTGCAGGTAA-3' were used as primers. Amplification reactions were performed with 500 ng genomic DNA in a total volume of 50 µL, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 µM of each primer, 200 µM each dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, and 2.5 units T*aq* DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden). The reaction was incubated for 1 min at 96°C, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C and 2 min at 70°C, and additional 5 min at 70°C and 55°C for 5 min. Amplifications were

carried out in a *Perkin-Elmer GeneAmp 2400* thermal cycler. In the second intron of IL-1ra gene, five alleles are defined by different numbers of a 86-bp segment repeat. Genotypes were determined by comparing the size of the bands with a molecular weigh ladder, with separation into allele 1 (4 repeats - 412 bp), allele 2 (2 repeats - 240 bp), allele 3 (3 repeats - 326 bp), allele 4 (5 repeats - 498 bp) and allele 5 (6 repeats - 584 bp).[22]

Gel electrophoresis

Restriction products were visualized by electrophoresis on vertical 10% non-denaturing polyacrylamide gels in 1X TBE (89 mM Tris-Borate, 89 mM boric acid, 2 mM EDTA), followed by silver staining (Bio-Rad Silver Stain Kit).

Statistical Analysis

The allele ratio and genotype distribution of periodontitis patients and healthy control subjects were analyzed with Chi-square test. A p-value <0.05 was considered significant.

Acknowledgements

This study was supported by FAPESP grants 99/05610-2 and 99/06094-8.

REFERENCES

1. Michalowics BS, Diehl SR, Gunsolley JC, Sparks BS, Brooks CN, Koertge TE, Califano JV, Burmeister JA, Schenkein HA (2000) Evidence of a substantial genetic basis for risk adult periodontitis. J Periodontol 71:1699-1707.

2. Offenbacher S (1996) Periodontal diseases: Pathogenesis. Ann Periodontol 1:821-878.

3. Roberts FA, Hockett RD Jr, Bucy RP, Michalek, SM (1997) Quantitative assessment of inflammatory cytokine gene expression in chronic adult periodontitis. Oral Microbiol Immunol 12:336-344.

4. Niimi T, Sato S, Tomita H, Yamada K, Akita K, Maeda H., Kawagushi H, Sugiura Y, Ueda R (2000) Lack of association with interleukin 1 receptor antagonist and interleukin-1β gene polymorphisms in sarcoidosis patients. Respir Med 94:1038-1042.

5. Pociot F, Molvig J, Wogensen L, Worsaae H, Nerup J (1992) A Taq I polymorphism in the human interleukin-1 beta (IL-1β) gene correlates with secretions in vitro. Eur J Clin Invest 22:396-402.

6. Wilson AG, Symons JA, McDowell TL, McDewitt HO, Duff GW (1997) Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. Proc Natl Acad Sci USA 94:3195-3199.

7. Shirodaria S, Smith J, McKay IJ, Kennett CN, Hughes FJ (2000) Polymorphisms in the IL-1A gene correlated with levels of interleukin-1α protein in gingival crevicular fluid of teeth with severe periodontal disease. J Dent Res 79:1864-1869.

8. Tarlow JK, Blakemore AIF, Lennard A, Solari R, Steinkasserer A, Duff GW (1993) Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-bp tandem repeat. Hum Genet 91:403-404.

9. Danis VA, Millington M, Hyland VJ, Grennan D (1995) Cytokine production by normal human monocytes: inter-subject variation and relationship to an IL-1 receptor-antagonist (IL-1Ra) gene polymorphism. Clin Exp Immunol 99:303-310.

10. Mansfield JC, Holden H, Tarlow JK, Di Giovine F, McDowell TL, Wilson AG, Holdsworth D, Duff GW (1994) Novel genetic association between ulcerative colitis and the anti-inflammatory cytokine interleukin-1 receptor antagonist. Gastroenterology 106:637-642.

11. Buchs N, di Giovine FS, Silvestr T, Vannier E, Duff GW, Miossec P (2001) IL-1B and IL-1Ra gene polymorphisms and disease severity in rheumatoid arthritis: interaction with their plasma levels. Genes Immunol 2: 222-228.

12. Shu KH, Lee SH, Cheng CH, Wu MJ, Lian JD (2000) Impact of interleukin-1 receptor antagonist and tumor necrosis factor-alpha gene polymorphism IgA nephropathy. Kidney Int 58:783-789.

13. Boiardi L, Salvarani C, Timms JM, Silvestri T, Macchioni PL, di Giovine FS (2000) Interleukin-1 cluster and tumor necrosis factor alpha gene polymorphisms in polymyalgia rheumatica. Clin Exp Rheumatol 18:675-681.

14. Tarlow JK, Clay FE, Cork MJ, Blakemore AIF, McDonagh AJG, Messenger AG, Duff GW (1994) Severity of alopecia areata is associated with a polymorphism in the interleukin-1 receptor antagonist gene. J Invest Dermatol 103:387-390.

15. Clay FE, Cork MJ, Tarlow JK, Blakemore AI, Harrington CI, Lewis F, Duff GW (1994) Interleukin-1 receptor antagonist gene polymorphism association with lichen sclerosus. Hum Genet 94:407-410.

16. Crusius JB, Pena AS, Van Oosten BW, Bioque G, Garcia A, Dijkstra CD, Polman CH (1995) Interleukin-1 receptor antagonist gene polymorphism and multiple sclerosis. Lancet 346:979.

17. Blakemore AIF, Tarlow JK, Cork MJ, Gordon C, Emery P, Duff GW (1994) Interleukin-1 receptor antagonist gene polymorphism as diseased severity factor in systemic lupus erythematosus. Arthritis Rheum 37:1380-1385.

18. Perrier S, Coussediere C, Dubost, JJ, Albuisson E, Sauvezie B (1998) IL-1 receptor antagonist (IL-1RA) gene polymorphism in Sjogren's syndrome and rheumatoid arthritis. Clin Immunol Immunopathol 87:309-313.

19. Huang D, Pirskanen R, Hjelmstrom P, Lefvert AK (1998) Polymorphisms in IL-1beta and IL-1 receptor antagonist genes are associated with myasthenia gravis. J Neuroimmunol. 81:76-81.

20. Santilla S, Savinainen K, Hurme M (1998) Presence of the IL-1RA allele 2 (IL-1RN*2) is associated with enhanced IL-1 beta production in vitro. Scand J Immunol 47:195-198.

21. Roberts FA, McCaffery KA, Michale SM (1997) Profile of cytokine mRNA expression in chronic adult periodontitis. J Dent Res 76:1833-1839.

22. Kornman KS, Crane A, Wang H-Y, di Giovine FS, Newman MG, Pirk FW, Wilson Jr. TG, Higginbottom FL, Duff GW (1997) The interleukin-1 genotype as a severity factor in adult periodontal disease. J Clin Periodontol 24:72-77.

23. Dubost JJ, Perrier S, Afane M, Viallard JL, Roux-Lombard P, Baudet-Pommel M, Begue C, Kemeny JL, Sauvezie B (1996) IL-1 receptor antagonist in saliva: Characterization in saliva and reduced concentrations in Sjogren's syndrome (SS). Clin Exp Immunol 106:237-242.

24. Trevilatto PC, Line SRP (2000) Use of buccal epithelial cells for PCR amplification of large DNA fragments. J Forensic Odontostomatol 18:6-9.

TABLE 1. Baseline clinical parameters of the subject population (n=113)

	Healthy (n=44)	Moderate (n=31)	Severe (n=38)
Age (years)			
Mean (± SD)	43.2 (±14.0)	36.9 (±11.2)	43.6 (±14.4)
Gender %			
Female	68.2	80.6	84.2
Male	31.8	19.4	15.8
Ethnic Group %			
Caucasoid	84.1	77.4	68.4
Afro-American	6.8	16.1	13.2
Mulatto	6.8	6.5	18.4
Japanese	2.3	0.0	0.0

TABLE 2. Distribution of the IL-1ra genotypes in the healthy group and in groups with moderate and severe chronic periodontitis

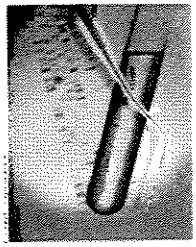
	Healthy		Moderate		Severe		*p* value
Genotype	n	(%)	n	(%)	n	(%)	
1/1	25	(56.8)	15	(48.4)	21	(55.2)	0.1536
1/2	16	(36.4)	14	(45.2)	8	(21)	
2/2	0	(0)	0	(0)	4	(10.5)	
1/3	1	(2.3)	0	(0)	0	(0)	
2/3	0	(0)	0	(0)	1	(2.7)	
1/4	2	(4.5)	1	(3.2)	3	(7.9)	
2/4	0	(0)	1	(3.2)	1	(2.7)	

TABLE 3. Distribution of the IL-1ra alleles in healthy, moderate and severe groups with chronic periodontitis

	Healthy		Moderate		Severe		*p* value
Allele	n	(%)	n	(%)	n	(%)	
1	69	(78.4)	45	(72.6)	53	(69.7)	0.799
2	16	(18.2)	15	(24.2)	18	(23.7)	
3	1	(1.1)	0	(0)	1	(1.3)	
4	2	(2.3)	2	(3.2)	4	(5.3)	
5	0	(0)	0	(0)	0	(0)	

TABLE 4. Frequencies (%) of the IL-1ra alleles in the Brazilian population* and in the population studied by Tarlow et al. (1993)[8]**

Allele	*	**	*p* value
1	73.9	73.6	0.1213
2	21.7	21.4	
3	0.9	0.7	
4	3.5	3.6	
5	0	0.7	



POLYMORPHISM AT POSITION -174 OF IL-6 GENE IS ASSOCIATED WITH SUSCEPTIBILITY TO CHRONIC PERIODONTITIS IN A CAUCASIAN BRAZILIAN POPULATION
Journal of Clinical Periodontology
v.5, 2003

Polymorphism at position -174 of IL-6 gene is associated with susceptibility to chronic periodontitis in a Caucasian Brazilian population

P. C. Trevilatto, R. M. Scarel-Caminaga, R. B. de Brito Jr, A. P. de Souza and S. R. P. Line

Department of Morphology, Dental School of Piracicaba, State University of Campinas, Piracicaba, SP, Brazil

Short title: *IL-6 polymorphism and chronic periodontitis*

Abstract

Background: Interleukin-6 (IL-6) is a multifunctional cytokine that mediates inflammatory tissue destruction. A G to C substitution at position -174 in the promoter of IL-6 influences in vitro transcription of IL-6. This polymorphism has been associated with inflammatory diseases like chronic arthritis.

Objective: The aim of this study was to investigate the association between the IL-6^{-174} polymorphism and susceptibility to chronic periodontitis in Brazilians.

Material and Methods: 84 non-smoking subjects over 25 years (mean age 42.4) were divided according to the severity level of periodontal disease: 37 healthy individuals (control group), 24 subjects with moderate and 24 with severe periodontitis. Genomic DNA was obtained from epithelial cells through a mouthwash with 3 % glucose and scraping of oral mucosa. The samples were analyzed for IL-6^{-174} polymorphism using PCR-RFLP. The significance of the differences in the frequencies of the polymorphism in the control and groups with periodontitis was assessed by Chi-square test ($p<0.05$).

Results: Differences were found between control and groups with periodontitis in the genotype ($p=0.0036$, OR=3.0) and in the allele ($p=0.0838$, OR=1.9) frequencies.

Conclusion: We concluded that the IL-6^{-174} polymorphism is associated with susceptibility to chronic periodontitis in the population studied.

Key words: chronic periodontitis; interleukin-6, polymorphism

Interleukin-6 (IL-6) plays a role in B-cell differentiation and T cell proliferation (Lotz et al 1988). It also stimulates hematopoiesis (Revel 1989) and accelerates bone resorption (Ishimi et al 1990). High levels of IL-6 in biological fluids and blood have been determined in infections, trauma, chronic inflammatory diseases and neoplasia (Hirano et al. 1990). It is involved in the pathogenesis of several inflammatory diseases such as rheumatoid arthritis (Hirano et al 1988, Houssiau et al. 1988) and psoriasis (Grossman et al 1989), therefore constituting a major mediator of the host response to injury and infection.

Higher levels of IL-6 were found in sites with gingivitis when compared to healthy sites (Kono et al. 1991, Matsuki et al. 1992, Takahashi et al 1994, Atilla & Kütükçüler 1998). Significantly higher expression of IL-6 mRNA was reported in diseased tissues when compared to healthy ones in periodontitis patients (Prabhu et al. 1996). Interleukin-6 levels were higher in culture supernatants of gingival tissues from gingivitis and periodontitis patients than in healthy control patients (Kamagata et al. 1989). It has been therefore suggested that this cytokine may be considered a useful indicator or a diagnostic marker for periodontal disease (Geivelis et al. 1993, Reinhardt et al. 1993).

Some polymorphisms in cytokine genes promoter sequences are associated with transcriptional activity (Pociot et al. 1992, Wilson et al. 1997, Olomolaiye et al. 1998, Shirodaria et al. 2000). A G to C substitution at position -174 in the promoter of IL-6 is located immediately upstream of the multiresponsive element (MRE), located at positions -173 to -151 relative to the transcription start site (Morse et al. 1999). The C allele was shown to alter the IL-6 gene transcription response to stimuli such as LPS and IL-1 (Fishman et al. 1998). The C allele creates an *Nla*III restriction site and has been assigned Genbank accession number AF005845 (Yasukawa et al. 1987, Ray et al. 1988, Olomolaiye et al. 1998, Morse et al. 1999).

Since cytokines like IL-6 seem to have key roles in the development of several chronic inflammatory diseases including periodontitis, in this study we investigated the possible association between IL-6^{-174} polymorphism and susceptibility to chronic periodontitis in Caucasian Brazilians.

Material And Methods

Subject Selection

A convenience sample of 84 unrelated, non-smoking Caucasian subjects >25 years of age (mean age 42.4), were recruited for study from the patient pool at the Dental Clinics of the Faculty of Dentistry of Piracicaba – UNICAMP

(approved by the Ethical Committee in Research at FOP/UNICAMP, protocol 63/99). The patients are from the Southeastern region of Brazil. The baseline clinical parameters for the subject population are presented in table 1. All subjects were in good general health and had at least 20 teeth in the mouth. Subjects did not have any of the following exclusion criteria: diseases of the oral hard or soft tissues except caries and periodontal disease; use of orthodontic appliances; need for pre-medication for dental treatment; chronic usage of anti-inflammatory drugs; a history of diabetes, hepatitis or HIV infection; immunosuppressive chemotherapy; history of any disease known to severely compromise immune function; present acute necrotizing ulcerative gingivitis or current pregnancy or lactation. Subjects completed personal medical and dental history questionnaires, and within a protocol approved by an Institutional Review Board, signed a consent form after being advised of the nature of the study. Diagnosis and classification of disease severity were made on the basis of clinical parameters and consisted of physical examination, medical and dental history, probing depth, assessment of clinical attachment loss (CAL), tooth mobility, gingival recession and observation of bleeding on probing. Measurements of probing depth and attachment level were recorded at 4 points around each tooth. Subjects were included in clinical categories according to periodontal disease severity:

1) *Healthy group*: Subjects found to exhibit no signs of periodontal disease as determined by the absence of clinical attachment loss and no sites with probing depth > 3 mm (n = 37),

2) *Moderate Periodontitis*: Patients with tooth sites exhibiting ≥ 3 mm and <7 mm CAL (n = 24),

3) *Severe Periodontitis*: Patients with teeth exhibiting ≥ 7 mm CAL (n = 24).

Sampling

The sampling of epithelial buccal cells was performed as described by Trevilatto & Line (2000). Briefly, 85 individuals undertook a mouthwash after 1 min, containing 5 ml 3 % glucose. Following mouthwash, a sterile wood spatula was used to scrape oral mucosa. The tip of the spatula was then shaked into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm for 10 min. The supernatant was discarded and the cell pellet resuspended in 500 µl of extraction buffer [10 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5 % SDS]. The samples were then frozen at -20°C until used for DNA extraction.

DNA Extraction

After defrosted, samples were incubated overnight (ON) with 100 ng/mL proteinase K (Sigma Chemical Co., St. Louis, MO, USA) at 37°C with agitation. DNA was then purified by sequential phenol/chloroform extraction and salt/ethanol precipitation. DNA was dissolved in 70 μL TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA]. The concentration was estimated by measurements of OD 260.

Polymerase Chain Reaction (PCR) and Restriction Fragment Polymorphism (RFLP)

Polymorphism in the IL-6 gene at position -174

The following primer pair was used for PCR amplification of genomic DNA samples: 5'-TTGTCAAGACATGCCAAGTGCT-3' (Forward) and 5'-GCCTCAGAGACATCTCCAGTCC-3' (Reverse). Amplification reactions were carried out with 500 ng genomic DNA in a total volume of 50 μL, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 μM of each primer, 200 μM each dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, and 2.5 units T*aq* DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden). The reaction was incubated for 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C and a final extension at 72°C for 5 min.

Amplifications were carried out in a *Perkin-Elmer GeneAmp 2400* thermal cycler. The products were digested with 1 U per 25 µL reaction of *Nla*III (CATG↓) at 37°C overnight (ON) to detect allele G (13 bp + 227 bp + 59 bp) and allele C (13 bp +118 bp + 109 bp + 59 bp).

Gel electrophoresis

Restriction products were visualized by electrophoresis on vertical 10 % non-denaturing polyacrylamide gels in 1X TBE (89 mM Tris-Borate, 89 mM boric acid, 2 mM EDTA), followed by silver staining (Bio-Rad Silver Stain Kit).

Statistical Analysis

The allele ratio and genotype distribution of periodontitis patients and healthy control subjects were analyzed with Chi-square test. A p-value <0.05 was considered significant.

Results

There was a significant difference in the genotype (p=0.0036) distribution and a less significant but still suggestive one in the allele frequency (p=0.0838) between healthy controls and periodontitis patients for the polymorphism

IL-6^{-174}. The distribution of the genotype and allele frequencies for the polymorphism studied is shown in tables 2 and 3, respectively. For genotypes G/G *vs* G/C together with C/C [odds ratio: control *vs* moderate/severe disease = 3.05 (1.27-7.52), p=0.0253]. For G allele *vs* C allele [odds ratio: control *vs* moderate/severe disease = 1.9 (0.97-3.71), p=0.0838]. Allele G of the IL-6^{-174} polymorphism was carried by 91.6% (77/84) of the study Caucasians; of these 48.8% (41/84) were homozygous [29.3% (12/41) belonged to the healthy group and 70.7% (29/41) to the diseased group]. The frequency of allele G in the Caucasians studied was 70.2% and of allele C was 29.8%, respectively.

Discussion

Cytokines are regulatory proteins believed to be involved in the initiation and progression of chronic inflammatory diseases, such as arthritis and periodontitis (Masada et al. 1990, Wilton et al. 1992, McDowell et al. 1995, Yavuzyilmaz 1995, Kornmann et al. 1997, Dongari-Bagtzoglou et al. 1998, Fishman et al. 1998, Bozkurt et al. 2000, McDewitt et al. 2000, Boch et al. 2001, Buchs et al. 2001, Papapanou et al. 2001). Many recent studies have been reporting positive, equivocal or negative associations between cytokine gene polymorphisms and periodontal disease (Roberts et al. 1997, Gore et al.

1998, Gaibraith et al. 1999, Armitage et al. 2000, Cullinan et al. 2000, Scarel-Caminaga et al. *in press*).

Interleukin-6 is a multifunctional cytokine that regulates immune responses and its effects overlap those of interleukin-1 (IL-1) and tumor necrosis factor (TNF). It is widely distributed among cells of the periodontium and is thought to play a role in the elevated B-cell response observed in the gingival tissues of patients with chronic periodontitis (Fujihashi et al. 1993).

In the study Caucasian Brazilians genotype G/G was statistically associated with susceptibility to chronic periodontitis (p=0.0036, OR=3.0). Fishman et al. (1998) have found G/G showing circulating IL-6 concentrations approximately twice as high as those homozygous for allele C. In fact, it was found a reduction in the frequency of the C/C genotype in patients with systemic-onset juvenile arthritis, suggesting that this genotype confers a protective influence against the development of the disease. The presence of the C allele therefore would result in a lower IL-6 expression after a given inflammatory stimulus compared with the G/G genotype (Fishman et al. 1998). On the contrary to what happens to the G/G genotype frequency, that augments as increases the degree of severity of periodontitis (33.3% in healthy, 50% in the moderate and 70.8% in the severe periodontitis), the frequency of heterozygous individuals (G/C) decreases as increases the degree

of severity of disease (58.3% with this genotype were healthy, 50% had moderate and only 12.5% had severe periodontitis) (Table 2). Maybe this represents a protective function of allele C, whose presence may reduce IL-6 production. The low frequency of the C/C genotype in all groups possibly is related to the low frequency of allele C in the population studied (29.8%). This low frequency is smaller than that of 41% found by Olomolaiye et al (1998) and 40.3% encountered by Fishman et al. (1998) in healthy Caucasian populations.

The G→C substitution in the promoter (position -174) of IL-6 gene has been considered to increase the in vitro transcription of IL-6 gene by two-fold (Morse et al. 1999). We found an association between allele G of the IL-6^{-174} polymorphism and susceptibility to chronic periodontal disease. If the C allele really increases the transcriptional rate of the IL-6 gene, the paradox of the association of IL-6 allele G with chronic periodontal disease, perhaps resides in the fact that beyond being pro-inflammatory, IL-6 stimulates interleukin-4 (IL-4) production, which is a potent anti-inflammatory mediator (Alexander & Damoulis 1994). Interleukin-4 has been reported to be a negative regulator of bone resorption by inhibiting the secretion of pro-inflammatory cytokines, such as IL-1, TNF and IL-6 (Shapira et al. 1992). Most cytokines are multifunctional, some of them having antagonic effects by interaction with

other inflammatory mediators, and this can be the case of IL-6 in the microenvironment of the periodontium. The decrease of heterozygous individuals (G/C) as increases the degree of severity of disease maybe would be representing a protective function of allele C, whose presence may decrease IL-4 production, which, at a time, inhibits IL-6 and other potent pro-inflammatory mediators production. However it is more probable that allele C decreases the IL-6 production because the polymorphism at position -174 is located in a negative regulatory domain (NRD), between -225 and -164 (Morse et al. 1999), having been previously reported to demonstrate a negative regulatory effect on gene expression (Ray et al. 1990).

The overall genotype distribution of the whole patient group was significantly different than the controls. When the genotype frequencies of the PD patients and the controls were compared, there was a statistically significant augment in G/G genotype frequency in the periodontitis patients. It was concluded that genotype G/G of the IL-6^{-174} polymorphism was significantly associated with susceptibility to chronic periodontal disease, suggesting that allele G may play a role in the pathogenesis and development of periodontal disease. To our knowledge this is the first report associating a polymorphism in the IL-6 gene with periodontal disease. The outcome and progression of periodontal disease constitute, however, an imbricate process

depending on the interaction of various cytokines pro- and anti-inflammatory of synergistic and antagonistic effects. Like IL-6, other pro-inflammatory mediators may play significant roles in the pathogenesis of chronic periodontitis. The study of cytokine gene polymorphisms may contribute to an understanding on the host mediator interactions that determine the disease phenotype.

Acknowledgements

This study was supported by FAPESP grants: 99/05610-2, 99/06094-8 and 00/07348-2.

References

Alexander, M. B. & Damoulis P. D. (1994) The role of cytokines in the pathogenesis of periodontal disease. *Current Opinion in Periodontology* 39-53.

Armitage, G. C., Wu, Y., Wang, H-Y, Sorrel, J., di Giovine, F. S. & Duff, G. W. (2000) Low prevalence of a periodontitis-associated interleukin-1 composite genotype in individuals of Chinese heritage. *Journal of Periodontology* **71**(2), 164-171.

Atilla, G. & Kütükçüler, N. (1998) Crevicular fluid interleukin-1β, tumor necrosis factor-α, and interleukin-6 levels in renal transplant patients receiving cyclosporine A *Journal of Periodontology* **69**, 784-790.

Boch, J. A., Wara-aswapati, N. & Auron, P. E. (2001) IL-1 signal transduction - current concepts and relevance to periodontitis *Journal of Dental Research* Review Feb **80**(2), 400-407.

Bozkurt, F. Y., Berker, E., Akkus, S. & Bulut, S. (2000) Relationship between interleukin-6 levels in gingival crevicular fluid and periodontal status in patients with rheumatoid arthritis and adult periodontitis *Journal of Periodontology* Nov**71**(11), 1756-1760.

Buchs, N., di Giovine, F. S., Silvestri, T., Vannier, E., Duff, G. W. & Miossec P. (2001) IL-1B and IL-1Ra gene polymorphisms and disease severity in rheumatoid arthritis: interaction with their plasma levels. *Genes Immunology* Jun **2**(4), 222-228.

Cullinan, M. P., Westerman, B., Palmer, J. E., Faddy & M. J., Seymor, G. J. (2000) IL-1 genotype and progression of periodontal disease in an Australian population *Journal of Dental Research* 79 (abstr.#220).

Dongari-Bagtzoglou, A. I. & Ebersole, J. L. (1998) Increased presence of interleukin-6 (IL-6) and IL-8 secreting fibroblast subpopulations in adult periodontitis *Journal of Periodontology* Aug **69**, 899-910.

Fishman, D., Faulds, G., Jeffery, R., Mohamed-Ali, V., Yudin, J. S., Humphries, S. & Woo, P. (1998) The effect if novel polymorphism in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma Il-6 levels, and an association with systemic-onset juvenile chronic arthritis. *Journal of Clinical Investigation* Oct **102**(7), 1369-1376.

Fujihashi, K., Kono, Y., Beagley, K. W., Yamamoto, M., McGhee, JR., Mestecky, J. & Kiyono, H. (1993) Cytokine and periodontal disease: immunopathological role of interleukins for B cell response in chronic inflamed gingival tissues. *Journal of Periodontology* **64**, 400-406.

Galbraith, G. M. P., Hendley, T. M., Sanders, J. J., Palesch, Y. & Pandey, J. P. (1999) Polymorphic cytokine genotypes as markers of disease severity in adult periodontitis. *Journal of Clinical Periodontology* **26**, 705-709.

Gore, E. A., Sanders, J. P., Pandey, J. P., Palesch, Y. & Galbraith, G. M. P. (1998) Interleukin-1β^{+3953} allele 2: association with disease status in adult periodontitis. *Journal of Clinical Periodontology* **25**, 781-785.

Geivelis, M., Turner, D. W., Pederson, E. D. & Lamberts, B. L. (1993) Measurements of interleukin-6 in gingival crevicular fluid from patients with destructive periodontal disease. *Journal of Periodontology* **64**, 980-983.

Grossman, R. M., Krueger, J., Yourish, D., Granelli-Piperno, A., Murphy, D. P., May, L. T., Kupper, T. S., Sehgal, P. B.& Gottlieb, A. B. (1989) Interleukin-6

is expressed in high levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes. *Proceedings of the National Academy of Sciences* (USA) Aug **86**(16), 6367-6371.

Hirano, T., Matsuda, T., Turner, M., Miyasaka, N., Buchan, G., Tang, B., Sato, K., Shimizu, M., Maini, R., Feldmann, M. et al. (1988) Excessive production of interleukin-6/ B cell stimulatory factor-2 in rheumatoid arthritis *European Journal of Immunology* Nov **18**(11), 1797-1801.

Hirano, T., Akira, S., Taga, T. & Kishimoto, T. (1990) Biological and clinical aspects of interleukin 6. *Immunology Today* **11**, 443-449.

Houssiau, F. A., Devogelaer, J., Van Damme, J., Nagant de Deuxchaisnes, C.& Van Snick, J. (1988) Interleukin-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides. *Arthritis & Rheumatism* Jun **31**(6), 784-788.

Ishimi, Y., Miyaura, C., Jin, C. H., Akatsu, T., Abe, E., Nakamura, Y., Yamaguchi, A, Yoshiki, Y., Matsuda, T., Hirano, T., Kishimoto, T. & Suda, T. (1990) IL-6 is produced by osteoblasts and induces bone resorption. *Journal of Immunology* Nov **145**(10), 3297-3303.

Kamagata, Y., Miyasaka, N., Inoue, H., hashimoto, J. & Iida, M. (1989) Cytokine production in inflamed human gingival tissues-interleukin-6 *Journal of Japanese Association of Periodontology*. Dec, **31**(4),1081-1087.

Kono, Y., Beagley, K. W., Fujihashi, K., McGhee, J. R., Taga, T., Hirano, T., Kishimoto, T. & Kiyono, H. (1991) Cytokine regulation of localized inflammation. Induction of activated B cells and IL-6-mediated polyclonal IgG and IgA synthesis in inflamed human gingiva. *Journal of Immunology* Mar 15, **146**(6), 1812-1821.

Kornman, K. S., Crane, A., Wang, H-Y., Di Giovine, F. S., Newman, M. G., Pirk, F. W., Wilson Jr. T. G., Higginbottom, F. L. & Duff, G. W. (1997) The interleukin-1 genotype as a severity factor in adult periodontal disease. *Journal of Clinical Periodontology* **24**, 72-77.

Lotz, M., Jirik, F., Kabouridis, P., Tsoukas, C., Hirano, T., Kishimoto, T. & Carson, D.A. (1988) B cell stimulating factor 2/ interleukin-6 is a costimulant for human thymocytes and T lymphocytes. *Journal of Experimental Medicine* Mar 1, **167**(3), 1253-1258.

Masada, M. P., Persson, R., Kenney, J. S., Lee, S. W., Page, R. C. & Allison, A. C. (1990) Measurement of interleukin-1α and -1β in gingival crevicular fluid: implications for the pathogenesis of periodontal disease. *Journal of Periodontal Research* **25**, 156-163.

Matsuki, Y., Yamamoto, T. & Hara, K. (1992) Detection of inflammation cytokine messenger RNA (mRNA)-expressing cells in human inflamed

gingiva by combined in situ hybridization and immunohistochemistry. *Immunology* May **76**(1), 42-47.

McDewitt, M. J., Wang, H. –Y., Knobelman, C., Newman, M. G., Di Giovini, F. S., Timms, J., Duff, G. W. & Kornman, K. S. (2000) Interleukin-1 genetic association with periodontitis in clinical practice. *Journal of Periodontology* **71**, 156-163.

McDowell, T. L., Symons, J. A., Ploski, R., Fárre, O. & Duff, G. W. (1995) A genetic association between juvenile rheumatoid arthritis and a novel interleukin-1 alpha polymorphism. *Arthritis & Rheumatism* **38**, 221-222.

Morse, H. R., Olomolaiye, O. O., Wood, N. A. P., Keen, L. J. & Bidwell, J. L. (1999) Induced heteroduplex genotyping of TNF-α, IL-1β, IL-6 and IL-10 polymorphisms associated with transcriptional regulation. *Cytokine* Oct **11**(10), 789-795.

Olomolaiye, O., Wood, N. A. P. & Bidwell, J. L. (1998) A novel *Nla*III polymorphism in the human IL-6 promoter. *European Journal of Immunogenetics* **25**, 207.

Papapanou, P. N., Neideud, A-M., Sandros, J. & Dahlén, G. (2001) Interleukin-1 gene polymorphism and periodontal status. *Journal of Clinical Periodontology* **28**, 389-396.

Pociot, F., Molvig, J., Wogensen, L., Worsaae, H. & Nerup, J. (1992) A Taq I polymorphism in the human interleukin-1 beta (IL-1β) gene correlates with secretions in vitro. *European Journal of Clinical Investigation* **22**, 396-402.

Prabhu, A., Michalowicz, B. S. & Mathur, A. (1996) Detection of local and systemic cytokines in adult periodontitis. *Journal of Periodontology* **67**, 515-522.

Ray, A., Tatter, S. B., May, L. T. & Sehgal, P. B. (1988) Activation of the human "β_2-interferon/ hepatocyte-stimulating factor/ interleukin 6" promoter by cytokines, viruses, and second messenger agonists. *Proceedings of the National Academy of Sciences* (USA) Sep **85**, 6701-6705.

Ray, A., La Forge, K. S. & Sehgal, P. B. (1990) On the mechanism for efficient repression of the interleukin-6 promoter by glucocorticoids: enhancer, TATA box, and RNA start site (Inr motif) occlusion. *Molecular and Cellular Biology* **10**, 5736-5746.

Reinhardt, R. A., Masada, M. P., Kaldahl, W. B., DuBois, L. M., Kornman, K. S., Choi, J. I., Kalkwarf, K. L. & Allison, A. C. (1993) Gingival fluid IL-1 and IL-6 levels in refractory periodontitis. *Journal of Clinical Periodontology* **20**, 225-231.

Revel, M. (1989) Host defence against infections of inflammations: Role of the multifunctional IL-6/IFN-beta2 cytokine. *Experientia* **45**, 549-557.

Roberts, F. A., Hockett, R. D. Jr, Bucy, R. P. & Michalek, S. M. (1997) Quantitative assessment of inflammatory cytokine gene expression in chronic adult periodontitis. *Oral Microbiology and Immunology* Dec **12**(6), 336-344.

Shapira, L., Van Dyke, T. E. & Hart, T. C. (1992) A localized absence of interleukin-4 triggers periodontal disease activity: a novel hypothesis. *Medicine Hipotheses* **39**, 319-322.

Scarel-Caminaga, R. M., Trevilatto, P. C., Souza, A. P., Brito Jr. R. B.& Line, S. R. P. (2002) Investigation of an IL-2 polymorphism in patients with different levels of chronic periodontitis. *Journal of Clinical Periodontology* **7**, *in press*.

Shirodaria, S., Smith, J., McKay, I. J., Kennet, C. N., & Hugh, F. J. (2000) Polymorphisms in the IL-1A gene are correlated with levels of interleukin-1 alpha protein in gingival crevicular fluid of teeth with severe periodontal disease. *Journal of Dental Research* Nov **79**(11), 1864-1869.

Takahashi, K., Takashiba, S., Nagai, A., Takigawa, M., Myoukai, F., Kurihara, H. & Murayama, Y. (1994) Assesment of interleukin-6 in the pathogenesis of periodontal disease. *Journal of Periodontology* Feb **65**, 147-153.

Trevilatto, P. C. & Line, S. R. P. (2000) Use of Buccal Epithelial Cells for PCR Amplification of Large DNA Fragments. *Journal of Forensic Odonto-Stomatology*. **18** (1): 6-9.

Wilson, A. G., Symons, J. A., McDowell, T. L., McDewitt, H. O. & Duff, G. W. (1997) Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. *Proceedings of the National Academy of Sciences* Apr 1, **94**(7), 3195-3199.

Wilton, J. M. A., Bampton, J. L. M., Griffiths, G. S., Curtis, M. A., Life, J. S., Johnson, N. W., Powell, J. R., Harrap, G. J. & Critchley, P. (1992) Interleukin-1 beta (IL-1β) levels in gingival crevicular fluid from adults with previous evidence of destructive periodontitis – a cross sectional study. *Journal of Clinical Periodontology* Jan **19**(1), 53-57.

Yasukawa, K., Hirano, T., Watanabe, Y., Muratani, K., Matsuda, T., Nakai, S. & Kishimoto, T. (1987) Structure and expression of human B cell stimulatory factor-2 (BSF-2/IL-6) gene. *The Embo Journal* **6**(10), 2939-2945.

Yavuzyilmaz, E., Yamalik, N., Bulut, S., Özen, S., Ersoy, F. & Saatçi, Ü. (1995) The gingival crevicular fluid interleukin-1β and tumor necrosis factor-α levels in patients with rapidly progressive periodontitis. *Australian Dental Journal* **40**(1), 46-49.

Table 1. Baseline clinical parameters of the subject population (n=84)

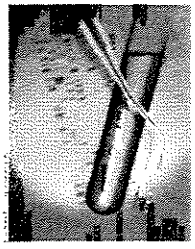
	Healthy (n=37)	**Moderate (n=24)**	**Severe (n=24)**	**Total (n=84)**
Age (years) Mean (± SD)	43.7 (14.7)	37.9 (11.5)	44.9 (11.6)	42.4 (13.2)
Gender (%)				
Female	25 (70.3)	18 (75.0)	20 (83.3)	63 (75.3)
Male	11 (29.7)	6 (25.0)	4 (16.7)	21 (24.7)

Table 2. Distribution of the IL-6 genotypes in the healthy group and in groups with moderate and severe chronic periodontitis in the Caucasian Brazilians

Genotype	***Healthy***		***Moderate***		***Severe***		***p value***
	n	***(%)***	***n***	***(%)***	***n***	***(%)***	
G/G	12	(33.3)	12	(50.0)	17	(70.8)	0.0036
G/C	21	(58.3)	12	(50.0)	3	(12.5)	
C/C	3	(8.4)	0	(0)	4	(16.7)	

Table 3 Distribution of the IL-6 alleles in the healthy and moderate/severe groups with chronic periodontitis in the Brazilian Caucasian individuals

Allele	***Healthy***		***Moderate/Severe***		***p value***
	n	***(%)***	***n***	***(%)***	
G	45	(62.5)	73	(76.0)	0.0838
C	27	(37.5)	23	(24.0)	



POLYMORPHISM IN THE TUMOR NECROSIS FACTOR-α (G-308A) GENE IS NOT ASSOCIATED WITH THE SUSCEPTIBILITY TO CHRONIC PERIODONTITIS IN A BRAZILIAN POPULATION
Journal of Periodontal Research, submitted

Polymorphism in the tumor necrosis factor-alpha (G-308A) gene is not associated with susceptibility to chronic periodontitis in a Brazilian population

Trevilatto PC[1], *de Brito Jr RB*[1], *Scarel-Caminaga RM*[1], *de Souza AP*[1], *Sallum AW*[2], *Line SRP*[1]

Departments of [1]Morphology and [2]Periodontology, Dental School of Piracicaba, State University of Campinas, Piracicaba, SP, Brazil

Running title: *TNF-α polymorphism and periodontitis*

Present address:
Sergio Roberto Peres Line
Faculdade de Odontologia de Piracicaba-UNICAMP
Departamento de Morfologia
Av. Limeira 901, CEP. 13414-018
Piracicaba - SP
Brazil
Tel: +55 (019) 3412-5333
Fax: +55 (019) 3412-5218
E-mail: sergio@fop.unicamp.br

Key words: chronic periodontitis; tumor necrosis factor; polymorphism; genetic risk factor

Abstract

Tumor necrosis factor-alpha (TNF-α) is a major mediator of the immuno-inflammatory response and may play an important role in the pathogenesis and progression of chronic periodontal disease (PD). Polymorphisms in the promoter of the TNF-α gene have been associated with some types of inflammatory diseases, such as asthma and rheumatoid arthritis. The aim of the present study was to investigate the association between a single-nucleotide polymorphism (SNP) of the TNF-α (G-308A) gene and severity of chronic periodontitis in Brazilians. One hundred and thirteen (113) non-smoking subjects over 25 years (mean age 41.6) were divided according to the severity level of periodontal disease: 44 healthy individuals (control group), 31 subjects with moderate and 38 patients with severe periodontitis. Genomic DNA was obtained from epithelial cells through a mouthwash with 3 % glucose and scraping of oral mucosa. The samples were analyzed for TNF-α (-308) polymorphism using polymerase chain reaction-restriction fragment length polymorphism techniques. The significance of the differences in the allele and genotype frequencies of the polymorphism between the control and groups with periodontitis was assessed by Chi-square test ($p<0.05$). No significant differences in the genotype distribution and allele frequency were found between control and groups with periodontitis. It was concluded that TNF-α (-308) polymorphism was not associated either with susceptibility to or with the severity of chronic periodontal disease. Other polymorphisms in this or/and other genes of the host inflammatory response might be involved in determining susceptibility to periodontitis in the study population.

Bacterial lipopolysaccaharides (LPS) activate monocytes of inflamed periodontal tissues to produce cytokines. Tumor necrosis factor-alpha (TNF-α) is an important immune mediator and a pluripotent proinflammatory cytokine, that is able to induce bone resorption (1). TNF-α gene is encoded on chromosome 6 inside the HLA region (2) and its biological activities have raised the possibility that polymorphisms within this gene might contribute to genetic association to diverse immuno-inflammatory diseases. In fact, this cytokine has been implicated in the pathogenesis of a large number of human diseases (3-7).

Within the promoter region of TNF-α gene, a biallelic polymorphic site at position -308 has been reported to influence the production of TNF-α protein (8). Allele A of TNF-α (-308) polymorphism has been positively associated with asthma (7) and ulcerative colitis (6).

Epidemiological studies indicate that periodontitis is widespread among the Brazilian population (9-13). TNF-α mRNA was significantly more frequent in diseased than in healthy gingival biopsies (14), suggesting a role of this cytokine in the development of periodontal disease. Increased production of TNF-α associated with the G/A genotype was detected in patients with advanced adult periodontitis (15). Because of the increasing evidence that genetic variations within the TNF-α locus are important in determining

susceptibility to periodontitis, this study aimed to investigate the possible link between the polymorphism at position -308 (G→A) of TNF-α gene promoter and chronic periodontitis in a Brazilian population.

Material and methods

Subject selection

A convenience sample of 113 unrelated, non-smoking subjects >25 years of age, were recruited for study from the patient pool at the Dental Clinics of the Faculty of Dentistry of Piracicaba - UNICAMP (approved by the Ethical Committee in Research at FOP/UNICAMP, protocol 63/99). The patients are from the Southeastern region of Brazil. The baseline clinical parameters for the subject population are presented in table 1. All subjects were in good general health and had at least 20 teeth in the mouth. Subjects did not have any of the following exclusion criteria: diseases of the oral hard or soft tissues except caries and periodontal disease; use of orthodontic appliances; need for pre-medication for dental treatment; chronic usage of anti-inflammatory drugs; a history of diabetes, hepatitis or HIV infection; immunosuppressive chemotherapy; history of any disease known to severely compromise immune function; present acute necrotizing ulcerative gingivitis or current pregnancy

or lactation. Subjects completed personal medical and dental history questionnaires, and within a protocol approved by an Institutional Review Board, signed a consent form after being advised of the nature of the study. Diagnosis and classification of disease severity were made on the basis of clinical parameters and consisted of physical examination, medical and dental history, probing depth, assessment of clinical attachment loss (CAL), tooth mobility, gingival recession and observation of bleeding on probing. Measurements of probing depth and attachment level were recorded at 4 points around each tooth. Subjects were included in clinical categories according to PD severity:

1) *Healthy group*: Subjects found to exhibit no signs of periodontal disease as determined by the absence of clinical attachment loss and no sites with probing depth >3 mm (n = 44),
2) *Moderate Periodontitis*: Patients with tooth sites exhibiting ≥3 mm and <7 mm CAL (n = 31),
3) *Severe Periodontitis*: Patients with teeth exhibiting ≥7 mm CAL (n = 38).

Sampling

The sampling of epithelial buccal cells was performed as described by Trevilatto & Line (16). Briefly, 113 individuals undertook a mouthwash after

1 min, containing 5 ml 3 % glucose. Following mouthwash, a sterile wood spatula was used to scrape oral mucosa. The tip of the spatula was then shaked into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm for 10 min. The supernatant was discarded and the cell pellet resuspended in 500 µl of extraction buffer [10 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5 % SDS]. The samples were then frozen at -20°C until used for DNA extraction.

DNA extraction

After defrosted, samples were incubated overnight (ON) with 100 ng/mL proteinase K (Sigma Chemical Co., St. Louis, MO, USA) at 37°C with agitation. DNA was then purified by sequential phenol/chloroform extraction and salt/ethanol precipitation. DNA was dissolved in 70 µL TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA]. The concentration was estimated by measurements of OD 260.

Polymorphism in the TNF-α gene at position –308

The oligonucleotides 5'- AGGCAATAGGTTTTGAGGGCCAT - 3' and 5'- TCCTCCCTGCTCCGATTCCG - 3' were used as primers. Amplification reactions were performed with 500 ng genomic DNA in a total volume of

50 μL, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 μM of each primer, 200 μM each dATP, dCTP, dGTP and dTTP, and 2.5 units T*aq* DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden). Cycling was 2 cycles of 3 min at 94°C, 1 min at 60°C and 1 min at 72°C, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C, with a final extension of 7 min at 72°C. The products were digested with 3 U per 25 μL reaction of N*co*I at 37°C ON to detect allele 1 [G] (20 bp + 87 bp) and allele 2 [A] (107 bp).

Gel electrophoresis

Restriction products were visualized by electrophoresis on vertical 10 % non-denaturing polyacrylamide gels in 1X TBE (89 mM Tris-Borate, 89 mM boric acid, 2 mM EDTA), followed by silver staining (Bio-Rad Silver Stain Kit).

Statistical analysis

The allele ratio and genotype distribution of periodontitis patients and healthy control subjects were analyzed with Chi-square test. A p-value <0.05 was considered significant.

Results

There was no significant difference in the genotype distribution between healthy controls and periodontitis patients in the polymorphism TNF-α^{-308} (p=0.289), neither was observed statistical variation in the frequency of the alleles TNF-α^{-308} (p=0.845). The distribution of the genotype and the frequencies of the alleles for the polymorphism studied are shown in tables 2 and 3, respectively. Allele 2 was carried by 19.5% (22/113) of the subjects, with 1.8% (2/113) of homozygous. When Caucasians were taken alone, the following allele 2 carriage rate for the TNF-α (-308) polymorphism was found: 20.7% (18/87), with 1% (1/87) of homozygous.

The alleles distribution in the study population was consistent with Hardy-Weinberg equilibrium.

Discussion

Because of their frequent presence in diseased sites, it has been postulated that cytokines such as interleukin-1 (IL-1) and TNF-α may be indicators of the active phase of periodontal disease (17). Recently, a number of single nucleotide polymorphisms (SNPs) have been identified in the TNF-α promoter, and the less common A allele of TNF-α (-308) has been associated

with certain chronic inflammatory diseases, including asthma (7), cerebral malaria (3), multiple sclerosis (4), Alzheimer disease (5), ulcerative colitis (6) and chronic bronchitis (18).

As can be noted in table 1, the majority (77%) of the individuals belonging to the population studied is of Caucasian heritage. We found an allele 2 carriage rate of 20.7% (18/87) for our Caucasians. In their study, Kornman *et al.* (19) found an allele 2 carriage rate of 28.5% at TNF-α (-308) locus. In neither studies was observed any link between this polymorphism and periodontal disease. However, a study has been reported in which the frequency of TNF-α (-308) allele G was significantly greater in patients with advanced periodontitis (20). This finding is intriguing once allele A has been found to give a two-fold greater level of transcription than the -308G form (21) and the nature of periodontitis is inflammatory. There is an evidence that allele A is over-represented in diseases where TNF-α levels are associated with poor prognosis (22). In cerebral malaria TNF-α is of major importance for the pathology. Individuals homozygous for the TNF-α (-308) A allele carry a 7-fold higher risk of death or severe neurological sequelae due to cerebral malaria.

Polymorphisms in certain host-response genes have been related to hypersecretion of several cytokines upon microbiological challenge (8, 23,

24). Allele A of TNF-α (-308) polymorphism has been found to increase in two-fold the production of TNF-α cytokine. Although the role TNF-α play in leading to tissue destruction, it is worth mentioning the need for evaluation of other potential candidate genes as contributors to periodontitis, since chronic periodontitis may possibly represent a polygenic disease. Analysis in other cytokine promoter genes in the same Brazilian patients have revealed positive associations with chronic periodontal disease (25-27). More studies on polymorphisms in genes of the host response can increase information with regard to which factors could indeed be implicated in the process of disease.

It was concluded that TNF-α (-308) polymorphism was not associated with the severity of chronic periodontal disease in the population studied. Although the prevalence of the polymorphism studied for the Brazilian population was similar to that reported for Europeans, we observed no significant differences in genotype and allele distributions of the TNF-α (-308) gene polymorphism between control and periodontitis groups. Tumor Necrosis Factor-α (-308) G-to-A polymorphism is not a useful marker for predicting susceptibility to chronic periodontal disease in the study population.

Acknowledgements

This study was supported by FAPESP grants 99/05610-2 and 99/06094-8.

References

1. Shapira L, Stabholz A, Rieckmann P, Kruse N. Genetic polymorphism of the tumor necrosis factor (TNF)-alpha promoter region in families with localized early-onset periodontitis. *J Periodont Res* 2001;**36**:183-186.
2. Wilson AG, de Vries N, Pociot F, di Giovine FS, van der Putte LB, Duff GW. An allelic polymorphism within the human tumor necrosis factor alpha promoter region is strongly associated with HLA A1, B8, and DR3 alleles. *J Exp Med* 1993;**177**:557-560.
3. McGuire W, Hill AV, Allsopp CE, Greenwood BM, Kwiatkowski D. Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. *Nature* 1994;**371**:508-510.
4. Braun N, Michel U, Ernst BP *et al.* Gene polymorphism at position -308 of the tumor-necrosis-factor-alpha (TNF-alpha) in multiple sclerosis and it's influence on the regulation of TNF-alpha production. *Neurosci Lett* 1996;**215**:75-78.
5. Perry RT, Collins JS, Wiener H, Acton R, Go RC. The role of TNF and its receptors in Alzheimer's disease *Neurobiol Aging* 2001;**22**:873-883.
6. Sashio H, Tamura K, Ito R *et al.* Polymorphisms of the TNF gene and the TNF receptor superfamily member 1B gene are associated with

susceptibility to ulcerative colitis and Crohn's disease, respectively. *Immunogenetics* 2002;**53**:1020-1027.

7. Witte JS, Palmer LJ, O'Connor RD, Hopkins PJ, Hall JM. Relation between tumour necrosis factor polymorphism TNFalpha-308 and risk of asthma *Eur J Hum Genet* 2002; **10**:82-85.
8. Wilson AG, Symons JA, McDowell TL, McDevitt HO, Duff GW. Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. *Proc Natl Acad Sci USA* 1997;**94**:3195-3199.
9. Gjermo P, Bellini HT, Marcos B. Application of the Community Periodontal Index of Treatment Needs (CPITN) in a population of young Brazilians. *Comm Dent Oral Epidemiol* 1983;**11:**342-346.
10. Gjermo P, Bellini HT, Pereira Santos V, Martins JG, Ferracyoli JR. Prevalence of bone loss in a group of Brazilian teenagers assessed on bite-wing radiographs. *J Clin Periodontol* 1984;**11:**104-113.
11. Pecora JD, da Cruz Filho AM. Study of the incidence of radicular grooves in maxillary incisors. *Braz Dent J* 1992;**3:**11-16.
12. Dini EL, Guimaraes LO. Periodontal conditions and treatment needs (CPITN) in a worker population in Araraquara, SP, Brazil. *Int Dent J* 1994;**44**:309-311.

13. Tinoco EM, Beldi MI, Loureiro CA *et al.* Localized juvenile periodontitis and *Actinobacillus actinomycetemcomitans* in a Brazilian population. *Eur J Oral Sci* 1997;**105:**9-14.

14. Roberts FA, Hockett RD Jr, Bucy RP, Michalek SM. Quantitative assessment of inflammatory cytokine gene expression in chronic adult periodontitis. *Oral Microbiol Immunol* 1997;**12**:336-344.

15. Galbraith GM, Steed RB, Sanders JJ, Pandey JP. Tumor necrosis factor alpha production by oral leukocytes: influence of tumor necrosis factor genotype *J Periodontol* 1998; **69**:428-433.

16. Trevilatto PC, Line SRP. Use of buccal epithelial cells for PCR amplification of large DNA fragments. *J Forensic Odontostomatol* 2000;**18**:6-9.

17. Yavuzyilmaz E, Yamalik N, Bulut S, Özen S, Ersoy F, Saatçi Ü. The gingival crevicular fluid interleukin-1β and tumor necrosis factor-α levels in patients with rapidly progressive periodontitis. *Aust Dent J* 1995;**40:**46-49.

18. Huang SL, Su CH, Chang SC. Tumor necrosis factor-alpha gene polymorphism in chronic bronchitis. *Am J Respir Crit Care Med* 1997;**156**:1436-1439.

19. Kornman KS, Crane A, Wang H-Y *et al.* The interleukin-1 genotype as a severity factor in adult periodontal disease. *J Clin Periodontol* 1997;**24**:72-77.

20. Galbraith GM, Hendley TM, Sanders JJ, Palesch Y, Pandey JP. Polymorphic cytokine genotypes as markers of disease severity in adult periodontitis. *J Clin Periodontol* 1999;**26**:705-709

21. Kroeger KM, Carville KS, Abraham LJ. The -308 tumor necrosis factor-alpha promoter polymorphism effects transcription. *Mol Immunol* 1997;**34**:391-399

22. Kwiatkowski D, Hill AV, Sambou I et al. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. *Lancet* 1990;**336**:1201-1204.

23. Pociot F, Molvig J, Wogensen L, Worsaae H, Nerup J. A Taq I polymorphism in the human interleukin-1 beta (IL-1β) gene correlates with secretions in vitro. *Eur J Clin Invest* 1992;**22:**396-402.

24. Shirodaria S, Smith J, McKay IJ, Kennet CN, Hugh FJ. Polymorphisms in the IL-1A gene are correlated with levels of interleukin-1 alpha protein in gingival crevicular fluid of teeth with severe periodontal disease. *J Dent Res* 2000;**79:**1864-1869.

25. Scarel-Caminaga RM, Trevilatto PC, Souza AP, Brito Jr. RB, Line SRP. Investigation of an IL-2 polymorphism in patients with different levels of chronic periodontitis. *J Clin Periodontol (In press)*.

26. Souza AP, Trevilatto PC, Scarel-Caminaga RM, Brito Jr. RB, Line SRP. MMP-1 promoter polymorphism: A risk factor for chronic periodontitis severity. *J ClinPeriodontol (In Press)*

27. Trevilatto PC, Scarel-Caminaga RM, Brito Jr RB, Souza AP, Line SRP. Polymorphism at position -174 of IL-6 gene is associated with susceptibility to chronic periodontitis in a Caucasian Brazilian population. *J Clin Periodontol (In press)*

Table 1. Baseline clinical parameters of the subject population (n=113)

	Healthy (n=44)	Moderate (n=31)	Severe (n=38)
Age (years)			
Mean (± SD)	43.2 (±14.0)	36.9 (±11.2)	43.6 (±14.4)
Gender %			
Female	68.2	80.6	84.2
Male	31.8	19.4	15.8
Ethnic Group %			
Caucasoid	84.1	77.4	68.4
Afro-American	6.8	16.1	13.2
Mulatto	6.8	6.5	18.4
Japanese	2.3	0.0	0.0

Table 2. Distribution of the TNF-α genotypes in the healthy group and in groups with moderate and severe chronic periodontitis.

Genotype	Healthy n (%)	Moderate n (%)	Severe n (%)	*p* value
G/G	37 (84.0)	24 (77.4)	31 (81.6)	0.289
G/A	7 (16.0)	7 (22.5)	5 (13.1)	
A/A	0 (0.0)	0 (0.0)	2 (5.3)	

Table 3. Distribution of the TNF-α alleles in healthy, moderate and severe groups with chronic periodontitis.

Allele	Healthy n (%)	Moderate n (%)	Severe n (%)	*p* value
G	81 (92.0)	55 (88.7)	67 (88.0)	0.674
A	7 (8.0)	7 (11.3)	9 (12.0)	

4. CONCLUSÃO

Artigo 1. A obtenção de DNA a partir de um bochecho com solução de glicose a 3 % e leve raspagem da mucosa jugal permite a execução de análises de suscetibilidade genética à doença periodontal.

Artigo 2. Os resultados microbiológicos e genéticos não foram suficientes para predizer a suscetibilidade à periodontite agressiva na família estudada.

Artigo 3. Os polimorfismos *IL-1A* (-889) e *IL-1B* (+3953), respectivamente nos genes da IL-1α e IL-1β, não se relacionaram à doença periodontal crônica na população estudada.

Artigo 4. A presença do alelo 2 do polimorfismo *IL-1B* (-511) no genótipo dos indivíduos negros e mulatos associou-se fortemente [OR: 18; p=0,049] à doença periodontal crônica, sugerindo a existência de um componente racial na determinação da suscetibilidade à periodontite.

Artigo 5. O alelo 2 do polimorfismo *IL-1RA* (intron 2) esteve associado à severidade da doença periodontal crônica na população estudada (p=0,029).

Artigo 6. Indivíduos apresentando o genótipo G/G do polimorfismo *IL-6*$^{-174}$ (G→C) mostraram aumento da suscetibilidade à doença periodontal crônica (OR=3,0; p=0,0036).

Artigo 7. O polimorfismo *TNFA* (-308) não apresentou correlação com a suscetibilidade à doença periodontal crônica na população estudada.

*REFERÊNCIAS BIBLIOGRÁFICAS

1. ALEXANDER, M.B.; DAMOULIS, P.D. The role of cytokines in the pathogenesis of periodontal disease. **Curr Opin Periodontol**, Philadelphia, p.39-53, 1994.

2. BERTOLINI, D.R. *et al.* Stimulation of bone resorption and inhibition of bone formation *in vitro* by human tumour necrosis factors. **Nature**, London, v.319, n.6053, p.516-518, Feb. 1986.

3. BOIARDI, L. *et al.* Interleukin-1 cluster and tumor necrosis factor alpha gene polymorphisms in polymyalgia rheumatica. **Clin Exp Rheumatol**, Pisa, v.18, n.6, p.675-681, Nov./Dec. 2000.

4. BUCHS, N. IL-1B and IL-1Ra gene polymorphisms and disease severity in rheumatoid arthritis: interaction with their plasma levels. **Genes Immun**, Houndmills, v.2, n.4, p.222-228, June 2001.

5. DANIS, V.A. *et al.* Cytokine production by normal human monocytes: inter-subject variation and relationship to an IL-1 receptor-antagonist (IL-1Ra) gene polymorphism. **Clin Exp Immunol**, Oxford, v.99, n.2, p.303-310, Feb. 1995.

*Referências de acordo com a NBR-6023, da Associação Brasileira de Normas Técnicas, de 2000. Abreviaturas dos periódicos em conformidade com *Medline*

6. GEMMELL, E.; SEYMOUR, G.J. Modulation of immune responses to periodontal bacteria. **Curr Opin Periodontol**, Philadelphia, p.28-38, 1994.

7. GENCO, R.J. Host responses in periodontal diseases: current concepts. **J Periodontol**, Chicago, v.63, 4 Suppl., p.338-355, 1992.

8. HABER, J. Smoking is a major risk factor for periodontitis. **Curr Opin Periodontol**, Philadelphia, p.12-18, 1994.

9. HONIG, J. *et al.* Increased interleukin-1 beta (IL-1β) concentration in gingiva tissue from periodontitis patients. **J Periodont Res**, Copenhagen, v.24, n.6, p.362-367, Nov. 1989.

10. HOWELL, T.H.; WILLIAMS, R.C. Nonsteroidal antiinflammatory drugs as inhibitors of periodontal disease progression. **Crit Rev Oral Biol Med**, Boca Raton, v.4, n.2, p.177-196, 1993.

11. JEFFCOAT, M.K. *et al.* Effects of nonsteroidal antiinflammatory drugs on bone loss in chronic inflammatory disease. **Ann N Y Acad Sci**, New York, v.696, p.292-302, Nov. 1993.

12. JOHNSON, G.K.; SLACH, N.A. Impact of tobacco use on periodontal status. **J Dent Educ**, Washington, v.65, n.4, p.313-321, Apr. 2001.

13. KORNMAN, K.S. *et al.* The interleukin-1 genotype as a severity factor in adult periodontal disease. **J Clin Periodontol**, Copenhagen, v.24, n.1, p.72-77, Jan. 1997.

14. KORNMAN, K.S.; DI GIOVINE, F.S. Genetic variations in cytokine expression: a risk factor for severity of adult periodontitis. **Ann Periodontol**, Copenhagen, v.3, n.1, p.327-338, 1998.

15. LINDEN, G.J.; MULLALLY, B.H.; FREEMAN, R. Stress and the progression of periodontal disease. **J Clin Periodontol**, Copenhagen, v.23, n.7, p.675-80, July 1996.

16. MANSFIELD, J.C. Novel genetic association between ulcerative colitis and the anti-inflammatory cytokine interleukin-1 receptor antagonist. **Gastroenterology**, New York, v.106, n.3, p.637-642, Mar. 1994.

17. MASADA, MP *et al.* Measurement of interleukin-1α and -1β in gingival crevicular fluid: implications for the pathogenesis of periodontal disease. **J Periodont Res**, Copenhagen, v.25, n.3, p.156-163, May 1990.

18. MEALEY, B.L. Diabetes and periodontal disease: two sides of a coin. **Compend Contin Educ Dent**, Jamesburg, v.21, n.11, p.943-946, Nov. 2000.

19. MEIKLE, M.C. *et al.* Gingival fibroblasts degrade type I collagen films when stimulated with tumor necrosis factor and interleukin 1: evidence that breakdown is mediated by metalloproteinases. **J Periodont Res**, Copenhagen, v.24, n.3, p. 207-213, May 1989.

20. NONNENMACHER, C.; MUTTERS, R.; DE JACOBY, L.F. Microbiological characteristics of subgingival microbiota in adult periodontitis, localized juvenile periodontitis and rapidly progressive periodontitis subjects. **Clin Microbiol Infect,** Oxford, v.7, n.4, p.213-217, Apr. 2001.

21. POCIOT, F. *et al.* A Taq I polymorphism in the human interleukin-1 beta (IL-1β) gene correlates with secretions in vitro. **Eur J Clin Invest**, Berlin, v.22, n.6, p.396-402, June 1992.

22. POTEMPA, J.; BANBULA, A.; TRAVIS, J. Role of bacterial proteinases in matrix destruction and modulation of host responses. **Periodontol 2000**, Copenhagen, v.24, p.153-192, Oct. 2000.

23. REINHARDT, R.A. *et al.* Gingival fluid IL-1 and IL-6 levels in refractory periodontitis. **J Clin Periodontol**, Copenhagen, v.20, n.3, p.225-231, Mar. 1993.

24. ROSSOMANDO, E.F. *et al.* Tumour necrosis factor alpha in gingival crevicular fluid as a possible indicator of periodontal disease in humans. **Arch Oral Biol**, Oxford, v.35, n.6, p.431-434, 1990.

25. SCHENKEIN, H.A. *et al.* Smoking and its effects on early-onset periodontitis. **J Am Dent Assoc**, Chicago, v.126, n.8, p.1107-1113, Aug. 1995.

26. SHIRODARIA, S. *et al.* Polymorphisms in the IL-1A gene are correlated with levels of interleukin-1 alpha protein in gingival crevicular fluid of teeth with severe periodontal disease. **J Dent Res**, Washington, v.79, n.11, p.1864-1869, Nov. 2000.

27. SHU, K.H. *et al.* Impact of interleukin-1 receptor antagonist and tumor necrosis factor-alpha gene polymorphism IgA nephropathy. **Kidney Int**, New York, v.58, n.2, p.783-789, Aug. 2000.

28. SLOTS, J.; KAMMA, J.J. General health risk of periodontal disease. **Int Dent J**, London, v.51, n.6, p.417-427, Dec. 2001.

29. STASHENKO, P. *et al.* Levels of interleukin 1β in tissue from sites of active periodontal disease. **J Clin Periodontol**, Copenhagen, v.18, n.7, p.548-554, Aug. 1991a.

30. STASHENKO, P. *et al.* Tissue levels of bone resorptive cytokines in periodontal disease. **J Periodontol**, Copenhagen, v.62, n.8, p.504-509, Aug. 1991b.

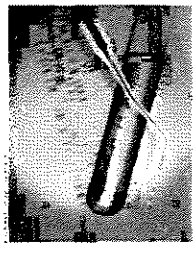
31. TARLOW, *et al.* Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of na 86-bp tandem repeat. **Hum Genet**, Berlin, v.91, n.4, p.403-404, May 1993.

32. THOMPSON, M.W., MCINNES, R.R., WILLARD, H.F. **Thompson & Thompson: Genetics in Medicine**. 5.ed. Pensilvania: Philadelphia, 1991. 500p.

33. TREVILATTO, P.C. *et al.* Polymorphism at position -174 of IL-6 gene is associated with susceptibility to chronic periodontitis in a Caucasian Brazilian population **J Clin Periodontol** [*In press*].

34. WACTAWSKI-WENDE, J. Periodontal diseases and osteoporosis: association and mechanisms. **Ann Periodontol**, Copenhagen, v.6, n.1, p.197-208, Dec. 2001.

35. WILSON, A.G. *et al.* Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. **Proc Natl Acad Sci USA**, Washington, v.94, n.7, p.3195-3199, Apr. 1997.



USE OF BUCCAL EPITHELIAL CELLS FOR PCR AMPLIFICATIONS OF LARGE DNA FRAGMENTS
The Journal of Forensic Odonto-Stomatology
v.18, n.1, June 2000, p.6-9

The Journal of Forensic Odonto-Stomatology

Forensic Odontology Unit
University of Adelaide
Adelaide, SA 5005
Australia

USE OF BUCCAL EPITHELIAL CELLS FOR PCR AMPLIFICATION OF LARGE DNA FRAGMENTS

P.C. Trevilatto, S.R.P. Line

Faculdade de Odontologia de Piracicaba - UNICAMP, Brazil

ABSTRACT

The analysis of human DNA is widely employed in the genetic studies of families and populations, and in most cases is performed with samples obtained from peripheral blood. The use of buccal epithelial cells as a source of DNA for PCR amplifications has several advantages over blood sampling but has only been used to amplify small fragments of DNA. Its use in forensic analysis has been limited to cases where the sampling of peripheral blood is not feasible. In the present study we show that buccal epithelial cells are a reliable source of DNA for the PCR amplification of high molecular mass fragments, which could be used in large-scale population sampling. Since most PCR gender-typing systems rely on the amplification and electrophoretic separation of the amelogenin gene, our results show that buccal epithelial cells may be the preferred source of DNA for gender -typing analysis. **(J Forensic Odontostomatol 2000; 18:6-9)**

Keywords: PCR, buccal epithelium, amelogenin.

INTRODUCTION

The analysis of human DNA is widely employed in genetic studies of families and populations and in most cases is performed with samples obtained from peripheral blood. Blood sampling is however an invasive procedure and sample collection may involve ethical problems in cases such as extreme illness, elderly persons and babies. Additionally, it requires medical supervision and specific equipment, which contribute to the increased overall costs of the procedure.

The polymerase chain reaction (PCR) provides a rapid and sensitive approach for the analysis of polymorphisms and mutations of the human genome. The use of buccal epithelial cells as a source of DNA for PCR reactions has been limited to studies of infectious agents present in oral mucosa;[1,2] and in forensic analysis, where the sampling of peripheral blood is not feasible.[3,4] Invariably, these studies report the amplification of small fragments of DNA (< 300 base pairs), which are suitable for the identification of viral and bacterial sequences, as well as the analysis of highly polymorphic loci in the human genome. However, amplification of larger DNA fragments may be desirable in some instances such as PCR-RLFP,[5] mismatch cleavage mutation analysis,[6,7] cloning, and sequencing of amplified sequences.[8] In the present study, we show that buccal epithelial cells are a reliable source of DNA for the PCR amplification of high molecular mass fragments, which could be used in large-scale population sampling and epidemiological studies, as well as in forensic analysis. As will be seen our results show that buccal epithelial cells may be the preferred source of DNA for gender -typing analysis.

MATERIALS AND METHODS

Sampling

A group of 83 consenting female subjects undertook a mouthwash containing 5 mL of 3 % sucrose rather than water in order to prevent osmotic imbalance tht would cause rupture of cells and loss of genomic DNA, for 2 min. A sterile wood spatula was then

used to scrape the buccal oral mucosa and the tip of the spatula shaken into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm for 10 min, the supernatant was discarded and the cell pellet resuspended in 500 uL of extraction buffer [10 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5 % SDS] with antibiotics. The samples were then frozen at –20°C until used for DNA extraction.

DNA extraction

After defrosting, the samples were incubated at 37°C with 100 ng/mL proteinase K*, and agitated overnight. DNA was then purified by sequential phenol/chloroform extraction and salt/ethanol precipitation and dissolved in 70 µL TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA]. The concentration was estimated by measurements of OD 260.

Polymerase chain reaction

Amplification reactions were performed with 300 to 700 ng DNA in a volume of 50 µL in reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, deoxyribonucleotides (200 µM each), 1 µM primers, 2U *Taq* DNA polymerase** and five pairs of primers were used. The primer sequences are as follows:

AMX1- 5' GGATTGGTTGTTACAGATGCC 3'
AMX2- 5' TTACTCACAGGCATGGCAAAAGCTGC 3'
AMX3- 5' CATTTCAGAACCATCAAGAAATGGG 3'
AMX4- 5' CTTTACAGAGCCCAGGGCATTG 3'
AMX5- 5' CCTCCCTGTAAAAGCTACCACC 3'
AMX6- 5' AATGTCTACATACCGGTGGCC 3'
AMX7- 5' GTAGAACTCACATTCTCAGGC 3'
AMX8- 5' GGCTTCAAAATATACTCACCACTTCC 3'
AMX9- 5' CCAGCCCCAGCCTGTTCAGCCAC 3'
AMX10- 5'TGTCTGCTAATGGTACTTTTTTAG 3'

Samples were heated initially to 95°C for 5 min, each cycle comprising denaturation at 95°C for 50 sec, primer annealing at 67°C for 1 min and polymerization at 72°C for 2 min. Samples were subjected to 35 cycles of amplification followed by a final extension of 72°C for 7 min. Amplification was carried out in a *Perkin-Elmer GeneAmp 2400 thermal cycler*#. Amplification products were visualized by electrophoresis on vertical 5% polyacrylamide gels in 1 X TBE (89 mM Tris-Borate, 89 mM boric acid, 2 mM EDTA), followed by silver staining¶.

RESULTS AND DISCUSSION

The use of buccal epithelial cells as a source of DNA for PCR amplifications has several advantages over blood sampling. The collection of material is fast and inexpensive, buccal samples can easily be obtained from people who are reluctant to donate blood, consent becomes simplified, there is no need of medical supervision during sampling, and the risk of contamination is reduced.[9,10]

The amplifications produced specific reaction products of 1690 base pairs [bp] (AMX1-AMX2), 1986 bp (AMX3-AMX4), 1550 bp (AMX5-AMX6), 879 bp (AMX7-AMX8), and 2039 bp (AMX9-AMX10), spanning the whole amelogenin X gene.

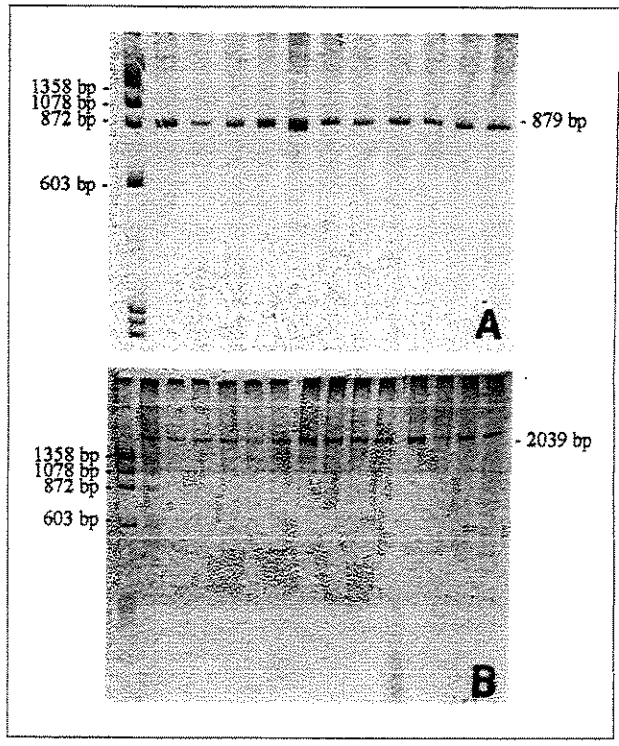


Figure 1. *Polyacrylamide gel electrophoresis showing PCR products from total genomic DNA derived from buccal epithelium. A: 879 bp product of amplification with primers AMX7 and AMX8. B: 2039 bp product of amplification with primers AMX9 and AMX10. Lane 1- ϕX-174-RF DNA Hae III digest molecular weight marker (Pharmacia).*

* Sigma Chemical Co., St. Louis, MO, USA
** Amersham Pharmacia Biotech, Uppsala, Sweden
\# Perkin-Elmer, Perkin-Elmer Co., Norwalk CT 06859, USA
¶ Bio-Rad Silver Stain Kit, Bio-Rad Laboratories, 200 Alfred

Fig. 1 illustrates typical PCR products from total genomic DNA derived from buccal epithelial cells showing that buccal epithelial cells can be used for the PCR amplification of large DNA fragments. The amount of DNA extracted ranged from 2.1 µg to 360 µg, which is sufficient to enable PCR amplification, with a success rate of identification of the gender gene, in this case the female, of around 90% of large fragments ranging from 879 to 2039 base pairs (Table 1). Failure to amplify DNA may result from degradation or from the presence of inhibitors which interfere with PCR reaction and can in most cases be overcome by adding bovine serum albumin to the PCR reaction,[11-13] or by repurification of DNA with Chelex extraction.[14] The use of small volumes of DNA extract is also recommended in order to reduce the amount of inhibitors.[3,14]

Primers	Fragment size (bp)	% positive
AMX1-AMX2	1690	91.5
AMX3-AMX4	1986	92.5
AMX5-AMX6	1550	88
AMX7-AMX8	879	88
AMX9-AMX10	2039	88

Table 1: *Positive amplification percentages of amelogenin X gene of 83 individuals.*

Gender determination can be a valuable piece of information in forensic investigations. Most PCR gender-typing systems rely on the amplification and electrophoretic separation of the amelogenin gene which produce small PCR products with 106-112 bp[15,16] or 330-218 bp[14] from the X and Y chromosomes respectively. However, the main difference between X and Y loci is a 177 bp insertion in the X gene[17] and the X and Y sequences can be amplified using a single set of primers which produce a 977 bp and 780 bp fragments,[18] which can be clearly distinguished in agarose or polyacrylamide gels. The results presented here show that buccal epithelial cells may be the preferred source of DNA for gender-typing analysis. Finally, it is worth mentioning that DNA extracted from this source has been routinely used in our laboratory for RFLP and direct sequencing of PCR products.

ACKNOWLEDGEMENTS

This project was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP, Grants 1995/4272-5 and 1996/11950-2.

REFERENCES

1. Robinson PA, High AS, Hume WJ. Rapid detection of human herpes simplex virus type I in saliva. Arch Oral Biol 1992; 37:797-806.

2. Otswald C, Muller P, Barten M, Rutsatz K, Sonnemberg M, Milde-Langosch K. Human papillomavirus DNA in oral squamous cell carcinomas and normal mucosa. J Oral Pathol Med 1994; 23:220-5.

3. Fridez F, Coquoz R. PCR DNA typing of stamps: evaluation of the DNA extraction. Forensic Sci Int 1996; 78:103-10.

4. Sweet D, Lorente JA, Valenzuela A, Lorente M, Villanueva E. PCR-based DNA typing of saliva stains recovered from human skin. J Forensic Sci 1997; 42:447-51.

5. Meltzer SJ. Determination of loss of heterozygosity using polymerase chain reaction. In: White BA. Ed. Methods in molecular biology. PCR Protocols. Current Methods and Applications.1993: 129-36.

6. Ellis TP, Humphrey KE, Smith MJ, Cotton RG. Chemical cleavage of mismatch: a new look at an established method. Hum Mutat 1998; 11:345-53.

7. Taylor GR, Deeble J. Enzymatic methods for mutation scanning. Genet Anal 1999; 14: 181-6.

8. Meltzer SJ. Direct sequencing of polymerase chain reaction products. In: White BA. Ed. Methods in molecular biology. PCR Protocols. Current Methods and Applications. 1993: 137-42.

9. Lench N, Stanier P, Williamson R. Simple non-invasive method to obtain DNA for gene analysis. Lancet 1988; 1356-8.

10. Meulenbelt I, Droog S, Trommelen GJM, Boomsma DI, Slagboom PE. High-yield noninvasive human genomic DNA isolation method for genetic studies in geographically dispersed families and populations. Am J Hum Genet 1995; 57:1252-4.

11. Hagelberg E, Sykes B, Hedges R. Ancient bone DNA amplified. Nature 1989; 342:485.

12. Gehrig C, Hochmeister MN, Budowle B, Reynolds R, Dirnhofer R. HLA-DQA1 subtyping data in the swiss population. Forensic Sci Int 1996; 83:27-30.

13. Hochmeister MN. A discussion of 'PCR DNA typing of stamps: evaluation of the DNA extraction'. Forensic Sci Int 1996; 83:75-7.

14. Faerman M, Filon D, Kahila G, Greenblatt CL, Smith P, Oppenheim A. Sex identification of archaeological remains based on amplification of the X and Y amelogenin alleles. Gene 1995; 167:327-32.

15. Sullivan KM, Mannucci A, Kimpton CP, Gill P. A rapid and quantitative DNA sex test: fluorescence-based PCR analysis of X-Y homologous gene amelogenin. Biotechniques 1993; 15:636-41.

16. Mannucci A, Sullivan KM, Ivanov PL, Gill P. Forensic application of a rapid and quantitative DNA sex test by amplification of the X-Y homologous gene amelogenin. Int J Legal Med 1994; 106:190-3.

17. Nakahori Y, Takenaka O, Nakagome Y. A human X-Y homologous region encodes amelogenin. Genomics 1991; 9:264-9.

18. Copelli SB, Bergadá C, Billerbeck AEC, Goldberg A.C, Kalil J, Damiani D, Targovnik HM. Molecular analysis of sex determination in sex-reversed and true hermaphroditism. Braz J Med Biol Res 1996; 29:743-8.

Address for correspondence:
Sergio R.P. Line
Av. Limeira 901
13414-018 Piracicaba-SP
Brazil
Email: serglin@fop.unicamp.br

COMITÊ DE ÉTICA

UNICAMP

COMITÊ DE ÉTICA EM PESQUISA

Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba
CEP-FOP-UNICAMP

CERTIFICADO

Certificamos que o Projeto de pesquisa intitulado "Suscetibilidade genética à severidade da doença periodontal em adultos e em famílias com perodontite de início precoce", sob o protocolo nº **63/99**, do Pesquisador(a) ***Paula Cristina Trevilatto,*** sob a responsabilidade do Prof(a). Dr(a). ***Sérgio Roberto Peres Line***, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa – FOP.

Piracicaba, 10 de setembro de 1999

We certify that the research projet with title "Genetic susceptibility to severity of periodontal disease in adults and in families with early-onset periodontitis", protocol nº **63/99**, by Researcher ***Paula Cristina Trevilatto***, responsibility by Prof. Dr. ***Sérgio Roberto Peres Line***, is in agreement with the Resotion 196/96 from National Committee of Health/Health Department (BR) and was approved by the Ethical Committee in Research at the Piracicaba Dentistry School/UNICAMP (State University of Campinas).

Piracicaba, SP, Brazil, 10 september 99

Prof. Dr. Pedro Luiz Rosalen
Secretário - CEP/FOP/UNICAMP

Prof. Dr. Antonio Bento Alves de Moraes
Coordenador - CEP/FOP/UNICAMP