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Estudo da associação entre Estomatite Ulcerativa Recorrente, Síndrome da Ardência Bucal e polimorfismos funcionais nos genes SLC6A4, IL1B,IL6,IL10, TNFA

Tese apresentada ao Curso de Pós-graduação em Farmacologia Bioquímica e Molecular do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Farmacologia Bioquímica e Molecular.

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‘’No meio da dificuldade, está a oportunidade’’

Afinal,

"O único lugar aonde o sucesso vem antes do trabalho é no dicionário."

(Albert Einstein)

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RESUMO

Este trabalho teve como objetivo geral investigar uma possível associação entre cinco polimorfismos genéticos funcionais em duas doenças bucais de etiologias diferentes em pacientes brasileiros. Para isto, a tese foi dividida em três artigos.

No primeiro, foi avaliada a associação entre os polimorfismos genéticos IL-1B +3954 (C/T) e 5HTTLPR com a Síndrome da Ardência Bucal (SAB). Para este estudo foi extraído o DNA de trinta pacientes com SAB e trinta e um controles. Não observamos diferença estatística entre os grupos quanto à distribuição do polimorfismo 5-HTTLPR (P=0.60), por outro lado, um aumento significante do genótipo heterozigoto, CT, foi encontrado nos pacientes com SAB (P=0.005). Concluímos então que há uma associação entre o genótipo alto produtor de IL-1β e SAB, embora mais estudos são necessários para identificar o papel desta citocina na etiologia da SAB.

Em seguida, estudamos a associação entre o polimorfismo IL-1B +3954 (C/T) e a EUR. Sessenta e dois pacientes com EUR e 62 voluntários saudáveis foram genotipados para o polimorfismo IL-1B +3954. Um aumento significante da presença do genótipo heterozigoto, CT, foi observado nos pacientes com EUR (p= 0.01). Estes resultados sugerem que há uma associação entre o genótipo que confere uma produção aumentada de IL-1β e EUR.

Por último, avaliamos pela primeira vez, através análise multivariada, a associação de polimorfismos genéticos nos genes *IL-B, IL-6, IL-10* e *TNFA* e EUR. Sessenta e quatro pacientes com EUR econtroles participaram deste estudo. Concluímos que existe uma associação entre os genótipos heterozigotos dos genes *IL-1* e *TNFA* (p= 0.03 e p=0.04, respectivamente) e EUR na população estudada.

SUMÁRIO

1- INTRODUÇÃO

1.1- Polimorfismos Genéticos

O conceito tradicional de polimorfismo presente nos livros textos é o de alterações na carga genética dos indivíduos, ocorrendo em uma freqüência de, no mínimo 1% de uma determinada população, que resultam em variações dentro de um padrão ainda considerado biologicamente normal, podendo causar ou não alterações na função da proteína e fenótipo. Existe uma proposta para se agrupar polimorfismos genéticos e mutações em um único grupo, porém na literatura encontramos uma grande variedade de nomenclatura destas variações nas seqüências genéticas (den Dunnen & Antonarakis, 2001).

Um polimorfismo na região promotora poderá alterar a proporção da transcrição de uma determinada proteína. Já quando localizado na região codificadora ou nos limites intron/exon, pode produzir proteínas incompletas ou inativas, como resultado de um splicing incorreto do ácido ribonucléico mensageiro (RNAm). Polimorfismos genéticos caracterizados por completas deleções gênicas eliminam qualquer atividade funcional da proteína, enquanto polimorfismos genéticos que são duplicações do gene inteiro podem resultar em elevados níveis de atividade (Miller e cols., 2001).

As técnicas mais usadas para se detectar a ocorrência de polimorfismos genéticos envolvem os polimorfismos genéticos de comprimento de fragmentos de restrição (RFLPs) (Botstein e cols., 1980), e os polimorfismos genéticos de número variável de repetições em tandem (VNTRs) (Moretti e cols., 2001). Alguns polimorfismos genéticos de ponto que criam ou destroem sítios de restrição enzima-específicos esta técnica e denominada de RFLP. Como as enzimas de restrição têm seqüências de reconhecimento específicas no DNA, as alterações da seqüência do DNA genômico acarretam na criação ou destruição de sítios de clivagem alterando, desse modo, o tamanho de um ou mais fragmentos de DNA oriundos da ação da enzima de restrição (Miller e cols, 2001). Os

polimorfismos genéticos por inserção/deleção que consistem numa série de comprimentos de fragmentos alélicos, relacionados entre si por um número variável de seqüências de DNA repetidas em tandem no intervalo entre dois sítios de restrição são os VNTRs (Moretti e cols, 2001).

Os VNTRs e os RFLPs detectam polimorfismos de forma similar, através da amplificação da região de interesse pela técnica da reação em cadeia da polimerase (PCR) e, se necessário, posterior tratamento com enzimas de restrição que reconhecem sítios específicos e originam fragmentos de DNA com comprimentos variados. Enquanto os RFLPs revelam polimorfismos genéticos devido à presença ou ausência de um sítio de restrição, os VNTRs revelam polimorfismos genéticos devido a números diferentes de repetições situadas entre o sítio de amplificação (Botstein e cols, 1980; Moretti e cols, 2001).

Polimorfismos genéticos funcionais em genes de citocinas e outros mediadores inflamatórios, que podem confirmar diferenças interindividuais na síntese e secreção dessas proteínas, têm sido associados a doenças que apresentam componentes inflamatórios (Parkhill e cols., 2000) ou comportamentais (Brydon e cols., 2005). Portanto, a investigação e a caracterização dos elementos específicos alterados podem proporcionar biomarcadores aplicáveis em diagnóstico e prognóstico, estimando o risco em indivíduos (Kornman e cols., 1997).

1.2- Resposta imune e Citocinas

As células e moléculas responsáveis pela imunidade constituem o chamado sistema imune e a resposta deste sistema frente a uma agressão é denominada resposta imune. Esta foi didaticamente dividida em resposta inata e adaptativa. A resposta inata se dá através dos mediadores inatos tais como toxinas bacterianas, neuropeptídeos,

peptídeos fibrinolíticos, cininas, fragmentos do sistema complemento, aminas vasoativas, enzimas lisossomais e citocinas. Em relação à resposta adaptativa, esta pode ser mediada por células, através das ações de linfócitos T (LT) e citocinas (resposta imune celular), ou mediada por anticorpos, produtos de linfócitos B (LB) ativados (resposta imune humoral)(Akira e cols., 2006)

Citocinas são geralmente proteínas ou glicoproteínas de peso molecular relativamente baixo (8 a 30kD) e, freqüentemente, consistem de uma única cadeia polipeptídica. Elas regulam processos biológicos importantes, tais como: crescimento e ativação celulares, inflamação, imunidade, reparo tecidual, fibrose e morfogênese (Van Wyk, 1992; Hopkins, 2003).

O termo citocinas já esteve baseado nos tipos celulares que as produziam; portanto, monocinas, linfocinas e interleucinas eram utilizadas para identificarem produtos de macrófagos, linfócitos e leucócitos, respectivamente. Algumas citocinas são fatores quimiotáticos para certos tipos celulares e são denominadas quimiocinas; já outras receberam a nomenclatura de acordo com suas funções biológicas. Com o advento das técnicas moleculares, tornou-se claro que a mesma proteína pode ser sintetizada por uma variedade de tipos celulares, incluindo células endoteliais e algumas células epiteliais. Por isso, o termo genérico citocinas tem sido preferido para designar essa classe de mediadores (Hopkins, 2003).

Algumas citocinas promovem inflamação e são chamadas de pró-inflamatórias, ao passo que outras, por suprimirem tal atividade, são chamadas de antiinflamatórias. Interleucina-4 (IL-4), (IL-10) e interleucina-13 (IL-13) são potentes ativadoras de linfócito B; entretanto, elas também são importantes agentes antiinflamatórios por possuírem a habilidade de suprimirem genes das citocinas pró-inflamatórias, como IL-1, TNF, e quimiocinas (Dinarello, 2000).

A secreção de citocinas é um evento breve e autolimitado, não existindo como moléculas pré-formadas, e sim necessitando de ativação transcricional. Uma vez sintetizadas, elas são rapidamente secretadas (Arai e cols., 1992).

As ações das citocinas se realizam através de ligações de alta afinidade a receptores específicos, localizados nas membranas das células-alvo (van e cols., 1992). Diferentes citocinas utilizam vias de sinalização especializadas, tal como a via Janus Kinases (JAKs) / Sinal Transducers and Activators of Transcription (STATs). A porção citoplasmática de muitos receptores de citocinas está associada aos membros dos receptores de tirosina-quinase da família das JAKs. Após a ligação, os JAKs tornam-se ativados por fosforilação. Uma vez ativados, eles fosforilam resíduos específicos de tirosina nos receptores de citocinas. Esses resíduos servem como porta para a entrada dos fatores de transcrição conhecidos como STATs. Proteínas STATs específicas e, até então, inativas são recrutadas aos receptores das citocinas e, então, fosforiladas. Ao mesmo tempo em que são liberadas do receptor, as STATs dimerizam-se e são translocadas para o núcleo. Nesse local, dímeros de STATs se ligam a seqüências específicas próximas aos promotores dos genes induzidos por citocinas, resultando na indução de sua produção (Leonard & O'Shea, 1998).

Citocinas possuem efeitos locais e sistêmicos, apresentando padrões de ação autócrinos, parácrinos e endócrinos (van e cols, 1992).

1.2.1- Interleucina-1β

A IL-1β madura é uma proteína de 17,5KD codificada pelo respectivo gene IL-1B e que se encontra arranjado em cluster, juntamente com os genes IL-A e IL-1RN, no braço longo do cromossomo 2 humano em uma região de 430 Kb (Nicklin e cols., 1994). Os

primeiros estudos sobre a citocina IL-1β apontaram a sua a capacidade de produzir febre (di Giovine & Duff, 1990). Estes resultados também foram confirmados posteriormente (ATKINS, 1960; Dinarello, 2000). Além do mais, a IL-1β tem se mostrado não só como potente pirógeno endógeno, mas também a citocina pró-inflamatória mais potente podendo então, influenciar a resposta do hospedeiro em inúmeras doenças (Merriman e cols., 1977).

A IL-1β está envolvida na ativação de células endoteliais e conseqüente aumento da expressão da molécula de adesão intercelular-1 (ICAM-1) e selectina-E, proporcionando a migração e recrutamento celular (Figueredo e cols., 1999). Os aumentos nos níveis destas moléculas de adesão em algumas doenças, como a EUR, podem estar associados com um polimorfismo genético associado à maior produção de IL-1β, o que pode favorecer o desenvolvimento das úlceras (Bazrafshani e cols., 2002a; Guimarães AL e cols., 2006a). Além disso, níveis aumentados de IL-1β têm se mostrado também no sangue e líquor de pacientes deprimidos (Maes e cols., 1991a; Maes e cols., 1991b).

Outros estudos também sugerem que essa citocina pode estimular a liberação de outros mediadores pró-inflamatórios, como a IL-8 e TNF-α (Figueredo e cols, 1999).

As fontes primárias de IL-1β são os monócitos, macrófagos e células dendríticas. Linfócitos B, células natural killer (NK) e queratinócitos também produzem essa citocina (Dinarello, 1989).

Recentemente, polimorfismos genéticos dentro dos genes desse cluster foram descritos e algumas dessas variações genéticas têm sido associadas a diferenças nos níveis produzidos de IL-1α e IL-1β (Molvig e cols., 1988; Endres e cols., 1989), levando alguns indivíduos a apresentarem uma resposta inflamatória mais exacerbada que outros, frente ao mesmo estímulo. Polimorfismos genéticos funcionais no locus –511 e +3954 do

gene da IL-1β já foram descritos (Pociot e cols., 1992). O polimorfismo funcional mais estudado é o single nucleotide polymorphism (SNP) que resulta na troca de uma citosina por uma timina na região codificadora, na posição +3954 do exon 5, destruindo assim, um sítio de restrição para a enzima de restrição Taq I (Pociot e cols, 1992). Nesse tipo de polimorfismo, os monócitos de indivíduos homozigotos para o alelo [T] produzem duas vezes mais IL-1β do que monócitos heterozigotos e quatro vezes mais do que monócitos dos indivíduos homozigotos para o alelo [C] após a estimulação com lipopolissacarídeo (LPS) (Pociot e cols, 1992). A resposta inflamatória que é direcionada em grande parte pela IL-1 é, portanto, geneticamente determinada, com alguns indivíduos tendo uma resposta mais exacerbada do que outros quando submetidos a um mesmo estímulo (Lang e cols., 2000). Diversos estudos avaliando a presença do alelo T têm relacionado este genótipo com inúmeras entidades patológicas, tais como artrite reumatóide, psoríase, doença periodontal, fibrose pulmonar e lúpus eritematoso (di Giovine & Duff, 1990; Pociot e cols, 1992; Kornman e cols, 1997; Cox e cols., 1999; Whyte e cols., 2000).

1.2.2- Interleucina-6

A IL-6 é uma glicoproteína com peso molecular variando de 20 a 30 KD, dependendo do tipo celular de origem que atua nas imunidades inata e adaptativa. O gene IL-6 se localiza no cromossomo 7. (Sehgal e cols., 1987). A transcrição dessa citocina é regulada pelos fatores de transcrição fator nuclear IL-6 (NFIL-6), NFκB, Fos/Jun, CRBP e receptor de glicocorticóide (Terry e cols., 2000)

Essa citocina não é espontaneamente produzida por células normais intactas (Kupper, 1990), sendo que sua síntese e liberação requerem estímulo inflamatório ou presença de outras citocinas como por exemplo, IL-1β e TNF-α (Kishimoto, 1989;

Littlewood e cols., 1991). A IL-6 é produzida por muitos tipos celulares, tais como linfócitos T e B, células NK, monócitos, macrófagos, adipócitos, mastócitos, células endoteliais e queratinócitos (Bauer & Herrmann, 1991).

A IL-6 possui várias ações na imunidade inata, onde estimula a síntese de proteínas de fase aguda por hepatócitos, contribuindo para os efeitos sistêmicos da inflamação (Crowl e cols., 1991). Ela também inibe a produção de TNF-α e IL-1 por células sanguíneas mononucleares, através da indução de seus receptores antagonistas (SCHINDLER, 1952; Tilg e cols., 1994).

Na imunidade adaptativa, essa citocina induz ativação, proliferação e diferenciação de linfócitos T, com produção de linfócitos T citotóxicos (LTc) e estimula o crescimento e diferenciação de linfócitos B (SCHINDLER, 1952). A IL-6 tem-se mostrado potente indutora de fosfolipase A2, intermediária na via final da produção de leucotrienos, PGs, e fator ativador de plaquetas (Crowl e cols, 1991). Entretanto, dados na literatura vêm também sugerindo um papel antiinflamatório para a IL-6 (SCHINDLER, 1952; Akira e cols., 1993; Tilg e cols, 1994; Ramsay e cols., 1994; Balto e cols., 2001). Uma vez que, essa citocina regula, positivamente, o inibidor tecidual das metaloproteinases-1 (TIMPs-1) (Sato e cols., 1990; Kopf e cols., 1994). Existem evidências de que proteínas de fase aguda, reguladas por IL-6, também possuem propriedades antiinflamatórias e imunossupressoras e atuam como antiproteinases e captadoras de oxigênio (Tilg e cols, 1994; Baumann & Gauldie, 1994; Jordan e cols., 1995; Tilg e cols., 1997).

Polimorfismos genéticos na região promotora do gene IL-6, como o situado na região -174 (G/C), podem resultar em variações inter-individuais no que diz respeito à expressão protéica (Ray e cols., 1990). Em um estudo onde se reproduziu in vitro as variantes alélicas deste polimorfismo, a variante G esta relacionada a uma maior produção de IL-6 em relação ao alelo C. Além disso, os níveis de expressão de IL-6 da

variável C não se alteraram após estimulação com LPS ou IL-1 (Fishman e cols., 1998). Uma explicação sugerida para este fato é a sua localização próxima ao sítio de ligação do receptor para glicocorticóide, um domínio de regulação negativa do promotor capaz de suprimir a transcrição do gene da IL-6 (Ray e cols, 1990). A presença do alelo G foi associada à várias doenças como artrite crônica juvenil precoce sistêmica (Fishman e cols, 1998), artrite reumatóide (Hirano e cols., 1988), osteoporose (Jilka e cols., 1992; Poli e cols., 1994) e psoríase (Grossman e cols., 1989). O alelo C mostra associação com riscos reduzidos para a doença de Alzheimer (Papassotiropoulos e cols., 1999).

1.2.3- Interleucina-10

IL-10 é uma citocina com peso molecular estimado em 18,647 KD (Vieira e cols., 1991) que se encontra profundamente envolvida na regulação das reações inflamatórias e respostas imunes. O gene da IL-10 humana está localizado no cromossomo 1q e consiste de cinco exons separados por quatro introns (Eskdale e cols., 1998).

A IL-10 afeta não somente o sistema imune como muitos processos incluindo angiogênese e tumorigênese. É uma molécula-chave no que diz respeito à diminuição do potencial patológico dos processos auto-imunes, através da inibição de muitos eventos da resposta inflamatória (de Waal e cols., 1992).

Ela apresenta efeitos inibitórios sobre a produção de citocinas pró-inflamatórias como IL-1α, IL-1β, IL-6, IL-8, IL-12 e TNF-α além de regular negativamente a expressão de moléculas ativadoras e coestimulatórias sobre monócitos e células dendríticas (Moore e cols., 1993; D'Andrea e cols., 1993; Macatonia e cols., 1993). A IL-10 possui atividades de fator de crescimento sobre linfócitos B e mastócitos, e pode tanto inibir quanto

aumentar as atividades dos linfócitos T, dependendo das condições de ativação e das subclasses destes linfócitos (Moore e cols, 1993).

IL-10 é produzida por linfócitos T virgem e os de memória, clones pertencentes aos sub-tipos T helper 1 (Th1) e T helper 2 (Th2), células NK, LB, linhagens de células B transformadas pelo vírus Epstein-Barr, monócitos, trofoblastos, células epiteliais bronquiais, e certas células tumorais incluindo melanomas e carcinomas de várias origens (de Waal e cols, 1992; Moore e cols, 1993).

Vários polimorfismos genéticos têm sido observados nesse gene na região flanqueadora 5'. Eles incluem duas áreas 6-11 (CA)n em regiões microssatélites, nas posições -1109 e -3942, assim como três SNP, nas posições -1082 (G/A), -819 (C/T) e -592 (C/A) (Eskdale e cols, 1998; Hurme e cols., 1998). Tais polimorfismos genéticos têm sido relacionados à produção aumentada de IL-10 por monócitos e células T e também por pacientes portadores ou sujeitos a desenvolverem lúpus eritematoso sistêmico (Mehrian e cols., 1998), doença meningocócica (Westendorp e cols., 1997), artrite reumatóide (Eskdale e cols, 1998) e pode ser um fator prognóstico para rejeição seguida de transplante renal (Sankaran e cols., 1999).

Em relação à funcionalidade, o polimorfismo -1082 (G/A) no gene IL-10 tem o seu alelo G associado a uma maior produção de IL-10 quando comparado ao alelo A (Turner e cols., 1997). A diferente produção de IL-10 entre as variantes polomórficas pode ser explicada pelo fato do lócus -1082 se situar dentro de uma região de ligação com o fator de transcrição proteína ativadora-1 (AP-1) (Kube e cols., 1995).

1.2.4- Fator de Necrose Tumoral-α

TNF-α é uma citocina de 17kD e é o principal mediador da resposta inflamatória contra as bactérias gram-negativas e outros microrganismos. O gene TNFA está

localizado na região de classe III do complexo de histocompatibilidade principal (MHC), no braço curto do cromossomo 6 (Stuber e cols., 1995).

O TNF-α é responsável por muitas das complicações sistêmicas de vários processos como a evolução da septicemia para falência múltipla dos órgãos. Essas reações do hospedeiro estão associadas a níveis séricos aumentados dessa citocina (Stuber e cols, 1995; Stuber e cols., 1996).

Pelo menos nove polimorfismos genéticos já foram descritos dentro da região promotora do TNFA até o momento, nas posições -1301, -863, -857, -575, -376, -308, -244 e -238, em relação ao sítio de transcrição (Bayley e cols., 2001). O polimorfismo na posição -308 (G/A) foi associado à produção alterada de linfócitos, estando o alelo com adenina [A] associado de seis a sete vezes à maior atividade transcricional no gene do que o alelo [G] (Wilson e cols., 1997).

1.3- Transmissão Serotoninérgica

A serotonina (5-hidroxitriptamina) é um neurotransmissor do sistema nervoso central e periférico, sendo sintetizada a partir de enzimas após o aminoácido precursor, triptofano, ser transportado para dentro do neurônio serotoninérgico. A bomba de transporte do triptofano é distinta do transportador de serotonina (5-HTT). Uma vez transportado para o interior do neurônio serotoninérgico, o triptofano é convertido em 5-hidroxitriptofano pela enzima triptofano hidroxilase e em seguida convertido em 5-hidroxitriptamina pela enzima aromática triptofano decarboxilase. A serotonina é armazenada em vesículas sinápticas onde permanece até ser liberada por um impulso neuronal. Depois da liberação de serotonina na sinapse cerebral, este neurotransmissor é recaptado pelo neurônio pré-sináptico via transportador de serotonina (Millan, 2003).

1.3.1- Polimorfismo do Gene Transportador de Serotonina (5-HTTLPR)

O gene do transportador de serotonina foi clonado em cérebro de ratos (Millan, 2003). Posteriormente, foi então identificado uma seqüência de cDNA placentário humano que possuía 92% de homologia em relação à seqüência encontrada no cérebro de rato. Estes dados possibilitaram que se descobrisse um único gene, denominado SLC6A4, responsável por codificar o transportador de serotonina e que se localiza no cromossomo 17q11.1-q12 (Ramamoorthy e cols., 1993a), flanqueado pelos marcadores D17S58 e D17S73. (Gelernter e cols., 1995). Este gene apresenta-se em uma seqüência de 31 KB e é organizado em 14 exons (Lesch e cols., 1994).

Dois polimorfismos genéticos já foram descritos no gene SLC6A4. O primeiro consiste em uma série de repetições dentro da região promotora do gene denominado por 5-HTTLPR, serotonin transporter-linked polymorfism (Heils e cols., 1995). O segundo ocorre no intron 2, onde se pode observar um número variável de repetições em tandem (VNTRs) de um segmento de 17 pares de base e que não tem sido relacionado a um efeito direto na expressão do gene (Ogilvie e cols., 1996).

O 5-HTTLPR é um polimorfismo que se localiza aproximadamente 1kb acima do sítio inicial de transcrição deste gene e é composto de 16 elementos de repetição, elementos estes ricos em CG e formados por 20 a 23 pares de base. Existem dois alelos comuns nesta região que diferem em 44 pares de base. A variante alélica longa (l) é composta pelos 16 elementos de repetição e a variante curta é formada pela deleção de 44 pb (Heils e cols., 1996). A presença do alelo curto foi associada com a redução da eficiência da transcrição do 5-HTTLPR. Além disso, foi observado que em linhagens linfoblásticas de células humanas homozigóticas para a variante longa, a concentração de RNA mensageiro do transportador de serotonina produzida é quase duas vezes maior que em células contendo uma ou duas cópias da variante curta (Lesch e cols., 1996). Estes

dados corroboram com estudo realizado posteriormente em plaquetas humanas (Greenberg e cols., 1999).

Alguns estudos têm mostrado o possível envolvimento do 5-HTTLPR com vários distúrbios psiquiátricos como comportamento suicida (Bellivier e cols., 2000; Bondy e cols., 2000; Courtet e cols., 2001), depressão (Bellivier e cols., 1998), ansiedade (Serretti e cols., 2002) e alcoolismo (Preuss e cols., 2000).

O alelo curto mostrou-se associado com o comportamento suicida violento (Bellivier e cols, 2000), enquanto que, uma ligação entre o alelo curto com o número de tentativas e tentativa mais violenta de suicídio foi observado em estudos que envolveram pacientes alcoólatras (Preuss e cols, 2000). Posteriormente, estudos em populações de pacientes psquiátricos corroboram estes dados (Courtet e cols, 2001).

Por outro lado, trabalhos também apresentam resultados diferentes. A associação do alelo longo com pacientes deprimidos que tentaram suicídio também foi observada (Du e cols., 1999; Russ e cols., 2000).

O polimorfismo 5HTTLPR pode estar associado com doenças bucais relacionadas ao stress. Recentemente foi observada uma associação deste polimorfismo com a EUR (Victoria e cols., 2005).

1.4- Estomatite Ulcerosa Recorrente (EUR)

A EUR, popularmente conhecida como afta, foi primeiramente descrita por Hipócrates (460-370 AC), sendo que o termo aphthai estaria relacionado a qualquer tipo de desordem bucal (Ship e cols., 2000). Hoje é uma das condições patológicas mais freqüentes da mucosa bucal, sendo caracterizada pelo desenvolvimento de ulcerações dolorosas, solitárias ou múltiplas na mucosa bucal. Tem sido estimado que pelo menos

20% da população geral vão sofrer episódios de EUR pelo menos uma vez em sua vida (Stanley, 1972; Axell & Henricsson, 1985). As lesões localizam-se em grande parte da mucosa bucal, sendo que a ocorrência dessas lesões na gengiva inserida e no palato duro é rara. A EUR começa como uma erosão superficial, única ou múltipla, coberta por uma membrana cinzenta. Geralmente tem margens bem circunscritas por um halo eritematoso. A lesão é bastante dolorosa podendo comprometer a alimentação por vários dias (Ship, 1996; Jurge e cols., 2006).

A classificação da EUR é fundamentalmente baseada no tamanho das lesões, distinguindo-se, portanto, as formas clínicas menores e maiores da doença. Múltiplas lesões puntiformes, pequenas e coalescentes, caracterizam uma terceira variante clínica, a forma herpetiforme (Ship, 1996; Natah e cols., 2004; Jurge e cols, 2006).

A EUR do tipo Menor é a forma mais comumente relatada, ocorrendo em cerca de 80% dos pacientes acometidos por EUR (Vincent & Lilly, 1992). As úlceras originam-se, quase que exclusivamente, na mucosa não-ceratinizada. Apresentam entre 3 e 10 mm de diâmetro, desaparecendo, sem deixar cicatrizes, em 7 a 14 dias (Greer, Jr. e cols., 1993). As mucosas jugal e labial são os sítios mais comumente envolvidos, seguidos pela superfície ventral da língua, fundo do vestíbulo, assoalho da boca e palato mole (Natah e cols, 2004; Jurge e cols, 2006). Estas ulcerações se iniciam, usualmente, na infância ou adolescência, e a freqüência das recorrências é altamente variável, oscilando de uma ulceração em poucos anos até mais de dois episódios por mês (Jurge e cols, 2006).

Na EUR do tipo Maior as lesões são maiores e mostram maior duração por episódio. As ulcerações são mais profundas que a variante menor, medindo de 1 a 3 cm de diâmetro, levando de duas a seis semanas para reparar, podendo deixar cicatriz (Natah e cols, 2004). O número de lesões varia de 1 a 10. Qualquer área de superfície bucal pode ser afetada, mas a mucosa labial, o palato mole e as fossas amigdalianas são

os sítios mais afetados. O início ocorre após a puberdade, e os episódios recorrentes podem continuar a se desenvolver por 20 anos ou mais (Natah e cols, 2004; Jurge e cols, 2006).

Na EUR do tipo Herpetiforme existe um número maior de lesões e as recorrências são mais freqüentes. As lesões individuais são pequenas, variando de 1 a 3 mm de diâmetro, em um único ataque podem surgir mais de uma dezena delas (Natah e cols, 2004; Jurge e cols, 2006). É comum ocorrer agrupamento de lesões individuais resultando na formação de ulcerações grandes e irregulares, que cicatrizam em 7 a 10 dias (Porter e cols., 1998). Qualquer superfície bucal pode estar envolvida. Observa-se predominância no sexo feminino com o seu início na fase adulta (Porter e cols, 1998).

Embora a etiologia da EUR não tenha sido esclarecida, diversos fatores sistêmicos predispõem ao desenvolvimento da EUR. As lesões podem ser encontradas na doença de Behçet (Rogers, 1997), neutropenia cíclica (Scully & Porter, 1989), em deficiências nutricionais, com ou sem distúrbios gastrointestinais (Grattan & Scully, 1986), em pacientes infectados pelo vírus HIV (Kerr & Ship, 2003) e portadores da Doença de Crohn (Plauth e cols., 1991). Diversos estudos mostram que deficiências de ferro, ácido fólico e vitamina B12 são bem mais freqüentes nos pacientes com EUR do que na população geral (Rogers, 1997; Porter e cols, 1998). Foi observado que após a terapia de reposição desses componentes, houve remissão completa ou pelo menos melhora das lesões (Wray, 1982).

A hipersensibilidade alimentar tem sido relacionada como um elemento predisponente à EUR (Nolan e cols., 1991; McCartan e cols., 1996b). Uma vez que eliminação de determinados alimentos da dieta resulta na melhora ou remissão das úlceras entre 25% a 75 % dos casos (Nolan e cols, 1991). Porém, outros estudos não corroboram com estes dados (Eversole e cols., 1982).

Diversos estudos sugerem associação de agentes microbianos com a EUR. Dentre estes, a bactéria H. pylori tem sido um microorganismo de grande interesse. Devido às similaridades histológicas entre a EUR e a úlcera gástrica, o envolvimento do H. pylori na etiologia da EUR tem sido muito estudado (Porter e cols, 1998; Birek e cols., 1999; Brozovic e cols., 2002; Victoria e cols., 2003). Tem se cogitado que a colonização da mucosa bucal por bactérias como *Streptococcus sanguis* e *Streptococcus mutans* e seus antígenos podem ser responsáveis pelo início da resposta imune (Hasan e cols., 1995; Sun e cols., 2002).

Uma possível associação entre sistema imune e EUR foi primeiramente considerada por volta de 1960 e confirmada por outros estudos posteriormente (Graykowski e cols., 1966; Lewkowicz e cols., 2003; Borra e cols., 2004). Além do mais uma produção aumentada de citocinas Th1 como, por exemplo, IL-2, TNF-α e IL-6 foi observada em células do sangue periférico de pacientes com EUR (Lewkowicz e cols., 2005). Recentemente foi observado que a expressão de genes Th1 estava aumentada em relação à Th2 nas lesões da EUR do que em mucosa normal (Borra e cols, 2004). Vários polimorfismos genéticos de interleucinas foram estudados na EUR e forte associação foi observada com genótipos alto produtores de IL-1β. Apesar disso, resultados descordantes são também encontrados (Bazrafshani e cols, 2002a; Bazrafshani e cols., 2002b; Bazrafshani e cols., 2003; Guimarães AL e cols, 2006a).

As evidências atuais indicam a presença de fatores genéticos associados com EUR uma vez que, pacientes com história familiar de EUR são mais suscetíveis a desenvolverem a doença precocemente e apresentarem quadro clínico mais grave do que os indivíduos sem história familiar (Ship, 1965; Miller e cols, 2001). Alem disso, há uma alta correlação entre EUR em gêmeos monozigóticos o que não é observado em gêmeos heterozigóticos (Miller & Ship, 1977).

Foi observado uma associação entre trauma na mucosa e o aparecimento de úlceras em pessoas susceptíveis à EUR (Wray, 1982). Um possível mecanismo para explicar esta associação seria a diminuição da barreira protetora da mucosa causada pelo traumatismo. Uma característica importante a se considerar é a relação inversa do uso do tabaco com a EUR bucal (Axell & Henricsson, 1985). Embora os efeitos dos subprodutos do tabaco no sistema imune permaneçam incertos, seu uso tem sido associado com o surgimento da ceratinização da mucosa, e sendo assim à baixa freqüência de EUR.

1.5- Síndrome da Ardência Bucal (SAB)

A SAB é uma condição caracterizada por queimação e sensação dolorosa na boca, porém a mucosa bucal encontra-se normal (Lamey & Lamb, 1989). A xerostomia (Gorsky e cols., 1987; Bergdahl & Bergdahl, 1999) e a disgeusia (Scala e cols., 2003) são outros sintomas comuns nos pacientes com SAB. Alem do mais, devido à diminuição da lubrificação bucal, estes pacientes tornam-se mais propensos a desenvolver infecções (Chen & Samaranayake, 2000).

O local mais comum de acometimento pela SAB é a língua, razão pela qual se identifica esta condição também como glossodinia ou glossopirose. O lábio, a mucosa jugal e o palato são locais que também podem ser acometidos (Basker e cols., 1978; Gorsky e cols, 1987). A SAB é encontrada principalmente nas mulheres, entre a quinta e sexta década de vida (Gorsky e cols, 1987; Gorsky e cols., 1991).

SAB pode ser classificada em suave, moderada e severa, sendo a SAB moderada mais freqüente. A SAB pode variar durante o decorrer do dia e três padrões diferentes foram propostos para os sintomas. O tipo 1 descreve períodos livres da sintomatologia pela manhã, aumentando gradativamente até à noite.O tipo 2 envolve a sensação de

queimação contínua durante o dia e o tipo 3 é caracterizado por períodos livres dos sintomas (Lamey & Lamb, 1989). A sintomatologia intermitente é mais comum que a contínua (Basker e cols, 1978)

A etiologia da SAB continua ainda bastante conflitante, sendo que fatores locais e sistêmicos são levantados. Dentre os fatores locais estão incluídos os tratamentos odontológicos, trauma, candidíase, infecções bacterianas, alergias e disfunções de glândulas salivares (Ship, 1996; Scala e cols, 2003). Aproximadamente 65% dos pacientes com SAB relataram não ter os sintomas antes do tratamento odontológico (Grushka, 1983). A candidíase tem sido um fator comum na etiopatogenia da SAB (Gorsky e cols, 1987; Gorsky e cols, 1991). Dentre as infecções bacterianas, a H. pylori tem sido um microorganismo de interesse no estudo da etiologia da SAB (Gall-Troselj e cols., 2001).

A queimação bucal sem a presença de lesão na mucosa ou pele representa um sintoma típico de dor neuropática crônica (Forssell e cols., 2002). A freqüente observação das alterações no paladar e xerostomia em pacientes com a SAB têm sugerido que esta síndrome pode refletir uma desordem neuropática (Grushka & Sessle, 1991). Alguns sintomas da síndrome têm um padrão similar aos observados em algumas condições inflamatórias neurais, considerando assim, que uma possível injúria periférica no nervo poderia estar relacionada com o aparecimento da SAB (Grushka e cols., 1998). A neuropatia das fibras trigeminais no local primário da lesão foi observada recentemente. Alem disso, esta neuropatia estava correlacionada com a duração dos sintomas (Lauria e cols., 2005). A falta de inibição entre as áreas de prolongamentos centrais dos nervos do paladar glossofaríngeo e corda timpânico, após esses nervos sofrerem injúria periférica, pode provocar confusão no paladar (Lehman e cols., 1995; Bartoshuk e cols., 1996). Vários autores acreditam que o aparecimento da SAB pode ser conseqüência de algum

trauma envolvendo o nervo trigêmio (Svensson e cols., 1993; Gao e cols., 2000; Heckmann e cols., 2001).

Os principais fatores sistêmicos envolvidos com a SAB são as deficiências nutricionais e hormonais. Foi demonstrado que 53% dos pacientes com SAB tinham deficiência de ferro (Brooke & Seganski, 1977). Deficiências de vitaminas, principalmente do complexo B foram relacionadas com a etiologia da SAB (Faccini, 1968).

Os fatores psiquiátricos têm sido extensivamente estudados como causa da SAB, entre eles, o estresse, a ansiedade e depressão (Rojo e cols., 1993; Al Quran, 2004). Alguns estudos consideram a depressão como a desordem psiquiátrica mais comum nos pacientes com a SAB (Browning e cols., 1987; Rojo e cols, 1993). Uma controvérsia observada na literatura é o fato da depressão e ansiedade serem primárias (Pokupec-Gruden e cols., 2000) ou secundárias (Grushka & Sessle, 1991) aos eventos relacionados à SAB.

2- ARTIGO I

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ORIGINAL ARTICLE

Association of interleukin-1β polymorphism with recurrent aphthous stomatitis in Brazilian individuals

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BACKGROUND: Recurrent aphthous stomatitis (RAS) is characterized by recurrent episodes of oral ulceration in an otherwise healthy individual. Some reports in the literature indicate that RAS may have immunological, psychological, genetic and microbiological bases. The purpose of the present study was to investigate the possible association between interleukin-1β (IL-1β) +3954 (C/T) genetic polymorphism and RAS in a sample of Brazilian patients.

SUBJECTS AND METHODS: Sixty-two consecutive subjects affected by minor and major forms of RAS and 62 healthy volunteers were genotyped at IL-1β (+3954). The chi-squared test was used for statistical analysis.

RESULTS: A significant increase in the high production of IL-1β genotype CT was observed in the group with RAS ($P = 0.01$). After stratifying RAS patients according to the mean number of lesions per episode, a significant difference was only observed between patients with ≥3 lesions in each episode and control.

CONCLUSION: There is an increased frequency of polymorphism associated with high IL-1β production in RAS patients.

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Keywords: recurrent aphthous stomatitis; cytokine; polymorphism; interleukin-1β; pathogenesis

Introduction

Recurrent aphthous stomatitis (RAS) is characterized by recurrent episodes of oral ulceration in an otherwise healthy individual (Porter *et al*, 1998). RAS has three different variants: minor aphthous ulcers, major aphthous ulcers and herpetiform ulcers (Stanley, 1972). It has been estimated that 20% of the general population will suffer from RAS at some time in their lives (Stanley, 1972; Axell and Henricsson, 1985). Possibly more than 40% of patients may have a familial history of RAS (Natah *et al*, 2004). Some investigators have correlated the onset of ulcers with exposure to certain foods (Thomas *et al*, 1973), but this has not been confirmed (Eversole *et al*, 1982). Many studies have suggested an association between RAS and psychological factors including anxiety and stress (McCartan *et al*, 1996; Chiappelli and Cajulis, 2004; Natah *et al*, 2004).

Interleukin-1 (IL-1) is a pro-inflammatory cytokine that plays a pivotal role in several chronic diseases (di Giovine and Duff, 1990). This cytokine is a primary activator of early chemotactic cytokines, as well as of the expression of endothelial cell adhesion molecules (ECAMs) that facilitate migration of leucocytes into tissues (Lang *et al*, 2000). In a recent study the expression of IL-1β cDNA was more abundant in RAS lesions than normal mucosa (Borra *et al*, 2004). Genetic polymorphisms have been described at IL-1β gene. A polymorphism of the IL-1β gene at +3954 (C/T) and at −511 was found to result an increased production of the cytokine (Pociot *et al*, 1992). Moreover, the IL-1β polymorphism in the region −511 was strongly associated with RAS (Bazrafshani *et al*, 2002a). As immunological and genetic factors have been implicated in the pathogenesis of RAS, the purpose of the present study was to investigate a possible association between the functional IL-1β + 3954 (C/T) genetic polymorphism with RAS in a sample of Brazilian patients.

Subjects and methods

Subjects and sample collection

Sixty-two consecutive subjects affected by minor and major forms of RAS (Table 1) and 62 age- and sex-matched control subjects (mean age = 36.9 years; range 8–84 years; standard deviation 16.5) were included in this study. There were 27 (43.5%) men and 35 (56.5%) women in the control group. The patients were recruited from the Oral Diagnosis Clinic at the Universidade Federal de Minas Gerais. Both experimental and control groups were from the same geographical area and had

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Table 1 Summary of the clinical data of RAS patients included in the study

Characteristics	*Values*
Age (years)	
Median	31.7
Range	7–69
Standard deviation	14.6
Gender, *n* (%)	
Male	26 (41.9)
Female	36 (58.1)
Mean number of lesions in each episode, *n* (%)	
<3 lesions	39 (62.9)
≥3 lesions	23 (37.1)

RAS, recurrent aphthous stomatitis.

identical socio-economic status. Ethnicity was not established as the hazards of judging Brazilians by colour, race and geographical origin was recently demonstrated (Parra *et al*, 2003).

The diagnosis of RAS was based on accepted clinical criteria (Ship *et al*, 2000). The control group was composed of patients without any history of RAS or systemic diseases. Exclusion criteria for both groups were the presence, apart from dental caries, of any other significant local or systemic diseases. Although periodontal disease was not an exclusion criterion, none of the individuals in both groups presented chronic periodontitis. The study protocol was approved by the local Ethics Committee and informed consent was obtained from all patients or from the parents when subjects were less than 18 years.

Oral mucosa swabs were taken once from the buccal mucosa of subjects. The swabs were taken with sterile plastic tips, placed immediately in Eppendorf microtubes containing 500 μl of Krebs buffer, and the pellet obtained after 5 min of centrifugation at 13 000 ***g*** was stored at −20°C until processing.

DNA isolation

DNA extraction was carried out as described by Boom *et al* (1990) and modified as below. We added 450 μl of lyses buffer (6.0 M GuSCN, 65 mM Tris–HCl pH 6.4, 25 mM EDTA, 1.5% Triton X-100) and 20 μl silica (SiO2, Sigma S-5631) to the microcentrifuge tube containing the oral mucosa swab pellet. The tube was vortexed and incubated for 10 min at 56°C, centrifuged at 3000 ***g*** for 1 min and the supernatant discharged. The pellet obtained (DNA adsorbed to the silica) was washed twice with 450 μl washing buffer (6.0 M GuSCN, 65 mM Tris–HCl), twice with 70% ethanol, once with 450 μl acetone and dried at 56° C for 10 min. Finally, 100 μl of TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) was added and incubated at 560°C for 10 min to elute the DNA. After incubation the solution was homogenized and centrifuged at 5000 ***g*** for 2 min and the supernatant containing DNA transferred to a new tube.

Genotyping

Interleukin-1β (+3954) polymorphisms were assessed by polymerase chain reaction (PCR) amplification and digestion. The sequences of PCR primers were 5′-CTCAGGTGTCCTCGAAGAAATCAAA-′3 and 5′-GCTTTTTTGCTGTGAGTCCCG-′3 with expected PCR product size of 194 bp, as described elsewhere (Moreira *et al*, 2005). PCR was carried out in a total volume of 50 μl, containing 10 μl of solution DNA, Premix buffer (50 mM KCl, 10 mM Tris–HCl pH 8.4, 0.1% Triton X-100, 1.5 mM MgCl2, deoxynucleoside triphosphates, *Taq* DNA polymerase (Phoneutria Biotecnologia, Belo Horizonte, Brazil) and primers (20 pmol/reaction). The amplification conditions consisted of 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 54°C for 35 s and 72°C for 30 s. The run was terminated by final elongation at 72°C for 5 min. Lid temperature was 103°. The products were digested with 5 U of *Taq*I at 65°C for 4 h and 97 + 85 + 12 bp DNA products were obtained for allele C and 182 + 12 bp DNA products for allele T. Visualization was performed in a 18 × 16 cm 10% polyacrylamide gel electrophoresis staining with ethidium bromide (0.5 μg ml^{-1}) (Figure 1).

Statistical analysis

Statistical significance of differences between case and control group distributions for alleles and genotypes was determined using the chi-squared test. A significance level of $P \leq 0.05$ was used. The observed genotype frequencies were compared with those calculated from Hardy–Weinberg equilibrium. All statistical analyses were performed using BioStat 3.0 software (Optical Digital Optical Technology, Belém, Brazil).

Results

The distribution of genotype frequencies of IL-1β polymorphism in patients with RAS and control is shown in Table 2. There was a higher frequency of the CT genotype in the RAS group than in the control ($P = 0.01$). After stratifying RAS patients according to the mean number of lesions per episode, a significant difference was only observed between patients with ≥3 lesions in each episode

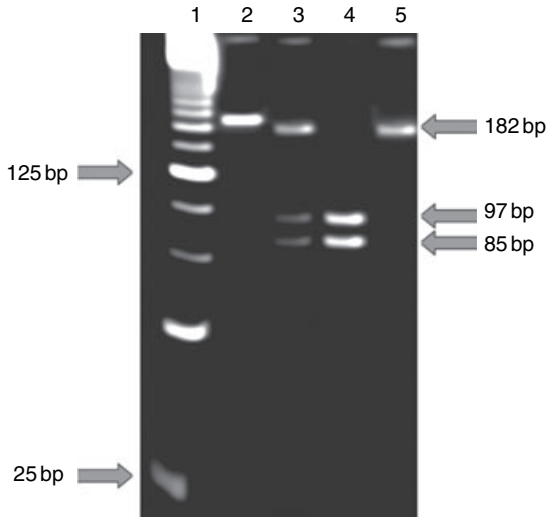

Figure 1 Electrophoresis in a 10% polyacrylamide gel. Lane 1, ladder 25 bp; lane 2, PCR product without digestion (194 bp); lane 3, genotype CT; lane 4, genotype CC; lane 5, genotype TT

Table 2 Distribution of the genotype of IL-1β +3954 polymorphism in patients with recurrent aphthous stomatitis (RAS; $n = 62$) and control subjects ($n = 62$)

	*RAS**	*RAS (<3 lesions)***	*RAS (≥3 lesions)****	*Control*
Genotypes, *n*(%)				
TT	0 (0)	0 (0)	0 (0)	0 (0)
CT	35 (57)	21 (54)	14 (61)	21 (44)
CC	27 (43)	18 (46)	09 (39)	41 (66)

**vs* control – $P = 0.01$; ***vs* control – not significant ($P = 0.07$); ****vs* control – $P = 0.04$.

and control ($P = 0.04$). The distribution of IL-1 β genotypes in the case group (TT:CT:CC, 0:35:27) was statistically different from those (5:25:31) expected from the Hardy–Weinberg equilibrium ($P = 0.002$). On the other hand, the distribution of IL-1β genotypes in the control group (TT:CT:CC, 0:21:41) was not statistically different from those (1:17:42) expected from the Hardy–Weinberg equilibrium ($P = 0.1084$).

Discussion

Recurrent aphthous stomatitis is a very common oral disease of unknown aetiology. Many local and systemic factors have been associated with the condition. Some reports in the literature indicate that RAS may have an immunological, psychological, genetic and microbiological bases (Porter *et al*, 1998; Ship *et al*, 2000; Natah *et al*, 2004). Although studies have tried to identify the role of the immune system in RAS, the immunopathogenesis remains to be established (Natah *et al*, 2000; Lewkowicz *et al*, 2003). Evidence suggests that ulceration results from an abnormal cytokine cascade in the oral mucosa, leading to enhanced cell-mediated immune response directed towards focal areas of the oral mucosa (Buno *et al*, 1998; Borra *et al*, 2004). Recently scanning with cDNA microarray analyses in RAS showed a more intense activity of Th1 gene cluster relative to the Th2 gene cluster (Borra *et al*, 2004).

Polymorphisms associated with cytokines have been used to investigate the pathogenesis of various diseases. A previous study showed that polymorphisms of tumor necrosis factor-α (TNF-α) or tumor necrosis factor-β (TNF-β) does not appear to be a significant factor in determining susceptibility to minor RAS (Bazrafshani *et al*, 2002b). In the current study we observed that the polymorphism at IL-1β +3954 was associated with RAS. In contrast to control, genotypes in the patient group were not distributed according to Hardy–Weinberg equilibrium. Bazrafshani *et al* (2002a) demonstrated an increased frequency of another polymorphism at IL-1β-511 region in RAS subjects. After stratifying RAS patients according to severity, only patients with ≥3 lesions per attack continued to show a significant association with the high production of IL-1β. Although polymorphism of IL-1β has been described in association with chronic periodontitis in Brazilian patients (Moreira *et al*, 2005), none of the individuals in the present study were affected by this condition.

Interleukin-1β is a proinflammatory cytokine. The relationship between local damage in RAS and IL-1β could be due to the activation of early chemotactic cytokines, and to the expression of ECAMs that facilitate migration of leucocytes into tissues (Lang *et al*, 2000; Dagia and Goetz, 2003). Increased levels of vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, are observed in the sera of RAS patients (Healy *et al*, 1997). Borra *et al* (2004) demonstrated higher IL-1β transcription in RAS lesions compared with normal mucosa. Therefore, the expression of adhesion molecules might be increased by increased levels of IL-1β present in individuals with the high producer genotype. Although RAS patients showed a genotype associated with high IL-1β production, other authors have shown that IL-1β production by peripheral blood mononuclear cells was not elevated in RAS (Lewkowicz *et al*, 2005).

Another mechanism explaining IL-1β polymorphism and RAS development should also be considered. It is well known that psychological factors are implicated in the pathogenesis of RAS (McCartan *et al*, 1996; Chiappelli and Cajulis, 2004). A previous study conducted by our group showed that RAS patients have a higher frequency of the serotonin transporter gene polymorphism (5-HTTLPR), associated with anxiety-related traits (Victoria *et al*, 2005). As IL-1β levels have been shown to be elevated in the blood or cerebrospinal fluid of depressed patients (Maes *et al*, 1991a,b), the high producer IL-1β genotype may be more susceptible to depression and RAS.

In conclusion, our findings demonstrate increased frequency of the CT variant form of +3954C/T polymorphism associated with high IL-1β production in RAS patients. Our findings provide additional support to a genetic basis for RAS development. Further studies are necessary to delineate the complex RAS immunopathogenesis.

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3- ARTIGO II

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Investigation of functional gene polymorphisms IL-1β, IL-6, IL-10 and TNF-α in individuals with recurrent aphthous stomatitis

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ABSTRACT

Recurrent aphthous stomatitis (RAS) is characterized by recurrent episodes of oral ulceration in an otherwise healthy individual. Some reports in the literature indicate that RAS may have immunological, psychological, genetic and microbiological bases.

Objective: The purpose of the present study was to investigate, using binary logistic regression analyses, a possible association between the functional IL-1β +3954 (C/T), IL-6 −174 (G/C), IL-10 −1082 (G/A) and TNF-α −308 (G/A) genetic polymorphism and RAS in a sample of Brazilian patients, using a multivariate statistical analysis.

Design: Sixty-four consecutive subjects affected by minor and major forms of RAS and 64 healthy volunteers were genotyped. To investigate the association between the single nucleotide polymorphisms and risk of RAS, binary logistic regression models were fitted. The associations were expressed by odd ratios (ORs) and adjusted for age and gender, with the corresponding 95% CIs. P-values less than 0.05 were considered significant.

Results: A significant increase in the IL-1β and TNF-α heterozygous genotypes were associated with an increased risk of RAS development (OR 2.40 and 3.07, respectively), in the multivariate model.

Conclusion: Our findings demonstrate that polymorphisms of high IL-1β and TNF-α production were associated with an increased risk of RAS development. Our findings also give additional support to a genetic basis for RAS pathogenesis.

1. Introduction

Recurrent aphthous stomatitis (RAS) is characterized by recurrent episodes of oral ulceration in an otherwise healthy individual.[1] Clinically, RAS includes three different variants, minor aphthous ulcers, major aphthous ulcers and herpetiform ulcers.[2,3] It has been estimated that 20% of the general population will suffer from RAS at some time in their lives.[2,4] Although the aetiology of RAS is unknown, current evidence indicates the presence of genetic factors in this disease. This has been confirmed by the finding that more than 40% of patients may have a family history of RAS.[5] The probability of a sibling developing RAS may also be influenced by the parents' RAS status.[6] Moreover, there is

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a high correlation of RAS in monozygote but not in dizygote twins.[7]

Psychological factors including anxiety and stress were associated with RAS in a considerable number of studies,[5,8] thus supporting the findings of our previous study in which RAS patients showed an increased frequency of serotonin transporter gene polymorphism (5-HTTLPR) associated with anxiety-related traits.[9]

Some forms of immune dysfunction appear to be related to RAS pathogenesis. An increased local expression of Th1 genes[10] and systemic production of cytokines, such as IL-2, TNF-α and IL-6 by peripheral blood mononuclear cells were observed in RAS patients.[11] In addition, decreased IL-10 mRNA levels were reported in RAS patients, which suggests a failure of the immune system to suppress inflammatory reaction to oral mucosa.[12]

A large number of functional genetic polymorphisms in the immunological system have been described in previous literature. Some genotypes of these polymorphisms can increase protein production[13,14] while other polymorphisms, such as IL-1β +3954 (C/T), were associated with RAS in a sample of British patients.[15] Considering that immunological alterations are reported in RAS pathogenesis, together with evidence demonstrating that genetic factors are associated with the disease, the purpose of the present study was to investigate a possible association between the functional IL-1β +3954 (C/T), IL-6 −174 (G/C), IL-10 −1082 (G/A) and TNF-α −308 (G/A) genetic polymorphisms and RAS in a sample of Brazilian patients.

2. Material and methods

2.1. Subjects and sample collection

Sixty-four consecutive subjects affected by minor and major forms of RAS (Table 1) and 64 age and sex matched control subjects (mean age = 36.9 years; range 8–84 years; standard deviation 16.5 years) were included in this study. The patients were recruited from the Oral Diagnosis Clinic at the Universidade Federal de Minas Gerais. Both the experimental and control groups were of the same geographic area and had identical socio-economic status. Ethnicity was not established, respecting the hazards of judging Brazilians by color, race and geographical origin as demonstrated in the past findings.[16]

Table 1 – Summary of the clinical data of RAS patients included in the study

Characteristics	Values
Age	
Median age (years)	31.7
Range of years	7–69
Standard deviation	14.3
Patient gender	
Male, no. (%)	28 (43.8)
Female, no. (%)	36 (56.2)
Number of lesions in aphthous episodes	
Less than 3 lesions in each aphthous episode, no. (%)	41 (64.1)
3 or more lesions in each aphthous episode, no. (%)	23 (35.9)
Commitment sites	
Floor of the mouth, no. (%)	15 (23.4)
Lips, no. (%)	21 (32.8)
Buccal mucosa, no. (%)	20 (31.3)
Tongue, no. (%)	7 (10.9)
Soft palate, no. (%)	1 (1.6)

The diagnosis of RAS was based on accepted clinical criteria.[17] The control group was comprised of patients with no history of RAS nor systemic diseases. Exclusion criteria for both groups included the presence of any other significant local or systemic diseases, excluding dental caries. No individual in either group presented chronic periodontitis. The study protocol was approved by the local Ethics Committee and informed consent was obtained from either the patients or the legal guardian when the patient was less than 18 years of age.

Oral mucosa swabs were removed once from the subjects' buccal mucosa. The swabs were performed using sterile plastic tips and placed immediately in Eppendorf microtubes containing 500 μl of Krebs buffer. The pellet was then obtained after 5 min of centrifugation at 13,000 × *g* and stored at −20 °C until processing.

2.2. DNA isolation

DNA extraction was carried out as aforementioned.[18] Initially, 450 μl of lyses buffer (6.0 M GuSCN, 65 mM Tris–HCl pH 6.4, 25 mM EDTA, 1.5% Triton X-100) and 20 μl silica (SiO_2, Sigma S-5631) were added to the microcentrifuge tube containing the oral mucosa swab pellet. The tube was mixed and incubated for 10 min at 56 °C, centrifuged at 3000 × *g* for 1 min and the supernatant discharged. The pellet with the DNA adsorbed in the silica was washed twice with a 450 μl washing buffer (6.0 M GuSCN, 65 mM Tris–HCl), twice with 70% ethanol, once with a 450 μl acetone and then dried at 56° C for 10 min. Finally, 100 μl of TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) was added and incubated at 56° C for 10 min to elute the DNA. After incubation the solution was vortexed and centrifuged at 5000 × *g* for 2 min and approximately 90 μl of supernatant containing DNA was transferred to a new tube.

2.3. Genotyping

The polymorphisms were assessed by polymerase chain reaction (PCR) amplification and digestion. The sequences of PCR primers are shown in Table 2. The PCR was carried out in a total volume of 50 μl, containing approximately 400 ng of DNA, primers (20 pmol/reaction), and 25 μl of Pre-mix buffer (Phoneutria Biotecnologia, Belo Horizonte, Brazil). According to the manufacturer, the Pre-mix buffer contained 50 mM KCl, 10 mM Tris–HCl pH 8.4, 0.1% Triton X-100, 1.5 mM $MgCl_2$, deoxynucleoside triphosphates and 1.25 units of Taq DNA polymerase. The conditions for amplification consisted of 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 54 °C for 35 s and 72 °C for 30 s. The run was terminated by final elongation at 72 °C for 5 min. In all steps the lid temperature was 103°. The products were digested with restriction enzyme according to manufacturer protocols (see Table 2). The

Table 2 – Primer sequence, reference and restriction enzymes used for each polymorphism

Genes	Primers (references)	Restriction enzyme (condition)	Genotypes
IL-1β +3954 (C/T)	5′-CTCAGGTGTCCTCGAAGAAATCAAA-3′ 5′-GCTTTTTTGCTGTGAGTCCCG-3′ (Pociot et al.[13])	*Taq*I[a] (65°/4 h)	TT–182 + 12 bp CT–182 + 97 + 85 + 12 bp CC–97 + 85 + 12 bp
IL-6 −174 (G/C)	5′-CAGAAGAACTCAGATGACCTG-3′ 5′-GTGGGGCTGATTGGAAACC-3′ (Klein et al.[32])	*hsp*92II[a] (37°/4 h)	CC–229 + 122 + 51 + 29 bp CG–229 + 173 + 29;bp GG–229 + 122 + 51 + 29 bp
IL-10 −1082 (G/A)	5′-CCAAGACAACACTACTAAGGCTCCTTT-3′ 5′-GCTTCTTATATGCTAGTCAGGTA-3′ (Koch et al.[33])	*Xag*I[b] (37°/8 h)	AA–280 + 97 bp GA–280 + 253 + 97 + 27 bp GG–253 + 97 + 27 bp
TNF-α −3O8 (G/A)	5′-AGGCAATAGGTTTTGAGGGCCAT-3′ 5′-TCCTCCCTGCTCCGATTCCG-3′ (Wilson et al.[14])	*Nco*I[a] (37°/12 h)	AA–107 bp GA–107 + 87 + 20 bp GG–87 + 20 bp

[a] Promega, Madison, USA.
[b] MBI Fermentas.

visualization of the product was performed in a 6.5% polyacrylamide gel electrophoresis staining with ethidium bromide (0.5 μg/ml).

2.4. *Statistical analysis*

The univariate analyses were performed using the Fisher exact test or the Chi-square test. To investigate the association between the single nucleotide polymorphisms and risk of RAS, binary logistic regression models were fitted. The associations were expressed by odd ratios (ORs) and adjusted for age (<26 and >26 years) and gender, with the corresponding 95% CIs. P-values less than 0.05 were considered significant. The multivariate analyses were assessed using SPSS (SPSS Inc., Chicago), version 14.0, and the univariate analyses were performed using BioStat 3.0 software (Optical Digital Optical Technology, Belém, Brazil)

3. Results

The distribution of genotype and allele frequencies of all polymorphisms in patients with RAS and control are shown in Tables3 (univariate analyses) and 4 (multivariate analyses). A significant increase in the IL-1β and TNF-α heterozygous genotypes was observed in the group with RAS in the univariate analysis ($P = 0.03$ and 0.04). In the multivariate model, adjusted for age and gender, the same genotypes of IL-1β and TNF-α shown were associated with an increased risk of RAS development (OR 2.40 and 3.07, respectively).

4. Discussion

RAS is a very common oral disease of unknown etiology. Many local and systemic factors have been associated with the condition. Some reports in the literature indicate that RAS may have immunological, psycologycal, genetic and microbiological bases.[1,3,5,17] Although studies have tried to identify the role of the immune system in RAS, the immunopathogenesis remains to be established.[19,20] Evidence suggests that the ulceration is the result of an abnormal cytokine cascade in the oral mucosa that leads to enhanced cell-mediated immune response directed toward focal areas of the oral mucosa.[10,12] Recent scanning with cDNA microarray analyses in RAS showed a more intense activity of the Th1 gene cluster relative to the Th2 gene cluster.[10]

Polymorphisms associated with cytokines have been used to investigate the pathogenesis of various diseases. In the current study, the univariate analysis showed that the polymorphism CT at IL-1β +3954 was associated with RAS. In the multivariate analysis, we observed that this genotype was associated with an increased risk of RAS development (OR 2.40). Previous literature has proven that this genotype has been associated with two-fold IL-1β production.[13] One such study, using a univariate analysis, also showed an increased frequency of IL-1β +3954 and −511 polymorphisms

Table 3 – Distribution of the genotypes in patients with recurrent aphthous stomatitis (RAS) and control subjects using an univariate analyses

Genotypes	RAS ($N = 64$)	Control ($N = 64$)	P-value
IL-1β +3954 (C/T) (N, %)			
CC	28 (56.2)	41 (64)	
CT	36 (43.8)	23 (36)	
TT	0 (0)	0 (0)	0.03
IL-6 −174 (G/C) (N, %)			
CC	1 (1.6)	0 (0)	
GC	25 (39)	24 (37.5)	
GG	38 (59.4)	40 (62.5)	0.58
IL-10 −1082 (G/A) (N, %)			
AA	31 (48.4)	31 (48.4)	
GA	26 (40.6)	23 (36)	
GG	7 (11)	10 (15.6)	0.70
TNF-α-308 (G/A)			
GG	38 (59.4)	47 (73.4)	
GA	22 (34.4)	10 (15.6)	
AA	4 (6.2)	7 (11)	0.04

P-values from Chi-squared test. A significance level of $P \leq 0.05$ was used.

Table 4 – Pooled genotype frequencies and risks for recurrent aphthous stomatitis (RAS) analyzed by single-nucleotide polymorphism

Characteristic	RAS (N = 64)		Control (N = 64)		OR[a]	95%CI[a]		
	N	%	N	%		Min	Max	
Polymorphism								
IL-1β +3954 (C/T)								
CC	28	43.7	41	64.0	Referent			
CT	36	56.2	23	35.9	2.40	1.11	5.20	0.03
TT	0	0	0	0	NA			
IL-6-174 (G/C)								
CC	1	1.5	0	0	NA			
GC	25	39.0	24	37.5	Referent			
GG	38	59.3	40	62.5	0.64	0.28	1.43	0.27
IL-10-1082 (G/A)								
AA	31	48.4	31	48.4	1.65	0.51	5.39	0.40
AG	26	40.6	23	35.9	1.85	0.55	6.22	0.32
GG	7	10.9	10	15.6	Referent			
TNF-α −308 (G/A)								
GG	38	59.3	47	73.4	Referent			
AG	22	34.3	10	15.6	3.07	1.22	7.74	0.02
AA	4	6.2	7	10.9	0.98	0.24	3.91	0.98

[a] Adjusted for gender and age. A significance level of $P \leq 0.05$ was used. NA: not applicable due to the low number of samples.

associated with a high cytokine producer genotype on RAS subjects.[15]

The relation between local damage in RAS and IL-1β can be explained by the action of this cytokine as a primary activator of early chemotactic cytokines, as well as of the expression of endothelial cell adhesion molecules which facilitate the migration of leucocytes into tissues.[21,22] Increased levels of vascular adhesion molecule-1 (VCAM-1) can be observed in the sera of RAS patients.[23] Moreover, a recent study demonstrated a higher IL-1β transcription in RAS lesions as compared to normal mucosa.[10] Therefore, we may speculate that the expression of the adhesion molecules may be induced by increased levels of IL-1β present in the individuals with the high producer genotype. Although RAS patients showed a genotype associated with high IL-1β production, other authors have shown that IL-1β production by peripheral blood mononudear cells was not increased in RAS.[11]

Another mechanism to explain IL-1β polymorphism and RAS development may also be considered. It is well-known that psychological factors are implied in the pathogenesis of RAS.[8] A previous study conducted by our group showed that RAS patients showed an increased frequency of serotonin transporter gene polymorphism (5-HTTLPR) associated with anxiety-related traits.[9] As IL-1β levels have proven to be higher in the blood or cerebrospinal fluid of depressed patients,[24,25] the high producer IL-1β genotype may be more susceptible to depression and RAS. It is interesting to note that IL-1β polymorphism has been related to psychosis susceptibility and early development of Alzheimer's disease.[26]

In the current study, we observed that TNF-α intermediary producer genotype was associated with an increased risk of RAS (OR = 3.07). The low number of individuals in case and control groups with a high TNF-α producer genotype may explain why this genotype was not associated with RAS. No previous studies demonstrate the association of TNF-α −308 (G/A) polymorphism and RAS.[27] TNF-α does indeed present some important immune regulatory activities and studies have suggested its relation to RAS. Likewise, elevated levels of TNF-α have been reported in the lesional mucosa and in the peripheral blood of RAS patients.[11,12,19,28] Enhanced cytotoxic destruction of epithelial cells by TNF-α produced by peripheral blood mononuclear cells[29] and leucocytes[28] in RAS subjects was demonstrated by in vitro studies. Moreover, RAS can be prevented by endogenous TNF-α synthesis inhibitors, such as thalidomide[30] and pentoxifylline.[19]

Although IL-6 and IL-10 were implied in RAS pathogenesis,[11,12,31] our data shows that polymorphisms on these genes were not associated with RAS. This is in contradiction to a previous study that demonstrated an association between IL-6 polymorphism and RAS.[27] This disparity is probably related to population heterogeneity.

In conclusion, our findings demonstrate that polymorphisms of high IL-1β and TNF-α production were associated with an increased risk of RAS development. Our findings also give additional support to a genetic basis for RAS pathogenesis.

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4- ARTIGO III

IL-1B, IL-6, IL-10 and TNFA functional gene polymorphisms in patients with recurrent aphthous stomatitis

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Abstract

Recurrent aphthous stomatitis (RAS) is characterized by recurrent episodes of oral ulceration in an otherwise healthy individual. Literature data suggest that RAS might have genetic background with a certain specific pattern of gene polymorphism inheritance.

Objective: The purpose of this study was to investigate, using binary logistic regression analyses, a possible association between the functional *IL-1B* +3954 (C/T), *IL-6* -174 (G/C), *IL-10* -1082 (G/A) and *TNFA* -308 (G/A) gene polymorphisms and RAS in a sample of Brazilian patients, using a multivariate statistical analysis.

Design: Sixty-four patients with RAS (mean age = 31.7 years; range 7-69 years) and 64 healthy volunteers (mean age = 36.9 years; range 8-84 years) were genotyped. Statistical analysis was based on the use of Chi square test together with Fischer test as well as binary logistic regression model (p-values below 0.05 were considered as significant).

Results: A significant increase in the *IL-1B* and *TNFA* heterozygous genotypes were associated with an increased risk of RAS development (OR 2.40 and 3.07, respectively) in the multivariate model

Conclusion: Our findings clearly demonstrate an association between inheritance *of IL-1B* and *TNFA* gene polymorphisms and RAS occurrence, thus giving additional support for genetic basis of this disease.

Key words**:** recurrent aphtous stomatitis; gene polymorphism, genetic

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Introduction

Recurrent aphthous stomatitis (RAS) is characterized by recurrent episodes of oral ulceration in an otherwise healthy individual [1]. Clinically, RAS includes three different variants, minor aphthous ulcers, major aphthous ulcers, and herpetiform ulcers [2]. It has been estimated that 20% of the general population will suffer from RAS at some time in their lives [3;4]. Although the etiology of RAS is unknown, current evidence indicates the presence of genetic factors in this disease. This has been confirmed not only by the finding that more than 40% of patients may have a family history of RAS [5], but also because there is a high correlation of RAS in monozygotic but not in dizygotic twins [6]. Moreover, if both parents have RAS, there is 90% probability that the child will be affected with RAS as well [7].

Psychological factors including anxiety and stress were associated with RAS in a considerable number of studies [5;8]. Previously we have reported increased frequency of serotonin transporter gene polymorphism associated with anxiety-related traits in RAS patients [9].

So far, data from the published literature indicate immune dysfunction in RAS pathogenesis. An increased expression of Th1 genes is observed in RAS lesions,[10] together with high levels of IL-2, TNF-α, and IL-6 secreted by circulating mononuclear cells of RAS patients [11]. In addition, decreased IL10 mRNA levels were reported in RAS patients, which suggests a failure of the immune system to suppress inflammatory reaction to oral mucosa [12]. Hematological, gastrointestinal, nutritional, allergic have also been implicated in RAS development [13]. There are now several examples of

polymorphisms occurring within cytokine genes that affect protein production, some of which are associated disease. [9;14].

Considering that immunological alterations are reported in RAS pathogenesis, together with evidence demonstrating that genetic factors are associated with the disease, the purpose of the present study was to investigate a possible association between the functional *IL-1B* +3954 (C/T), *IL-6* -174 (G/C), *IL-10* -1082 (G/A) and *TNFA* -308 (G/A) gene polymorphisms and RAS in a sample of Brazilian patients.

Material and methods

Subjects and sample collection

Sixty-four consecutive subjects affected by minor and major forms of RAS (Table 1) and 64 age and sex matched control subjects (mean age = 36.9 years; range 8-84 years; standard deviation 16.5 years) were included in this study. The patients and controls were recruited from the Oral Diagnosis Clinic and Restorative Dentistry Clinic, respectively, at the Universidade Federal de Minas Gerais. Both the experimental and control groups were of the same geographic area and had identical socio-economic status. Ethnicity was not established, respecting the hazards of judging Brazilians by color, race and geographical origin [15].

The diagnosis of RAS was based on accepted clinical criteria [16]. The control group comprised of participants with no history of RAS nor clinical evidence of systemic diseases. Exclusion criteria for both groups included the presence of any other significant local or systemic diseases, excluding dental caries. No individual in either group presented chronic periodontitis. The study protocol was approved by the local

Ethics Committee and informed consent was obtained from either the patients or the legal guardian when the patient was less than 18 years of age.

Oral mucosa swabs were removed once from the subjects' buccal mucosa. The swabs were performed using sterile plastic tips and placed immediately in Eppendorf microtubes containing 500 µl of Krebs buffer. The pellet was then obtained after 5 min of centrifugation at 13000 g and stored at -20ºC until processing.

DNA isolation

DNA extraction was carried out as aforementioned [17]. Initially, 450 µl of lyses buffer (6.0 M GuSCN, 65 mM Tris HCl pH 6.4, 25 mM EDTA, 1.5% TritonX-100) and 20 µl silica (SiO2, Sigma S-5631) were added to the microcentrifuge tube containing the oral mucosa swab pellet. The tube was mixed and incubated for 10 min at 56 ºC, centrifuged at 3,000 g for 1 min and the supernatant discharged. The pellet with the DNA adsorbed in the silica was washed twice with a 450 µl washing buffer (6.0 M GuSCN, 65 mM Tris HCl), twice with 70% ethanol, once with a 450 µl acetone, and then dried at 56º C for 10 min. Finally, 100 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) was added and incubated at 56° C for 10 min to elute the DNA. After incubation the solution was vortexed and centrifuged at 5,000 g for 2 minutes and approximately 90µL of supernatant containing DNA was transferred to a new tube.

Genotyping

The polymorphisms were assessed by polymerase chain reaction (PCR) amplification and digestion. The sequences of PCR primers are shown in Table 2. The PCR was

carried out in a total volume of 50 µl, containing approximately 400 ng of DNA, primers (20 pmol/reaction), and 25 µl of Pre-mix buffer (Phoneutria Biotecnologia, Belo Horizonte, Brazil). According to the manufacturer, the Pre-mix buffer contained 50 mM KCl, 10 mM Tris-HCl pH 8.4, 0.1% Triton X-100, 1.5 mM MgCl2, deoxynucleoside triphosphates, and 1.25 units of Taq DNA polymerase. The conditions for amplification consisted of 94ºC for 3 min followed by 35 cycles of 94ºC for 30 s, 54ºC for 35 s and 72ºC for 30 s. The run was terminated by final elongation at 72ºC for 5 min. In all steps the lid temperature was 103º. The products were digested with restriction enzyme according to manufacturer protocols (see table 2). The visualization of the product was performed in a 6.5% polyacrylamide gel electrophoresis staining with ethidium bromide (0.5 µg/ml).

Statistical Analysis

The univariate analyses were performed using the Fisher exact test or the chi-square test. To investigate the association between the single nucleotide polymorphisms and risk of RAS, binary logistic regression models were fitted. The associations were expressed by odd ratios (ORs) and adjusted for age (<26 and ≥ 26 years) and gender, with the corresponding 95% CIs. *P*- values less than 0.05 were considered significant. The multivariate analyses were assessed using SPSS (SPSS Inc., Chicago), version 14.0, and the univariate analyses were performed using BioStat 3.0 software (Optical Digital Optical Technology, Belém, Brazil)

Results

The distribution of genotype and allele frequencies of all polymorphisms in patients with RAS and control are shown in Tables 3 (univariate analyses) and 4 (multivariate analyses). A significant increase in the *IL-1B* and *TNFA* heterozygous genotypes were observed in the group with RAS in the univariate analysis (p= 0.03 and p=0.04, respectively). In the multivariate model, adjusted for age and gender, the same genotypes of *IL-1B* and *TNFA* were associated with an increased risk of RAS development (OR 2.40 and 3.07, respectively).

Discussion

Some reports in the literature indicate that RAS may have immunological, psychological, genetic, and microbiological bases [1;5;13;16]. Albeit there is substantial evidence upon certain abnormalities of immune system in the pathogenesis of RAS, still its exact mechanism is unknown [18;19]. Evidence suggests that the ulceration is the result of an abnormal cytokine cascade in the oral mucosa that leads to enhanced cell-mediated immune response directed toward focal areas of the oral mucosa [10;12]. Recent scanning with cDNA microarray analyses in RAS showed a more intense activity of the Th1 gene cluster relative to the Th2 gene cluster [10].

In the current study, the univariate analysis showed that the polymorphism CT at *IL-1B* +3954 was associated with RAS. The multivariate analysis confirmed that this genotype was associated with an increased risk of RAS development (OR 2.40). Although no individual in both groups presented the genotype TT, which is associated with the highest IL-1β production, the heterozygous (CT) subjects has been associated with two-fold IL-1β production compared to the genotype CC [20]. Another study, using a

univariate analysis, also showed an increased frequency of *IL-1B* + 3954 and -511 polymorphisms associated with a high cytokine producer genotype on RAS subjects [14].

The relation between local damage in RAS and IL-1β can be explained by the action of this cytokine as a primary activator of early chemotactic cytokines, as well as of the expression of endothelial cell adhesion molecules which facilitate the migration of leukocytes into tissues [21;22]. Increased levels of vascular adhesion molecule -1 (VCAM-1) can be observed in the sera of RAS patients [23]. Moreover, a recent study demonstrated a higher *IL-1B* transcription in RAS lesions as compared to normal mucosa [10]. Therefore, we may speculate that the expression of the adhesion molecules may be induced by increased levels of IL-1β present in the individuals with the high producer genotype. Despite IL-1β production by peripheral blood mononuclear cells in RAS subjects is not increased [11], increased transcription of IL-1B gene in RAS lesions has been demonstrated [10].

As increased levels of anxiety are found in some patients with RAS [8], and mononuclear cells of patients with stress and anxiety show increased amounts of IL-1β, the high producer IL-1β genotype may be more susceptible to anxiety, stress and RAS [24]. We have previously demonstrated association between RAS and 5-HTTLPR genotype [9], another anxiety-related polymorphism.

The low number of individuals with the high TNF-α producer genotype restricted the analysis of this genotype in relation to RAS development. Any way, we observed that the TNF-α intermediary producer genotype was associated with an increased risk of RAS (OR =3.07). Although this finding is in contrast to a previous study [25], many investigations using saliva, serum or tissues indicate that TNF-α has an important role in

RAS [11;12;18;26]. Moreover, enhanced cytotoxic destruction of epithelial cells by TNF-α produced by peripheral blood mononuclear cells [27] and leukocytes [26] in RAS subjects was demonstrated by *in vitro* studies.

In the current study no association between *IL-6* -174 (G/C) high producer genotype and RAS was observed in contrast to earlier observations [14]. We might speculate that this disparity might be related to the population heterogeneity. On the other hand, the present study confirms that *IL-10* gene polymorphism is not related to RAS patients development [28]. Although IL-6 and IL-10 were implicated in RAS pathogenesis [11;12;14;29], our data show that *IL-6* and *IL-10* gene polymorphisms were not associated with RAS. Therefore, other biological or environmental factors were involved with altered production of these cytokines.

There is an important limitation of the present study that should be considered. As our investigation was restricted to a genetic analysis, no speculation could be performed about the functional relations between cytokines, such as synergistic effects. In conclusion, our findings clearly demonstrate an association between inheritance of *IL-1* and *TNFA* gene polymorphisms and RAS occurrence, thus giving additional support for genetic basis of this disease.

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Table 1- Summary of the clinical data of RAS patients included in the study.

Characteristics	*values*
Age	
Median age,	31.7 years
range of years	7 - 69
standard deviation	14.3
Patient gender	
- Male, n° (%)	28 (43.8)
- Female, n° (%)	36 (56.2)
Number of lesions in aphthous episodes	
Less than 3 lesions in each aphthous episode n° (%)	41 (64.1)
3 or more lesions in each aphthous episode, n° (%)	23 (35.9)
Commitment sites	
Floor of the mouth, n° (%)	15 (23.4)
Lips, n° (%)	21 (32.8)
Buccal mucosa, n° (%)	20 (31.3)
Tongue, n° (%)	7 (10.9)
Soft palate, n° (%)	1 (1.6)

Table 2- Primer sequence, reference, and restriction enzymes used for each polymorphism.

Genes	*Primers* (references)	Restriction Enzime (condition)	Genotypes
IL-1B +3954 (C/T)	5' CTCAGGTGTCCTCGAAGAAATCAAA 3' 5' GCTTTTTTGCTGTGAGTCCCG 3' [20]	*TaqI*[§] (65º/4hs)	TT-182+12 bp CT-182+97+85+12 bp CC-97+85+12 bp
IL-6-174(G/C)	5' CAGAAGAACTCAGATGACCTG 3' 5' GTGGGGCTGATTGGAAACC 3' [30]	*hsp92II*[§] (37º/8hs)	CC-229 + 122 + 51 + 29 GC-229 + 173 + 122 + 51 + 29 GG-229 + 173 + 29
IL-10-1082(G/A)	5' CCAAGACAACACTACTAAGGCTCCTTT 3' 5' GCTTCTTATATGCTAGTCAGGTA 3' [31]	*XagI*[#] (37º/4hs)	AA-280+97 bp GA-280+253+97+27 bp GG-253+97+27 bp
TNFA-308(G/A)	5' AGGCAATAGGTTTTGAGGGCCAT 3' 5' TCCTCCCTGCTCCGATTCCG 3' [32]	*NcoI*[§] (37º/12hs)	AA-107 bp GA-107+87+20 bp GG-87+20 bp

[§]Promega, Madison, USA. [#] MBI Fermentas,

Table 3- Distribution of the genotypes in patients with recurrent aphthous stomatitis (RAS) and control subjects using an univariate analyses.

Genotypes	RAS(*n*=64)	Control(*n*=64)	*P*-value
IL-1B+3954(C/T)			
n(%)			
CC	28(56.2%)	41(64%)	
CT	36(43.8%)	23(36%)	
TT	0(0%)	0(0%)	0.03
IL-6-174(G/C)			
n(%)			
CC	1(1.6%)	0(0%)	
GC	25(39%)	24(37.5%)	
GG	38(59.4%)	40(62.5%)	0.58
IL-10-1082(G/A)			
n(%)			
AA	31(48.4%)	31(48.4%)	
GA	26(40.6%)	23(36%)	
GG	7(11%)	10(15.6%)	0.70
TNFA-308(G/A)			
n(%)			
GG	38(59.4%)	47(73.4%)	
GA	22(34.4%)	10(15.6%)	
AA	4(6.2%)	7(11%)	0.04

P-values from chi-squared test. A significance level of $P \leq 0.05$ was used

Table 4- Distribution of the genotypes in patients with recurrent aphthous stomatitis (RAS) and control subjects and analysis by a binary logistic regression.

Characteristic	RAS (N=64)		Control (N=64)		OR[§]	95%CI[§]		[§]
	N	(%)	N	(%)		Min	Max	
Polymorphism								
IL-1B +3954 (C/T**)**								
CC	28	43.7	41	64.0	Referent			
CT	36	56.2	23	35.9	2.40	1.11	5.20	0.03
TT	0	0	0	0	NA			
IL-6 -174 (G/C)								
CC	1	1.5	0	0	NA			
GC	25	39.0	24	37.5	Referent			
GG	38	59.3	40	62.5	0.64	0.28	1.43	0.27
IL-10 -1082 (G/A)								
AA	31	48.4	31	48.4	1.65	0.51	5.39	0.40
AG	26	40.6	23	35.9	1.85	0.55	6.22	0.32
GG	7	10.9	10	15.6	Referent			
TNFA -308 (G/A*)*								
GG	38	59.3	47	73.4	Referent			
AG	22	34.3	10	15.6	3.07	1.22	7.74	0.02
AA	4	6.2	7	10.9	0.98	0.24	3.91	0.98

[§] Adjusted for gender and age. A significance level of $P \leq 0.05$ was used.

NA=not applicable due to the low number of samples.

5- CONSIDERAÇÕES FINAIS

Existem, na literatura, numerosas propostas para se explicar o mecanismo etiológico da EUR. Apesar de existirem fortes evidências apontando disfunção do sistema imune na EUR, a causa desta doença permanece desconhecida. Alguns polimorfismos genéticos nos genes *IL-1B, IL-6, IL-10 e TNFA* são responsáveis por um aumento na expressão dos mesmos genes. Com base nisto investigamos a associação entre polimorfismos genéticos destes genes e EUR.

Estudos prévios mostraram a associação entre o polimorfismo genético na região +3954 do gene *IL-1B* e EUR na população inglesa (Bazrafshani e cols, 2002a). No presente estudo, observamos que a variante polimórfica CT do gene *IL-1B* foi associada com a EUR não só quando avaliada separadamente, mas também na análise com os outros polimorfismos. Este genótipo confere um risco aumentado de desenvolvimento de EUR (OR 2.40). Nenhum dos dois grupos estudados apresentou o genótipo TT que é o responsável pela maior produção de IL-1β. Porém o genótipo CT produz duas vezes mais IL-1β in vitro que o homozigoto CC (Pociot e cols, 1992). A distribuição dos genótipos do nosso grupo controle foi semelhante a de estudos realizados com populações na mesma região (Moreira e cols, 2005).

Vários fatores são usados na literatura para tentar explicar a etiologia da EUR incluindo trauma local, deficiências nutricionais, disfunção imunológica, doenças psiquiátricas e agentes microbianos. A produção aumentada de IL-1β diante de qualquer um destes fatores pode levar uma predisposição aumentada para o desenvolvimento da EUR, uma vez que, a IL-1β estimula a produção de outras citocinas inflamatórias e moléculas de adesão em células endoteliais (Lang e cols, 2000; Dagia & Goetz, 2003) . Níveis Aumentados da molécula de adesão vascular-1 (VCAM-1) (Healy e cols, 1997) e níveis locais aumentados de cDNA de IL-1□. (Borra e cols, 2004) observados em pacientes com EUR dão um suporte adicional para estas especulações. A IL-1β pode

também agir de forma central. Estudos demonstram que é observado aumento na expressão de IL-1β em pacientes ansiosos e estressados (Brydon e cols, 2005). Além disso, foi relatada a associação entre EUR e o 5HHTLPR, um outro polimorfismo relacionado com ansiedade (Victoria e cols, 2005).

Devido ao pequeno número de indivíduos com genótipo alto produtor de TNF-α (AA) deste estudo, a associação entre este genótipo e a EUR não foi observada. De toda forma evidenciou-se a associação entre EUR e indivíduos heterozigotos (GA), que têm produção intermediária de TNF-α. Apesar destes dados conflitarem com estudos em outras populações (Bazrafshani e cols, 2002b), muitos trabalhos têm atribuído um importante papel do TNF-α na etiologia da EUR (Dolby, 1969; Taylor e cols., 1992; Buno e cols, 1998; Lewkowicz e cols, 2005).

A variante polimórfica C situada no lócus- 174 do gene *IL-6,* não alterara os níveis de expressão de IL-6 mesmo após estimulação com LPS ou IL-1 (Fishman e cols, 1998). Com base neste dado era de se esperar que grande parte das doenças autoimunes e inflamatórias, assim como na EUR, estariam sempre associadas com a variante polimórfica G, que confere maior produção de IL-6. Fato este observado previamente (Bazrafshani e cols, 2002a), mas não no presente estudo. Uma possível explicação para este fato seria uma heterogeneidade da população estudada. Por outro lado, os nossos resultados confirmam a não associação do polimorfismo -1082 no gene *IL-10* e EUR (Bazrafshani e cols, 2003).

Com base nestes fatos concluímos que os polimorfismos genéticos dos genes IL-1B e TNFA estão associados com a EUR em nossa população evidenciando a base genética desta doença.

A ausência de estudos sobre polimorfismos na SAB, aliado ao fato de que os polimorfismos IL1B +3954 e 5HTTLPR estão relacionados com alterações na percepção de dor e alterações psiquiátricas, nos levaram a investigar estes genótipos em pacientes com SAB.

No presente estudo, nenhum dos dois grupos estudados apresentou o genótipo TT que é o responsável pela maior produção de IL-1β. Porém, o genótipo CT, que leva também a uma produção aumentada de IL-1β em relação ao homozigoto CC (Pociot e cols, 1992), mostrou associação com a SAB nesta população. Evidências mostram associação entre aumento na expressão de IL-1β em pacientes acometidos por stress e ansiedade (Brydon e cols, 2005). Além disso, stress e depressão são observados frequentemente associados com a SAB (Grushka e cols, 1998; Pokupec-Gruden e cols, 2000). Uma possível forma de atuação desta citocina na SAB seria devido à capacidade de desencadear hiperalgesia não só em doenças inflamatórias, mas também em doenças possivelmente neuropáticas, como a SAB, (Opree & Kress, 2000). Por outro lado, a variante polimórfica curta do polimorfismo 5HTTLPR, que esta associada com ansiedade (Serretti e cols, 2002), não mostrou relação com a SAB. Apesar disso, outros mecanismos envolvidos na síntese de serotonina devem ser estudados posteriormente.

Dois pontos importantes devem ser considerados durante a análise do estudo de polimorfismos genéticos. Primeiro devemos lembrar que o fenótipo, por definição, é o resultado da interação entre o genótipo e o meio ambiente. Com isto, vale a pena lembrar que uma limitação de estudos que avaliam exclusivamente o status genético das populações é a impossibilidade de inferências qualitativas e quantitativas sobre as proteínas codificadas por estes genes.

Segundo, outros polimorfismos genéticos alto produtores de uma proteína antagonista podem estar em desequilíbrio de ligação com o polimorfismo de estudo. Isto

normalizaria a ação destas proteínas. A solução para este problema só é possível através de consórcios internacionais de mapas haplóticos (Hapmap), que avaliam a distribuição de vários polimorfismos em várias populações (*, 2005). Com base nisso, o objetivo geral deste trabalho foi realizar uma investigação inicial na busca de variações genéticas na EUR e SAB.

Os estudos de associação entre polimorfismos genéticos e doenças têm se mostrado uma importante ferramenta não só para investigar a etiologia das doenças, mas também para prognóstico dos tratamentos a serem instituídos nas mesmas. Um exemplo disso é que pacientes com genótipos que conferem uma maior produção de IL-1β têm maior sucesso ao tratamento anti viral para hepatite B (Chan e cols., 2006). Em doenças onde não se observa um único agente etiológico definido, como a EUR e a SAB, a possibilidade de se encontrar um guia terapêutico, baseado em possível genotipagem, poderá trazer um impacto positivo para qualidade de vida dos portadores destas enfermidades.

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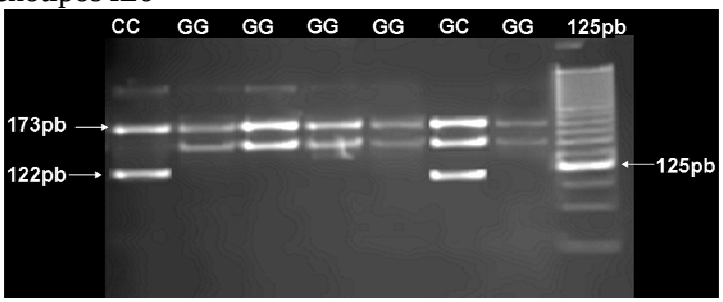
6- ANEXOS

Anexo I (ASPECTOS ÉTICOS)

A realização do presente estudo foi aprovada pelo Comitê de Ética em Pesquisa da UFMG – COEP, conforme protocolo Nº 009/00.

Anexo II (Genótipos)

Genótipos *IL6*



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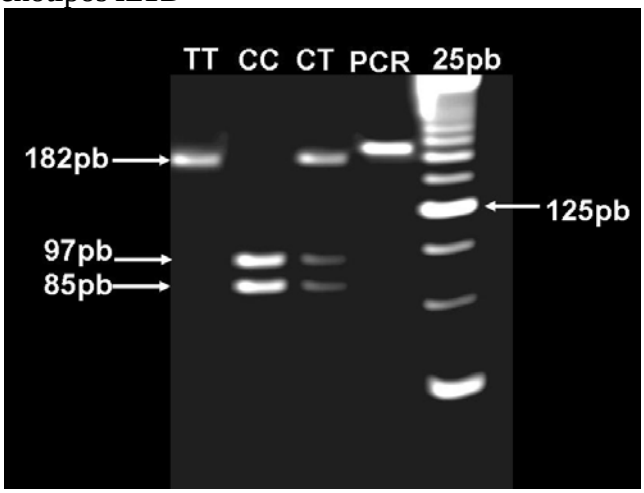

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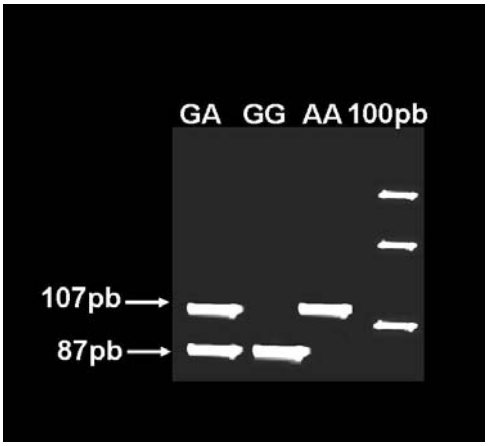

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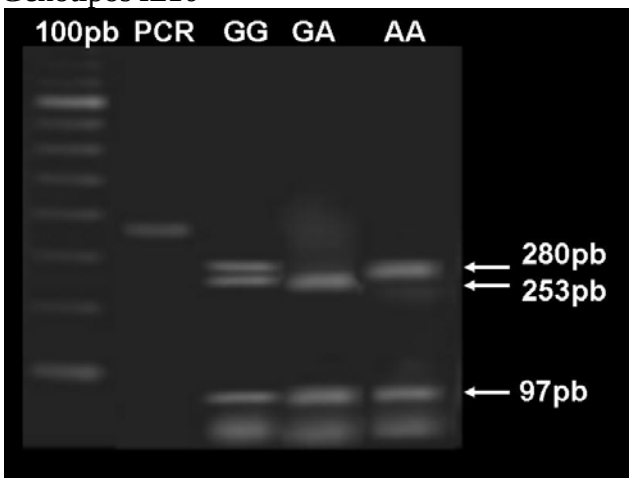
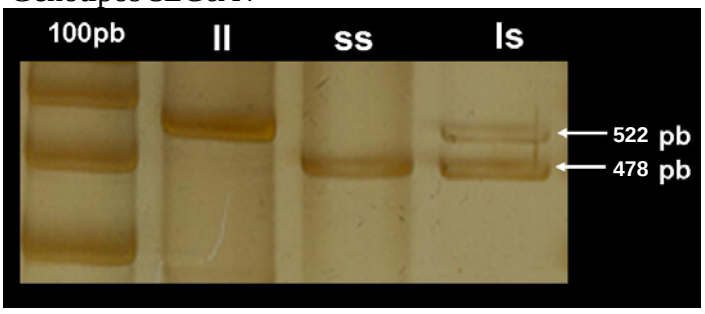


Genótipos *SLC6A4*



Anexo III (Sequências dos polimorfismos genético)

Os primers estão sublinhados e os sítios de restrição apontados por setas