Oral Diseases (2006) 12, 580–583. doi:10.1111/j.1601-0825.2006.01243.x

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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.

Oral Diseases (2006) **12**, 580–583

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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Received 9 August 2005; revised 18 November 2005, 12 December 2005; accepted 23 December 2005

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

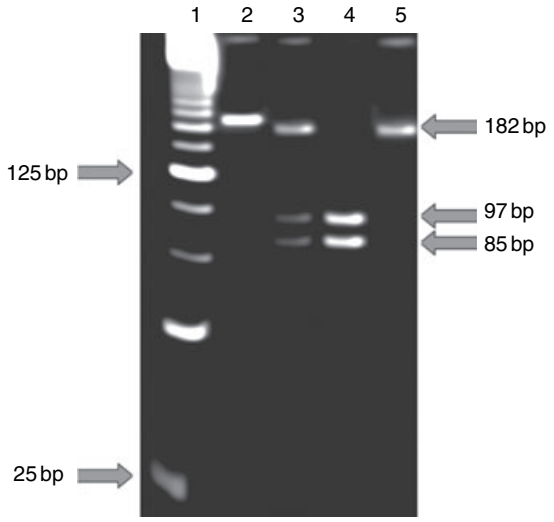

Figure 1 Electrophoresis in a 10% polyacrylamide gel. Lane 1, ladder 25 bp; lane 2, PCR product without digestion (194 bp); lane 3, genotype CT; lane 4, genotype CC; lane 5, genotype TT

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.

Acknowledgements

This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Brazil. Dr RS Gomez is a research fellow of CNPq.

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