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Hyper-reactive mononuclear cells and neutrophils in chronic periodontitis

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Abstract

Objectives: Stimulated mono- and polymorphonuclear cells from patients with periodontitis have shown increased release of interleukin-1β (IL-1β) and oxygen radicals, respectively. The aim was to study whether this hyper-reactivity could be found both in mono- and polymorphonuclear cells from the same patient, and whether there was a relation to the gene coding for IL-1β (IL-1β^{+3953}).

Material and Methods: Peripheral mononuclear cells from 14 non-smoking and well-treated patients and pair-matched controls were incubated with opsonized *Staphylococcus aureus* and lipopolysaccharide (LPS). Released IL-1β and tumour necrosis factor (TNF)-α were determined with ELISA. Generation of oxygen radicals from the Fcγ-receptor-stimulated neutrophils was measured with chemiluminescence and the polymorphism at IL-1β^{+3953} was measured with polymerase chainreaction.

Results: The mononuclear cells from the patients released more IL-1β after incubation with LPS ($p<0.001$) and with bacteria ($p<0.05$). The release of TNF-α tended to be higher in the patient group. The peripheral neutrophils from the patients generated more oxygen radicals ($p<0.06$). We found no differences between the study groups regarding the IL-1β^{+3953} polymorphism.

Conclusion: The similarity in systemic inflammation between patients and controls suggests that the increased release/generation of IL-1β and oxygen radicals from peripheral leukocytes in periodontitis patients is of a constitutional nature and of pathogenic relevance.

Key words: IL-1β; oxygen radicals; neutrophils; mononuclear cells; periodontitis

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It is generally accepted that periodontitis results from an exuberant inflammation induced by oral microorganisms. The tissue destruction of the tooth-supporting tissues is associated with the release of many proteolytic enzymes and reactive oxygen species (ROS), predominantly from activated neutrophils (Weiss 1989, Tervahartiala et al. 2000). Several in vitro studies have shown hyper-reactive neutrophils in patients with periodontitis (Fredriksson et al. 2003), which may lead to an excessive release of ROS and proteolytic enzymes followed by tissue destruction.

The inflammation may depend on constitutional factors modulated by proinflammatory cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α). Higher concentrations of proinflammatory cytokines such as IL-1β have been associated with deep pockets and attachment loss (Figueredo et al. 1999, Faizuddin et al. 2003). Mononuclear leukocytes (monocytes and lymphocytes) are major sources of these cytokines. An increase in the release of IL-1β but not of TNF-α from in vitro lipopolysaccharide (LPS)-stimulated mononuclear cells has been reported in patients with chronic periodontitis (McFarlane et al. 1990). During the last decade, several studies have shown a possible association between periodontitis and polymorphisms of the genes coding for IL-1β synthesis. Some authors have focused on allele 2 of the IL-1β^{+3953} polymorphism, which has been associated with an increased IL-1β production (Pociot et al. 1992). Kornman et al. (1997) and Gore et al. (1998) found a correlation between a genotype including this allele and a higher risk of severe periodontitis, but others could not confirm this, especially in non-smokers (Hodge et al. 2001, Meisel et al. 2004).

The aim of this leukocyte study in chronic periodontitis patients was to measure the release of IL-1β and TNF-α from in vitro-activated peripheral mononuclear leukocytes in relation to that of oxygen radicals from neutrophils

in the same blood samples. The patients were otherwise healthy and periodontally well-treated non-smokers, a prerequisite to minimize the effect of various inflammatory reactions and the local disease activity on the leukocytes. Another aim was to relate the IL-1β release to the distribution of allele 2 of the IL-1B^{+3953} polymorphism.

Material and Methods

Participants

Fourteen non-smokers including five women (mean age 51.1 ± 6.2 years) having a periodontal status of at least six sites with marked attachment loss (clinical attachment loss >5 mm) and 14 non-smoking healthy, age- and gender-matched controls (staff members, mean age 53.3 ± 7.6 years) participated in this study. The patients had received comprehensive periodontal treatment that included training about how to achieve meticulous oral hygiene and scaling, with or without periodontal surgery, resulting in few shallow pockets and negligible gingivitis. All participants were healthy without ongoing systemic diseases or infections, no allergies or heart diseases and had taken no medication or antioxidant drugs during the last week.

To assess the general health of the participants, the blood counts and the levels of some acute-phase proteins relevant to inflammation were determined.

All subjects gave informed consent to participate, and the study was approved by the Ethics Committee at Huddinge University Hospital.

Blood status and plasma proteins

Venous blood was taken with EDTA vacutainer tubes (Becton-Dickinson, Rutherford, NUJ, USA) from all participants. Leukocytes and differential counts in blood samples were performed with a cell counter (Coulter STAKES Analyzer, Coulter Electronics Inc., Hialeah, Fl, USA). Plasma was obtained from the EDTA-blood after centrifugation at $1700 \times g$ for 10 min. and frozen at -70°C, pending analysis. The plasma haptoglobin and α-1-antitrypsin proteins were measured with a nephelometer analyser (Behringwerke AG Diagnostica, Marburg, Germany) using polyclonal antibodies. Capsular reactive protein (CRP) was determined with a high-sensitive commercial kit (DADA Bering, Deerfield, IL, USA)

Preparation of leukocytes

Neutrophils and mononuclear cells were separated with density centrifugation (1500 r.p.m., 10 min. at room temperature) after lysis of the red blood cells with 0.83% NH_4Cl, using a single Percoll gradient ($\delta = 1.079$) (Pharamacia, Uppsala, Sweden). The cells were washed twice in cold phosphate-buffered saline (PBS), counted in a cell counter and stored at $+4^{\circ}$C until use within 3 h.

Stimulation of mononuclear cells

The mononuclear cells were incubated for 20 h in 1 ml RPMI buffer (Gibco, Invitrogen, Carlsbad, CA, USA) at 37°C, with (i) *Staphylococcus aureus* opsonized with commercial γ-globulin as described earlier (Bergström & Åsman 1993), 150 bacteria per mononuclear cell, (ii) 1 ng/ml LPS (from *Escherichia coli* 0111:84, Sigma-Aldrich, Inc., St. Louis, MO, USA) or (iii) buffer alone.

After incubation, the cells were centrifuged at $+4^{\circ}$C with 350 g for 10 min. and the supernatant was harvested and frozen at 70°C, pending analysis of IL-1β and TNF-α.

Free oxygen radicals

The generation of oxygen radicals after Fcγ-receptor-mediated activation with *S. aureus* opsonized with γ-globulin was measured by luminol-enhanced chemiluminescence, using the method described by Bergström & Åsman (1993). Briefly, the neutrophils were mixed with luminol and bacteria (cell: bacteria = 1: 200), and the maximal light intensity was recorded with a luminometer (Victor II, Wallac, Turku, Finland).

The amounts of IL-1β and TNF-α released from mononuclear cells were measured with commercial ELISA kits (R&D System Europe Ltd., Abingdon, UK) in accordance with the manufacturer's instructions.

Analysis of *IL-1β*$^{+3953}$ polymorphism

IL-1β^{+3953} genotypes were determined with the polymerase chain reaction using a slight modification of the method described by Gore et al. (1998). The primers were: 5′-CTC AGG TGT CCT CCA AGA AAT CAAA-3′ and 5′-GCT TTT TTG CTG TGA GTC CCG-3′ (Cybergene, Huddinge, Sweden)

Statistical analyses

The blood counts and concentrations of acute-phase reactants are given as mean values and standard deviation, while the release of IL-1β and TNF-α is expressed as median and interquartile range (IR). The significance of the differences between patients and controls was determined with the Wilcoxon signed-rank test. The correlation between oxygen radical generation from in vitro-activated peripheral neutrophils and cytokine release from the mononuclear cells was determined with the Spearman rank order correlation.

Results

The blood counts and plasma concentrations of acute-phase reactants were all within normal ranges, and no significant differences were noted between the patients and the controls (Table 1). This excludes the possible influence of inflammatory factors on the leukocytes studied.

The incubation medium alone (including Ca^{2+}) caused a slight release of cytokines. The release of IL-1β from mononuclear cells of patients was higher than from the cells of healthy controls during incubation with bacteria and with LPS. The difference was most marked when the cells were incubated with LPS. This activation was stronger in the

Table 1. Blood count and plasma concentrations of the acute-phase reactants – i.e. capsule-reactive protein (CRP), haptoglobin and α-1-antitrypsin – in venous blood from 14 patients with chronic periodontitis and 14 periodontally healthy controls, mean values (standard deviation)

	Patients	Controls
White blood cells (10^9/l)	5.7 (1.1)	5.8 (1.2)
Neutrophils (10^9/l)	3.0 (0.6)	3.2 (0.9)
Lymphocytes (10^9/l)	1.9 (0.6)	1.5 (0.4)
Monocytes (10^9/l)	0.34 (0.10)	0.36 (0.11)
CRP (mg/l)	2.2 (3.4)	1.4 (1.3)
Haptoglobin (g/l)	1.1 (04)	0.84 (0.24)
α-1-antitrypsin (g/l)	1.2 (0.13)	1.04 (0.25)

No significant differences were noted between patients and controls (Wilcoxon' signed-rank test).

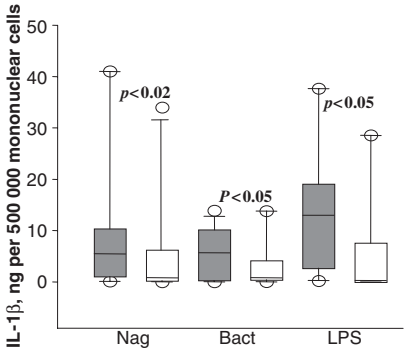

Fig. 1. Release of interleukin-1β (IL-1β) from 0.5×10^6 mononuclear cells during incubation for 20 h with opsonized *Staphylococcus aureus* (Bact, 150 bacteria/mononuclear cell) and lipopolysaccharide (LPS) (1 ng/ml). The cells referred to as no activating agent (Nag) have been incubated without an activating agent. Significance of the differences was calculated with the Wilcoxon signed-rank test. Filled boxes indicate patients. $N = 14$ pairs.

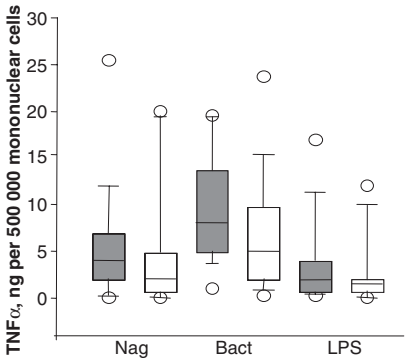

Fig. 2. Release of tumour necrosis factor (TNF)-α from 0.5×10^6 mononuclear cells during incubation for 20 h with opsonized *Staphylococcus aureus* (Bact, 150 bacteria/mononuclear cell) and lipopolysaccharide (LPS) (1 ng/ml). The cells referred to as no activating agent (Nag) have been incubated without an activating agent. Significance of the differences was calculated with the Wilcoxon signed-rank test. Filled boxes indicate patients. $N = 14$ pairs.

patient group (Fig. 1). Although the release of TNF-α was usually higher from the patients the differences were not significant (Fig. 2).

The generation of oxygen radicals from in vitro Fc-γ receptor activated neutrophils, measured as peak chemiluminescence, was higher in the patient group (median (IR) 372 (145) mV) than in the control group, 294 (163) mV, $p = 0.06$. However, no correlations between neutrophil reactivity and monocyte reactivity were found in this study (data not shown).

Table 2. Distribution of interleukin (IL)-1 genotypes and mean (SD) release of IL-1β from peripheral mononuclear cells during incubation with lipopolysaccharide (LPS) (ng/0.5 $\times$ 10^6 mononuclear cells)

	Homozygous for allele 1	Heterozygous	Homozygous for allele 2
Patients	8	5	0
Controls	8	5	0
All participants	16	10	0

$n = 13$ patients with chronic periodontitis and 13 periodontally healthy controls.

The frequencies of the alleles of IL-1β^{+3953} polymorphism are shown in Table 2. The distribution of alleles 1 and 2 was the same in the patient and control groups. No participant was homozygous for allele 2 (Table 2), and the release of IL-1β did not differ significantly between the genotypes.

Discussion

This study indicates that mononuclear cells and neutrophils from patients with adult periodontitis are hyper-reactive. Fc-γ receptor- or LPS-activated mononuclear cells from patients released significantly more IL-1β, and Fc-γ receptor-activated peripheral neutrophils from the same blood samples generated more reactive oxygen species. The latter has been shown in several studies (Fredriksson et al. 1998). Some authors have reported a reduced, and others a similar or even no release of IL-1β from mononuclear cells after LPS stimulation in periodontitis (Garrison & Nichols 1989, Shapira et al. 1994, Fokkema et al. 2002, Mahanonda et al. 2004). An increase in the release of IL-1β from mononuclear cells after LPS stimulation is reported in patients with chronic periodontal disease (McFarlane et al. 1990). These various findings may be related to the presence of lymphocytes, the method of stimulation or the effect of systemic/local inflammatory factors including smoking. However, this is the first time that an increase in the release of IL-1β has been shown together with Fc-γ receptor-activated neutrophil generation of oxygen radicals in non-smoking periodontitis patients with negligible systemic and local inflammation. Our stimulation methods using *E. coli* LPS and opsonized *S. aureus* were chosen, as in earlier studies we have found significant differences in neutrophil responsiveness with these activators (Fredriksson et al. 1998).

The hyper-reactivity of the leukocytes indicated by the increased production of IL-1β and oxygen radicals by mononuclear cells and neutrophils, respectively, suggests that they may be involved in the aetiology and pathogenesis of periodontitis with the cytokine as an inflammatory stimulator and the radicals as tissue-destructive elements. This finding also accords with the in vivo findings of increased concentrations of IL-1β in GCF (Figueredo & Gustafsson 1998, Engebretson et al. 2002).

The proposed hyper-reactivity of the peripheral leukocytes may be secondary to the inflammatory reactions from ongoing periodontitis, e.g. primed by proinflammatory cytokines leaking from the local inflammation. An indication of such an interaction between tissue compartments is the decrease in IL-8 and macrophage chemoattractant protein after full-mouth extraction (Fokkema et al. 2003). The study failed to show an effect on the release of IL-1β but a study by Nakamura et al. (2004) has shown a strong effect of LPS priming on IL-1β release from monocytes. However, the patients in our study had been adequately treated and had only a slight periodontal inflammation locally, which hardly could have had any systemic effect on the peripheral cells. Furthermore, the inflammatory blood and plasma parameters were similar in both groups of non-smoking patients and pair-matched controls. Therefore, patients with periodontitis seemed to have a constitutionally different host response as regards the leukocytes.

Although we found no association between the release of IL-1β and the IL-1β^{+3953} polymorphism, in contrast to Pociot et al. (1992), it is reasonable to believe that leukocyte reactivity is influenced by a number of genetic polymorphisms (Kinane & Hart 2003, Shapira et al. 2005), and this remains the most possible mechanism explaining the leukocyte hyper-reactivity.

In conclusion, the similarity in systemic inflammatory parameters between

patients with well-treated periodontitis and pair-matched controls suggests that the increased release/generation of IL-1β and oxygen radicals from peripheral leukocytes in periodontitis patients is of a constitutional nature and of pathogenic relevance in chronic periodontitis.

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Clinical Relevance

Scientific rationale: Several earlier studies have shown that peripheral leukocytes from patients with periodontitis are hyper-reactive and respond more to in vitro stimulation. The objects of this study were to investigate whether this also is the case for leukocytes from successfully treated patients.

Principal findings: Both neutrophils and mononuclear cells from the patients responded more to in vitro stimulation, i.e. these cells released more oxygen radicals and cytokines. However, this was not related to IL-1β^{+3953} polymorphism.

Practical implications: Our findings imply that even successfully treated patients with no remaining pockets and little gingivitis still must maintain a good plaque control in order to avoid a relapse of the disease since they seem to have an intrinsic characteristic for hyper-reactivity to bacteria and bacterial products.