Induction of interleukin-1 alpha production by *Porphyromonas gingivalis* in mononuclear blood cell cultures from periodontitis patients

M. URQUIÁ¹, A. RODRÍGUEZ-ARCHILLA², A. PALACIOS-CÓRDOBA³, R. ASENCIO⁴, J.M. FERRER⁴

¹ Professor of Oral Medicine. Oral Immunology Research Laboratory.
² Associate Professor of Oral Medicine. Oral Immunology Research Laboratory.
³ Resident. Department of Anaesthesiology. Hospital «San Cecilio» (Granada).

SUMMARY

The capacity of mononuclear blood cells to produce interleukin-1 alpha (IL-1 alpha) after stimulation with *Porphyromonas gingivalis* in cell culture was studied. The results obtained with cells from periodontitis patients were compared with those from a control population. The concentration of IL-1 alpha in serum and saliva was also determined and compared with the concentration in mononuclear blood cell cultures. No significant relationship was found between the incidence of periodontitis or severity of the lesions and IL-1 alpha production in the presence of *P. gingivalis*. Nevertheless, 11 of 30 periodontitis patients, showed levels >30 pg/ml of IL-1 alpha in mononuclear blood cell cultures stimulated by *P. gingivalis*, whereas only three healthy control showed these titers of IL-1 alpha.

KEY WORDS:

Interleukin-1 alpha, periodontal disease, *Porphyromonas gingivalis*.

RÉSUMÉ

Nous avons étudiés la production d'interleukine-1 alpha (Il-1 alpha) par des monocytes sanguins en culture après stimulation par *Porphyromonas gingivalis*. Les résultats observés chez des patients atteints de parodontose ont été comparés avec ceux obtenus chez des témoins. La concentration de Il-1 alpha dans le sérum et la salive a également été comparée avec celle des cultures de monocytes. Nous n'avons pas trouvé de différences significatives entre la sévérité des lésions parodontales et les concentrations d’Il-1 alpha. Cependant, 11 des 30 patients atteints de parodontose présentaient des niveaux d’Il-1 alpha supérieurs à 30 μg/ml, tandis que seulement 3 témoins avaient des niveaux élevés.

MOTS CLÉS:

Interleukine-1 alpha, maladie parodontale, *Porphyromonas gingivalis*.
INTRODUCTION

Local and systemic immune response to some bacterial antigens can play a significant role in the origin, course and prognosis of periodontal disease. The nature of the immune system's contribution to periodontitis has not been completely elucidated. The direct destruction of tissues by bacterial action and by histologic enzymes, eg collagenase, Bacteroides fragilis aminopeptidases (Suido et al., 1986) and other proteases released by bacteria, have both been described. One study (Lamster et al., 1987) has reported a higher concentration of putrescine in the crevicular fluid of teeth with severe periodontitis, as compared with control subjects.

In addition to the existence of bacteria-induced lesions, some of the lesions observed arise from the action of humoral or cellular components of the immune system. In this connection, fibroblastic alterations and enhanced macrophage activation accompanied by the release of proteolytic enzymes have been reported. In certain advanced stages of the diseases, diverse pathogens may have immunosuppressive effects (Shenker, 1987). Farida et al. (Farida et al., 1986) reported raised titers of anti-actinomyces temcomitans IgG antibodies in juvenile periodontitis, and an even more significant increase in anti-Porphyromonas gingivalis IgG antibodies in rapidly progressing periodontitis.

Recent studies have shown the group of substances known as IL-1 to have other properties in addition to their well known action as a mediator of the inflammatory process (Alarcon-Riquelme and Alarcon-Segovia, 1988). Apart from its involvement in regulating the immune response, many observations suggest that this cytokine may play an important role in the pathogenesis of processes that destroy the periodontium. Dewhirst et al. (Dewhirst et al., 1985) noted that the molecular structure of osteoblast activating factor was similar to that of IL-1. Littlewood et al. (Littlewood et al., 1991) described the promotion of IL-6 production by IL-1, and called attention to the presence of IL-6 receptors on osteoblasts. In gingival tissues, Bartold et al. (Bartold and Haynes, 1991) observed that IL-6 production by gingival fibroblasts increased in the presence of IL-1 (Clement-Lacroix et al., 1991).

The production of other cytokines is also influenced by IL-1. Kristensen et al. (Kristensen et al., 1991) have shown that IL-1 enhances IL-8 production markedly in endothelial cells, moderately in fibroblast and monocyte cultures, and weakly in keranocyte cultures. This cytokine is also thought to be responsible for the production of prostaglandin E-2 (Tatakis et al., 1991), which acts directly on osteoblasts (Otterness et al., 1991), osteoclasts and vascular endothelium (Kasahara and Mukaida, 1991), and facilitates osteoclast activity (Nguyen et al., 1991). IL-1 has also been shown to be actively involved in the destruction of collagen (Hauptmann et al., 1991), which acts directly on gingival tissue.

The findings summarized above suggest that the group of substances termed IL-1 are involved in the pathogenesis and appearance of periodontal disease (Meikle et al., 1990), either as a modulator of the immune response (antigen presentation) or as an agent that acts directly or indirectly on part of the bone and vascular lesions that appear in periodontitis. The present study was designed to evaluate the capacity of circulating nucleated cells to produce IL-1 alpha in cell culture with P. gingivalis. The concentration of IL-1 alpha produced in cell culture was compared with the concentrations in serum and saliva, with particular attention to the relationship between cytokine production and the severity of periodontal disease.

MATERIAL AND METHODS

Blood samples were obtained from a group of 30 patients (mean age 55.1 ± 4.7 years) with periodontitis of different degrees of severity: 8 with gingivitis, 11 with moderate periodontitis (periodontal pocket depth 3-6 mm) and 11 with severe periodontitis (periodontal pocket depth > 6 mm) and from 20 healthy control subjects (mean age 55.8 ± 5 years) free of systemic disease.

1 ml of unstimulated saliva and 10 ml of peripheral blood samples were collected from both groups. The blood was processed under sterile conditions. Defibrination with glass pellets was followed by separation of the two blood cell series by Ficoll-Paque density gradient centrifugation (density 1082). The halo of nucleated cells was adjusted in a Neubauer chamber to a suspension of 5×10⁶ cells/ml. P. gingivalis (ATCC 33277) were obtained through the courtesy of Professor J. Liebana, Department of Microbiology, University of Granada, Spain. P. gingivalis was grown under an atmosphere
of 80% N₂: 10% CO₂: 10% H₂ on plates consisting of trypsinase soy agar (DIFCO Laboratories) supplemented with 0.1% yeats extract, 0.01% menadione and 0.05% equine hemin III. Bacteria were suspended in Dulbecco’s phosphate buffered saline (DPBS) pH 7.4. Blood cells were incubated during 24 hours in glutamine enriched RPMI-1640 medium in the presence of a constant concentration of P. gingivalis (1 x 10⁶ bacteria/ml).

IL-1 alpha in the supernatant was detected with a commercial assay: a sandwich immunoassay with peroxidase-conjugated anti-IL-1 alpha as the second antibody (Quantikine™, Research and Diagnostics Systems). The manufacturers instructions were carefully adhered. 200 µl of each supernatant obtained after incubation was added in each well of in 96-well U-bottomed microtiter plates, previously coated with anti-IL-1 alpha antibody and incubate for 2 hours at room temperature.

After washing with wash buffer solution, 200 µl of IL-1 alpha conjugate was added to each well and incubate for 1 hour at room temperature. After washing again, another 200 µl of substrate solution (tetramethylbenzidine and hydrogen peroxide) was added to each well and incubate for 20 minutes at room temperature. Finally, 50 µl of stop solution was added to each well. The wells were read by spectrophotometry at 450 nm. Results were obtained by reference to a standard curve generated with different standard samples provided in the commercial assay, which was linear in the range of 0-250 pg/ml of IL-1 alpha. So, the minimum detectable dose of IL-1 alpha using this standard curve is 0.3 pg/ml. The Microstat integrated statistics package was used to analyze the results. The results obtained were distributed in three groups: low (<30 pg/ml); moderate (30-100 pg/ml) and high values of IL-1 alpha (100-200 pg/ml).

RESULTS

IL-1 alpha values >30 pg/ml were found in serum in 6 patients, in saliva in 6 patients and in the supernatant of cultures exposed to P. gingivalis in 14. With the exception of one sample positive finding in saliva, all values found in saliva were <100 pg/ml. In contrast, several of the values in supernatant were high. The subjects with serum IL-1 alpha levels >30 pg/ml did not show high values of IL-1 alpha in saliva. Figure 1 shows the distribution of patients and controls with different levels of interleukin-1 alpha expression in serum, saliva and supernatant.

Figure 2 compares the results obtained in saliva and in the supernatant of mononuclear cell fraction incubated with P. gingivalis for the group of periodontitis patients. The subjects who yielded levels >30 pg/ml of IL-1 alpha in saliva generally matched those with high concentrations in supernatant.

No significant association between the degree of severity of periodontitis and IL-1 alpha concentration in supernatant, was seen. Figure 3 shows the distribution of periodontitis patients according to the severity of the disease and the concentration of IL-1 alpha. However, this figure shows that cell cultures from patients with more severe degrees of periodontitis produced the largest amounts of IL-1 alpha after stimulation by P. gingivalis.
**DISCUSSION**

Recent studies have suggested that other cells (e.g., Langerhans cells) in addition to members of the monocyte/macrophage series may be involved in IL-1 production.

Weinstein et al. (Weinstein et al., 1987) found IL-1 on the membrane of B lymphocytes and fibroblasts, but was unable to determine whether this type of membrane constituted a stage previous to IL-1 secretion. Endothelial, epithelial, NK and mesangial cells, neutrophils and some T cell clones have also been found to secrete IL-1. As noted by Markinkiewicz (Markinkiewicz, 1991), only certain subpopulations of monocytes and macrophages produce this cytokine, and IL-1 secreting cells of this lineage play different roles in immune response.

Although this study was not intended to tell us whether the IL-1 alpha we detected was secreted only by antigen presenting cells, we suspect that the contribution of other cell types is minor compared to that of monocytes. Because of the difficulty in isolating pure series of cells by density gradient centrifugation, and due to the equivocal results this method yields, we used mononuclear cell fractions in the present study. This decision does not weaken the significance of our findings, since our objective was to characterize the IL-1 alpha producing capacity in response to stimulation by *P. gingivalis* antigens, rather than to compare IL-1 production by different types of cells.

We chose *P. gingivalis* because it is commonly present in periodontitis (Pertuiset et al., 1987), and because of its marked antigenic capacity. Thus, previous works of Wilton et al. (Wilton et al., 1992) and Matsubara (Matsubara, 1990) emphasize the important role of this microorganism in the release of inflammatory mediators in periodontal disease. It has been shown that endotoxin released by *P. gingivalis* is a potent signal for the production of IL-1 by macrophages (Hanazawa et al., 1985). The involvement of these microorganisms (either directly or indirectly [Page, 1991], via lesions caused by their endotoxin) in the appearance of periodontitis (Saglie et al., 1987) and the severity of the lesions (Williams et al., 1987) makes them worthy of further study.

Most subjects who reported a history of treatment with antibiotics, frequent infections or some degree of (humoral or cellular) hyperergy were excluded from the study.

Stimulation of IL-1 alpha production in the presence of the bacteria was much more marked in the patient.
group (14 vs 30) than in controls (3 vs 20). These values were much lower than those reported by Lindemann and Economou (Lindemann and Economou, 1988), who found increased IL-1 production by monocytes from nearly all their periodontitis patients. In their study, nearly all Gram negative bacteria stimulated the production of IL-1 and tumor necrosis factor (TNF) to a similar degree. However, these authors did not give the results of stimulation assays with material from control subjects, nor did they say whether the stimulation achieved in cells from periodontitis patients was comparable to that in control monocytes. Their controls consisted of populations of monocytes not subjected to antigenic stimulation. Moreover, these authors used an indirect method to measure IL-1 concentrations after cell culture; they added the supernatant to thymocyte culture and observed the stimulation and proliferation capacity of these cells by measuring tritiated thymidine uptake.

The number of periodontitis patients whose mononuclear cell fraction reacted to *P. gingivalis* stimulation by producing IL-1 alpha was greater in our study than in the series reported by Yamazaki *et al.* (Yamazaki *et al.*, 1989). None of the periodontopathic bacteria used by these authors was able to significantly stimulate neutrophils to produce IL-1, and increased concentrations of cytokine were found in only 4 out of their 18 subjects.

Yamazaki *et al.* (Yamazaki *et al.*, 1989) used enriched populations of polymorphonuclear leukocytes obtained by Ficoll-Paque density gradient centrifugation, and containing less than 2% monocytes. As in the above mentioned study (Lindemann and Economou, 1988), IL-1 concentration in the supernatant was determined indirectly by quantifying thymocyte stimulation. This method may give misleading results due to the production by PMNs of factors that inhibit IL-1; thymocyte stimulation would thus be reduced not because of the presence of less IL-1, but because the action of the cytokine would be curtailed. The direct measurement of IL-1 in the supernatant avoids these possible sources of interference. We believe the greater number of positive tests in saliva among periodontitis patients in comparison to controls reflects the greater degree of unspecific antigenicity due to deficient oral hygiene in patients. However, Figure 2 illustrates that those patients in which IL-1 alpha production was markedly stimulated in supernatant also had concentrations >30 pg/ml of cytokine in saliva.

Patients with titers >30 pg/ml of IL-1 in serum did not match those with titers >30 pg/ml in saliva, suggesting that IL-1 alpha production by perioral tissues is independent.

The IL-1 molecules are readily susceptible to destruction and are markedly unstable in aqueous media as well as in phosphate or citrate buffers. The mechanism of breakdown is temperature-dependent (Gu *et al.*, 1991): at 39°C breakdown occurs by oxidation of two cysteine residues, while maximum stability is achieved in cultures at 3°C. These temperature requirements may explain part of the discrepancies between the results published by different authors. Moreover, one study has described an anti-IL-1 activity in the supernatant of cell cultures and in serum, that causes proteolysis and interferes with the determination of IL-1 with ELISA (Mae *et al.*, 1991). These two impediments to the accurate measurement of IL-1 *in vivo* and *in vitro* make caution necessary in evaluating the results published to date.

Figures 3 shows the distribution of patients according to the severity of periodontitis and IL-1 alpha concentration. Although there was no significant difference between patients with mild and severe lesions in IL-1 alpha concentration, the percentage of patients with raised titers was greater among patients with moderate or severe periodontitis. It is noteworthy that in these patients, oral hygiene tended to be deficient. A study of MacFarlane *et al.* (MacFarlane *et al.*, 1990) shows that unstimulated monocytes from periodontitis patients released significantly more IL-1 beta than controls. There was a wide variation in the amount of IL-1 beta released (0.45-13.00 ng/ml per 10⁶ cells) which did not correlate with either the degree of bone loss or pocket formation observed clinically. However, the findings of Wang and Stashenko (Wang and Stashenko, 1993) indicate that bone resorption stimulated by bacterial infection is primarily mediated by IL-1 alpha in a rat model system.

ACKNOWLEDGEMENTS

This study was supported by the Andalusian Regional Government through the Research Team Training Program (Plan de Consolidación de Grupos de Investigación). We thank Ms Gertrudis Gómez Villaescusa for her technical assistance, and Ms Karen Shaskok for translating the original manuscript into English.
REFERENCES


