Levels of Interleukin-1β, -8, and -10 and RANTES in Gingival Crevicular Fluid and Cell Populations in Adult Periodontitis Patients and the Effect of Periodontal Treatment

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Background: Various cytokines have been identified at sites of chronic inflammation such as periodontitis. Cytokines are synthesized in response to bacteria and their products, inducing and maintaining an inflammatory response in the periodontium. The purpose of the present study was to investigate the involvement of interleukin-1β (IL-1β), IL-8, and IL-10 and RANTES (regulated on activation, normally T cell expressed and secreted) and the cell populations associated with the immune response in destructive periodontitis, as well as the effect of periodontal therapy on cytokine levels in gingival crevicular fluid (GCF).

Methods: Data were obtained from 12 patients with moderate to advanced periodontitis and 6 healthy controls. Patients presenting at least 2 sites with ≥2 mm clinical attachment loss were included in the destructive periodontitis group. After monitoring for 4 months, only 6 patients showed destructive periodontitis and GCF samples and soft tissues biopsies were collected from these patients. GCF samples and biopsies were collected both from active (12 CGF samples and 6 biopsies) and inactive (12 CGF samples and 6 biopsies) sites. The comparison with healthy controls was carried out by collecting GCF samples from 6 healthy volunteers (12 samples) and biopsies during the surgical removal of wisdom teeth. In periodontal patients, clinical data and GCF samples were obtained prior to periodontal treatment (72 samples) and 2 months after periodontal therapy (72 samples). GCF was collected using a paper strip; eluted and enzyme-linked immunoabsorbent assays (ELISA) were performed to determine cytokine levels. The inflammatory infiltrate was analyzed by immunohistochemistry of gingival biopsy samples with monoclonal antibodies against CD3, CD8, CD4, CD11c, and CD19 antigens.

Results: Cellular components of the inflammatory infiltrate include B and T lymphocytes and monocyte/macrophages. Active sites contained a higher number of B lymphocytes and macrophages. IL-8 and IL-1β and RANTES in GCF were detected in the majority of sites from periodontal patients (100%, 94% and 87%, respectively); IL-10 was found in only 43%. IL-8 was the only cytokine detected in the GCF (75%) of the control group. Moreover, IL-1β levels were significantly higher in active sites versus inactive sites (P <0.05). IL-8 and IL-10 and RANTES were increased in active sites; however, differences were not significant (P >0.05). A positive correlation between the IL-8 and RANTES (r = 0.677, P <0.05) was observed in periodontitis patients. Periodontal therapy reduced the total amount of IL-1β, IL-8, and IL-10 and RANTES. Data showed a weak correlation between the clinical parameters and the total amount of cytokines in periodontitis.

Conclusions: These data suggest that the amount of crevicular IL-1β, IL-8, and IL-10 and RANTES is associated with periodontal status. Removal of the bacterial plaque reduces the antigenic stimuli and consequently could modulate the chemokines present in GCF. We propose that the dynamic interactions between cytokines, their production rates, and their quantity could represent factors controlling the induction, perpetuation, and collapse of the cytokine network present in the periodontal disease. J Periodontol 2000;71:1535-1545.

KEY WORDS

Immune response; interleukin 1-β; interleukin-8; interleukin-10; RANTES; cytokines; gingival crevicular fluid; periodontitis/etiology; periodontitis/therapy.
Periodontal disease results from the interaction of the host defense mechanisms with the plaque microorganisms. The immune response underlying the pathogenesis of the disease has been studied for the past 30 years. Histological studies support the notion that the immune system reacts against plaque microorganisms. The infiltrate present in periodontal disease contains mononuclear cells, mainly transmigrated mononuclear phagocytes and lymphocytes. Whereas T lymphocytes predominate in the established chronic lesion, the proportion of B cells and plasma cells increases with disease progression.1-4

There is abundant evidence that major tissue destruction in established periodontitis lesions results from recruitment of host cells via activation of monocytes/macrophages, lymphocytes, fibroblasts, and other cell types. Considerable effort has been made to study the cytokines released by different host cells when exposed to components of periodontopathogenic bacteria.5,6 These studies have demonstrated that a number of pro-inflammatory cytokines are synthesized in response to periodontopathogenic bacteria and their products, hence inducing and maintaining an inflammatory response in the periodontium.7,8

IL-1 is a pro-inflammatory cytokine which enables the recruitment of cells towards infection sites, promotes bone resorption, and stimulates prostaglandin (PGE2) release by monocytes and fibroblasts and the release of metalloproteinases that degrade extracellular matrix proteins.9 The predominant form of IL-1 found in the periodontal tissues is IL-1β, which is primarily produced by macrophages.10,11 Increased levels of interleukin-1 have been reported in the inflammatory gingival tissue of patients with periodontitis.12,13 IL-1 was also detected in gingival crevicular fluid (GCF)14,15 from diseased, inflammatory periodontal tissues and elevated levels have been associated with active sites and with the progression of the disease.16

The cytokine IL-10 is produced by T helper 2 (Th2) lymphocytes and inhibits cytokine production by activated T helper 1 (Th1) clones.17 The ability of IL-10 to inhibit cytokine synthesis by both T cell types was found to be due to its inhibitory effect on macrophage-monocytes.18 IL-10 also modulates the expression of cytokines of myeloid origin, with important functional consequences on the activation and maintenance of the immune response. The effect of IL-10 on different cell types suggested anti-inflammatory or immunosuppressive activities in vivo, which have been tested in a variety of experimental models19-21 and may exert potent regulatory effects on immune responses in periodontal disease.

During the last decade, a family of structurally related cytokines with chemotactic activity for specific types of leukocyte populations has been identified, and are presently known as chemokines.8 The molecular hallmark of the chemokine family is the conservation of 4 cysteine residues which affect the tertiary structure of the proteins. Depending on the number of amino acids separating these 2 cysteines, the chemokines can be divided into CXC, CC, C, and CX3C subfamilies. CXC chemokines mainly attract and activate neutrophils, whereas CC chemokines attract and activate monocytes, lymphocytes, basophils, eosinophils, natural killer cells, and dendritic cells.22,23 IL-8 is one of the CXC chemokines and is considered a relevant mediator of granulocyte accumulation.24 IL-8 has been detected in GCF25-27 and in the gingival tissue of periodontal patients.28,29 RANTES (regulated on activation, normal T cells expressed and secreted) is a member of the CC chemokines, with significant chemotactic activity for eosinophils,30 monocytes,31 and CD45+ T cells.32 The previous data suggest a role for RANTES in both acute and chronic stages of inflammation.

The aim of our study was to determine the total amount and local concentration of the pro-inflammatory cytokine IL-1β, the CXC chemokine IL-8, the CC chemokine RANTES, and the immunosuppressive cytokine IL-10 in GCF samples; we also identified the cell populations present in lesions undergoing episodic attachment loss from gingival tissue biopsies and evaluated short-term variations of IL-1β, IL-8, and IL-10 and RANTES in GCF when periodontal therapy was provided.

MATERIALS AND METHODS

Patient Population
The study group consisted of 12 adult patients (4 men and 8 women; 36 to 62 years old; mean age 47.16 ± 11 years) with moderate to advanced periodontitis selected from the Primary Attention Service, Dentistry School of the University Complutense, Madrid, Spain. All patients had at least 14 natural teeth excluding third molars (10 or more posterior teeth), 5 to 6 teeth with probing depth ≥ 3 mm and attachment loss ≥ 3 mm, and radiographic evidence of moderate to advanced periodontal disease. Subjects did not suffer from systemic illness and had not received antibiotics or non-steroid anti-inflammatory therapy in the 6 months prior to the study; furthermore none had received periodontal therapy at the time of examination. Six periodontal healthy subjects were recruited for the control group (2 men and 4 women; 35 to 51 years old; mean age 40.1 ± 3 years). They did not have periodontal disease as determined by the absence of clinical attachment loss and no sites had probing depth ≥ 5 mm. Prior to the study, all subjects received supragingival prophylaxis to remove gross calculus and allow probing access. The protocol was clearly explained to all patients and Institutional Review Board-approved informed consents were signed. The protocol stated that, within 2 weeks of the detection of disease activ-
ity, patients would be provided with periodontal treatment.

**Clinical Measurements**

Clinical parameters were evaluated in all teeth, excluding third molars, and included: probing depth (PD), clinical attachment loss and dichotomous measurements of supragingival plaque accumulation (PI), and bleeding on probing to the base of the crevice (BOP) (Table 1). Six sites were examined for each tooth: mesiobuccal, buccal, distobuccal, distolingual, lingual, and mesiolingual. An automated disk probe was used for attachment level recordings and for probing depths. Attachment level and probing depth data were taken again 7 days after the beginning of the study. Disease activity was defined by the tolerance method. The active sites exhibited attachment loss ≥2.0 mm during the following 2-month period. Patients were monitored for 4 months, and all clinical measurements were taken at 2-month intervals until at least 2 sites showed ≥2 mm clinical attachment loss and again after 2 months of periodontal therapy. One calibrated examiner monitored the patients and collected the clinical reports.

Six of the 12 periodontitis patients exhibited at least 2 sites with ≥2 mm attachment loss. GCF and soft tissue gingival biopsy samples were collected from active and inactive sites in these 6 patients. Inactive sites were defined as those sites with PD and BOP equivalent to active sites, but without attachment loss.

In order to monitor the effect of periodontal therapy on cytokine levels, clinical parameter measurements and the GCF collection were also performed after 2 months of periodontal therapy, evaluating all sites selected at the beginning of the study. Periodontal therapy consisted of scaling, root planing, and oral hygiene instruction.

**Collection of Gingival Crevicular Fluid**

After isolating the tooth with a cotton roll, supragingival plaque was removed with curets, avoiding the marginal gingiva. The crevicular site was then gently dried with an air syringe. GCF was collected by placing filter paper strips into the sulcus/pocket until mild resistance was sensed and left in place for 30 seconds. Strips contaminated by saliva or blood were excluded from the sampled group.

GCF samples were obtained from 12 independent sites (72 samples), from 3 locations based on probing depth: 2 sites with ≤3 mm, 2 sites with 4 to 6 mm, and 2 sites with ≥6 mm of randomly selected teeth.

GCF samples were collected again 2 months after periodontal therapy from the same sites to evaluate the effect of therapy on cytokine levels (72 samples). GCF samples were also obtained from 2 sites in the 6 healthy controls (12 samples) at the mesiobuccal gingival sulci (teeth 16 and 26). Cytokine levels from GCF samples collected from the active (12 samples) versus inactive sites (12 samples) were compared in the 6 patients with severe periodontitis.

**GCF Analysis**

When GCF was collected, the volume of the sample (Table 1) on the filter paper strips was measured using a calibration unit. A standard curve correlating digital read out to volume was constructed for each calibration with standard human serum. Each GCF sample was applied 3 times to a filter paper strip and the corresponding units were recorded. No re-calibration of the calibration unit was necessary throughout the study period. The readings were converted to an actual volume (µl) by reference to the standard curve. After GCF volume measurements, strips were placed in Eppendorf vials with 50 µl of phosphate buffered saline with 0.05% Tween-20 (PBS-T). GCF was extracted by centrifugation at 10,000 g for 5 minutes at 4°C and the procedure was repeated 3 times.

**Quantification of Cytokines**

Aliquots of each GCF sample were assayed by an enzyme linked immunosorbent assay (ELISA) to determine the levels of IL-1β, IL-8, and IL10 and RANTES using matched antibody pairs and according to the manufacturer’s recommendations. Briefly, 96 well micro plates were coated with the anti-human monoclonal antibody overnight at 4°C. They were then washed 3 times with washing buffer (50 mM TRIS,
0.2% Tween-20, pH 7.9 to 8.1) and 15 µl of GCF samples in 100 µl phosphate buffered saline-tween-20 (PBS-T) were added to the plate in duplicates and incubated for 1 hour at room temperature (RT). One hundred µl of appropriate diluted biotin-labeled antibody‡‡ was added to each well, covered, and incubated for 1 hour at RT. Plates were again washed 3 times and incubated with 100 µl HRP-conjugated streptavidin†† for 30 minutes at RT. After extensive washing, 100 µl TMB (1,1′-trimethyl-ene-bis-4-formylpyridinium bromide)†‡ substrate solution were added. The reaction was stopped after 30 minutes by the addition of 50 µl 0.1 M sulfuric acid and color was measured at 450 nm using an automated micro-plate spectrophotometer.|| The amount of each cytokine was determined in picograms (pg). Cytokine concentrations were calculated with a standard curve (15.6 to 1000 pg) obtained with a standard recombinant cytokine.††† Cytokine values lower than the detection limit in our assay (below 15.6 pg) were considered undetectable. Cytokine concentration (pg/µl) was calculated from the volume of GCF estimated from the calibration unit reading, according to the following formula:

\[
\text{cytokine concentration (pg/µl)} = \frac{\text{total cytokine (pg)}}{\text{volume (µl)}}
\]

**Collection of Gingival Tissues**

Gingival tissue was obtained from the 6 patients with destructive periodontitis: one gingival specimen from each active site and one marginal soft-tissue biopsy from each inactive site (12 biopsies total). One biopsy was collected from each of the 6 healthy controls (2 men and 4 women; 35 to 51 years) during the surgical removal of wisdom teeth. The incisions were made 1 to 2 mm subgingivally; therefore, the specimens consisted of the gingival margin, sulcular epithelium, and gingival connective tissue. Soft tissue biopsy specimens were immediately embedded in tissue freezing medium OCT compound¶¶ and snap-frozen in liquid nitrogen slurry. Cryostat sections (4 to 6 µm thick) were obtained,||| briefly prefixed in acetone,*** and stored desiccated at −70°C until use.

**Monoclonal Antibodies**

Monoclonal antibodies employed included anti-CD3 (SPT-T3b),35 anti-CD8 (B.9.4.2),36 anti-CD4 (Hp2.6),37 anti-CD11c (HC1/1)38 and anti-CD19 (BU12).39

**Immunohistochemistry**

Tissue sections were fixed for 15 minutes in acetone at 4°C. A standard 3-stage immunoperoxidase avidin-biotin-peroxidase complex technique was used to detect CD3, CD19, CD4, CD8, and CD11c. Briefly, nonspecific tissue binding was blocked by incubation with 1% horse serum for 30 minutes. Specimens were incubated for 60 minutes with 40 µl of diluted primary antibody. All rinsing steps were performed with tris-buffered saline (TBS pH 7.2-7.6). Biotinylated horse anti-mouse IgG secondary antibodies were incubated at 1:200 dilution for 30 minutes.††† Endogenous peroxidase activity was quenched by exposure for 30 minutes to 0.3% H2O2 in methanol. Sections were then incubated for 45 minutes with peroxidase biotin-horseradish peroxidase macromolecular complexes.††† A black color was developed by exposure for 6 to 8 minutes to 0.5 mg/ml of the chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB).‖‖ Sections where counter-stained with hematoxylin Carazzi, dehydrated, and permanently mounted. Positive and negative controls were processed with each series. Standard tissue sections of human tonsils were used as positive controls for immunohistochemistry. Supernatant from X63-myeloma cells was used as a negative control. Specificity and sensitivity of each immunohistochemical staining were determined by comparison with the appropriate positive and negative controls included in each series.

**Data Analysis**

The clinical parameters and the total amount and concentration of each cytokine at healthy and diseased sites were calculated as subject mean ± standard deviation. Sites were used as the experimental unit of observations. Clinical measurements and the total amount and concentration of each cytokine were compared between healthy and diseased sites using unpaired Student t test. The significance of differences within each group was assessed using the ANOVA test. The significance (alpha = 0.05) of differences was assessed using the test. The correlation of cytokine levels with clinical parameters, probing depth, and degree of activity in healthy and diseased subjects was calculated using Pearson’s correlation.

The topographical distribution of positive cells with monoclonal antibodies in the infiltrating connective tissue (ICT) was established by analysis of consecutive tissue sections as previously described.40,41 A range between 0 and 4 was used to establish the degree of antibody-positive cells present in the inflammatory infiltrate: 0 = no detectable cells; 1 = less than 10%; 2 = approximately 25%; 3 = approximately 50%; and 4 = ≥50%. The degree of histological inflammation in the connective tissue underlying the junctional epithelium (JE) was established by hematoxylin and eosin staining, according to the density and distribution of the inflammatory cells of stained sections and a Tagge inflammation score (TIS) was assigned.42 Briefly, each
histological section was scored according to the following criteria: 0 = scattered areas of chronic inflammatory cells adjacent to the crevicular epithelium; 1 = accumulation of chronic inflammatory cells forming a thin, continuous band and the infiltrate extended between and around the epithelial rete ridges; 2 = a dense infiltration of chronic inflammatory cells replacing the gingival fibers and extending towards the oral side; 3 = a generalized, dense accumulation of chronic inflammatory fibers in all areas. All the slides were coded by the same person (AS) and analyzed by another (JG), making the measurements double blind. The results were verified by another examiner (AA).

RESULTS

Degree of Histological Inflammation

Periodontitis is a well characterized disease resulting in gingival inflammation. Periodontitis is associated with an infiltration of inflammatory cells in the gingiva, which were detected in all histological samples analyzed. Inflammatory associated cells were detected in the peri-vascular area underneath the JE and adjacent to the bacterial plaque. Tissue biopsies from controls showed a low degree of inflammation (TIS 1), which is characterized by the presence of a poorly defined infiltration of cells. A significantly higher degree of inflammation could be observed in the gingiva of periodontal patients TIS 2/3 degree of inflammation was observed in biopsies derived from inactive sites, with a well organized and dense cell infiltration in the connective tissue, however, extensions through the oral epithelium were not detected. Finally, the highest degree of inflammation (TIS 3) was observed in biopsies derived from active sites and inflammatory infiltration cells extended through the apical and JE. Furthermore, a clear infiltration was present in the connective tissue, reaching to areas underneath the oral epithelium (Table 2, Fig. 1A to 1C).

Table 2.

Characterization of Inflammatory Infiltrate From Gingival Biopsies in Periodontitis Patients and Controls

<table>
<thead>
<tr>
<th>Group</th>
<th>Degree of Inflammation</th>
<th>Inflammatory Infiltrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>TIS Score</td>
</tr>
<tr>
<td>Patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active sites</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Inactive sites</td>
<td>6</td>
<td>2/3</td>
</tr>
<tr>
<td>Controls</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

* 0 = no detectable cells; 1 = <10%; 2 = approximately 25%; 3 = approximately 50%; 4 = ≥50%.

Characterization of the Infiltrate

A significant infiltration of inflammatory cells was observed in the connective tissue of periodontal disease. The cellular composition of the infiltrate was defined by immunohistochemistry with antibodies. Table 2 shows the degree of histological inflammation and characterization of inflammatory infiltrate according to the source of the biopsy and distribution of the antibodies employed. The highest TIS 3 inflammation score was observed in active sites; where, in addition, the highest cellularity in the infiltrate was present. Biopsies from active sites had an increased number of B cells (CD19) and macrophages (CD11c) (Table 2; Fig. 1D and 1F). Finally, a significant amount of helper T cells (CD4) were also detected (Fig. 1E).

Cytokines in GCF Collected from Periodontal Patients and Controls

Levels of IL-1β, IL-10, and IL-8 and RANTES cytokines were analyzed by ELISA in the GCF. The majority of sites in periodontitis patients had detectable levels of IL-1β (94%, 68/72), IL-8 (100%, 72/72) and RANTES (87%, 63/72), whereas only 43.05% (31/72) had detectable IL-10. On the other hand, IL-8 (75%, 9/12) was the only detectable cytokine in GCF of the healthy group.

Total amounts and concentrations of the different cytokines analyzed are shown in Table 3. The presence of certain cytokines was clearly characteristic of disease. In controls, IL-1β, IL-10, and RANTES were undetectable (<15.6 pg), whereas significant levels of these cytokines were present in periodontitis patients. IL-8 was present in GCF of the control group; however, a significant increase was observed in the periodontitis group (P <0.05). Consequently, periodontitis disease courses with the specific secretion of inflammatory cytokines (IL-1β), immune regulatory cytokines (IL-10), and chemokines (RANTES and IL-8) although IL-8 is also detected in healthy GCF but at lower levels. Association of cytokine secretion with severity of periodontitis was evaluated according to probing depth (Table 4). Total amount and concentration of IL-1β, IL-8, and IL-10 and RANTES was measured as reported in Materials and Methods. Total amount of IL-1β and IL-8 was higher in pockets with PD >6 mm than PD <3 mm (P >0.05). Total amount of RANTES showed no significant variations according to the different probing depths. However, the concentration of IL-1β and IL-8 and RANTES cytokines decreased with PD (P >0.05). In contrast, IL-10 concentration was higher in sites with PD of 4 to 6 mm than in <3 mm and not detected in pockets >6 mm.

One of the main concerns in adult
**Figure 1.**

A. Diagram illustrating localization of the sections in panels B and C. JE, junctional epithelium; SE, sulcular epithelium; OGE, orogingival epithelium; ICT, infiltrated connective tissue. B. Chronic inflammatory cells in a gingival section located subjacent to the junctional epithelium in a periodontitis patient (hematoxylin and eosin; original magnification x20). C. Chronic inflammatory cells in a gingival section located subjacent to the orogingival epithelium in a periodontitis patient (hematoxylin and eosin; original magnification x20). D. Frozen section of a gingival biopsy from a periodontitis patient stained with anti-human CD11c using a DAB chromogen and hematoxylin counterstain. Staining is evident in infiltrating macrophages (original magnification x20). E. Frozen section of a gingival biopsy from a periodontitis patient stained with an anti-human CD4 (T cells) using a DAB chromogen and hematoxylin counterstain. Staining is evident in infiltrating T cells (original magnification x20). F. Frozen section of a gingival biopsy from a periodontitis patient stained with an anti-human CD19 (B cells) using a DAB chromogen and hematoxylin counterstain. Staining is evident in infiltrating B cells (original magnification x20).

**Table 3.**

*Cytokine Quantitation in GCF From Periodontitis Patients and Controls (mean ± SD)*

<table>
<thead>
<tr>
<th></th>
<th>IL-1ß</th>
<th>IL-8</th>
<th>IL-10</th>
<th>RANTES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Periodontitis</td>
<td>Control Periodontitis</td>
<td>Control Periodontitis</td>
<td>Control Periodontitis</td>
</tr>
<tr>
<td>Total amount (pg)</td>
<td>&lt;15.6</td>
<td>59.6 ± 23</td>
<td>44.8 ± 17*</td>
<td>212.5 ± 133*</td>
</tr>
<tr>
<td>Range</td>
<td>21.8 – 196.9</td>
<td>28.9 – 75.8</td>
<td>24.1 – 534.1</td>
<td></td>
</tr>
<tr>
<td>Concentration (pg/µl)</td>
<td>&lt;15.6</td>
<td>103.8 ± 90</td>
<td>188.9 ± 98†</td>
<td>316.7 ± 209†</td>
</tr>
<tr>
<td>Range</td>
<td>19.3 – 516.1</td>
<td>96.3 – 361.6</td>
<td>36.1 – 886</td>
<td></td>
</tr>
<tr>
<td>Observations</td>
<td>12</td>
<td>72</td>
<td>12</td>
<td>72</td>
</tr>
</tbody>
</table>

* Total amount in periodontitis versus control; P value 0.025.
† IL-8 concentration in periodontitis versus controls; P value 0.03.
periodontal disease is the definition of active sites, where tissue destruction, and probably secretion of certain cytokines, occur. Active sites are identified by an increase in probing depth during the length of study. In periodontal patients, the levels of IL-1β detected in active sites are clearly higher than those found in inactive sites, when measured either as total amount or as concentration (85.8 pg versus 34.5 pg, \(P\) value = 0.03; 86.4 pg versus 44.8 pg, \(P\) value = 0.005, respectively; (Table 5). IL-8 and IL-10 and RANTES are more abundant in active sites versus inactive sites, although differences were not so clear (\(P > 0.05\)).

Considering that cytokines could be related to the level of disease, levels of certain cytokines should revert to control values following periodontal treatment. To investigate this hypothesis, we determined the levels of these cytokines in sites before and after periodontal treatment. Table 6 shows that the total amount of IL-8 in the post-treatment sites decreased significantly (\(P < 0.05\)), while IL-1β concentration increased significantly (\(P < 0.05\)). IL-10 and RANTES were undetectable after periodontal treatment. Periodontal treatment resulted in a significant decrease in GCF volume (\(P = 0.001\)).

Correlation Between Cytokines and Clinical Parameters
The correlation between total amount and concentration of cytokines and clinical parameters is presented in Tables 7 and 8. A positive correlation between the total amount of IL-8 and RANTES \((r = 0.677; P < 0.05)\) was noted. Moreover, good correlations between IL-1β and RANTES concentrations \((r = 0.531; P > 0.05)\) and between total amount of IL-10 and IL-1β concentration \((r = 0.798; P > 0.05)\) were obtained. Table 8 shows a

**Table 4.**

<table>
<thead>
<tr>
<th>Probing Depth</th>
<th>N</th>
<th>IL-1β (pg)</th>
<th>IL-8 (pg)</th>
<th>IL-10 (pg)</th>
<th>RANTES (pg)</th>
<th>GCF (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Amount</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3mm</td>
<td>24</td>
<td>48.8 ± 1</td>
<td>193.3 ± 155</td>
<td>43.9 ± 16</td>
<td>42.1 ± 28</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>4-6mm</td>
<td>24</td>
<td>56.8 ± 16</td>
<td>194.4 ± 97</td>
<td>38.5 ± 13</td>
<td>38.6 ± 20</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>&gt;6mm</td>
<td>24</td>
<td>72.5 ± 3</td>
<td>256.1 ± 148</td>
<td>&lt;15.6</td>
<td>41.6 ± 16</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Concentration (pg/µl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3mm</td>
<td>139.8 ± 13</td>
<td>304.2 ± 244</td>
<td>48.8 ± 27</td>
<td>65.8 ± 45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-6mm</td>
<td>88.5 ± 43</td>
<td>339.8 ± 198</td>
<td>60.7 ± 53</td>
<td>63.5 ± 44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;6mm</td>
<td>98.6 ± 84</td>
<td>302.8 ± 201</td>
<td>&lt;15.6</td>
<td>54.3 ± 35</td>
<td></td>
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</tr>
</tbody>
</table>

**Table 5.**

<table>
<thead>
<tr>
<th>Site</th>
<th>Total Amount (pg)</th>
<th>Concentration (pg/µl)</th>
<th>Clinical Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1β</td>
<td>IL-8</td>
<td>IL-10</td>
</tr>
<tr>
<td>Active</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 12)</td>
<td>85.0 ± 22*</td>
<td>231.2 ± 184</td>
<td>39.3 ± 16</td>
</tr>
<tr>
<td>Inactive</td>
<td>34.5 ± 3*</td>
<td>119.2 ± 47</td>
<td>36.7 ± 9</td>
</tr>
</tbody>
</table>

* Active versus inactive site, \(P\) value 0.03.
† Active versus inactive site, \(P\) value 0.005.

**Table 6.**

<table>
<thead>
<tr>
<th></th>
<th>Total Amount (pg)</th>
<th>Concentration (pg/µl)</th>
<th>GCF (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1β</td>
<td>IL-8</td>
<td>IL-10</td>
</tr>
<tr>
<td>Before</td>
<td>59.6 ± 23</td>
<td>212.5 ± 133*</td>
<td>41.7 ± 13</td>
</tr>
<tr>
<td>After</td>
<td>53.4 ± 21</td>
<td>85.4 ± 49*</td>
<td>&lt;15.6</td>
</tr>
</tbody>
</table>

* Total IL-8 before versus after treatment, \(P\) value 0.011.
† IL-1β concentration before versus after treatment, \(P\) value 0.027.
‡ GCF before versus after treatment, \(P\) value 0.001.
Table 7. Correlation Matrix Between Total Amount and Concentration of Cytokines

<table>
<thead>
<tr>
<th></th>
<th>Total amount (pg)</th>
<th>Concentration (pg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1β</td>
<td>IL-10</td>
</tr>
<tr>
<td>Total amount (pg)</td>
<td>1.000</td>
<td>-0.042</td>
</tr>
<tr>
<td>IL-1β</td>
<td>-0.042</td>
<td>1.000</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.155</td>
<td>-0.647</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.294</td>
<td>0.729</td>
</tr>
<tr>
<td>RANTES</td>
<td>-0.121</td>
<td>0.460</td>
</tr>
</tbody>
</table>

* P value, <0.05.

Table 8. Correlation Between Total Amount of Cytokines and Clinical Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total amount</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1β</td>
</tr>
<tr>
<td>PI</td>
<td>0.019</td>
</tr>
<tr>
<td>BOP</td>
<td>0.081</td>
</tr>
<tr>
<td>PD</td>
<td>0.175</td>
</tr>
<tr>
<td>GCF</td>
<td>0.291</td>
</tr>
</tbody>
</table>

* P value, <0.05.

A weak correlation between clinical parameters and total amount of cytokines. However, a significant correlation between the total amounts of IL-8 and RANTES and GCF volume was obtained (r = 0.706, r = 0.736; P < 0.05).

DISCUSSION

The host immune response to periodontopathic bacteria involves a complex series of events not completely understood. The immune response is mediated by the release of cytokines involved in inflammatory reactions which are characteristic of periodontitis. This study demonstrates that the development of chronic adult periodontitis is related to the secretion of specific cytokines, such as IL-1β, regulatory cytokines (IL-10), or chemokines (IL-8 and RANTES) into the crevicular sulci and with the presence of a specific cellular inflammatory infiltrate in the gingival tissue. Periodontal treatment, which consisted of the removal of bacterial plaque, reduced the degree of inflammation and the total amount of cytokines present in the GCF.

Bacterial products induce an immune response involving T and B lymphocytes and macrophages. In fact, clear differences in the degree of inflammation and secreted cytokines were observed when periodontitis patients were compared with healthy controls. Periodontal disease histopathological classification includes 4 different stages.43-45 Our results confirm that there is a higher degree of inflammation and increased number of B cells and macrophages in the inflammatory infiltrate from biopsies of periodontitis patients as compared to healthy controls. CD19+ cells are more abundant in active sites than at inactive sites, whereas the difference was not so evident for T cells and macrophages. Additionally, the infiltrate of the inactive sites predominantly contains T lymphocytes; however, an increase in the number of B cells and plasma cells is characteristic of the active site.4 These data are in agreement with other authors, suggesting that the episodic loss of connective tissue in periodontitis patients is related to the massive presence of B cells and macrophages in the inflammatory infiltrate.46

The following cytokines were detected in the GCF of periodontitis patients: IL-1β (in 94% sites), IL-8 (100%), IL-10 (43%), and RANTES (87%). IL-8 was the only cytokine detected in GCF from healthy individuals (75%). Potentially, the presence of IL-8 could be related to the steady state of the gingiva. This is a site of permanent antigenic insult requiring the presence of cells such neutrophils, macrophages, and antigen-presenting cells that could be chemoattracted towards the gingival micro-environment by IL-8. The increase in IL-8 levels in periodontitis patients, in agreement with our findings, has been previously described.26,27 However, other authors found that IL-8 concentrations were increased in healthy donors.34,47 As previously suggested,47 one possible explanation could be attributed to the sample collection method employed. Additionally, the increase could be due to the GCF volume found in diseased sites, which in our study varied significantly between periodontitis patients and healthy controls. This difference in GCF volume could depend on the length of sample collection; in one study47 GCF was collected with filter strips for a 3-minute period, while in another report,27 strips were left in the crevice for 20 seconds. In our study, GCF was collected with filter strips for 30 seconds, in order to avoid cytokine
secretion induced by the mechanical irritation provoked by a longer collection period. In fact, in our study, the increase in the total amount of IL-8 was directly related with the adjacent sulcular depth (Table 4) and with the presence of an active site that implies attachment loss (Table 5).

Gingival inflammation is initiated when endotoxin or bacterial lipopolysaccharides (LPS) induce the synthesis of inflammatory cytokines, such as IL-1β, TNF-α, and IL-6. Therefore, IL-1β levels are indicators of the state of the gingival tissue and the degree of inflammation. Our data show that IL-1β, undetectable in healthy gingiva, was present in GCF of periodontitis patients (total amount 64 pg) and decreased after 2 months of periodontal treatment. However, the reduction in probing depth and, consequently, the reduction of GCF volume resulted in an increase in IL-1β concentration. The observation that the IL-1β concentration increased progressively with the probing depth reduction is clear; however, the explanation for this finding is not obvious (Table 3). Our data are in agreement with other authors reporting the presence of IL-1β in biopsies of human periodontal connective tissue.

Our results show that both IL-10 and RANTES cytokines are detected exclusively in periodontitis patients. IL-10 was detected in 43% of the GCF samples from periodontal patients and little variation was observed according to active sites or the sulcular depth. Consequently, IL-10 levels could be due to certain “high” producer patients, or to a specific Th1/Th2 response. IL-10 is a cytokine inhibiting factor that regulates the production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α, and INF-γ. Therefore, the presence of IL-10 in GCF of periodontitis patients could perform a relevant role in the regulation of local immune response. Although the results of the present study demonstrate variable amounts of IL-10 production in pockets with different probing depths, the relationship of these findings with disease progression remains to be established. It is possible to speculate that the local increase in IL-10 production may select the migration of Th2 T cells.

RANTES is the other cytokine specifically found in periodontitis patients. It is a member of CC chemokines, which activate monocytes, eosinophils, and basophil leukocytes, inducing chemotaxis and release of other cell mediators. The presence of RANTES in GCF could be involved in the development of the gingival inflammatory response by mediating leukocyte recruitment and activation. It has been clearly established that the production of specific chemokines is associated with the selective migration of subsets of effector T cells. Activated T cells, expressing CCR3 (CC chemokine receptor-3) and CCR5 (CC chemokine receptor-5), are specifically attracted by RANTES, MCP-1 (monocyte chemotactic protein-1) and MIP-1β (macrophage inflammatory protein-1β) chemokines, which have been reported to be ligands for these receptors. Recent data demonstrate that RANTES is an efficient chemoattractant for Th1 cells (but not for Th2 cells), inducing a dose response transmigration of Th1 cells. Therefore, RANTES may play a significant role in the regulation of local immune reactions controlling the balance between pro-inflammatory and anti-inflammatory T cell subsets. The presence of T cells and macrophages observed in biopsies of connective tissue (Table 2; Fig. 1) implies their potential role in the mechanisms of tissue destruction associated with destructive periodontitis, suggesting that RANTES could contribute to the increased infiltration of macrophage/monocytes observed in the periodontal tissues of this pathological condition.

The marked reduction of cytokines in GCF following treatment clearly suggests a relationship between active disease and cytokine production. Successful therapy resulted in lower cytokine levels together with an evident recovery of the periodontal tissues. The reduction of GCF volume at sites subject to periodontal therapy may account for the apparent increase in IL-1β concentration observed.

In our results, higher cytokine levels were detected in the GCF from inflamed sites than in healthy sites; moreover, cytokine levels in GCF could not be correlated with any of the clinical parameters tested in the present study. These findings are in agreement with others studies and could be explained by the fact that clinical parameters such as probing depth, attachment loss, and bleeding on probing do not necessarily reflect current disease activity. These results indicate that cytokine production does not merely reflect the presence of gingival inflammation and, furthermore, are consistent with the previously reported divergence between clinical signs of inflammation and disease activity.

Our study demonstrates a significant correlation between IL-8 and RANTES. IL-8 can be induced by a variety of stimuli including cytokines (e.g., IL-1), bacterial products (e.g., LPS), or viral products. Secretion of chemokines in response to IL-1 is mediated by common responsive elements, such as the nuclear factor-kB consensus motif, considered as potential gene transactivation domains. Production of chemokines by cells localized at inflammatory sites could induce strong adhesive interactions between the rolling leukocytes and the endothelium. Consequently, cells could then be attracted to the inflammatory site by the chemokine concentration gradient.
ogenic bacteria and cellular components of the immune system could enable activation of reactive cells and cytokine secretion. Regulatory immune response mechanisms to antigenic stimulus could generate complex networks among cytokines and cellular components producing inflammation and tissue destruction. One of the best examples for cytokine network mechanisms is provided by IL-8 and IL-1β, where IL-1β controls the local levels of IL-8. Therefore, further studies to correlate the function of cytokines implicated in the disease, the activated components present in the inflammatory infiltrate, and the potential role of these cellular components in tissue destruction are needed to understand the pathogenesis of the periodontal disease and to establish pathways for novel therapeutic approaches.

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