Susceptibility to periodontitis. Studies with LPS-stimulated whole blood cell cultures
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CHAPTER 2

A Type 2 Response in LPS-Stimulated Whole Blood Cell Cultures from Periodontitis Patients

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ABSTRACT

It is acknowledged that periodontitis results from the interaction of the host immune response with bacteria accumulating on the tooth surfaces. Although bacteria are essential, they are insufficient to cause the disease. Despite this knowledge it remains unclear why certain individuals are more susceptible to periodontitis than others. Therefore the present study investigated whether differences exist in the actual immune response between periodontitis patients and controls after stimulation of peripheral blood cells. Whole blood cell cultures (WBCC) were stimulated with LPS from *E. coli* during 18 hours and the release of PGE$_2$, IL-1$\beta$, IL-6, IL-8, IL-10, IL-12p40, IL-12p70 and TNF$\alpha$ were measured. The levels of PGE$_2$ were two fold higher in the WBCC from periodontitis patients than from controls. In contrast, the levels of IL-12p70 in WBCC from patients were two fold lower. Furthermore, WBCC from patients secreted lower levels of IL-1$\beta$ and higher levels of IL-8 when compared to WBCC from controls. No differences were observed with respect to IL-6, IL-10, IL-12p40 and TNF$\alpha$ production. It is known from the literature that LPS-stimulated WBCC reflect specifically the behavior of the monocytes and that monocytes are peripheral precursors of antigen-presenting cells (APCs). Therefore it is concluded that the monocytes in the present WBCC from periodontitis patients are responsible for the higher levels of PGE$_2$ and lower levels of IL-12p70. Since, it is has been shown that APC-derived IL-12p70 induces Th1 cells that promote cellular immunity, while APC-derived PGE$_2$ induces Th2 cells that promote humoral immunity, it is postulated that APCs from periodontitis patients may have a bias in directing type 2 helper (Th2) responses and thereby promoting the humoral immunity in periodontitis.
INTRODUCTION

Periodontitis is a chronic destructive inflammatory disease of the teeth supporting tissues, i.e. connective tissue from the periodontal ligament and alveolar bone. This inflammatory condition will, if left untreated, eventually lead to loosened teeth and subsequent exfoliation. Periodontitis is a multifactorial disease in which host factors and environmental factors play an important role (1). It is widely accepted that periodontitis results from interaction of the host's defense mechanisms with bacteria accumulating on the tooth surface (2). The prevalence of periodontal pathogens in the general population is moderate to high: 27% for A. actinomycetemcomitans (3) to 100% for Fusobacterium nucleatum (4). Despite this prevalence it is recognized that not everyone is equally susceptible to periodontitis (5, 6). Therefore, although bacteria are essential in the induction of the inflammatory response in the periodontal tissues, they are insufficient to cause destructive periodontal disease (1). This implicates differences in susceptibility and intrinsic differences in the host immune response.

It is generally accepted that LPS, derived from gram-negative bacteria that accumulate on the tooth surfaces, penetrate the periodontal tissues and subsequently recruit and activate immune cells (1). Histological studies have shown that the immune response results in a periodontal lesion that consists of lymphocytes, monocytes/macrophages and plasma cells (2). Triggering by (pro)-inflammatory stress signals, like LPS, tissue cells as well as immune cells start to secrete inflammatory mediators such as cytokines, chemokines and prostaglandins. These released molecules may mediate the inflammatory response and the destruction of the periodontal tissues but may also affect the functional status of specific immune cells in the periodontal lesion. Such different effects of the induced mediators on the function of cells in the immediate neighborhood determines the course of the immune response and hence the resistance or the susceptibility to the disease (7).

In several diseases and inflammatory conditions LPS responsiveness of peripheral blood cells has been studied as a measure of the host immune capacity. Therefore the
purpose of the present study was to investigate, in a whole blood cell culture system, differences in the nonspecific cellular immune response to LPS between periodontitis patients and controls.

MATERIALS AND METHODS

Selection of study subjects
Since smoking is recognized as an important risk factor in the pathogenesis of periodontitis, the possible influence of this environmental factor on the immune response was excluded (8). Therefore a total of 19 non-smoking patients with chronic untreated periodontitis were recruited from patients, who were referred to the Academic Center for Dentistry Amsterdam (ACTA) for diagnosis and/or treatment. Non-smokers were defined as those who had never smoked or had ceased smoking more than 10 years ago. All patients showed on dental radiographs periodontal bone loss of > 1/3 of the total length of the root on ≥ 2 teeth per quadrant. Nineteen control subjects, patients of ACTA referred for the treatment of dental caries, were matched for age and gender. The selected controls did not suffer from periodontitis and did not show loss of alveolar bone as was confirmed on dental radiographs.

All participants were free from systemic diseases and had no clinical symptoms of bacterial, viral or parasitic infections at the time of the study. None of the subjects in the study had taken any form of medication that could affect their periodontal status, such as anti-inflammatory agents, antibiotics and immuno-suppressants during at least the preceding 6 months. Approval by the Institutional Review Board on human studies was obtained and all subjects signed an informed consent. To further characterize patients and controls, subgingival bacterial samples were taken and subsequently cultured in a standardized way as previously described (9) (Table 1).
### Table 1. Demographical, bacteriological and blood cell data of the two study groups

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 19)</th>
<th>Patients (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong> b (yrs)</td>
<td>38 ± 5</td>
<td>37 ± 6</td>
</tr>
<tr>
<td>No. Caucasians</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>No. Females / Males</td>
<td>11 / 8</td>
<td>11 / 8</td>
</tr>
</tbody>
</table>

<table>
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<th>Bacteriological Data</th>
<th>Controls (n = 19)</th>
<th>Patients (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Actinobacillus</em> actinomycetemcomitans</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td><em>Porphyromonas</em> gingivalis</td>
<td>4</td>
<td>12*</td>
</tr>
<tr>
<td><em>Bacteroides</em> forsythus</td>
<td>8</td>
<td>18**</td>
</tr>
<tr>
<td><em>Prevotella</em> intermedia</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td><em>Fusobacterium</em> nucleatum</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td><em>Peptostreptococcus</em> micros</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td><em>Campylobacter</em> rectus</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blood Cell Data</th>
<th>Controls (n = 19)</th>
<th>Patients (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>5.89 ± 1.33</td>
<td>6.11 ± 1.73</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.41 ± 0.12</td>
<td>0.36 ± 0.08</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.82 ± 0.65</td>
<td>1.88 ± 0.59</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>3.50 ± 1.00</td>
<td>3.72 ± 1.41</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.11 ± 0.06</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.04 ± 0.02</td>
<td>0.03 ± 0.02</td>
</tr>
</tbody>
</table>

*Values are means ± SD.

b The sampling was carried out in total at 4 sites and the deepest sites of each quadrant of the dentition were selected.

*, P < 0.05 and **, P < 0.01 as analyzed by the Fisher’s exact test.

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**Whole blood cell cultures (WBCC)**

Venous blood was collected by venepuncture in sodium heparin-containing blood collecting tubes (VT 100SH tubes; Venoject; Terumo Europe, Leuven, Belgium). The
blood was diluted five-fold in endotoxin-free RPMI 1640 medium containing L-glutamine and 25 mM HEPES (Gibco BRL, Life Technologies B.V., Breda, NL) and was supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. The diluted whole blood was cultured in duplicate in non-pyrogenic 24-well flat-bottom tissue culture plates (Costar, Cambridge, MA, USA) in the absence or presence of LPS from *Escherichia coli* O127:B8 (Difco, Detroit, MI, USA) in various concentrations for 18 hours at 37°C. The period between the blood collection and the start of the incubation was 1.5 hour. Following culturing supernatants were collected and stored at -80°C until determination of cytokines and PGE₂ concentrations.

In order to determine the most optimal LPS concentration for the stimulation of the WBCC, the blood samples were incubated with 6 different concentrations of LPS: 0, 0.01, 0.1, 1, 10 and 100 ng/ml.

Venous blood of all participants was also collected in an EDTA(K₃) containing tube (Becton Dickinson Vacutainer System Europe, Meylan, F) for the determination of the leukocyte and the leukocyte differentiation counts, which were performed in standard automated procedures.

**Cytokine and PGE₂ determinations**

The IL-1β, IL-6, IL-8, IL-10 and tumor necrosis factor (TNF)-α levels were measured in the supernatants using commercially available ELISAs (PeliKine Compact™ human ELISA kits, CLB, Amsterdam, NL) according to the manufacturers instructions. The sensitivity of the kits for the various cytokines used varied from 2 to 9 pg/ml.

Measurements of IL-12p70 and IL-12p40 levels were performed by a specific solid-phase sandwich ELISA as described previously (10). The IL-12p70 ELISA detects biologically active IL-12. The detection limit of the IL-12 assays was 3 pg/ml for IL-12p70 and 20 pg/ml for IL-12p40.

PGE₂ concentrations were determined using the ACE™ competitive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI), which had a detection limit of 15 pg/ml.
Statistical analysis
SPSS package (version 9.0 for Windows, Chicago, IL, USA) was used for data analysis and box plot generation. Since the concentration of the released mediators showed a non-normal distribution, the data were log-transformed for statistical analysis. The influence of the demographical, bacteriological and blood cell count parameters, as listed in Table 1, on the cytokine and PGE\textsubscript{2} secretion in the cultures were evaluated by using a forward linear regression analysis. Comparisons between patients and controls for the blood cell counts and the log-transformed cytokine and PGE\textsubscript{2} concentrations were performed by means of the unpaired $t$-test and an analysis of covariance respectively. $P$-values $< 0.05$ were considered significant.

RESULTS

The production of the inflammatory mediators IL-1$\beta$, IL-6, IL-8, IL-10, IL-12p40, TNF-$\alpha$ and PGE\textsubscript{2} was measured in the supernatants of the WBCC after 18 hours of stimulation with various concentrations of LPS from \textit{E. coli}. The cells that were incubated without LPS, only in case of IL-8 detectable levels of the cytokine were found. Stimulation with increasing LPS concentrations induced a dose dependent production of all the mediators tested and provided sigmoidal curves. The paths of all curves were similar for both the patient and the control group. As the curves of the mediators reached a saturation-level at 1 ng/ml LPS, it was decided to compare patients and controls for the production of mediators at this LPS dose. In this respect the only exception relates to IL-12p70. For this cytokine no dose response curves could be obtained, since this cytokine is produced in low concentrations in LPS stimulated conditions. Therefore, the production of IL-12p70 was only measured in the supernatants of the cell cultures that were stimulated with the highest level of LPS used, i.e. 100 ng/ml.

Demographical, bacteriological and blood cell data of the study groups are presented in Table 1. With regard to the blood cell data, analyses showed no
differences between the periodontitis patients and the control subjects. The levels of mediators are presented in Figures 1A and 1B. Linear regression analyses, including all the demographical, bacteriological, clinical and blood cell data, revealed that the number of monocytes, the age and the race of the subjects were significant confounders for the production of mediators. Therefore, in the comparisons between patients and controls these confounding factors were introduced as covariates in the analyses of variance.

Results showed no differences between the two study groups with regard to the levels of the released cytokines IL-6, IL-10, IL-12p40 and TNF-α in the supernatants (Figure 1). However, the levels of IL-1β measured in the supernatants of the periodontitis patients were one and a half fold lower than the levels measured in the supernatants of the controls. The periodontitis patients proved to produce two fold higher concentrations of PGE₂ in the supernatants in comparison to the control group (Figure 1A). Within the patient group it was interesting to note that several patients secreted very high levels of PGE₂: 7 out of the 19 patients produced > 5000 and up to 11,000 pg/10⁶ monocytes, which was three to six fold higher than the mean of the control group. Furthermore, the mean levels of IL-8 in the supernatants of the periodontitis patients were one and a half fold higher than those in the controls (Figure 1B).

The concentrations of IL-12p70 were measured in 100 ng/ml LPS stimulated cultures (Figure 1B). IL-12p70 is the bioactive heterodimer that is regulated differently from IL-12p40, the biologically inactive form of IL-12 (11). Detectable levels of IL-12p70 were found for 11 subjects in the control group and for 11 in the patient group. The assay revealed for these patients a mean concentration of 12.0 ± 6.6 pg/ml of IL-12p70, while the corresponding value for the control subjects was 24.4 ± 16.7 pg/ml. Showing that cells of the periodontitis patients produced half of the IL-12p70 levels from the controls.
Fig. 1. LPS induced mediator production in 19 periodontitis patients and 19 controls. For the measurements of IL-1β, IL-6, IL-8, IL-10, IL-12p40, TNFα, and PGE₂, the whole blood cell cultures were stimulated with 1 ng/ml LPS from E. coli for 18 hours, where as the cultures for IL-12p70 were stimulated with 100 ng/ml LPS from E. coli for 18 hours. Data are means ± SEM and are expressed as cytokine or PGE₂ production per 10⁶ monocytes. The individual data per subject were the mean of two different cultures. * , P < 0.05, **, P < 0.01 as determined by analysis of covariance, with age, race and number of monocytes as covariates. □, controls; ■, patients.
DISCUSSION

In the present study the differences in the production of inflammatory mediators by innate immune cells from patients suffering from periodontitis and controls were studied. For this purpose, whole blood cell cultures (WBCC) were stimulated with LPS from *E. coli*. The present data show that the production of PGE$_2$ and IL-8 was elevated and the production of IL-12p70 and IL-1β was reduced in WBCC harvested from periodontitis patients. Concomitant production of IL-6, IL-10, IL-12p40 and TNF-α was comparable between patients and controls, indicating that the peripheral blood cells of patients with periodontitis showed a similar competence as control subjects to produce these latter cytokines.

It has been extensively studied and shown in parallel cultures of whole blood and freshly isolated monocytes as well as in kinetics that WBCC stimulated with LPS specifically reflect the behavior of the monocytes (10, 12, 13-15). It was shown that the purified monocytes but not CD14-depleted peripheral blood mononuclear cells (PBMC) or granulocytes were responsible for the production of cytokines following the stimulation with LPS (10, 14). Furthermore, the reflected performance of monocytes in LPS-stimulated WBCC was found for relatively low levels of LPS, since the cytokine production by neutrophils required much higher amounts of LPS (15). Therefore it is highly likely that the levels of the inflammatory mediators found in the WBCC in the present study reflect the behavior of the monocytes.

In the present study LPS from *E. coli* was used as a non-specific stimulant to activate the peripheral blood cells. *E. coli*-derived LPS was used to make valid comparisons possible with previous studies and studies outside the field of periodontology, since it is the most common and most widely studied source of LPS and above all very reliable. In addition, not all patients and all controls were colonized by the major periodontal pathogens. Furthermore, the findings in the present study corroborate the results of a study using freshly isolated monocytes purified from the peripheral blood of periodontitis patients and control subjects, which were also stimulated with *E. coli*-derived LPS (16). Their results showed that the isolated
monocytes from the periodontitis patients produced also higher levels of PGE₂ and lower levels of IL-1β compared to the controls, while the levels of TNF-α and IL-6 were comparable. Unfortunately, data on the release of the chemokine IL-8 and the cytokines IL-10, IL-12p40 and IL-12p70 were not available in that particular study.

Immunity depends on two major types of specific immune responses: cellular and humoral responses. The balance between these responses is orchestrated by cytokines produced by CD4⁺ Th cells. Th1 cells make the 'type 1' cytokine interferon (IFN)-γ and Th2 cells the 'type 2' cytokines IL-4 and IL-5 (17). It is acknowledged now that factors associated with APCs highly determine the tuning of the balance between type 1 and type 2 cytokines (18, 19). However, functional studies with APCs harvested from the peripheral tissues are very difficult if not impossible. Since studies on the role of APCs are based on in vitro experiments with peripheral blood precursors, i.e. the monocytes, and which resemble the in vivo types (20-22), it is tempting to extrapolate the data of the present study towards the possible role of APCs in periodontitis.

In fact, APCs play a central role as they transfer information from an infected microenvironment to the T cells. This information is crucial to select the most effective immune response to the pathogenic antigens associated with that microenvironment (18). APCs provide T cells not only with an antigen-specific stimulatory signal and a series of co-stimulatory signals, but also with polarizing signals, i.e. soluble molecules. The expression of such polarizing APC factors is triggered by (pro)inflammatory stress ('danger') signals, such as microbial products like LPS, which may affect the APC either directly or indirectly via the activation of neighboring tissue cells (19).

The most clearly defined factors determining Th1 and Th2 differentiation from a T cell precursor are mediators present at the initiation of the immune response at the stage of ligation of the T cell receptor (23, 24). In this respect IL-12 and PGE₂ are important factors. IL-12 is a dominant factor in directing the development of Th1 cells producing high levels of IFN-γ (25-27). PGE₂ selectively inhibits IFN-γ production and
is reported to favor the development of Th2 cells (28). Furthermore, it has been proposed that the ratio of IL-12 to PGE$_2$ produced by APCs during T cell activation is highly predictive for the level of IFN-$\gamma$ production by Th cells (29). Because the present study showed both higher levels of PGE$_2$ and lower levels of IL-12p70 in cultures of the patients, these results suggest a type 2 promoting phenotype of APCs from patients with periodontitis. Accompanying this, a decreased type 1 response, i.e. reduced levels of IFN-$\gamma$ produced by PBMC, has been reported for periodontitis patients (30, 31). This relationship between monocyte-derived PGE$_2$ and IL-12 levels and decreased IFN-$\gamma$ production by PBMC has also been shown for several other diseases, like atopic dermatitis, atopic asthma, rheumatoid arthritis and HIV infection (13, 14, 32-43).

In support to this suggested Th2-promoting phenotype of APCs from periodontitis patients, are the observations that T cells in periodontal lesions express higher levels of IL-4 and IL-5 mRNA, and produce more IL-4 and have a lower IL-2/IL-4 ratio compared to controls (44-46). These Th2 cells, whose development is induced by IL-4, have been implicated in humoral immune responses due to their production of B cell growth and differentiation factors (47). Indeed, the infiltrate in the periodontal lesion seems to be dominated by B cells and plasma cells and T cell regulated polyclonal B cell responses are believed to be important in the pathogenesis of the progressive periodontal lesion (2, 48).

The suggested Th2-promoting phenotype of APCs from periodontitis patients implies either an intrinsic characteristic or a different priming of the monocytes, due to an altered tissue environment, i.e. the inflammatory periodontal lesion. This suggested Th2-promoting phenotype of APCs from periodontitis patients may be an important factor in the susceptibility to the disease.
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REFERENCES


CHAPTER 2


