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Publication date
2002

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Citation for published version (APA):

Fokkema, S. J. (2002). *Susceptibility to periodontitis. Studies with LPS-stimulated whole blood cell cultures*. [Thesis, fully internal, Universiteit van Amsterdam].

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CHAPTER 3

Increased Release of IL-12p70 by Monocytes after Periodontal Therapy

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ABSTRACT

Differences in the susceptibility to periodontitis suggest differences in the host immune response. A previous study showed that LPS-stimulated whole blood cell cultures (WBCC) from periodontitis patients produced higher levels of prostaglandin E₂ (PGE₂) and lower levels of IL-12p70 compared to controls. The present study investigated whether this different immune response of periodontitis patients is intrinsic or acquired. Therefore the release of inflammatory mediators was measured in *E. coli* LPS-stimulated WBCC of 12 periodontitis patients before and after non-surgical periodontal therapy. The levels of IL-12p70 increased two times and the levels of PGE₂ showed a trend towards reduction after therapy, whereas the levels of IL-1 β , IL-6, IL-8, IL-10, IL-12p40 and TNF- α did not change. Since it is known from the literature that LPS-stimulated WBCC reflect the behavior of the monocytes, it is concluded that, after periodontal therapy, the different functional phenotype of peripheral blood monocytes from patients resembles that of subjects without periodontitis.

INTRODUCTION

It is widely believed that the inflammatory and immune responses in periodontitis are initiated and perpetuated mainly by a small group of gram-negative bacteria, including *A. actinomycetemcomitans*, *P. gingivalis*, *B. forsythus*. Although these periodontal pathogens are essential in the induction of the inflammatory response in the periodontal tissues, they are insufficient to cause destructive periodontal disease, since it is recognized that not everyone is equally susceptible (1). However, it remains unclear why certain individuals are more susceptible to periodontitis than others.

These acknowledged differences in susceptibility to periodontitis implicate intrinsic differences in the host immune response. In a previous study the host immune capacity of periodontitis patients was compared to controls by studying the LPS responsiveness of peripheral white blood cells (2). In that study WBCC were stimulated with *E. coli*-derived LPS. Results showed that the cultures from periodontitis patients produced higher levels of IL-8 and PGE₂ and lower levels of IL-12p70 in comparison to the controls, while the levels of IL-1 β , IL-6, IL-8, IL-10, IL-12p40 and TNF- α did not differ. To elucidate whether the nature of this immune response in periodontitis patients was an intrinsic or acquired characteristic of the peripheral blood cells, the present study investigated the effect of periodontal therapy on the LPS responsiveness of WBCC from periodontitis patients.

MATERIALS AND METHODS

Selection of patients

Twelve non-smoking patients (mean age 34 years; range 26-42 years) with untreated periodontitis were recruited from patients, who were referred to the Department of Periodontology of the Academic Center for Dentistry Amsterdam (ACTA) for diagnosis and treatment. According to the diagnostic criteria recently introduced (3), 6 patients were diagnosed as generalized severe post-adolescent periodontitis (aggressive periodontitis), 4 patients as generalized severe adult periodontitis and 2 patients as generalized moderate adult periodontitis (chronic periodontitis). Furthermore, all patients were free from systemic diseases and usage of medication, and reported no clinical symptoms of bacterial, viral or parasitic infections at the time of the study. Approval by the Institutional Review Board on human studies was obtained and all subjects signed an informed consent.

Periodontal therapy

For this specific study each patient received a non-surgical periodontal therapy, which aimed at maximal reduction of the periodontal infection within a short period of time. After all baseline procedures were performed, teeth that were judged as lost were first extracted. Next, all patients were motivated and instructed for daily oral hygiene procedures on an individual level. For maximal plaque control throughout the entire period of the study, i.e. 3 months, the patients were instructed to rinse twice daily with 0.2% chlorhexidine (Corsodyl®). In all patients a thorough full mouth scaling and root planing (SRP) under local anesthesia was performed during 2 sessions within 24 hours. In conjunction with the SRP systemic antibiotics (AB), Amoxicillin 375 mg TID and Metronidazol 250 mg TID, were given for 1 week, starting one day before the first SRP session. Approximately one month later patients were recalled for oral hygiene control and removal of staining, and SRP at sites with a probing depth of > 3 mm and/or at sites showing bleeding on probing. Three months after the full mouth SRP plus AB therapy all patients returned for the final evaluation.

To evaluate the effect of the periodontal therapy clinical measurements, microbiological samples and peripheral blood collections were performed at baseline and 3 months after therapy.

Clinical measurements and bacteriological sampling

An experienced examiner (CS) carried out a full-mouth periodontal examination at 4 sites per tooth both at baseline as well as 3 months after therapy. The clinical measurements included probing depth (PD) and bleeding on probing (BOP); for the bacteriologically sampled sites it also included probing attachment level (PAL). The PD and PAL were recorded to the nearest millimeter.

The sampling and culturing procedures were performed in a standardized way, as described previously (4). In each patient the 4 deepest sites per quadrant were selected for the subgingival sampling at baseline and the same sites were sampled again after therapy. In case the selected site was at a tooth that was scheduled for

extraction, an additional alternative deepest site in the same quadrant was selected for the sampling.

Stimulation of whole blood cell cultures (WBCC)

The venous blood was collected by venepuncture in sodium heparin-containing blood collecting tubes (VT 100SH tubes; Venoject; Terumo Europe, Leuven, Belgium) and was performed prior to the bacteriological sampling and clinical measurements. 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 for 18 hours at 37°C in duplicate in non-pyrogenic 24-well flat-bottom tissue culture plates (Costar, Cambridge, MA, USA) in the absence or presence of LPS from *E. coli* O127:B8 (Difco, Detroit, MI, USA) in various concentrations. The choice for this non-specific stimulant to activate the WBCC was motivated on the basis of previous experiments, which revealed that not all patients and controls were always colonized by the same major periodontal pathogens. Moreover, since many studies have used the common available and highly purified source of *E. coli*-LPS to activate peripheral blood cells (2, 5-10), it facilitates comparisons with other studies. 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 previous experiments, the most optimal LPS concentration for the stimulation of the WBCC was determined. But for the purpose of the present study the effect of the periodontal therapy on the paths of the produced sigmoidal curves was also evaluated. Again, for all but one (IL-12p70) of the tested mediators an optimum was reached at 1 ng/ml LPS and the paths of the curves were the same at baseline and after therapy. The production of IL-12p70 was only measured at 100 ng/ml LPS, since this cytokine is released in extreme low levels in < 100 ng/ml LPS stimulated WBCC.

Venous blood of all patients was also collected in an EDTA(K₂) containing tube (Becton Dickinson Vacutainer System Europe, Meylan, F) for the determination of total leukocyte and leukocyte differentiation counts, which were performed in standard automated procedures.

Cytokine and PGE₂ determinations

The IL-1 β , IL-6, IL-8, IL-10 and 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.

Measurement of IL-12p70 and IL-12p40 levels was performed by a specific solid-phase sandwich ELISA as described previously (6). 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 10.0 for Windows, Chicago, IL, USA) was used for data analysis. Comparisons at baseline and after therapy for the peripheral blood cell counts were performed by means of a paired *t*-test. Since the concentration of the released mediators showed a non-normal distribution, the data were log-transformed. Analyses of data for the log-transformed cytokine and PGE₂ concentrations at baseline and after therapy were performed by means of an analysis of covariance for matched pairs with the number of monocytes as a covariate. *P*-values < 0.05 were considered significant.

RESULTS

Clinical and microbiological outcomes of the periodontal therapy

Clinically all patients showed a good treatment response. The overall mean PD for the whole study group was 5.1 mm at baseline and 2.9 mm 3 months after therapy. The overall mean BOP scores for all patients were 82% at baseline and 28% after therapy. Table 1 shows that the mean percentage of shallow PD (≤ 3 mm) increased nearly 3 times after periodontal therapy and reciprocally the mean percentages of deep PD decreased. In line with the resolution of the inflammation in the periodontal tissues after therapy, there was a decrease in the bleeding tendency for all PD categories (Table 1). Furthermore, the mean PD and PAL of the bacteriologically sampled sites were reduced after periodontal therapy (Table 2).

The effect of periodontal therapy on the prevalence of periodontal pathogens is shown in Table 2. *A. actinomycetemcomitans*, *P. gingivalis* and *B. forsythus* were no longer detectable after therapy. The other cultured bacteria were detectable in a decreased number of patients after the therapy.

Table 1. Mean percentages of the prevalence of 3 probing depth (PD) categories and the corresponding mean bleeding on probing (BOP) values before and after therapy

		Baseline	After therapy
PD	1 – 3 mm	33	80
	4 – 5 mm	34	18
	≥ 6 mm	33	2
BOP for PD categories			
	1 – 3 mm	66	13
	4 – 5 mm	85	25
	≥ 6 mm	94	48

Table 2. Prevalence of periodontal pathogens and the mean probing depth (PD), probing attachment level (PAL) and bleeding on probing (BOP) of the sampled sites before and after therapy

	Baseline	After therapy
	number of patients with positive culture (n=12)	
<i>Actinobacillus actinomycetemcomitans</i>	4	0
<i>Porphyromonas gingivalis</i>	7	0
<i>Bacteroides forsythus</i>	10	0
<i>Prevotella intermedia</i>	10	4
<i>Fusobacterium nucleatum</i>	12	9
<i>Peptostreptococcus micros</i>	12	3
<i>Campylobacter rectus</i>	2	3
Mean PD (mm)	8.4	3.9
Mean PAL (mm)	7.5	4.1
Mean BOP (%)	82	28

Effect of periodontal therapy on blood cell number and function

The total number of white blood cells was decreased after periodontal therapy (Table 3). On the basis of leukocyte differentiation, it was observed that the numbers of lymphocytes were reduced after therapy. A trend for reduction was present after therapy for the numbers of neutrophils, eosinophils and basophils. The number of monocytes did not change after therapy. Also the differential percentages of the various types of white blood cells did not change after therapy (data not shown).

Table 3. The total and differential white blood cell counts before and after periodontal therapy

	Baseline	After therapy	<i>P</i> -values
WBC	6.64 ± 0.48	5.71 ± 0.42	0.04
neutrophils	4.03 ± 0.45	3.44 ± 0.31	0.09
eosinophils	0.13 ± 0.05	0.09 ± 0.03	0.07
basophils	0.04 ± 0.007	0.03 ± 0.004	0.08
lymphocytes	2.02 ± 0.13	1.78 ± 0.17	0.04
monocytes	0.41 ± 0.04	0.38 ± 0.05	0.5

WBC represents the total number of the peripheral white blood cells.

Data are expressed as the means ± SEM in 10⁹ / L.

P-values are determined by a paired *t*-test.

The production of the cytokine IL-6, the chemokine IL-8 and the biologically inactive form of IL-12, IL-12p40, did not change after periodontal therapy (Figure 1B). However, the production of IL-12p70, the bioactive heterodimer of IL-12, in the LPS-stimulated WBCC was on average two times higher after periodontal therapy: 36 ± 8 versus 70 ± 18 pg per 10⁶ monocytes (Figure 1B). In 7 out of the 12 patients the production of IL-12p70 increased up to three times after therapy and in 1 patient even four times. There were no differences detected for the release of IL-12p70 between the various types of patients before as well as after periodontal therapy. Furthermore, the IL-12p70 levels secreted by the patients after therapy were now not significantly different from the levels secreted by control subjects without destructive periodontal disease; on average the controls secreted 57 ± 10 pg IL-12p70 per 10⁶ monocytes (2).

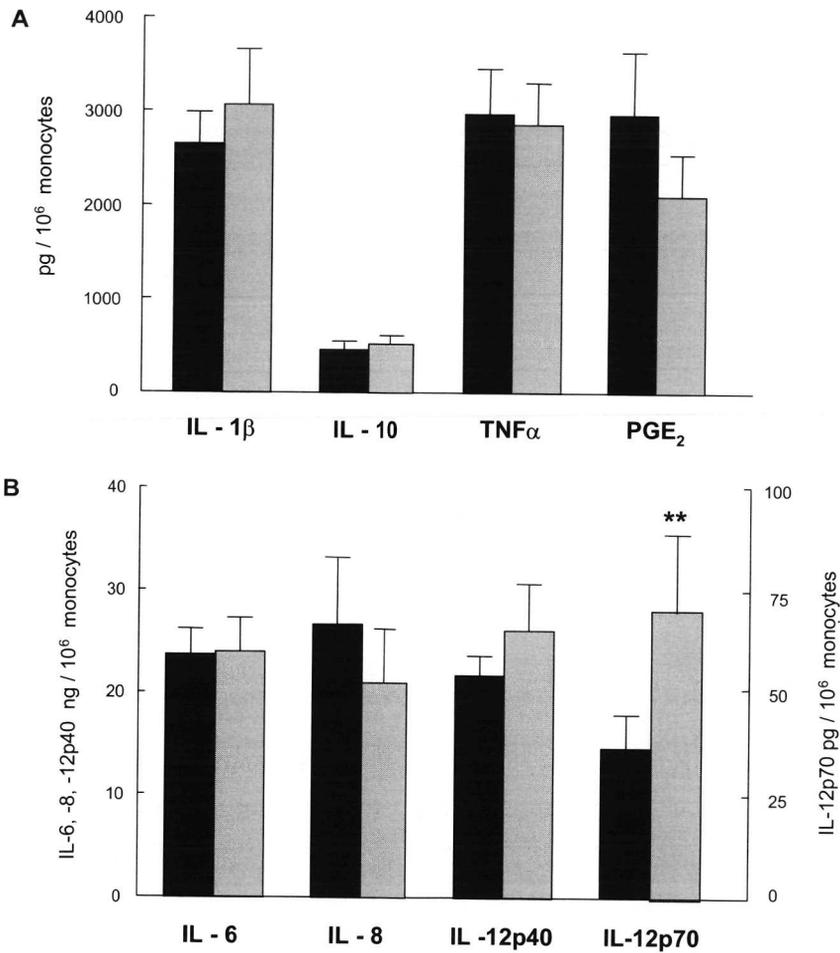


Fig. 1. LPS induced production of inflammatory mediators in 12 generalized periodontitis patients before and after periodontal therapy. For the production of IL-1 β , IL-6, IL-8, IL-10, IL-12p40, TNF- α and PGE₂, whole blood cell cultures were stimulated with 1 ng/ml and for IL-12p70 with 100 ng/ml LPS from *E. coli* for 18 hours. Data are means \pm SEM and are expressed as cytokine or PGE₂ production per 10⁶ monocytes. The individual data per subject were the mean of two different cultures. **, $P = 0.003$ as determined by analysis of covariance for matched pairs with the number of monocytes as a covariate. ■, before therapy; □, after therapy.

DISCUSSION

Successful periodontal therapy results clinically in shallow probing depths and a low tendency towards bleeding on probing and microbiologically in a low detection level of periodontal pathogens (11). In addition it reduces the size of the inflamed lesion, causes a decrease in the inflammatory cell subsets, like B-cells, T-cells and plasma cells (12, 13, 14). As in the present study these clinical and microbiological outcomes of the therapy have been accomplished, it may be suggested that the number of inflammatory cells in the lesions decreased. This may have resulted in a decrease of the recruitment of granulocytes and lymphocytes and as a consequence lower number of these cells were observed in the peripheral blood after therapy.

The results of the present study showed that after periodontal therapy, LPS-stimulated WBCC from periodontitis patients produced increased levels of IL-12p70 and a trend towards reduced levels of PGE₂, whilst the levels of IL-1 β , IL-6, IL-8, IL-10, IL-12p40 and TNF- α remained unchanged. In a previous study it has been shown that identical LPS-stimulated WBCC from untreated periodontitis patients produced a lower level of IL-12p70 and a higher level of PGE₂ in comparison to controls, i.e. subjects without destructive periodontal disease (2). Interestingly, the IL-12p70 levels, measured after periodontal therapy in the present study, were comparable to those from control subjects. WBCC stimulated with LPS specifically reflect the behavior of the monocytes as has been shown in parallel cultures of whole blood and freshly isolated monocytes (6, 9, 10, 15, 16). Therefore, it was concluded that the monocytes were responsible for the release of the inflammatory mediators(2).

It is well acknowledged that mediators produced by antigen-presenting cells (APCs), including the monocytes, macrophages and dendritic cells, highly determine the commitment of T-helper (Th) cells toward either a Th1 or Th2 phenotype (17). In this respect IL-12p70 is a determining factor. APC-derived IL-12p70 is a dominant and crucial factor in directing the development of Th1 cells and in maintaining Th1 responses; Th1 cells are high producers of IFN- γ and promote cellular immune

responses (18-19). Therefore, in the previous study of Fokkema and coworkers it was suggested that APCs from periodontitis patients have a Th2-promoting phenotype. This suggested that APCs from periodontitis patients might have an intrinsic characteristic in driving specific Th2 cell responses and thereby promoting the humoral immunity in periodontitis. However, the current results show that after periodontal therapy the IL-12p70 levels released in the WBCC from patients increased towards the levels of control subjects. This implicates that the suggested Th2-promoting phenotype of the monocytes from periodontitis patients may not be an intrinsic characteristic of these immune cells. An explanation for the changed response after periodontal therapy could be that in an untreated periodontitis patient, the monocytes in the peripheral blood are differently primed compared to healthy conditions. This priming may have occurred due to alterations in the inflamed peripheral periodontal tissues. Recent studies have shown that uncommitted immature DCs, exposed to IFN- γ or either PGE₂ at the moment of induction of their maturation or shortly thereafter, develop the capacity to produce high and respectively low levels of IL-12p70 (20, 21). This same feature has also been shown for peripheral blood monocytes that were pre-incubated with either IFN- γ or PGE₂ (Kaliński, unpublished data). These data suggest that the actual IL-12p70-producing capacity of peripheral blood monocytes may reflect the ratio of IFN- γ to PGE₂ in particular peripheral tissues; in case of the present study it may reflect the ratio in the inflamed periodontal tissues.

In conclusion, after periodontal therapy the different functional phenotype of peripheral blood monocytes from patients resembles that of subjects without destructive periodontal disease. Subsequently, the increased levels of IL-12p70 after therapy may promote the development of cellular immune responses while inhibiting the generation of Th2 cells. Moreover, after therapy the numbers of the total peripheral blood leukocytes are decreased.

ACKNOWLEDGEMENTS

We would like to thank Dr. A.J. van Winkelhoff of the Department of Oral Microbiology of the ACTA for the analysis of the microbiological samples. Prof. Dr. M.L. Kapsenberg of the Department of Cell Biology and Histology, Academic Centre of the University of Amsterdam is kindly acknowledged for providing the IL-12p40 and IL-12p70 ELISAs. This study was supported in part by a grant of the Netherlands Institute for Dental Sciences (IOT).

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