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A type 2 response in lipopolysaccharide (LPS)-stimulated whole blood cell cultures from periodontitis patients

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SUMMARY

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 *Escherichia coli* during 18 h and the release of prostaglandin E_2 (PGE_2), IL-1β, IL-6, IL-8, IL-10, IL-12p40, IL-12p70 and tumour necrosis factor-alpha (TNF-α) was 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-1β and higher levels of IL-8 when compared with WBCC from controls. No differences were observed with respect to IL-6, IL-10, IL-12p40 and TNF-α production. It is known from the literature that LPS-stimulated WBCC reflect specifically the behaviour of the monocytes and that monocytes are peripheral precursors of antigen-presenting cells (APC). 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 type (Th1) cells that promote cellular immunity, while APC-derived PGE_2 induces type 2-helper (Th2) cells that promote humoral immunity, it is postulated that APC from periodontitis patients may have a bias in directing Th2 responses and thereby promoting the humoral immunity in periodontitis.

Keywords periodontitis monocytes PGE_2 IL-12p70 antigen-presenting cells

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 defence mechanisms with bacteria accumulating on the tooth surface [2]. The prevalence of periodontal pathogens in the general population is moderate to high: 27% for *Actinobacillus 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.

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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 neighbourhood determine the course of the immune response and hence the resistance or susceptibility to the disease [7].

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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 (WBCC) system, differences in the non-specific cellular immune response to LPS between periodontitis patients and controls.

SUBJECTS 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 Centre 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 two or more 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 immunosuppressants 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 characterize further patients and controls, subgingival bacterial samples were taken and subsequently cultured in a standardized way as previously described [9] (Table 1).

Table 1. Demographic, bacteriological and blood cell data of the two study groups

	Controls ($n = 19$)	Patients ($n = 19$)
Age† (years)	38 ± 5	37 ± 6
No. Caucasians	15	13
No. females/males	11/8	11/8
No. subjects culture positive for‡		
Actinobacillus actinomycetemcomitans	5	8
Porphyromonas gingivalis	4	12*
Bacteroides forsythus	8	18**
Prevotella intermedia	15	18
Fusobacterium nucleatum	19	19
Peptostreptococcus micros	17	17
Campylobacter rectus	1	4
No. ($\times 10^9/l$) of†		
Leucocytes	5·89 ± 1·33	6·11 ± 1·73
Monocytes	0·41 ± 0·12	0·36 ± 0·08
Lymphocytes	1·82 ± 0·65	1·88 ± 0·59
Neutrophils	3·50 ± 1·00	3·72 ± 1·41
Eosinophils	0·11 ± 0·06	0·10 ± 0·05
Basophils	0·04 ± 0·02	0·03 ± 0·02

†Values are means ± s.d.

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

*$P < 0·05$; **$P < 0·01$ as analysed by Fisher's exact test.

Whole blood cell cultures

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, The Netherlands) 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-bottomed tissue culture plates (Costar, Cambridge, MA) in the absence or presence of LPS from *Escherichia coli* O127 : B8 (Difco, Detroit, MI) in various concentrations for 18 h at 37°C. The period between the blood collection and the start of the incubation was 1·5 h. Following culturing, supernatants were collected and stored at –80°C until determination of cytokines and prostaglandin E_2 (PGE_2) concentrations.

In order to determine the most optimal LPS concentration for the stimulation of the WBCC, the blood samples were incubated with six 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(K_3) containing tube (Becton Dickinson Vacutainer System Europe, Meylan, France) for the determination of the leucocyte and the leucocyte differentiation counts, which were performed in standard automated procedures.

Cytokine and PGE_2 determinations

The IL-1β, IL-6, IL-8, IL-10 and tumour necrosis factor-alpha (TNF-α) levels were measured in the supernatants using commercially available ELISAs (PeliKine Compact™ human ELISA kits; CLB, Amsterdam, The Netherlands) according to the manufacturer's instructions. The sensitivity of the kits for the various cytokines used varied from 4 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.

PGE_2 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) 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 demographic, bacteriological and blood cell count parameters, as listed in Table 1, on the cytokine and PGE_2 secretion in the cultures was 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_2 concen-

trations were performed by means of the unpaired *t*-test and an analysis of covariance, respectively. $P < 0.05$ was considered significant.

RESULTS

The production of the inflammatory mediators IL-1β, IL-6, IL-8, IL-10, IL-12p40, TNF-α and PGE_2 was measured in the supernatants of the WBCC after 18 h of stimulation with various concentrations of LPS from *E. coli*. For cells that were incubated without LPS, only in case of IL-8 were detectable levels of the cytokine 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 groups. 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.

Demographic, 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 Fig. 1a,b. Linear regression analyses, including all the demographic, 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 co-variants 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 (Fig. 1). However, the levels of IL-1β measured in the supernatants of the periodontitis patients were 1·5-fold lower than the levels measured in the supernatants of the controls. The periodontitis patients proved to produce two-fold higher concentrations of PGE_2 in the supernatants in comparison with the control group (Fig. 1a). Within the patient group it was interesting to note that several patients secreted very high levels of PGE_2: seven out of the 19 patients produced >5000 and up to 11 000 pg/10^6 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 1·5-fold higher than those in the controls (Fig. 1b).

The concentrations of IL-12p70 were measured in 100 ng/ml LPS-stimulated cultures (Fig. 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. This showed that cells of the periodontitis patients produced half of the IL-12p70 levels of the controls.

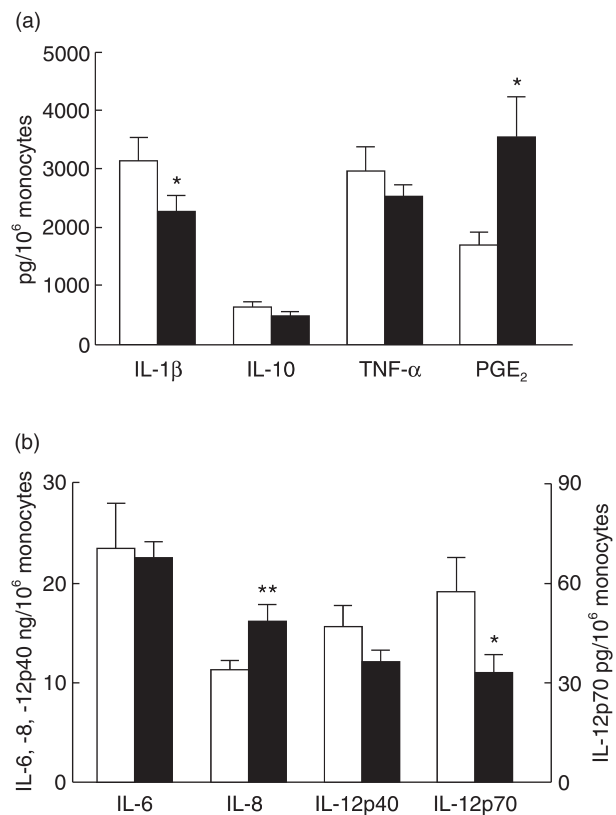

Fig. 1. LPS-induced mediator production in 19 periodontitis patients and 19 controls. For the measurements of IL-1β, IL-6, IL-8, IL-10, IL-12p40, tumour necrosis factor-alpha (TNF-α) and prostaglandin E_2 (PGE_2) the whole blood cell cultures were stimulated with 1 ng/ml LPS from *Escherichia coli* for 18 h, whereas the cultures for IL-12p70 were stimulated with 100 ng/ml LPS from *E. coli* for 18 h. Data are means ± s.e.m. and are expressed as cytokine or PGE_2 production per 10^6 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 co-variants. □, 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, 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 behaviour of the monocytes [10,12–15]. 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 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 behaviour of the monocytes.

In the present study LPS from *E. coli* was used as a non-specific stimulant to activate the peripheral blood cells. *Escherichia 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 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 also produced higher levels of PGE_2 and lower levels of IL-1β compared with 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-gamma (IFN-γ) and Th2 cells the 'type 2' cytokines IL-4 and IL-5 [17]. It is acknowledged now that factors associated with APC highly determine the tuning of the balance between type 1 and type 2 cytokines [18,19]. However, functional studies with APC harvested from the peripheral tissues are very difficult, if not impossible. Since studies on the role of APC 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 APC in periodontitis.

In fact, APC 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]. APC provide T cells not only with an antigen-specific stimulatory signal and a series of costimulatory 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 neighbouring 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 PGE_2 are important factors. IL-12 is a dominant factor in directing the development of Th1 cells producing high levels of IFN-γ [25–27]. PGE_2 selectively inhibits IFN-γ production and is reported to favour the development of Th2 cells [28]. Furthermore, it has been proposed that the ratio of IL-12 to PGE_2 produced by APC during T cell activation is highly predictive of the level of IFN-γ 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 APC from patients with periodontitis. Accompanying this, a decreased type 1 response, i.e. reduced levels of IFN-γ 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-γ production by PBMC has also been shown for several other diseases, like atopic dermatitis, allergic asthma, rheumatoid arthritis and HIV infection [13,14,32–43].

In support of this suggested Th2-promoting phenotype of APC 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 with 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 APC 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 APC from periodontitis patients may be an important factor in the susceptibility to the disease.

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