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Evaluation of selected peripheral blood leukocyte functions in patients with various forms of periodontal disease

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

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Summary

Introduction:

Periodontitis (P) is an infectious disease that develops in the supporting tissues of the tooth. One of the risk factors leading to it may be dysfunction of some immune system cells. Therefore, the object of the study was to assess selected functions of peripheral blood leukocytes in patients with various forms of P. As leukocytes are able to secrete interleukin (IL)-4 and IL-6, concentrations of their soluble receptors and the expression of their membrane receptors were investigated.

Materials and Methods:

Twenty generally healthy subjects with aggressive (AP) and chronic periodontitis (CP) were enrolled in the study. The control group consisted of 8 healthy subjects, with no changes in periodontium. Peripheral blood mononuclear cells (PBMCs) were isolated and cultured. Levels of IL-4, IL-6, and their soluble receptors sIL-4R and sIL-6R were determined in the supernatant by ELISA. The expressions of cell surface IL-4R and IL-6R were assayed on PBMC using flow cytometry.

Results:

No statistically significant differences were found in the selected parameters between people with periodontal disease and healthy controls. However, in subjects with AP, there was an increasing tendency in IL-6 concentration and IL-4R expression on PBMCs.

Conclusions:

Our results show that leukocytes play a significant part in P and their activity is probably lesion-dependent. Estimation of the cytokines secreted by leukocytes may facilitate differentiation and prognosis of the disease progression.

Key words:

periodontitis • IL-4 • IL-6 • receptors

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INTRODUCTION

Periodontitis (P) is an infectious disease that develops in the supporting tissues of the tooth. The inflammatory process affecting periodontal tissues leads to the formation of periodontal pockets, resorption of the alveolar bone, and eventual tooth loss. Certain bacteria of dental plaque are the primary etiological factor responsible for the disease progression. It is believed, however, that some people are particularly vulnerable to the disease and its severe and rapid course¹¹. The nature of local and general responses of the organism to pathogenic bacteria is likely to affect the clinical course of P. A number of earlier studies have focused on the search of risk factors that predispose to the development of the disease^{15, 16, 24}. In many cases of P in young subjects, with progression to a rapid loss of bone, general dysfunctions of the immune system can be observed, including impairment of certain functions of neutrophils (adherence, chemotaxis, destruction of bacteria)²⁴. The role of leukocytes in the pathomechanism of P has been recently emphasized. The simultaneous monitoring of the expression of surface receptors, release of cytokines, and concentration of soluble receptors may elucidate the role of leukocytes in the immune response in periodontal tissue.

The present investigation was to evaluate selected functions of peripheral blood leukocytes in patients with various forms of P compared with controls. The study focused on the capacity of leukocytes to secrete interleukin (IL)-4 and IL-6, their soluble receptors, and the expression of IL-4 and IL-6 membrane receptors.

MATERIALS AND METHODS

Twenty generally healthy subjects with P aged 21–48 were enrolled in the study. The patients with P were divided into two groups according to clinical and radiological diagnosis. The first group consisted of 10 subjects, aged 21–41, with aggressive periodontitis (AP) and advanced lesions in periodontal tissue. The second group included 10 patients aged 40–48 with chronic periodontitis (CP) and stable lesions. The control group (C) consisted of 8 generally healthy subjects, aged 20–45, with no periodontal lesions.

Isolation of mononuclear cells from peripheral blood

Venous peripheral blood samples (8 ml) were drawn into test-tubes containing sodium heparin Vacutainer CPT (cell preparation tube; Becton Dickinson, USA). Mononuclear cells were isolated by centrifugation at $1500 \times g$ for 20 min. Then the tubes with contents were gently shaken. The supernatant was collected in plastic tubes with a Pauter's pipette and

centrifuged again in $750 \times g$ for 5 min. Next, 6 ml PBS without Ca and Mg ions was added to the sediment (Biomed, Serum and Vaccine Plant, Lublin, Poland). Washing in the same conditions was repeated twice. The sediment was suspended in RPMI (MEM Alpha Medium, Gibco BRL, USA). Its density was determined using a Coulter Maxm hematological apparatus, reaching 1,000,000 cells/ μ l.

Culture

Mononuclear cells were cultured on Sarstedt plates. Four cultures from each patient were carried out simultaneously. 100 μ l suspension (10,000/ μ l density) and 900 μ l medium (85% MEM Alpha Medium + 15% fetal bovine serum; Gibco BRL, USA) were added to each culture. 10 μ l PHA/1 ml of culture was used for stimulation. The cultures were incubated for 72 h at 37°C and in an atmosphere of 5% CO₂ in an incubator (Lab – Line Instruments Inc., USA). Cell viability was determined using trypan blue to be higher than 90%. Following incubation, the culture sample was carefully mixed, collected into a plastic tube with a Pauster's pipette, and centrifuged in $750 \times g$ for 5 min. The supernatant was obtained and frozen at –80°C (to determine cytokines and their soluble receptors). The supernatant was rinsed twice in PBS, Gibco BRL, and suspended in 300 μ l PBS.

Preparation of samples for determination of receptors using a cytometer

The suspension (35 μ l) was transferred to plastic tubes (12 $\times$ 75 mm) and then 10 μ l samples of "biotinylated cytokine reagent": rh IL-4 and rh IL-6 labeled with biotin (R&D Systems, USA) were added. Concentrations of IL-4, IL-6, and their respective soluble receptors sIL-4R and sIL-6R in the supernatant were determined by ELISA using the Quantikine Human IL-4 Immunoassay kit, Quantikine Human IL-4sR Immunoassay kit, Quantikine Human IL-6 Immunoassay kit, and Quantikine Human IL-6sR Immunoassay kit (R&D Systems, USA).

The methodology was accepted by the Committee of Bioethics, Medical Academy of Białystok.

The results were subjected to statistical analysis using the SPSS 8.0 PL package. The U-Mann-Whitney test was used to compare groups. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Mean concentrations of IL-4 in the culture supernatant of peripheral blood mononuclear cells

(PBMCs) obtained from the subjects with AP and CP did not differ from the mean concentration of IL-4 in controls. The mean IL-6 levels in groups CP and C were analogous. The mean concentration of IL-6 in group AP was over twice as high as in controls, although the difference was not statistically significant. Soluble IL-4R was present in the supernatant only in 7 subjects, including 2 in group C, 2 in AP, and 3 in CP. The mean levels of this receptor in patients were lower than in healthy subjects, the difference being statistically non-significant. The mean concentrations of sIL-6R in the supernatant in groups CP and C were similar, being lower, though not significantly, in AP patients. Data are presented in Table 1.

The mean fluorescence value of IL-4R on CD3, CD19, CD4 and CD8 cells obtained from AP subjects were elevated compared with groups C and CP, but because of the high standard deviations, the differences were not statistically significant. The mean fluorescence values for IL-6R on PBMCs in groups AP, CP and C were comparable. It seems, however, that the mean values of this parameter on CD19 cells in group AP showed a growing tendency, though not statistically significant, compared with groups C and CP. Mean fluorescence values and standard deviations are presented in Table 2.

DISCUSSION

Many recent studies have tried to explain the role of B lymphocytes and respective subpopulations of T lymphocytes in periodontal inflammation. According to Seymour et al.²², stable lesions in periodontal tissue, common in CP patients, show a predominance of T helper (Th)1 cells. Th2 cells seem to play a role in advanced progressive lesions²¹. The cytokines secreted by these cells affect the activation of B lymphocytes and thus influence the production of antibodies. B lymphocytes and plasmatic cells predominate in the inflammatory infiltration in advanced stages of P²¹. Disease progression and rapid destruction of periodontal tissue may be antibody dependent. If the antibodies are protective in nature, inflammation will not spread. Otherwise, further activation of B lymphocytes will occur, leading to uncontrolled secretion of IL-1 and the associated bone destruction^{7, 9, 20}. Increased Th2 activity in P subjects as compared with healthy controls was observed by Aoyagi et al.¹ and Lundqvist et al.¹⁴, who noted high concentrations of IL-4 in the culture supernatant after stimulation of peripheral blood lymphocytes. A significant increase in the level of IL-4 as well as predominance of IL-4⁺ have been also observed in periodontal tissue in P patients^{2, 7, 26}. The level of IL-4 is likely to increase with the progression of periodontal lesions².

Table 1. Mean values of IL-4, IL-6, sIL-4R and sIL-6R concentrations in blood serum in the aggressive periodontitis (AP), chronic periodontitis (CP) and control (C) groups

Parameter	AP	CP	C
IL-4 (pg/ml)	17.4 ± 10.6	16.6 ± 22.1	14.0 ± 10.6
IL-6 (ng/ml)	71.9 ± 52.3	84.0 ± 34.7	72.3 ± 37.6
sIL-4R (pg/ml)	4.2 ± 7.7	7.4 ± 11.9	10.7 ± 15.5
sIL-6R (pg/ml)	117.6 ± 59.2	188.5 ± 105.4	188.9 ± 145.4

Table 2. Mean fluorescence values of IL-4R and IL-6R on PBMCs in the aggressive periodontitis (AP), chronic periodontitis (CP) and control (C) groups

Parameter	AP	CP	C
IL-4R/CD3	6.6 ± 6.9	3.3 ± 4.2	2.7 ± 3.3
IL-4R/CD19	8.0 ± 9.4	4.2 ± 3.4	3.8 ± 4.7
IL-4R/CD4	5.3 ± 6.1	7.7 ± 13.4	2.6 ± 3.5
IL-4R/CD8	4.6 ± 5.0	3.6 ± 5.3	2.5 ± 3.4
IL-6R/CD3	3.5 ± 3.2	2.8 ± 3.1	2.4 ± 2.6
IL-6R/CD19	4.8 ± 5.5	4.2 ± 5.2	2.6 ± 2.7
IL-6R/CD4	3.7 ± 3.8	2.3 ± 2.5	2.2 ± 2.3
IL-6R/CD8	3.6 ± 3.9	2.1 ± 2.3	1.9 ± 2.2

However, other authors have not found the expression of IL-4 in periodontal tissues, perhaps because of the rapid breakdown of this cytokine^{5, 14, 25}. Similar concentrations of IL-4 have been observed in the supernatants of PBMCs obtained from healthy subjects and from patients with gingivitis or periodontitis⁷.

The activity of lymphocytes can also be expressed by their capacity to secrete IL-6. This interleukin has a number of effects, including the ability to stimulate differentiation of active B lymphocytes towards cells that release immunoglobulins^{13, 19}. Gingival mononuclear cells (GMCs) from pathologically altered periodontal tissues can produce IL-6 spontaneously, while PMBCs from the same subjects produce the interleukin only after stimulation¹². There are many reports on the presence of IL-6 in the gingiva pocket fluid and its substantial release by PBMCs and GMCs^{6, 8, 10, 12, 18, 25}. It has been frequently emphasized that the expression of this cytokine is more intense in the inflamed periodontal tissues than in PBMC cultures of the same subjects. The increase in IL-6 concentration in periodontal tissues is also correlated with elevated levels of IgG, IgA and IgM¹². However, according to Takahashi et al.²³, there is no difference in the concentrations of this cytokine between PBMC cultures and the sera of patients with P or healthy subjects.

We found that PBMCs in P patients produced IL-4 and IL-6. Mean IL-4 concentrations in P patients

were within the norm. However, there was a slight increase in the level of this interleukin in AP patients with advanced changes in periodontal tissues. The mean IL-6 level in CP patients was analogous to that noted in healthy subjects. However, in AP patients its concentration in the supernatant was over twice as high as in the CP and C groups. Thus, our results on the secretory capacity of leukocytes find confirmation in earlier literature data showing that advanced periodontal lesions may be accompanied by excessive activation of Th2 and B lymphocytes, the cells likely to be responsible for the disease progression.

The behavior of interleukins is, however, dependent on a number of factors and therefore difficult to evaluate. The secretion of cytokines is mainly affected by mutual interactions as well as interactions with cellular and soluble receptors^{3, 27}. There are still few data on interleukin receptors in P patients. Kono et al.¹² believe that it is IL-4 and IL-5 that affect the process of isotopic changes or IL-6R induction on B lymphocytes, and not IL-2. These authors found a high expression of this receptor on resting and active T lymphocytes and on active B cells from periodontal tissues. However, this receptor was not observed on PBMCs isolated from the same patients. In our study, the mean fluorescence value of IL-6 in P patients was similar to controls. However, in AP patients this

parameter showed slightly higher values. The most pronounced increase in the mean fluorescence value of IL-6R was noted on B lymphocytes. The sIL-6R level in the same group was lower than in the CP and C groups, most likely due to its binding by IL-6.

According to Collins et al.⁴, an increase in IL-4R is observed on CD3 cells. This is in agreement with our results showing a growing tendency in IL-4R fluorescence values, particularly on CD3 lymphocytes in AP patients. The increase in IL-4R on lymphocytes may be due to stimulation by IL-4 itself¹⁷. However, it is difficult to interpret the results regarding sIL-4R concentrations, since it was present in culture supernatant only in a few P and C subjects.

In conclusion, our results, as well as literature data, show that leukocytes play a significant part in P. Their activity is, however, lesion-dependent. Estimation of the cytokines secreted by leukocytes may facilitate differentiation and prognosis of the disease progression. This, however, requires further data on cytokines, their agonists and antagonists, soluble and cellular receptors, and their involvement in P. Elucidation of these relationships will provide more information on the immune reactions that occur in periodontal tissues in patients with various forms of the disease, which may prevent periodontal tissue destruction.

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