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Evidence for a specific crevicular lymphocyte profile in aggressive periodontitis

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Background and Objective: It is undisputed that the periodontal pocket is a particular region of the host defense that is dominated by polymorphonuclear leukocytes. However, little is known about the lymphocytes in the crevice. It was the aim of this study to analyse the proportions of T cells ($CD3^+$), T-helper cells ($CD4^+$), T-suppressor cells ($CD8^+$), and B cells ($CD20^+$) in the crevice of patients with localized aggressive periodontitis (LAP), generalized aggressive periodontitis (GAP), and generalized chronic periodontitis (CP). The results were compared with those obtained from periodontally healthy controls.

Material and Methods: Crevicular cells were collected according to a previously described method. The lymphocyte subpopulations were analysed by using an indirect immunofluorescence method.

Results: Significant differences were established between the test groups and the controls regarding the mean number of $CD8^+$ lymphocytes (LAP > CP and controls; $p < 0.05$) and $CD20^+$ lymphocytes (LAP/GAP > CP, $p < 0.05$ and LAP/GAP > controls; $p < 0.001$). Significant variations in the $CD4^+/CD8^+$ ratio were observed (LAP < controls and GAP < controls; $p < 0.01$), as well as a correlation between the number of T cells and the degree of inflammation.

Conclusion: In the present study, patients with LAP and patients with GAP were found to have increased numbers of crevicular T-suppressor/cytotoxic and B cells. This supports the hypothesis of a changed immune pathology in patients with aggressive periodontitis.

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The analysis of lymphocyte subpopulations in blood and other body fluids or tissues is a well-established method for using to characterize immunological profiles in various infectious diseases. In addition, in patients with periodontal diseases, analyses have been carried out to investigate the proportions of lymphocyte subpopulations in the peripheral blood (1–3) as well as in biopsies of periodontal tissue (4–7). Little is known, however, about the proportions of crevicular lymphocytes in different forms of periodontitis (8). In contrast, there are extensive data on polymorphonuclear leukocytes, which constitute another important cell fraction that dominates the healthy crevice and the periodontal pocket (9,10).

It is generally accepted that the crevice is an important region of the local host defence (11). The occurrence of different lymphocyte patterns in this area has been hitherto little investigated. Although different studies on crevicular fluid cells report the presence of lymphocytes, to date the crevicular lymphocyte subpopulations have not been examined (3,10,12,13).

It is known that the healthy gingiva contains a $CD4^+/CD8^+$ ratio comparable with that of the peripheral blood (14). However, a reduced $CD4^+/CD8^+$ ratio has been described in periodontal lesions, compared with that of the peripheral blood (15–17). Several researchers assume that T lymphocytes are predominant in gingivitis, whereas B-cell dominance develops during the formation of a periodontal lesion (18,19). Pietruska *et al*. observed T lymphocytes more frequently in aggressive periodontitis lesions and B cells more frequently in generalized chronic periodontitis lesions (20). Suarez *et al*., who compared periodontally healthy patients to patients with aggressive periodontitis, employing immunohistochemistry and reverse transcription–polymerase chain reaction (RT–PCR) for cytokines, reported that the tissue of patients with aggressive periodontitis contains fewer $CD3^+$ and $CD4^+$ cells, but only a minimal difference in the number of $CD8^+$ cells was observed between the groups (21). They propose that the role of $CD8^+$ cells in aggressive periodontitis lesions should be investigated, and Petit *et al*., also demand further research on the role of $CD8^+$ cells (22).

Therefore, the objective of this study was to characterize crevicular lymphocyte subpopulations in patients with chronic and aggressive periodontitis.

Material and methods

Study population

Those taking part in the investigations were consecutively recruited (i.e. they were patients with aggressive and chronic periodontitis who were referred to our department, by colleagues, for further diagnosis and therapy).

Periodontally healthy patients of appropriate age and gender, who showed no signs of plaque or infection, and were also completely free of any signs of periodontitis (probing depth < 3.5 mm) served as controls. Systemic disease and antibiotic therapy over the previous 12 months were excluded by anamnesis. All subjects were nonsmokers or ex-smokers who had ceased smoking at least 18 months before the start of the study.

The crevicular cells were sampled from the deepest site in each of the four quadrants and pooled. The periodontal pockets chosen for sampling had the same values regarding the clinical scores of plaque index, sulcus bleeding index, and probing depth category (localized and generalized aggressive periodontitis 6–9 mm; generalized chronic periodontitis 4–6 mm; control group 1–3.5 mm). The patients were diagnosed following the guidelines of the AAP 1999 (23) and recruited into the four groups listed below.

(i) Localized aggressive periodontitis (LAP) (11 patients: seven women, four men; 20–30 years of age; average age 26.7 years). All patients in this group were characterized by the microbiological presence of *Actinobacillus actinomycetemcomitans*.

(ii) Generalized aggressive periodontitis (GAP) (14 patients: six women, eight men; 30–40 years of age; average age 37.1 years). The diagnosis was based on clinical and X-ray data. *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *A. actinomycetemcomitans* were detected in the subgingival plaque samples of these subjects.

(iii) Generalized chronic periodontitis (CP) (13 patients: nine women and four men; 39–77 years of age; average age 54.3 years). *Fusobacterium nucleatum* and *Prevotella intermedia* were verified in all patients of this group.

(iv) Control group (12 patients: six women and six men; 18–58 years of age; average age 34.5 years). All patients in this group displayed a healthy periodontal status, documented by a low mean plaque score (plaque index: 0.01) and inflammation score (sulcus bleeding index: 0.11). Periodontopathogenic bacteria were not observed in these subjects.

Crevicular cell sampling

The collection of crevicular cells was carried out using a method described by Sigusch *et al*. (10). In brief, after the designated area of collection had been dried out with cotton rolls and compressed air, the test site was rinsed with a sterile 0.9% (v/v) saline solution. Then, an Eppendorf pipette (10 µl), fitted with a conventional standard pipette tip, was placed at the crevice or pocket entrance to effect the application of the rinsing fluid whilst protecting the soft tissues. The cell suspension gained by the ensuing suction was collected in an Eppendorf tube, pooled, and washed twice. For the following examinations, the cell count was set to 1×10^6 cells/ml.

To ascertain the cell count, 200 µl of Türk's solution was added to 20 µl of the cell suspension; 10 µl of the resulting solution was transferred to a counting chamber, and the concentration of cells was determined using an optical microscope at a magnification of ×400. The proportion of living cells was established by using the Trypan Blue exclusion test (24).

Immunofluorescence method

To analyse the lymphocyte subpopulations, 30 µl of each cell suspension was applied to epoxy-coated slides and then air-dried. These slides were fixed with acetone for 2 min, then rehydrated for 5 min at room temperature in Tris-buffered saline. Then, 30 µl of the respective primary antibodies [$CD3^+$ (pan T cells), $CD4^+$ (T-helper cells), $CD8^+$ (T-suppressor/cytotoxic cells), and $CD20^+$ (B cells)] was applied to the samples (the concentration of which had been set to 1×10^6 cells/ml) and the carriers were incubated for 30 min under moist conditions. Subsequently, each carrier was washed twice, in phosphate-buffered saline (PBS), for 5 min. The concentration of the primary antibodies, as used above, lay within the range recommended by the manufacturer. After a further drying step, the samples were incubated for 30 min with 30 µl of the secondary antibody conjugated to fluorescein isothiocyanate (FITC).

The primary antibody against $CD56^+$ [natural killer (NK)] cells was also used in pretrials. However, no crevicular NK cells were detected and

therefore we did not carry out any further testing for $CD56^+$.

Finally, the region surrounding the application area was dried, the test zone covered with a mounting medium (ready-to-use DAKO Fluorescent Mounting Medium; DakoCytomation GmbH, Hamburg, Germany), and sealed with a covering glass top, by way of preparation for microscopic evaluation.

Statistical analysis

Statistical analysis was performed using the Kruskal–Wallis one-way analysis of variance (ANOVA). With the calculated test size it was possible to determine significant differences between the groups.

Whenever the Kruskal–Wallis one-way ANOVA provided a significant result ($p < 0.05$), the pairs of groups between which the particular differences were of consequence were established by using the chi-square approximation.

The tests of Pearson, Spearman, and Kendall were also used to analyse whether there was a correlation between the crevicular lymphocytes and the periodontal inflammation score.

Results

The key results, listed below, were established after performing descriptive statistical evaluations.

(i) The four groups showed the following mean values of crevicular cell viability: controls, 85.8 ± 3.5%; CP, 80.5 ± 4.4%; GAP, 79.0 ± 5.4%; and LAP, 76.1 ± 8.3%. The differences between the groups were statistically significant (chi-square = 15.59; $p < 0.01$). Notable differences were found among the LAP, GAP, and control groups (Fig. 1).

(ii) Analysis of the lymphocyte subpopulations showed the highest median $CD3^+$ cell count in the LAP group ($n = 967$), and the lowest in the CP group ($n = 400$). However, the difference between the groups was not significant. Essentially identical median values for $CD4^+$ cells were determined for all four groups (data not shown).

(iii) The highest median value for $CD8^+$ cells was found in the LAP group ($n = 267$) and the lowest was found in the control group ($n = 83.5$). Marked differences were demonstrated between the groups (chi-square = 8.6; $p < 0.05$), with the difference between the LAP and CP groups being statistically significant (Fig. 2).

(iv) The mean $CD4^+/CD8^+$ ratio was highest for the control group, decreasing progressively in the order CP > LAP > GAP (i.e. patients with aggressive periodontitis displayed a clearly lower $CD4^+/CD8^+$ ratio of crevicular lymphocytes in comparison to patients with chronic periodontitis). However, only the differences between the control group and the LAP group, and the control group and the GAP group, were significant (Fig. 3).

Differences between the groups were also identified when the number of B cells ($CD20^+$) was compared. The lowest median cell count (16.5) was observed in the control group. The cell count median of 216.5 for the group of LAP patients was 13 times higher than that of the control group. A $CD20^+$ cell count of 173.5 was observed for the GAP patients. Although still higher than that of the control group, the value of 67 was clearly lower for patients suffering from chronic periodontitis when compared with the LAP and GAP groups. The differences between the groups were highly significant (chi-square = 16.9; $p < 0.001$). The comparison produced significant differences between the paired groups LAP and CP/controls, as well as between the GAP and CP/controls (Fig. 4).

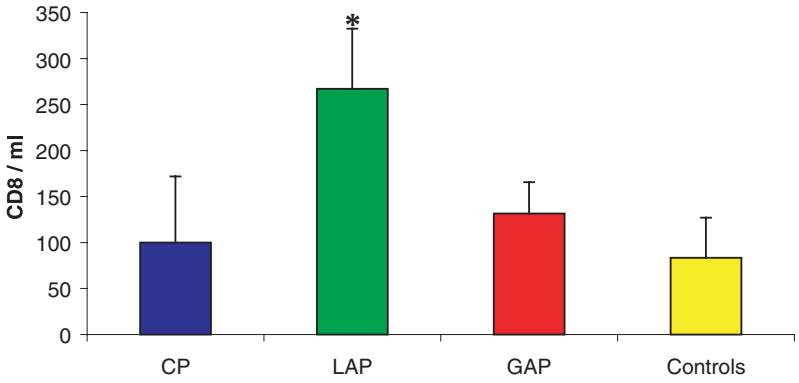

Fig. 2. $CD8^+$ lymphocytes. Significant differences between the test groups localized aggressive periodontitis (LAP) and generalized chronic periodontitis (CP), and the controls ($p < 0.05$).

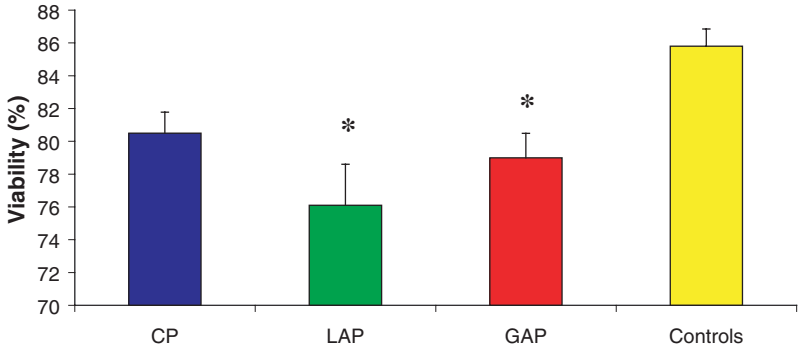

Fig. 1. Crevicular cell viability. Significant differences in crevicular cell viability between the test groups localized aggressive periodontitis (LAP) and generalized aggressive periodontitis (GAP), and the control group (*$p < 0.01$).

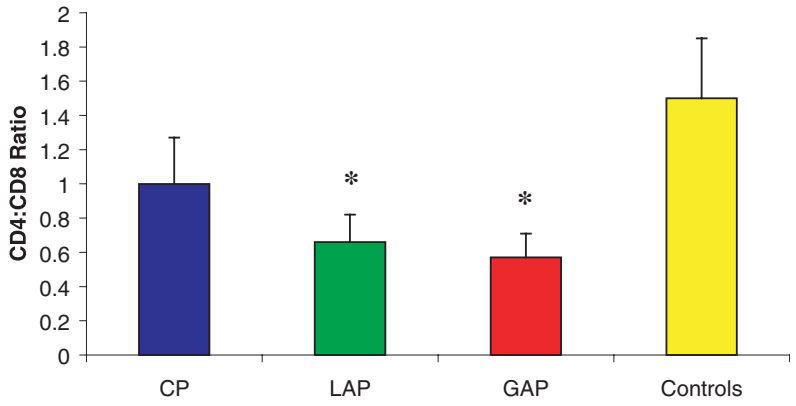

Fig. 3. $CD4^+/CD8^+$ ratio. Significant differences between the test groups localized aggressive periodontitis (LAP) and generalized aggressive periodontitis (GAP), and the control group ($p < 0.01$).

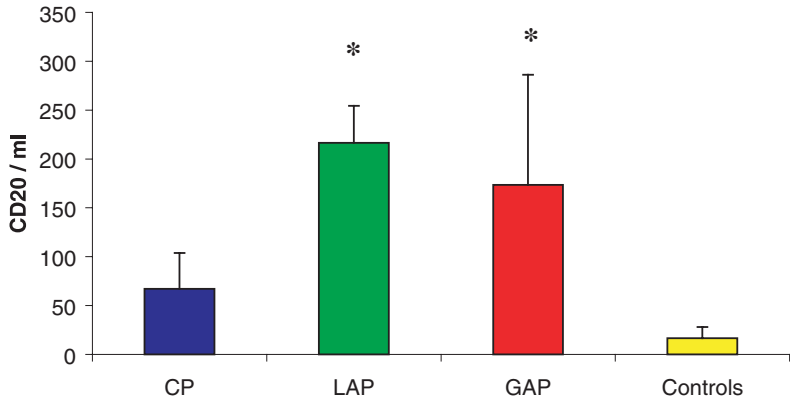

Fig. 4. $CD20^+$ cells. Significant differences between the test groups localized aggressive periodontitis (LAP) and generalized aggressive periodontitis (GAP), and generalized chronic periodontitis (CP) ($p < 0.05$), and the test groups LAP/GAP, and controls ($p < 0.001$).

Furthermore, a correlation was identified between the $CD3^+$ cell count and the inflammation score (SBI) for the groups with CP and GAP (Table 1).

Discussion

To date, no comparative study of crevicular lymphocyte subpopulations in different forms of periodontitis exists in the published literature. In contrast to previous studies analysing immune cell infiltration in periodontitis, in which the samples were mostly investigated by means of biopsy (25–27), the present study examined the lymphocytes of the crevicular fluid. These were recovered by utilizing a noninvasive method (10).

Some studies exist that determined the proportion of mononuclear cells in the crevicular fluid without differentiating between the lymphocyte subpopulations (9,12).

Kennett *et al.* likewise took crevicular samples from patients with chronic periodontitis using micropipettes (12). They were able to establish the following cell populations: 70–80% granulocytes, 10–20% monocytes/macrophages, 5% mast cells, and 5% T lymphocytes, but no B lymphocytes.

The presence of NK cells, amongst others, was analysed in the crevicular fluid during the pre-experimentation work for this study. It is known that the proportion of NK cells in the total peripheral blood lymphocytes is $\approx 15\%$. However, no NK cells were found in the crevicular fluid in the different diseased and healthy sites. Because other authors (2) have reported a particularly elevated proportion of NK cells in the peripheral blood of patients with aggressive periodontitis, it can be assumed – on the basis of the results of the current investigation – that these cells do not migrate into the crevicular region.

In work carried out in the 1980s, a reduced $CD4^+/CD8^+$ ratio in the periodontal lesion was described (14–17). In the present study, a reduction of the $CD4^+/CD8^+$ ratio in crevicular lymphocytes was also observed in patients

Table 1. Results of correlation analysis between CD3 and periodontal inflammation score for the groups chronic periodontitis (CP), localized aggressive periodontitis (LAP), and generalized aggressive periodontitis (GAP)

	CP		LAP		GAP	
	Mean	SEM	Mean	SEM	Mean	SEM
CD3	714.15	201.10	1093.80	204.60	661.50	94.9
sulcus bleeding index	1.30	0.30	1.80	0.30	1.00	0.20
Pearson	$r = 0.74$	$p = 0.003$*	$r = 0.34$	$p = 0.166$	$r = 0.69$	$p = 0.003$*
Spearman	$r = 0.68$	$p = 0.007$*	$r = 0.23$	$p = 0.259$	$r = 0.48$	$p = 0.039$*
Kendall	$r = 0.53$	$P = 0.001$*	$r = 0.20$	$p = 0.249$	$r = 0.37$	$p = 0.027$*

*Statistically significant for $p < 0.05$.
SEM, standard error of the mean.

with aggressive periodontitis (LAP and GAP), when compared with healthy controls. Furthermore, it was noticeable that the $CD4^+/CD8^+$ ratio in patients with LAP and GAP, when compared with the $CD4^+/CD8^+$ ratio of patients in the control group, was markedly lower than in patients with CP.

Because the total T-cell ($CD4^+$) count in all four groups showed nearly identical median values, the change in the $CD4^+/CD8^+$ ratio might be explained by the presence of a higher number of T-suppressor/cytotoxic cells ($CD8^+$).

A positive correlation between the $CD3^+$ cell count and the degree of inflammation (quantified by sulcus bleeding index) for the CP and GAP groups was identified (i.e. the number of T cells in the crevicular fluid increases with an increasing level of clinical inflammation parameters). These findings correspond to those of the tissue samples in cases of gingival inflammation (20,28,29).

Lappin *et al.* examined the distribution of lymphocyte subpopulations in biopsies from periodontal lesions, with reference to the probing depth, in patients with chronic and aggressive periodontitis (30). They observed marked variations in the findings of both forms of periodontitis. The results were interpreted as suggesting a differing pathogenesis for chronic periodontitis and aggressive periodontitis. The authors demonstrated distinctly higher T- and B-cell counts in patients with aggressive periodontitis than in patients with chronic periodontitis.

The results of the present study support the assumption of a possible variance in the immune pathology of both forms of periodontitis, especially when considering the high $CD20^+$ cell count and the low $CD4^+/CD8^+$ ratio of crevicular mononuclear cells in aggressive periodontitis cases.

Seymour *et al.* investigated the expression of CD29 on gingival CD4-positive lymphocytes in tissue samples of patients with CP and LAP (28). They likewise observed a lowered $CD4^+/CD8^+$ ratio in the periodontal lesions of subjects with LAP. The results of this study also confirm these findings for the periodontal pocket.

The proportion of $CD8^+$ cells in the crevicular fluid was found to be higher in all periodontitis groups than in the healthy controls. In the LAP group, this increase was statistically significant. These findings are interesting on the background that herpesvirus infections have recently been in focus as a result of the effects that they exert on the immune defense (31). Herpesviruses have been shown to be present at high frequencies in patients with LAP and GAP (32). The immune system response to a herpesvirus challenge changes from a predominantly $CD4^+$ response early in infection to a $CD8^+$ response in the latent infection. Furthermore, it is known that $CD4^+$ cells contribute to the expansion of cytotoxic $CD8^+$ T lymphocytes (31). Human cytomegalovirus can modulate antigen-specific T-lymphocyte functions, resulting in a relative increase in $CD8^+$ suppressor cells, which, in turn, may lead to an impairment of cell-mediated immunity (33,34). Consistent with immune responses of a herpesvirus infection, aggressive periodontitis has been related to low $CD4^+/CD8^+$ ratios (35,36) and, within the $CD8^+$ lymphocytes, a shift towards cytolytic T (Tc) lymphocytes (22).

Even though there was no differentiation between T-suppressor and T-cytotoxic cells in our study, it is interesting to note that patients who were suffering from LAP presented the highest $CD8^+$ cell counts and also showed a significantly reduced CD4/CD8 ratio. This reduced CD4/CD8 ratio was also present in patients with GAP and was not caused by a change in $CD4^+$ cell numbers.

It is known that the cytokines interleukin-4 and interleukin-5, which are produced by T-helper 2 cells, possess an important regulatory function in the immunoglobulin production of B cells. An increase in the expression of interleukin-4 and interleukin-5 in aggressive periodontitis has been demonstrated by various authors (37,38). Nakajima *et al.* postulated a close association between humoral immunity and allergic reactions, on the one hand, and the specific response of T-helper 2 cells, on the other (39). Interleukin-4 in particular, is considered to play a decisive role in regulating the antibody production in B cells.

In the present work, the lowest median value for the B-cell counts in the periodontal pocket was calculated for the control group (5,16). In the LAP group, the median of 216.5 was more than 13 times higher than in the controls. For GAP, a significant difference was also observed.

Amer *et al.* ascertained an increase in the proliferation of B cells in the peripheral blood in patients with aggressive periodontitis (40). Yamazaki *et al.* showed a distinct increase in the $CD19^+/CD3^+$ ratio within the periodontal lesion (41). This suggests that the periodontal lesion is also dominated by B cells in the tissue. This was substantiated by the results of Afar *et al.*, who demonstrated a marked increase in a B-cell subpopulation – namely the autoantibody-producing $CD20^+$ $CD5^+$ cells – of patients with aggressive periodontitis (2).

Epstein–Barr virus may act as a potent polyclonal B-lymphocyte activator, capable of inducing the proliferation and differentiation of immunoglobulin-secreting cells, features associated with the progression of some types of periodontal disease (42).

B-cell expansion could not be verified on the basis of our data; however, high B-cell counts in the LAP and GAP groups argue for this pathogenetic background.

The results of this study prove that crevicular immune cells (i.e. $CD8^+$ T-suppressor/cytotoxic cells and $CD20^+$ B cells) can be observed to occur at significantly higher numbers in the crevice of patients with LAP and GAP forms of periodontitis when compared to patients with chronic periodontitis and healthy control subjects. These results are consistent with the hypothesis that the high $CD8^+$ and B-cell counts may arise from a herpesvirus infection. The hypothesis of a changed immune pathology, which is reflected by elevated $CD8^+$ and B-cell counts, is supported by the findings of this study.

References

1. Katz J, Goultschin J, Benoliel R, Schlesinger M. Peripheral T lymphocyte subsets

in rapidly progressive periodontitis. *J Clin Periodontol* 1988;**15:**266–268.
2. Afar B, Engel D, Clark EA. Activated lymphocyte subsets in adult periodontitis. *J Periodont Res* 1992;**27:**126–133.
3. Gemmell E, Woodford V, Seymour GJ. Characterization of T lymphocyte clones derived form porphyromonas gingivalis infected subjects. *J Periodont Res* 1996;**31:**47–56.
4. Cobb CM, Singla O, Feil PH, Theisen FC, Schultz RE. Comparison of NK-Cell (Leu-7+ and Leu-11b+) populations in clinically healthy gingiva, chronic gingivitis and chronic adult periodontitis. *J Periodont Res* 1989;**24:**1–7.
5. Celenligil H, Kansu E, Ruacan S, Evatalay K, Caglayan G. Immunohistological analysis of gingival lymphocytes in adult periodontitis. *J Clin Periodontol* 1990;**17:**542–548.
6. Fujita S, Takahashi H, Okabe H, Ozaki Y, Hava Y, Kato I. Distribution of natural killer cells in periodontal diseases: an immunohistochemical study. *J Periodontol* 1992;**63:**686–689.
7. Celenligil H, Kansu E, Ruacan S, Evatalay K, Caglayan G. *In situ* characterization of gingival mononuclear cells in rapidly progressive periodontitis. *J Periodontol* 1993;**64:**120–127.
8. Takeuchi Y, Sakurai K, Ike I, Yoshie H, Kawasaki K, Hara K. ICAM-1-expressing pocket epithelium, LFA-1-expressing T cells in gingival tissue and gingival crevicular fluid as features characterizing inflammatory cell invasion and exudation in adult periodontitis. *J Periodont Res* 1995;**30:**426–435.
9. Thurre C, Robert M, Cimasoni G, Baehni P. Gingival sulcular leukocytes in periodontitis and in experimental gingivitis in humans. *J Periodont Res* 1984;**19:**457–468.
10. Sigusch B, Klinger G, Holtz H, Suss J. In vitro phagocytosis by crevicular phagocytes in various forms of periodontitis. *J Periodontol* 1992;**63:**496–501.
11. Cimasoni G. Crevicular fluid updated. *Monogr Oral Sci* 1983;**12:**1–152.
12. Wilton JM, Renggli HH, Lehner T. The isolation and identification of mononuclear cells from gingival crevice in man. *J Periodont Res* 1976;**11:**262–268.
13. Kennett CN, Cox SW, Eley BM. Investigations into the cellular contribution to host tissue proteases and inhibitors in gingival crevicular fluid. *J Clin Periodontol* 1997;**24:**424–431.
14. Stoufi ED, Taubmann MA, Ebersole JL, Smith DJ, Stashenko PP. Phenotypic analyses of mononuclear cells recovered from healthy and diseased human periodontal tissues. *J Clin Immunol* 1987;**7:**235–245.
15. Okada H, Kida T, Yamagami H. Characterization of the immunocompetent cells in human advanced periodontitis. *J Periodont Res* 1982;**22:**187–189.
16. Taubman MA, Stoufi ED, Ebersole JL, Smith DJ. Phenotypic studies of cells from periodontal disease tissues. *J Periodont Res* 1984;**19:**587–590.
17. Taubman MA, Stoufi ED, Seymour GJ, Smith DJ, Ebersole JL. Immunoregulatory aspects of periodontal disease. *Adv Dent Res* 1988;**2:**228–333.
18. Seymour GJ. Importance of the host response in the periodontium. *J Clin Periodontol* 1991;**18:**421–426.
19. Ishii T, Mahanonda R, Seymor GJ. The establishment of human T cell lines reactive with specific periodontal bacteria. *Oral Microbiol Immunol* 1992;**7:**225–229.
20. Pietruska MD, Zimnoch L, Waszkiel D, Stokowska W, Duraj E. Evaluation of lymphocyte populations and subpopulations extracted from inflamed periodontal tissues. *Rocz Akad Med Bialymst* 2002;**47:**218–225.
21. Suarez LJ, Ocampo AM, Duenas RE, Rodriguez A. Relative proportions of T-cell subpopulations and cytokines that mediate and regulate the adaptive immune response in patients with aggressive periodontitis. *J Periodontol* 2004;**75:**1209–1215.
22. Petit MDA, Hovenkamp E, Hamann D, Roos MTL, van der Velden U, Miedema F, Loos BG. Phenotypical and functional analysis of T cells in periodontitis. *J Periodont Res* 2001;**36:**214–220.
23. Armitage G. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* 1999;**4:**1–6.
24. Fallon HJ, Freie R, Davidson JD, Trier JS, Burk D. Leukocyte preparations from human blood: evaluation of their morphologic and metabolic state. *J Lab Clin Med* 1962;**59:**779–791.
25. Johannessen AC, Nilsen R, Knudsen GE, Kristofersen T. *In situ* characterisation of mononuclear cells in human chronic marginal periodontitis using monoclonal antibodies. *J Periodont Res* 1986;**21:**113–127.
26. Malberg K, Molle A, Streuer D, Gangler P. Determination of lymphocyte populations and subpopulations extracted from chronically inflamed human periodontal tissues. *J Clin Periodontol* 1992;**19:**155–158.
27. Gemmell E, Feldner B, Seymour GJ. CD45RO and CD45RA positive cells in peripheral blood and periodontal lesions before and after stimulation with *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. *Oral Microbiol Immunol* 1992;**7:**84–88.
28. Seymour GJ, Taubman MA, Eastcott JW, Gemmell E, Smith DJ. CD29 expression on CD4+ gingival lymphocytes supports migration of activated memory T lymphocytes to diseased periodontal tissue. *Oral Microbiol Immunol* 1997;**12:**129–134.
29. Itoh H, Ohsawa Y, Yoshie H, Yamazaki K. Oligoclonal accumulations of T-cell clones in gingivitis and periodontitis lesions. *Oral Microbiol Immunol* 2002;**17:**324–329.
30. Lappin DF, Koulouri O, Radvar M, Hodge P, Kinane DF. Relative proportions of mononuclear cell types in periodontal lesions analyzed by immunohistochemistry. *J Clin Periodontol* 1999;**26:**183–189.
31. Slots J. Herpesviruses in periodontal diseases. *Periodontol 2000*, 2005;**38:**33–62.
32. Saygun I, Yapar M, Ozdemir A, Kubar A, Slots J. Human cytomegalovirus and Epstein-Barr virus type 1 in periodontal abscesses. *Oral Microbiol Immunol* 2004;**19:**83–87.
33. Sester M, Sester U, Gartner BC, Girndt M, Meyerhans A, Kohler H. Dominance of virus-specific CD8 T cells in human primary cytomegalovirus infection. *J Am Soc Nephrol* 2002;**13:**2577–2584.
34. Wikby A, Johansson B, Olsson J, Lofgren S, Nilsson BO, Ferguson F. Expansion of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study. *Exp Gerontol* 2002;**37:**445–453.
35. Kinane DF, Johnston FA, Evans CW. Depressed helper-to-suppressor T-cell ratios in early-onset forms of periodontal disease. *J Periodont Res* 1989;**1:**401–405.
36. Mathur A, Michalowicz BS. Cell-mediated immune system regulation in periodontal diseases. *Crit Rev Oral Biol Med* 1997;**8:**76–89.
37. Kamma JJ, Giannopoulou C, Vasdekis VG, Mombelli A. Cytokine profile in gingival crevicular fluid of aggressive periodontitis: influence of smoking and stress. *J Clin Periodontol* 2004;**31:**894–902.
38. Sigusch B, Pfitzner A, Klinger G, Schütze J, Glockmann E. Th2 cytokine pattern – indication on an allergic component in the genesis of periodontitis. *Dtsch Zahnärztl Z* 2003;**58:**304–307. [In German.]
39. Nakajima A, Hirose S, Yagita H, Okumura K. Roles of IL-4 and IL-12 in the development of Lupus in NZB/WF1 Mice1. *J Immunol* 1997;**158:**1466–1472.
40. Amer A, Singh G, Darke C, Dolby AE. Spontaneous lymphocyte proliferation in severe periodontal disease: role of T and B cells. *J Oral Pathol Med* 1990;**19:**49–52.
41. Yamazaki K, Nakajima T, Aoyagi T, Hara K. Immunohistological analysis of memory T lymphocytes and activated B lymphocytes in tissues with periodontal disease. *J Periodont Res* 1993;**28:**324–334.
42. Gemmel E, Seymour GJ. Cytokine profiles of cells extracted from humans with periodontal diseases. *J Dent Res* 1998;**77:**16–26.