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B-1a cells and plasma cells in periodontitis lesions

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Background and Objective: Host response mechanisms in periodontal tissues are complex and involve numerous systems of interactions between cells. The B-cell lineage seems to predominate in chronic periodontitis lesions. The aim of the present investigation was to study the correlation between inflammatory cells and some functional markers in gingival lesions obtained from subjects with severe chronic periodontitis.

Material and Methods: Thirty-eight Caucasian subjects volunteered to take part in the study. A gingival biopsy from one randomly selected diseased proximal site (probing pocket depth > 6 mm and bleeding on probing positive) was obtained from each patient. Immunohistochemical preparation was used to identify inflammatory cells and functional markers. Correlations between the different percentages of cell markers were analyzed by pairwise correlation.

Results: B cells (B-1a and B-2 cells) occurred in larger proportions than T cells and plasma cells. A statistically significant correlation was found between the percentage of B-1a cells and plasma cells and between all B lymphocytes and plasma cells. About 60% of B lymphocytes exhibited autoreactive features.

Conclusion: It is suggested that B-1a cells constitute a significant part of the host response in periodontitis lesions and that plasma cells may develop from both B-2 and B-1a cells.

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In a recent review (1) on aspects of the adaptive host response in periodontitis, a meta-analysis was carried out with regard to the cell composition in periodontitis lesions. It was reported that plasma cells represent about 50% of the cells present in lesions, while B cells comprise about 18%. The proportion of B cells was larger than that of all T cells, and T-helper cells were present in larger numbers than T-cytotoxic cells. Polymorphonuclear leukocytes and macrophages were present at a proportion that represented about 5% of all cells. However, in this meta-analysis some of the studies applied stereological techniques to assess the proportion of cells that were identified using morphological criteria (2–6). Progress in the development of immunohistochemical markers has made it possible to obtain more detailed information of cells in periodontitis lesions and, hence, phenotypic characterization of the cells was performed in parallel to the assessment of additional features of the cells such as receptor and memory functions. Although the techniques used to detect cells varied between studies, overall, cells of the B-cell lineage (B lymphocytes and plasma cells) seem to dominate periodontitis lesions. Data reported from our laboratory and other groups revealed that a subtype of B cells (i.e. B-1a cells) with autoreactive properties is found in high proportions in periodontitis lesions (7–9). Recent reports indicate that B cells have several functions in the host response and are important in the regulation of the immune system through antigen presentation and the production of cytokines (10–12).

CD14 is a well-known receptor with high affinity for lipopolysaccharide, an endotoxin produced by gram-negative bacteria. This receptor is mostly expressed on the surface of the monocyte cell lineage comprising macrophages and dendritic cell precursors. There is evidence that CD14 is present also on B-1a cells (13–15).

Apoptosis, the process of programmed cell death, plays a central role in the immune system in both the maintenance of self-tolerance and the homeostatic control of lymphocyte populations. In lymphocytes, apoptosis is mostly recognized to be induced by two major pathways, one of which is associated with mitochondrial permeability changes that lead to the release of cytochrome *c* and activation of a cysteine protease called caspase-9, which is responsible for the DNA cleavage, nuclear fragmentation and eventually cell death. Bcl2, a member of the Bcl family of proteins, specifically prevents this pathway of apoptosis by blocking the release of cytochrome *c* (16). Recent publications (17,18) reported an association between the Bcl2 proteins and forms of chronic and aggressive periodontitis. The aim of the present investigation was to study the correlation between inflammatory cells and some functional markers (such as CD14 and Bcl2) in gingival lesions obtained from subjects with severe chronic periodontitis.

Material and methods

Thirty-eight Caucasian subjects volunteered to take part in the study. Twenty-eight subjects (15 men and 13 women; mean age 52.2 ± 7.5 years, range 43–69 years) were recruited from the Clinic of Periodontics, Public Dental Services Göteborg. Ten patients (six men and four women; mean age 55.9 ± 7 years, range 43–67 years) were enrolled from the Clinic of Reconstructive Dentistry, University of Basel, Switzerland. The study protocol was approved by the Swedish and Swiss local human review boards in 2004 and, prior to enrollment, all subjects received comprehensive information regarding the purpose and outline of the study and signed an informed consent form.

All subjects had generalized, severe chronic periodontitis, exhibited bone loss of > 50% at all teeth, demonstrated probing pocket depths of > 6 mm and showed evidence of bleeding on probing at > 80% of proximal sites.

Biopsy and immunohistochemical preparation

A gingival biopsy from one randomly selected diseased proximal site (probing pocket depth > 6 mm and bleeding on probing positive) was obtained from each patient. The biopsies were retrieved as reported previously (19,20). In brief, following local anesthesia, two parallel incisions, 4 mm apart, were made through the soft tissue until bone contact was achieved. The two incisions were connected with a perpendicular incision that was placed about 4 mm apical of the gingival margin. The biopsies were carefully removed and prepared for immunohistochemical analysis.

The biopsies were embedded (OCT Compound, Tissue Tek®; Miles, Elkhart, IN, USA), snap frozen in liquid nitrogen and stored at −70°C. From each tissue portion, 5-μm-thick sections were prepared in a cryostat and stained using immunohistochemical techniques. The panel of antibodies used is presented in Table 1. The CD4 and CD8 monoclonal antibodies were used to identify T-helper cells and T-cytotoxic cells, respectively. The CD19 marker detected B cells and the CD138 marker identified plasma cells. Monoclonal antibodies were also used to identify elastase (a neutrophil protease), CD14 (a specific receptor for lipopolysaccharides) and Bcl2 oncoprotein (a blocker of apoptotic cell death). A standard avidin-biotin peroxidase (ABC) method (21) was applied for the staining (Vectastain®, Elite® ABC Kit, Vector®; Vector Laboratories, Inc., Burlingame, CA, USA. The DAB Peroxidase Kit was used as chromogene and the sections were counterstained with Methyl Green (Vector®; Vector Laboratories, Inc.). In each staining procedure performed, negative controls without primary antibodies were included. Sections of human tonsils were used as positive controls.

The identification of autoreactive B cells was performed as previously reported (9). In brief, Texas Red–Avidin and FITC–Avidin (Vector®; Vector Laboratories, Inc., Burlingame, CA, USA) were used in a double-staining method to detect CD5-positive and CD19-positive cells. The sections were dried and mounted using Vectashield® mounting medium (Vector Laboratories) with 4',6-diamidino-2-phenylindole, to preserve fluorescence and to counterstain cell nuclei.

Histological analysis

Histological analysis of the inflammatory cell infiltrate was performed using a Leitz DM-RBE microscope equipped with a Leica Q-500 MC® image system (Leica, Wetzlar, Germany). The size of the infiltrated connective tissue was measured using a mouse cursor (magnification ×50). For assessment of the density of labeled cells, a point counting procedure was applied as previously described (19,20). Thus, a 400-point lattice was superimposed over the tissue area at a magnification of ×400 and the number of cross-points on positive cells was counted. In addition, the number of positive cross-points was related to the total number of points and finally expressed as a percentage of the tissue area.

Table 1. Specificity of monoclonal mouse anti-human antibodies used for immunohistochemical analysis

Antibodies (clone)	Specificity	Dilutions	Isotype
CD5 (D23)[a]	T cells, B-cell subsets	1:50	IgG1
CD19 (HD37)[a]	B cells	1:25	IgG1
CD4 (MT310)[a]	T-helper/inducer cell	1:20	IgG
CD8 (DK25)[a]	T-suppressor/cytotoxic cells	1:50	IgG1
CD138 (B-54)[b]	Plasma cells	1:500	IgG1
Elastase (NP57)[a]	Neutrophils	1:400	IgG1
CD14 (TÜK4)[a]	Lipopolysaccharide receptor	1:20	IgG2a
Bcl2 (124)[a]	Anti-apoptosis protein	1:100	IgG1

[a]DakoCytomation, Glostrup, Denmark.
[b]Serotec, Oxford, UK.

A fluorescence microscope (Leitz DM-RXA; Leica) equipped with a COHU CCD camera that was connected to LEICA Q-FISH software (Leica Microsystems Imagin Solutions, Cambridge, UK) was used to obtain digital images from sections that were double stained for CD5 and CD19. Cells positive for both markers were evident in composite images by the presence of both the red (CD5) and the green (CD19) color (Fig. 1). The number of positive cells for both markers (CD5 + CD19) was related to the overall number of CD19-positive cells and was expressed as a percentage, as previously described (9).

Statistical analysis

Mean values ± standard deviations were calculated for each variable using the subject as the experimental unit. Correlations between the different percentages of cell markers were analyzed by pairwise correlation analyses (Pearson correlation coefficients) ($p < 0.05$).

Results

The results from the histological analyses are reported in Tables 2 and 3. The percentage of infiltrated connective tissue surface area occupied by CD19-positive cells was 8.19%. The corresponding values for the B-1a cells (CD19 + CD5), T-helper (CD4) and T-cytotoxic (CD8) lymphocytes, plasma cells (CD138) and elastase-positive cells were 5.0, 4.04, 2.16, 4.36 and 2.56%, respectively (Table 2). The densities of CD14-positive and Bcl2-positive cells were 3.65% and 2.96%, respectively. B cells (B-1 and B-2 cells) were present in larger proportions than T cells and plasma cells.

Table 2. Results from the immunohistochemical analysis

Variable	Mean percentage	Standard deviation
B-1 cells (CD5 + CD19)	5.00	3.86
CD138	4.36	2.22
CD19	8.19	5.21
CD4	4.04	2.49
CD8	2.16	1.30
Elastase	2.56	1.02
CD14	3.65	2.22
Bcl2	2.96	1.57

The mean percentage and standard deviation is shown for the cells staining positive for various phenotype markers (i.e. CD4, CD8, CD138, CD19, CD5 + CD19, elastase) and functional markers (i.e. CD14, Bcl-2).

The pairwise correlation analysis (Table 3) revealed a statistically significant correlation between the percentages of B-1a cells (CD5 + CD19) and plasma cells and between all B lymphocytes (CD19) and plasma cells (Fig. 2A,B and Fig. 3A,B). A significant correlation was also observed between the densities of T-helper cells and T-cytotoxic cells. The proportion of cells that expressed the functional marker CD14 correlated with the fraction of B lymphocytes, plasma cells, T-helper cells and T-cytotoxic cells. Correlations were also found between Bcl2-positive cells and T-helper and T-cytotoxic cells.

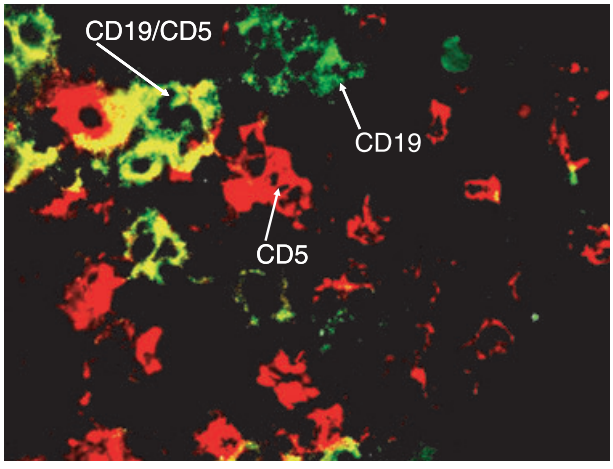

Fig. 1. Fluorescence light micrograph of the infiltrated connective tissue (original magnification ×1000). Composite image following staining with Texas Red–avidin (CD5) (red) and FITC–avidin (CD19) (green). The double-staining of CD19/CD5 illustrated the presence of B-1a cells.

Discussion

In the present study, the proportion of various cell markers was analyzed in gingival biopsies retrieved from subjects with severe chronic periodontitis. It was demonstrated that the proportion of B lymphocytes was larger than the proportions of T lymphocytes, plasma cells and neutrophils. Furthermore, about 60% of the B lymphocytes exhibited both the CD19 and the CD5 markers and therefore they were classified as B-1a cells. The proportion of B-1a cells correlated with that of plasma cells. It is suggested that B-1a cells constitute a significant part of the host response in periodontitis lesions and that plasma cells may develop from both B-2 and B-1a cells.

The finding that B cells and plasma cells dominate among cells in periodontitis lesions is in agreement with data reported previously. Berglundh & Donati (1) performed a meta-analysis of the distribution of leukocytes in periodontitis lesions and concluded that plasma cells outnumbered other cells in the lesions. This observation is, however, not in line with data reported in the present study. We demonstrated that the proportion of B lymphocytes was considerably larger than that of plasma cells. The reason for this difference may be explained by the different techniques used (stereological technique/morphological criteria vs. immunohistochemistry) for the detection of inflammatory cells in the studies referred to and in the current experiment.

Proportion of B-1a cells

Assessment of the number of autoreactive B lymphocytes (B-1a cells) using the double-staining technique

Table 3. Results from the correlation analysis

CD5 + CD19	CD138	CD4	CD8	Elastase	CD14	Bcl2	
0.9197	0.6285	0.7579	0.0650	−01766	0.4784	−0.1755	CD19
< 0.0001 (38)	< 0.0001 (38)	0.0937 (38)	0.6982 (38)	0.2611 (30)	0.0027 (37)	0.2919 (38)	
	0.6985	0.2217	−0.0278	−0.1690	0.3160	−0.2032	CD5 + CD19
	0.0001 (37)	0.1808 (30)	0.8680 (38)	0.3719 (30)	0.0567 (37)	0.2210 (38)	
		0.1521	0.2904	−0.2118	0.5492	−0.0026	CD138
		0.3619 (38)	0.0769 (38)	0.2611 (30)	0.0004 (37)	0.9874 (38)	
			0.5783	0.2593	0.4254	0.3998	CD4
			0.0001 (38)	0.1664 (30)	0.0087 (37)	0.0129 (38)	
				0.2521	0.5332	0.5381	CD8
				0.1789 (30)	0.0007 (37)	0.0005 (38)	
					−0.0969	0.2719	Elastase
					0.6168 (29)	0.1459 (30)	
						0.0561	CD14
						0.7412 (37)	

Values are given as Pearson correlation coefficients, *p*-values and number of observations.

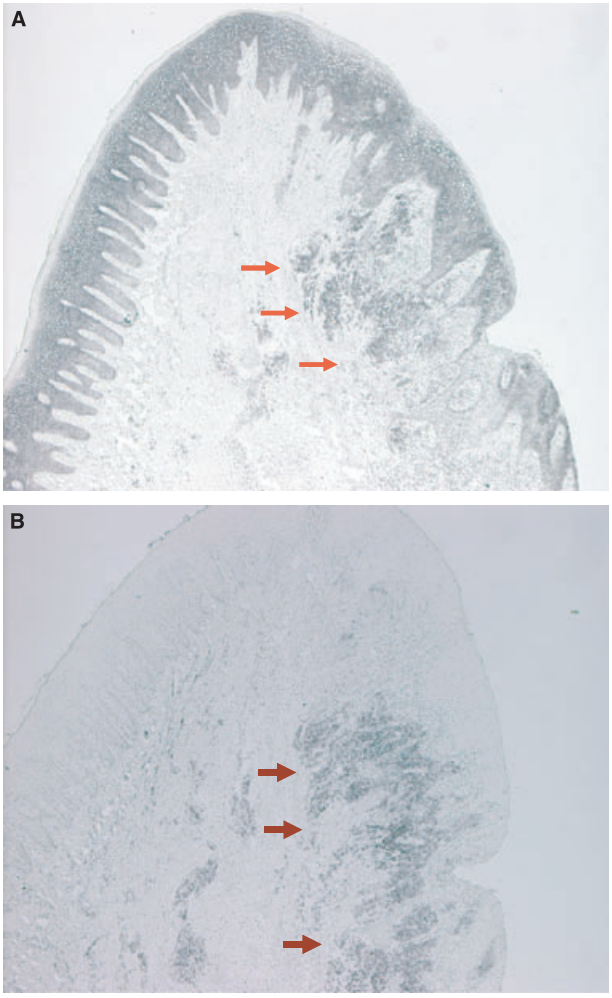

Fig. 2. (A) Biopsy from patient 026. Positive cells stained for CD138 (red arrows) (Magnification ×5). (B) Biopsy from patient 026. Positive cells stained for CD19 (red arrows) (magnification ×5).

(CD19 + CD5), revealed that almost two-thirds of the B lymphocytes (CD19) were also positive for the CD5 marker. The presence of such autoreactive B lymphocytes (B-1a cells) in periodontitis lesions was previously demonstrated. Sugawara *et al*. (7) studied CD5-positive B cells in gingival biopsies and reported that about 37% of the B cells in the lesion expressed the CD5 marker. Aramaki *et al*. (8) analyzed the B-1a cells present in blood samples from 21 periodontitis subjects and six healthy controls, and in gingival biopsies from 10 chronic periodontitis patients. The authors reported that the ratio of autoreactive B cells related to the total number of B cells in the peripheral blood did not differ between periodontitis patients and healthy controls. Analysis of autoreactive B cells in gingival biopsies, however, revealed that 48.9% of the B cells in the lesions exhibited the CD5 marker. Similarly, Berglundh *et al*. (9) examined 21 subjects with severe chronic periodontitis, seven young subjects with localized aggressive periodontitis and 26 periodontally healthy subjects. Blood samples were obtained from each subject, whereas gingival biopsies were obtained from the periodontitis subjects only. About 40–50% of the B cells in the peripheral blood of the periodontitis-susceptible individuals expressed markers of autoreactive features, whereas less than 15% of the

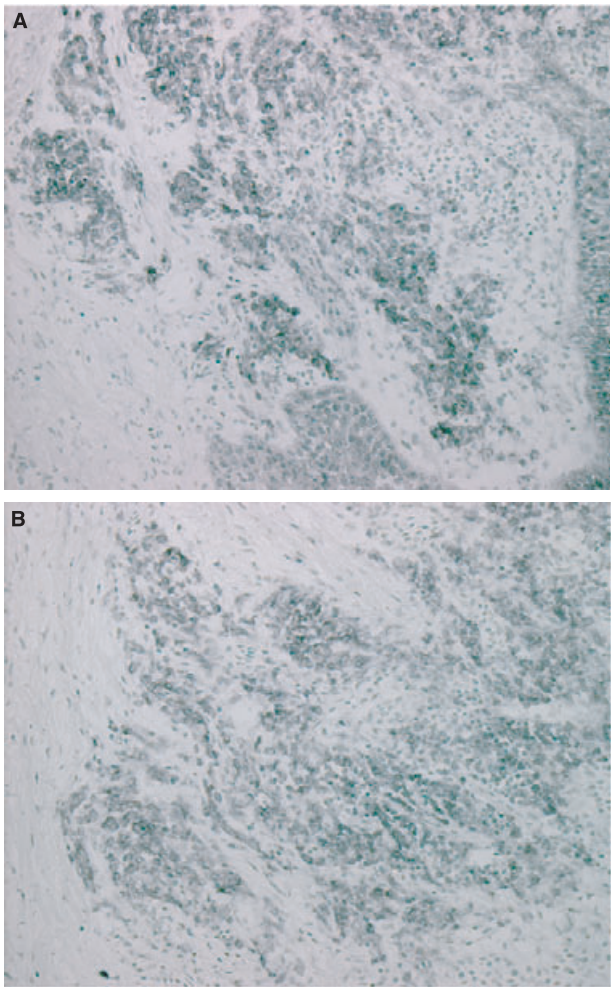

Fig. 3. (A) Biopsy from patient 026. Positive cells stained for CD138 (magnification ×20). (B) Biopsy from patient 026. Positive cells stained for CD19 (magnification ×20).

circulating B cells in the healthy subjects exhibited such markers. Berglundh *et al.* (9) reported that about 30% of the B cells found in the periodontitis lesions were B-1a cells. The difference between the percentages of B-1a cells in periodontitis lesions reported in previous studies (7–9) and that reported in the current study is, at present, not understood.

Correlations

One of the main objectives of the current study was to evaluate correlations between different cell proportions in the periodontitis lesion. Significant correlations were found between B-1a cells and plasma cells, and between B lymphocytes and plasma cells. This observation may thus indicate that plasma cells in periodontitis may develop from both B-1a and B-2 cells. Antibodies produced by plasma cells may, consequently, also be autoantibodies, as previously demonstrated (22–24). A significant correlation was also found between CD14-positive cells and B cells/plasma cells. There is convincing evidence that B cells serve as antigen-presenting cells in periodontitis (11,25) and in other diseases (26–28). Mahanonda *et al.* (25), in a study on periodontitis, observed a significant up-regulation of CD86 and of the dendritic cell marker, CD83, on B cells in periodontitis lesions. Gemmell *et al.* (11) also reported that B cells served as antigen-presenting cells in periodontitis. Gemmell *et al.* (11) analyzed biopsies obtained from 25 subjects with periodontitis and reported that B cells were the predominant type of antigen-presenting cells. The findings in the present study regarding the correlation between CD14-positive cells and B cells may further advocate a role for B lymphocytes as antigen-presenting cells.

The results of the present investigation also highlighted a significant correlation between CD14 and cells of the T-cell lineage (T-helper/T-cytotoxic cells). To our knowledge, there are no previous reports on such a correlation. Future studies using a double-staining technique with markers for CD14 and B cells or T cells may provide further information in this regard.

Moreover, the present study demonstrated a significant correlation between Bcl2 and T lymphocytes of both helper and cytotoxic types. Gamonal *et al.* (17) studied the apoptotic markers in gingival tissue from subjects with chronic periodontitis and in gingival tissue from healthy controls. The expression of caspase-3, Fas, FasL, Bcl2 and p53 was determined using immunohistochemistry analyses. The results demonstrated that only Bcl2-positive cells were present at significantly higher numbers in biopsies from periodontitis patients than in control tissues. This finding was confirmed in a recent study by Bulut *et al.* (18), who analyzed clinical features and apoptosis proteins (e.g. p53, Bcl2 and caspase-3) in gingival tissues retrieved from eight subjects with generalized aggressive periodontitis and from 10 controls. The immunohistochemical analysis showed that while the expression of caspase-3 and p53 did not differ between diseased and healthy subjects, the number of Bcl2-positive cells was significantly higher in lesions from subjects with generalized aggressive periodontitis than in controls. The fact that B cells, together with plasma cells, dominate among leukocytes in periodontitis lesions, and that these cells appear to be long-lasting, indicate a down-regulated apoptosis. In the present study, the lack of a correlation between these cell groups and Bcl2-positive cells may point to other regulatory mechanisms of apoptosis.

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