The gingival plasma cell infiltrate in renal transplant patients on an immunosuppressive regimen

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Treatment with immunosuppressive agents inhibits gingival inflammation and progression of periodontitis in humans. We examined the numbers and the isotype distribution of immunoglobulin-producing plasma cells by immunohistochemistry in gingival specimens taken from renal transplant recipients receiving immunosuppressive agents (IS), and from otherwise comparable systemically healthy patients. The immunosuppressed patient group had significantly ($P < 0.05$) fewer IgG-, IgA-, IgG1-, IgG2-, and IgG4-producing plasma cells in the connective tissue adjacent to the pocket epithelium. The reduced numbers of such patients with quiescent periodontal disease support the contention that high counts of plasma cells are indicative of more severe disease. □ *Cyclosporin A; immunosuppression; inflammation; periodontitis; steroids*

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Schuller et al. (1) were the first to show that the immunosuppressive agents prednisone and azathioprine inhibit clinical signs of gingivitis in humans. Later, Tollefsen & Johansen (2) showed in a long-term study that renal allograft recipients, maintained on such a drug regimen, developed significantly less alveolar bone loss than a matched systemically healthy group.

Chronic marginal periodontitis (CMP) is triggered by bacteria in dental plaque. Disease progression is episodic, and longer periods of quiescence probably alternate with shorter periods of aggressive disease (3). The extent, density, and composition of the cellular infiltrate have been taken as measures of the severity of periodontal diseases (4–11). Numbers of plasma cells and activated lymphocytes predominate in the advanced or aggressive stages of periodontitis, while gingivitis lesions contain low counts of B cells and activated B cells (10, 12–15). Patients under immunosuppressive (IS) medication with a history of periodontitis that is burnt out, but with a potentially pathogenic bacterial load, may constitute an excellent reference when elucidating the mechanisms underlying the disease. To complement the approaches whereby patients with aggressive disease were examined (7–15), we here want to assess the relevance of numbers of gingival isotype-specific plasma cells as an indicator of periodontal disease activity in a group of IS patients with established, but quiescent, disease. If plasma cell numbers have any indicative value, low counts might be anticipated in this group when compared with systemically healthy persons with chronic marginal periodontitis.

Materials and methods

Selection of subjects

The test group consisted of 10 renal transplant outpatients with chronic marginal periodontitis. The patients had been under treatment for their renal diseases for 1 to 6 years at the Lillehammer Regional Hospital, Norway, and received individualized doses of prednisolone (7.5–10 mg per day), combined with azathioprine (50–125 mg per day) and cyclosporin A (CyA, 100–400 mg per day). Two women and eight men participated (mean age, 46 years; range, 29–62 years); none had received professional periodontal treatment. The reference group comprised 10 systemically healthy subjects with periodontitis (6 women and 4 men; mean age, 43 years; range, 20–61 years) from a private dental practice. None of the subjects in this group had received professional periodontal treatment or taken any drugs for at least 6 months. The study followed the guidelines given in the Declaration of Helsinki.

Clinical procedures

The extent of gingival inflammation and dental plaque deposits were graded on all teeth (16). Probing pocket depth was scored adjacent to all surfaces, and full-mouth intraoral radiographs were taken; on these, alveolar bone loss was assessed as the distance in millimeters from the interproximal cementoenamel junction to the most coronal position of the alveolar bone. The mean values

Table 1. Periodontal characteristics of the patients in the study. Geometric mean values and standard deviations (*s*)

	Immunosuppressed patients		Systemically healthy persons	
	Geometric mean	*s*	Geometric mean	*s*
Tooth surfaces with plaque index > 1 (%)	51.0	18.06–83.47	49.6	35.48–63.80
Bleeding gingival units ≥ 2 (%)	11.5*	0.04–22.20	28.8*	14.15–46.12
Pocket depth (mm)	2.5*	2.06–3.15	3.7*	2.90–4.67
Alveolar bone loss (mm)	3.5	2.64–4.75	4.3	3.28–5.72

* Significant difference between groups (*t* test, $P < 0.025$).

and standard deviations of the periodontal measurements are listed in Table 1. The corresponding scores of the biopsy sites are given in Table 2.

Immunosuppressed patients have generally shallow periodontal pockets (Table 1), and for this study the deepest pockets available were sought. A biopsy was performed, including the entire soft tissue wall of the pocket to be examined. Seven specimens came from the palatinal aspect of maxillary first molars; three were taken buccaly to lower first molars. Specimens from the systemically healthy group were selected on the basis of similarity of site, plaque load (not different by more than a score of 1), pocket depth, and loss of support (not different by more than 1 mm) (Table 2).

Immunohistochemistry

Specimens were first washed under gentle agitation in ice-cold phosphate-buffered saline (PBS), pH 7.4, for 48 h at 4°C, then fixed in 70% ethanol, dehydrated, embedded in paraffin wax, and processed by routine histologic procedures. Longitudinal sections, 4 mm thick, were cut at right angles to the tooth surface. The first sections were stained with hematoxylin-eosin, and the rest were processed for immunohistochemical staining, as follows:

1) Fluorescein isothiocyanate (FITC)-labeled polyclonal rabbit anti-human IgG (DAKO, Glostrup, Denmark) was diluted 1:100, and tetramethylrhodamine isothiocyanate (TRITC)-labeled polyclonal rabbit anti-human IgA (DAKO) was diluted 1:125 in PBS before overlaying the sections. The slides were incubated in a dark, humidified chamber overnight at 4°C. Finally, the slides were washed for 10 min in PBS and mounted in ImmunoMount mounting medium (Shandon, Pittsburgh, Pa., USA).

2) Monoclonal mouse anti-human IgM and IgD immunoglobulins (DAKO) were diluted 1:20 in PBS and applied on the sections for 8 h. After washing for 10 min in PBS, the slides were reincubated for 12 h at 4°C with biotinylated horse anti-mouse immunoglobulins (Vector Laboratories, Burlingame, Calif., USA) at a 1:100 dilution, washed, and then incubated for 30 min at ambient temperature with TRITC-conjugated avidin D. After final washing and drying, the slides were mounted with ImmunoMount.

3) Sections with monoclonal anti-human IgG subclass immunoglobulins (Zymed Laboratories Inc., San Francisco, Calif., USA) were incubated overnight at 4°C. After

Table 2. Periodontal conditions at the biopsy sites. Proportions or geometric mean values and standard deviations (*s*)

	Immunosuppressed patients		Systemically healthy persons	
Tooth surfaces with plaque index > 1	10 of 10*		5 of 10*	
Bleeding gingival units ≥ 2	2 of 10*		8 of 10*	
	Geometric mean	*s*	Geometric mean	*s*
Pocket depth (mm)	4.2	3.6–4.7	4.6	3.8–5.6
Alveolar bone loss (mm)	4.1	3.7–5.5	4.5	3.3–4.8

* Significant difference between groups (chi-square test, $P < 0.025$).

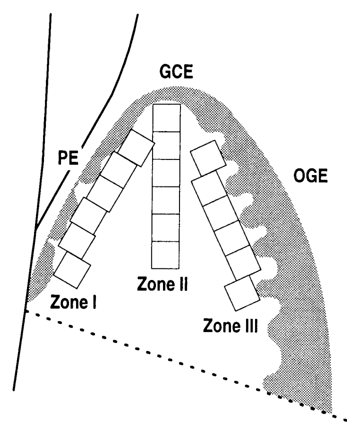

Fig. 1. The three zones into which plasma cells were counted, and placement of the ocular counting grid. The broken line indicates the horizontal incision made to remove the specimen. PE: pocket epithelium. GCE: gingival crevicular epithelium. OGE: oral gingival epithelium.

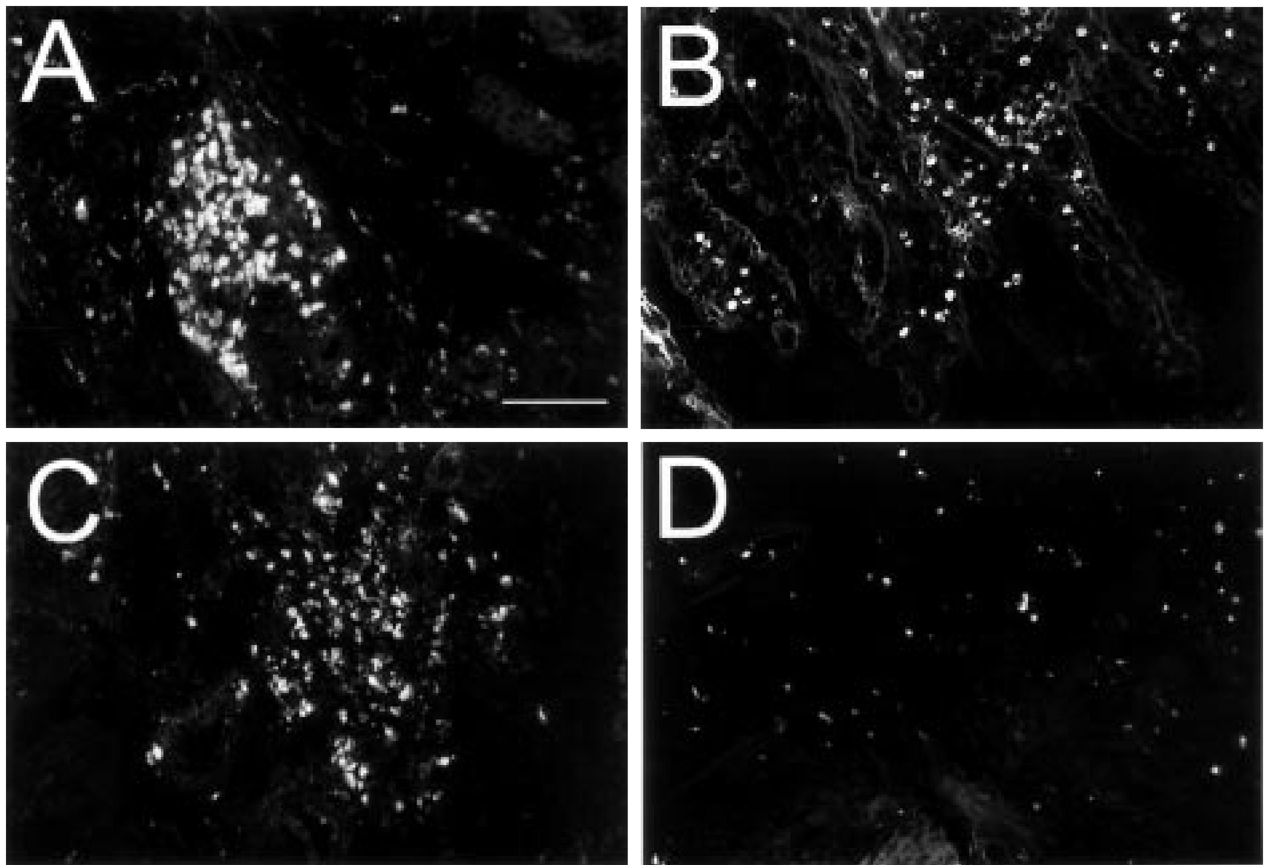

Fig. 2. Plasma cells, labeled by immunohistochemistry, producing IgG (A, C) or IgA (B, D) in the connective tissue below the junctional epithelium in periodontal lesions from systemically healthy persons (A, B) and from patients under immunosuppressive medication (C, D). The bar indicates 100 μm.

washing for 10 min in PBS, the slides were incubated for 12 h at 4°C with a second layer of biotinylated horse anti-mouse IgG (Vector Laboratories) at a dilution of 1:100. The sections were then washed, incubated with FITC-avidin for 1 h, and finally mounted with ImmunoMount.

Negative control sections were prepared by substituting normal rabbit or horse sera for the specific antisera used.

Fluorescence microscopy

Labeled cells were counted at 200 × final magnification by aid of an ocular grid, in a randomly chosen section from each specimen, within 3 connective tissue zones (Fig. 1): zone I, along the pocket epithelium; zone II, within the lamina propria; and zone III, beneath the oral epithelium. The grid was placed coronally in the connective tissue and in contact with the lower layers of the epithelium (Fig. 1), and all fluorescence-positive plasma cells within the field indicated by the grid were counted. For zone II the grid was then moved apically and the adjoining field counted. For zones I and III the grid was moved apically while maintaining contact with the pocket and gingival epithelium, respectively. The mean value from all measurable consecutive fields was computed and ascribed to that zone. Cell counts were carried out in a single-blind fashion.

Statistical methods

To obtain equality of variances, the clinical data were logarithmically transformed and compared by using *t* tests. Cell counts were compared by means of Kruskall–Wallis and Mann–Whitney tests. Frequencies were compared by using chi-square tests.

To evaluate the reproducibility of the cell counts, countings were repeated on sample sections before the start of the study. Additionally, all plasma cells in sections from two patients were counted three times, at intervals of about 2 months. The counts were evaluated by analysis of variance for paired comparisons. None of the variance ratios computed reached statistical significance ($F < 0.83$).

Results

All sections showed features of chronic inflammation. Fields with plasma cells were often collagen-poor, although

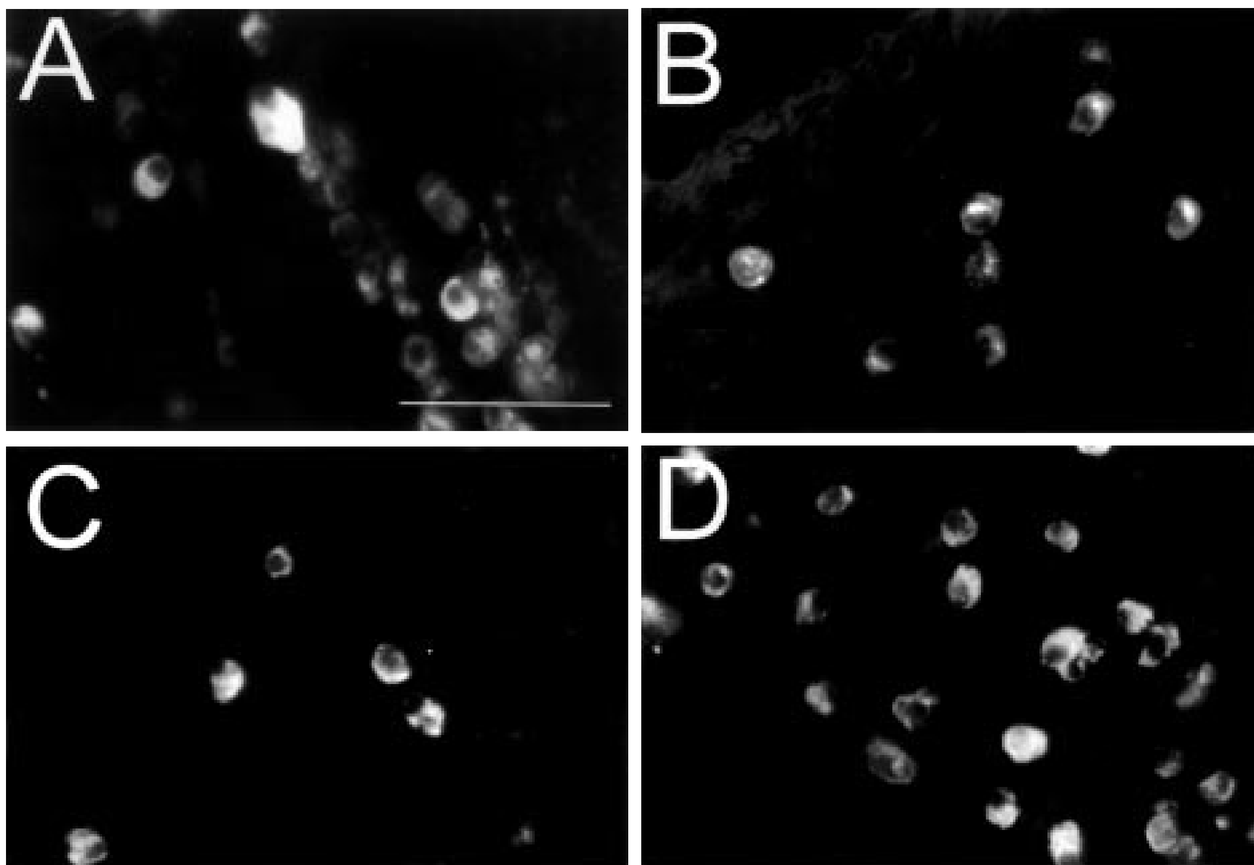

Fig. 3. Immunohistochemical labeling of IgG subclass–producing plasma cells. A: IgG1. B: IgG2. C: IgG3. D: IgG4. Cells in the connective tissue below the junctional epithelium in periodontal lesions are shown. The bar indicates 50 μm.

well-defined collagen bundles could surround such areas. While most of the plasma cells showed a densely staining cytoplasm, some had features of degeneration with nuclear pyknosis and cellular fragmentation. Examples of representative stainings are shown in Fig. 2.

The specimens from the systemically healthy group showed many IgG-producing plasma cells, often occurring in large and dense clusters. In contrast, discrete clusters of IgG-producing plasma cells were seen beneath the pocket epithelium and in the lamina propria in specimens taken from the immunosuppressed (IS) patients. Representative sections stained for the four IgG subclasses are shown in Fig. 3A–D. Significant differences in the numbers of IgG-producing plasma cells between the groups were found in zone I (Fig. 4A, Mann–Whitney test, $P < 0.05$). The data from the IgG subclass counts (Fig. 4B) ranked IgG1 > IgG2 > IgG4 > IgG3 in both groups. The IS group had significantly lower numbers of IgG2-producing cells in all three zones as compared with the systemically healthy group and lower IgG1- and IgG4-producing cells in zone I (Kruskall–Wallis and Mann–Whitney tests, $P < 0.05$).

Clusters of IgA-producing cells were seen in specimens from the systemically healthy group, mostly situated beneath the dentogingival epithelium, but solitary cells were found throughout the entire section (Fig. 2). In specimens from the immunosuppressed group, IgA-producing cells mostly occurred singly, but small clusters were sometimes seen beneath the dentogingival epithelium. Significant differences between the groups were found in zone I (Fig. 4A, Mann–Whitney test, $P < 0.05$).

IgM-producing plasma cells were generally few in number and occurred as solitary cells in most specimens. Very few IgD-producing plasma cells were found in specimens from the immunosuppressed patients, whereas small aggregates occasionally were seen in the specimens from the systemically healthy group. No significant differences between the two groups were found for IgM- and IgD-producing plasma cells (Fig. 4A).

The percentage distributions among the IgG, IgA, and IgM median values in zone I were 74%, 17%, and 9% for the systemically healthy group and 75%, 20%, and 5% for the transplant recipients. These match the values observed by others (17). For IgG1, IgG2, IgG3, and IgG4, the respective values were 38%, 31%, 6%, 25% and 38%, 23%, 13%, 26%. The distributions showed no statistically significant differences between groups (chi-square test).

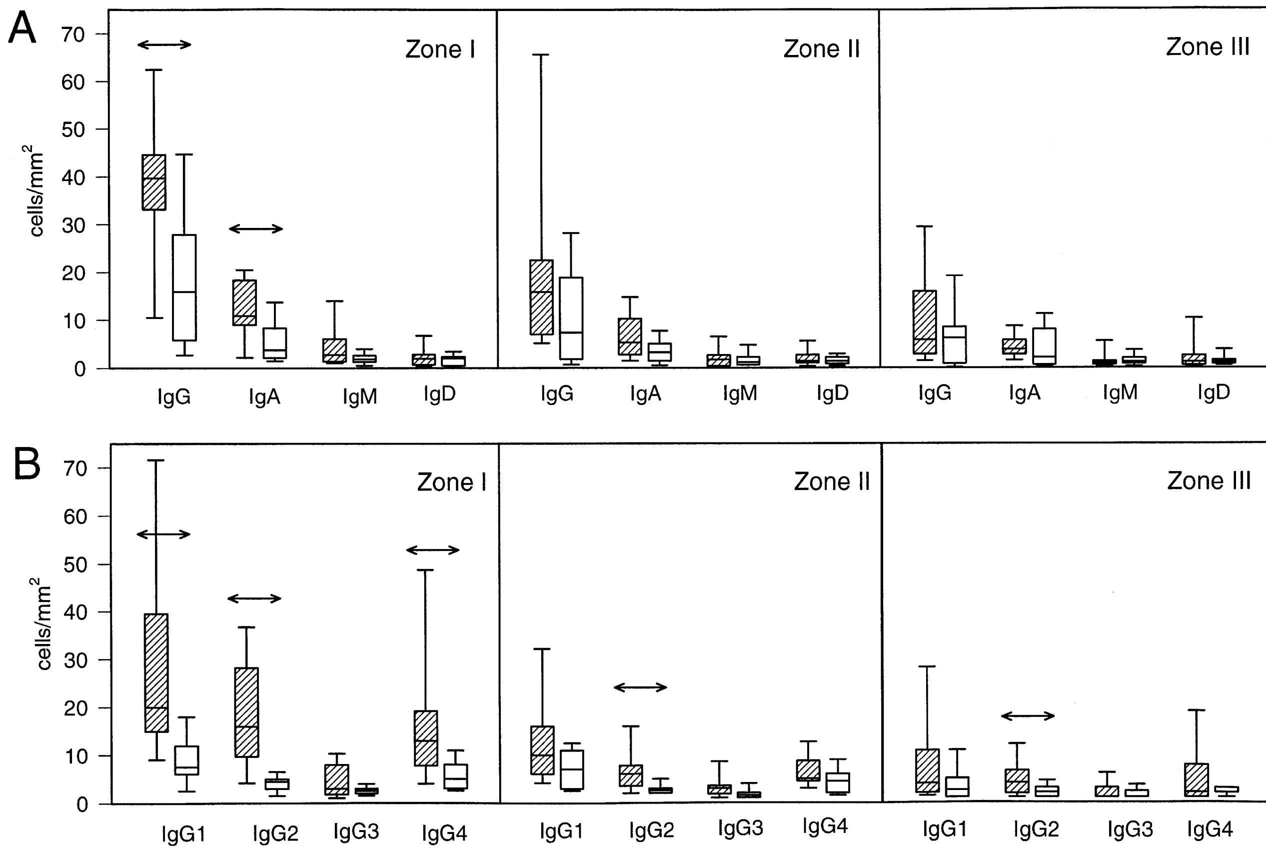

Fig. 4. Distribution of immunoglobulin-producing plasma cell counts from systemically healthy persons (hatched bars) and from patients under immunosuppressive medication (open bars) in three gingival connective tissue zones (see Fig. 1). Median (horizontal bar), 75% confidence interval (box), 95% confidence interval (vertical bars), and statistically significant differences between the groups (Mann–Whitney tests, $P < 0.05$, double arrows) are indicated. A: total IgG-, IgA-, IgM-, or IgD-producing cells. B: IgG subclass–producing cells.

Discussion

Established gingivitis lesions in humans contain few activated B cells and plasma cells, while increased influx of B blasts, followed by their differentiation to plasma cells, is associated with signs of periodontal breakdown (7, 10, 12, 15, 18). Lesions that recently experienced attachment loss show the highest numbers of B cells and plasma cells (9, 11, 13, 14). The predominant isotype produced by the local plasma cells is IgG, followed by IgA (8, 17, 19–21), and this was also found in the systemically healthy group in this study. Medication with azathioprine and prednisone has been shown to reduce gingival inflammation distinctly without adversely affecting periodontal attachment (22, 23). The present results extend the observations by Tollefsen et al. (24), who found significantly fewer methyl-green pyronin staining plasma cells near the advancing front of periodontal lesions in specimens taken from transplant recipients than in corresponding samples from healthy persons: compared with their healthy counterparts, our group of IS patients had significantly ($P < 0.05$) fewer IgG- and IgA-producing gingival plasma cells in the connective tissue zone below the pocket epithelium (Fig. 4A). The present results corroborate the contention that numbers of plasma cells are indicative of periodontal disease activity (8–10, 13, 14).

Our results do not indicate that the ratio IgG:IgA is of importance for the severity of the periodontal lesions, as suggested by Kilian et al. (21), because this ratio was similar in both of our patient groups. In distinct contrast with the present findings and our earlier independent observations (24), transplant patients treated with CyA and prednisone (25), as well as dogs treated with CyA only (26), were shown to have markedly increased total numbers of plasma cells in their gingiva as compared with untreated subjects. The latter studies did not assess cell numbers in zones (Fig. 1) or by immunoglobulin isotypes produced, but differences in medication (our patients also received azathioprine) and time period elapsed after start of the medication (long in our patients) remain the most likely explanations for this variance.

Local production of IgG4 antibodies may increase significantly under active periodontitis (27). The authors of that article suggested that levels of IgG4 antibodies above normal may provide a marker of active periodontitis. Increased numbers of IgG4- (and IgA2-) secreting cells are

also found in advanced stages of periodontitis (8). We therefore compared the IgG subclass plasma cell distribution in our two groups (Fig. 4B). Numbers of IgG4-producing plasma cells in zone I were significantly lowered in the IS group, confirming that high counts of those cells indicate severe disease.

The drug regimen of the patients in this study comprised prednisone, azathioprine, and cyclosporin A. Steroids suppress monocyte/macrophage functions (secretion of TNFα, IFNγ, IL-1), lower the expression of adhesion molecules, and depress the generation of cytotoxic effector cells (28). Azathioprine has little effect on ongoing immune responses, but affects monocyte and lymphocyte production through its antimitotic activities (29). CyA preferentially inhibits clonal expansion and cytokine secretion of T helper cells (mainly Th2 type), which are necessary for activation, maturation, and differentiation of B cells to plasma cells (30). CyA also inhibits expression of the CD40 ligand on activated T cells, meaning that transplant patients receiving this drug might be deficient in this aspect of T-cell signaling to B cells (31). As the combination of drugs may operate at several points, the reduced numbers of plasma cells in the IS patients could be due to 1) a reduced reservoir of B lymphocytes, 2) a reduced recruitment of B blasts from gingival blood vessels, or 3) impaired terminal differentiation into plasma cells.

In conclusion, in slowly progressing periodontal disease, the infiltrate in the human connective tissue adjacent to the pocket epithelium is characterized by markedly reduced numbers of plasma cells producing IgG, IgA, IgG1, IgG2, and IgG4. This lends support to the contention that high numbers of such cells are indicative of more severe and aggressive periodontal disease forms (8–10, 13, 14).

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