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Immunohistological analysis of T cell functional subsets in chronic inflammatory periodontal disease

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SUMMARY

IL-2, interferon-gamma (IFN-γ), IL-4 and IL-6 producing T cells in periodontitis and gingivitis-affected human tissues were investigated by immunohistochemistry to clarify the relationship between T cell functional subsets and disease entity. Using alkaline-phosphatase anti-alkaline-phosphatase technique, the relative proportions of each cytokine-producing T cell were calculated in the crevicular 1/3, middle 1/3 and oral 1/3 areas selected in the connective tissue of sections. CD19:CD3 and CD4:CD8 ratios were determined on the serial sections. Compared with gingivitis tissues, the proportion of cytokine-producing cells in periodontitis-affected samples was higher overall in the crevicular 1/3 ($P < 0.02$). The middle 1/3 exhibited a higher percentage of cytokine-producing cells, except for IL-6-producing cells. Frequencies of cytokine-producing cells in the oral 1/3 did not differ. IL-4 was the prominent cytokine in periodontitis-affected tissues, with the highest proportion detected in the crevicular 1/3. The CD19:CD3 ratio was higher in periodontitis tissues irrespective of the location, indicating a B cell dominance in periodontitis lesions. Furthermore, a significant positive correlation between the proportion of IL-4-producing cells and the CD19:CD3 ratio was noted. The CD4:CD8 ratio consistently exceeded 2·0 in both periodontitis and gingivitis. These results suggest that immunoregulation of both periodontitis and gingivitis are T cell-dependent, but in periodontitis type 2 helper T cells predominate and thereby control B cell activation.

Keywords Th1 Th2 periodontal disease immunohistology

INTRODUCTION

T cells play pivotal roles in immune responses, whereby they regulate polyclonal B cell activation as well as antigen-specific antibody formation [1]. It has been suggested that T cells dominate the gingivitis lesion, with an increase in the number of B cells and plasma cells in the periodontitis lesion [2], albeit under T cell control. Conversion of a stable T cell lesion to a progressive B cell lesion has been hypothesized as being associated with the pathogenesis of chronic inflammatory periodontal disease [3]. Studies have focused on whether the conversion from gingivitis to periodontitis is associated or not with distinct phenotypes of T cell infiltrate. Previous phenotypic analysis of T cells revealed reduced [4,5] or unchanged [6] CD4:CD8 ratios in the periodontitis tissues compared with those in peripheral blood and gingivitis tissue, while the proportion of memory T cells was dominant both in gingivitis and periodontitis, suggesting that gingivitis and periodontitis can barely be discriminated by the phenotype of T cell infiltrate [7,8].

Recently, functional subsets of T cells have been proposed according to their cytokine synthesis profile in mice. Th1 clones secrete IL-2, interferon-gamma (IFN-γ) and tumour necrosis factor-beta (TNF-β), whereas Th2 clones secrete IL-4, IL-5, IL-6 and IL-10. Th1 and Th2 clones also harbour distinct functions, in that Th1 but not Th2 clones participate in DTH and cytotoxicity response, whereas Th2 clones provide more efficient help for B cells to differentiate into immunoglobulin-producing cells [9]. Furthermore, it has been demonstrated that different antigens derived from a given organism induce distinct functional subsets in different strains of mice, resulting in a distinct clinical entity [10]. Similar classification of functional subsets of T cells is now applicable for humans [11], and has implications in infectious and allergic diseases [12].

The purpose of the present study was to determine, by means of immunohistological procedure, the predominant functional subsets of T cells in periodontitis and gingivitis, providing possible involvement of T cell functional subsets in the clinical entity of the disease.

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Table 1. Clinical profiles of biopsy sites

Clinical parameters	Periodontitis ($n = 19$)	Gingivitis ($n = 7$)
Gingival index	1·4 ± 0	0·7 ± 0·8
Probing depth (mm)	6·2 ± 1·6	2·9 ± 0·7
Loss of attachment (mm)	7·1 ± 1·7	3·0 ± 0
Bleeding on probing (% positive sites)	94·7	0
Bone loss	51·8 ± 21·5	3·8 ± 2·9

Clinical assessments were made after initial therapy for the periodontitis group and before treatment for the gingivitis group. Each value represents the respective mean ± s.d. scores of clinical parameters measured on each sampling site per patient. Bone loss value represents mean ± s.d. of the scores measured at the mesial and distal approximal sites of each sampling site per patient.

PATIENTS AND METHODS

Patients and gingival biopsy sampling

Nineteen patients with moderate to advanced adult periodontitis (AP) referred to Niigata University Dental Hospital took part in this study (age 39–59 years, mean 49·2 years). Gingival biopsies were obtained at the time of periodontal surgery after completion of initial therapy, which included motivation, oral hygiene instruction, scaling and root planing or extraction of severely involved teeth. As control, seven gingivitis tissues showing no supporting tissue destruction were obtained from premolar sites requiring extraction for orthodontic treatment, or from third molar sites at the time of extraction due to pericoronitis. Clinical assessments of the sampling sites are shown in Table 1. Informed consent was obtained from all patients, in conformity with the Helsinki Declaration, before inclusion in the study.

The biopsies were taken as described before [8]. The tissue was immediately embedded in OCT compound (Miles Labs, Elkhart, IN), quenched and stored in liquid nitrogen until use.

Serial cryostat sections (6 μm thick) were cut from the central part of each specimen in a plane parallel to the long axis of the teeth, and oriented so that the pocket epithelium, oral epithelium and connective tissues were exposed over the same section. For immunohistological study, the sections were air-dried and fixed in equal parts of chloroform/acetone for 5 min. Sections were then individually wrapped in plastic film and stored at −20°C.

Immunohistochemistry

Monoclonal anti-IL-2, anti-IFN-γ, anti-IL-4 and anti-IL-6 were obtained from Genzyme (Boston, MA). Anti-CD3, anti-CD4, anti-CD8 and anti-CD19 were purchased from Dako (Glostrup, Denmark). An alkaline-phosphatase anti-alkaline-phosphatase (APAAP) method was used to detect intracytoplasmic cytokines. Double staining was carried out using an avidin-biotin immunoperoxidase (ABC-PO; ABC kit; Vector Labs, Burlingame, CA) technique together with an APAAP method to determine the predominant cell types (e.g. CD4 *versus* CD8, CD3 *versus* CD19).

Sections were incubated with first primary MoAb at a predetermined dilution followed by rabbit anti-mouse IgG (Dako) and finally with monoclonal mouse APAAP (Dako). Colour was developed with Substrate III (Vector). For double staining, the sections were first incubated with biotinylated goat anti-mouse IgG (Vector) and finally with ABC-PO after application of the first primary antibodies. After colour development using 0·005% 3,3′-diaminobenzidine in Tris–HCl buffer pH 7·2 containing 0·01% hydrogen peroxide, the specimens were incubated with normal rabbit serum (Vector) and reacted with the second primary MoAb to the sections. Subsequent procedures followed the single staining steps using APAAP. Each incubation was of 30 min at room temperature and followed by washing for 10 min in Tris-buffered saline (TBS; pH 7·2). Nuclei were counter-stained with Lillie's haematoxylin.

Endogenous peroxidase and alkaline phosphatase activities were blocked whenever necessary by 0·17% NaN_3 and 1 mM levamisole, respectively. To confirm the specificity of the procedure, unrelated MoAbs and TBS were substituted for primary MoAbs.

Cell analysis

Cell analysis of selected areas was performed as previously described [8]. Briefly, relocatable areas of significant round cell infiltrate in the connective tissue comprising the crevicular 1/3, middle 1/3 and oral 1/3 of each section were selected using histologic landmarks on haematoxylin and eosin-stained slides (Fig. 1). Positive cell counts were performed for these selected foci with an ocular grid (0·04 mm^2) at a magnification of ×400. The six serial sections from each specimen were processed using the above methodology and in the following combinations: (i) anti-IL-2; (ii) anti-IFN-γ; (iii) anti-IL-4; (iv) anti-IL-6; (v) anti-CD3 anti-CD19; (vi) anti-CD4 anti-CD8. Since the distribution of IL-2^+, IFN-γ^+ and IL-4^+ cells coincided with that of CD3^+ cells, each cytokine-positive fraction in the total CD3^+ cell of the sections was calculated by dividing the number of cytokine-positive cells by the number of CD3^+ cells. For IL-6, positive cells localized as coinciding with CD3^+ cells were counted, then divided by the total number of CD3^+ cells to calculate the

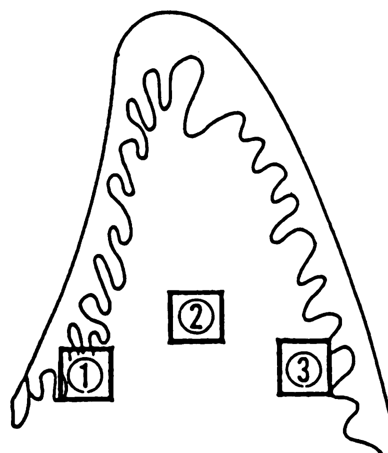

Fig. 1. Diagram of analytical procedure of infiltrating cells. Connective tissue was divided into three portions: crevicular 1/3 (1), middle 1/3 (2) and oral 1/3 (3).

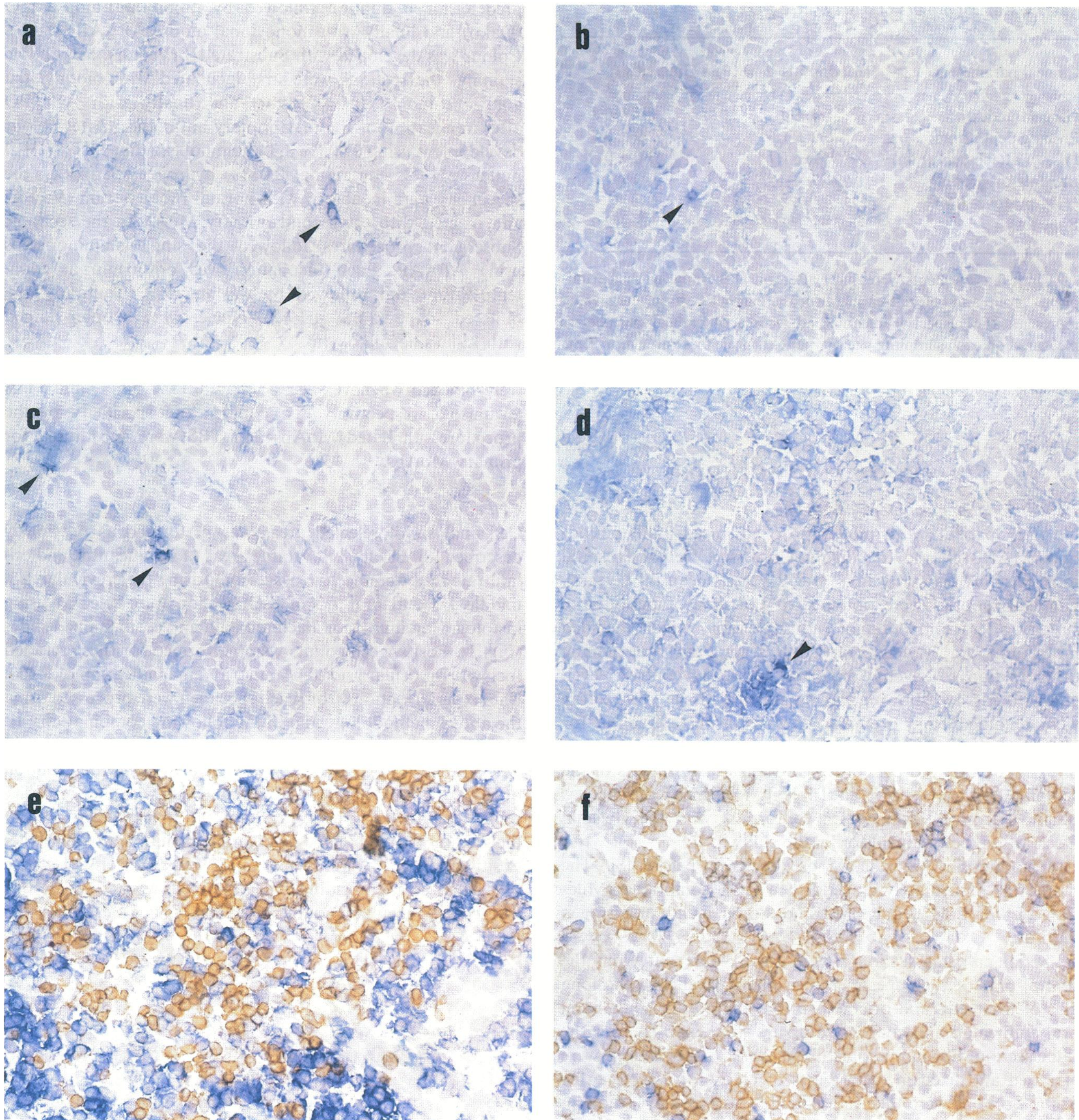

Fig. 2. Immunoreactivity for cytokines and phenotypic analysis in the periodontitis lesion. Single staining for IL-2 (a), IFN-γ (b), IL-4 (c) and IL-6 (d), and double staining for CD3/CD19 (e) and CD4/CD8 (f) were performed on the sequential sections. Cytokine immunoreactivities (arrowhead) were visualized using alkaline-phosphatase anti-alkaline-phosphatase (APAAP) and Substrate kit III (Vector). For double staining, CD3 and CD4 were immunolabelled with peroxidase, whereas CD19 and CD8 were immunolabelled with alkaline phosphatase.

fraction of IL-6 producing $CD3^+$ cells. The CD19:CD3 ratio and the CD4:CD8 ratio were also calculated.

Statistical analysis

The significance of difference between periodontitis and gingivitis was tested using the Mann–Whitney *U*-test. The difference of the proportion of each cytokine-positive cell within the same disease category was analysed using paired *t*-test. The relationships between the percentages of each cytokine-positive cell and the CD19:CD3 or CD4:CD8 ratios in each location were analysed using Spearman rank correlation. The null-hypothesis was rejected at $P < 0{\cdot}05$.

RESULTS

Immunohistological findings

IL-2. IL-2 was observed either in the cytoplasm or on the membrane rim (Fig. 2a). The number of cases exhibiting IL-2 immunoreactivity in periodontitis amounted to 16, 13 and 5 of 19 for the crevicular 1/3, middle 1/3 and oral 1/3, respectively. However, those in gingivitis were 3, 1 and 0 for the crevicular 1/3, middle 1/3 and oral 1/3, respectively (Fig. 3). The mean percentage of IL-2$^+$ cells was significantly higher in the crevicular 1/3 and middle 1/3 of periodontitis tissues than in those of gingivitis tissues ($P < 0{\cdot}02$ and $P < 0{\cdot}05$, respectively; Table 2). Furthermore, analysis with respect to the area revealed that the number of IL-2$^+$ cells was higher in the crevicular 1/3 than in the oral 1/3 ($P < 0{\cdot}05$), with the middle 1/3 ranking in between.

IFN-γ. The staining pattern of IFN-γ was similar to that of IL-2, albeit to a lesser extent (Fig. 2b). Frequency of cases presenting IFN-γ$^+$ cells found in periodontitis and gingivitis was distributed as follows: 11/19 *versus* 1/7 in the crevicular 1/3; 14/19 *versus* 0/7 in the middle 1/3; 6/19 *versus* 0/7 in the oral 1/3 (Fig. 3). The proportion of IFN-γ$^+$ cells rated the lowest among the cytokine expressions, regardless of the area or types of diseases, in spite of the significantly higher proportion of positive cells in periodontitis lesions compared with the gingivitis lesions, in the crevicular and middle 1/3 ($P < 0{\cdot}02$ and $P < 0{\cdot}01$, respectively; Table 2). However, in one particular case IFN-γ$^+$ cells rated the highest, and reached a percentage as high as IL-4$^+$ or IL-6$^+$ cells.

IL-4. Intensive staining of IL-4 was one of the characteristic features in the present study (Fig. 2c). Frequency of cases showing IL-4$^+$ cells in periodontitis and gingivitis was as

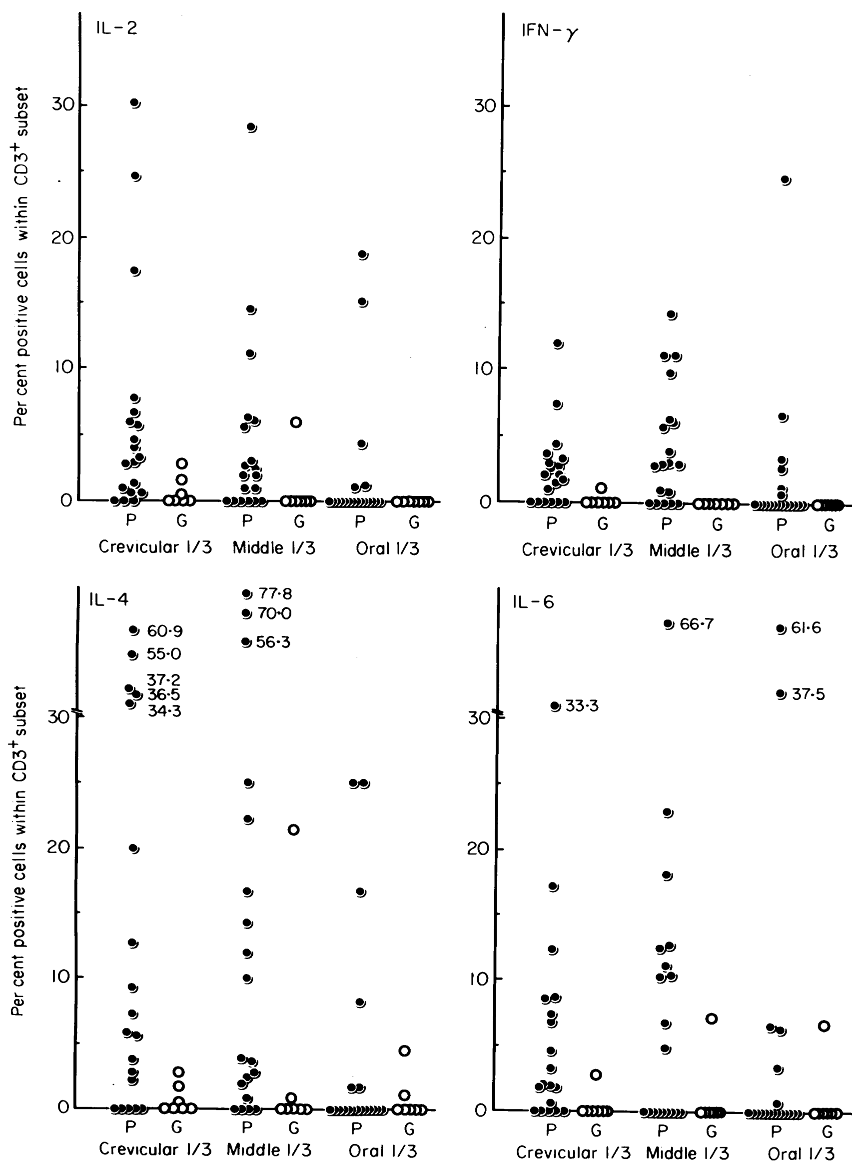

Fig. 3. Percentages of cytokine-immunoreactive cells in gingivitis group (G: $n = 7$) and periodontitis group (P: $n = 19$). The sections were immunostained for each cytokine and CD3. The positive cells were counted in each area of the section. Relative percentages were calculated by dividing the total number of cytokine-immunoreactive cells by the total number of CD3$^+$ cells.

Table 2. The proportion of cytokine-positive cells (positive % of $CD3^+$ cells), CD19:CD3 and CD4:CD8 ratios in each area associated with the disease categories

	Gingivitis ($n = 7$)	Periodontitis ($n = 19$)	P*
Crevicular one-third			
IL-2	0·70 ± 0·41	6·33 ± 1·98	<0·02
IFN-γ	0·16 ± 0·16	2·46 ± 0·69	<0·02
IL-4	0·70 ± 0·42	15·44 ± 4·49**	<0·02
IL-6	0·40 ± 0·40	5·81 ± 1·88	<0·02
CD19:CD3	0·16 ± 0·49	1·33 ± 0·31	<0·01
CD4:CD8	28·03 ± 18·86	6·89 ± 1·66	>0·05
Middle one-third			
IL-2	0·84 ± 0·84	4·51 ± 1·61	<0·05
IFN-γ	0	4·29 ± 1·02	<0·01
IL-4	3·18 ± 3·04	18·41 ± 6·55***	<0·05
IL-6	1·02 ± 1·02	9·29 ± 3·58	>0·05
CD19:CD3	0·35 ± 0·16	4·00 ± 1·60	<0·01
CD4:CD8	4·27 ± 0·94	3·08 ± 0·57	>0·05
Oral one-third			
IL-2	0	2·13 ± 1·23	>0·05
IFN-γ	0	2·07 ± 1·32	>0·05
IL-4	0·79 ± 0·63	4·12 ± 1·93	>0·05
IL-6	0·95 ± 0·95	6·11 ± 3·66	>0·05
CD19:CD3	0·03 ± 0·01	2·20 ± 1·53	<0·01
CD4:CD8	2·90 ± 0·34	2·05 ± 0·46****	>0·05

Values were expressed as the mean ± s.e.m.
*Difference between gingivitis and periodontitis was analysed by Mann–Whitney U-test. **IL-4 was significantly higher than any other cytokine ($P < 0·05$) within periodontitis in crevicular 1/3; ***IL-4 was significantly higher than IL-2 and IFN-γ ($P < 0·05$) within periodontitis in middle 1/3; ****significantly lower than in crevicular 1/3 within periodontitis ($P < 0·01$).

follows; 14/19 *versus* 3/7 in the crevicular 1/3, 15/19 *versus* 2/7 in the middle 1/3, 6/19 *versus* 2/7 in the oral 1/3 (Fig. 3). Like other cytokine-expressing cells, the proportion of $IL\text{-}4^+$ cells was low in gingivitis. However, in periodontitis lesions the $IL\text{-}4^+$ cell population was dominant, with a significantly higher proportion than other cytokines in the crevicular 1/3 and the middle 1/3 ($P < 0·02$ and $P < 0·05$, respectively; Table 2). Comparative analysis of cytokine-producing cells within periodontitis revealed that the proportion was highest in the crevicular 1/3 ($P < 0·05$), and was significantly higher than $IL\text{-}2^+$ and $IFN\text{-}\gamma^+$ cells in the middle 1/3 ($P < 0·05$; Table 2).

IL-6. Staining of IL-6 was less intense than that of IL-2 or IL-4 (Fig 2d). For periodontitis cases, immunoreactivity for IL-6 was identified in 14/19 in the crevicular 1/3, 10/19 in the middle 1/3, and 6/19 in the oral 1/3, whereas 1/7 in any area in gingivitis (Fig. 3). Although the difference of the proportion of $IL\text{-}6^+$ cells between periodontitis and gingivitis was only significant in the crevicular 1/3 ($P < 0·02$; Table 2), there were a few cases showing high IL-6 immunopositivity (>40%) in the middle 1/3 and the oral 1/3. Most $IL\text{-}6^+$ cells were identified as mononuclear cells, whereas fibroblast-like cells and epithelial cells were also positive for IL-6 in some cases (Fig. 4).

CD19:CD3 ratio. Although the CD19:CD3 ratio was significantly higher in all areas of periodontitis compared with gingivitis ($P < 0·01$; Table 2), significant numbers of T cells were found in the inflammatory infiltrates (Fig. 2e). Spatial analysis in periodontitis demonstrated a higher ratio in the middle 1/3 than in the crevicular 1/3 ($P < 0·05$; Table 2). Some sites of periodontitis tissues showed T cell-dominant infiltration, particularly in the oral 1/3. Only three cases displayed a CD19:CD3 ratio exceeding 1·0 in that area (Fig. 5).

CD4:CD8 ratio. Most of the periodontitis tissues (18/19 in the crevicular 1/3 and 13/19 in the middle 1/3) had a CD4:CD8 ratio greater than 2·0 (Fig. 2f), which is similar to the ratio in peripheral blood, indicating that the proportion of $CD4^+$ cells exceeded that of $CD8^+$ cells. In contrast, only 7/19 cases showed a CD4:CD8 ratio >2·0 in the oral 1/3 (Fig. 5). Mean ratio in the oral 1/3 was significantly lower than in the crevicular 1/3 ($P < 0·01$; Table 2).

Correlation between the proportion of each cytokine-positive cell and B cell:T cell ratio. As shown in Table 3, no positive correlation was found for IL-2 and IL-6 in any area of the sections. Positive correlations were found for IL-4 in both the crevicular 1/3 and oral 1/3, and for IFN-γ in the middle 1/3.

DISCUSSION

T cell functions are determined by their soluble factors, cytokines, which are synthesized following antigenic stimulation [9]. Phenotypic analysis of T cells in periodontitis-affected tissues has demonstrated up-regulation of the IL-2 receptor [13] and HLA-DR [14], a shift of CD4 and CD8 populations [4,5], and elevation of CD45RO expression with a concomitant decrease of CD45RA [7,8]. However, these findings failed

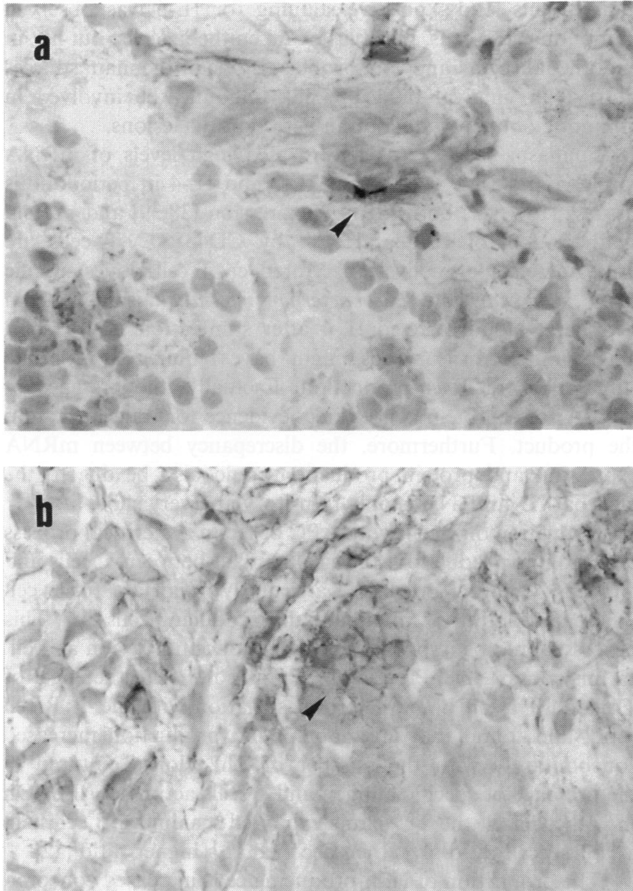

Fig. 4. IL-6 immunoreactivity of fibroblast-like cells (a) and epithelial cells (b) (arrowhead).

to provide an explanation for the intrinsic difference in immunological mechanisms discriminating between gingivitis and periodontitis.

Functional subsets were defined by the cytokine profile in mouse T cells [15]. Evidence supports the extension of this classification to humans [11]. Inducible functional subsets could be determined by antigen properties or different types of antigen-presenting cells (APC) [16]. Furthermore, a close association between distinct patterns of cytokine production and disease susceptibility to *Mycobacterium* infection was reported, in which mRNAs for IL-4, IL-5 and IL-10 predominated in the multi-bacillary form of the disease, whereas mRNAs for IL-2 and IFN-γ were evident in the lesion of the resistant form [12].

The present study has clearly shown the presence of cells with immunoreactivity for IL-2, IFN-γ, IL-4 and IL-6 both in periodontitis- and gingivitis-affected gingiva, albeit to a much lesser extent in gingivitis. The periodontitis tissues were obtained at the time of periodontal surgery which was undertaken after initial therapy. The initial therapy consisted of scaling, root planing and oral hygiene instruction. Despite this, almost all tissues exhibited bleeding on gentle probing from the base of the periodontal pocket. There was, however, a reduction in clinical severity of gingival inflammation. Early study on the nature of the cellular infiltrates in periodontal disease has shown that these infiltrates consist of predominantly plasma cells [17], even after initial therapy, and represent periodontitis tissues not affected by severe marginal gingivitis. In periodontitis, IL-2^+ and IFN-γ^+ cells, seen in Th1-mediated DTH resulting from *Mycobacterium* infection in humans [18] and mouse models [19], were lower than IL-4^+ and IL-6^+ cells. An immunohistological study of experimental gingivitis in humans demonstrated that the lesions consisted mainly of T cells. Most of these expressed MHC class II antigen, indicating

Table 3. Correlation between the proportion of cytokine-positive cells and CD19:CD3 ratio

CD19:CD3 ratio in the respective area	IL-2	IFN-γ	IL-4	IL-6
Crevicular 1/3	0·30	0·08	0·55*	0·21
Middle 1/3	0·19	0·68**	0·14	0·09
Oral 1/3	0·01	0·08	0·68**	0·49

Relative correlation coefficients are shown.
Values are significant at $*P < 0{\cdot}02$ and $**P < 0{\cdot}01$, respectively.

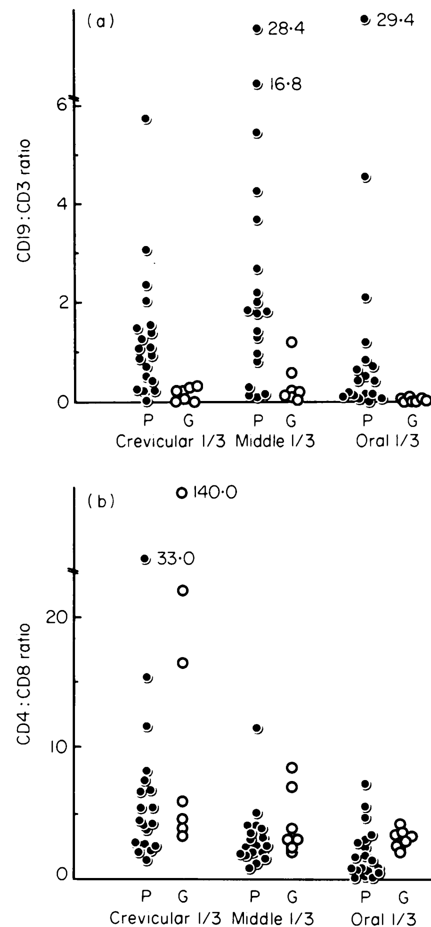

Fig. 5. Ratios of CD19:CD3 (a) and CD4:CD8 (b) in gingivitis group (G: $n = 7$) and periodontitis group (P: $n = 19$). Dual immunolabelling for either CD19 and CD3 or CD4 and CD8 was carried out on the sections from each subject. The positive cells were counted in each area of the section and the ratios were calculated.

that the lesion was similar to well controlled skin DTH lesions [20]. This led us to speculate that Th1 predominated in gingivitis. However, our result, showing a low percentage of $IL-2^+$ and $IFN-\gamma^+$ cells, contradicts the above findings. On the other hand, the elevated proportion of $IL-4^+$ and $IL-6^+$ cells, which was characteristic in this study, would seem to indicate that B cells and plasma cells preferentially infiltrate, and lead to elevated local IgG production in periodontitis.

Mycobacterium-reactive human T cell clones have been reported to show distinct cytokine production profiles. One clone produced IL-2 in response to antigen and irradiated peripheral blood mononuclear cells (PBMC), but not with anti-CD3, whereas the other clone displayed the opposite [21]. This indicates that the cytokine production profile may depend on the nature of the stimulus. Therefore, several explanations for low percentages of $IL-2^+$ and $IFN-\gamma^+$ cells in periodontitis lesions can be formulated: (i) a limited number of Th1 cells was recruited to the periodontitis lesion; (ii) antigens derived from periodontopathic bacteria may not induce IL-2 and/or IFN-γ from Th1 and Th0. Furthermore, IFN-γ from Th1 and IL-10 from Th2 cross-regulate the inhibition of cytokine production by reciprocal subset [22]. Although it is not now being investigated, a significant number of Th2 cells in the periodontitis lesion might have produced IL-10 and have caused inhibition of Th1 cytokine production. Prostaglandin E_2 (PGE_2) is known to inhibit IL-2 and IFN-γ production, but not IL-4 and IL-5 [23], which leads us to consider that PGE_2 could also be responsible for the Th2 predominance in periodontitis lesions. Furthermore, an *in vivo* study showed that the PGE_2 level in gingival tissue of periodontitis is high enough to induce bone resorption *in vitro* [24].

IL-4 and IL-6 play important roles in B cell activation [25]. IL-4 up-regulates the CD23 expression of B cells [26] as well as IgG4 synthesis [27]. In this context, the number of $CD23^+$ activated B cells increased in periodontitis lesions compared with gingivitis [8]. Higher levels of IgG4 in gingival crevicular fluid from active periodontitis patients have been reported than in those from stable periodontitis patients or serum from patients with both types of disease [28]. Most of the T cells infiltrating into connective tissue subjacent to the pocket epithelium of inflammatory gingival tissue are $CD45RO^+$ helper T cells. They are stimulated to synthesize more IL-4 than do $CD45RA^+$ cells [29]. Furthermore, it has been demonstrated that PBMC separated from periodontitis patients produced more IL-4 upon stimulation with *Porphyromonas gingivalis*, a representative periodontopathic bacterium, than do PBMC from gingivitis patients [30]. This supports our results, in which the proportion of $IL-4^+$ cells was found to be elevated in periodontitis lesions. However, the possibility that not all of the IL-4 immunoreactive cells identified in the present study are T cells cannot be excluded. Even though mast cells and eosinophilic leucoyctes can also synthesize IL-4 [31], mast cells are scarce in inflamed gingiva and exhibit different distribution, infiltrating mainly beneath the oral epithelium [32]. Moreover, we have demonstrated by the immunohistochemical double-labelling method that IL-4-producing cells belong to the $CD45RO^+$ subset of T cells [33]. So, most IL-4 immunoreactive cells in gingival lesions are likely to be T cells.

IL-6 is produced by several types of cells, including epidermal cells, endothelial cells, fibroblasts and also T and B cells [34]. In this study, positive staining for IL-6 was observed in mononuclear cells, fibroblasts and epithelial cells, but not in endothelial cells. Gingival fibroblast is one of the main sources of IL-6 in gingival tissue [35,36], and may be involved in immunoglobulin production in periodontitis lesions.

Fujihashi *et al.* [37] demonstrated high levels of mRNA for IL-5 and IL-6 but not for IL-2 and IL-4 in periodontitis tissues. However, both mRNA expression [38,39] and protein synthesis [40] of IL-2 and IL-4 by $CD45RO^+$ T cells are up-regulated upon activation. Moreover, T cell clones specific for *P. gingivalis* and *Fusobacterium nucleatum* isolated from peripheral blood secreted IL-4 after stimulation [30]. Hence, gingival mononuclear cells might have required appropriate stimulation for IL-4 production. Factually, the presence of mRNA for a protein is not direct evidence for translation into the product. Furthermore, the discrepancy between mRNA expression and protein production could also be due to the fact that IL-4 mRNA is only transitory and very short-lived.

Activation of T cells with APC or lectin is a critical process for cytokine production. Physical contact between T cells and antigen-presenting B cells is particularly important for B cell differentiation into immunoglobulin-producing cells [41]. This process would be important in the periodontitis lesion as well. In this context, Del Prete *et al.* examined *in vitro* the effect of Th1 and Th2 on B cell help in association with the T : B cell ratio, and found that immunoglobulin production increased concomitantly with the number of Th2 clones cultured in the presence of specific antigen and B cells acting as APC. On the other hand, the Th1 clone exhibited maximum B cell help at a T : B cell ratio of 1, then declined at a higher ratio [42].

The proportion of IL-4-producing cells increased concomitantly with the B : T cell ratio in the crevicular 1/3 portion, where the large amount of antigens in dental plaque would stimulate T cells continuously. $IFN-\gamma^+$ cells also showed a statistically significant correlation with the B : T cell ratio in the middle 1/3, but the finding is not necessarily significant from a biological viewpoint because of their low number.

Although our findings suggest the infiltration of type 2 helper T cells as a characteristic feature of periodontitis lesions, accumulating further evidence supporting this view would seem to be required. Understanding the function and regulatory mechanisms relevant to the activation of Th1 and Th2 subsets despite their complexity may lead to an improved control of anti-bacterial immune responses in chronic inflammatory periodontal diseases.

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