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Cloning, Characterization, and Antigen Specificity of T-Lymphocyte Subsets Extracted from Gingival Tissue of Chronic Adult Periodontitis Patients

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Chronic periodontitis is characterized by dense infiltrations of B and T lymphocytes within the gingival connective tissue. Distinct anaerobic gram-negative bacteria as well as autoimmunity to collagen have been reported to play a role in the etiology and the pathogenesis of this disease. Here we describe the cloning and characterization of $CD4^+$ and $CD8^+$ T lymphocytes isolated from inflamed gingival tissue obtained from four patients with chronic periodontitis. Clones were raised with phytohemagglutinin and interleukin-2 and tested for proliferation in response to whole-cell antigens of *Porphyromonas gingivalis*, *Prevotella intermedia*, *Actinobacillus actinomycetemcomitans*, human collagen type I, and two bacterial heat shock proteins. $CD4^+$ T-cell clones reactive with collagen type I were obtained from all four patients. Eighty percent of these clones had phenotypes resembling the mouse type 2 T helper (Th) phenotype, i.e., they produced high levels of interleukin-4 and low levels of gamma interferon. No collagen-type-I-reactive $CD8^+$ clones were obtained. Bacterial-antigen-reactive $CD4^+$ and/or $CD8^+$ T-cell clones were also obtained from each patient, and the majority of the clones showed a Th0-like cytokine pattern and produced equal amounts of interleukin-4 and gamma interferon. Although most clones were reactive with *P. intermedia*, it seems that the immune response is not strictly directed against this particular microorganism, as clones reactive with one of the other bacteria were also obtained from two patients. We propose that collagen-specific $CD4^+$ Th2-like T cells contribute to the chronicity of periodontitis but that their modes of activation might be controlled by Th0-like T cells specific for periodontitis-associated bacteria.

Chronic periodontitis is an inflammatory disease of the supporting tissues of the teeth, including gingivae, periodontal attachment fibers, and the alveolar bone. Along with several other oral bacteria, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, and *Prevotella intermedia* have been implicated in the initiation and progression of different forms of this disease (18). The immunopathogenic mechanism involved in periodontitis is not understood, although the interaction between bacterial antigens and T lymphocytes in inflamed oral lesions has been suggested to be of importance in the pathogenesis of the disease in human (12, 39) and experimental rat (16, 44, 50) models. In addition to bacterial antigens, autoantigens have also been considered to play a role in the pathogenesis of periodontitis (1, 4). Periodontal tissues destroyed by chronic inflammation contain mainly type I collagen which may have been enzymatically degraded (3, 29). As in other diseases in which collagen is degraded, both cellular and humoral immune responses to collagen have been reported for periodontal diseases (2, 14, 15, 22, 43).

To help delineate the T-cell events involved in the regulation of periodontitis, it is important to determine the functional difference and modes of activation of T-cell subsets which might be involved in mediating the disease.

Studies over the past few years have revealed that the function of T cells is related to the cytokines they produce. On the basis of this cytokine production, cloned mouse $CD4^+$ T helper (Th) cells have been divided into Th1, Th2, and Th0 cells (10, 28).

Th1 cells produce interleukin-2 (IL-2) and gamma interferon (IFN-γ) but not IL-4, IL-5, or IL-10 and are involved in cellular antimicrobial immunity by activating macrophages. They are responsible for inflammation and delayed-type hypersensitivity. Th2 cells secrete IL-4, IL-5, and IL-10 but not IL-2 or IFN-γ and are associated with efficient antibody generation, especially of the immunoglobulin G1 (IgG1), IgE, and IgA isotypes. Cells of the third subset, Th0 cells, produce a mixture of both Th1 and Th2 cytokines, and it has been hypothesized that they are early memory $CD4^+$ T cells which differentiate further into Th1 or Th2 cells upon antigenic stimulation (42).

Human antigen-specific $CD4^+$ T-cell clones can also be characterized in relation to the mouse Th1, Th2, and Th0 cytokine profiles (23, 32, 36, 49).

In addition, identical cytokine profiles have recently been described for $CD8^+$ T-cell clones, suggesting that cytotoxic T cells which exhibit inflammatory properties produce IFN-γ, whereas noncytotoxic $CD8^+$ T cells exhibiting suppressor properties are IL-4 producers (36, 43).

It appears that the in vivo type of immune response to certain infectious organisms is determined by the outcome of a balance between type 1 and type 2 cytokines. The net cytokine effect will favor regression or progression of local tissue inflammation (26). There is some evidence that the profiles of lymphokines secreted by T-cell subsets in vivo may overlap with, but are not identical to, the profiles of in vitro-cloned T

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cells (35, 37). Because periodontal lesions comprise high numbers of $CD4^+$ and $CD8^+$ T lymphocytes within the adjacent connective tissue (7, 8, 38), experiments on the clone-based classification of these T cells might provide some information on the type of cytokines and the type of immunity that are involved in mediating periodontitis.

Here we report the cloning and characterization of $CD4^+$ and $CD8^+$ T cells isolated from inflamed gingival tissue of four patients with chronic periodontitis. Established T-cell clones were tested for their proliferative responses to whole-cell antigens of *P. gingivalis*, *P. intermedia*, *A. actinomycetemcomitans*, human collagen type I, and two bacterial heat shock proteins (HSP) and for their production of cytokines.

MATERIALS AND METHODS

Isolation and cloning of T lymphocytes. Inflamed gingival tissue, excised from four patients with chronic adult periodontitis undergoing therapeutic periodontal surgery, was placed in petri dishes containing cold phosphate-buffered saline (PBS) supplemented with penicillin (500 IU/ml), streptomycin (500 mg/liter), and nystatin (40 IU/ml). Diagnosis of chronic adult periodontitis was made on the basis of clinical and radiographic criteria according to the American Association for Periodontology's consensus (1989). All patients had more than one pocket of ≥5 mm and at least one pocket with a ≥4-mm loss of attachment. Their ages ranged between 28 and 45 years. Petri dishes were transported and kept on ice until the cell extraction procedures were started. One piece of tissue was quick-frozen in liquid nitrogen and stored at −80°C until use. The other pieces of tissue were washed three times in cold PBS to remove blood clots and adherent erythrocytes and were minced with scissors into fragments of 1 to 3 mm^2. The fragments were then transferred to petri dishes containing Hanks' balanced salt solution, 2% fetal calf serum, and 25 IU of collagenase IV (Worthington, Freehold, N.J.) per ml. After 90 min of incubation at 37°C with 5% CO_2, the fragments were sieved and washed three times with Hanks' balanced salt solution. Finally, the cell pellet was resuspended in 1 ml of Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated pooled human serum (HS) and 80 μg of gentamicin (culture medium) per ml, and viable lymphocytes were counted.

First, fibroblasts, neutrophils, keratinocytes, and epithelial cells were removed by density centrifugation on Lymphoprep (Nycomed, Oslo, Norway). However, the recovery of the lymphoid cells was low, i.e., less than 40% of the total number of lymphoid cells counted before centrifugation. To avoid selection within the lymphocyte population as much as possible, we cloned the T lymphocytes directly from the mixed cell suspension. Cells were plated so that the lymphocytes were seeded under limiting dilution conditions of one lymphocyte cell per well or less. Cells were cultured in 96-microwell flat-bottom plates in culture medium in the presence of a feeder mixture containing 10 U of human recombinant IL-2 (rIL-2) per ml, 1 μg of phytohemagglutinin (PHA) per ml, 2.10^5 irradiated (3,000 rads) allogeneic peripheral blood lymphocytes (a mixture from two nonrelated donors) per well, and 10^4 irradiated (3,000 rads) Epstein-Barr virus-transformed B cells from the JY line (American Type Culture Collection, Rockville, Md.) per well. Fourteen days later, the seeding plates were scored for proliferation. Microcultures were considered clones on the basis of the frequency of proliferating microcultures (i.e., cultures with growth in less than 30% of the 96 wells). Clones were further expanded and maintained in 24-well plates. Fresh medium (IMDM, 10% HS, and 10 U of rIL-2 per ml) was added at 3-day intervals. Fresh feeder mixture was added every 14 days.

FACS analysis. For fluorescence-activated cell sorter (FACS) analysis, cloned T lymphocytes were labeled with appropriate monoclonal antibodies (MAbs) and rabbit anti-mouse Ig $F(ab')_2$–fluorescein isothiocyanate in a two-step procedure. Samples were analyzed with a FACScan (Becton Dickinson). The MAbs used were CD3, CD4, and CD8 specific (all from Ortho Diagnostic Systems, Raritan, N.J.). The monomorphic αβ-T-cell-receptor (TCR)-specific antibody BMA031 was purchased from T Cell Sciences, Cambridge, Mass. The monomorphic γδ-TCR-specific MAb 11F2 was kindly provided by J. Borst, National Cancer Institute, Amsterdam, The Netherlands.

Antigens. *P. gingivalis* (HG1491), *P. intermedia* (HG1490), and *A. actinomycetemcomitans* (HG1492) strains were cultured in liquid BM medium (46). Bacteria were fixed overnight at 4°C in PBS containing 0.5% formaldehyde. Cultures were kindly provided by T. J. M. van Steenbergen, Department of Oral Microbiology, Academic Center for Dentistry Amsterdam (ACTA). Before use, bacteria were washed three times in PBS and heated at 100°C for 20 min. Finally, bacteria were resuspended in IMDM plus 10% HS at a protein concentration of 200 μg/ml. Collagen type I was isolated from human skin by T. Bos, Department of Experimental Periodontology, Academic Center for Dentistry Amsterdam, according to the method of Chung and Miller (6). Collagen was dissolved in 0.5 M acetic acid to a concentration of 10 mg/ml and was sterilized under UV light (distance, 10 cm) for 10 min. For T-cell stimulation a concentration of 100 μg/ml in IMDM was used. Recombinant HSP from *Mycobacterium bovis* (65 kDa) and *Mycobacterium tuberculosis* (71 kDa) were obtained from J. D. A. van Embden, Department of Molecular Biology, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands.

Both HSP were used at a final concentration of 10 μg/ml. For reactivity to the superantigen, *Staphylococcus aureus* enterotoxin A (SEA), SEB, and SEC were mixed at final concentrations of 10 ng/ml for SEA and 100 ng/ml for SEB and SEC.

Cytokine induction. Ten days after the feeder mixture was added, cloned T lymphocytes (10^5 per well) were stimulated for 24 h with a combination of PHA (1 μg/ml) and phorbol myristate acetate (PMA; 1 ng/ml) in the absence of IL-2. Control cells were cultured in medium alone. Supernatants were collected and stored in aliquots at −20°C until cytokine production was analyzed. Stimulation was measured in a proliferation assay by [^{3}H]thymidine incorporation. All T-cell clones tested showed a stimulation index (proliferation of T cells in the presence of PHA-PMA/proliferation without PHA-PMA) of >15.

T-cell proliferation assay. The proliferative response of the cloned T lymphocytes to PHA-PMA was measured after 40 h. For the last 16 h, the cells were cultured in the presence of 0.33 μCi of [^{3}H]thymidine per well. The samples were harvested on fiberglass filters and counted in a liquid scintillation counter.

For antigen-specific proliferation, T cells (10^4 per well) were cocultured with autologous or HLA-matched irradiated peripheral blood mononuclear cells (PBMC) (10^5 per well) in the presence of bacterial antigens (10 μg per well) or collagen type I (2 μg per well) for 3 days, the last 16 h of which the cells were in the presence of [^{3}H]thymidine. An antigen response was considered positive if the stimulation index (proliferation of T cells in the presence of antigen/proliferation without antigen) was 4 or more in repeated experiments. To exclude T-cell activation by bacterial superantigens, the need for antigen processing was tested with fixed PBMC. Irradiated PBMC were treated with 0.006% (final concentration) glutaraldehyde for 30 s on ice. Fixation was stopped by the addition of 0.2 M (final concentration) glycine. After 10 min the cells were washed twice with Hanks' balanced salt solution.

Cytokine measurements in vitro. IL-4 and IFN-γ production by the cloned T lymphocytes was measured with specific solid-phase sandwich enzyme-linked immunosorbent assays (ELISA) with MAbs 4F2 (anti-IL-4) and MD2 (anti-IFN-γ) as catching antibodies and MAb 5A4 (anti-IL-4) and MAb MD1 (anti-IFN-γ) as detection antibodies. The MAbs and assays have already been described (5, 45). Human rIL-4 and rIFN-γ were used as references. The lowest levels of detection were 100 pg/ml for IL-4 and 100 pg/ml for IFN-γ.

Cytokine detection in situ. Serially frozen sections of tissue with thicknesses of 4 to 6 μm were made as previously described (47). Slides were air dried for 1 h, fixed for 10 min in acetone, and again air dried for at least 10 min. For immunohistochemical staining, the sections were rehydrated in PBS–10% HS–10% horse serum for 5 min. The tissue sections were incubated with anti-IL-4, anti-IFN-γ, anti-CD4, or anti-CD8 MAb for 1 h at room temperature. Slides were rinsed and incubated with a biotinylated horse anti-mouse Ig for 30 min at room temperature. After rinsing of the slides, the antibody-biotin conjugates were detected with streptavidin-alkaline phosphatase. Enzyme activity was detected with a fuchsin solution, and the reaction was stopped after 30 min with distilled water. Washing procedures and antibody dilutions were performed in PBS–1% HS–1% horse serum. Anti-IL-4 MAb 1-41-1 was a gift from Sandoz, Basel, Switzerland. Anti-IFN-γ MAb MD1 was the same as that used in ELISA. The MAbs specific for CD4 or CD8 are the same as those used for FACS analysis. For the fuchsin solution (final pH 8.2), reagent A was mixed with reagent B just before use. Reagent A contained 250 μl of acid fuchsin (4% in 2 N HCl), 250 μl of sodium nitrite (4%), and 35 mg of levamisole in 100 ml of 0.2 M Tris-HCl (pH 8.0). Reagent B contained 50 mg of naphthol AS-BI sodium salt (Sigma N 2250) in 1 ml of dimethylformamide.

RESULTS

Cloning of T lymphocytes. Highly efficient random cloning of T cells has been described for the cloning method used (27, 41). Table 1 shows as a representative example the proliferation of T-cell cloning 14 days after seeding of unfractionated gingiva cells obtained from patient 1. Growing microcultures from the seedings of three and one cells per well were further expanded with IL-2. All cultures were analyzed for the expression of CD3, CD4, CD8, and the αβ or γδ TCR. Table 2 shows that cloning of gingival lymphocytes by limiting dilution resulted in the generation of a variety of T-cell clones expressing the αβ or γδ TCR. This reflects a random selection of gingival T cells. To obtain stable T-cell clones, microcultures of αβ TCR^+ T cells were stimulated once in 2 weeks with IL-2, PHA, and feeder cells. Stable T-cell clones expressing the αβ TCR and CD4 or CD8 were also obtained from three other patients (Table 3).

TABLE 1. Cloning of gingival T lymphocytes upon stimulation with PHA

No. of gingiva cells seeded per well[a]	No. of proliferating microcultures[b]
10[c]	31
10[c]	30
3	19[d]
3	17[d]
1	8[d]
1	7[d]

[a] Unfractionated cells obtained from gingival tissue of patient 1 were seeded in 96-well flat-bottomed microtiter plates.
[b] Number of proliferating microcultures in 96 seeded wells that responded to PHA and IL-2 after 14 days of culture.
[c] The estimated cell density was one lymphocyte per well.
[d] The probability of clonality is more than 96%, i.e., the frequency of proliferating microcultures in the seeded wells is less than 30%.

Antigen specificity of CD4$^+$ and CD8$^+$ αβ TCR$^+$ T-cell clones. Established T-cell clones from all four patients were tested for their proliferative responses to a panel of antigens including the 65- and 71-kDa HSP, human collagen type I, and formaldehyde- and heat-treated bacterial suspensions of *P. gingivalis*, *P. intermedia*, and *A. actinomycetemcomitans*. Table 3 shows that although the clones are non-antigen-specifically raised, some of the CD4$^+$ and CD8$^+$ T-cell clones were reactive with one of the selected antigens. Table 4 shows their proliferative responses. The most frequently recognized antigens are *P. intermedia* and collagen type I. For each patient, CD4$^+$ but no CD8$^+$ collagen-type-I-reactive T cells were cloned. For three of these patients bacterial-antigen-reactive CD4$^+$ T-cell clones were also generated.

CD8$^+$ T-cell clones reactive with *P. intermedia* were raised from samples from two patients (Table 4). None of the collagen-type-I-reactive T-cell clones responded to any of the bacteria and vice versa. The bacterial-antigen-reactive T cells were not cross-reactive, and no reactivity against HSP was observed. To exclude the possibility that the T-cell clones were reactive with bacterial superantigens, proliferative responses were measured with fixed-antigen-presenting cells. Because superantigens bind directly to both major histocompatibility complex class II molecules and TCRs, they do not require antigen processing by non-fixed-antigen-presenting cells. Table 5 shows that for stimulation of the T-cell clones, antigen processing is necessary.

Cytokine profiles of antigen-specific CD4$^+$ and CD8$^+$ αβ TCR$^+$ T-cell clones. To compare the cytokine production of antigen-specific T-cell clones with the production of non-antigen-specific T-cell clones, cytokine production was measured after stimulation with a combination of PHA and PMA for 24 h. When the production of IFN-γ was less than 10% of the production of IL-4, the cytokine pattern was considered type 2 (Th2 like); the majority of collagen-reactive CD4$^+$ T-cell clones showed a dominating Th2-like production pattern, producing high levels of IL-4 and almost no IFN-γ (Table 6). This is in contrast with the CD4$^+$ T-cell clones reactive with one of the bacterial antigens; the majority of these CD4$^+$ T-cell clones produced almost the same level of IL-4 and IFN-γ, resembling the type 0 (Th0-like) secretion pattern. The two CD8$^+$ T-cell clones reactive with *P. intermedia* produced no detectable amounts of IL-4 or IFN-γ.

Cytokine profiles of non-antigen-specific CD4$^+$ and CD8$^+$ αβ TCR$^+$ T-cell clones. Fifty percent of the non-antigen-specific CD4$^+$ T-cell clones from each patient showed type 2 cytokine production, with high levels of IL-4 and low levels of IFN-γ (Table 7). Only three clones raised from samples from

TABLE 2. Phenotypic analysis of proliferating microcultures after stimulation with PHA

Clone type	No. of cultures (n = 51[a])			
	CD4$^+$	CD8$^+$	CD4$^-$ CD8$^-$	Died
αβ TCR^{+b}	22	9	4	5
γδ TCR^{+b}	0	4	7	
Established clones[c] of αβ TCR$^+$	22	9		

[a] See Table 1 for experiment specifications and the source of the cultures.
[b] For FACS analysis proliferating microcultures were transferred to 2-ml wells and cultured in the presence of 10 U of human rIL-2 for several days. The probability of clonality is more than 96%, i.e., the frequency of proliferating microcultures in the seeded wells is less than 30%.
[c] Of all clonal growing microcultures, only those expressing the αβ TCR and CD4 or CD8 were expanded further to obtain stable T-cell clones.

TABLE 3. Summary of established and antigen-reactive T-cell clones isolated from chronically inflamed gingival tissue of four patients with adult periodontitis

Patient	No. of αβ TCR$^+$ T-cell clones[a]			
	Established		Antigen reactive[b]	
	CD4$^+$	CD8$^+$	CD4$^+$	CD8$^+$
1	22	9	5	0
2	22	6	7	1
3	16	7	2	0
4	15	2	1	1

[a] Of all clonal growing microcultures, only those expressing the αβ TCR and CD4 or CD8 were further cultured to obtain stable T-cell clones.
[b] Antigen reactivity was tested with a panel of antigens. For proliferative responses, see Table 4.

TABLE 4. Proliferative responses of gingival αβ TCR$^+$ T-cell clones to various antigens[a]

Cell type and clone	Proliferative response to antigens						
	None	HSP65	HSP71	Col	Pi	Pg	Aa
CD4$^+$							
VE02	0.2	0.1	0.3	**3.3**	0.2	0.5	0.1
VE05	0.3	0.1	0.4	**4.3**	0.5	0.2	0.2
VE26	0.2	0.3	0.4	0.3	0.3	0.2	**2.7**
VE50	0.3	0.5	0.3	0.4	0.4	**3.9**	0.2
VE44	0.2	0.5	0.1	0.1	**2.2**	0.4	0.4
BR04	0.1	0.1	0.1	**2.6**	0.1	0.1	0.1
BR06	0.3	0.2	0.1	**3.7**	0.4	0.4	0.2
BR09	0.3	0.3	0.3	**3.4**	0.1	0.5	0.4
BR13	0.4	0.6	0.5	**2.8**	0.3	0.3	0.5
BR10	0.3	0.5	0.4	0.6	0.6	0.7	**5.1**
BR17	0.2	0.4	0.7	**8.0**	0.3	0.6	0.5
BR22	0.5	0.6	0.3	0.4	**9.3**	0.5	0.4
LO23	0.2	0.5	0.4	**2.7**	0.1	0.3	0.1
LO31	0.4	0.4	0.6	0.4	**4.8**	0.5	0.3
GA15	0.2	0.1	0.2	**5.6**	0.4	0.2	0.5
CD8$^+$							
GA06	0.5	0.5	0.6	0.3	**9.2**	0.6	0.6
BR29	0.4	0.4	0.5	0.3	**12.9**	0.5	0.4

[a] Antigen preparations: HSP65, HSP71, collagen type I (Col), *P. intermedia* (Pi), *P. gingivalis* (Pg), and *A. actinomycetemcomitans* (Aa). Results are 10^3 counts per minute and are means for triplicate cultures. The standard deviation was less than 10% in all cases. Positive responses (stimulation index, >4) are in boldface type.

TABLE 5. Requirement of proliferative responses of gingival T-cell clones to bacterial antigens for active antigen processing[a]

Clone	Proliferative response to antigen								
	None	Pi		Pg		Aa		SA	
		Normal	Fixed[b]	Normal	Fixed	Normal	Fixed	Normal	Fixed
VE26	0.2	NT[c]	NT	0.5	0.4	**3.9**	0.6	**6.7**	**7.5**
BR10	0.3	NT	NT	0.4	0.6	**5.1**	0.6	**11.3**	**12.9**
VE44	0.4	**4.1**	0.5	0.5	0.6	NT	NT	**9.1**	**7.2**
BR22	0.4	**6.6**	0.3	0.4	0.3	NT	NT	**8.9**	**8.5**
LO31	0.4	**5.4**	0.4	0.6	0.6	NT	NT	**8.8**	**10.5**
GA06	0.5	**7.2**	0.6	0.5	0.6	NT	NT	**10.7**	**11.2**
BR29	0.6	**6.8**	0.5	0.7	0.5	NT	NT	**18.4**	**16.7**
VE50	0.2	0.5	0.5	**4.7**	1.0	NT	NT	**10.9**	**11.2**

[a] Antigen preparations: *P. intermedia* (Pi), *P. gingivalis* (Pg), *A. actinomycetemcomitans* (Aa), and a mixture of SEA, SEB, and SEC (SA). Results are 10^3 counts per minute and are means for triplicate cultures. The standard deviation was less than 10% in all cases. Positive responses (stimulation index, >4) are in boldface type.
[b] PBMCs were fixed with 0.006% glutaraldehyde (see Materials and Methods).
[c] NT, not tested.

two different patients showed a typical type 1 secretion pattern, with large amounts of IFN-γ and almost no IL-4 production. The remaining clones produced more or less equal amounts of IFN-γ and IL-4 and are therefore defined as type 0 (Th0-like) cells. Apparently, there are at least two major cytokine patterns among the $CD4^+$ T-cell clones from all four patients: (i) a distinct Th2-like pattern with a relatively high level of IL-4 and a low level of IFN-γ production and (ii) a Th0-like pattern with more or less equal levels of production of IFN-γ and IL-4.

TABLE 6. Cytokine profiles of antigen-specific αβ TCR^+-positive T-cell clones from gingival tissue

Cell type and clone	Proliferative response[a] (cpm, 10^3)		Cytokine production (ng/ml)		Type[b]	Reactivity[c]
	PMA-PHA$^-$	PMA-PHA$^+$	IFN-γ	IL-4		
$CD4^+$						
VE02	0.2	47.2	0.60	15.30	2	Col
VE05	0.4	23.5	<0.10[d]	1.50	2	Col
VE26	0.3	30.9	<0.10	0.50	2	Aa
VE50	0.4	16.6	<0.10	<0.10[d]	?	Pg
VE44	0.3	23.7	1.00	0.90	0	Pi
BR04	0.2	17.8	<0.10	1.30	2	Col
BR06	0.3	32.0	<0.10	3.80	2	Col
BR09	0.2	17.6	<0.10	68.80	2	Col
BR13	0.4	18.9	<0.10	0.50	2	Col
BR10	0.4	16.9	1.00	1.70	0	Aa
BR17	0.4	15.8	1.90	0.80	0	Col
BR22	0.2	23.9	2.10	6.30	0	Pi
LO23	0.3	18.9	1.30	18.60	2	Col
LO31	0.4	16.0	1.90	1.20	0	Pi
GA15	0.2	23.7	1.60	1.80	0	Col
$CD8^+$						
GA06	0.4	16.9	<0.10	<0.10	?	Pi
BR29	0.9	15.8	<0.10	<0.10	?	Pi

[a] T cells (10^5 per well) were stimulated with PHA-PMA ($^+$) or not stimulated ($^-$) for 24 h, and cytokine in the supernatants was measured by ELISA. Reactivity of the T-cell clones was confirmed by a [^{3}H]thymidine incorporation assay.
[b] Cytokine secretion profile of PHA-PMA-stimulated T cells. When the production of IL-4 was less than 10% of the production of IFN-γ, the cytokine pattern was determined to be type 1. When the production of IFN-γ was less than 10% of the production of IL-4, the cytokine pattern was determined to be type 2. The cytokine pattern was determined to be type 0 when a mixture of IFN-γ and IL-4 was produced. Unstimulated T cells produced no cytokines (type 0).
[c] Proliferative response to collagen type I (Col), *A. actinomycetemcomitans* (Aa), *P. intermedia* (Pi), and *P. gingivalis* (Pg).
[d] Below detection level (100 pg/ml).

TABLE 7. Cytokine profiles of non-antigen-reactive T-cell clones isolated from chronically inflamed gingival tissue

Cell type and clone	Cytokine production (ng/ml)		Type[a]	Cell type and clone	Cytokine production (ng/ml)		Type[a]
	IFN-γ	IL-4			IFN-γ	IL-4	
$CD4^+$							
VE17	1.90	1.30	0	BR19	<0.10	1.20	2
VE13	0.60	1.50	0	BR16	<0.10	1.20	2
VE18	2.30	1.90	0	BR23	<0.10	1.90	2
VE21	0.60	2.60	0	BR02	<0.10	3.90	2
VE12	0.70	2.50	0	BR31	<0.10	8.90	2
VE37	0.90	3.10	0	LO17	1.80	2.70	0
VE39	<0.10	<0.10	?	LO06	1.00	2.70	0
VE47	0.50	<0.10	1	LO24	2.50	2.80	0
VE09	<0.10	0.80	2	LO21	1.60	0.80	0
VE34	<0.10	1.70	2	LO18	<0.10	<0.10	0
VE35	<0.10	2.60	2	LO20	2.20	<0.10	1
VE42	<0.10	4.20	2	LO26	2.20	<0.10	1
VE20	<0.10	7.30	2	LO25	<0.10	1.80	2
VE45	<0.10	8.30	2	LO12	<0.10	2.20	2
VE14	0.40	7.60	2	LO09	<0.10	7.20	2
VE08	0.90	13.30	2	LO11	<0.10	7.60	2
VE10	1.30	13.70	2	LO10	<0.10	7.90	2
GA02	1.90	3.50	0	LO16	<0.10	12.90	2
GA03	1.80	1.20	0	LO28	<0.10	14.10	2
GA05	1.30	1.90	0				
				$CD8^+$			
GA10	1.50	1.40	0	VE36	<0.10	<0.10	?
GA13	1.50	1.30	0	VE28	1.40	<0.10	1
GA17	2.80	3.80	0	VE29	1.00	<0.10	1
GA16	4.10	9.50	0	VE24	0.50	<0.10	1
GA18	3.00	5.70	0	VE30	>5.00	<0.10	1
GA09	<0.10	2.20	2	VE25	<0.10	0.80	2
GA04	<0.10	4.80	2	VE27	<0.10	1.70	2
GA08	<0.10	9.70	2	VE01	<0.10	4.50	2
GA07	<0.10	15.00	2	VE31	0.50	8.50	2
GA19	0.50	10.70	2	GA12	1.20	2.40	0
GA01	0.80	16.50	2	LO22	<0.10	<0.10	?
BR18	3.50	1.50	0	LO30	2.90	0.80	0
BR26	1.50	3.10	0	LO03	0.40	0.70	0
BR21	1.30	8.30	0	LO15	1.10	<0.10	1
BR01	4.50	1.80	0	LO07	<0.10	1.10	2
BR15	3.50	9.50	0	LO19	<0.10	2.20	2
BR20	2.60	6.10	0	LO04	<0.10	25.40	2
BR05	1.70	0.80	0	BR11	0.90	0.90	0
BR14	1.50	2.40	0	BR07	1.20	0.40	0
BR12	<0.10	1.10	2	BR25	1.10	<0.10	1
BR03	<0.10	1.20	2	BR24	<0.10	1.40	2
				BR28	<0.10	1.70	2

[a] See Table 6, footnote *b*.

On the basis of IFN-γ and IL-4 production, $CD8^+$ αβ TCR^+ T-cell clones could also be divided into three different secretion types according to the type 1, type 2, and type 0 patterns (Table 7). The type 1 cytokine pattern is more frequently observed among $CD8^+$ T-cell clones than among $CD4^+$ T-cell clones. For two patients, IFN-γ-producing T-cell clones remained few in number among the $CD8^+$ T-cell clones also, while type 0 and type 2 patterns were equally distributed among $CD8^+$ T-cell clones. One patient showed a balance between type 1 and type 2 $CD8^+$ T-cell clones. However, the number of isolated $CD8^+$ T-cell clones was relatively low,

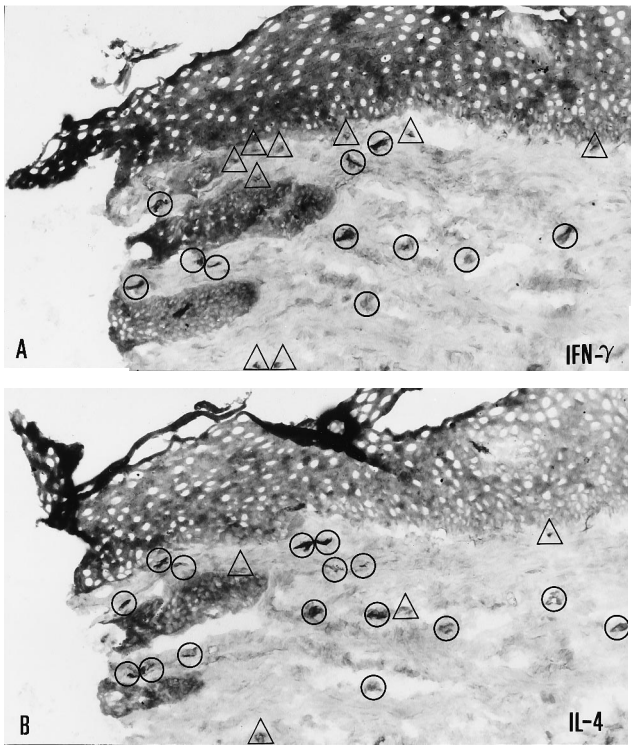

FIG. 1. Immunohistochemical staining of chronically inflamed gingival tissue of patient 1. Two sets of three adjacent sections of tissue with thicknesses between 4 and 6 nm were made. Sections from one set were stained for CD4, CD8, and IFN-γ (A). Sections from the other set were stained with CD4, CD8, and IL-4 (B). Both $CD4^+$ and $CD8^+$ T cells are present within the connective tissue. Circles indicate cells positive for CD4; triangles indicate cells positive for CD8. The majority of the $CD8^+$ cells are good IFN-γ producers. The majority of the $CD4^+$ T cells produce both IL-4 and IFN-γ. A small number of the $CD4^+$ T cells produce only IL-4. $CD4^+$ T cells producing only IFN-γ appear not to be present. For technical details and estimates, see reference 47.

which made it difficult to compare their cytokine pattern with those of the $CD4^+$ T-cell clones.

In situ localization of IL-4 and IFN-γ. To examine whether cytokine production by $CD4^+$ and $CD8^+$ T-cell clones reflects lymphocyte activation in vivo, we analyzed local cytokine production in a portion of the gingival tissue used for T-cell cloning. Figure 1 shows a representative example of an immunohistochemical staining for IL-4 and IFN-γ in two sets of serial gingiva sections from patient 1. $CD4^+$ and $CD8^+$ T cells are both located in the connective tissue and just under the basement membrane. The majority of the $CD4^+$ cells were stained for IL-4 as well as for IFN-γ. These double-producing cells fit the Th0-like phenotype. $CD4^+$ T cells producing only IL-4 are considered Th2-like cells. No $CD4^+$ cells producing only IFN-γ were observed. This is in contrast with the majority of the $CD8^+$ T cells, which produce only IFN-γ. A few cells also stained positive for IL-4. $CD8^+$ T cells producing only IL-4 were rare but present.

DISCUSSION

In order to analyze different subsets of T lymphocytes which may be involved in periodontitis, we have cloned and characterized T lymphocytes isolated from chronically inflamed gingival tissue obtained from patients with chronic periodontitis. Clones were raised with PHA and IL-2 as initial stimulants and tested for proliferation on four antigens which have been implicated in the disease, namely, *P. gingivalis*, *P. intermedia*, *A. actinomycetemcomitans*, and human collagen type I. The last antigen was included because type I collagen is the most abundant protein in periodontal ligaments, the gingival connective tissue, and the alveolar bone (3, 29), and immune responses to this type of collagen have been described for patients with chronic periodontitis (2, 14, 15, 22, 43). In addition, all T-cell clones were tested for reactivity to HSP.

Phenotypic analysis of 51 T-cell microcultures raised from samples from patient 1 suggests a random selection, as at least four different subtypes of T cells were present among the cultures. These subtypes were distributed with a $CD4^+$ αβ TCR^+/$CD8^+$ αβ TCR^+/$CD4^-$ $CD8^-$ αβ TCR^+/$CD4^-$ $CD8^{-/+}$ γδ TCR^+ ratio of 27:12:3:9. A relatively high number of γδ TCR^+ lymphocytes were cloned. This is in accordance with the reported frequency of γδ TCR^+ T cells found within the epithelium of chronically inflamed gingiva tissue (20). However, in this study we focused on long-term $CD4^+$ and $CD8^+$ T-cell clones expressing the αβ TCR.

In all four patients the CD4/CD8 ratio of the clones does not completely match the CD4/CD8 ratios found in gingival lymphocytes isolated from patients with adult periodontitis (20). It can be argued that the cloning procedure used favors the proliferation of $CD4^+$ clones. However, other studies suggest that a preferential growth of $CD8^+$ clones is obtained by nearly the same cloning method (48).

Our results show that in the gingival tissue of four patients suffering from chronic periodontitis, at least two antigen-specific $CD4^+$ T-cell populations were present. One was reactive with whole-cell antigens of periodontitis-associated bacteria, and the other was reactive with the autoantigen collagen type I.

These results support the concept that an autoimmune component might be involved in the pathogenesis of periodontitis (1, 4). One explanation for the presence of collagen-type-I-reactive T cells might be shared antigenic determinants on periodontitis-associated bacteria and collagen type I (1, 21). Although the T-cell epitopes involved are not yet known, molecular mimicry between collagen type I and the bacterial antigens seems unlikely, as none of the clones obtained showed any cross-reactivity. Common bacterial antigens like HSP are also not cross-reactive agents, as the T-cell clones were specific for one particular bacterium and were not reactive with HSP65 and HSP71. Another explanation might be an enhanced presentation of collagen type I peptides through inappropriate expression of HLA class II molecules (1, 33). Conditions conducive to such an increased presentation of collagen would be created by environmental factors like bacteria, toxins, or local cytokine production within the periodontal tissue. In situ staining for IL-4 and IFN-γ indeed suggests that local cytokine production might be involved in increased antigen presentation, as IFN-γ is known to enhance major histocompatibility complex expression on many cell types.

The collagen-type-I-reactive T-cell clones showed mainly a cytokine pattern characterized by relatively high levels of IL-4 and low levels of IFN-γ production, indicating a Th2-like pattern. The cytokine pattern of $CD4^+$ T-cell clones reactive with periodontitis-associated bacteria resembled that of Th0-like cells. This implies that endogenous antigens like collagen type I may contribute to the chronicity of the disease, whereas the exogenous bacterial antigens *P. intermedia*, *P. gingivalis*, and *A. actinomycetemcomitans* may be involved in early stages of periodontitis. This concept is consistent with the study of Hirsch et al. (14), who found that responses to collagen type I are induced by fragments released during degradation of periodontal tissue by bacterial proteases. The concept also corre-

lates with recent models of the induction and progression of other autoimmune diseases (25, 30).

Th0- and Th2-like lymphocytes were also observed among the non-antigen-specific $CD4^+$ T-cells clones, suggesting dominant T-cell subsets within the tissue.

Recently, two groups reported the absence of IL-4 mRNA in gingival mononuclear cells isolated from periodontitis patients (11, 19). This finding contrasts with our isolation of IL-4-producing T-cell clones from the periodontal lesions. However, it has been suggested by Yamazaki et al. (51) that mRNA for IL-4 is short-lived within the gingival tissue, since they were unable to detect IL-4 mRNA by in situ hybridization, while abundant IL-4 was present after immunohistochemical staining of the same tissue sections. It is interesting that for the $CD45RO^+$ subset, the percentage of IL-4-producing T cells was higher in periodontal lesions than in gingival tissue (24, 51).

From the rat model it was suggested that Th2 cells were protective against the development of periondontal disease (9, 50). However, we find Th2 cells dominant in chronically inflamed tissue. Adult periodontitis is a slowly proceeding disease, and it might well be that this slow process is due to protection by Th2 cells. In terms of our experiments, it is possible that production of IL-4 (by collagen-specific T cells) at sites of antigen specificity (production of IFN-γ by bacterial-antigen-specific T cells) will increase local B-cell activity and cause the switch to an Ig class type useless for opsonization because of the lack of C1q complement binding sites. Recently, two groups have shown that the isotype switch variant IgG4 is 24 times more frequent at sites of periodontitis than in serum (34, 40). Furthermore, IL-4 downregulates Th1 cells by blocking IL-2-dependent proliferation and IFN-γ secretion and thus has an indirect anti-inflammatory effect. Tissue damage therefore is limited to a slow ongoing process instead of to a local delayed-type hypersensitivity reaction with increased local tissue destruction. Disturbing the local IL-4 balance will inflame the disease and induce progression of the disease.

In addition to $CD4^+$ T-cell clones we were able to clone $CD8^+$ T cells. The clones exhibited a pattern similar to that of the $CD4^+$ clones. The immune regulatory role of $CD8^+$ T cells is difficult to interpret, but the results show that there are distinct subsets of $CD8^+$ cells that produce different cytokine patterns. Recently, it has been suggested that $CD8^+$ T cells play an important role in determining the pattern of cytokines produced by $CD4^+$ T cells and the isotypes of Igs expressed by B cells (17, 43). $CD8^+$ T cells appear to be active in early immune responses and so influence the direction that the responses take. However, the mechanisms of how $CD8^+$ T cells are recruited and activated and how they produce their effects are not known.

The recognition of bacterial antigens, including *Mycobacterium leprae*, *Listeria monocytogenes*, and *Streptococcus mutans* (13, 36, 48), by $CD8^+$ T cells via major histocompatibility complex class I molecules has been described. These bacteria are able to live and multiply intracellularly for some time. Recently, active internalization by receptor-mediated endocytosis and intracellular growth has also been described for *P. gingivalis* (31). Whether this is also the case for *P. intermedia* remains to be resolved.

The pathogenesis of chronic periodontal diseases is complex and depends on the host response and the route of sensitization. Chronic exposure to microorganisms and their products results in a continual release of inflammatory mediators, enzymes, growth factors, and cytokines, which triggers a cascade of immunological and nonimmunological reactions resulting in tissue destruction and bone resorption alternating with periods of healing. Our study suggests that cellular immunity directed against either periodontitis-associated bacteria or collagen type I might be at least one part of this process.

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