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Cytokine Profiles of Cells Extracted from Humans with Periodontal Diseases

E. Gemmell* **and G.J. Seymour**

Immunopathology Laboratory, Oral Biology and Pathology, Department of Dentistry, The University of Queensland, Queensland 4072, Australia; *to whom correspondence should be addressed

Abstract. FACS analysis was used to determine the percent interferon (IFN)-gamma-, interleukin (IL)-4-, IL-10-, and CD30-positive T-cells extracted from adult periodontitis (AP) and healthy/gingivitis (H/G) subjects. Additionally, the percentages of IL-1β-, IL-10- and IL-12-producing B-cells and macrophages were ascertained. The percent IL-10+ CD8 cells extracted from AP lesions was decreased compared with H/G tissues ($p = 0.033$), and the percent CD30+ CD4 ($p = 0.001$) and CD30+ CD8 ($p = 0.028$) cells was higher in AP than in H/G tissues. The percentages of IL-1β+ macrophages ($p = 0.003$) and IL-12+ B-cells ($p = 0.034$) were both higher in AP lesions than in H/G tissues. The specific effect of *Porphyromonas gingivalis* on the cytokine profiles of peripheral blood mononuclear cells isolated from *P. gingivalis*-infected AP and H/G patients was also determined. While there were no significant differences in the percent cytokine-positive T-cells after stimulation with *P. gingivalis* outer membrane antigens (OM) for 6 days compared with cells incubated in medium only, the percent CD30+ CD4 cells increased significantly ($p = 0.047$ and $p = 0.063$ for AP and H/G groups, respectively). There was also an increase in the percent IL-1β+ B-cells from AP patients ($p = 0.029$), and the percent IL-12+ monocytes from AP and H/G subjects was higher than the percent IL-12+ B-cells, both after stimulation with *P. gingivalis* OM ($p = 0.005$ for the AP group and $p = 0.058$ and therefore not quite significant for the H/G group) and when incubated in medium alone ($p = 0.016$ and $p = 0.015$ for AP and H/G groups, respectively). This study has shown that IL-10+ CD8 cells may be significant in gingival lesions, and that CD30+ T-cells indicative of Th2 or Th0 cells may play a role in progressive periodontal disease. This study has also shown that B-cells produce IL-1 in the gingival lesion and that *P. gingivalis* may be significant in the induction of B-cell-induced IL-1.

Key words: cytokines, T-lymphocytes, periodontal diseases, B-lymphocytes, macrophages.

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Introduction

Cytokines produced locally have an influence on the development of a particular immune response. Major advances have been made in the field of cytokine regulation of T-cell function, and so-called Type 1 and Type 2 cytokines may play predominant roles in determining the outcome of infection (Yamamura *et al.*, 1992). Originally classified in the mouse, Th1 cells produce IL-2, interferon (IFN)-gamma, and tumor necrosis factor (TNF)-beta, while Th2 cells are defined by the production of IL-4, IL-5, IL-6, IL-10, and IL-13. Both cell types produce IL-3, TNF-alpha, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Mosmann and Coffman, 1989; Kelso, 1995). Human CD4+ T-cells have cytokine patterns and functions comparable with those that exist in mice, although the synthesis of IL-2, IL-6, IL-10, and IL-13 is not as restricted to a single subset as in the mouse T-cell (Mosmann and Sad, 1996). The net effect of the Th1 cytokines IL-2 and IFN-gamma is to enhance cell-mediated responses, while that of the Th2 cytokine IL-4 is to suppress cell-mediated responses and hence enhance the resistance associated with humoral immunity (Modlin and Nutman, 1993). *In vivo* studies with cytokines and anti-cytokine antibodies provide support for the concept that the functional abilities of the Th1 and Th2 subsets are due mainly to the cytokines they produce (Street and Mosmann, 1991). There is no doubt that the balance between local levels of cytokines is important in determining the outcome of an immune response, and although many immune responses seem to involve predominant Th1 or Th2 cytokines (Kelso, 1995), the functional outcome may best be predicted by the cytokines involved and their respective antagonists.

IFN-gamma is found in sites of delayed-type hypersensitivity (DTH) reactions (Yamamura *et al.*, 1992; Tsicopoulos *et al.*, 1992), and the developing gingival lesion has been shown to follow a pattern similar to that of a controlled DTH response (Seymour *et al.*, 1988). Possible lower IL-2 and IFN-gamma levels in periodontal disease lesions suggest decreased Th1 responses (Gemmell and Seymour, 1994). IFN-gamma is produced during an immune response by antigen-specific T-cells and natural killer (NK) cells recruited by IL-2. The regulatory effects of IFN-gamma include activation of macrophages to enhance their phagocytic and tumor-killing capability as well as

activation and growth enhancement of cytotoxic T-lymphocytes (CTL) and NK cells (Morris, 1988; O'Garra, 1989). IFN-gamma appears to be a requisite for the induction of the Th1 subset (reviewed in Scott, 1993). Another cytokine IL-12, produced by monocytes/ macrophages, B-cells, and other accessory cells, has pleiotropic effects on NK cells and T-cells, including the induction of transcription and secretion of cytokines and enhancement of cytotoxicity activity. IL-12 also induces IFN-gamma production and plays a role in Th1 induction (Trinchieri, 1993).

In chronic inflammatory periodontal disease, the predominant lymphocyte in the stable lesion is the T-cell, while increased proportions of B-cells and plasma cells can be demonstrated in the progressive lesion (Seymour, 1987, 1991), indicating a role for Th2 cells in the progressive lesion. B-cells appear to be activated locally in the gingival lesion (Gemmell and Seymour, 1991), suggesting the presence of B-cell proliferation and differentiation factors such as the type 2 cytokine IL-4, which is required for the clonal expansion of antigen-specific B-cells. The cytokine IL-10 is also a growth and differentiation factor for activated B-cells (Rousset *et al.*, 1992). However, IL-10 plays a major role in suppressing immune and inflammatory responses (de Waal Malefyt *et al.*, 1992). It suppresses T-cell responses by inhibiting the antigen-presenting capacity of macrophages by down-regulating Class II MHC expression as well as IFN-gamma-induced macrophage-mediated destruction of pathogens (de Waal Malefyt *et al.*, 1993). IL-10 plays a role in diminishing DTH reactions and other cell-mediated responses and therefore may be significant in periodontitis.

IL-1 mediates tissue destruction *via* a number of pathways (Page, 1991) and has been reported to act as a co-stimulatory signal for Th2 but not Th1 cells (Chang *et al.*, 1990; Germann *et al.*, 1993). IL-1 levels are elevated in AP subjects compared with healthy individuals (Honig *et al.*, 1989), and this may lead to the proliferation of Th2 cells, resulting in B-cell proliferation. The most likely source of IL-1 in the gingival tissues has been reported to be the macrophage (Matsuki *et al.*, 1993); however, as yet, the contribution of B-cell-produced IL-1 in progressive periodontal lesions has not been determined.

Several studies have reported on the presence of cytokines in periodontal disease. Manhart *et al.* (1994) used a quantitative cell-blotting method to determine individual gingival cell IL-2 and IL-4 production. However, most reports have used the reverse-transcriptase polymerase chain-reaction (RT-PCR) method to determine the message in entire tissue specimens (Takeichi *et al.*, 1994; Ebersole and Taubman, 1994; Fujihashi *et al.*, 1996; Prabhu *et al.*, 1996). These techniques fail to identify the cell types, and there is an apparent lack of quantitation with the RT-PCR method. The short half-life of some mRNA's such as IL4 means that the results do not necessarily reflect actual cytokine production. While the ELISA technique has also been used extensively to measure large amounts of secreted cytokine, again this method does not recognize individual cell types. The aim of this study was to determine the local cytokine profiles in the gingival tissues using an intracytoplasmic tehnique to determine actual individual cytokine-producing cells. After cells were stained, flow cytometry was used to ascertain the percent IFN-gamma-, IL-4-, and IL-10-producing T-cells and IL-1β-, IL-10-, and IL-12-producing B-cells and macrophages extracted from AP and H/G subjects. Since *P. gingivalis* has been implicated as a major periodontopathogen (Genco and Slots, 1984; Mayrand and Holt, 1988; Okuda and Takazoe, 1988), the specific effect of this organism on the cytokine profiles of peripheral blood mononuclear cells isolated from *P. gingivalis*-infected AP and H/G patients was also determined. Additionally, the expression of CD30, which may be a marker of Th2 cells, was determined on T-cells extracted from AP and H/G tissues as well as on peripheral blood T-cells after stimulation in culture with *P. gingivalis* OM.

Materials and methods

Subjects

Gingival tissue was obtained from a total of 88 patients undergoing periodontal surgery for both disease- and non-disease-related reasons at a private dental clinic in Brisbane, Australia. Thirty-nine biopsies taken from patients undergoing surgery for non-disease-related reasons displayed minimum periodontal disease, with probing attachment levels of < 4 mm from the cemento-enamel junction, exhibited minimal bleeding when probed, and were classified as a H/G group. Previous studies have shown that apparently clinically healthy gingivae display histological evidence of inflammation similar to that seen in marginal gingivitis (Seymour *et al.*, 1983, 1988), so that clinically healthy and marginal gingivitis samples were grouped together. Tissues obtained from 49 patients showed moderate to advanced disease and were classified as an AP group with probing attachment levels > 5 mm from the cemento-enamel junction. All subjects in this group had previous oral hygiene in-struction and scaling and root planing prior to surgery. All sites in this group had the presence of bleeding when gently probed from the base of the periodontal pockets at the time of surgery. The informed consent of each patient, to use tissue which would otherwise be discarded, was obtained at the time of surgery. Institutional ethics review committee approval for the study was obtained.

Peripheral blood and plaque samples were collected from subjects attending the Periodontal Clinic, Dental School, The University of Queensland, and the Oral Care Research Programme in the Department of Dentistry at the University of Queensland. They were divided into two groups on the basis of past disease activity. The H/G group had very little periodontal breakdown, with minimal bone loss and minimal loss of attachment from the cemento-enamel junction, as determined by x-ray. The AP group had loss of attachment of > 5 mm in at least 4 sites, and all had significant radiological bone loss of at least 6 mm as measured from the cemento-enamel junction in 4 sites. All subjects were in good general health and had not taken antibiotics or anti-inflammatory drugs within 3 months prior to examination. A written explanation of the purpose of the study was provided for each subject, and signed consent was obtained according to the Helsinki Declaration. Institutional ethics review committee approval for the study was obtained. Subjects were selected further on the basis of the presence of *P. gingivalis* in the plaque samples and the presence of anti-*P. gingivalis* anti-

bodies in the serum, so that all subjects finally selected had been exposed to *P. gingivalis* infection (Gemmell *et al.*, 1995a). Thirty-one subjects (18 males and 13 females) comprised the AP group, and 31 (20 males and 11 females) comprised the H/G group.

Bacteria and outer membrane preparation

The periodontopathic bacterium *Porphyromonas gingivalis* ATCC 33277 was used in this study. The organism was cultured anaerobically as described by Bird and Seymour (1987). The outer membrane (OM) was prepared by a modification of the methods of Watanabe *et al.* (1989) and Deslauriers *et al.* (1990). Briefly, 10 g of *P. gingivalis* (wet weight) was suspended in 20 mL of 10 mM Tris-HCl, pH 7.5, containing 0.05 mM phenylmethanesulfonyl fluoride (PMSF). The cells were sonicated (Sonics & Materials Vibra-Cell, Danbury, CT, USA) for 16 min at two-minute intervals on ice. The sonicated preparation was centrifuged at 10,000 x *g*, and the supernatant containing the crude membrane was further centrifuged at 80,000 x *g* for 2 hrs. The crude membrane preparation was re-suspended in 5 mL of 10 mM Tris-HCl containing PMSF and mixed with an equal volume of 4% Triton X-100 to dissolve the inner membrane. The suspension was stirred for 30 min at room temperature and then centrifuged for 2 hrs at 100,000 x *g* to pellet the OM. The OM was washed twice in distilled water by centrifugation at 100,000 x *g* for 2 hrs prior to being freeze-dried. The protein concentration was determined by means of the BCA protein assay (Pierce, Rockford, IL, USA). Gemmell *et al.* (1995b) have demonstrated that specific antibodies in the sera of both healthy subjects and periodontal disease patients who have *P. gingivalis* in their plaque recognized 32 antigens in the *P. gingivalis* OM preparation, and these ranged in molecular weight from 4.2 to 169.2 kDa. Fifteen of these in the molecular weight range of 4.2 to 47.9 kDa were attributed to LPS, so that this OM preparation contains both protein and LPS antigens.

Detection of *P. gingivalis* and serum anti-*P. gingivalis* antibodies

Several sites with deepest probing depths were sampled after removal of supragingival plaque and then pooled. The presence of *P. gingivalis* in the plaque samples was determined by means of an ELISA previously reported by Bird (1992). Briefly, 96-well high-binding Maxisorb Immunoplates (Nunc, Roskilde, Denmark) were coated with the plaque samples at a 1/2 dilution in carbonate buffer, pH 9.6, followed by the addition of a monoclonal anti-*P. gingivalis* antibody CB5.C5 (1 mg/mL) (1/2000) to detect *P. gingivalis* in the samples and then biotin-conjugated rabbit anti-mouse Ig (Amersham International, Buckinghamshire, UK) (1/10,000) and finally streptavidin horseradish peroxidase (DAKO, Glostrup, Denmark). After incubation with substrate containing 0.0075% H_2O_2 and 2.5 mM O'Tolidine (Eastman Kodak, Rochester, NY, USA), the optical densities of the wells were read at an absorbance of 450 nm. Protein concentrations of *P. gingivalis* were determined (BCA protein assay), and dilutions of *P. gingivalis* were used on each plate to construct a standard curve from which the μg/mL *P. gingivalis* protein in the plaque samples were determined (Gemmell *et al.*, 1995b).

To determine the presence of anti-*P. gingivalis* antibodies, we used an ELISA similar to the one used above (Gemmell *et al.*, 1995b). Serum was separated from venous blood obtained from each subject and added to immunoplates coated with OM of *P. gingivalis* (0.5 μg protein/mL). After the addition of biotin-conjugated sheep anti-human immunoglobulins (Amersham International, Buckinghamshire, UK) (1/10,000), streptavidin horseradish peroxidase was then added, and the optical densities of wells were determined as above. Anti-*P. gingivalis* antibodies in the test samples were determined from a standard curve of dilutions of the monoclonal anti-*P. gingivalis* antibody (CB5.C5) (Bird, 1992; Ni Eidhin and Mouton, 1993).

Cell preparation

Mononuclear cells were isolated from the peripheral blood of the AP and H/G subjects by Ficoll-Paque centrifugation. Gingival mononuclear cells from the AP and H/G surgeries were extracted as described by Daly *et al.* (1983). Briefly, each biopsy was cut into approximately 1-mm fragments and then incubated in RPMI-1640 medium (Flow Laboratories, North Ryde, Australia) containing 10% heat-inactivated pooled human AB serum, 1% glutamine (Sigma Chemical Co., St. Louis, MO, USA), 50 IU/ mL penicillin, 50 μg/mL streptomycin (Flow Laboratories), 1.25 μg/mL fungizone, and 1 mg/ mL collagenase Type 2 (Sigma) for 90 min with gentle rolling at 37ºC. The ratio of tissue to medium was 100 mg:1 mL. The fragments were then worked through a stainless steel grid (0.5-mm mesh size) to obtain a single cell suspension. These cells were washed twice to remove the collagenase and then centrifuged on a Ficoll-Paque gradient to remove keratinocytes, fibroblasts, and tissue debris. Both peripheral blood and

Table 1. Clinical data of AP and H/G groups

Study	Clinical Status	Mean Age ± SE[a]	Sex M	Sex F	Attachment Level from Cemento-enamel Junction
T-cell cytokines and CD30	AP (n[b] = 15)	42.3 ± 2.6	8	7	> 5 mm in at least 4 sites
	H/G (n = 13)	44.9 ± 3.7	5	8	< 4 mm
B-cell and macrophage IL-1β and IL-10	AP (n = 18)	46.7 ± 2.5	8	9	> 5 mm in at least 4 sites
	H/G (n = 14)	44.0 ± 3.4	4	10	< 4 mm
B-cell and macrophage IL-12	AP (n = 16)	51.8 ± 3.0	11	5	> 5 mm in at least 4 sites
	H/G (n = 12)	42.6 ± 4.2	8	4	< 4 mm

[a] Standard error of the mean.
[b] Number of gingival tissue samples.

gingival mononuclear cells were counted and viabilities determined by ethidium bromide and acridine orange (20 μg/mL).

Cell culture

Peripheral blood mononuclear cells were suspended in RPMI-1640 medium supplemented with 10% pooled human AB serum, glutamine, and antibiotics as described above and cultured at a concentration of 5 x 10^5 lymphocytes/200-μL well in round-bottomed 96-well tissue culture plates (Nunc, Roskilde, Denmark). The cells were stimulated with the OM of *P. gingivalis* at a concentration of 1.0 μg/well for 6 days in a humidified atmosphere of 5% CO_2 in air at 37ºC. A previous study has shown that 1.0 μg/well *P. gingivalis* OM is the optimum concentration, and incubation for 6 days is the optimum incubation period for peripheral blood T-cell proliferation under these conditions (Gemmell *et al.*, 1996). Control cells were treated as above in the absence of *P. gingivalis* OM.

Flow cytometric analysis

A technique based on that of Andersson *et al.* (1990) was used to determine the percent CD4+ and CD8+ T-cells staining positive for cytoplasmic IL-4, IFN-gamma, and IL-10 and the percent CD19+ B-cells and CD14+ monocytes/ macrophages staining positive for IL-1β, IL-10, and IL-12. We achieved surface membrane staining of T-cells, B-cells, and monocytes/ macrophages in freshly isolated gingival cell suspensions and peripheral blood cells cultured with *P. gingivalis* OM for 6 days by washing the cells with PBS/0.1% azide (PBS/azide) and then incubating them for 30 min at 4ºC with either phycoeryrthrin (PE)-conjugated mouse anti-human CD4, PE-conjugated mouse anti-human CD8, PE-conjugated mouse anti-human CD19, or PE-conjugated mouse anti-human CD14, all purchased from DAKO (Glostrup, Denmark). For intracellular cytokines to be detected, cells have to be fixed and permeabilized to allow antibodies to penetrate the cell membrane and the membranes of the endoplasmic reticulum and Golgi organelle (Sander *et al.*, 1991). Fixation by paraformaldehyde has been shown to be gentle, to preserve leukocyte membrane stability and cell morphology, as well as to retain antigenicity and to maintain the light-scattering properties of leukocytes being analyzed by flow cytometry (reviewed in Sander *et al.*, 1991). Therefore, membrane-stained CD4 or CD8 cells were fixed in 4% paraformaldehyde in PBS for 5 min at room temperature, followed by permeabilization with proteinase K in 20 mM TRIS/EDTA buffer (10 μg/mL) for 10 min at 37ºC (Gemmell *et al.*, 1995a). The cells were then incubated for 30 min at 4ºC with rabbit anti-human IL-1β, sheep anti-human IL-4 rabbit anti-human IFN-gamma (Genzyme,

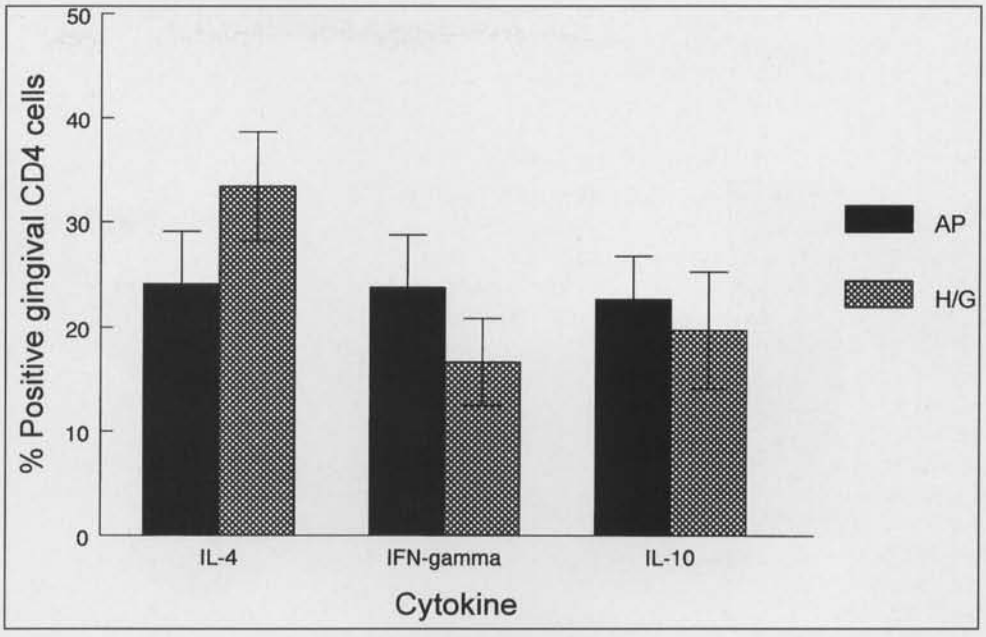

Figure 1. FACS analysis demonstrated no differences in the percent IL-4+, IFN-gamma+, and IL-10+ CD4 T-cells extracted from the gingival tissues of AP (N = 15) and H/G (N = 13) subjects.

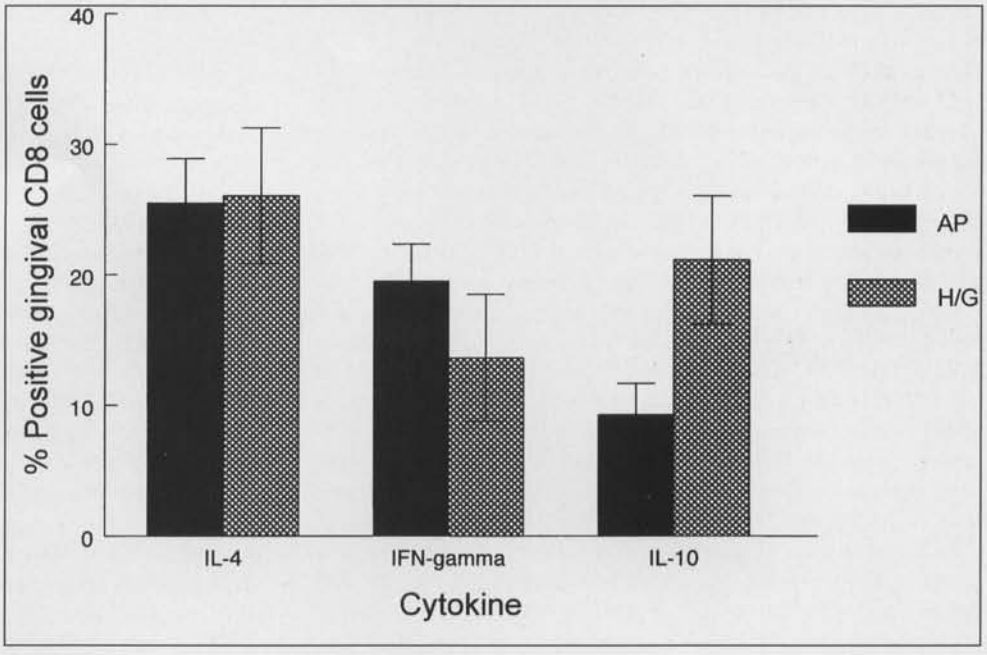

Figure 2. Gingival mononuclear cells were extracted from AP (N = 15) and H/G (N = 13) subjects and the percent IL-4+, IFN-gamma+, and IL-10+ CD8 T-cells determined by FACS analysis. The percent IL-10+ CD8 cells was higher in H/G tissues than in AP tissues.

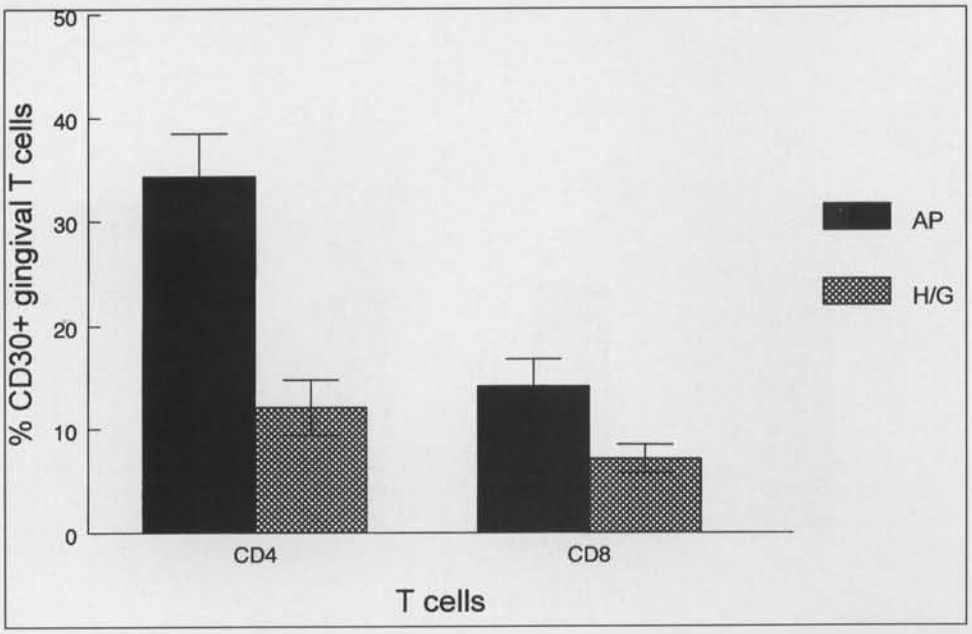

Figure 3. Mononuclear cells were extracted from the gingival lesions of AP (N = 15) and H/G (N = 13) subjects, and FACS analysis showed that the percent CD30+ CD4 and CD8 T-cells was higher in AP than H/G subjects.

Boston, MA), rat anti-human IL-10, or mouse anti-human IL-12 (p35/p7O) (Pharmingen, San Diego, CA), followed by biotinylated swine anti-rabbit Ig, biotinylated rabbit anti-goat Ig (cross-reacts with sheep antibodies), biotinylated rabbit anti-rat Ig, or biotinylated rabbit anti-mouse Ig (DAKO) and then fluorescein-conjugated (FITC) streptavidin (DAKO). For each test sample, a control was performed in which the CD4, CD8, CD19, or CD14 cells were incubated in PBS in place of the anti-cytokine antibody after fixation and permeabilization to assess non-specific binding of the final two layers. A PE-conjugated specific mouse Ig isotype antibody was used in place of the CD4, CD8, CD19, or CD14 antibodies for assessment of non-specific binding of mouse monoclonal antibodies to human cell-surface antigens. Ten thousand stained cells from each sample were analyzed by dual-color flow cytometry on a FACS Analyzer (Becton Dickinson, Mountain View, CA, USA) after cell debris was gated out by side-scatter and volume parameters. We determined the percent cells positive for intracytoplasmic staining by subtracting the percentage of positive cells in the control from the percentage of positive cells in the test sample. Gemmell *et al.* (1995a) have previously validated local perinuclear FITC cytoplasmic staining of cytokines by fluorescent microscopy, which also confirmed the lack of FITC membrane staining.

Statistics

We analyzed results with a one-way analysis of variance using the Minitab package (Minitab Inc., State College, PA, USA).

Results

Gingival tissues

Gingival mononuclear cells were extracted from 15 AP and 13 H/G subjects, and the percentages of CD4+ and CD8+ cells producing IL-4, IFN-gamma, and IL-10 as well as of CD30+ T-cells were determined. The percentages of CD19+ B-cells and CD14+ macrophages positive for IL-1β, IL-10 (18 AP and 14 H/G), and IL-12 (16 AP and 12 H/G) were also determined. The mean ± standard error of $11.7 \times 10^5 \pm 1.5$ and $9.2 \times 10^5 \pm 3.2$ cells were extracted from AP and H/G gingival tissues, respectively. The mean percent ± standard error of CD4 and CD8 cells recovered was 7.9 ± 1.3 and 8.1 ± 1.0, respectively, and that for CD19 and CD14 cells was 6.3 ± 0.6 and 8.3 ± 0.8, respectively. The mean percent of each cell type recovered from the gingival mononuclear cell suspensions was approximately 0.6 to 0.9×10^5 cells. The clinical data of the AP and H/G subjects from which the gingival samples were taken are presented in Table 1. There were no differences in the mean ages between the AP and H/G groups in all studies.

There were no significant differences between the percent IL-4+ or IFN-gamma+ CD4 and CD8 cells or IL-10+ CD4 cells extracted from AP or H/G lesions. There was, however, a significant decrease in the percent IL-10+ CD8 cells extracted from AP lesions compared with H/G tissues ($p = 0.033$) (Figs. 1, 2). The percent CD30+ CD4 ($p = 0.001$) and CD30+ CD8 ($p = 0.028$) cells was significantly higher in AP than in H/G tissues (Fig. 3). The CD4/CD8 ratio in the AP group (0.6 ± 0.1) was significantly lower than in the H/G group (1.4 ± 0.2) ($p = 0.001$), confirming the clinical status of the two groups. There did not appear to be any relationship between the percent cytokine-positive T-cells in individual lesions and the CD4/CD8 ratio.

There were no significant differences between the percent IL-10+ CD19+ and CD14+ cells extracted from AP or H/G lesions. However, the percent IL-1β+ CD14 cells was significantly higher in AP lesions than in H/G tissues ($p = 0.003$), although there were no differences in the percent IL-1β+ CD19 cells (Figs. 4, 5). The CD14/CD19 ratio rose from 1.1 ± 0.1 in AP tissues to 1.8 ± 0.3 in H/G tissues ($p = 0.025$), reflecting the increased percent B-cells in AP lesions.

The percent IL-12+ CD19 cells was higher in AP than in H/G tissues ($p = 0.034$), and although the percent IL-12+ CD14 cells was also higher in AP lesions, this was not significant at the 0.05 level (Figs. 4, 5). Again, the CD14/CD19 ratio increased from 1.1 ± 0.2 in AP tissues to 1.7 ± 0.3 in H/G tissues, although this difference was not significant.

Peripheral blood

We isolated peripheral blood mononuclear cells from *P. gingivalis*-positive AP and H/G subjects and stimulated them with *P. gingivalis* OM for 6 days to determine whether

there was any difference in the effect of this organism on cytokine-producing cells.

There were no significant differences in the percentages of IL-4+, IFN-gamma+, or IL-10+ CD4 or CD8 cells from nine AP and ten H/G subjects after stimulation with *P. gingivalis* OM compared with cells incubated in medium only (Tables 2, 3). Fig. 6 details the FACS profiles of CD8 cells from a periodontal disease patient demonstrating only small increases in the percent IFN-gamma- and IL-10-positive CD8 cells after specific antigen stimulation. The mean age ± SE of the two groups was 46.9 ± 3.7 (AP) and 51.0 ± 5.3 (H/G).

The percent CD30+ CD4 cells isolated from 11 AP and 11 H/G subjects increased significantly after stimulation with *P. gingivalis* OM compared with cells incubated with medium alone ($p = 0.047$ and $p = 0.063$ for AP and H/G groups, respectively) (Fig. 7). There was no effect on the percent CD30+ CD8 cells. Again, there was no difference in the mean age ± SE of 45.2 ± 3.2 and 51.4 ± 4.8 between the AP and H/G groups, respectively.

Peripheral blood was isolated from a further 11 AP and 10 H/G subjects (mean age ± SE of 41.4 ± 2.9 and 41.1 ± 3.0, respectively). There was no difference in the percent IL-10+ CD19 or CD14 cells isolated from either AP or H/G subjects after stimulation *in vitro* with or without *P. gingivalis* OM. There was, however, an increase in the percent IL-1β+ CD19 cells from AP patients after stimulation with *P. gingivalis* OM compared with cells incubated in medium alone ($p = 0.029$). The percent IL-1β+ CD19 cells was higher than the percent IL-1β+ CD14 cells after *P. gingivalis* stimulation, although this was not quite significant ($p = 0.058$) (Table 4).

The percent IL-12+ CD14 cells from AP subjects was higher than the percent IL-12+ CD19 cells, both after stimulation with *P. gingivalis* OM ($p = 0.005$) and when incubated in medium alone ($p = 0.016$). Similarly, there was a higher percent of IL-12+ CD14 cells than IL-12+ CD19 cells from H/G subjects when cells were incubated in medium only ($p = 0.015$), and although this was also the case for cells stimulated with *P. gingivalis* OM, this was not quite significant ($p = 0.058$) (Tables 4, 5).

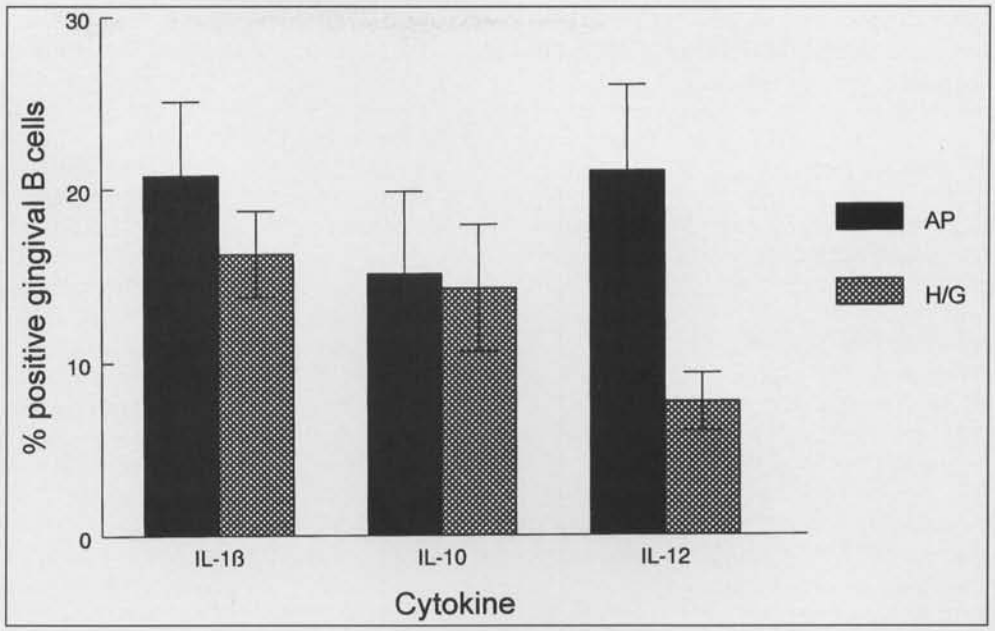

Figure 4. Gingival mononuclear cells were extracted from AP and H/G subjects, and the percentages of IL-1β+, IL-10+ (AP, N = 18; H/G, N = 14), and IL-12+ (AP, N = 16; H/G, N = 12) CD19 B-cells were determined by FACS analysis. The percent IL-12+ CD19 cells was higher in AP than in H/G lesions.

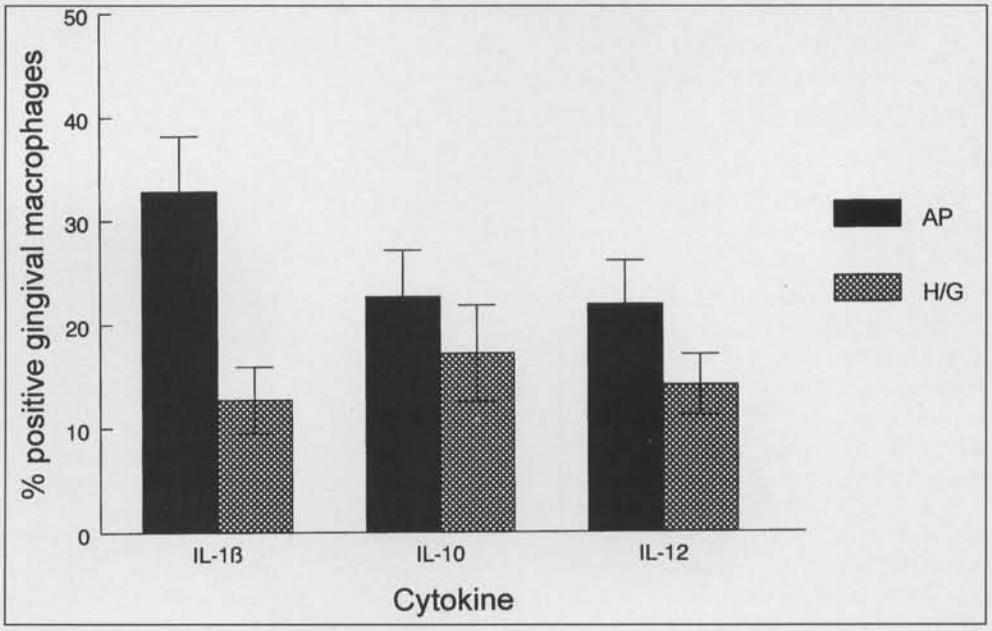

Figure 5. Gingival mononuclear cells were extracted from AP and H/G subjects, and the percentages of IL-1β+, IL-10+ (AP, N = 18; H/G, N = 14), and IL-12+ (AP, N = 16; H/G, N = 12) CD14 macrophages were determined by FACS analysis. The percent IL-1β + CD14 cells was higher in AP than in H/G legions.

Discussion

The increased numbers of B-cells and plasma cells in

Table 2. Mean percent (± SE[a]) IL-4-, IFN-gamma-, and IL-10-producing peripheral blood CD4 and CD8 T-cells isolated from AP subjects (n = 9) after stimulation with *P. gingivalis* OM

Cytokine-positive Cells	Cells Incubated in Medium Only	Cells Stimulated with *P. gingivalis* OM
IL-4+ CD4 cells	50.0 ± 7.4	44.9 ± 8.2
IFN-gamma+ CD4 cells	21.0 ± 8.4	20.9 ± 7.9
IL-10+ CD4 cells	32.6 ± 9.4	22.2 ± 6.2
IL-4+ CD8 cells	62.5 ± 8.7	60.0 ± 7.8
IFN-gamma+ CD8 cells	10.1 ± 5.8	14.7 ± 8.9
IL-10+ CD8 cells	14.3 ± 4.8	19.1 ±6.5

[a] Standard error of the mean.

Table 3. Mean percent (± SE[a]) IL-4-, IFN-gamma-, and IL-10-producing peripheral blood CD4 and CD8 T-cells isolated from H/G subjects (n = 10) after stimulation with *P. gingivalis* OM

Cytokine-positive Cells	Cells Incubated in Medium Only	Cells Stimulated with *P. gingivalis* OM
IL-4+ CD4 cells	63.7 ± 7.6	56.4 ± 9.2
IFN-gamma+ CD4 cells	21.0 ± 6.6	22.9 ± 9.1
IL-10+ CD4 cells	26.2 ± 6.5	25.1 ± 5.8
IL-4+ CD8 cells	51.9 ± 10.6	60.5 ± 11.3
IFN-gamma+ CD8 cells	17.4 ± 8.0	16.7 ± 8.9
IL-10+ CD8 cells	14.9 ± 5.5	21.4 ± 7.4

[a] Standard error of the mean.

progressive lesions (Seymour and Greenspan, 1979) suggest an increased presence of B-cell proliferation and differentiation cytokines such as IL-4. However, the present study demonstrated that there was no difference in the percentages of IL-4+ CD4 or CD8 cells extracted from either AP or H/G tissues. Although there were no differences in the percentages of IL-4+ T-cells in AP and H/G gingival tissues, other cytokines such as IL-13, with roles in B-cell responses, may be significant in the periodontal lesion. Similarly, with the reported suppression of T-cell-mediated responses in periodontal disease (Cole *et al.*, 1987; Evans *et al.*, 1989), a decrease in IFN-gamma could be predicted. Again, this was found not to be the case in the present study, although reduced or negligible levels of IFN-gamma as measured by an immunoassay in gingival mononuclear cell culture supernatants have been reported (Gemmell and Seymour, 1994).

The data in the present study indicate that both Th1 and Th2 as well as/or Th0 cells are found in both gingivitis and periodontitis tissues, or may offer support for the concept that such distinction of T-cells in humans does not in reality exist (Kelso, 1995). This is also suggested by the studies of Ebersole and Taubman (1994) and Takeichi *et al.* (1994), who demonstrated the presence of message for both Th1 and Th0 cytokines in diseased gingival tissues, and of Prabhu *et al.* (1996), who showed IL-2, IL-4, IL-6, IL-10, and IFN-gamma expression in diseased or healthy gingivae with no skewing toward a Th1 or Th2 profile. Although there were no differences in the profiles of the Th2 cytokine IL-4 and the Th1 cytokine IFN-gamma, the results of the present study showed that a higher percent CD30+ CD4 and CD8 cells were extracted from AP lesions than from H/G tissues, and also that *P. gingivalis* induced an increase in the percent peripheral blood CD30+ CD4 cells *in vitro*. CD30 is a member of the tumor necrosis factor/nerve growth factor receptor family (Smith *et al.*, 1990; Durkop *et al.*, 1992) and was originally described as a surface molecule on Hodgkin's and Reed-Sternberg cells in patients with Hodgkin's disease (Schwab *et al.*, 1982). Under normal conditions, CD30+ cells are not observed in the peripheral blood but are found in the proximity of B-cell follicles of lymphoid tissues as well as on the edge of germinal centers (Stein *et al.*, 1985). However, peripheral blood cells activated *in vitro* with Th2-inducing antigens such as TES (*Toxocara canis* excretory/secretory antigen) have been shown to express CD30, whereas Th1-inducing antigens such as PPD do not. CD30 has been reported to be preferentially expressed on Th2 and Th0 cells (Del Prete *et al.*, 1995), and even though the percentages of positive cells were small in this study, these cells may be significant in the activation of B-cells which occurs locally in the periodontitis lesion (Gemmell and Seymour, 1991). A role for CD30+ T-cells has been suggested in a number of diseases, including SLE and HIV, in which elevated serum levels of soluble CD30 have been detected and in which Th2 responses predominate (Del Prete *et al.*, 1995).

IL-10 appears to have a number of often-conflicting actions on cells. Not only does it contribute to the clonal expansion of antigen-specific B-cells (Rousset *et al.*, 1992), but also it suppresses Th1 and other cell-mediated responses and may therefore exacerbate inflammatory conditions. IL-10 has also been shown to inhibit the actions of pro-inflammatory cytokines such as IL-1 and IL-6 (de Waal Malefyt *et al.*, 1993), which induce tissue destruction and bone resorption (Stashenko *et al.*, 1987; Roodman, 1992), and to induce an increase in the production of IL-1 receptor antagonist (IL-1ra) (de Waal Malefyt *et al.*, 1993), which would result in dampening of the destructive immune response. The results of the present study have shown a lower percent gingival IL-10+ CD8 cells extracted from AP lesions compared with H/G tissues. Not only is there a greater proportion of CD8 cells than CD4 cells in progressive lesions (Taubman *et al.*, 1984; Cole *et al.*, 1987), but also a lower percent produce IL-10. Therefore, in H/G tissues, IL-10 may contribute to decreased inflammation and a stable lesion by dampening down macrophage responses with a consequent reduction in tissue destructive cytokines such as IL-1, whereas in AP lesions, lower amounts of IL-10 may contribute to disease progression. Furthermore, the

results of this study demonstrated that in individual cases, a high percent of IL-10+ T-cells was extracted from some tissue samples, either AP or H/G lesions, while a very low percent or no IL-10+ T-cells were extracted from others. Again, the presence of IL-10+ T-cells may indicate a stable lesion, whereas a low percent may indicate a progressive lesion. A role for IL-10 has also been suggested by another study which used RT-PCR to demonstrate two profiles for CD4 cells isolated from inflamed periodontal tissues. One pattern showed the presence of IFN-gamma, IL-6, IL-10, and IL-13 mRNA, while the other pattern was similar with the exception of a lack of IL-10 mRNA (Yamamoto *et al.*, 1997). Correlation of clinical data with the presence or absence of IL-10 may resolve this issue.

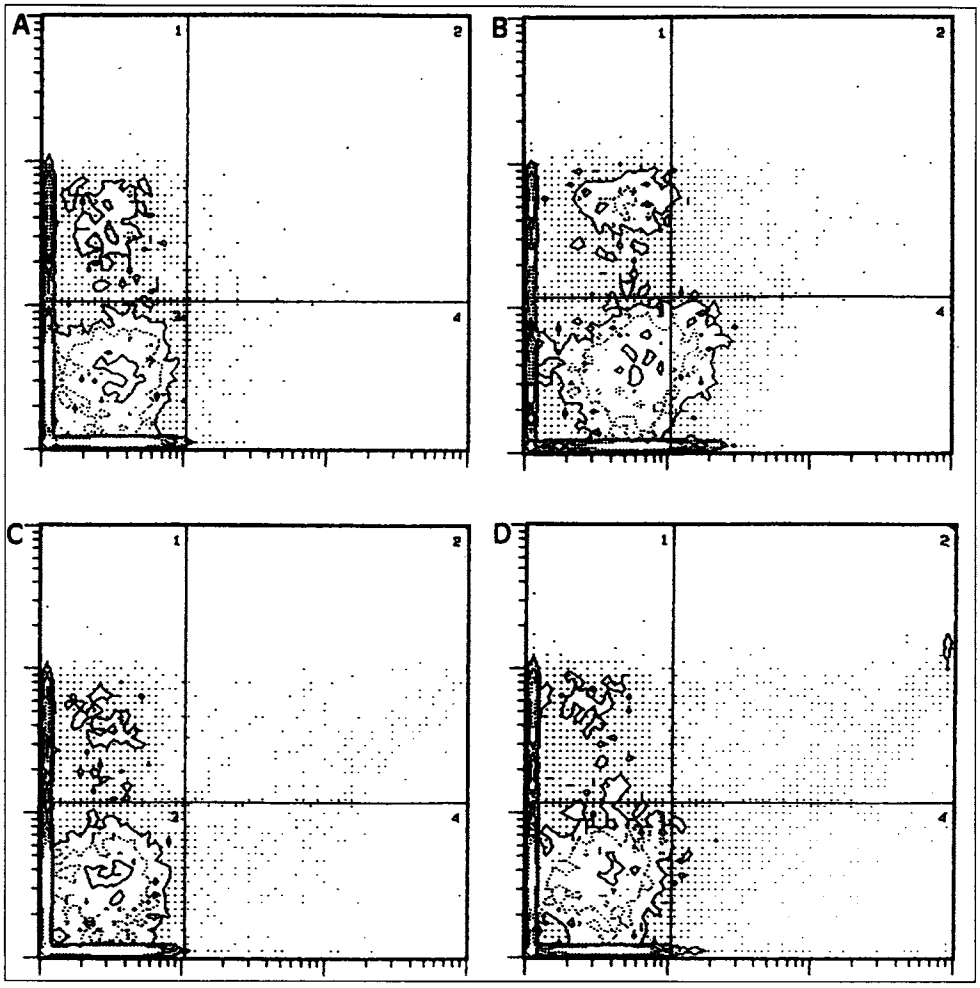

Figure 6. Peripheral blood mononuclear cells from a periodontitis subject were stimulated with and without *P. gingivalis* OM for 6 days, and the FACS profiles for IFN-gamma+ CD8 cells and IL-10+ CD8 cells after incubation in medium alone (A and C, respectively) and after incubation with OM antigens (B and D, respectively) are demonstrated. There was a slight increase in both the percent IFN-gamma and IL-10+ cells from this particular patient only after specific antigen stimulation.

Several studies have used RT-PCR to detect a range of cytokines in gingival periodontal disease (Takeichi *et al.*, 1994; Ebersole and Taubman, 1994; Fujihashi *et al.*, 1996; Prabhu *et al.*, 1996; Yamamoto *et al.*, 1997). In determining what is actually going on in the gingival tissues, investigators must relate the presence of message to the actual production of cytokines. In this context, the present study used an intracytoplasmic technique to detect the presence of individual cytokine-producing cells and demonstrated that all the cytokines studied were produced by cells extracted from periodontal diseased tissues and confirms the results of the above studies, showing no clear pattern of cytokines. These results could be in agreement with the concept that a clear distinction between Th1 and Th2 does not exist (Kelso, 1995) or suggest that both Th1 and Th2 cells are involved in the pathogenesis of periodontal disease.

P. gingivalis had no stimulatory effect on the percent IL-4+, IFN-gamma+, or IL-10+ peripheral blood CD4 or CD8 cells *in vitro*. Mahanonda *et al.* (1989) demonstrated that the median precursor T-cell frequency to *P. gingivalis* in the peripheral blood of both healthy and periodontal disease subjects was of the order of $50/10^6$ cells. If *P. gingivalis* activates antigen-specific T-cells, because of the low numbers in the circulation, any changes in the numbers of cytokine-positive cells may not be detectable. *P. gingivalis* did induce a significant increase in the percent CD30+ CD4 cells from both AP and H/G subjects compared with cells incubated in medium alone, further implicating the CD30+ subset and possibly Th2 and Th0 cells in periodontal disease.

In addition to T-cells, macrophages and B-cells are also major sources of cytokines. In the present study, neither B-cells nor macrophages contributed to any differences in IL-10+ cells in either AP or H/G lesions, nor did *P. gingivalis* induce any increase or decrease in the percent IL-10+ cells. The present study has shown, therefore, that any specific role played by IL-10 in the periodontal lesion may be due to the CD8 subpopulation of T-cells.

IL-12 provides a link between the natural resistance mediated by phagocytic cells and NK cells and the adaptive immunity mediated by helper T-cells, cytolytic T-cells, and B-cells. IL-12 is reported to play a role in the differentiation of Th1 cells (Trinchieri, 1993). In the present study, macrophages extracted from both AP and H/G tissues were the major producers of IL-12 compared with B-cells. This was also the case for peripheral blood macrophages from either AP or H/G subjects after stimulation *in vitro* with or

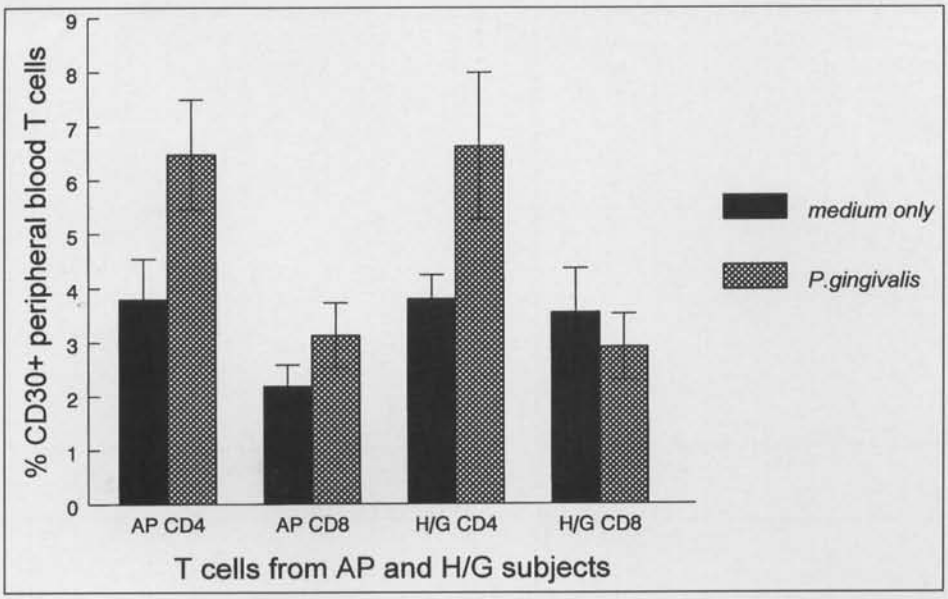

Figure 7. Mononuclear cells were isolated from the peripheral blood of AP (N = 11) and H/G (N = 11) subjects infected with *P. gingivalis* and stimulated in the presence or absence of *P. gingivalis* OM *in vitro*. After 6 days in culture, FACS analysis demonstrated that the percent CD30+ CD4 but not CD8 T-cells from both AP and H/G subjects increased after specific antigen stimulation.

Table 4. Mean percent (± SE[a]) IL-1β-, IL-10-, and IL-12-producing peripheral blood CD19+ B-cells and CD14+ monocytes isolated from AP subjects (n = 11) after stimulation with *P. gingivalis* OM

Cytokine-positive Cells	Cells Incubated in Medium Only	Cells Stimulated with *P. gingivalis* OM
IL-1β+ CD19 cells	20.8 ± 5.5	36.4 ± 3.7
IL-10+ CD19 cells	36.1 ± 6.6	30.7 ± 5.6
IL-12+ CD19 cells	37.2 ± 7.8	28.0 ± 6.3
IL-1β+ CD14 cells	17.4 ± 6.4	20.1 ± 6.7
IL-10+ CD14 cells	35.3 ± 6.5	33.1 ± 7.9
IL-12+ CD14 cells	62.5 ± 5.3	55.6 ± 5.6

[a] Standard error of the mean.

Table 5. Mean percent (± SE[a]) IL-1β-, IL-10-, and IL-12-producing peripheral blood CD19+ B-cells and CD14+ monocytes isolated from H/G subjects (n = 10) after stimulation with *P. gingivalis* OM

Cytokine-positive Cells	Cells Incubated in Medium Only	Cells Stimulated with *P. gingivalis* OM
IL-1β+ CD19 cells	35.2 ± 5.3	40.8 ± 6.6
IL-10+ CD19 cells	41.6 ± 6.3	40.1 ± 8.6
IL-12+ CD19 cells	37.7 ± 7.2	40.2 ± 8.0
IL-1β + CD14 cells	27.9 ± 8.3	20.8 ± 8.7
IL-10+ CD14 cells	40.1 ± 7.6	49.5 ± 7.4
IL-12+ CD14 cells	66.0 ± 7.4	63.1 ± 7.8

[a] Standard error of the mean.

without *P. gingivalis* OM. These results suggest that differences between clinical states may not be due to IL-12, which therefore may not be a significant factor in periodontal disease. In any case, recent reports have demonstrated that the addition of IL-12 to T-cell cultures during activation resulted in the promotion of the Th2 cytokine IL-4 as well as IFN-gamma with or without IL-10 (Lamont and Adorini, 1996) and to the potentiation of established Th2 responses by increasing the proliferation and IL-4 production of established Th2-like T-cell clones (Jeannin *et al.*, 1995), bringing into question the central role of IL-12 in Th1 commitment. As well as being protective, IL-12 can also promote detrimental effects. Administration of doses of IL-12, reportedly beneficial in other infections, during experimental viral infections resulted in adverse effects, including inhibition of CTL activity and virus-induced CD8+ T-cell expansion, and the accompanied induction of TNF was a pivotal factor in the ensuing pathology (Orange *et al.*, 1994). This study demonstrated the potential complications arising from IL-12 administration during an ongoing immune response.

IL-1 is a key mediator of chronic inflammatory disease with the potential to initiate tissue destruction and bone loss in periodontal disease (Birkedal-Hansen, 1993). In the present study, both B-cells and macrophages were IL-1 β -positive in both AP and H/G tissues, although the percent positive macrophages was higher in AP than in H/G lesions. Since there are higher numbers of B-cells in AP lesions, the contribution of IL-1β by these cells could be considerably greater than that in H/G tissues. This is confirmed by the results showing an increase in the percent IL-1β+ B-cells from AP patients after stimulation with *P. gingivalis* compared with cells incubated in medium alone, and the percent positive B-cells was higher than the percent positive macrophages after *P. gingivalis* stimulation. The concept of polyclonal B-cell activation in periodontal disease was first introduced in the 1980's, and a wide variety of bacterial products, including cell wall components such as LPS in Gram-negative bacteria, has the ability to stimulate human B-cells polyclonally (Tew *et al.*, 1989). Therefore, in the present study, even though the results indicate that *P. gingivalis* may have stimulated T-cells in an antigen-specific way, the IL-1 results suggest

a polyclonal stimulation of B-cells.

This study has shown that IL-10+ CD8 cells may be significant in gingival lesions. It has also shown that CD30+ T-cells indicative of Th2 or Th0 cells may play a role in progressive periodontal disease and furthermore, that the periodontopathogen *P. gingivalis* may induce this T-cell subset. Additionally, this study has demonstrated that B-cells may play an important role in the presence of the destructive cytokine IL-1 in the lesion, and that *P. gingivalis* may be significant in the induction of B-cell production of this cytokine.

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