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สำนักงานกองทุนสนับสนุนการวิจัย

ทุนพัฒนานักวิจัยประจำปี 2538 จาก สกว.
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คุณสมบัติของทีเซลล์โคลนส์จากเหงือกที่มีความจำเพาะต่อ *พอร์ไฟโรโมแนส จินจิวาลิส* (*Porphyromonas gingivalis*) ในผู้ป่วยโรคปริทันต์อักเสบ

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บทคัดย่อ

โรคปริทันต์อักเสบมีลักษณะของการมีทีเซลล์และบีเซลล์อย่างหนาแน่นภายในเนื้อเยื่อเกี่ยวพันของเหงือก และมีความเกี่ยวข้องกับแบคทีเรียแกรมลบในคราบจุลินทรีย์ใต้เหงือก *พอร์ไฟโรโมแนส จินจิวาลิส* เป็นเชื้อหลักในการก่อโรค วัตถุประสงค์ของการวิจัยนี้เพื่อทำการสร้างทีเซลล์ลายส์และทีเซลล์โคลนจากเหงือกที่มีความสัมพันธ์กับแบคทีเรียที่ทำให้เกิดโรคปริทันต์ (*พอร์ไฟโรโมแนส จินจิวาลิส*) ในผู้ป่วยโรคปริทันต์อักเสบขั้นรุนแรงและตรวจสอบฟีโนไทป์บนผิวเซลล์ คุณสมบัติจำเพาะ รวมทั้งชนิดของไซโตคายน์ ตอนเริ่มงานวิจัยไม่สามารถเลี้ยงเซลล์เม็ดเลือดขาวจากเหงือกได้ และเซลล์จากเหงือกตายภายใน 2 สัปดาห์ ในระหว่างนั้นสามารถสร้างทีเซลล์ลายส์จากเลือดจำนวน 2 ลายส์ของผู้ป่วยโรคปริทันต์อักเสบขั้นรุนแรงจำนวน 2 คน โดยเริ่มที่กระตุ้นด้วยแอนติเจน และเลี้ยงต่อไปในไฟโตฮีแมกกลูตินิน อินเตอร์ลูคิน-2 และบีเซลล์ลายส์ ที่ทำให้เปลี่ยนแปลงโดยอิพสตายน์-บาร์ ไวรัส (เป็นเซลล์ที่มีความสามารถในการส่งผ่านแอนติเจน) ทำการทดสอบความจำเพาะของแต่ละลายส์เป็นระยะๆ โดยวิธีโพรลิเฟอเรชัน จากการวิเคราะห์โดยใช้โฟล ไซโตเมตรี พบว่า เซลล์ลายส์หนึ่งมี ทีเซลล์ชนิดที่เป็น ซีดี4 เป็นหลัก ส่วนอีกเซลล์ลายส์หนึ่งมีทีเซลล์ชนิด ซีดี8 เป็นหลัก อย่างไรก็ตามการวัดไซโตคายน์ที่สร้างจากเซลล์ลายส์ทั้งสอง โดยใช้วิธีอิไลซ่า พบว่าผลิตอินเตอร์เฟอรอนแกมมา แต่ไม่ผลิตอินเตอร์ลูคิน-4 หลังจากทำการกระตุ้นด้วย *พอร์ไฟโรโมแนส จินจิวาลิส* จากความพยายามที่จะเลี้ยงทีเซลล์ที่มีความจำเพาะต่อแอนติเจนจากเหงือกของผู้ป่วยโรคปริทันต์อักเสบ สุดท้ายเราสามารถสร้างได้หนึ่งทีเซลล์ลายส์ และหนึ่งทีเซลล์โคลนจากผู้ป่วยโรคปริทันต์อักเสบขั้นรุนแรง โดยการหาสภาวะที่เหมาะสมต่อการตอบสนองของทีเซลล์โดยใช้ เดนไดร์ติคเซลล์ ซึ่งเป็นเซลล์ที่มีความสามารถส่งผ่านแอนติเจนได้เป็นอย่างดีเยี่ยม เดนไดร์ติคเซลล์ที่เปลี่ยนแปลงมาจากโมโนไซต์ที่เกาะติดจานเพาะเลี้ยงโดย จีเอ็ม-ซีเอสเอฟ และ อินเตอร์ลูคิน-4 นำเดนไดร์ติคเซลล์ ที่เตรียมได้มาสร้างทีเซลล์ที่มีความจำเพาะโดยกระตุ้นด้วย *พอร์ไฟโรโมแนส จินจิวาลิส* แล้วนำไปเพาะเลี้ยงร่วมกับทีเซลล์จากเหงือกในอาหารเลี้ยงเซลล์ที่มีอินเตอร์ลูคิน-2 แล้วกระตุ้นต่อเป็นระยะด้วยเดนไดร์ติคเซลล์ที่ได้รับการกระตุ้นด้วยแอนติเจน พบว่าทีเซลล์ลายส์จากเหงือกประกอบด้วย ทีเซลล์ชนิด ซีดี4 87% และ ซีดี8 3 % และแสดงความจำเพาะต่อ *พอร์ไฟโรโมแนส จินจิวาลิส* เป็นอย่างดี การสร้างโคลนจากทีเซลล์ลายส์จากเหงือกทำด้วยวิธีลิมิต ไดลูชั่น สามารถโคลนได้ 5 โคลน มี 3 โคลนไม่เสถียรและตายในที่สุด ส่วน 2 โคลนที่เหลือเสถียร แต่หลังจากการทดสอบความจำเพาะ มีเพียง 1 โคลนเท่านั้นที่แสดงการตอบสนองที่ดีต่อ *พอร์ไฟโรโมแนส จินจิวาลิส* โคลนที่ได้จากเหงือกนี้ผลิตไซโตคายน์แบบเดียวกับเซลล์ที่มีความจำเพาะต่อแอนติเจนที่ได้จากเลือด ได้แก่ อินเตอร์เฟอรอนแกมมาจำนวนมาก แต่ไม่ผลิตอินเตอร์ลูคิน-4 ดังนั้นผลของทีเซลล์ลายส์ที่มีความจำเพาะต่อแอนติเจนจากเลือดและเหงือกของผู้ป่วยโรคปริทันต์อักเสบขั้นรุนแรงสนับสนุนบทบาทของไซโตคายน์ทีเอช1 ในการเกิดโรคปริทันต์อักเสบ อย่างไรก็ตาม ยังมีความจำเป็นในการพัฒนาและสร้างทีเซลล์โคลนเอมาศึกษาเพิ่มเติม โดยจะมีนิสิตปริญญาเอกดำเนินงานวิจัยชิ้นนี้ต่อ

Abstract

Periodontitis is characterized by dense infiltrations of T and B cells within the gingival connective tissue in association with Gram negative bacteria in subgingival plaque. *Porphyromonas gingivalis* has been implicated as a major pathogen. The aims of the present study was to establish and characterize human gingival T cell lines (TCLs) and clone (TCC) reactive with periodontopathic bacteria, *P.gingivalis* in severe periodontitis patients and also to investigate these cells in terms of their surface phenotypes, specificity and cytokine profiles. At the beginning of the project, growing gingival lymphocytes was not a success and the gingval cells died within two weeks. In the meantime two peripheral blood TCLs reactive with *P.gingivalis* derived from two patients with severe adult periodontitis were established. They were initially grew in antigen stimulated culture and subsequently maintained in Phytohemagglutinin, interleukin-2 and Epstein – Barr Virus (EBV) transformed B lymphoblastoid cell lines (antigen presenting cells). The specificity of each peripheral blood TCL was assessed periodically by proliferation assay. Flow cytometric analysis showed that the majority of one peripheral TCL were CD4+ cells whereas the other were CD8+ cells. However, both of them produced IFN-γ but no IL-4 after stimulation with *P.gingivalis* as measured by ELISA. Attempts have been made to grow antigen specific T cells from periodontitis tissues. Finally, we could obtain one gingival TCL and TCC from a severe periodontitis patient by trying to optimize T cell response in culture using dendritic cells (DC), the most potent professional antigen presenting cells. DC were derived from adherent monocytes using Granulocyte macrophage- colony stimulating factor (GM-CSF) and IL-4. To generate antigen specific T cells, *P.gingivalis* pulsed monocyte-derived DC were co-cultured with gingival T cells and then expanded in culture medium containing IL-2. Stimulation process was repeated periodically with antigen pulsed DC. The gingival TCL consisted of 87% CD4+ and 3%CD8+ and showed good specificity to *P.gingivalis* Cloning from this gingival TCL were carried out by limit dilution technique. Five clones were obtained and 3 of which were not stable and died. The other 2 clones are stable but after testing specificity, only one clone showed good response to *P.gingivalis*. This gingival clone also produced similar cytokine pattern to those antigen specific cells derived from peripheral blood ie. high IFN-γ production but not IL-4. Hence, the results of antigen specific T cells from both peripheral blood and gingival tissue of severe periodontitis patients seem to suggest the role of Th1 cytokine in the pathogenesis of periodontitis. However, more gingival T cell clones need to be generated. At present the work is being continued by a PhD student.

Key words: T cells, *Porphyromonas gingivalis*, periodontitis, cytokines

Introduction

Periodontal disease is chronic inflammation of tooth supporting structures including gingiva, connective tissue and alveolar bone (Williams, 1990) . It is caused by complex interaction between host defenses and microorganisms in dental plaque (Seymour, 1991). Clinically, periodontal disease could be defined into two distinct groups, gingivitis and periodontitis (Caton, 1989). Gingivitis, a relatively stable form, is characterized by increased redness, swelling and bleeding of gingiva during brushing and probing. Without any periodontal treatment, the lesion may be confined to the marginal tissues and does not endanger the life of the dentition. On the other hand, periodontitis, an advanced form shows the clinical appearance of gingival inflammation, deepened gingival sulcus and loosing of the tooth due to destruction of attachment apparatus (Hirschfeld and Wasserman, 1978; McFall, 1982). This periodontal breakdown appears to be cyclical or periodic (Goodson et al., 1982).

Etiology: Plaque microorganisms

It has long been recognized that microorganisms in dental plaque and their products are the etiologic agents of periodontal disease (Socransky and Haffajee, 1992). At healthy and gingivitis sites, composition of microbial plaque are quite similar and the majority are Gram positive facultative bacteria, such as *Streptococci* and *Actinomyces* (Slots, 1977). In contrast, plaque associated with periodontitis appears to be a specific group of bacteria or critical pathogens in mixed infection. Gram negative anaerobes such as *P.gingivalis*, *Bacteroides forsythus*, and *A.actinomycetemcomitans* cause most cases of periodontitis (American Academy of Periodontology, 1996). *P.gingivalis* is a Gram negative, anaerobic, non motile, asaccharolytic rod. It is a member of the much investigated black-pigmented *Bacteroides* group which have long history associated with periodontal disease. These species produce an unusually large array of virulent factors such as collagenase, an array of proteases (including those that destroy immunoglobulins), endotoxin, fatty acids, NH_3, H_2S, indole etc. (Macdonald et al., 1956; 1963). *P. gingivalis* is more frequently detected in destructive forms of periodontal disease but uncommon and in low numbers in health or gingivitis. The species have been shown to be reduced in successfully treated sites but is commonly encountered in sites that exhibit recurrence

of disease post-therapy (Bragd et al., 1987; Van Winkelhoff et al., 1988). Interestingly, *P. gingivalis* has been shown to be able to invade human gingival epithelial cells *in vitro* (Duncan et al., 1993). This intracellular shelter would make these microorganisms possible to escape not only from host defense mechanisms but also from mechanical removal out of periodontal pocket via scaling and root planing.

Pathogenesis: Host Response to Plaque Microoganisms

Although microbial plaque are known to be essential to initiate disease and fuel progression, it is insufficient to explain the prevalence and severity of periodontitis. Host factors such as host defense mechanisms to dental plaque and environmental factors such as smoking are equally as important as determinants of disease occurrence and severity outcome.

Immunohistological studies have shown that the predominant lymphocytes in the infiltrate of the stable lesion are the T cells with increasing numbers of B cells and plasma cells in the progressive lesion (reviewed in Seymour, 1987; 1991). T cells play a major role in protection against infection and also in inducing the damage associated with the infection (Romagnani, 1993).

Significant numbers of T cells are found in progressive periodontal disease lesions. the majority of which co-express HLA-DR (Okada et al, 1988; Reinhardt et al, 1988) and CD45RO antigens (Gemmell, 1992) suggesting that they are activated cells. It is well established that T cells are central to the activation of B cells and other immunocompetent cells found in progressive lesions. Previous studies have suggested that a local T cell immunoregulatory imbalance may exist in human periodontal disease (reviewed in Seymour, 1987; Taubman, 1988) such that the study of T cells extracted form periodontal lesions is necessary to elucidate further the nature of T cell control operating in the periodontal lesion. Antigen specific T cells reactive with *P. gingivalis* have been demonstrated in the peripheral blood of patients with periodontal disease (Mahanonda et al, 1989) and treatment of these patients has resulted in a decrease in the frequency of antigen-specific T cells presumably as a result of the reduction in antigenic load (Mahanonda et al, 1991) suggesting that the response of patients with advanced disease to *P. gingivalis* may be antigen specific.

Cytokines are central to the mechanism by which helper T cells regulate the immune response (Swain, 1991) and the production of appropriate cytokines is necessary for the

development of protective immunity. The immune response to infection would appear to be regulated by the balance between Th1 and Th2 cytokines, these two pathways often being mutually exclusive, with one resulting in protection and the other in progression of disease (Cox and Liew, 1992). On activation, antigen specific naïve T cells are believed to progress into Th0 cells which secrete a combination of Th1 and Th2 cytokines and then differentiate further into Th1 or Th2 cells with repeated antigen stimulation (reviewed in Modlin and Nutman, 1993). Th1 cells differentially produce IL-2 and IFN-γ whereas Th2 cells secrete IL-4, IL-5 and IL-6 after activation by antigen or mitogen (reviewed in Mosmann, 1991). Differentiation of Th0 cells into Th1 cells is dependent on IFN-γ whereas differentiation into Th2 cells is dependent on the presence of IL-4, an important factor in the clonal expansion of antigen specific B cells (Scott, 1993). These two cytokines reciprocally inhibit the induction of the other T cell subset. The net effect of the Th1 cytokines is to enhance cell-mediated responses, while that of the Th2 cytokines IL-4 is to suppress cell-mediated response and enhance humoral immune response (Modlin and Nutman, 1993). Murine IL-2 and IL-4 producing clones can be derived from the same cells, suggesting that the cytokine phenotype is acquired during T cell differentiation and is not secondary to the expansion of distinct subpopulations predetermined to produce a specific cytokine pattern (Rocken et al, 1992).

Whether the Th1 and Th2 paradigm exits and perhaps may be a major determinant of the progression of periodontitis remains to be investigated. It has been studies by measuring the levels of various cytokines in gingival crevicular fluid and extracts of healthy and diseased gingival tissues, determining levels of cytokine messenger RNA in the tissue using immunocytochemical techniques or polymerase chain reaction on tissue extracts and by measuring cytokine production by mononuclear cells harvested from gingival tissue. Based on their work and published studies by others (Aoyagi et al., 1995), Gemmell et al., (1997) postulated that the Th1 response is associated with stable periodontal lesions, whereas a Th2 response leads to the production of nonprotective antibody and disease progression. Recently, more consistant findings of Th2 cytokines including IL-10 and IL-13 have been reported in periodontitis tissues, thus supporting the role of Th2 subset in the pathogenesis of periodontal disease (Yamazaki et al., 1997); Aramaki in press). However, not all the data support the Th2 response, other investigators observed the mixture of Th1 and Th2 responses in the diseased tissues (Fujihashi et al., 1996).

Hence, the data regarding the Th1 and Th2 paradigm in periodontal disease appear to be inconclusive.

Dendritic Cells: Potent Antigen Presenting Cells (APC)

Dendritic cells (DC) are unique subsets of leukocytes with their important roles in initiating and maintaining immune reactivity. Their main function is to present processed antigen to T cells. When compared with other professional APCs (monocytes and B cells), DC are the most potent APC in both primary and seconday immune response (reviewed by Hart et al., 1997). During T cell activation, at least two signals are required which DC actively involve. One is antigen signal where DC uptake, process and present antigen together with major histocompatibility complex on their surface. The other signal is co-stimulatory signal where mature DC express high level of co-stimulatory molecule, CD80, CD86 and CD40 (Ni and O'Neill, 1997). Without co-stimulation, specific antigen triggering of T cell may lead to anergy or cell death (Schwartz, 1996).

Prior DC studies had difficulties due to restrictin by the relatively limiting numbers of these cells from any one site in tissue or blood. However, at present the problems have been recently overcome by new knowledge available in application of cytokines ie. GM-CSF and IL-4 or TNFα to grow and maintain these cells in culture (Lutz, 1996; Sallusto and Lanzavecchia, 1994).

Objectives

(a). To generate gingival T cell lines and clones specific for *Porphyromonas gingivalis* from periodontitis patients.

(b). To characterize *P. gingivalis* specific T cell lines and clones in terms of their cell surface phenotypes and cytokine profiles.

Materials and Methods

1.Bacterial Preparation

Whole bacteria, *P. gingivalis* strain FDC-381 (from JJ. Zambon in SUN YaB were kindly provided by Dr.T.Koseki ,Tokyo Medical and Dental University, Japan) was used. The organisms were kindly grown by Associate Professor Jintakorn Kuwatanasuchart, Department of Microbiology, Faculty of Dentistry, Chulalongkorn University.

Briefly *P.gingivalis* was grown at 37° C anaerobically in the anaerobic chamber (Forma Scientific, USA) at 10% H_2 and 90% N_2 in Trypticase soy broth which the media consisted of following :-

Trypticase soy broth (Difco laboratories,USA)	30 grams/ 1 ml
Hemin (1mg/ml) (Sigma, USA) + Vitamin K (10mg/ml) (Sigma), stock solution	10 ml
Distilled water to 1000 ml	

The purity of *P.gingivalis* was checked by Gram stain smear ,colony morphology and pigment production on CDC anaerobic blood agar plates which consisted of the following :-

Trypticase soy agar (Difco laboratories)	40.0 grams
Hemin (1mg/ml) (Sigma, USA) + Vitamin K (10mg/ml) (Sigma), stock solution	10 ml
Sterile lysed horse blood	50 ml
Kanamycin SO_4	4 ml
Distilled water to 1000 ml	

The bacteria were harvested by centrifugation (model GS-6R ; Beckman Instruments, USA) at 2,500 rpm for 10 min, washed three times in sterile Phosphate - buffered saline (PBS , 0.15 M , pH 7.2). For heat-killed whole cell preparation, the bacteria were heat-killed at 100°C for 15 min. For whole cell sonicate preparation, the microorganisms were subjected to sonication with high intensity ultrasonication (High Intensity Ultrasonic Processor, microprocessor controlled

600-Watt Model, Sonic and Material Inc., USA) at 4 °C for 20 minute-elapsed time, with pulse on 2.5 seconds and pulse off 2 seconds. The sonicates were examined microscopically for complete breakage of cells. Then, the protein concentration of the organism was determined by using the Bio-Rad protein assay, and the bacteria stock was aliquoted and stored at -20 °C until use.

2. Subject Selection

Three adult periodontitis patients, two males named CC (35 years old) and the other named SA (45 years old) and one female named WA (54 years old), were selected for the present study according to the severity of their periodontal conditions. There was no history of systemic disease and neither had taken antibiotics or steroids in the past 3 months. All of them were diagnosed with generalized adult periodontitis with a few hopeless teeth. Such teeth had probing pocket depth deeper than 6 mm with periodontal attachment loss at least 5 mm. The level of alveolar bone support was less than one third of root length and clinically mobile (third degree mobility) as demonstrated by radiographic appearance. They were to be extracted due to periodontal cause.

All subjects were volunteers and willing to donate peripheral blood as well as gingival biopsies for research. Biopsies of gingival tissues attached to the teeth with "hopeless" prognosis due to severe periodontitis were obtained.

3. Peripheral Blood T Cell Line Study

3.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Heparinized peripheral blood was collected from the median cephalic vein. The PBMC were isolated by Isoprep® density gradient centrifugation. The cells were washed two times (300g, 15°C, 5 min) in lymphocyte culture medium(RPMI), RPMI-1640 (Gibco BRL,USA) supplemented with 2 mM glutamine (Seromed, Germany), penicillin (50 international unit, IU/ml), streptomycin (50 μg/ml) (Seromed). Viability of isolated cells was tested using Trypan blue.

3.2. Establishment of Espstein - Barr Virus (EBV) Transformed B Lymphoblastoid Cell Lines (LCLs)

Isolated PBMC from two patients, CC and SA, were transformed with EBV 3 ml of 10×10^6 PBMC in RPMI were resuspended gently with 0.5 ml of the supernatant of the EBV - producing marmoset lymphoblastoid cell line B95-8 (starter culture cells kindly donated by Dr.Busarawan, Ministry of Public Health, Bangkok, Thailand) and 1 ml FCS (Gibco) in 25 cm^3 tissue culture flask (Nunc, Denmark). For T-cell suppression, 0.5ml of cyclosporin A (1 μg/ml) were added to the cell suspension to make a final concentration of 100 ng/ml. The cells were incubated at 37°C, 5% CO_2 in air in an upright position undisturbed for 3 weeks. Monitoring for cell growth was observed under an inverted phase microscope (Olympus CK2 , Japan). These transformed B cells were fed twice a week by RPMI with 20%FCS and maintained to a density of 0.5 - 1.0 x 10^6 cell/ml in RPMI medium containing 20% FCS. (Walker 1990, Weinhold 1992). These transformed B cells were fed twice a week by diluting to a density of 0.5 - 1.0 x10^6 cells/ml in 25 cm^3 tissue culture flask. Before utilising both LCLs, the phenotype of these cells was examined by immunofluorescence technique staining with anti-Leu 12 monoclonal antibody (MAb, Becton Dickinson, USA).

The cells were cultured under the above conditions for 2 months before testing for an appropriate radiation dose for LCLs to stop proliferation and serve as antigen presenting cells. Varying doses of radiation such as 0, 20, 30, 40 Gray (Gy) were tested on LCLs. 5 x 10^5 cells/ml LCLs were cultured in RPMI with 10% FCS in 96 well U - bottomed plates and incubated at 37°C, 5% CO_2, for 3 and 7 days. In the wells with no radiation, the cells were cultured with or without *P. gingivalis* (8 μg/ml). Five replicate cultures were established at each radiation dose. At Day3 and Day7, ^{3}H-thymidine(Amersham, England) was added to each microwell 4 hours(hr) prior to harvesting onto glass fibre discs using Titertek cell harvester (Titertekplus A1, ICN flow, England). ^{3}H-thymidine incorporation was measured in a 1219 Rackbeta, liquid scintillation counter (LKB, Finland). The results were expressed as mean counts per minutes (CPM) ± standard error of the mean (SEM).

3.3 Establishment of T Cell Lines (TCLs) from Peripheral Blood

PBMC ($1x10^6$/ml) from CC and SA were stimulated in 24 well plates(Costar,USA) over a 10 day period with 8 ug protein/ml of *P. gingivalis* in RPMI containing 10% FCS without recombinant Interleukin 2 (rIL-2). Live blast cells reactive to *P. gingivalis* were then enriched using Isoprep® density gradient centrifugation. The cells (2×10^6) were cultured in 2 ml conditioned medium (which contains RPMI, 10% FCS, 20 U/ml r-IL-2 (Beringer Manheime,Germany)) without antigen stimulation in 24 well plates for 7 days. After a 7 day resting period, the cells were washed and restimulated with 25 µg/ml Phytohaemaglutinin (PHA) in the presence of 5×10^5/ml irradiated (30 Gy) autologous LCLs. These TCLs were maintained at a concentration of 10^5 - 10^6 /ml in this conditioned medium in 24 well tray on a cycle of resting (without LCLs or PHA stimulation) for 1 week followed by stimulation (with 5×10^5/ml irradiated autologous LCLs and 25 µg/ml PHA) for 1 week.

The periodical restimulation with PHA permitted T blast cells to be maintained up to 42 days. After the 1st (day 17), 2nd (day 31) round of stimulation and rest, the cells were tested for antigen responsiveness in a proliferative assay. The phenotypic analysis was carried out at Day17.

3.4 Specificity of Peripheral Blood TCLs

PBMC culture in this study was incubated at 37 ° C in a humidified atmosphere of 5% CO_2 in air. $5x10^5$/well TCL were cultured in the presence of 8ug/ml *P.gingivalis* and $1x10^5$/well irradiated autologous LCLs in 96U-well microtitre plates in the absence of rIL-2. In the negative control wells, the cells were cultured with LCLs and medium only, while in the positive control well, the cells were cultured with LCLs and 25 ug/ml PHA. Cell culture were set up in RPMI plus 10% heat inactivated FCS (56°C, 30 min) in 96 well U- bottomed microtitre plates. After the prescribed culture period, 0.5 µci/ml ^{3}H-thymidine was added to each microwell 4 hr prior to harvesting onto glass fibre discs. ^{3}H- thymidine incorporation was measured in a Beckman LS 2800 scintillation counter. The results were expressed as CPM. Due to a very low number of TCL, only one culture was set up at a time but analysis of their specficity was carried out two consecutive times.

3.5 Phenotypic Analysis of Peripheral Blood TCLs

Murine MAbs Conjugated with fluorescein isothiocyanate (FITC) phycoerythrin (PE) were used as listed in table 1. These fluorochromes were well characterized for use with immunofluorescent probes and suitable for dual - color analysis because of their separate emission spectral characteristics.

Double labelling immunofluorescence was carried out to analyse the CD3, CD4, CD8 and CD19 positive cells. TCLs were harvested from the culture on Day 17. Then the cells were ficolled and washed two times in RPMI. Approximately 10^4 TCL (100 µl) were aliquoted into microcentrifuge tubes and then were stained with the following marker combinations; 1) FITC-conjugated anti-CD4 + PE-conjugated anti-CD8; 2) FITC-conjugated anti-CD3 + PE-conjugated anti-CD19. Cells were stained at 4°C for 30min, washed with PBS containing 0.1% albumin and 0.01% sodium azide and then fixed with 1% formaldehyde. Eight thousand stained cells, gated on forward and side scatter, were analysed for fluorescence intensity on the FACScan (Becton Dickinson, Mountain View, CA). Control samples stained with mouse isotype antibody (PE or FITC labeled) were used for quadrant setting.

MAb	Specificity	Population
anti-Leu-2a*(PE)	CD 8	T cell subset
anti-Leu-3a*(FITC)	CD4	T cell subset
anti-Leu-4* (FITC)	CD3	pan T cells
anti-Leu-12 (PE)	CD19	pan B cells
mouse IgG_1* (FITC,PE)	mouse IgG_1	-
mouse IgG_{2a}* (PE)	mouse IgG_{2a}	-

*MAb were purchased from Becton Dickinson

Table 1: Monoclonal antibodies used for flow cytometric analysis of peripheral blood TCLs

3.6 Cytokine Profiles of Peripheral Blood TCLs

After the first , second and third rounds of stimulation and rest, the TCLs from the two subjects were ficolled and washed. For the cytokine analysis, $5x10^5$/well TCL were cultured in the presence of 8ug/ml *P.gingivalis* and $1x10^5$/well irradiated autologous LCLs in 96U-well microtitre plates in the absence of rIL-2. In the negative control wells, the cells were cultured with LCLs and medium only, while in the positive control well, the cells were cultured with LCLs and 25 ug/ml PHA. The supernatants were collected after 6 hr, 1 day and 3 days in culture and stored in aliquots at -70°C. IL4 and IFN-γ production by the TCLs was measured with specific solid-phase sandwich enzyme-linked immunosorbent assays (ELISA) using the Quantikine™ Human IL-4 Immunoassay (Genzyme, Cambridge, USA) and InterTest-γ™ (Genzyme) ELISA kit respectively. The assay was performed according to commercial instructions. Cytokine concentrations were calculated by comparison with a standard curve of each cytokine.

4. Gingival T Cell Line Study

4.1 Preparation of Gingival T Cells

Gingival tissue sample was collected from all three subjects (CC, SA and WA) with generalized severe periodontitis. Biopsies of gingival tissues attached to the teeth with "hopeless" prognosis due to severe periodontitis were obtained. No other dental diseases such as pulpal disease was involved. The excised tissues were immediately transferred in transport medium, kept on ice and processed in the laboratory within 24 hr.

The methods of gingival cell extraction followed those described by Daly et al.(1983). Briefly, the tissues were washed thoroughly in RPMI then cut into small fragments approximately 1 - 2 mm^3 segments. These fragments were incubated in 10% FCS in RPMI containing 2 mg/ml of collagenase (Clostridial collagenase CLSIII grade, Sigma). The ratio of medium to tissues were 1 ml : 100 mg of tissue. Following incubation for 90 minutes at 37°C, in an atmosphere of 5% CO_2 in air, the residual tissue fragments were dissagregated further by gentle filtering through filter of mesh size 70 μm (Becton Dickinson , Franklin lakes, NJ). The cell suspension was washed twice

and resuspended in RPMI. The lymphocytes were counted in haemocytometer and viability assessed by trypan blue exclusion.

4.2 Preparation of Dendritic Cells (DC)-Derived Mononuclear Cells

The problems experienced with gingival mononuclear cells obtained from subjects CC and SA were cell death which occurred within two weeks after stimulation with *P.gingivalis* or PHA. Therefore, attempts have been made to optimize gingival cell culture conditions in order to establish gingival TCL from a periodontitis patient, WA. Approaches have been made by using DC as antigen presenting cells (APC) in culture. DC are characterized by their potent capacity to provide critical APC activity for initiating specific T cell activation and proliferation. Large numbers of monocyte derived DC could be generated *in vitro* using GM-CSF (granulocyte-macrophage colony stimulating factor) and IL-4 (Sallusto and Lanzavecchia, 1994; Lutz et al., 1996). The DC obtained by this method in the present study were found to highly express CD40, CD80 and CD86 on cell surface and weekly express CD14 (Figure 7).

DC from subject VN were generated from adherent monocytes. Briefly, T cells were removed from PBMC by rosetting with neuraminidase-treated sheep erythrocytes. Non-rosetting cells (T cell depleted mononuclear cells) were resuspended in RPMI with 10% heat inactivated autologous serum, and allowed to adhere to 24-well plates (1×10^6 cells per ml). After 2 hr at 37°C the nonadherent cells were removed, washed twice in culture medium and the adherent cells (monocytes) were cultured in medium containing cytokines [GM-CSF (40 ng per ml) and IL-4 (50 ng per ml)]. DC cultures were fed with fresh medium and cytokines every 3 days and cell differentiation were monitored under light microscope (modified from Zhou and Tedder 1996). Approximately after 6 day culture, monocyte-derived DC were ready to be pulsed with antigen, *P.gingivalis* sonicates (10 μg/ml) for 24 hr at 37 °C, then washed twice, before co-culturing with gingival T cells.

4.3 Generation of Gingival T Cell Line and T Cell Clone

Gingival cells from subject VN were cultured in RPMI containing rIL-2 (100 U/ml) for 3 days. Isolation of T cells was achieved by negative selection of anti-CD19 (PE) stained cells and anti-CD56 (PE) (Becton Dickinson) stained cells respectively using flow cytometry, ie. removal of B cells and NK cells. Purified T cells ($1x10^6$/ml) were co-cultured with *P. gingivalis* pulsed DC (ratio DC: T cells = 1:5) in the absence of rIL-2 in 24 well plates for 5 days and after that rIL-2 (100 U/ml) was added to the culture every other day for approximately 1 week. These antigen specific T cells were further expanded by a cycle of a 5 day stimulation period with *P.gingivalis* pulsed DC and a 7 day in rIL-2 containing medium. The cells continued to grow in culture up to 40 days and were subsequently tested for specificity, cell surface phenotypes and cytokine profiles.

Cell cloning from gingival TCL was carried out using limit dilution technique.

4.4Specificity of gingival TCL and TCC

Specificity of gingival TCL was tested against different periodontopathic bacteria such as *P.gingivalis*, *A. actinomycetemcomitans*, *Prevotella intermedia*, *Actinomyces viscosus* using proliferation assay. For VN2 clone, it was only tested for proliferative response to *P.gingivalis*. The antigen specific T cells ($5x10^5$ cells/ml) were cultured with and without bacteria pulsed DC ($1x10^5$ cells/ml) in triplicate for 4 days in 96 U bottom microtiter plates. Cultures were pulsed with 0.5 μCi of [^{3}H] thymidine for 24 hours and the uptake were measured in a liquid scintillation counter.

4.5 Phenotypic Analysis of gingival TCL

The gingival TCL (VN-TCL) were harvested from the culture, and stained with the following marker combinations; 1) FITC-conjugated anti-CD4 + PE-conjugated anti-CD8; 2) FITC-conjugated anti-TCR-γ/δ-1 + PE-conjugated anti-Leu-19. The Mab used were listed in Table 2. Cell staining procedures were as described earlier.

MAb	Specificity	Population
anti-Leu-2a*(PE)	CD 8	T cell subset
anti-Leu-3a*(FITC)	CD4	T cell subset
anti-TCR-γ/δ-1* (FITC)	γ/δ TCR	T cell subset
anti-Leu-19 (PE)	CD56	NK cells
mouse IgG_1* (FITC,PE)	mouse IgG_1	-
mouse IgG_{2a}* (PE)	mouse IgG_{2a}	-

*MAb were purchased from Becton Dickinson

Table 2: Monoclonal antibodies used for flow cytometric analysis of gingival TCL

4.6 Cytokine Profiles of gingival TCC

4.6.1 ELISA method

For measurement of cytokine, VN2 clone ($5x10^5$ cells/ml) were cultured with and without *P. gingivalis* pulsed DC ($1x10^5$ cells/ml) for 3 days in 24 well plates. Supernatants were collected and stored in aliquots at -20°C until use. The levels of IL-4, and IFN-γ production were measured by commercial ELISA kits as previously described.

4.6.2 *Intracellular cytokine staining*

Intracellular cytokine expression (IL-4, and IFN-γ) of VN2 clone were determined according to the method described by Waldrop et al. (1997). $1.5x10^6$ cells/ml VN2 clone were cultured with *P.gingivalis* pulsed DC ($3x10^5$ cells/ml) for 6 hr in the presence of 2μg/ml Brefeldin A (Sigma). The cultured cells were stained with FITC conjugated anti-CD3 for 15min, fixed with FACS Lysing Solution (Becton Dickinson) for 10min, and permeabilized with FACS Permeabilizing Solution (Becton Dickinson) for 10min. Then the cells were washed, stained with PE-conjugated anti- IL-4 and PE-conjugated anti- IFN-γ for 30min, fixed with 1% paraformaldehyde, and analysed by flow cytometry.

Results

1. Peripheral blood T cell lines

Selection for appropriated radiation dose of LCLS

Flow cytometric analysis showed that more than 95% of these cells were CD19+ (B cells) and less than 0.1% were CD3+ cells (T cells).

The results of mean CPM of irradiated CC-LCLs and irradiated SA-LCLs with varying radiation doses on Day 3 and Day 7 of culture were presented in Table 3. Both LCLs without radiation (0 Gy) showed up to 5 fold higher CPM than those with radiation and a higher proliferative responses were seen on Day 3. With 20 Gy, high background as much as $5.01x10^3$ CPM could be detected on day 3 whereas lower CPM as $2.38x10^3$ and $3.94x10^3$ CPM resulted from 30Gy radiation of CC-LCLs and SA-LCLs respectively. 30 Gy and 40 Gy radiation provided similar outcome of proliferative responses. When irradiated LCLs (30Gy) were cultured in the presence of *P.gingivalis* or PHA, very low proliferative response was resulted as compared with non-irradiated LCLs (Table 4). Therefore, 30 Gy were selected as an appropriate radiation dose for LCLs to support lymphocyte cultures.

	Mean $x10^3$ CPM ± SE			
CC-LCL	**0 Gray**	**20 Gray**	**30 Gray**	**40 Gray**
Day 3	17.05±1.35	3.81± 0.32	2.38 ± 0.15	2.03 ± 0.21
Day 7	12.15 ± 0.49	0.34 ± 0.02	0.21 ± 0.02	0.13 ± 0.03
SA-LCL	**0 Gray**	**20 Gray**	**30 Gray**	**40 Gray**
Day 3	31.14 ± 2.27	5.01 ± 0.54	3.94 ± 0.53	3.93 ± 0.56
Day 7	8.43 ± 0.42	1.90 ± 0.14	1.94 ± 0.27	2.22 ± 0.51

Table 3: Testing for appropiate irradiation dose for LCLs. $5x10^5$/ml LCLs with varying radiation doses were cultured in RPMI with 10%FCS for 3 and 7 days without *P.gingivalis* or PHA. Proliferative responses are expressed as mean CPM±SE.

	Mean x10^3 CPM ± SE			
	P.gingivalis	PHA	*P.gingivalis*	PHA
CC-LCL	**0 Gray**	**0 Gray**	**30 Gray**	**30 Gray**
Day 3	17.76±1.59	11.41±1.56	2.37 ± 0.10	1.31 ± 0.15
Day 7	12.11 ± 1.18	9.21 ± 0.19	0.19 ± 0.01	0.18 ± 0.01
SA-LCL	**0 Gray**	**0 Gray**	**30 Gray**	**30 Gray**
Day 3	29.17 ± 3.73	21.33 ± 1.66	4.32 ± 0.76	3.93 ± 0.19
Day 7	14.65 ± 1.99	12.25 ± 4.28	2.05 ± 0.34	1.54 ± 0.14

Table 4: 30 Gy radiation effect on LCL proliferation. 5x10^5/ml LCLs (30 Gy) were cultured in the presence of *P.gingivalis* or PHA for 3 and 7 days. Proliferative responses are expressed as mean CPM±SE.

Specificity of peripheral blood TCLs

After the 1st round of stimulation and rest (day 14), CC-TCLs and SA-TCLs showed a proliferative response to *P. gingivalis* comparable to PHA. Although, the relatively high background in the negative control wells were noticed, the CPM in such wells were lower than those in experimental wells (Figure 1). Increased response to the organism in both TCLs could further be seen after the 2nd round of stimulation and rest (day 28) (Figure 1). The results, therefore, indicate specificity of the TCLs.

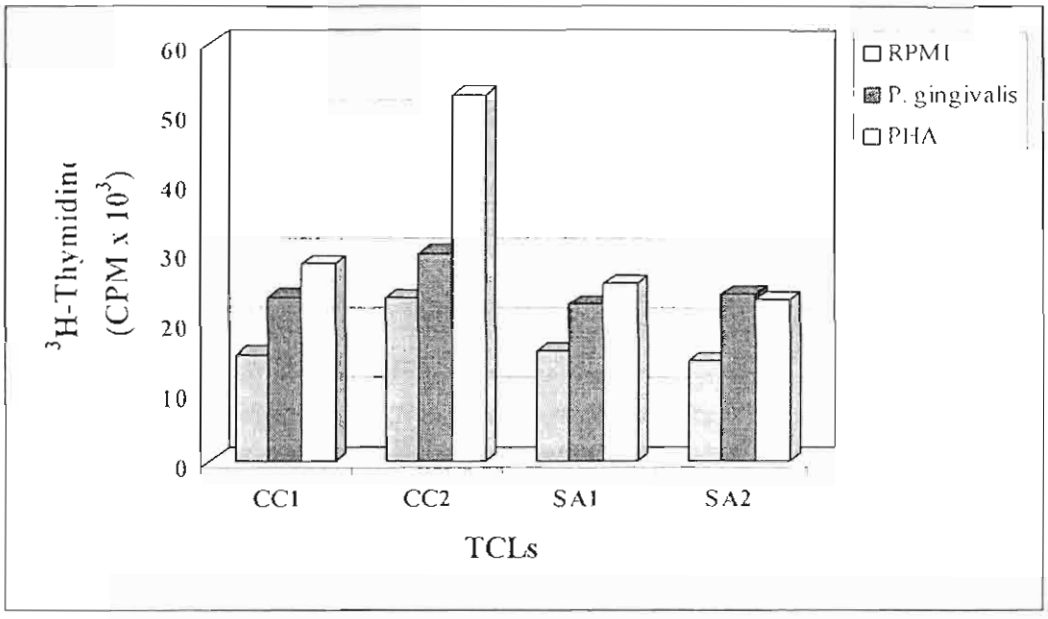

Figure 1:Proliferative response of CC-TCLs and SA-TCLs to *P. ginigvalis* and PHA.
CC1 = CC-TCLs after the first round of stimulation and rest (day 14).
CC2 = CC-TCLs after the second round of stimulation and rest (day 28).
SA1 = SA-TCLs after the first round of stimulation and rest (day 14).
SA2 = SA-TCLs after the second round of stimulation and rest (day 28).

Phenotypic analysis of TCLs

The phenotypic analysis of CD3, CD4, CD8 and CD19 positive cells in both TCLs were shown in Table 5. CC-TCL at the resting stage after the second round of stimulation and rest (day 28), consisted of 67.54% CD4+CD3+ cells, 34.99% CD8+CD3+ cells and less than 5% CD19+ cells while SA-TCL had predominantly CD8+CD3+ cells (73.31%), 13.57% CD4+CD3+ cells and less than 6% CD19+ cells.

PHENOTYPE	CC-TCL	SA-TCL
CD3+	95.71 %	84.03 %
CD4+CD3+	67.54 %	13.57 %
CD8+CD3+	34.99 %	73.31 %
CD19+	4.09 %	5.03 %

Table 5: Phenotypic analysis of CC-TCL and SA-TCL.

Cytokine analysis

Quantikine™ human IL-4 immunoassay and InterTest-γ™ human IFN-γ kit were used to determine IL-4 and IFN-γ production, respectively. Cytokines which taken from supernatants of *P. gingivalis* specific TCLs were measured after stimulation with *P. gingivalis* , PHA, and also without *P. gingivalis* as a negative control for 6 hr., 1 day, and 3 day culture. The measurement of both cytokines was carried out three consecutive time at day 28(CC1, SA1), 42(CC2, SA2), 56(SA3). One measurement for each cytokine could be performed due to limited cell numbers. The results were presented in Figures 2-6.

It was found that IL-4 as measured by immunoassay could not be detected in any cultures with or without stimulation by the bacteria or PHA. In contrast, a certain amount of IFN-γ could be detected in both CC-TCLS and SA-TCLS after 6 hr. stimulation with *P. gingivalis* and continued to be detected on Day 1 and Day 3 which is suggestive of type 1 T-cell response . The kinetics of IFN-γ secretion did not show a consistent pattern in both TCLs. The levels of cytokine in *P. gingivalis* stimulated cultures were higher than those of control cultures except for CC2 at 6 hr. and SA1 at Day 3, but always lower than the PHA stimulated cultures. In general, SA-TCLs seemed to produce higher amount of IFN-γ after bacterial stimulation than CC-TCLs and the

highest amount detected was 2379 pg/ml found on Day 3 after the fourth round of stimulation and rest.

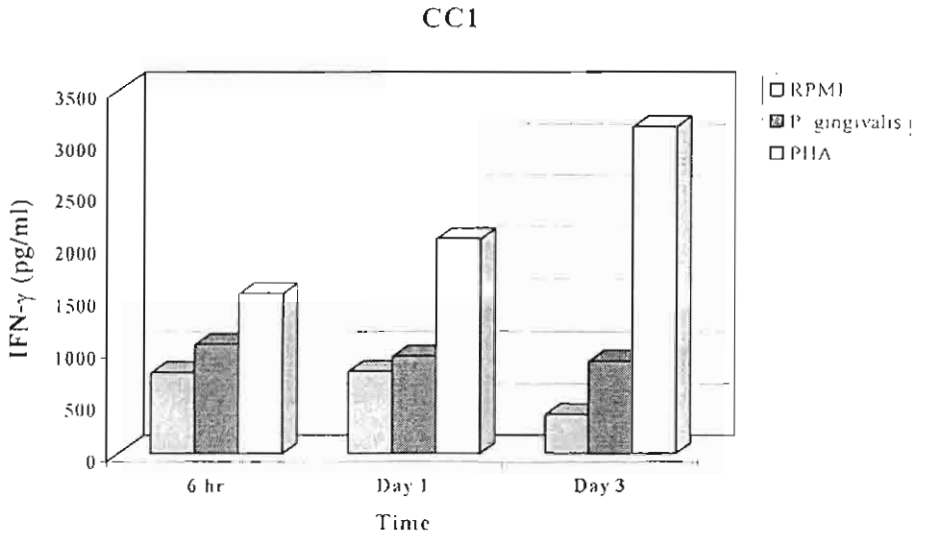

Figure 2:IFN-γ production form CC-TCLs (Day 28) stimulated with *P. gingivalis*, PHA or non-stimulated was measured at different incubation periods.

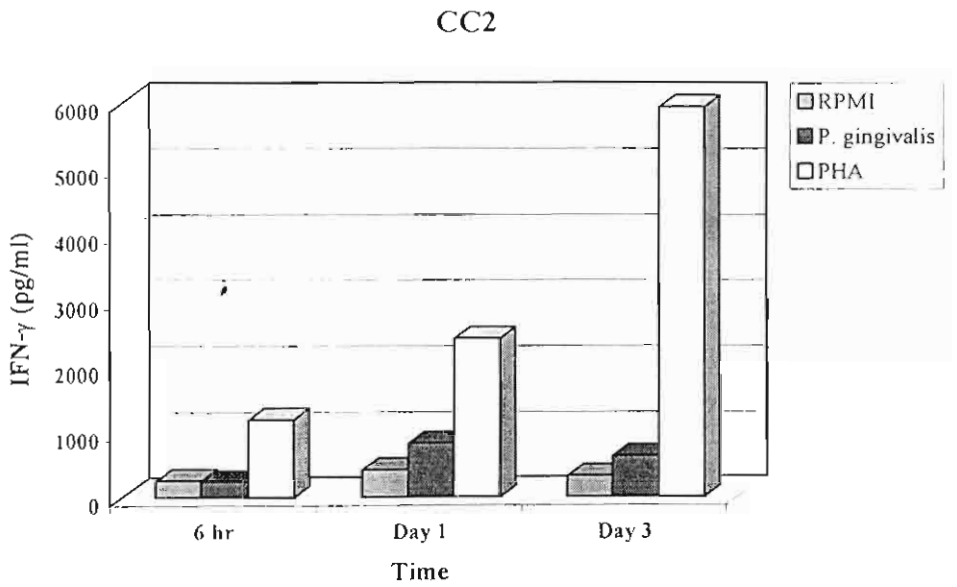

Figure 3:IFN-γ production form CC-TCLs (Day 42) stimulated with *P. gingivalis*, PHA or non-stimulated was measured at different incubation periods.

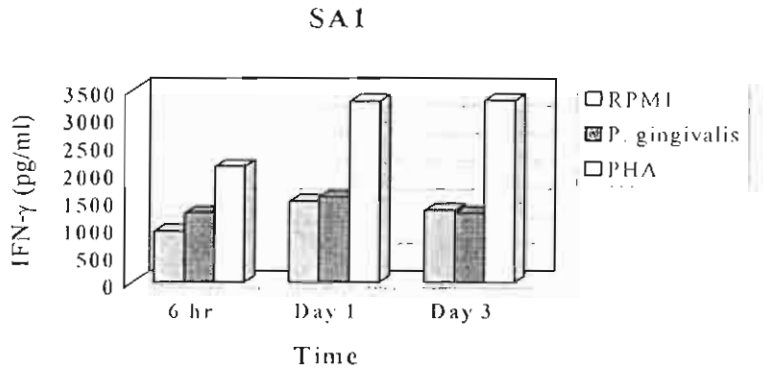

Figure 4:IFN-γ production form SA-TCLs (Day 28) stimulated with *P. gingivalis*, PHA or non-stimulated was measured at different incubation periods.

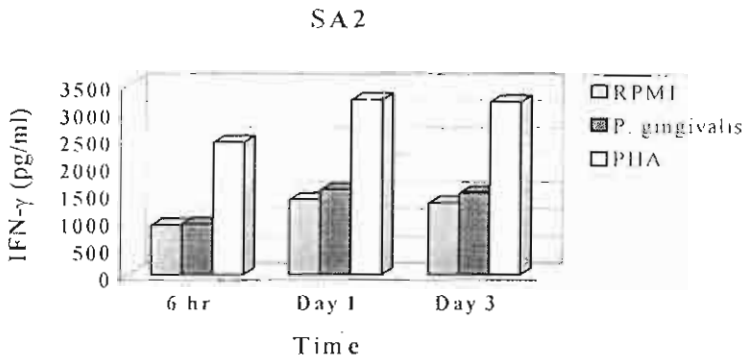

Figure 5: IFN-γ production form SA-TCLs (Day 42) stimulated with *P. gingivalis*, PHA or non-stimulated was measured at different incubation periods

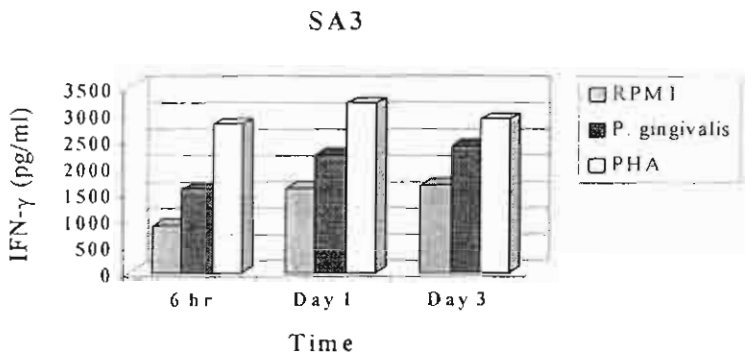

Figure 6:IFN-γ production form SA-TCLs (Day 56) stimulated with *P. gingivalis*, PHA or non-stimulated was measured at different incubation periods.

2. Gingival T cell line and T cell clone

One gingival tissue-derived T cell line (VN-TCL) and T cell clone (VN-TCC) specific for *P.gingivalis* from a severe periodontitis patient were established by the use of monocyte-derived DC. These DC generated *in vitro* using GM-CSF and IL-4 were found to highly express co-stimulatory molecules: CD40, CD80 and CD86 but very low CD14 (Figure7). VN-TCL were grown up to 40 days in culture with good specificity to *P.gingivalis* but not other bacteria such as *A.actinomycetemcomitans*, and *A.viscosus* (Figure 8). Phenotypic analysis revealed that this cell line was 87% CD4+, 3% CD8+, <1% TCRγδ+ and <2% CD56+ cells (NK cells) (Table6). Five gingival TCC were initially generated from this TCL by limit dilution technique, however three of them were unstable and died after 2 weeks. The other two TCCs (VN1 and VN2) grew rapidly but only VN2 showed good proliferative response to *P.gingivalis* (Figure 9). Cytokine measurement by ELISA showed that VN2 clone, after *P.gingivalis* stimulation, secreted IFN-γ (3,862 pg/ml) , but not IL-4. This IFN-γ production was confirmed by the presence of intracytoplasmic cytokine in VN2 clone. Figure 10 demonstrated that there were 4.28% of IFN-γ producing CD3+ TCC.

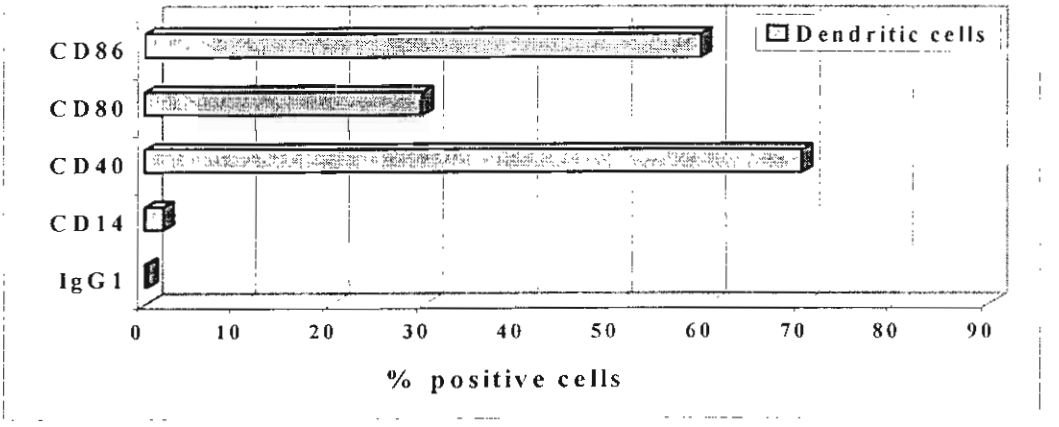

Figure7: High expression of co-stimulatory molecules on monocyte-derived DC.

Specificity of gingival TCL

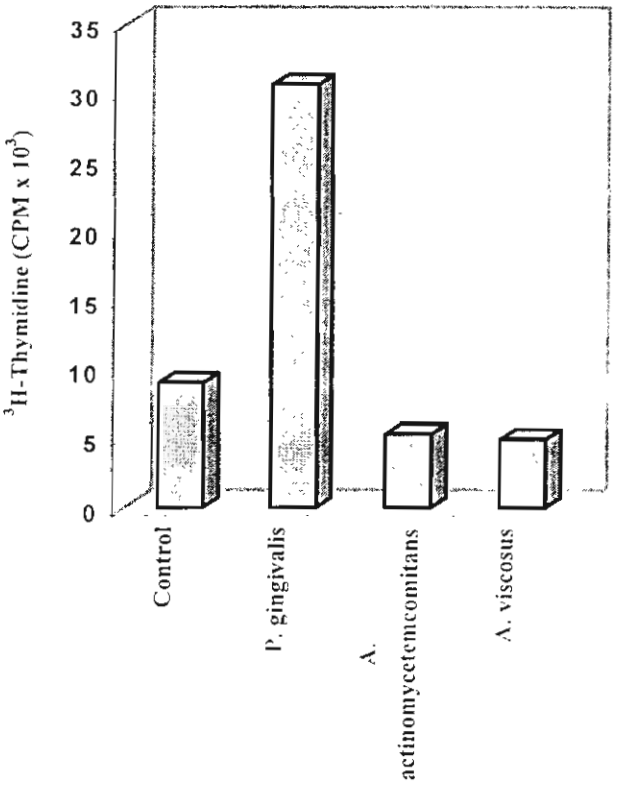

Figure8: Proliferative response of VN-TCL to *P.gingivalis*, *A.actinomycetemcomitans* and *A.viscosus*.

Phenotypic analysis of gingival TCL

PHENOTYPE	**VN-TCL**
CD4+	87 %
CD8+	3%
TCRγδ	<1%
CD56	<2%

Table6: Phenotypic analysis of VN-TCL

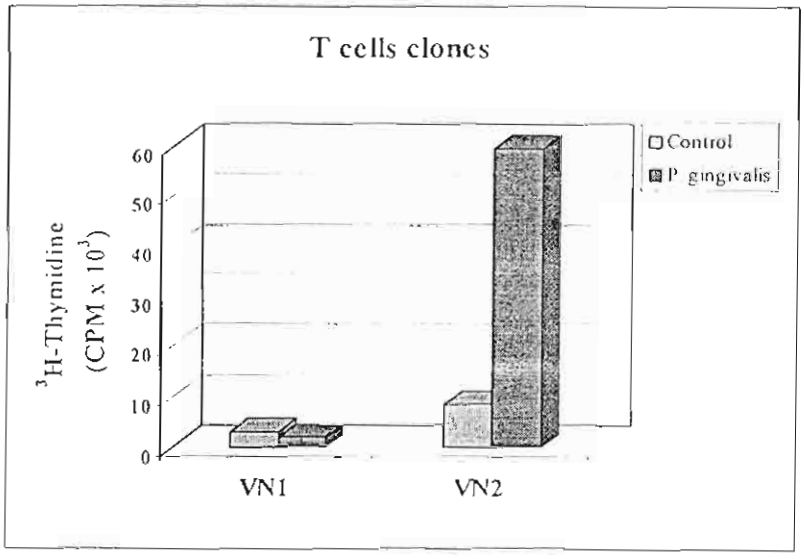

Figure9: Proliferative response of VN1-TCC and VN2-TCC to *P.gingivalis*

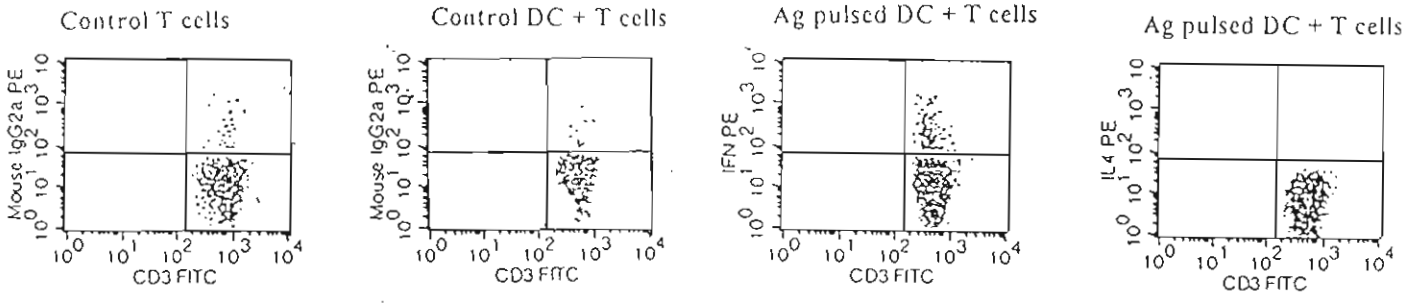

Figure10: Intracellular cytokine staining of VN2 clone.

Discussion

The activation of T cells induced by periodontopathic bacteria in inflamed gingival tissues has been well recognized to play an important role in the pathogenesis of periodontal disease (Seymour, 1991). High levels of some cytokines are found in gingival crevicular fluid and extracts of diseased periodontal tissues from periodontitis patients (Gemmell et al., 1997). Conflicts do exist as periodontal researcher try to relate types of cytokines, Th1 and Th2, with diseased and non-diseased status. Some reported Th2 response is associated with periodontitis (Manhart et al., 1994, Yamazaki et al., 1997) but others (Fujihashi et al., 1996) reported the mixture of Th1 and Th2 response.

Up to date, there have been only two studies of antigen specific T cells derived from periodontitis tissues which provide information of cytokine profiles. Gemmell et al. (1995) showed that T cell clones specific for *P.gingivalis* derived from one periodontitis patient produced high level of IL-4. The other study (Wassenaar et al., 1992) indicated that the majority of established clones were Th0 subset which produced equal amount of IL-4 and IFN-γ. Our results demonstrated that T cell lines generated from both peripheral blood and inflamed gingival tissues as well as gingival T cell clone, all derived from severe adult periodontitis patients, produced IFN-γ but not IL-4. The role of Th subset development and their contribution to pathogenesis of periodontitis remain unclear. Generation of more T cell lines and clones specific to *P.gingivalis* and their cytokine profiles would provide a better understanding on the role of T cell activation in relationship to disease.

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TITLE: THE IMMUNE MODULATION OF B CELL RESPONSES BY *PORPHYROMONAS GINGIVALIS* AND INTERLEUKIN-10

Chantrakorn Champaiboon[1], Kosol Yongvanitchit[2], Sathit Pichyangkul[3], Rangsini Mahanonda[4]

[1] MSc, [4] Ph.D. Department of Periodontology, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand.

[2] BSc, [3] Ph.D Department of Immunology and Medicine, US Army Medical Component, AFRIMS, Bangkok, Thailand.

Address correspondence to :

Rangsini Mahanonda, Ph.D.

Department of Periodontology, Faculty of Dentistry, Chulalongkorn University, Henry Dunant Rd., Bangkok10330, Thailand.

Tel: 011-662-218-8621

Fax: 011-662-218-8851

E-mail: <mrangsin@netserv.chula.ac.th>

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Running title: **The effect of *P.gingivalis* and IL-10 on B cells**

ABSTRACT

A unique characteristic of the advanced periodontal lesion is the presence of large number of B cells and plasma cells. Polyclonal B cell activation induced by periodontopathic bacteria has been cited as being important for these elevated number of B cells, but their role in the pathogenesis of periodontal disease remains unknown. In this study we used an in vitro model to investigate the activation of immune cells by the periodontopathic bacterium, *Porphyromonas gingivalis* in healthy subjects. Peripheral blood mononuclear cells (PBMC) cultured with sonicated extracts of *P. gingivalis* led to a large number of activated B and NK cells as monitored by CD69 expression using flow cytometry while a small number of activated γδ and αβ T cells were observed. It was further demonstrated that the bacterium itself could directly activate only B cells but not the others when positively sorted cells were used. Measurement of B cell regulatory cytokine production in *P. gingivalis*-stimulated PBMC cultures by ELISA revealed a large amount of IL-10 but no IL-12 or IL-15. High levels of IL-10 have been consistently found in inflamed gingival tissues, however the cellular source of this cytokine has not yet been determined. In our *P. gingivalis*-stimulated PBMC cultures, the major producing cells were monocytes, not B cells or αβ T cells. When IL-10 was added to B cells in the presence of bacteria, significantly increased B cell proliferative responses were observed. These results suggest that *P. gingivalis,* both directly and indirectly via macrophage IL-10, may play an important role in polyclonal B cell activation associated with periodontal disease.

Key words : *Porphyromonas gingivalis* / B cells / Periodontal disease / IL-10

INTRODUCTION

The nature of periodontitis, an advanced form of chronic inflammatory periodontal disease, has been well recognized as a predominantly B cell lesion associated with Gram negative bacterial pathogens in subgingival plaque.[1,2] Early phenotypic studies have shown that the majority of B cells in periodontitis lesions are in an activated stage.[3] To date, the underlying mechanisms of this predominantly B cell lesion remain largely unknown although polyclonal B cell activation (PBA) induced by periodontopathic bacteria has been cited as one possible mechanism.[4,5] The majority of these periodontopathic bacteria are Gram negative anaerobes and includes *Porphyromonas gingivalis*, recognized as an important pathogen in various types of periodontitis.[6,7,8] Stimulation of PBMC cultures with periodontopathic bacteria leads to the activation of B cells and the production of polyclonal antibodies and an increased proliferative response.[9,10] It is becoming increasingly clear that the enhanced antibody production associated with PBA seems to be of little value to the host. In fact much of this nonprotective antibody may be locally produced auto-antibodies against gingival tissue components (collagen).[11,12]

B cell regulatory cytokines also play an important role in B cell activation, many of which have been found present in high levels in inflamed gingival tissues and in gingival crevicular fluid from periodontitis patients. Studies by Fujihashi et al.[13] demonstrated high levels of IL-5 and IL-6 mRNA expression in gingival mononuclear cells isolated from severe periodontitis patients but IL-2 and IL-4 mRNA expression were not detected. While some studies have reported IL-4 in the gingival tissues[14,15] others[16,17,] have been unable to detect it

in inflamed gingival tissues. Recently, the cytokine IL-10 has been consistently reported in periodontitis lesions either by the measurement of mRNA expression or protein concentration.[15,18,19,20] IL-10 is produced by B cells, T cells and macrophages.[21] While it plays a major role in suppressing immune and inflammatory response, it is also a potent growth and differentiation factor for activated human B cells.[22]

In the present study, we investigated the expression of the activation marker CD69 on PBMC and sorted B-cell populations after stimulation with *P. gingivalis*. The production of B cell regulatory cytokines (IL-10, IL-12, and IL-15) by *P. gingivalis*- treated PBMC cultures was also investigated. Finally, the combined effect of *P. gingivalis* and IL-10 on polyclonal B cell activation with regard to B cell proliferation was determined.

MATERIALS AND METHODS

Chemical Reagents and Monoclonal Antibodies

RPMI 1640 was obtained from Gibco, Flow Laboratories, Grand Island, NY. Gentamycin was purchased from SoloPak Laboratories Inc., Elk Grove Village, IL. Sodium bicarbonate, D-glucose, HEPES, fetal bovine serum (FBS), phosphate-buffered saline (PBS), dimethyl sulphoxide (DMSO) and Ficoll-Hypaque (Histopaque 1.077) were the products of Sigma Chemical Co., St. Louis, MO. Recombinant human IL-10 was obtained from Genzyme Co., Cambridge, MA. [^{3}H] thymidine was purchased from Amersham Life Science, Buckinghenshire, UK. Mouse anti-human CD4, anti-CD8, anti-19, anti-CD20, anti-TCR-α/β-1, anti-TCR-γ/δ-1, anti-CD56, anti-CD14, anti-CD69 and mouse IgG1 monoclonal antibodies

(mAbs) labeled with either phycoerythrin (PE) or fluorescein (FITC) were obtained from Becton Dickinson, San Jose, CA (Table 1).

Bacterial Preparation

Sonicated extracts of *Porphyromonas gingivalis* FDC-381 (kindly provided by Ms. Nantana Aroonrerk, Department of Conservative & Prosthodontics, Faculty of Dentistry, Srinakarinwirot University) were used. The bacteria were cultured in trypticase soy broth at 37 °C under anaerobic chamber (Forma Scientific, USA). The bacteria were harvested by centrifugation (Beckman Instruments, USA) at 2060 × g for 15 minutes, washed twice in PBS. The purity was assessed by Gram staining and colony morphology on trypicase soy blood agar. The microorganisms were subjected to sonication with high intensity ultrasonication (High Intensity Ultrasonic Processor, microprocessor controlled 600-Watt Model, Sonic and Material Inc., USA) at 4 °C for 20 minute-elapsed time, with pulse on 2.5 seconds and pulse off 2 seconds. The sonicates were examined microscopically for complete breakage of cells. Then, the protein concentration of the organism was determined by using the Bio-Rad protein assay (Bio-Rad, CA), and the bacterial stock was aliquoted and stored at –20 °C until use.

Cell Isolation

Heparinized peripheral blood was obtained from 15 healthy adult volunteers with no sign of periodontal disease. These subjects had not taken antibiotics or anti-inflammatory drugs within the past 3 months. None of them had symptoms of infection.

PBMC were prepared as previously described by Boyum.[23] Briefly, heparinized peripheral blood was layered on Ficoll-Hypaque , and centrifuged for 30 minutes at 700 × g at 25 °C. PBMC were washed twice and then resuspended in RPMI 1640 medium with 10% heat inactivated autologous serum. For purification of lymphocyte subpopulations, PBMC were further separated into T cell- enriched (Er+) and T cell- depleted (Er-) population by rosetting with neuraminidase-treated sheep erythrocytes. To purify B cells and monocytes, Er- population was stained with mAbs against CD20(FITC) and mAbs against CD14(PE). The stained cells were washed twice with PBS and then CD20 positive cells (B cells) and CD14 positive cells (monocytes) were sorted on FACStar Plus (Becton Dickinson, Mountain View, CA). Purified NK cells, αβ and γδ T cells were positively sorted from the Er+ population. The mAbs used to label NK cells, αβ and γδ T cells were anti-CD56(PE), and anti-TCR-α/β-1 (PE) respectively. Normally, the cells purified by this method were about 95-98% pure on subsequent flow cytometric analysis.

Cell Stimulation

PBMC (2×10^6 cells/ml) were cultured with or without sonicated extracts of *P. gingivalis* (0.016, 0.4 and 10 μg/ml). For purified lymphocyte subpopulation studies, they (1×10^6 cells/ml) were stimulated with 10μg/μl of *P. gingivalis* sonicated extracts. After 24 hours, PBMC and purified cell populations were harvested and analysed for the expression of a very early activation marker (CD69) by flow cytometry (FACScan, Becton Dickinson). While, the culture supernatants from *P. gingivalis*-stimulated PBMC cultures were also collected for evaluation of cytokine production (IL-10, IL-12, and IL-15).

In kinetic studies of IL-10 production, PBMC (2×10^6 cells/ml) were cultured with or without sonicated extracts of *P. gingivalis* (10 μg/ml). The incubation periods were varied from 0, 24, 48, 72 and 96 hours. After each incubation period, the culture supernatants from *P. gingivalis*-stimulated PBMC cultures were collected for evaluation of IL-10 production.

Cellular Source of IL-10 in *P. gingivalis*-Stimulated PBMC Cultures

In cell depletion studies, removal of monocytes, αβ T cells or B cells from PBMC cultures were achieved by negative selection of anti-CD14 (PE) stained cells, anti-TCR-α/β-1 (PE) stained cells, or anti-CD20 (PE) stained cells, respectively. Control undepleted PBMC were stained with isotype control antibody and then negatively sorted by flow cytometry under the same condition. For cell subset purification, αβ T cells, monocytes and B cells were positively sorted as described earlier. Flow cytometric removed cell cultures and sorted populations were stimulated with *P. gingivalis* sonicated extracts at 10 μg/ml. After 24 hours of incubation, the supernatants were collected for analysis of IL-10 production by ELISA.

Effect of *P. gingivalis* and IL-10 on Purified B cells

For the measurement of proliferative responses, purified B cells (1×10^6 cells/ml) were cultured under following conditions; 1) B cell control; 2) B cells + *P. gingivalis* (10 μg/ml); 3) B cells + IL-10 (500 ng/ml); and 4) B cells + *P. gingivalis* (10 μg/ml) + IL-10 (500ng/ml). Recombinant human IL-10 was always added at day 3.[24] Cell cultures were pulsed with [^{3}H] thymidine (0.5 μCi/ 200 μl/ well) at day 4 and incubated for another 18-24 hours. Cells were harvested onto glass fibers (Mach II harvester ; Tomtec, Orange, CT) and

radioactivity uptake was measured using a liquid scintillation counter (Beta Plate, Wallace, Turku, Finland).

Detection of Cytokines

The levels of IL-10, IL-12 and IL-15 production were measured by commercial ELISA kits (Genzyme Co.). The assay was performed according to commercial instructions. Cytokine concentrations were calculated by comparing with a standard curve of each cytokine. The detection limits of IL-10, IL-12 and IL-15 were 8, 25 and 47 pg/ml, respectively.

Phenotypic Analysis of Activated Cells by Flow Cytometry

Approximately 1×10^5 cells in a 100 μl of RPMI 1640 medium were aliquoted and then stained with the following markers. 1) FITC-conjugated anti-TCR- α/β-1 + PE-conjugated anti-CD69; 2) FITC-conjugated anti-TCR-γ/δ-1 + PE-conjugated anti-CD69; 3) PE-conjugated anti-CD56 + FITC-conjugated anti-CD69; 4) PE-conjugated anti-CD19 + FITC-conjugated anti-CD69. Cells were stained at 4°C for 30 minutes, washed with PBS containing 0.1% albumin and 0.01% sodium azide and then fixed with 1% paraformaldehyde. Fifty thousand stained cells, gated on forward and side scatter, were analyzed for fluorescence intensity on the FACScan. A control sample stained with mouse isotype antibody (PE and FITC labeled) was used for quadrant setting.

Statistic Analysis

The data were analyzed using the computer program SigmaStat for DOS (Jandel Scientific, San Rafael, CA). Results were expressed as mean ± S.E. Mann-Whitney's non-parametric rank sum test and Student's t test were used where appropriate. *P* values of 0.05 or less were regarded as significant.

RESULTS

Activation of Mononuclear Cell Populations in *P. gingivalis*- Stimulated PBMC Cultures

In this experiment, we assessed sonicated extracts of *P. gingivalis* for their ability to induce CD69 expression by peripheral blood lymphocytes. PBMC isolated from healthy donors were cultured and stimulated with different concentrations of *P. gingivalis* (0.016, 0.4 and 10 μg/ml) for 24 hours. As shown in Figure 1, large number of B cells and NK cells expressed CD69 after *P. gingivalis* stimulation. A smaller increase in number of γδ T cells and αβ T cells expressing CD69 was observed. In purified population studies, positively sorted cells were stimulated with *P. gingivalis* sonicated extracts. After 24 hours, expression of CD69 was measured by flow cytometry. The results expressed in Figure 2 show that B cells but not αβ T cells, γδ T cells or NK cells were selectively activated by *P. gingivalis* to induce the expression of CD69.

Cytokine Production by PBMC Stimulated with *P. gingivalis*

We tested the ability of *P. gingivalis* sonicated extracts to induce the B cell regulatory cytokines IL-10, IL-12,and IL-15 in PBMC cultures. Results presented in Figure 3 show that a large amount of IL-10 was consistently detected in *P. gingivalis*-stimulated PBMC cultures. The production of IL-10 was found to be dose-dependent. There was no IL-12 or IL-15 detected in culture supernatants. The time course of *P. gingivalis* stimulated IL-10 production showed that the level of IL-10 peaked at 24 hours, and then declined to baseline at 96 hours (Figure 4).

Cellular Source of IL-10 in *P. gingivalis*-Stimulated PBMC Cultures

The cellular source of IL-10 in *P. gingivalis*-stimulated PBMC cultures was further investigated. Depletion of monocytes from PBMC cultures reduced the production of IL-10 in response to *P. gingivalis* stimulation by 95%. In contrast, depletion of αβ T cells or B cells did not have any observable effect on IL-10 production (Figure 5). To further evaluate the contribution of monocytes in *P. gingivalis* induced IL-10 production, we stimulated flow cytometric sorted monocytes, αβ T cells and B cells and assayed for IL-10 production. The results in Figure 6 show that high levels of IL-10 were detected in *P. gingivalis*-stimulated monocyte cultures, but not in *P. gingivalis*-stimulated αβ T cell or B cell cultures.

Effect of *P. gingivalis* and IL-10 on B cell Activation

This experiment was designed to evaluate the effect of *P. gingivalis* and IL-10 on the B cell proliferative response. Results shown in Figure 7 indicate that *P. gingivalis* alone moderately induce B cells to proliferate whereas IL-10 alone does not have any observable effect. A significant increase in B cell proliferation however, was observed in B cell cultures stimulated with *P. gingivalis* in combination with IL-10 ($P < 0.05$).

DISCUSSION

A large number of many activated T cells, B cells and other types of immune cells are known to infiltrate inflamed gingival tissues, however, the role of these cells in the pathogenesis of periodontal disease remains unclear. Understanding the mechanisms of how infiltrated mononuclear cells become activated is essential to prevent or/and reduce immune-mediated tissue destruction in periodontitis. In this study, we used an in vitro model to study the activation of immune cells by the periodontopathic bacterium, *P. gingivalis.* PBMC cultures derived from healthy donors incubated with sonicated extracts of *P. gingivalis* led to a dose-dependent activation of different lymphocyte subpopulations, as monitored by the expression of CD69. CD69 expression is the earliest inducible cell surface protein acquired during lymphoid activation both in vitro and in vivo under physiological conditions and inflammation.[26,27] A large increase in the number of CD69+ cells was consistently observed in B cells, NK cells, and to a lesser degree in γδ T cells and αβ T cells. When flow cytometric sorted cells were used, it was found that only B cells and not αβ T cells, γδ T cells nor NK cells were directly activated to increase expression of CD69 in response to *P. gingivalis* .

Taken together, the data suggest that the activation of αβ T cells, γδ T cells and NK cells in *P. gingivalis*-stimulated PBMC cultures is not directly mediated by bacterial products. It is possible that soluble mediator(s) produced in *P. gingivalis*-stimulated PBMC cultures or cell to cell contact is necessary for the activation of NK cells while T cells require antigen processing and presentation for activation. Further studies are required to investigate these mechanisms.

Hyperactivation of B cells or polyclonal B cell activation is a hallmark of progressive adult periodontitis lesions.[4,5] High levels of the B cell regulatory cytokines, IL-5 and IL-6 have been detected in gingival mononuclear cell cultures of adult periodontitis patients and it has been suggested that these are critical mediators in the induction of hyperresponsive B cells.[28] To elucidate further the role of other B cell regulatory cytokines, we assessed the production of IL-10, IL-12 and IL-15 in *P. gingivalis*-stimulated PBMC cultures. Large amounts of IL-10 were detected in the culture supernatants, while IL-12 and IL-15 were not detected. These findings support recent studies by Yamazaki et al.[15] and Aramaki et al.[20] which showed increased levels of IL-10 mRNA and IL-10 protein in inflamed gingival tissues. The cellular source of IL-10 detected in gingival tissues is not clear. IL-10 producing T cells have been detected in gingival tissues.[16,19,29] However, the present in vitro model indicated that monocytes were the major cellular source of IL-10 in *P. gingivalis*-stimulated PBMC cultures. The production was rapid and peaked within 24 hours. B cells and αβ T cells did not contribute to the *P. gingivalis* induced IL-10 production.

IL-10 was initially described as a cytokine synthesis inhibition factor.[30,31] Later investigations have suggested the critical role of IL-10 in B cell proliferation and antibody

production.[22,32] High levels of IL-10 have been linked to the pathogenesis of autoimmune diseases including systemic lupus erythematosus, Sjogren's syndrome and rheumatoid arthritis.[33,34] In periodontitis, Stein and his colleagues[35] have recently suggested that increased IL-10 production leads to expansion of CD5+ B cells and enhances production of auto-antibodies directed against gingival tissue components. The role of IL-10 in suppressing cell-mediated immune responses in periodontitis is still unknown. Recent data have also shown that IL-10 inhibits neutrophil phagocytic and bactericidal activity[36], such that an increased production of IL-10 in gingival tissue may suppress phagocytic cell function against periodontopathic bacteria and lead to disease progression. In this experiment, we have shown that the stimulation of purified B cells with *P. gingivalis* sonicated extracts resulted in moderate B cell proliferation. The proliferative response of B cells was found to be significantly enhanced in the presence of IL-10. The results strongly support the role of IL-10 in B cell activation and more important, it provides the mechanism underlying the predominance of B cells in periodontitis lesions. In response to *P. gingivalis* infection, host immune cells, possibly monocytes and antigen reactive T cells produce IL-10. We hypothesize that this IL-10 may in turn augment the activation of *P. gingivalis* stimulated B cells, resulting in the rapid proliferation of B cells and the production of potentially destructive auto-antibodies. Furthermore, IL-10 may suppress the defensive function of phagocytic cells and result in increased susceptibility to infection.

The role of hyperresponsive B cells or polyclonal B cell activation in periodontitis remains elusive. It is perceived as a non-protective reaction and hence contributes to the pathogenesis of the disease. Anti-collagen antibody has been consistently detected and

suggested to be involved in gingival tissue destruction.[11,12,37] In addition to the well known role in antibody production, B cells also have the determinative role as antigen presenting cells (APCs) in the generation of T cell mediated immune responses.[38] Recent studies have shown that B cells are critical in the pathogenesis of T cell mediated autoimmune diseases.[39,40] They serve as APCs in the generation of autoreactive T cells involved in autoimmune disease.[41] It has been shown that a high frequency of collagen-specific T cell clones can be established from the gingival tissues of chronic adult periodontitis patients[42] and that lymphocytes isolated from periodontal lesions exhibited a high degree of cytotoxicity against fibroblast.[43] Taken together, these data suggest the presence of autoreactive T cells in the inflamed tissues normals/patients.

The development of T helper (Th) subsets appears to be controlled at level of APCs.[44] Dendritic cells produce IL-12 and thus, influence the development of Th1 cells,while B cells influence the development of Th2 cells since they fail to produce IL-12.[45] A strong Th2 response has been detected in inflamed human gingival tissues.[16,29] Although B cells contribute the majority of infiltrated immune cells in periodontitis lesions, there is no study so far to analyze the function of tissue infiltrated B cells as APCs. The speculative role of B cells to serve as APCs to drive Th2 cell development and to promote autoreactive T cell mediated tissue destruction in periodontitis is provocative and more studies are needed to test this concept.

In conclusion, we have shown that stimulation of PBMC isolated from naive donors with sonicated extracts of *P. gingivalis* leads to the activation of B cells, NK cells, γδ and αβ T cells. *P. gingivalis* sonicated extracts directly stimulate B cells but not NK, γδ and αβ T cells

to express CD69. IL-10 production is detected in *P. gingivalis*-stimulated PBMC cultures. Upon exposure of B cells with *P. gingivalis* sonicated extracts and IL-10, the proliferative response of B cells is significantly increased. IL-10 may prove to be the critical cytokine involved in polyclonal B cell activation associated with periodontal disease.

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Table 1: Monoclonal antibodies used for flow cytometric analysis and cell sorting.

Monoclonal antibodies(mAbs)	*Specificity*	*Populations*
Anti-Leu-2a (FITC)	CD8	T cell subset
Anti-Leu-3a (FITC)	CD4	T cell subset
Anti-TCR-α/β-1 (FITC,PE)	α/β TCR	T cell subset
Anti-TCR-γ/δ-1 (FITC,PE)	γ/δ TCR	T cell subset
Anti-Leu-12 (FITC,PE)	CD19	Pan B cells
Anti-Leu-16 (FITC,PE)	CD20	Pan B cells
Anti-Leu-19 (PE)	CD56	NK cells
Anti-Leu-M3 (PE)	CD14	Monocytes
Anti-Leu-23 (FITC, PE)	CD69	Activation antigen
Mouse IgG1 (FITC, PE)	-	-

* All mAbs were purchased from Becton Dickinson, San Jose, CA.

FITC= fluorescein isothiocyanate; PE= phycoerythrin;

TCR = T cell receptor.

Figure Legends

Figure 1 Flow cytometric analysis of CD69 expression on lymphocyte subpopulations in *P. gingivalis* stimulated PBMC cultures. PBMC cultures were stimulated with various concentrations of *P. gingivalis* (0.016, 0.4 and 10 μg/ml). After 24 hours of incubation, cells were harvested and stained with 1) FITC-conjugated anti-TCR-α/β-1 + PE-conjugated anti-CD69; 2) FITC-conjugated anti-TCR-γ/δ-1 + PE-conjugated anti-CD69; 3) PE-conjugated anti-CD56 + FITC-conjugated anti-CD69; 4) PE-conjugated anti-CD19 + FITC-conjugated anti-CD69 as described in methods. Gates were set with isotype control antibodies conjugated with either FITC and PE. M1 region consists of CD69+ cells. Numbers in each histogram represent % of cells that express CD69. Similar results were obtained in 5 independent experiments.

Figure 2 Flow cytometric analysis of CD69 expression on purified lymphocyte subpopulation, purified αβ T cells, γδ T cells, NK cells and B cells were stimulated with sonicated extracts of *P. gingivalis* (10 μg/ml). After 24 hours of incubation, cells were harvested and then stained with anti-CD69 FITC or PE. Gates were set with isotype control antibody conjugated with FITC. M1 region consists of CD69+ cells. Numbers in each histogram represent % of αβ T cells, γδ T cells, NK and B cells that express CD69. Similar results were obtained in 3 independent experiments.

Figure 3 Cytokine production by *P. gingivalis* treated PBMC. PBMC cultures were stimulated with (0.016, 0.4 and 10 μg/ml) of sonicated extracts of *P. gingivalis*. Culture supernatants were collected after 24 hours of incubation and assessed for IL-10, IL-12 and IL-15 production by ELISA.

Figure 4 Kinetic study of IL-10 production from *P. gingivalis* stimulated PBMC cultures. PBMC were cultured with or without sonicated extracts of *P. gingivalis* (10 μg/ml). The incubation period was varied from 0, 24, 48, 72 and 96 hours. The culture supernatants were collected at 24-hour intervals for analysis of IL-10 production by ELISA.

Figure 5 Effect of cell depletion in PBMC on IL-10 production in response to *P. gingivalis* stimulation. Removal of monocytes, αβ T cells or B cells from PBMC cultures were achieved as described in Materials and Methods. Both depleted population and undepleted PBMC were stimulated with *P.gingivalis* sonicated extracts 10μg/ml. Culture supernatants were collected after 24 hours of incubation, and then assay for IL-10 production by ELISA.

Figure 6 Analysis of IL-10 production from sorted monocytes, αβ T cells and B cells in response to *P. gingivalis* stimulation. Monocytes, αβ T cells and B cells ($1x10^6$ cells/ml) were positively sorted as described in Materials and Methods and then cultured with or without 10 μg/ml of sonicated extracts of *P. gingivalis*. After 24 hours of incubation, the supernatnants were collected for analysis of IL-10 production by ELISA.

Figure 7 The proliferative response of IL-10 and *P. gingivalis* stimulated B cells. Purified B cells were cultured under various conditions: 1) B cell control; 2) B cells + *P. gingivalis* (10 μg/ml); 3) B cells + IL-10 (500 ng/ml); and 4) B cells + *P. gingivalis* + IL-10. DNA synthesis was measured by [^{3}H] thymidine incorperation. Results are expressed as the mean of triplicates ± S.E. from 5 independent experiments.

*, $P < 0.05$ compared with controls

**, $P < 0.05$ compared with controls and *P. gingivalis* treated B cells

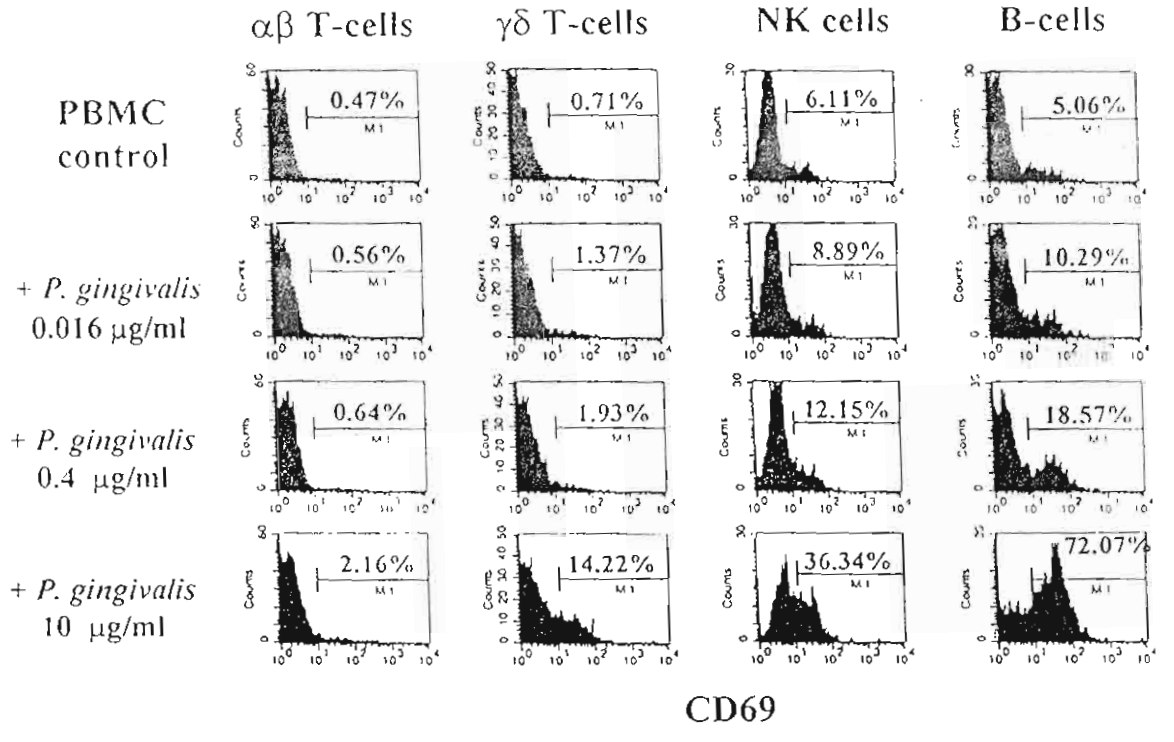
αβ T-cells
γδ T-cells
NK cells
B-cells
PBMC control
+ P. gingivalis 0.016 μg/ml
+ P. gingivalis 0.4 μg/ml
+ P. gingivalis 10 μg/ml
0.47%
0.71%
6.11%
5.06%
0.56%
1.37%
8.89%
10.29%
0.64%
1.93%
12.15%
18.57%
2.16%
14.22%
36.34%
72.07%
CD69

Figure 1

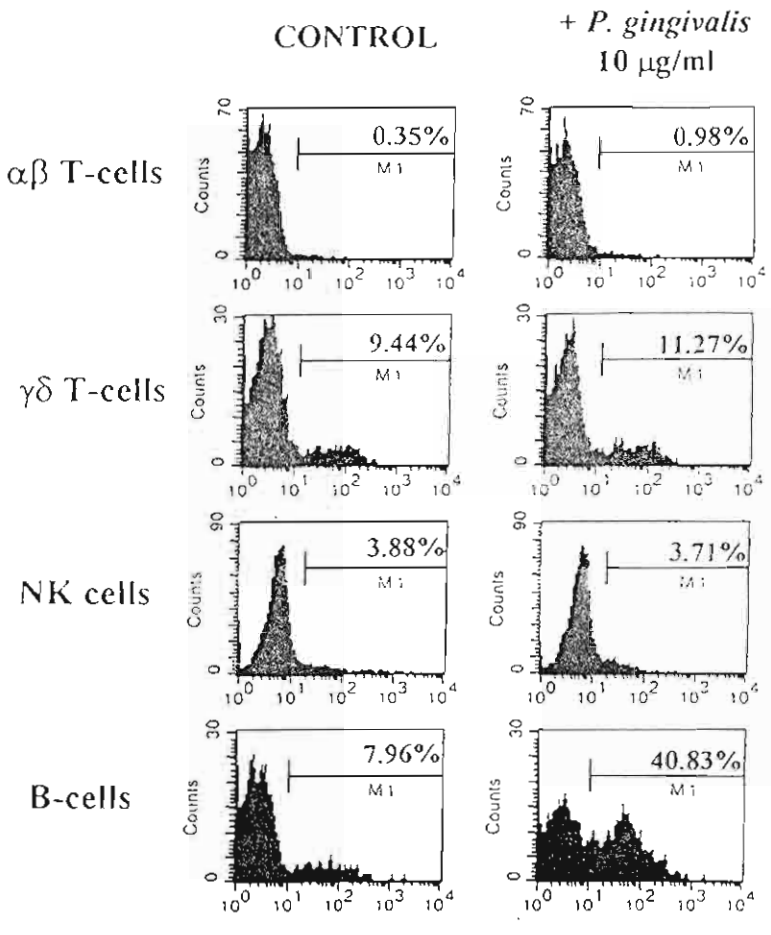
CONTROL
+ P. gingivalis
10 μg/ml
αβ T-cells
γδ T-cells
NK cells
B-cells
0.35%
0.98%
9.44%
11.27%
3.88%
3.71%
7.96%
40.83%
M1
Counts
CD69

Figure 2

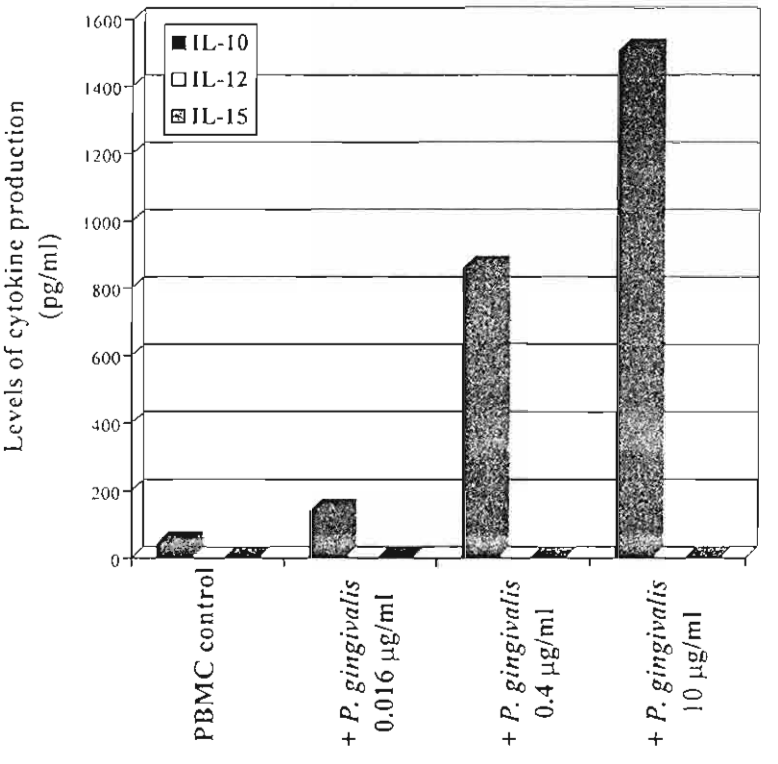
Levels of cytokine production (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
IL-10
IL-12
IL-15
PBMC control
+ P. gingivalis 0.016 µg/ml
+ P. gingivalis 0.4 µg/ml
+ P. gingivalis 10 µg/ml

Figure 3

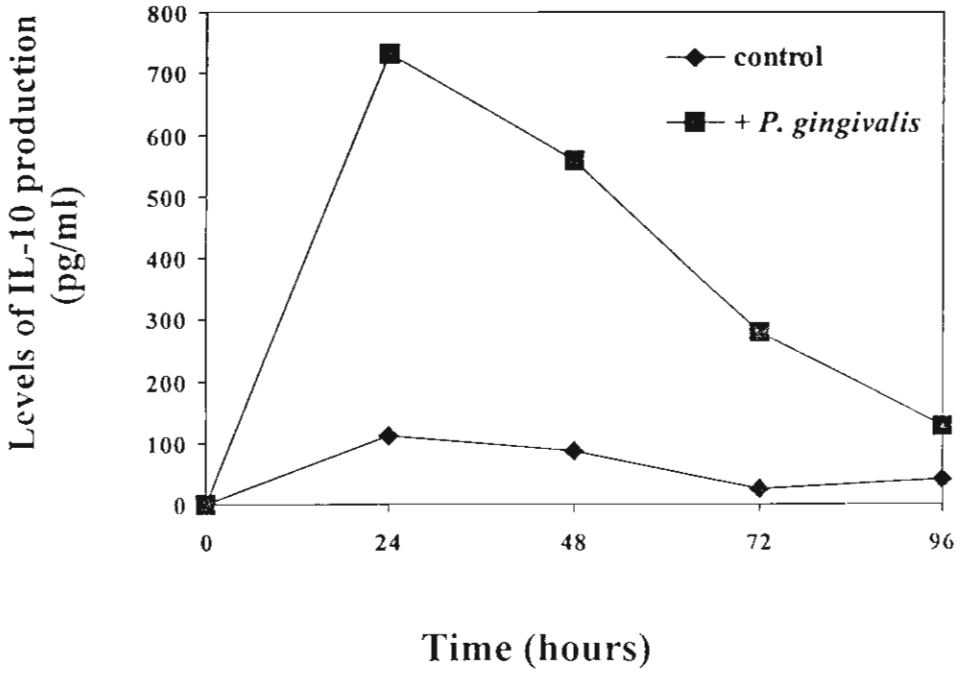
Levels of IL-10 production (pg/ml)
800
700
600
500
400
300
200
100
0
0
24
48
72
96
control
+ P. gingivalis
Time (hours)

Figure 4

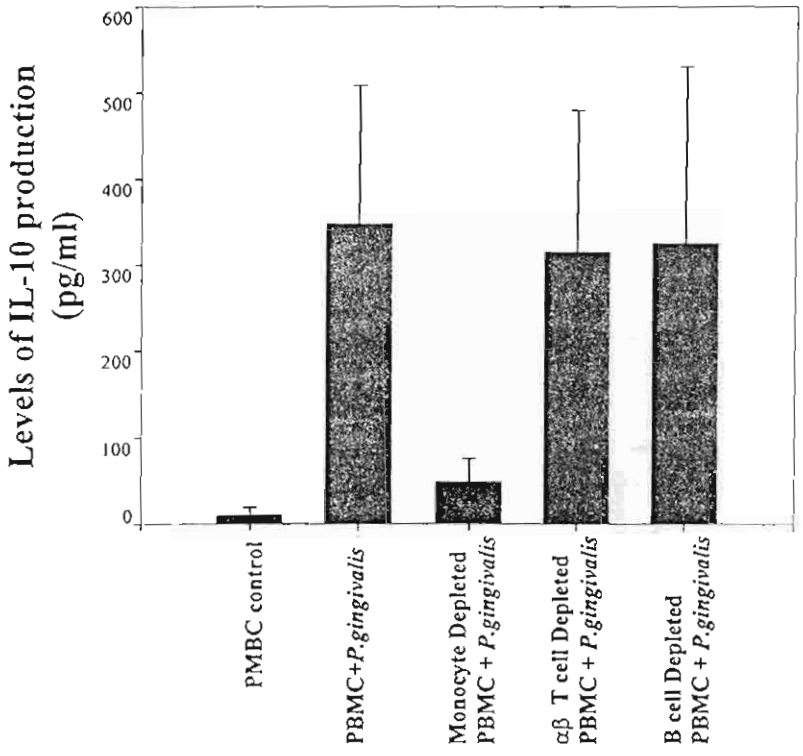
Levels of IL-10 production (pg/ml)
600
500
400
300
200
100
0
PMBC control
PBMC+P.gingivalis
Monocyte Depleted PBMC + P.gingivalis
αβ T cell Depleted PBMC + P.gingivalis
B cell Depleted PBMC + P.gingivalis

Figure 5

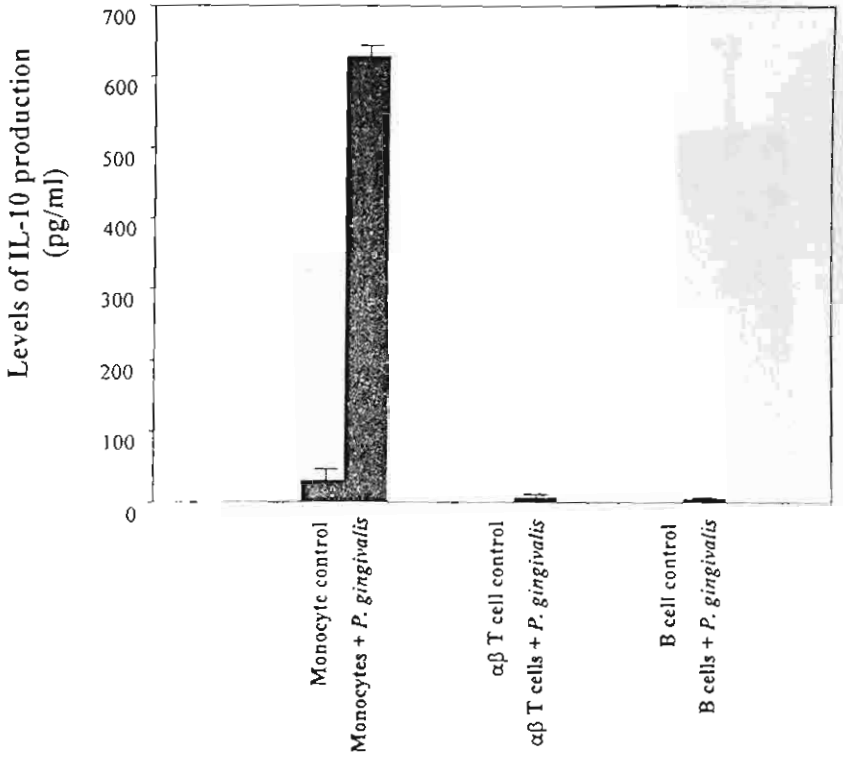
Levels of IL-10 production (pg/ml)
700
600
500
400
300
200
100
0
Monocyte control
Monocytes + P. gingivalis
αβ T cell control
αβ T cells + P. gingivalis
B cell control
B cells + P. gingivalis

Figure 6

^{3}H Thymidine incorporation (CPM)
2500
2000
1500
1000
500
0
*
**
B cell control
B cells + IL-10
B cells +P. gingivalis
B cells+IL-10 P.gingivalis

Figure 7