CHAPTER 3

Materials and Methods

3.1 Materials

3.1.1 Chemical Reagents

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 calf serum, collagenase (Clostridial Collagenase CLS III grade), 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, NA.

3.1.2 Medium

The culture medium used in these studies was RPMI1640 supplemented with 2 mM L-glutamine, 2 mg/ml of sodium bicarbonate, 2 mg/ml of D-glucose, 5.94 mg/ml of HEPES and 20 μg/ml gentamycin.

3.1.3 Monoclonal Antibodies

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 labeled with either phycoerythrin or fluorescein (as described in Table 1) were obtained from Becton Dickinson, San Jose, CA.

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.

3.2 *Bacterial Preparation*

Sonicated extracts of *Porphyromonas gingivalis* FDC-381 (kindly provided by Ms. Nantana Aroonrerk, Department of Conservatives & Prosthodontics, Faculty of Dentistry, Srinakarinwirot University) were used. Stock cultures were maintained at –70°C in skim milk containing 1% dimethyl sulphoxide (DMSO). The bacteria were grown in trypticase soy broth at 37 °C under anaerobic chamber (Forma Scientific, USA). The purity of bacterial species was examined by colony morphology on trypticase soy blood agar as well as by Gram's staining. The bacteria were harvested by centrifugation (Beckman Instruments, USA) at 2060 × g for 15 minutes, washed twice in sterile PBS (0.15 M: pH 7.2-7.4) (Ishii et al., 1992). 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 bacterial stock was aliquoted and stored at –20 °C until use.

3.3 *Phenotypic Characterization of Mononuclear Cells Recovered from Periodontitis Lesions*

3.3.1 Gingival Tissue Samples

Gingival tissue samples were collected from three subjects with generalized severe periodontitis attending dental treatment at Siriraj Hospital. Each patient had no history of periodontal treatment for the past 12 months. Most of their teeth showed at least 6 mm. probing depth with gingival inflammation. Biopsies of gingival tissues attached to the teeth with "hopeless" diagnosis due to severe periodontitis were obtained. No other dental diseases such as pulpal disease were involved. Their systemic health was good and no antimicrobial drugs or anti-inflammatory drugs were taken within the past 3 months. The excised tissues were immediately placed in steriled tubes containing RPMI 1640 medium, kept on ice and transferred to the laboratory within 24 hours for gingival cell extraction.

3.3.2 Preparation of Gingival Cells

The method of obtaining cells from the gingival tissues was that described by Daly et al. (1983a) and Seymour et al. (1985). Briefly, the tissues were washed thoroughly in RPMI 1640 medium and then they were cut into small fragments approximately 1 - 2 mm^3 segments. These fragments were incubated in 10% FCS in RPMI 1640 medium containing 2 mg/ml of collagenase. The ratio of medium to tissues was 1 ml : 100 mg of tissue. Following incubation for 90 minutes at 37 ^{o}C, in an atmosphere of 5% CO_2 and air, the residual tissue fragments were disaggregated 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 1640 medium. The lymphocytes were counted in haemocytometer and viability assessed by trypan blue exclusion.

3.3.3 Phenotypic Analysis of Gingival Cells by Flow Cytometry

Approximately 1 × 10^5 cells in a hundred microliter of RPMI 1640 medium were aliquoted into microfuge tubes and then were stained with the following marker combinations; 1) FITC-conjugated anti-CD4 + PE-conjugated anti-CD69; 2) FITC-conjugated anti-CD8 + PE-conjugated anti-CD69; 3) FITC-conjugated anti-CD19 + PE-conjugated anti-CD69. Cells were stained at 4^{o}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 (Becton Dickinson, Mountain View, CA). A control samples stained with mouse isotype antibody (PE and FITC labeled) was used for quadrant setting.

3.4 *In vitro Studies of Peripheral Blood Mononuclear Cells from Healthy Subjects*

3.4.1 Subject Selection

Heparinized peripheral blood (50-180 ml) was obtained from 15 healthy adult volunteers. These subjects had not taken antibiotics or anti-inflammatory drugs during a period of three months prior to the beginning of the study. None of them had symptoms of infection.

3.4.2 Peripheral Blood Mononuclear Cell Preparation

PBMC were prepared as previously described by Boyum (1968). Briefly, heparinized peripheral blood was layered on Ficoll-Hypaque (Histopague), and centrifuged for 30 minutes at 700 × g at 25 ^{o}C. PBMC were washed twice with PBS and then resuspended in RPMI 1640 medium with 10% heat inactivated autologous serum. Cells were counted in haemocytometer and cell viability was assessed by trypan blue exclusion.

3.4.3 Purification of Monocytes, αβ T-cells, γδ T-cells, NK cells and B-cells

T-cells were removed from PBMC by rosetting with neuraminidase-treated sheep erythrocytes. Non-rosetting cells (T-cell depleted mononuclear cells) were stained with anti-CD20 (FITC) mAb and then positively sorted by flow cytometry (FACStar; Becton Dickinson, Mountain View, CA). Purified monocytes were collected the same time by positive sorting of CD14+ cells (stained with anti-CD14 (PE) mAb).

Purified NK cells, αβ T-cells and γδ T-cells were also positively sorted from rosetting cells (T-cell fraction). The mAbs used to label NK cells, αβ T-cells and γδ T-cells are anti-CD56 (PE) , anti-TCR-α/β-1 (PE) and anti-TCR-γ/δ-1 (PE), respectively. Normally, cells purified by this method was about 95-98% pure on subsequent flow cytometric analysis.

3.4.4 Stimulation of PBMC Cultures with P. gingivalis

PBMC cultures (2 $\times 10^6$ cells/ml) were stimulated with or without sonicated extracts of *P. gingivalis* (0.016, 0.4 and 10 μg/ml) in a humidified atmosphere of 5% CO_2 in air at 37 ^{o}C. After 24 hours, the cells were harvested and analyzed for the expression of very early activation marker (CD69) by flow cytometry (FACScan). The culture supernatants from *P. gingivalis*-stimulated PBMC cultures were also collected for evaluation of cytokine production (IL-10, IL-12 and IL-15).

3.4.5 Studies of P. gingivalis -Stimulated Purified αβ T-cells, γδ T-cells, NK cells and B-cells

Purified αβ T-cell, γδ T-cell, NK cell and B-cell cultures (1 $\times 10^6$ cells/ml) were stimulated with or without sonicated extracts of *P. gingivalis* (10 μg/ml) in a humidified atmosphere of 5% CO_2 in air at 37 ^{o}C. After 24 hours, the cells were harvested and analyzed for the expression of very early activation marker (CD69) by flow cytometry (FACScan).

3.4.6 Kinetic Studies of IL-10 Production by P. gingivalis-Stimulated PBMC

PBMC (2×10^6 cells/ml) 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 in a humidified atmosphere of 5% CO_2 in air at 37 ^{o}C. After each incubation period, the culture supernatants from *P. gingivalis*-stimulated PBMC cultures were collected for evaluation of IL-10 production.

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

a) Depletion studies

To identify the cellular source of IL-10 in *P. gingivalis*-stimulated PBMC cultures, cell depletion experiment was performed. 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. Flow cytometric removed cell cultures were stimulated with *P. gingivalis* sonicated extracts at 10 μg/ml. Culture supernatants were collected after 24 hours of incubation, and then assayed for IL-10 production by ELISA.

b) Sorted population

αβ T-cells, monocytes and B-cells were positively sorted as described earlier and they were cultured (1 $\times 10^6$ cells/ml) with or without 10 μg/ml of sonicated extracts of *P. gingivalis*. After 24 hours of incubation, the supernatants were collected for analysis of IL-10 production by ELISA.

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

For the measurement of proliferative response, Purified B-cells (1 $\times 10^6$ 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* + IL-10. Recombinant human IL-10 was always added at day 3 (Itoh and Hirohata, 1995). Cells cultures were pulsed with [^{3}H] thymidine (0.5 μCi/ 200 μl/ well) at day 4 and were 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).

3.4.9 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 comparison with a standard curve of each cytokine. The detection limits of IL-10, IL-12 and IL-15 are 8, 25 and 47 pg/ml, respectively.

3.4.10 Flow Cytometric Analysis

The mAbs used in this study include 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. Cell staining procedures as well as flow cytometric analysis were similar to those described in section 3.3.3.

3.5 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.