CHAPTER 3

Materials and Methods

3.1 Bacterial preparation

3.1.1 *Porphyromonas gingivalis*

Whole bacteria, *P. gingivalis* strain FDC-381 (from JJ. Zambon in SUN YaB were kindly provided by Dr.Takeyoshi Koseki ,Tokyo Medical and Dental University, Japan) was used.

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

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

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

Trypticase soy agar (Difco laboratories)	40 grams
Hemin (1 mg/ml) + Vitamin K (10 mg/ml) stock solution	10 ml
Sterile lysed horse blood	50 ml
Kanamycin SO_4	4 ml
Distilled water to 1000 ml	

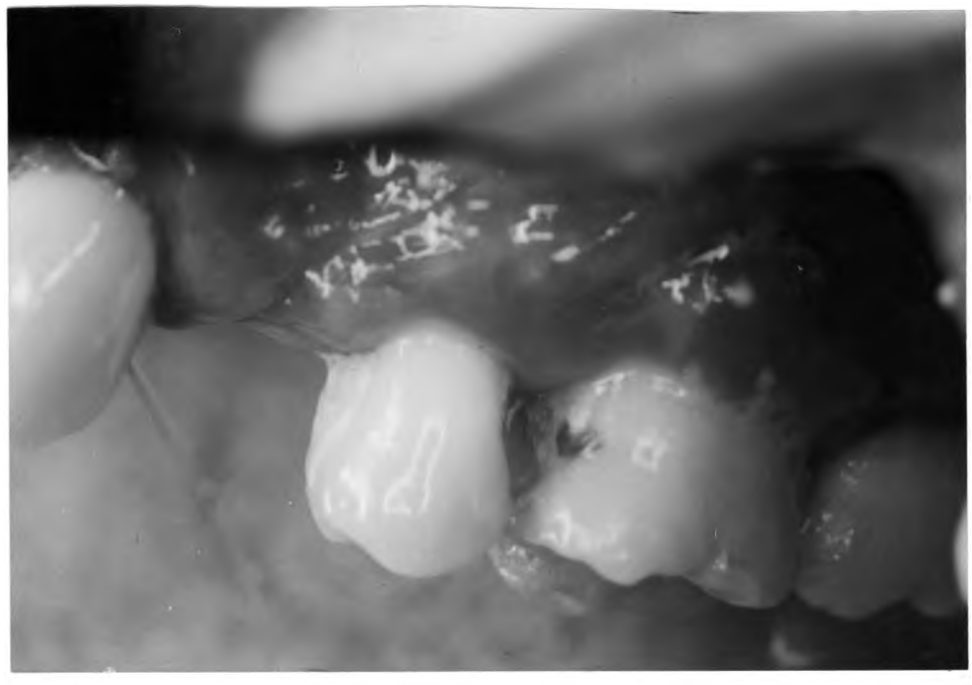
The bacteria were harvested by centrifugation (Beckman Instruments, USA) at 300xg for 10 min, washed three times in sterile phosphate - buffered saline (PBS , 0.15 M , pH 7.2) and heat - killed at 100 °C for 15 min. Stock solutions of the organism were adjusted to a final protein concentration of 0.5 mg/ml as determined by the method of Lowry (1951) and stored at - 20 °C .

3.2 Subject selection

Two adult periodontitis patients, one male named CC (35 years old) and the other male named SA (45 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. Both of them were diagnosed as generalized adult periodontitis with a few hopeless teeth (Figure 2). 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 these teeth were clinically mobile with third degree mobility as demonstrated by radiographic appearance (Figure 3). However, these hopeless teeth did not have periapical lesion. They were to be extracted due to periodontal cause.

Both subjects were volunteers and willing to donate peripheral blood for research.

A



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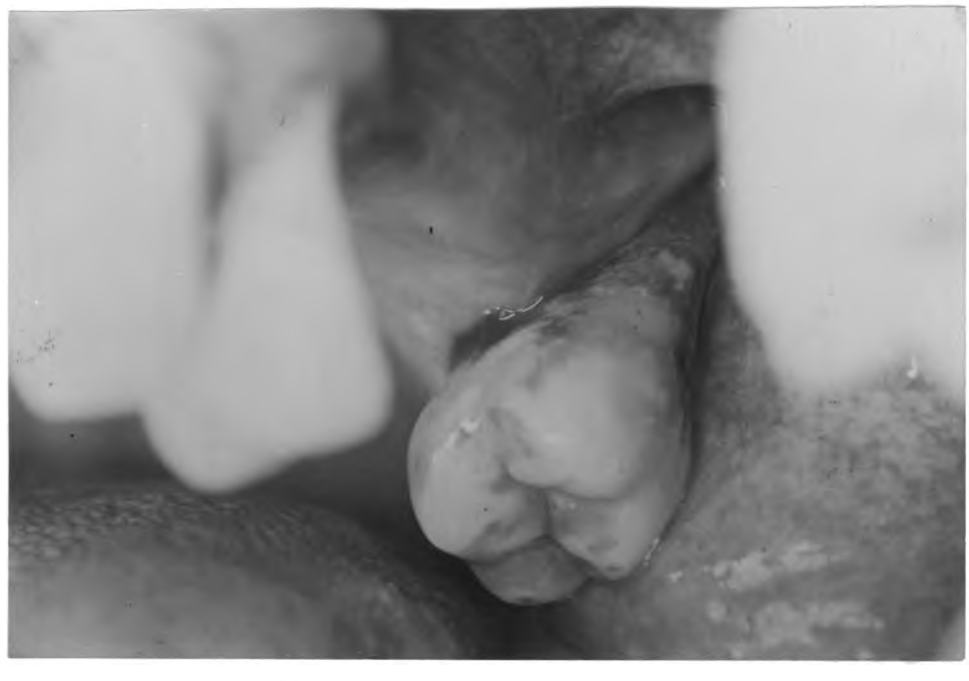
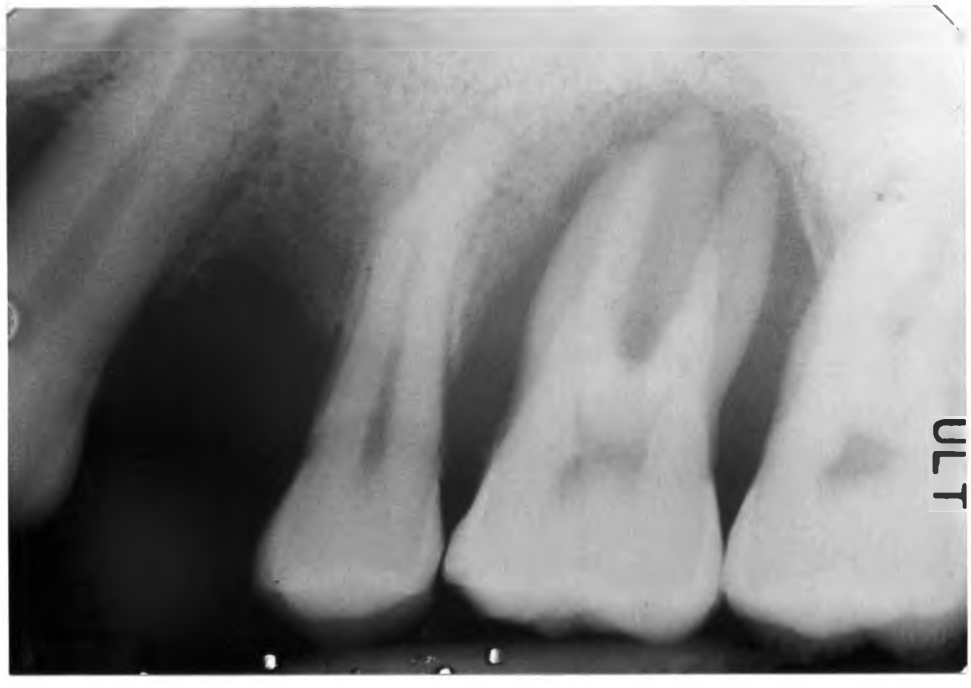


Figure 2: Both patients (A: patient CC; B: patient SA) participated in this research had good physical health except for severe periodontitis which had a few hopeless teeth. Their clinically peridontal conditions were red and edematous with supragingival plaque deposited.

A



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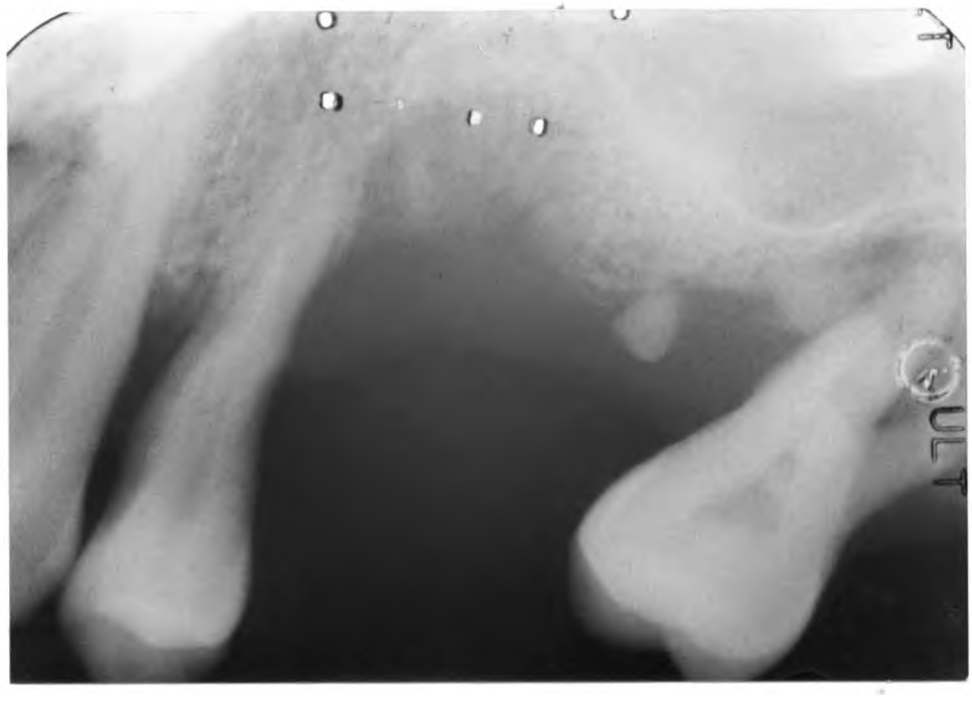

Figure 3 : The level of alveolar bone support of both patients (A: patient CC; B: patient SA)conditions radiographically appeared less than one third of root length. And these teeth were clinically mobile with third degree mobility. The hopeless teeth had no periapical lesion.

3.3 Isolation of peripheral blood mononuclear cells (PBMC)

Heparinized peripheral blood was collected from the median cephalic vein. The PBMC were isolated by Isoprep® (Robbins Scientific, CA., USA) density gradient centrifugation (300xg, 15°C, 25 min) as described by Boyum (1964). The cells were washed two times (300xg, 15°C, 5 min) with lymphocyte culture medium(RPMI), RPMI-1640(Gibco BRL, NY., USA) supplemented with 2 mM glutamine (Seromed, Berlin, Germany), penicillin (50 international unit, IU/ml), streptomycin (50 µg/ml) (Seromed).

Viability of isolated cells was tested using Trypan blue (Sigma). The viable cells appeared clear under microscope compared to the blue nonviable cells. The percentage of viable cells was determined using a haemocytometer (Klaus, 1987).

3.4 Establishment of Epstein - Barr Virus (EBV) transformed B lymphoblastoid cell line (LCLs)

3.4.1 Testing LCLs by immunofluorescence technique staining and flow cytometry

Isolated peripheral blood mononuclear cells 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 provided by Dr.Busarawan Sriwanthana , Ministry of Public Health, Bangkok, Thailand) and 1 ml of fetal bovine serum (FBS) (Gibco BRL) in 25 cm^3 tissue culture flask (Nunc, Kamstrup, Denmark). For T-cell suppression, 0.5ml of cyclosporin A (1 µg/ml)

was added to the cell suspension to make a final concentration of 100 ng/ml. The cells were incubated at 37°C, 5% CO_2 (Sheldom Manufacturing Inc., USA) 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% FBS and maintained to a density of 0.5 - 1.0 x 10^6 cells/ml in RPMI medium containing 20% FBS (Walker, 1990; Weinhold, 1992). Before utilizing both LCLs, the surface immunoglobulin (sIg) phenotype of these cells was examined by direct immunofluorescence staining technique with anti-IgG and anti-IgM fluorescein conjugated monoclonal antibody(MAb) (Becton Dickinson, CA., USA). By a density of 0.5-1.0 x10^6 cells/ml of cell suspension were incubated with fluorescein conjugated anti-IgG+IgM and anti-IgM at 20°C for 30 minutes, and washed twice with cold PBS. The stained cells were studied under dark field and fluorescence microscope. In addition, cell phenotype was assessed by flow cytometric analysis. Anti-Leu 12 monoclonal antibody was used. These cells were CD 19 positive(+) or B-cells. It was found to be > 90% CD 19+ and nearly < 0.1% CD3+ (anti-Leu4, Becton Dickinson).

3.4.2 Testing for an appropriate radiation dose for LCLs

The cells were cultured under the above conditions for 2 months before testing for an appropriate radiation dose for LCLs to 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 irradiated-LCLs were cultured in RPMI with 10% FBS 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 Day 3 and Day 7, ^{3}H-thymidine (Amersham, Buckinghamshire, England) was added to

each microwell 4 hours(hr) prior to harvesting onto glass fibre discs using Titertek cell harvester (ICN Flow, England). ^{3}H-thymidine incorporation was measured in a liquid scintillation counter (LKB, Finland). The results were expressed as mean counts per minute (CPM) ± standard error of the mean (SEM).

3.5 *Establishment of T-cell lines (TCLs) from Peripheral blood*

PBMC ($1x10^{6}$ cells/ml) from CC and SA were stimulated in 24 well plates (Costar, MA., USA) over a 9 day period with 8 ug protein/ml of *P. gingivalis* in RPMI containing 10% FBS 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} cells/ml) were then entered resting period and were cultured in 2 ml conditioned medium which contained RPMI, 10% FBS, 20 U/ml r-IL-2 (Beringer Manheime, GmbH, Germany) without antigen stimulation in 24 well plates for 5 days. After a 5 day resting period, the cells were washed and restimulated with 25 μg/ml Phytohaemagglutinin(PHA)(Gibco BRL) in the presence of 5×10^{5} cells/ml irradiated (30 Gy) autologous LCLs in the same medium. These TCLs were maintained at a concentration of 10^{5}-10^{6} cells/ml in this conditioned medium in a 24 well tray on a cycle of resting (without LCLs or PHA stimulation) for 7 days followed by stimulation (with 5×10^{5}/ml irradiated autologous LCLs and 25 μg/ml PHA) for 7 days (Figure 4).

The periodical restimulation with PHA permitted T blast cells to be maintained up to 42 days in culture for CC and 56 days for SA. After the 1st (day 14), 2nd (day 28) round of stimulation and rest for CC and SA, the cells were phenotyped and tested for antigen responsiveness in a proliferative assay.

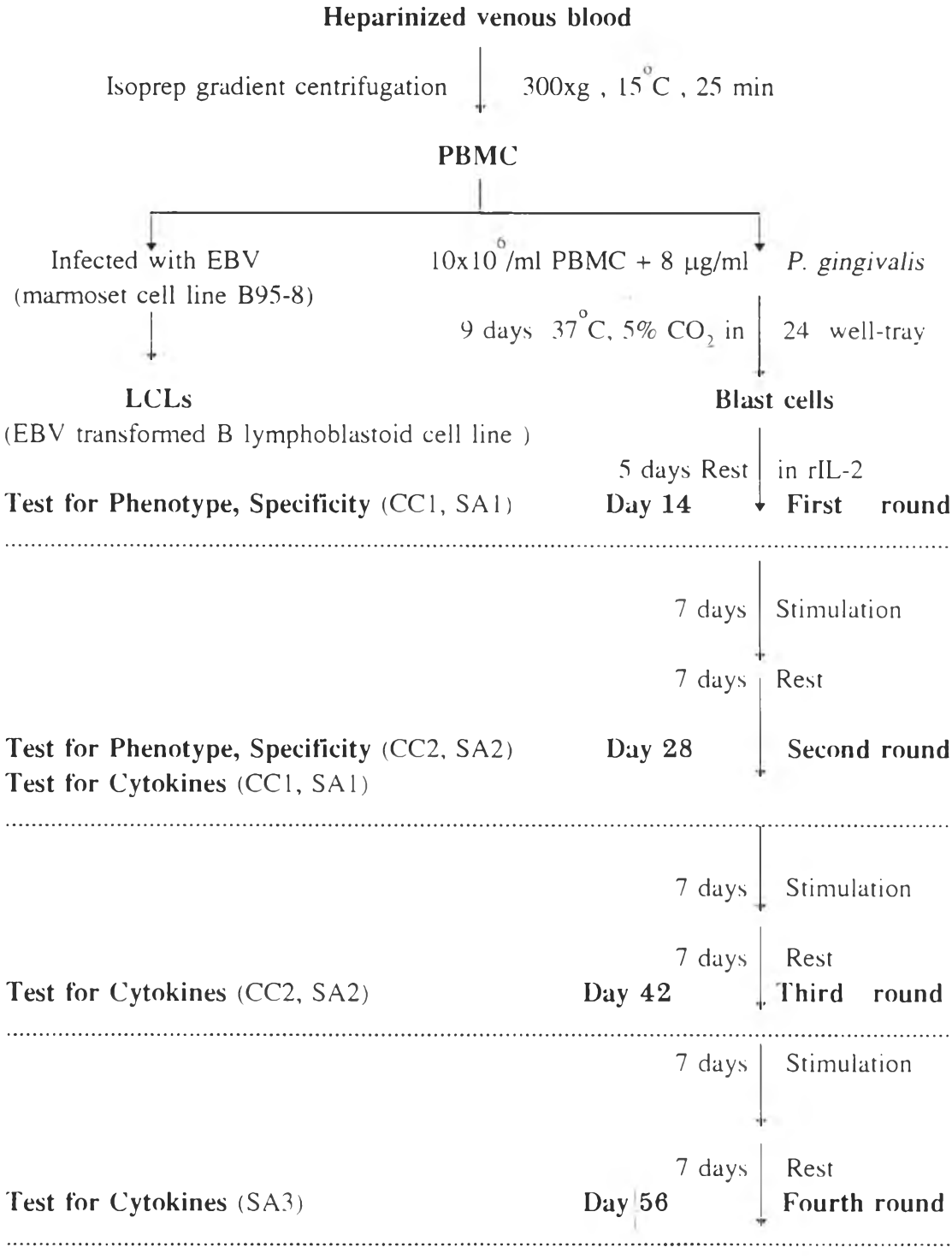

Figure 4: Diagram of the establishment of *P. gingivalis* specific T-cell lines of both CC-TCLs and SA-TCLs which demonstrated the round of stimulation and rest up to 56 days.

3.6 Proliferative response of TCL

Every PBMC culture in this research was incubated at 37 °C in a humidified atmosphere of 5% CO_2. Also in every proliferative assay, each culture was set up in RPMI plus 10% heat inactivated FBS in 96 well U- bottomed microtitre plates both TCLs stimulated with *P. gingivalis*, PHA and unstimulated as control. After the drescribed culture period, 0.5 μci/ml ^{3}H-thymidine was added to each microwell 4 hours prior to harvesting onto glass fibre discs using a Titertex cell harvester. ^{3}H- thymidine incorporation was measured in a liquid scintillation counter. The results were expressed as CPM. The proliferative response data were derived from a single experiment.

3.7 Phenotypic study

3.7.1 Staining of PBMC with monoclonal antibodies

Murine MAb conjugated with fluorescein isothiocyanate (FITC) and phycoerythrin (PE) were used as listed in Table 2. 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. 10^{4} PBMC (100 μl, in PBS/azide/BSA) were incubated with 10 μl of different MAb for 30 min at 4 °C in a direct immunofluorescence technique and washed once in RPMI plus 10% FBS. FITC and/or PE conjugated mouse Ig G_1, (3 μl) and Ig G_{2a} (3 μl) (Table 2) were used as controls for nonspecific binding of the MAb. The stained cells were fixed in PBS

containing 1.3% formalin. The fixed cells were either analysed immediately or refrigerated overnight and analysed the following day.

Table 2 : Monoclonal antibodies used for flow cytometric analysis

MAb	Specificity	Reference
anti-Leu-2a*(FITC)	CD8	Engleman et al., 1981.
anti-Leu-3a*(FITC)	CD4	Kotzin et al., 1981.
anti-Leu-4* (PE, FITC)	CD3	Ledbetter et al., 1981. Kan et al., 1983.
anti-Leu-12*(PE)	CD19	Loken et al., 1987.
mouse IgG_1* (FITC,PE)	mouse IgG_1	
mouse IgG_{2a}* (PE)	mouse IgG_{2a}	

*MAb was purchased from Becton Dickinson

3.7.2. Flow cytometric analysis

Flow cytometry is the measurement of flowing cells. It is uniquely powerful in its ability to quantitate the amount of fluorescence emitted, light scattered by individual flowing cells and subcellular components. Stained PBMC were analysed on a Becton Dickinson FACS Scan analyser. The laser beam was obtained from a high power source.The desired excitation wave length of 488 nm was obtained by laser source.

The FITC and PE signals were split by a 550 nm short pass dichroic mirror placed in the fluorescence emission beam orthogonal (90°) to the exciting beam and the hydrodynamically focused, coaxial jet stream of cells. The green/FITC signal was isolated using a 530 nm short pass dichroic mirror and the red/PE signal was isolated using a 570 nm long pass dichroic mirror. The instrument was calibrated using calibrate flow cytometry alignment beads (Becton Dickinson).

The lymphocyte population was gated using standard two dimensional light scatter histogram of forward angle light scatter (cell volume/size) versus orthogonal light scatter (internal structure/granularity). The gated population contained small and large lymphocytes but excluded dead cells and erythrocytes.

Data was collected on 20,000 ungated events to ensure a gated population of approximately 10,000 - 15,000 cells. The data was analysed by means of the BDIS Cell Quest program and was collected as frequency histograms and contour plots, the original data was retained on disc for future analysis. Cells expressing the CD3, CD4, CD8 and CD19 antigen were expressed as a percentage of the cell line populations minus background fluorescence. Negative controls were established using cells stained with mouse IgG fluorescence controls of identical isotype to the monoclonal antibodies used.

Due to the cost of MAb, the phenotypic data from each staining cell were derived from a single experiment.

3.8 Cytokine study

After the second, third and fouth rounds of stimulation and rest (Day 28, Day 42, Day 56), the TCLs from the two subjects were separated and washed. For the cytokine study, 5×10^5 cells/ml TCLs were cultured in the presence of 8 μg/ml *P. gingivalis* and 1×10^5 cells/ml LCLs in 96 U-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 wells, the cells were cultured with LCLs and 25 μg/ml PHA. The supernatants were collected after 6 hours, 1 day, and 3 days in culture and stored in aliquots at -70 °C until cytokine production was analysed. The cytokine data were derived from a single experiment.

IL-4 and IFN-γ production by the TCLs were measured by specific solid-phase sandwich enzyme-linked immunosorbent assays (ELISA) using the Quantikine™ Human IL-4 Immunoassay (R&D Systems, MN., USA), and the InterTest-γ™ Human IFN-γ Kit (Genzyme, MA., USA) respectively.

3.8.1 Testing for Interleukin 4 (IL-4)

The principle of the assay were briefly described as following. A MAb specific for IL-4 has been coated onto the microtiter plate provided in the kit. Standards and samples were pipetted into the wells, and any IL-4 present was bound by the immobilized antibody. After washed away unbound sample proteins, an enzyme-linked polyclonal antibody specific for IL-4 was added to the wells to "sandwich" any IL-4 immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of IL-4 bound in the

initial step. The color development was stopped by 2N Sulfuric acid and the intensity of the color was measured.

A curve was prepared, plotting the optical density versus the concentration of IL-4 in the standard wells. By comparing the optical density of the samples to this standard curve, the concentration of the IL-4 in the unknown samples was then determined. Methods in details for measuring IL-4 concentration was in laboratory manual of the Quantikine™ Human IL-4 Immunoassay.

3.8.2 Testing for Interferon - γ (IFN-γ)

The principles of the IFN-γ assay are similar to those of IL-4. First, a 96-well microtiter plate, coated with MAb specific for human IFN-γ, was used to capture IFN-γ present in standard samples and unknown specimens. Then, a goat polyclonal antibody which binds to multiple epitopes on the IFN-γ contained on the solid phase was added.

Next, a third antibody, biotin-labelled donkey-anti-goat Ig, which binds to the goat polyclonal antibody already bound to IFN-γ, was then applied.

Finally, streptavidin-peroxidase was added, which binds to the biotin on the third antibody. The peroxidase enzyme acted with peroxide substrate and OD chromagen to produce increased absorbance at 492 mm indicating the presence of IFN-γ

Increased absorbance due to bound, immunoreactive IFN-γ was quantitated by an ELISA reader. The measured absorbance was proportional to the concentration of IFN-γ that was present in the original sample. A reference curve

was obtained by plotting the IFN-γ concentration of several dilutions of standard versus absorbance. The IFN-γ concentrations in experimental samples could be determined by comparison of their absorbances with those obtained from the known amounts of human IFN-γ in the standard dilutions. Methods in details for measuring IFN-γ concentration was in laboratory manual of the InterTest-γ™ Human IFN-γ Kit.