CHAPTER 4

Results

4.1 Phenotypic Characterization of Mononuclear cells Recovered from Periodontitis Lesions

Gingival cells were extracted from three adult periodontitis subjects. All gingival tissue sample analyses were found to harbor an infiltrate rich in activated lymphocytes (Figure 1). Results presented in Table 2 show that the mean percentage of CD19+ B-cells, CD4+ T-cells and CD8+ T-cells were 49.03%, 18.25% and 7.2%, respectively. CD19+ B-cells were the predominant lymphoid cells in the periodontal lesions. The majority of immune cells found in inflamed tissues is activated cells as monitored by the expression of CD69 (an early activation antigen). The mean percentage of CD69+ B-cells is 56.24% of total B-cells. The mean percentages of CD69+, CD4+ T-cells and CD69+, CD8+ T-cells are 58.04% and 76.06 % of total CD4+ and CD8+ T-cells, respectively.

Table 2: Phenotypic Characterization of Mononuclear Cells Recovered from Periodontitis Lesions

	The percentages of each population in gingival tissues (%)		
	CD4+ T-cells	CD8+ T-cells	B-cells
Subject 1	23.66 (89.39)*	7.23 (95.85)*	40.89 (67.05)*
Subject 2	18.98 (62.96)*	8.12 (42.73)*	43.64 (58.94)*
Subject 3	12.1 (21.79)*	6.32 (89.59)*	62.55 (42.72)*
Mean ± S.E.	**18.25 ± 4.6** **(58.04 ± 27.8)***	**7.2 ± 0.7** **(76.06 ± 23.7)***	**49.03 ± 9.6** **(56.24 ± 10.1)***

* % of CD69 expression on each population

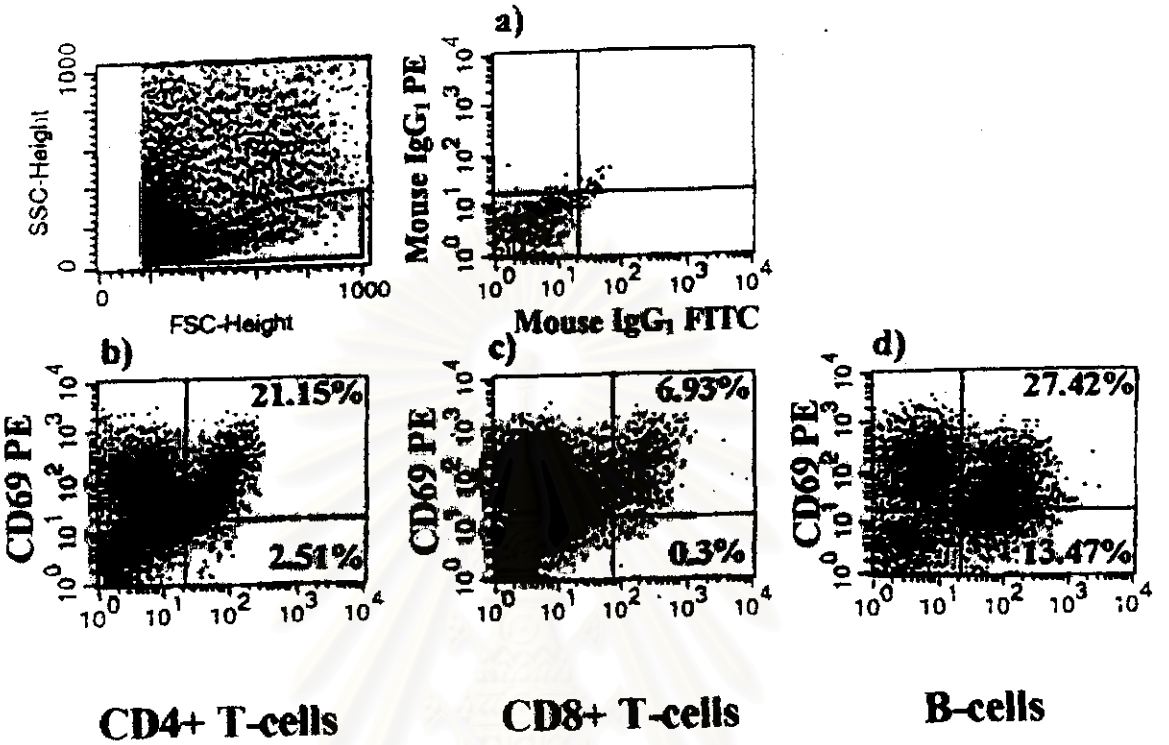


Figure 1: Flow cytometric analysis of monomuclear cells from periodontitis lesions. Cells were obtained from gingival tissues and then doubled stained with (b) FITC-conjugated anti-CD4 + PE-conjugated anti-CD69; (c) FITC-conjugated anti-CD8 + PE-conjugated anti-CD69; (d) FITC-conjugated anti-CD19 + PE-conjugated anti-CD69 as described in methods. Gates were set with isotype control antibodies conjugated with either FITC and PE (a). Numbers in upper right corner of each dot plot represent % of cells that expressed CD69+. Similar results were obtained in 3 separate experiments. PSC, forward scatter; SSC, side scatter.

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

Several infectious microorganisms have been shown to non-specifically activate PBMC cultures *in vitro* (Ivanyi and Lehner, 1970; 1971; Donaldson et al.,1982b). In this experiment, we assessed sonicated extracts of *P. gingivalis* for its ability to induce cell activation. 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 2, large number of B-cells and NK cells became activated after exposure to *P. gingivalis* sonicated extracts, as monitored by the expression of CD69. Smaller increase in number of cells expressing CD69 was observed on αβ T-cells and γδ T-cells. Cell activation in this experiment represents non-specific activation rather than antigen-specific activation since PBMC used were isolated from healthy donors. Next, we investigated whether *P. gingivalis* could directly activate B-cells, αβ T-cells, γδ T-cells or NK cells. 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 3 show that B-cells but not αβ T-cells, γδ T-cells or NK cells were selectively activated by *P. gingivalis* to induce expression of CD69.

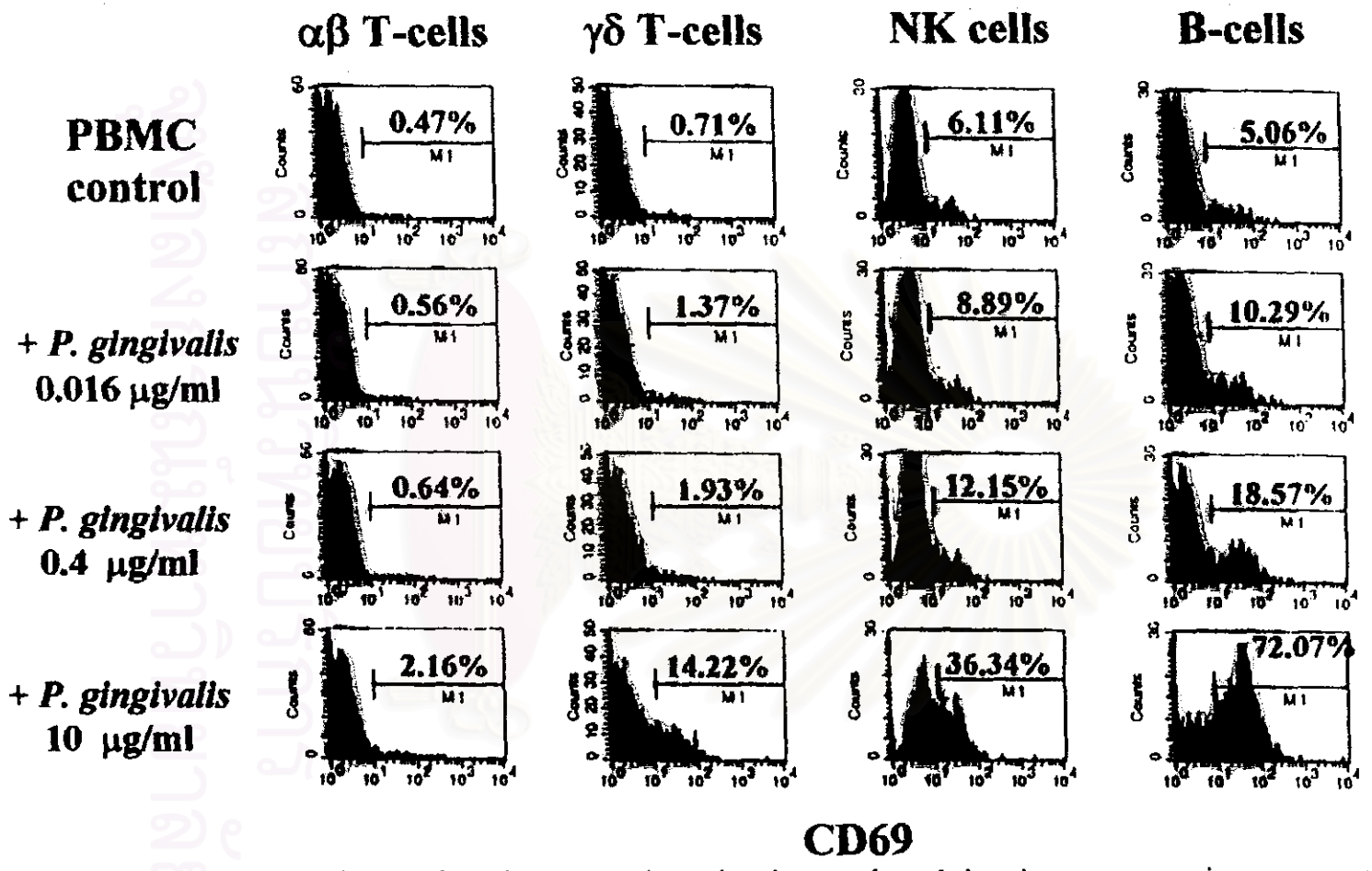


Figure 2: Flow cytometric analysis of CD69 expression on lymphocyte subpopulations in response to various concentrations of *P. gingivalis* (0.016, 0.4 and 10 μg/ml). After 24 hours of incubation, cells were harvested and stained with 1) FITC-conjugated anti-TCR-α/β-1 + PE-conjugated anti-CD69; 2) FITC-conjugated 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.

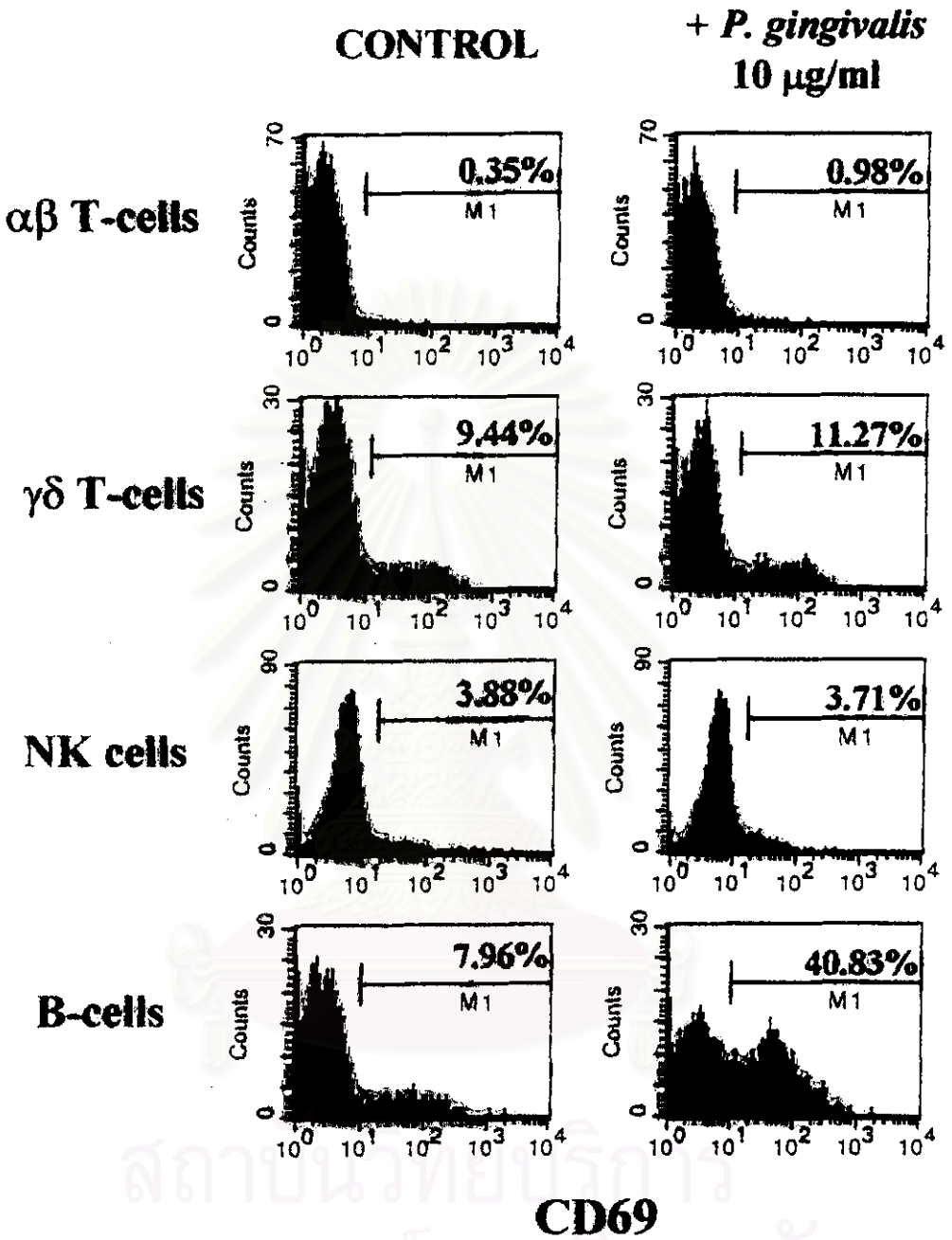


Figure 3: Flow cytometric analysis of CD69 expression on purified αβ T-cells, γδ T-cells, NK cells and B-cells in response to sonicated extracts of *P. gingivalis* (10 μg/ml). After 24-hour incubation, cells were harvested and then stained with anti-CD69 FITC or PE. Numbers in each histogram represent % of αβ T-cells, γδ T-cells, NK cells and B cells that express CD69. Similar results were obtained in 3 independent experiments.

4.3 *Cytokine Production by PBMC Stimulated with P. gingivalis*

Increased levels of B-cell regulatory cytokines, IL-5, IL-6 but not IL-2 and IL-4 have been detected in gingival mononuclear cells extracted from periodontitis patients (Fujihashi et al., 1993a; 1993b). We tested the ability of *P. gingivalis* sonicated extracts to induce other B-cell regulatory cytokine production in PBMC cultures. Results presented in figure 4 show that large amount of IL-10 were consistently detected in *P. gingivalis*-stimulated PBMC cultures. The production of IL-10 was found to be dose-dependent. There was no IL-12 and 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 5).

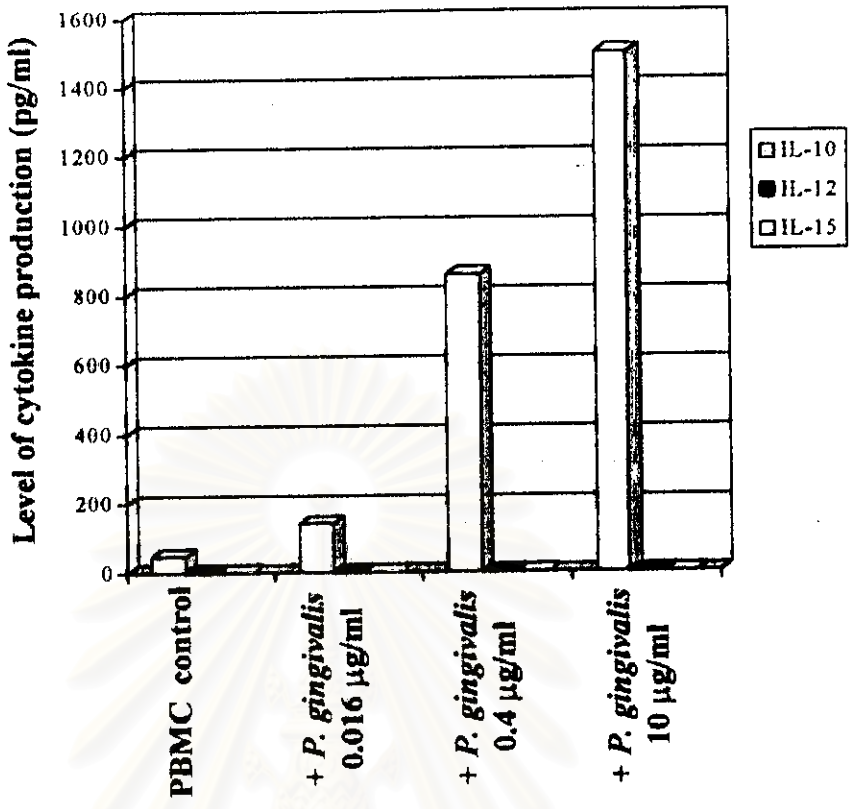


Figure 4: Cytokine production by *P. gingivalis*-treated PBMC. PBMC cultures were stimulated with (0.016, 0.4 and 10 µg/ml) of sonicated extracts of *P. gingivalis*. Cultures supernatants were collected after 24 hours of incubation and assessed for IL-10, IL-12 and IL-15 production by ELISA.

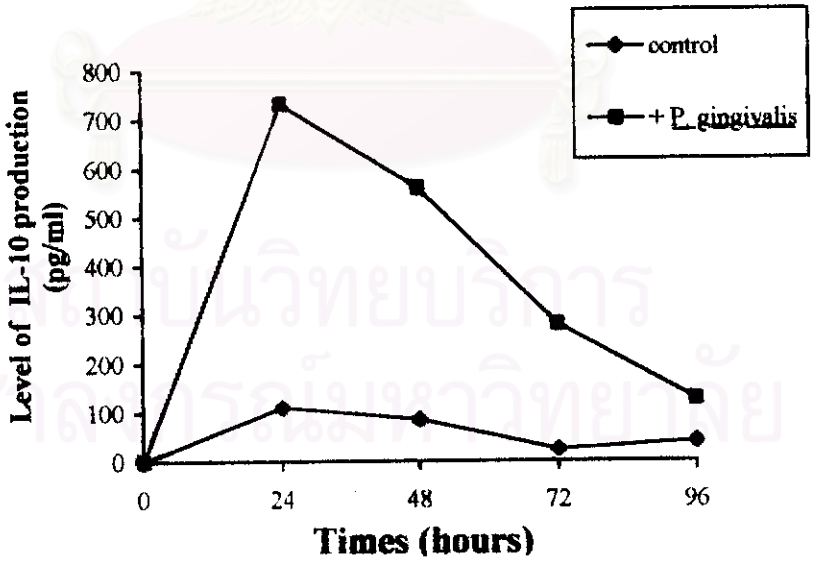


Figure 5: Kinetic study of IL-10 production from *P. gingivalis*-stimulated PBMC cultures. PBMC were cultured with or without sonicated extracts of *P. gingivalis* (10 µg/ml). The incubation period was varied from 0, 24, 48, 72 and 96 hours. The culture supernatants were collected at 24-hour intervals for analysis of IL-10 production by ELISA.

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

Different cell types including; monocytes, B-cells and T-cells are known to produce IL-10 (De Waal Melefyt et al., 1992; Mosmann, 1994). In this experiment, we evaluated the cellular source of IL-10 in *P. gingivalis*-stimulated PBMC cultures. Depletion of monocytes from PBMC cultures greatly 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 6). To further evaluation the contribution of monocytes in *P. gingivalis* induced IL-10 production, we stimulated flow cytometric sorted monocytes, αβ T-cells and B-cells for IL-10 production. The results in figure 7 show that high level of IL-10 was detected in *P. gingivalis*-stimulated monocyte cultures, but not in *P. gingivalis*-stimulated αβ T-cell and B-cell cultures.

Figure 6: 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 by negative selection of anti-CD14 (PE), anti-TCR-αβ-1 (PE), 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. 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.

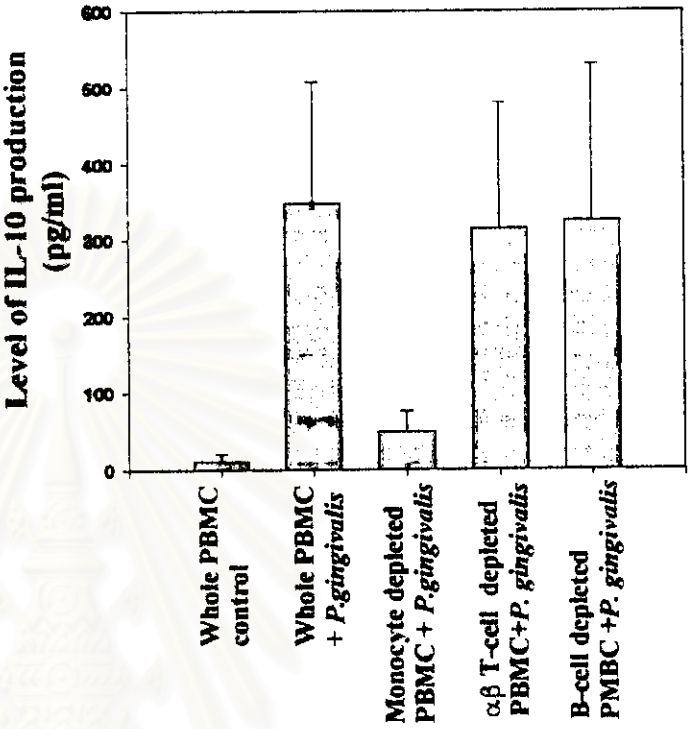
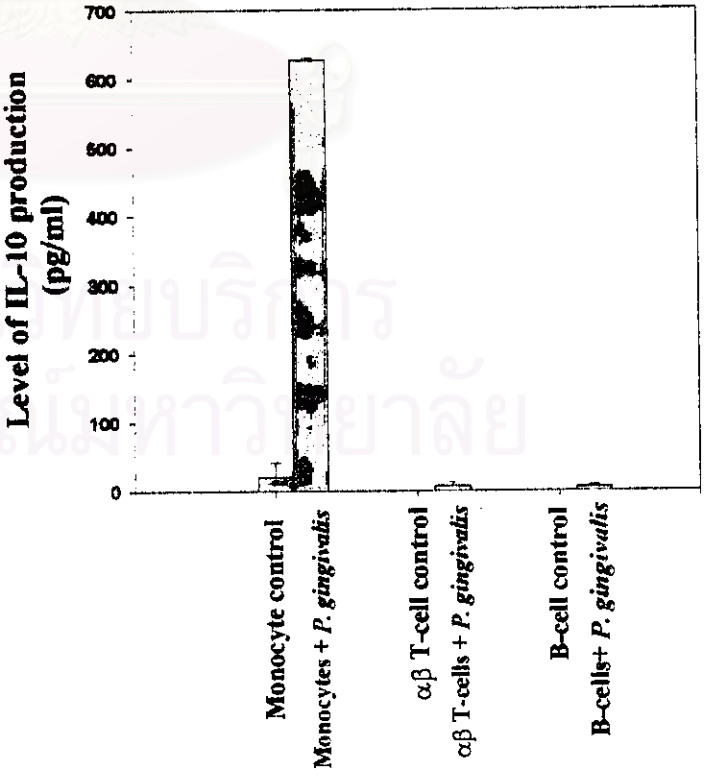


Figure 7: Analysis of IL-10 production from sorted monocytes, αβ T-cells and B-cells in response to *P. gingivalis* stimulation. Monocytes, αβ T-cells and B-cells ($1x10^5$ cells/ml) were positively sorted as described earlier and then cultured with or without 10 μg/ml of sonicated extracts of *P. gingivalis*. After 24 – hour incubation, the supernatnants were collected for analysis of IL-10 production by ELISA.



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

The increased number of infiltrated B-cells is consistently observed in periodontitis lesion (Stoufi et al., 1987; Seymour and Greenspan, 1979; Matsuo et al., 1996). The mechanism underlying a predominance of B-cells in inflamed tissues remains unclear. Monocyte derived IL-10 is known to be involved in B-cell activation (Rousset et al., 1992; Itoh and Hirohata, 1995). This experiment was designed to evaluate the effect of *P. gingivalis* and IL-10 on B-cell proliferative response. Results shown in figure 8 indicate that *P. gingivalis* moderately induce B-cells to proliferate. Significantly increase in B-cell proliferative response was observed in B-cell cultures stimulated with *P. gingivalis* in combination with IL-10.

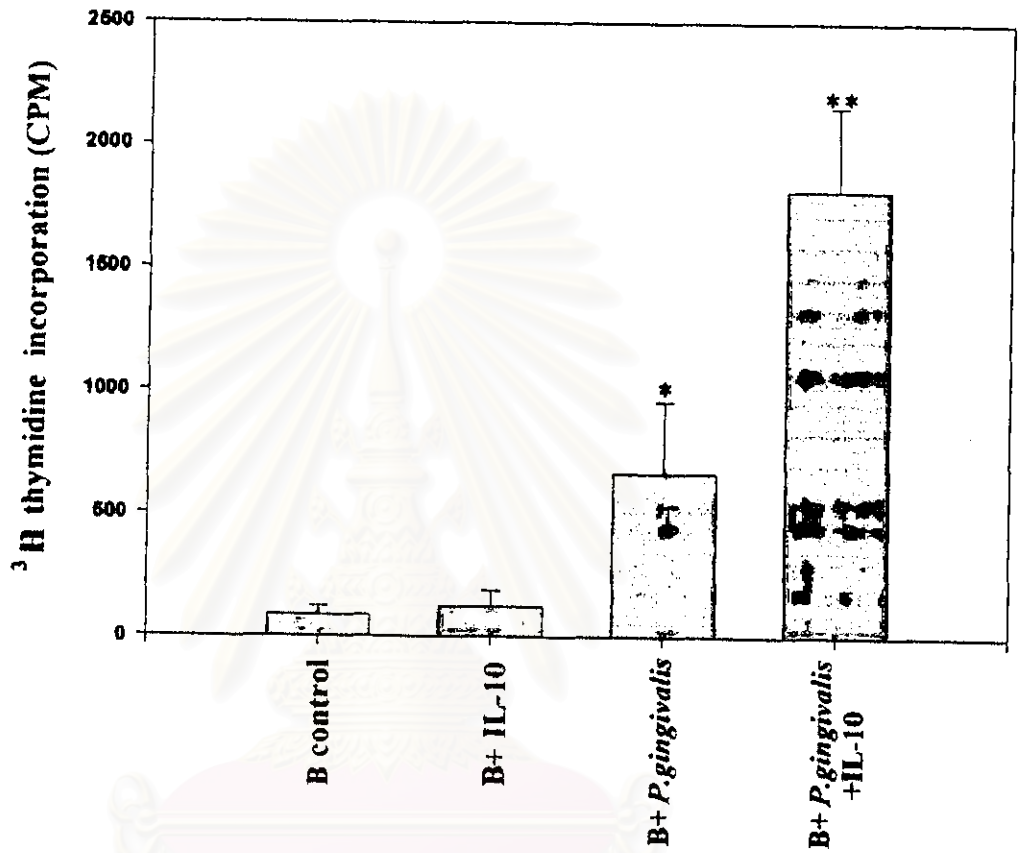


Figure 8: The proliferative response of IL-10 and *P.gingivalis* treated B-cell under various conditions: 1) B-cell control; 2)B-cells +*P. gingivalis*(10 μg/ml); 3) B-cells + IL-10 (500 ng/ml); and 4) B-cells + *P. gingivalis* + IL-10. Purified B-cells were cultured for 5 days. DNA synthesis was measured by [^{3}H] thymidine incorporation. Results are expressed as the mean of triplicates +/- S.E. from five independent experiments. CPM, count per minute.
*, $P < 0.05$ compared with controls
**, $P < 0.05$ compared with controls and *P. gingivalis* treated B-cells