CHAPTER 4

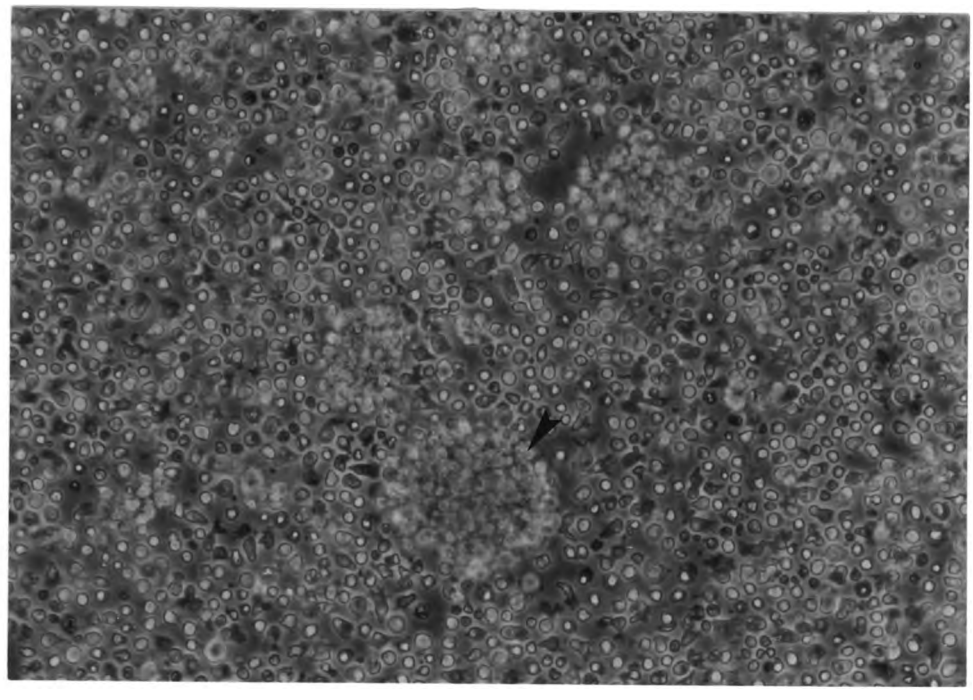
Results

4.1 Selection appropriated radiation dose for LCLs

Both LCLs of CC and SA were similar in appearance. They were large, round and clear cells which might be seen in cluster or solitary. These LCLs were in 4 months culture which seen under inverted phase microscope in 4x5x4 of magnification (Figure 5). Both CC-LCLs and SA-LCLs were examined by immunofluorescence staining technique with fluorescein conjugated anti-IgG+ IgM. The stained cells were seen under dark field and fluorescence microscpoe as shown in "patching" appearance in 4x5x4 of magnification (Figure 6). Cell phenotype of CC-LCLs and SA-LCLs was assessed by flow cytometric analysis. MAb-CD19 PE was used. These cells were CD19 positive(CD19+) or B-cells. It was found to be more than 95% CD19+ and nearly less than 0.1% CD3+ (Figure 7). The results confirmed that both LCLs were B-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 90 fold higher CPM than those with radiation and a higher proliferation was on day 3. With 20 Gy, some cells, i.e. SA-LCLs still survived and provided high background as much as 5.01×10^{3} CPM on day 3. At the same day, the 30Gy irradiated CC-LCLs and SA-LCLs showed 2.38×10^{3} and 3.94×10^{3} CPM respectively which were similar to 40 Gy irradiated cells. Therefore, 30 Gy were selected as an appropriate radiation dose for LCLs to support lymphocyte cultures (Figure 8 and 9).

A



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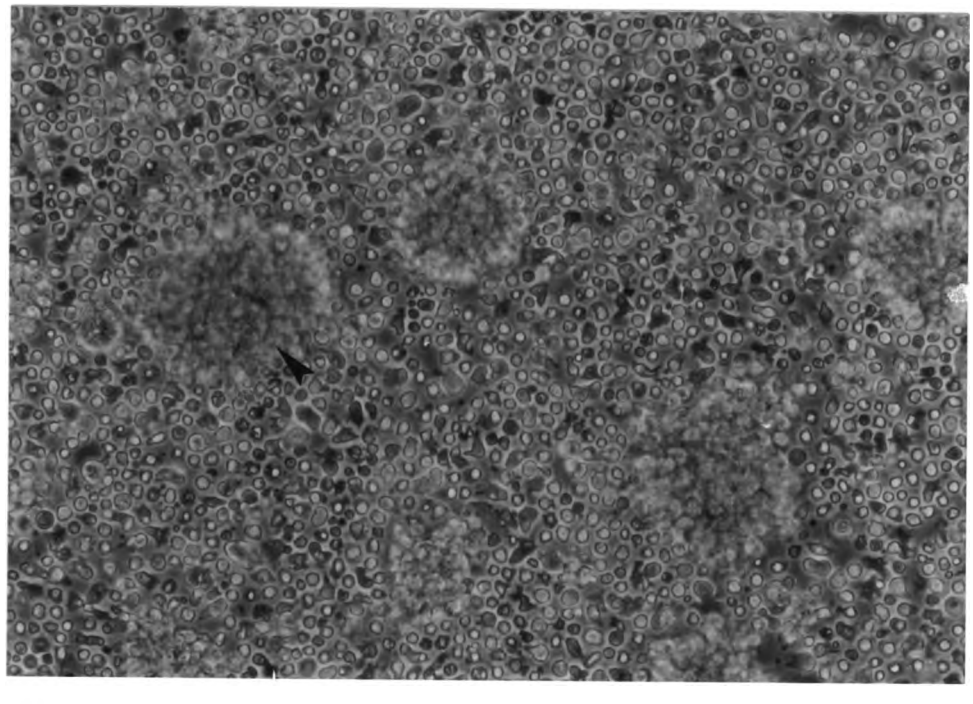


Figure 5: Both CC-LCLs (Figure A) and SA-LCLs (Figure B) were in 4 months culture which seen under inverted phase microscope in 4x5x4 of magnification. These were similar in appearance. They were large, round and clear cells which might be seen in cluster or solitary.

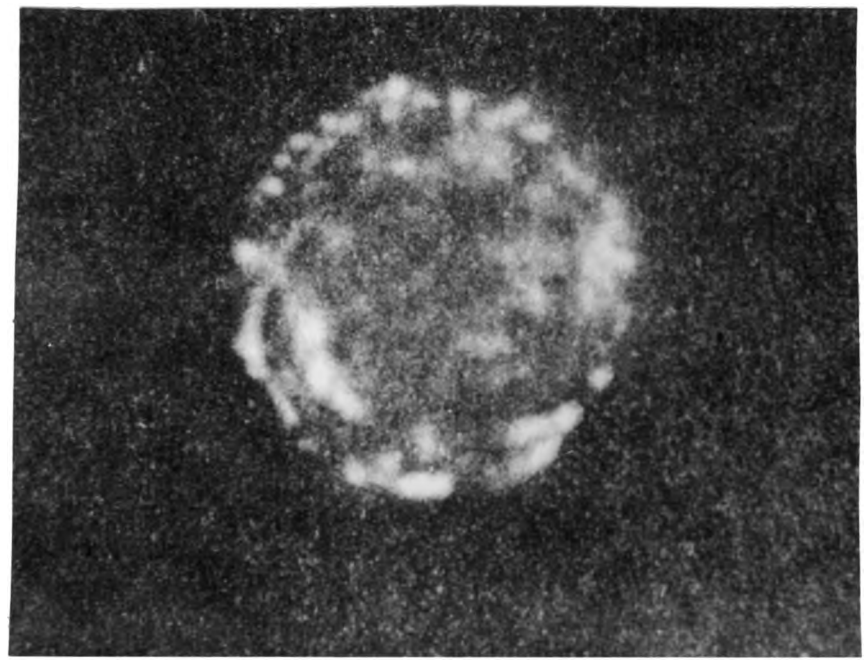
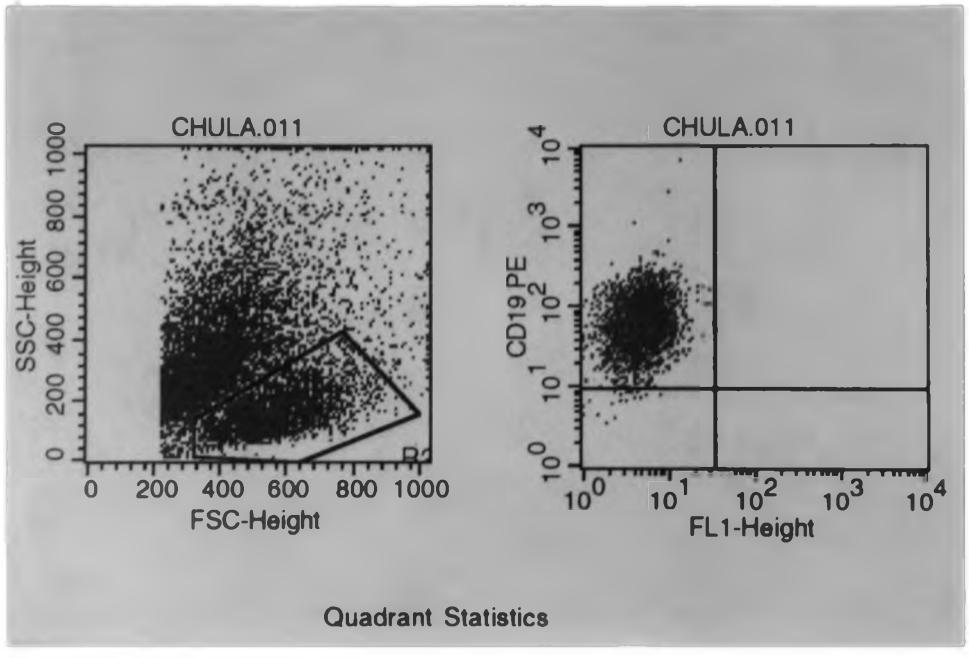


Figure 6: Both CC-LCLs and SA-LCLs were examined by immunofluorescence staining technique with fluorescein conjugated anti-IgG+IgM. The stained cells were seen under dark field and fluorescence microscope as shown in "patching" in 10x100x4 of magnification.

A



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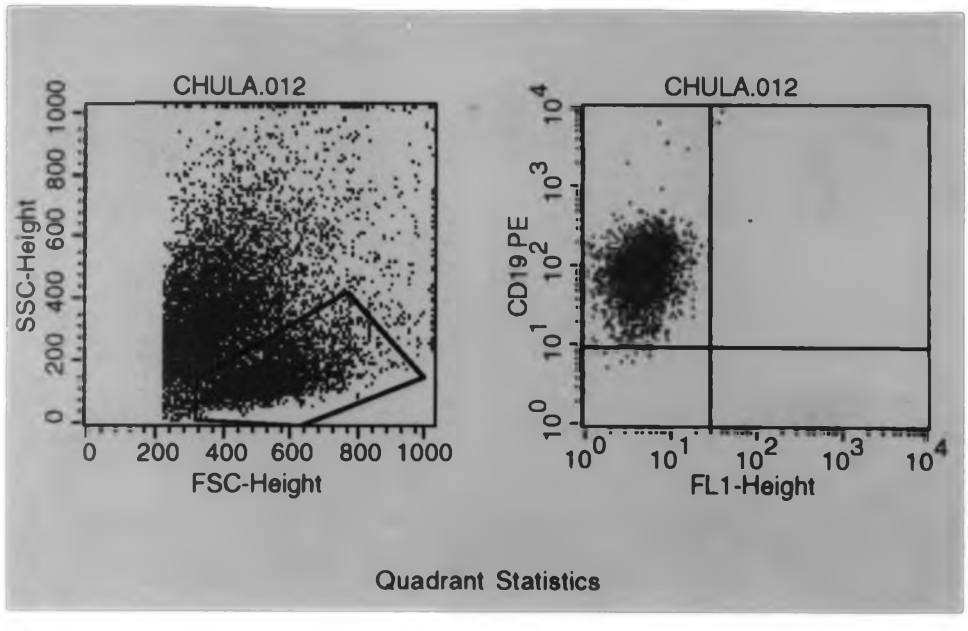


Figure 7: Cell phenotypes of CC-LCLs (A) and SA-LCLs (B) was assessed by flow cytometric analysis. MAb-CD19 PE was used. These cells were CD19 positive (CD19+) or B-cells. It was found to be more than 95% CD19+ and nearly less than 0.1% CD3+.

	Mean $\times 10^3$ 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 appropriate irradiation dose for LCLs as antigen presenting cells. Varying doses of radiation tested on LCLs which were cultured without *P. gingivalis* or PHA.

	Mean $\times 10^3$ CPM ± SE			
	P.g.	**PHA**	***P.g.***	**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	2.15 ± 0.19
Day 7	14.65 ± 1.99	12.25± 4.28	2.05 ± 0.34	1.54 ± 0.14

Table 4: Testing for appropriate irradiation dose for LCLs as antigen presenting cells. Varying doses of radiation tested on LCLs which were cultured with *P. gingivalis* or PHA.

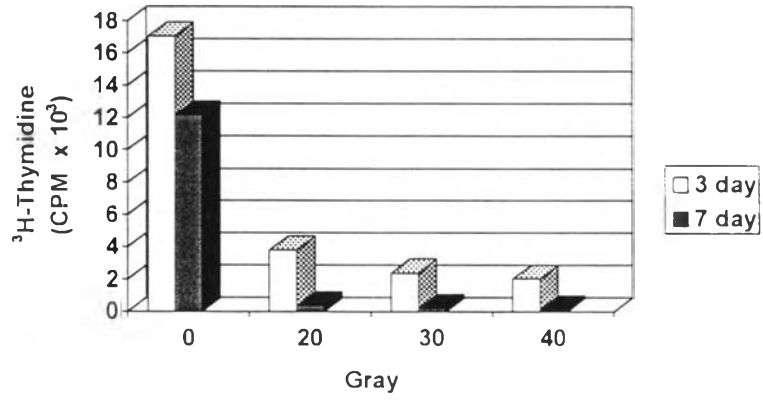


Figure 8: Testing for an appropriate radiation dose for CC-LCLs without prior stimulation

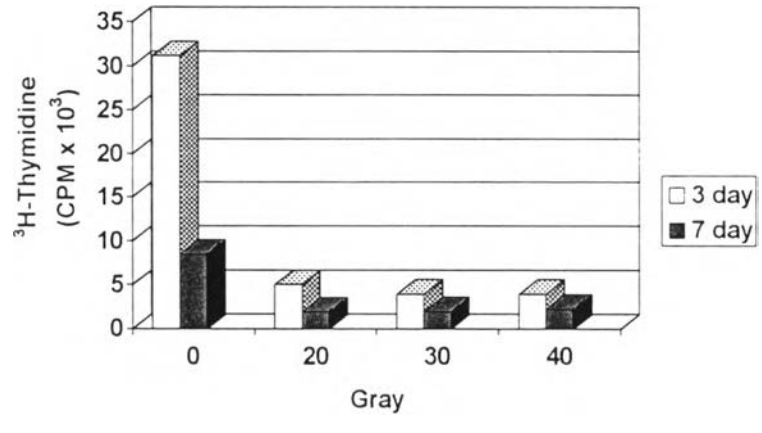
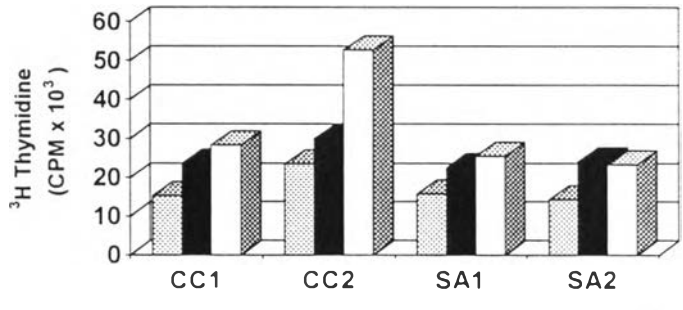


Figure 9: Testing for an appropriate radiation dose for SA-LCLs without prior stimulation

4.2 Specificity of 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 10).

After the 2nd round of stimulation and rest (day 28), both lines showed an increased response to the organism (Figure 10). In addition the high mitogen response with PHA was shown by CC-TCLs. The results, therefore, indicate specificity of the TCLs.



	CC1	CC2	SA1	SA2
RPMI	15.31	23.35	15.72	14.29
Pg	23.62	29.88	22.52	24.04
PHA	28.21	52..71	25.40	23.18

Figure 10: Proliferative response of CC-TCLs and SA-TCLs to *P. gingivalis* 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-TLCs after the first round of stimulation and rest(day 14).

SA2= SA-TCLs after the second round of stimulation and rest(day 28).

4.3 Phenotypic analysis of TCLs

Due to cells death on the first phenotypic test (day 14), so the results on the second phenotypic test(day 28) were representative for the phenotypic analysis of CD3, CD4, CD8 and CD19 positive cells (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 with 34.99% being CD8+CD3+. Less than 5% of the cells were CD19+ (Figure 11). While SA-TCL had predominantly CD8+CD3+ population with 73.31% being CD8+CD3+ and 13.57% being CD4+CD3+. Less than 6% were CD19+ (Figure 12).

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 : Both TCLs had different phenotype. CC-TCLs had a majority of CD4+ cells, while SA-TCLs had a majority of CD8+ cells.

Figure 11: Phenotypic analysis of PHA-stimulated-CC-TCLs after second round of stimulation and rest (day 28) by staining with mouse IgG (PE/FITC) as control (A), MAb to CD3(PE) / CD4(FITC) (B), CD3(PE) / CD8(FITC) (C), CD3(FITC) / CD19(PE) (D)

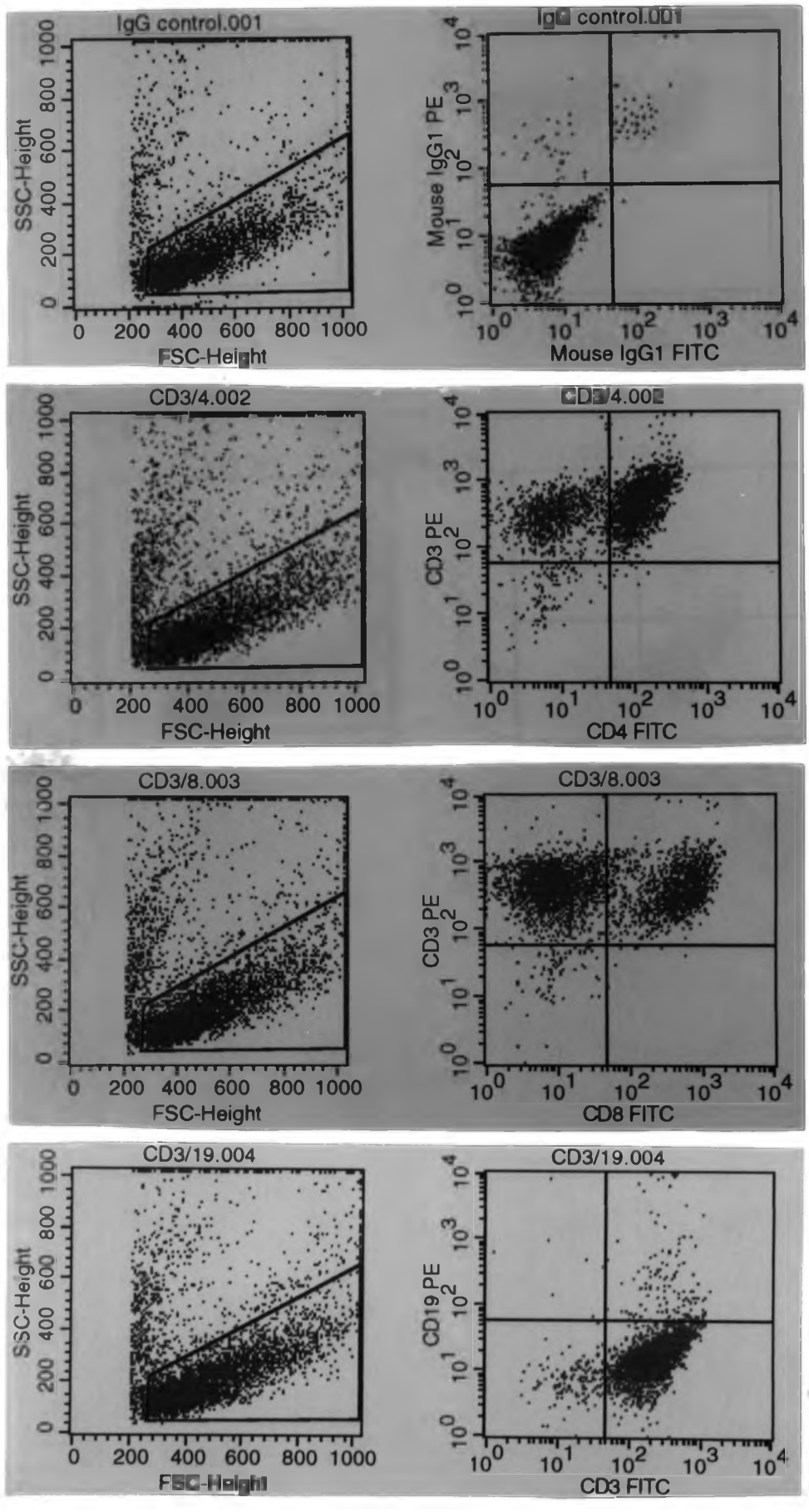
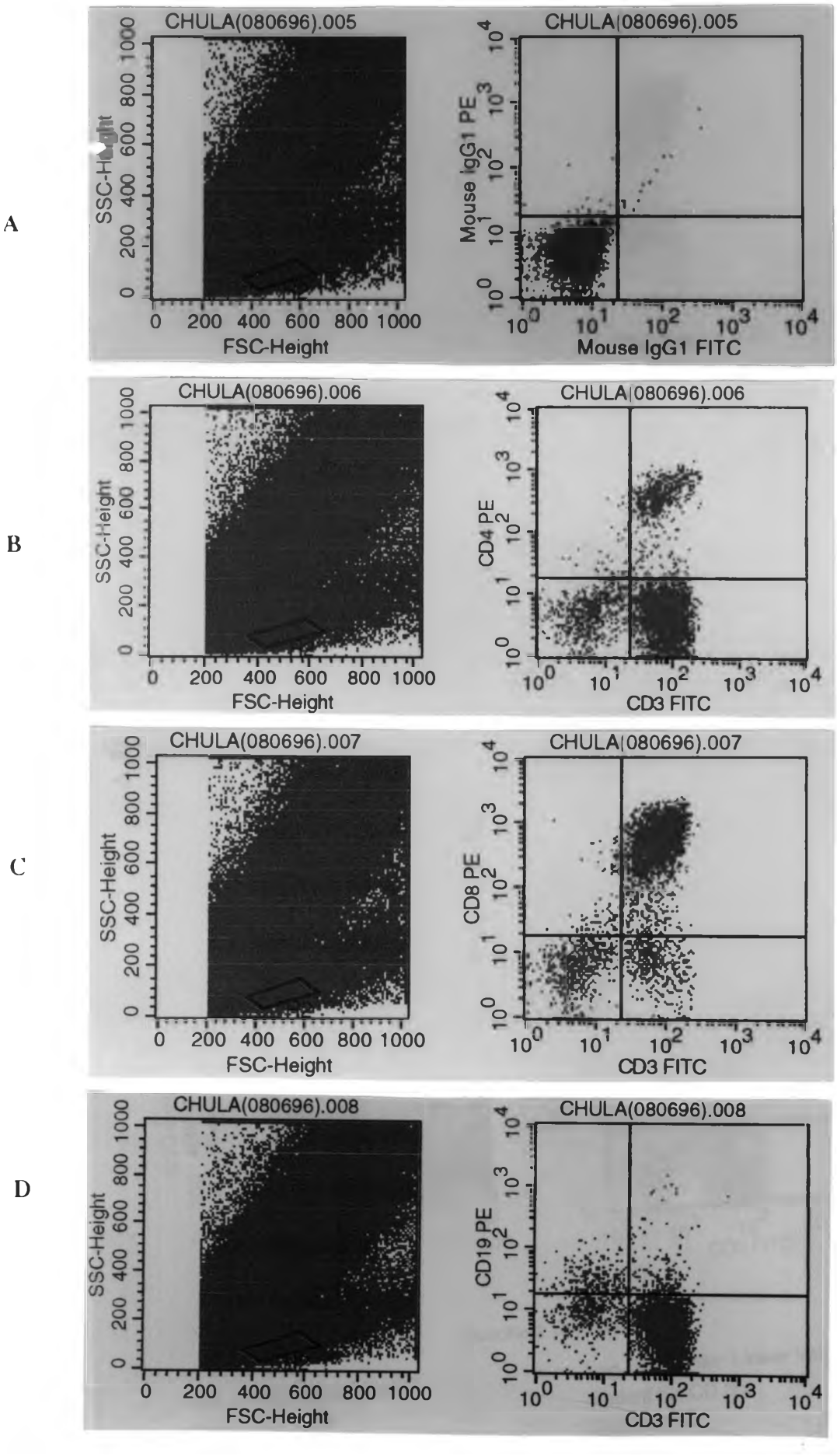


Figure 12: Phenotypic analysis of PHA-stimulated-SA-TCLs after second round of stimulation and rest (day 28), by staining with mouse IgG (FITC / PE) as control (A), MAb to CD3(FITC) / CD4(PE) (B) , CD3(FITC) / CD8(PE) (C) , CD3(FITC) / CD19(PE) (D)



4.4 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 each time. These were single well tested due to inadequate TCLs. The results were presented in Table 6 and Figure 13-17.

It was found that IL-4 as measured by immunoassay could not be detected in both TCL cultures with or without stimulation by the bacterium or PHA. Incontrast, a certain amount of IFN-γ was detected in both CC-TCLs and SA-TCLs after 6 hr. stimulation with *P. gingivalis* and continued to be detected on Day1 and Day3 which is suggestive of Type 1 T-cell response (Figure 13-17). The kinetics of IFN-γ secretion did not show a consistent pattern in both TCLs. Somehow, the level of the cytokine in *P. gingivalis* stimulated culture was higher than the standards except for CC2 at 6 hr. and SA1 at Day3, but always lower than the PHA stimulated culture. 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 Day3 after the fourth round of stimulation and rest.

	CC1			CC2		
	6 hr	Day 1	Day 3	6 hr	Day 1	Day 3
RPMI	781.7	794.9	377.7	261.0	418.8	318.9
Pg	1055.0	939.5	896.7	234.7	823.8	627.4
PHA	1542.0	2057.0	3154.0	1192.0	2420.0	5.940

	SA1			SA2		
	6 hr	Day 1	Day 3	6 hr	Day 1	Day 3
RPMI	943.5	1482.0	1323.0	917.7	1385.0	1321.0
Pg	1278.0	1569.0	1274.0	939.5	1561.0	1513.0
PHA	2119.0	3292.0	3314.0	2436.0	3237.0	3193.0

	SA3		
	6 hr	Day 1	Day 3
RPMI	889.7	1583.0	1652.0
Pg	1570.0	2222.0	2379.0
PHA	2791.0	3191.0	2892.0

Table 6 : IFN-γ production from CC-TCLs and SA-TCLs in single well culture as detected by ELISA (pg/ml)

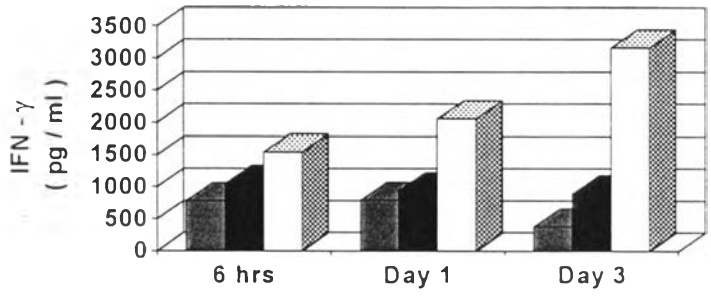
CC1= Day 28 of CC-TCL culture

CC2= Day 42 of CC-TCL culture

SA1= Day 28 of SA-TCL culture

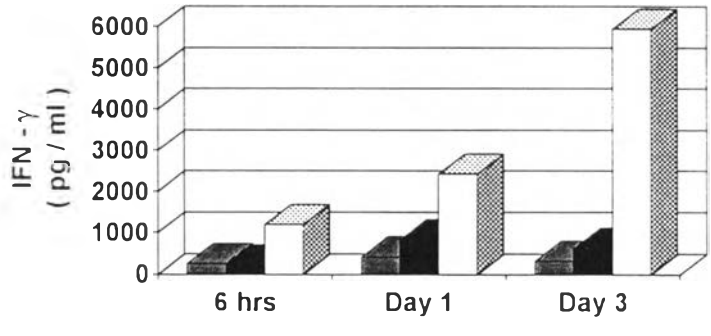
SA2= Day 42 of SA- TCL culture

SA3= Day 56 of SA-TCL culture



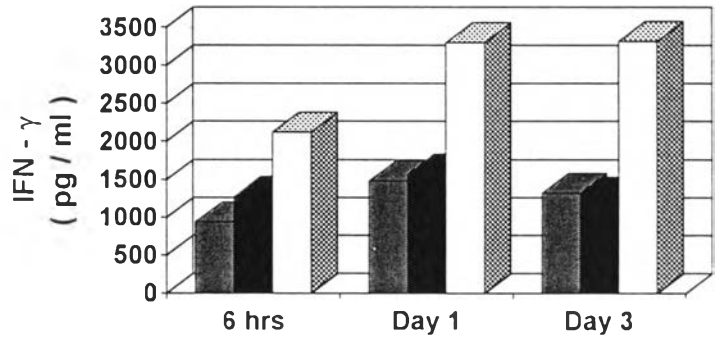
RPMI	781.7	794.9	377.7
Pg	1055.0	939.5	896.7
PHA	1542.0	2057.0	3154.0

Figure 13: IFN-γ production from CC-TCLs(Day 28) stimulated with *P. gingivalis*, PHA or non-stimulated was measured at different incubation periods.



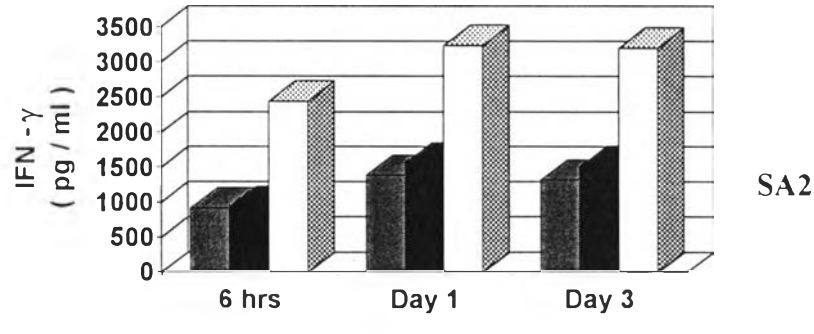
RPMI	261.0	418.8	318.9
Pg	234.7	823.8	627.4
PHA	1192.0	2420.0	5940.0

Figure 14 : IFN-γ production from CC-TCLs(Day 42) stimulated with *P. gingivalis*, PHA or non-stimulated was measured at different incubation periods.



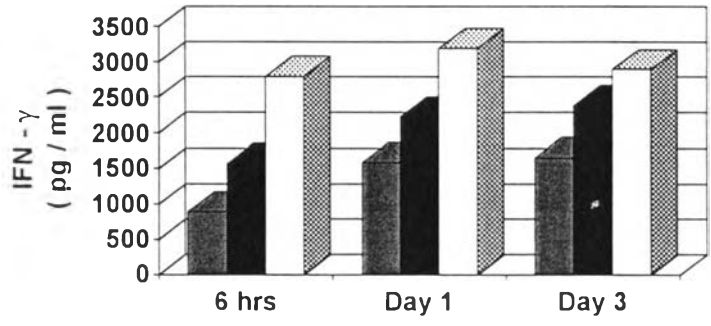
RPMI	943.5	1482.0	1323.0
Pg	1278.0	1569.0	1274.0
PHA	2119.0	3292.0	3314.0

Figure 15 : IFN-γ production from SA-TCLs(Day 28) stimulated with *P. gingivalis*, PHA or non-stimulated was measured at different incubation periods.



RPMI	917.7	1385.0	1321.0
Pg	939.5	1561.0	1513.0
PHA	2436.0	3237.0	3193.0

Figure 16 : IFN-γ production from SA-TCLs(Day 42) stimulated with *P. gingivalis*, PHA or non-stimulated was measured at different incubation periods.



RPMI	889.7	1583.0	1652.0
Pg	1570.0	2222.0	2379.0
PHA	2791.0	3191.0	2892.0

Figure 17 : IFN-γ production from SA-TCLs(Day 56) stimulated with *P. gingivalis*, PHA or non-stimulated was measured at different incubation periods.