IV. RESULTS

1. Expression of 1B2 molecules on haematopoietic cell lines

1B2 mAb is an antibody obtained from hybridomas derived from BALB/c mouse spleen cells after immunization with the human haematopoietic cell lines, U-937. Preliminary studies suggested that this mAb reacted with an antigen expressed on several haematopoietic cell lines. Therefore, it was of interest, whether this molecule is expressed on all haematopoietic cell lines. To find the answer, various haematopoietic cell lines from several origins including the T cell lines HPB-ALL and SupT-1; the B cell line JY; the myeloid cell lines KG-1, U-937 and HL-60, and the erythro/myeloid cell line K-562 were tested for the expression of the 1B2 mAb recognized molecule by means of an indirect immunofluorescent technique. As shown in Table 2, all haematopoietic cell lines tested were positive with 1B2 mAb when analyzed by a fluorescent microscope. Photographs of positive staining were shown in Figure 1. They were also different in patterns of reaction, some were homogeneous while others were speckled.

To confirm these results, the stained cells were also analyzed on a FACScan. The immunofluorescence analysis of the reactivities of 1B2 mAb with each cell line is shown in Figure 2. Again, it was shown that all haematopoietic cell lines tested reacted with 1B2 mAb in different fluorescent intensities.

2. Expression of 1B2 molecules on non-haematopoietic cell lines

As the result from previous experiments showed that all haematopoietic cell lines tested were positive with 1B2 mAb, the question of whether human non-haematopoietic cell lines also expressed this molecule was raised. In order to substantiate this, three human non-haematopoietic cell lines, including HEp-2, PC9 and PC14, were tested for an expression of 1B2 molecules by an immunofluorescent technique and analyzed by a fluorescent microscope. It was demonstrated that all non-haematopoieitc cell lines tested were also positive with 1B2 mAb (Table 3). They also reacted in different intensities and patterns, as shown in Figure 1.

Table 2. Expression of 1B2 molecule on haematopoietic cell lines

Cell types	Designation	Monoclonal antibody	
		1B2	IgG2b
T cell lines	HPB-ALL	+	-
	SupT-1	+	-
B cell line	JY	+	-
Myeloid cell lines	U-937	+	-
	HL-60	+	-
	KG-1	+	-
Erythro/myeloid cell line	K-562	+	-

Table 3. Expression of 1B2 molecules on non-haematopoietic cell lines

Designation	Monoclonal antibody	
	1B2	IgG2b
HEp-2	+	-
PC9	+	-
PC14	+	-

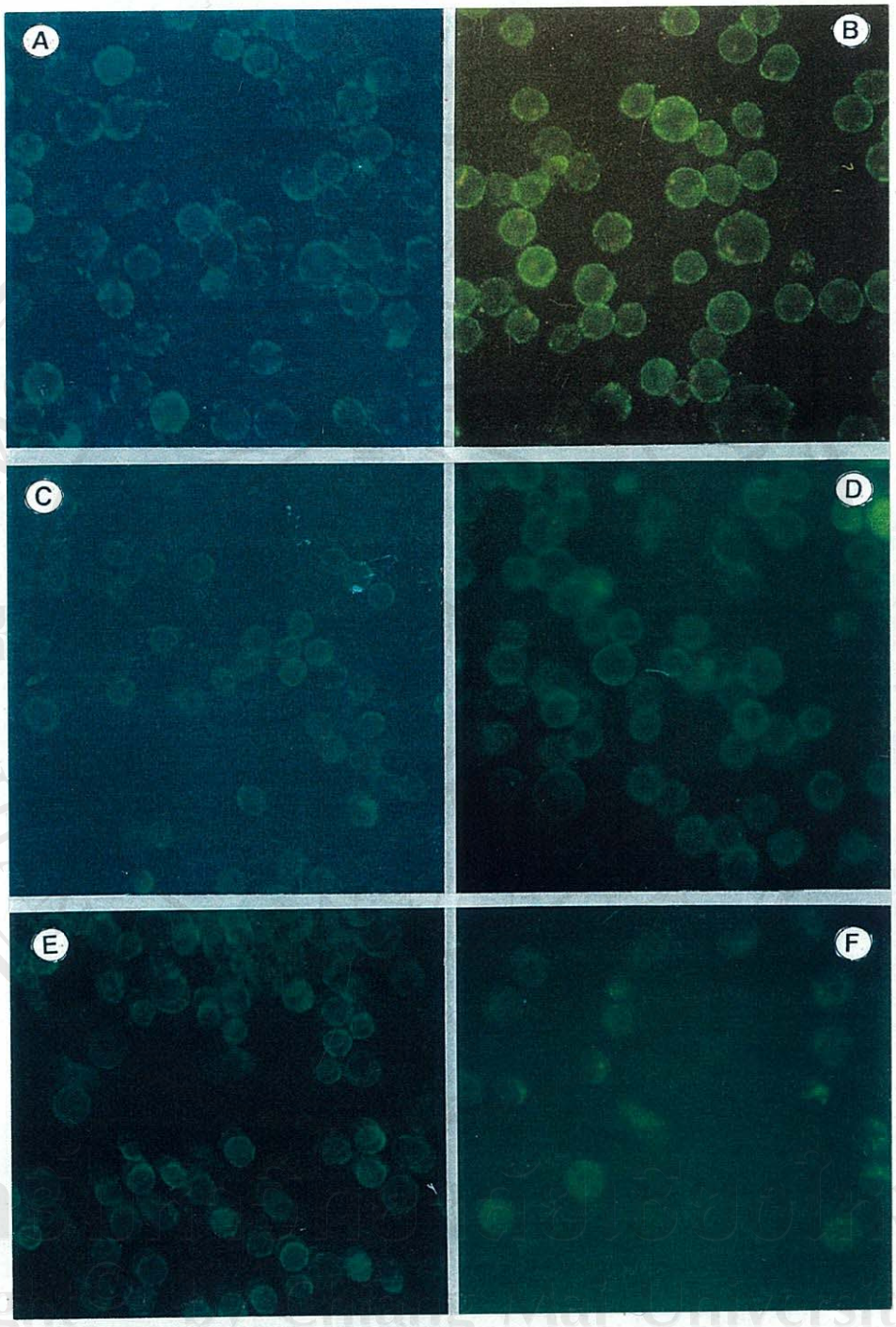

Figure 1. Photographs of cell lines given positive reaction with 1B2 mAb

Human haematopoietic cell lines including U-937 (A), K-562 (B), SupT-1 (C) and non-haematopoietic cell lines including HEp-2 (D), PC9 (E) and PC14 (F) were stained with 1B2 mAb and conjugated sheep F(ab')2 anti mouse IgG+IgM by the indirect immunofluorescent technique. (Magnification 400x)

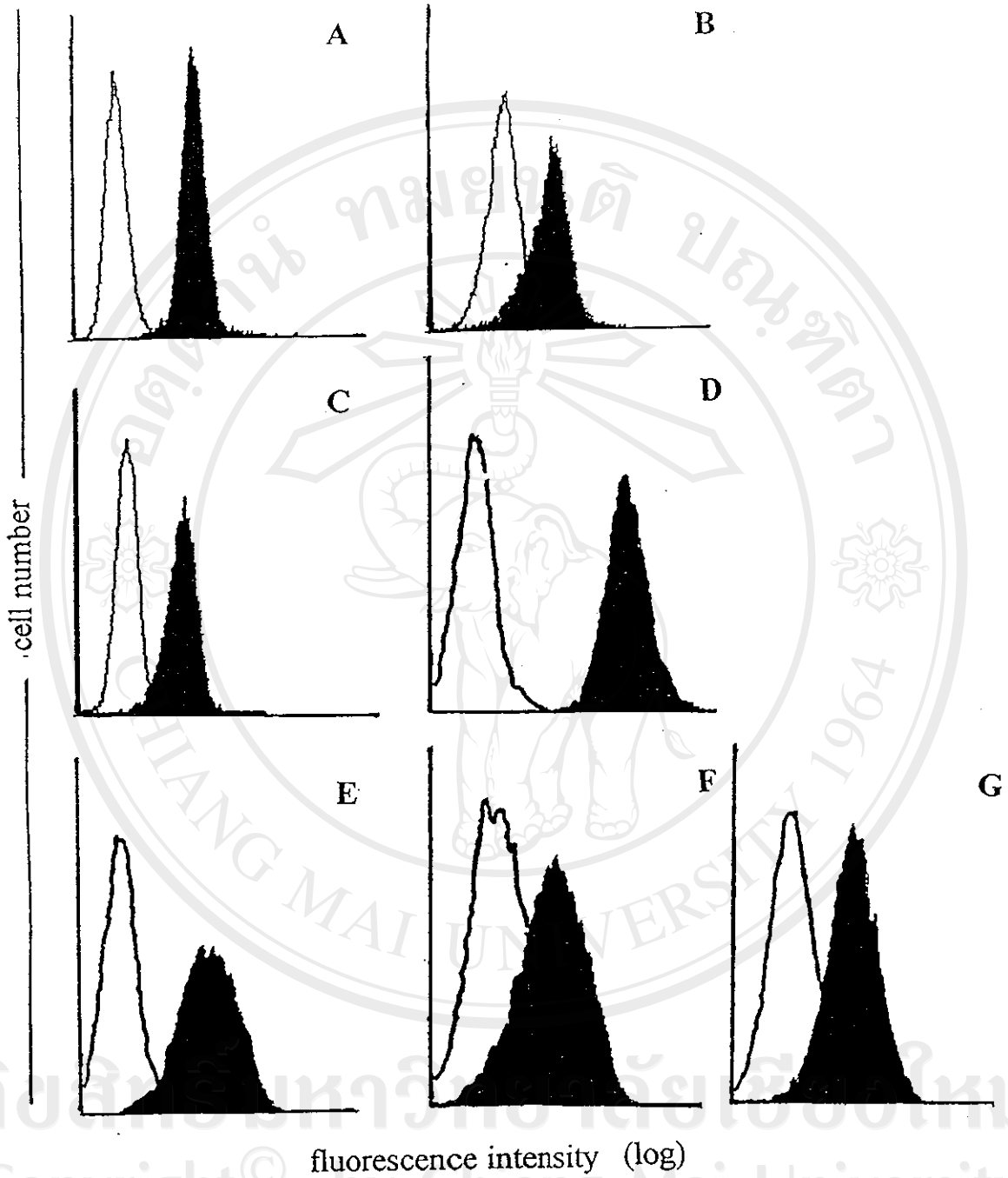

Figure 2. Expression of 1B2 molecules on haematopoietic cell lines

Haematopoietic cell lines including U-937 (A), K-562 (B), SupT-1 (C), KG-1 (D), HL-60 (E), HPB-ALL (F) and JY (G) were stained with 1B2 mAb or isotype matched control mAb. Shaded peaks represent the immunofluorescence profiles of cells stained with 1B2 mAb, and unshaded peaks represent the background fluorescence of isotype matched control mAb.

3. Expression of 1B2 molecules on peripheral blood cells

Previous studies indicated that all cell lines tested expressed 1B2 molecules. To investigate whether peripheral blood cells express this molecule, an expression of 1B2 molecules was determined by an indirect immunofluorescent technique. PBMC and granulocytes were isolated from the heparinized blood of 15 healthy donors by the Ficoll-Hypaque centrifugation and 6% Dextran sedimentation, respectively. Both cell types were stained with 1B2 mAb, MEM18 (anti-CD14), Leu-4 (anti-CD3), 3E10 (undefined mAb) and VIT6 (anti-CD1a). The immunofluorescent reactivities were analyzed by a fluorescent microscope. It was found that neither PBMC nor granulocytes reacted with 1B2 mAb (Table 4). The results also showed that PBMC was strongly positive with MEM18 and all the stained cells were negative with VIT6. Immunofluorescence of the reactivities were also analyzed on a FACScan, and the lymphocyte, monocyte and granulocyte populations were gated according to size and granularity. Again, all the cells were negative with 1B2 mAb, but recorded a strong positive with their own specific mAb, i.e. monocytes were positive with MEM18 (anti-CD14), granulocytes were positive with their specific 3E10 mAb and lymphocytes were positive with Leu-4, which was anti-CD3. The reaction patterns were similar for each donor, and one of which is demonstrated in Figure 3.

4. Expression of 1B2 molecules on activated PBMC

Several cell surface molecules can be induced to express on antigen, mitogen or cytokine activated PBMC. To study the possible induction of 1B2 molecule expressions on activated PBMC, the PBMC isolated from 5 healthy donors were cultured at the final concentration of $1x10^6$ cells/ml in the presence or absence of mitogen (PHA 2.0 μg/ml or ConA 40 μg/ml) for 1 and 3 days, or in the presence or absence of antigen (PPD 30 μg/ml) for 1, 3, and 5 days. The activated PBMC were stained with 1B2 and other controls mAb, i.e. MEM18 (anti-CD14), Leu-4 (anti-CD3), CD25-8D8 (anti-CD25) and IgG2bκ isotype matched control followed by FITC labelled sheep $F(ab')_2$ anti mouse IgG+IgM. The stained cells were analyzed for immunofluorescent reaction with a fluorescent microscope. It was found that PHA activated PBMC, ConA activated PBMC and PPD activated PBMC were all negative with 1B2 mAb. Cells tested with Leu-4, MEM18 or CD25-8D8 were positive, but all those

Table 4. Expression of 1B2 molecule on peripheral blood cells

Donor number	Cell types	Monoclonal Antibody		
		1B2	MEM18	VIT6
1	PBMC	-	+	-
	Granulocytes	-	ND	-
2	PBMC	-	+	-
	Granulocytes	-	ND	-
3	PBMC	-	+	-
	Granulocytes	-	ND	-
4	PBMC	-	+	-
	Granulocytes	-	ND	-
5	PBMC	-	+	-
	Granulocytes	-	ND	-
6	PBMC	-	+	-
	Granulocytes	-	ND	-
7	PBMC	-	+	-
	Granulocytes	-	ND	-
8	PBMC	-	+	-
	Granulocytes	-	ND	-
9	PBMC	-	+	-
	Granulocytes	-	ND	-
10	PBMC	-	+	-
	Granulocytes	-	ND	-
11	PBMC	-	+	-
	Granulocytes	-	ND	-
12	PBMC	-	+	-
	Granulocytes	-	ND	-
13	PBMC	-	+	-
	Granulocytes	-	ND	-
14	PBMC	-	+	-
	Granulocytes	-	ND	-
15	PBMC	-	+	-
	Granulocytes	-	ND	-

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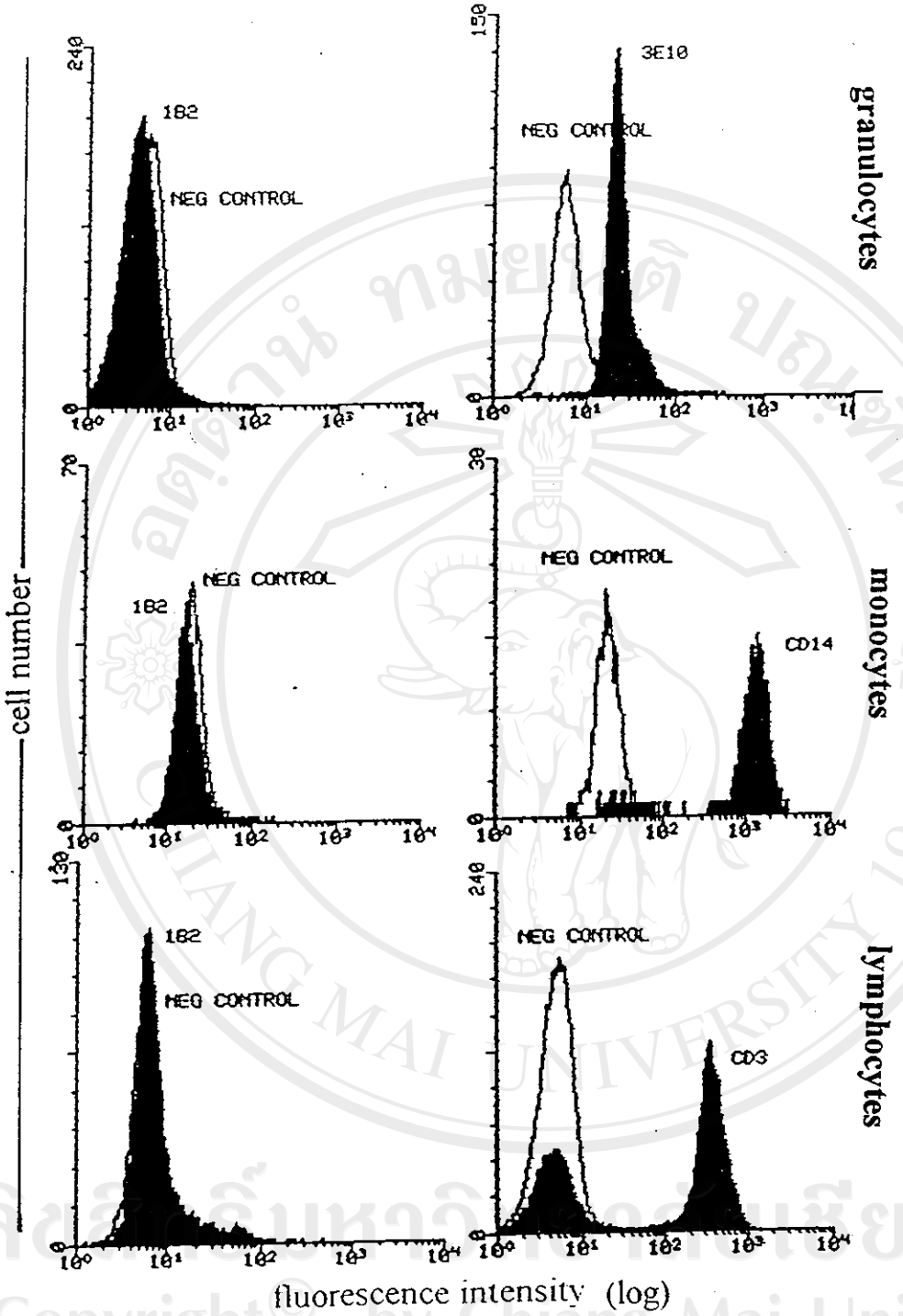

Figure 3. Expression of 1B2 molecules on peripheral blood cells

Immunofluorescence analysis of the reactivity of 1B2 mAb with freshly isolated peripheral blood cells. The lymphocyte, monocyte, and granulocyte populations were gated according to size and granularity, and fluorescent intensity was analyzed on a FACScan. Shaded peaks represent the immunofluorescence profiles of cells stained with indicated mAb and unshaded peaks represent the background fluorescence of negative control mAb.

stained with IgG2b isotype matched control were all negative (Table 5-7). The stained cells were also analyzed for immunofluorescent reactivity on a FACScan. PHA activated cells from both 1 and 3 days stimulation were negative with 1B2 mAb, but positive with CD25-8D8 (anti-IL2Rα) and Leu-4 (anti CD3). A similar reaction also occurred on ConA and PPD activated cells, regardless of the stimulation period. The reaction patterns of the mAb were similar for each experiment, one of which is demonstrated in Figures 4, 5 and 6 for PHA activated PBMC, ConA activated PBMC and PPD activated PBMC, respectively.

To determine whether cytokine stimulation can induce the expression of 1B2 molecules, similar experiment were performed. Instead of using antigen or mitogen, the PBMC from 5 healthy donors were cultured for 1 and 3 days in the presence of cytokines, including rTNFα (40 ng/ml) and rGM-CSF (100 ng/ml). The stimulated cells were then analyzed for the expression of 1B2 molecules, by an indirect immuno-fluorescent technique and staining with 1B2 mAb and other mAb controls, including MEM18, Leu-4, CD25-8D8 and IgG2b isotype matched control. The stained cells were then analyzed by fluorescent microscope and FACScan for their immunofluorescent reactivities. The results shown in Tables 8 and 9 indicate that both the rTNFα and rGM-CSF activated cells of all donors were negative with 1B2 mAb. The FACS profiles of one donor, representative of 5 donors, were shown in Figures 7 and 8 for rTNFα and rGM-CSF activation, respectively.

5. <u>Expression of 1B2 molecules on leukemic blood cells</u>

Since the results from previous experiments indicated that only the cell lines expressed 1B2 molecules, and whether they might express on the cell surface of leukemic blood cells which are abnormal in both proliferation and differentiation, was of interest. To demonstrate such a possibility, the heparinized whole blood from 6 patients with ALL or AML were isolated for leukocytes, by treating with NH_4Cl-Tris pH 7.2 in order to lyse the red blood cells. The leukocytes were then stained with mAb including 1B2, IgG2b isotype matched control, MEM18 and Leu-4, and followed by FITC labelled sheep $F(ab')_2$ anti mouse IgG+IgM. The reaction patterns were observed on a fluorescent microscope. As shown in Table 10, only one AML proved positive, while the others gave a negative reaction with 1B2 mAb. All cells tested showed a negative reactivity with

Table 5. Expression of 1B2 molecules on PHA activated PBMC

Donor number	Stimulation time (days)	Monoclonal antibody				
		1B2	IgG2b	MEM18	Leu-4	CD25-8D8
1	1	-	-	+	+	ND
	3	-	-	+	+	ND
2	1	-	-	+	+	ND
	3	-	-	+	+	ND
3	1	-	-	ND	+	+
	3	-	-	ND	+	+
4	1	-	-	ND	+	+
	3	-	-	ND	+	+
5	1	-	-	ND	+	+
	3	-	-	ND	+	+

ND : not done

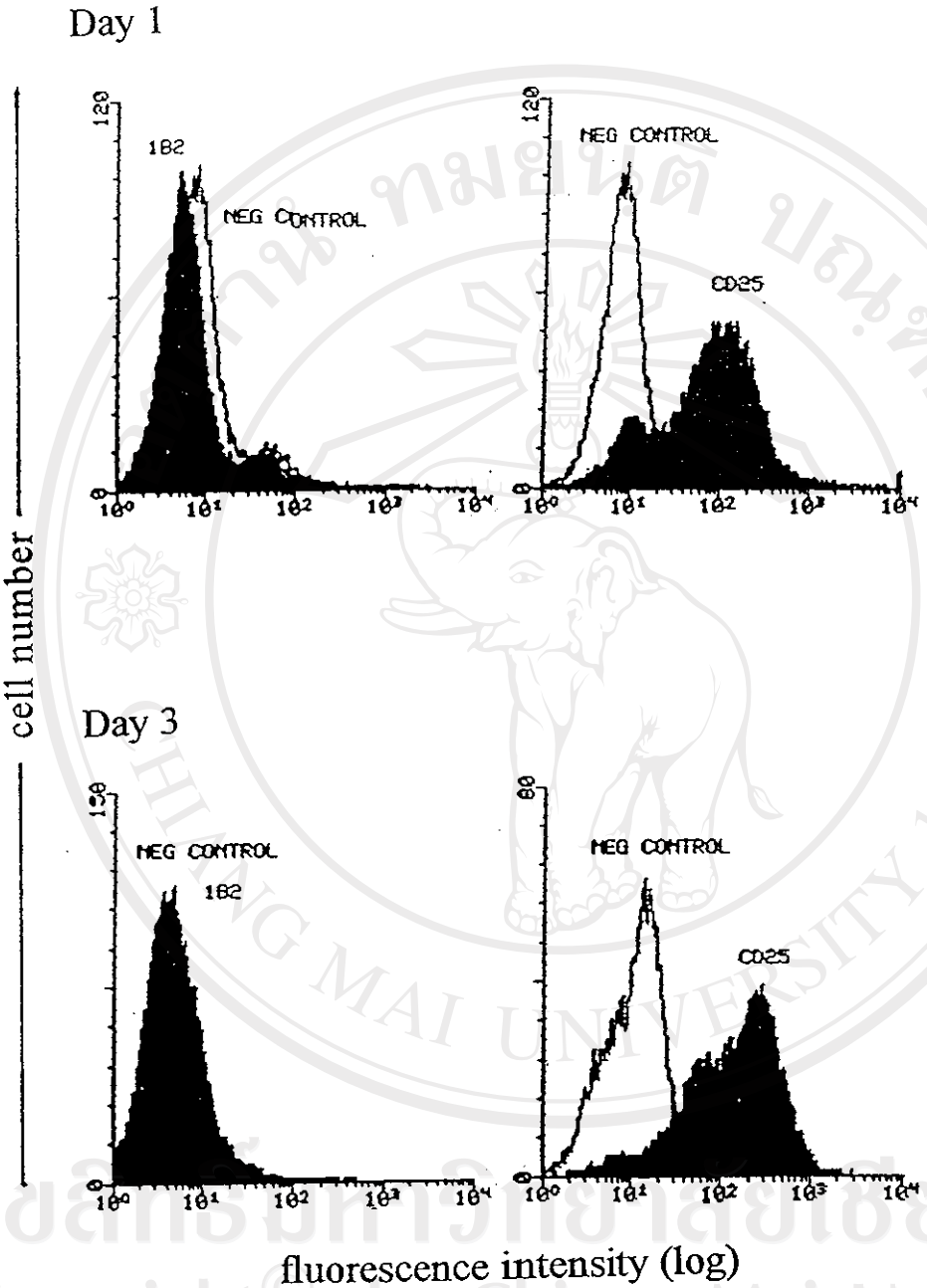

Figure 4. Expression of 1B2 molecules on PHA activated PBMC

Freshly isolated PBMC was activated with PHA for 1 and 3 days before staining with 1B2 mAb. The fluorescent intensity was analyzed on a FACScan. Shaded peaks represent the immunofluorescence profiles of activated cells stained with indicated mAb and unshaded peaks represent the background fluorescence of isotype matched control mAb.

Table 6. Expression of 1B2 molecules on ConA activated PBMC

Donor number	Stimulation time (days)	Monoclonal antibody				
		1B2	IgG2b	MEM18	Leu-4	CD25-8D8
1	1	-	-	+	+	ND
	3	-	-	+	+	ND
2	1	-	-	+	+	ND
	3	-	-	+	+	ND
3	1	-	-	ND	+	+
	3	-	-	ND	+	+
4	1	-	-	ND	+	+
	3	-	-	ND	+	+
5	1	-	-	ND	+	+
	3	-	-	ND	+	+

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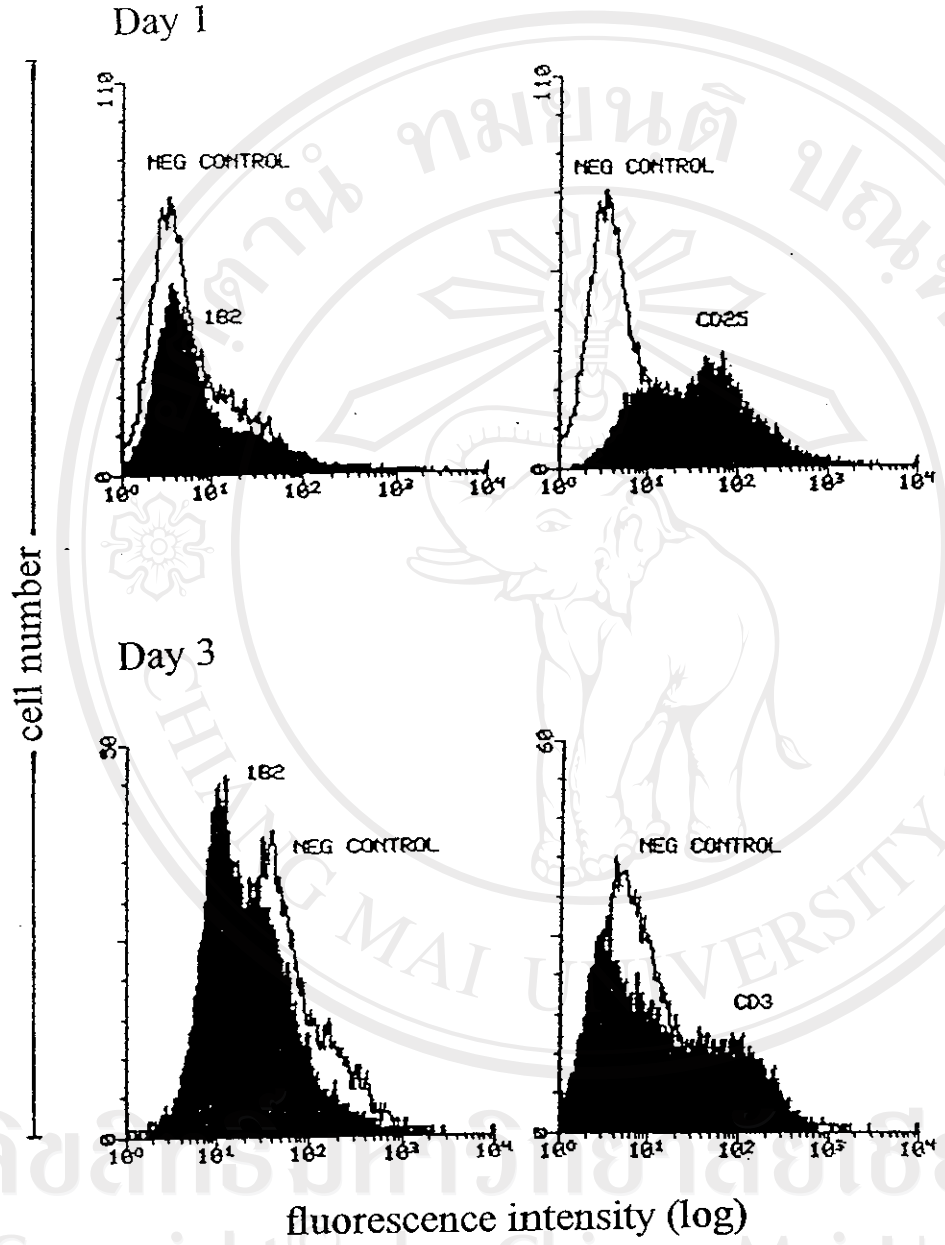

Figure 5. Expression of 1B2 molecules on ConA activated PBMC

Freshly isolated PBMC was activated with ConA for 1 and 3 days before staining with 1B2 mAb. The fluorescent intensity was analyzed on a FACScan. Shaded peaks represent the immunofluorescence profiles of cells stained with indicated mAb and unshaded peaks represent the background fluorescence of isotype matched control mAb.

Table 7. Expression of 1B2 molecules on PPD activated PBMC

Donor number	Stimulation time (days)	Monoclonal antibody				
		1B2	IgG2b	MEM18	Leu-4	CD25-8D8
1	1	-	-	+	+	ND
	3	-	-	+	+	ND
2	1	-	-	+	+	ND
	3	-	-	+	+	ND
3	1	-	-	ND	+	+
	3	-	-	ND	+	+
4	1	-	-	ND	+	+
	3	-	-	ND	+	+
5	1	-	-	ND	+	+
	3	-	-	ND	+	+
	5	-	-	ND	+	+

ND : not done

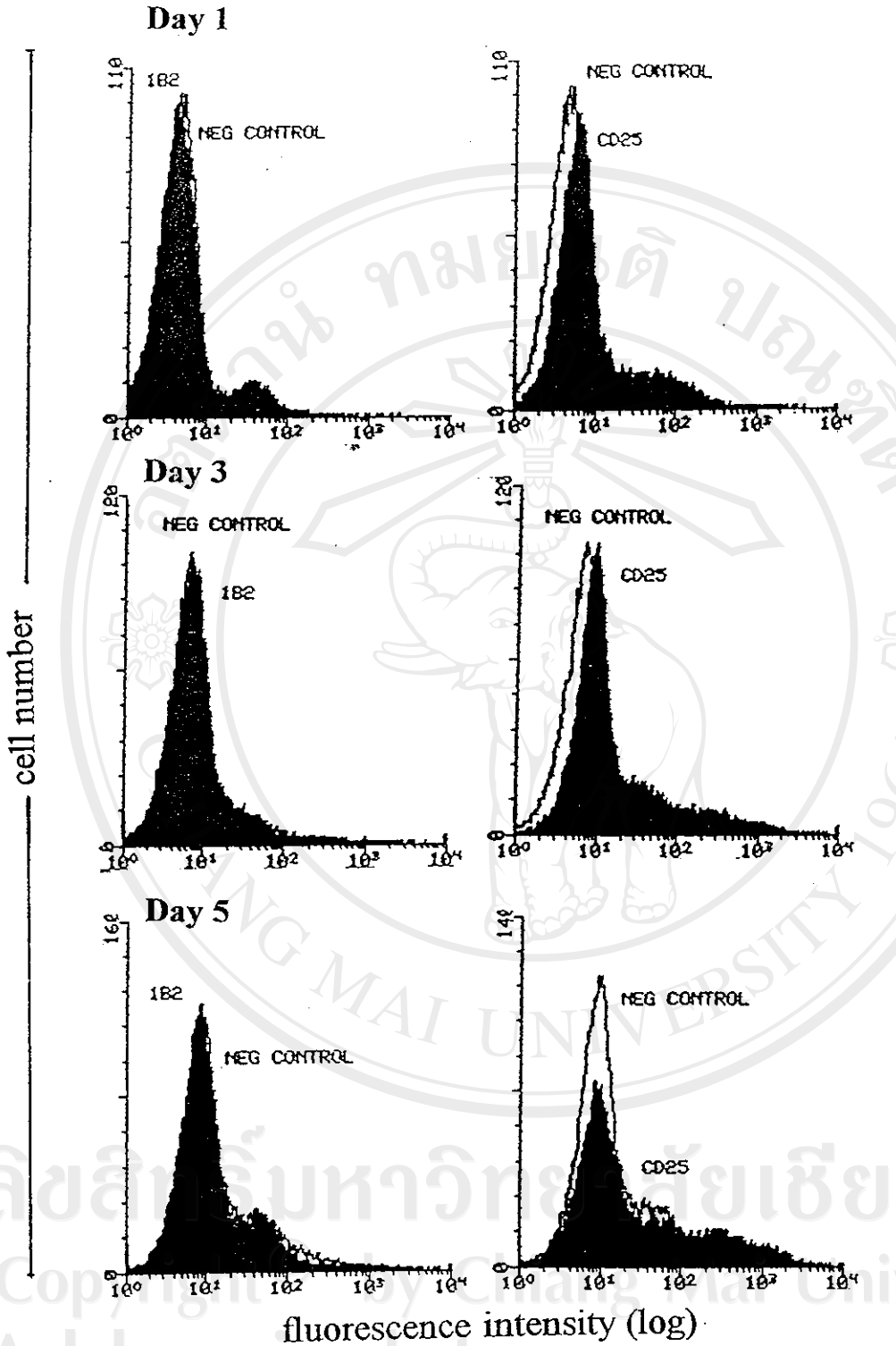

Figure 6. Expression of 1B2 molecules on PPD activated PBMC

Freshly isolated PBMC was activated with PPD for 1, 3 and 5 days before staining with 1B2 mAb. The fluorescent intensity was analyzed on a FACScan. Shaded peaks represent the immunofluorescence profiles of cells stained with indicated mAb and unshaded peaks represent the background fluorescence of isotype matched control mAb.

Table 8. Expression of 1B2 molecule on rTNFα activated PBMC

Donor number	Stimulation time (days)	Monoclonal antibody				
		1B2	IgG2b	MEM18	Leu-4	CD25-8D8
1	1	-	-	+	+	ND
	3	-	-	+	+	ND
2	1	-	-	+	+	ND
	3	-	-	+	+	ND
3	1	-	-	ND	+	+
	3	-	-	ND	+	+
4	1	-	-	ND	+	+
	3	-	-	ND	+	+
5	1	-	-	ND	+	+
	3	-	-	ND	+	+

ND : not done

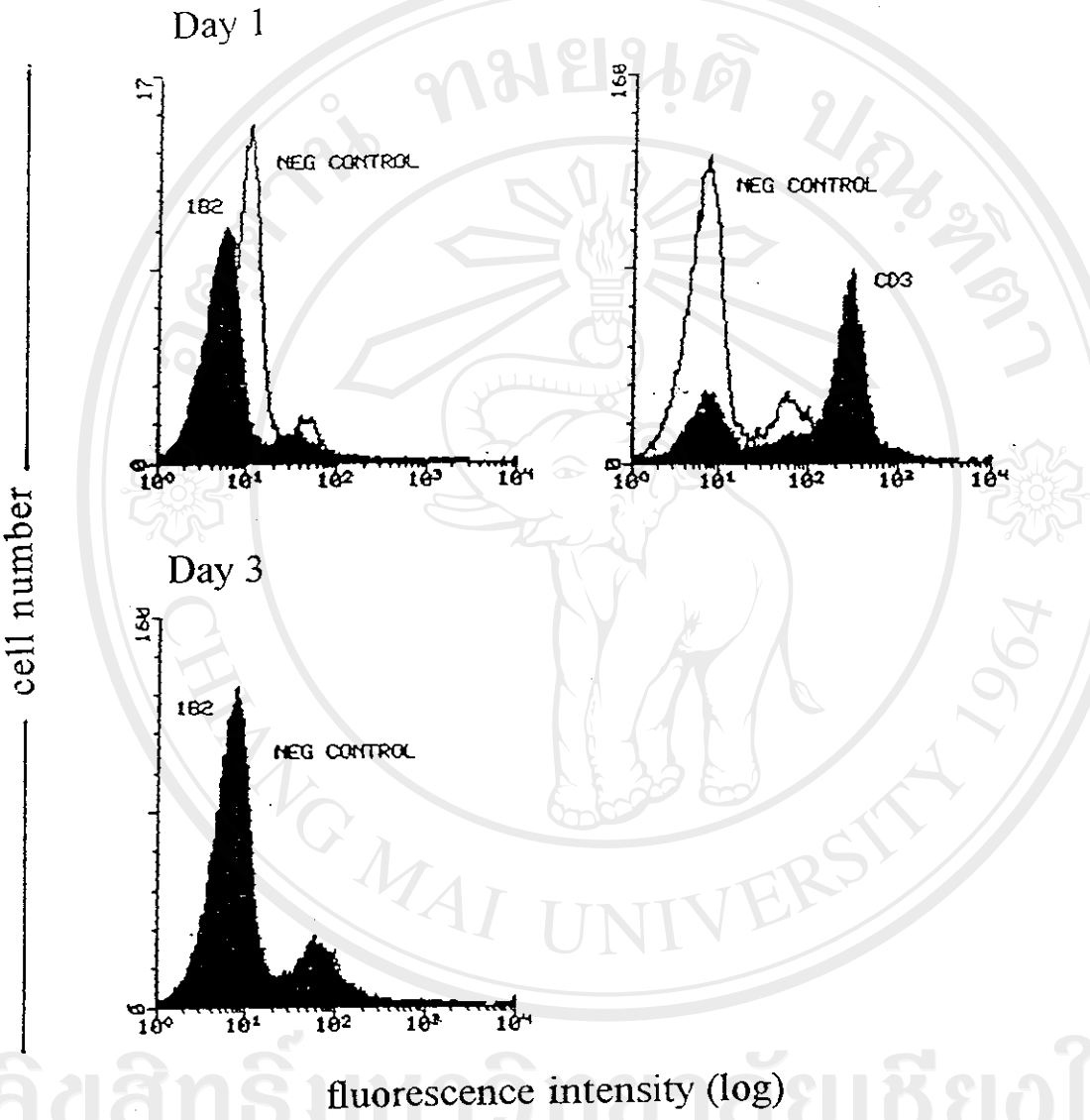

Figure 7. Expression of 1B2 molecules on rTNFα activated PBMC

Freshly isolated PBMC was activated with rTNFα for 1 and 3 days before staining with 1B2 mAb. The fluorescent intensity was analyzed on a FACScan. Shaded peaks represent the immunofluorescence profiles of cells stained with indicated mAb and unshaded peaks represent the background fluorescence of isotype matched control mAb.

Table 9. Expression of 1B2 molecules on rGM-CSF activated PBMC

Donor number	Stimulation time (days)	Monoclonal antibody				
		1B2	IgG2b	MEM18	Leu-4	CD25-8D8
1	1	-	-	+	+	ND
	3	-	-	+	+	ND
2	1	-	-	+	+	ND
	3	-	-	+	+	ND
3	1	-	-	ND	+	+
	3	-	-	ND	+	+
4	1	-	-	ND	+	+
	3	-	-	ND	+	+
5	1	-	-	ND	+	+
	3	-	-	ND	+	+

ND : not done

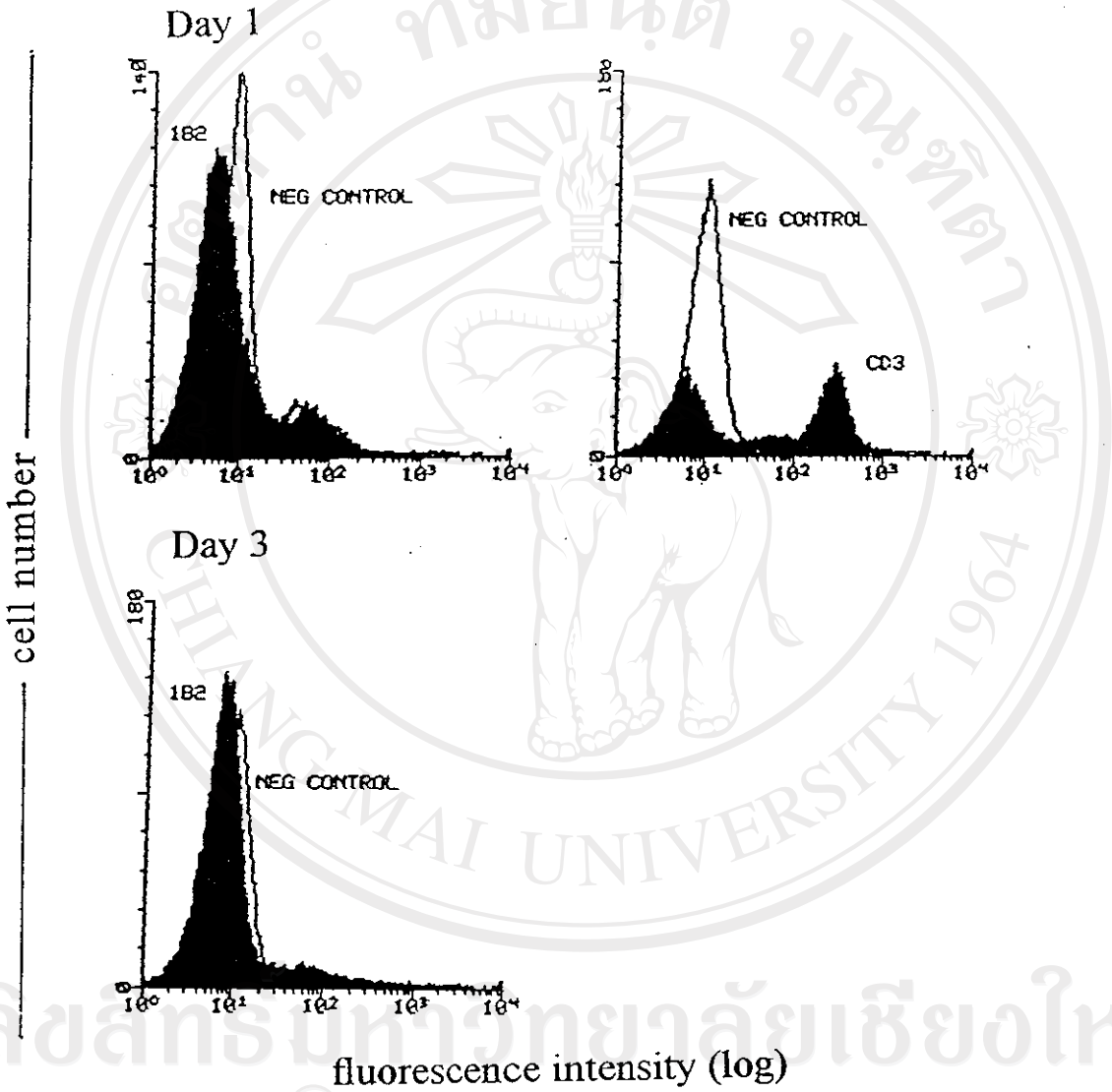

Figure 8. Expression of 1B2 molecules on rGM-CSF activated PBMC

Freshly isolated PBMC was activated with rGM-CSF for 1 and 3 days before staining with 1B2 mAb. The fluorescent intensity was analyzed on a FACScan. Shaded peaks represent the immunofluorescence profiles of cells stained with indicated mAb and unshaded peaks represent the background fluorescence of isotype matched control mAb.

IgG2b, whereas, the positive controls for this systems, including MEM18 and Leu-4, showed correct results on the cells tested.

6. The 1B2 molecules involved in cellular proliferation

The results from all previous experiments indicated that 1B2 molecules expressed only on cell lines or some leukemic cells. Therefore, their function was very interesting as to whether they might be involved in cellular proliferation. The functional study of 1B2 molecules was then performed. The first experiment was to find the answer as to whether 1B2 mAb might inhibit the proliferation of activated PBMC, so that the suboptimal concentration of antigen and mitogen used to activate cells were titrated. PBMC from 4 healthy PPD-positive donors were cultured in 96-well plates for 3 days in the presence or absence of various concentrations of mitogens, PHA and ConA, and 7 days in the presence or absence of PPD antigen. The cultures were pulsed with [^{3}H]thymidine at a final concentration of 0.4 μCi/well for 18 hrs. before harvesting. The [^{3}H]thymidine uptakes were measured in a liquid scintillation counter and calculated by means of the c.p.m. of triplicate cultures. As shown in Figures 9, 10 and 11, the suboptimal concentration of PHA, ConA and PPD antigen were 0.03125 μg/ml, 2.5 μg/ml and 1.25 μg/ml, respectively.

The effect of 1B2 mAb on activated PBMC was then studied. PBMC from two normal PPD-positive donors were stimulated in 96-well plates with PHA (0.03125 μg/ml), ConA (2.5 μg/ml) or PPD (1.25 μg/ml) in the presence or absence of 1B2 mAb (5.0 μg/ml). After 3 days for PHA and ConA, or 7 days for PPD activation, [^{3}H]thymidine incorporation was determined. As shown in Table 11 and Figure 12, 1B2 mAb had no effect on the proliferation of either mitogen or antigen activated PBMC.

As 1B2 mAb has no effect on the proliferation of activated PBMC, other experiments were tried out to study the effect of 1B2 mAb on cell lines. Haematopoietic cell lines, U-937 and K-562, which reacted strongly with 1B2 mAb, were selected for this study. U-937 and K-562 were individually cultured in 96-well plates in the presence or absence of 1B2 mAb (5, 10 and 20 μg/ml) at the final cell concentration of $1x10^5$ and $1x10^6$ cells/ml, together with [^{3}H]thymidine (0.2 μCi/well). The cells were cultured for 3 and 5 hrs. After each period, they were harvested and the [^{3}H]thymidine uptake was determined.

In order to substantiate that only 1B2 mAb was involved in the cell proliferation, and this role was not the effect of other molecules that

Table 10. Expression of 1B2 molecules on leukemic blood cells

Donor number	Type of abnormality	Monoclonal Antibody			
		1B2	IgG2b	MEM18	Leu-4
1	AML	-	-	ND	ND
2	AML	-	-	ND	ND
3	AML	+	-	ND	ND
4	ALL	-	-	ND	ND
5	ALL	-	-	+	+
6	ALL	-	-	+	+

ND : not done
AML: acute myeloblastic leukemia
ALL : acute lymphoblastic leukemia

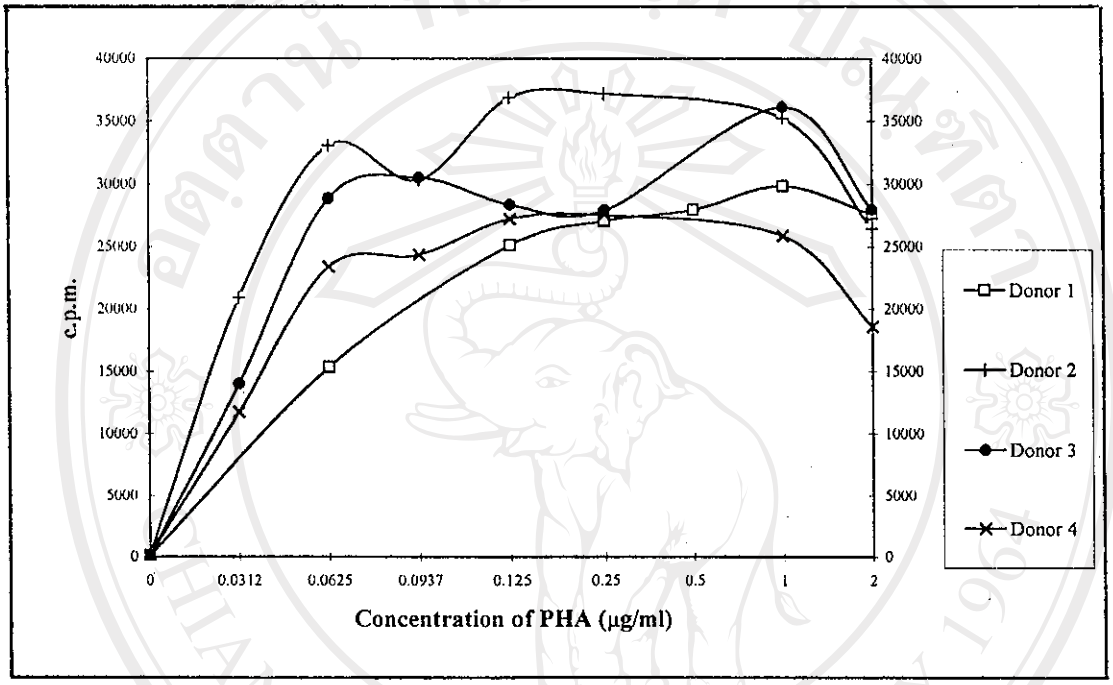

Figure 9. Determination of the suboptimal concentration of PHA

Freshly isolated PBMC from 4 healthy donors were cultured for 3 days in the presence or absence of various concentrations of PHA (0-2 µg/ml) at the final cell concentration of $1x10^6$ cells/ml. [^{3}H]thymidine (0.4 µCi/well) was pulsed for 18 hrs. before harvesting and the radioactive incorporation was determined. Each point represents the mean counts per minute of incorporated [^{3}H]thymidine from triplicate cultures.

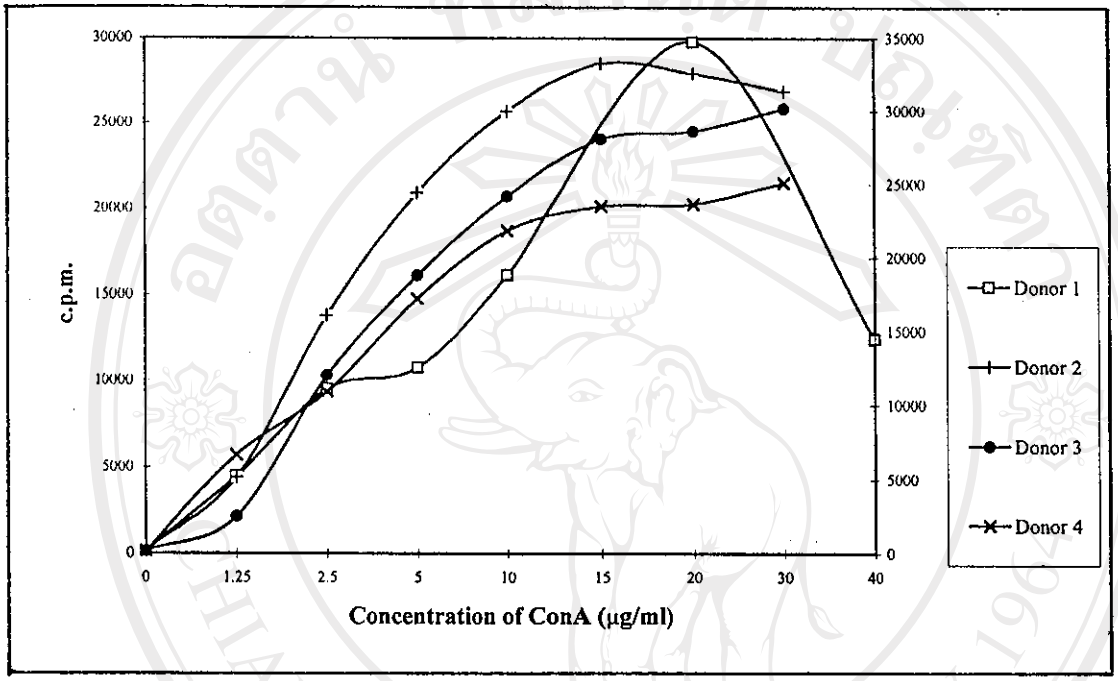

Figure 10. Determination of the suboptimal concentration of ConA

Freshly isolated PBMC from 4 healthy donors were cultured for 3 days in the presence or absence of various concentrations of ConA (0-40 µg/ml) at the final cell concentration of $1x10^6$ cells/ml. [^{3}H]thymidine (0.4 µCi/well) was pulsed 18 hrs. before harvesting and the radioactive incorporation was determined. Each point represents the mean counts per minute of incorporated [^{3}H]thymidine from triplicate cultures.

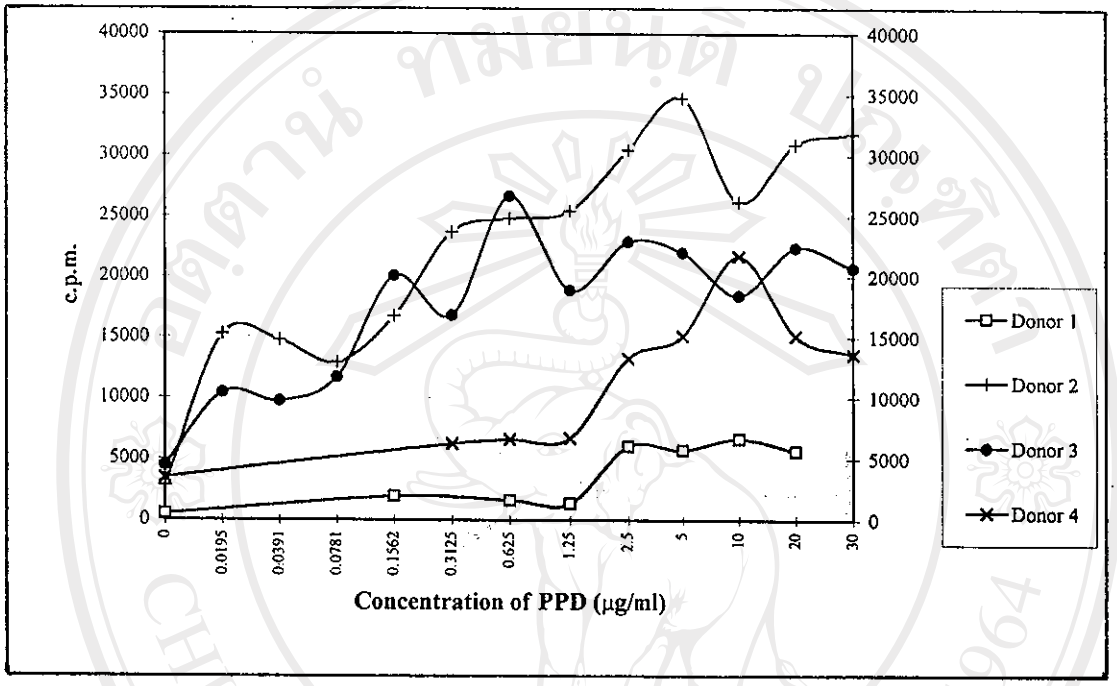

Figure 11. Determination of the suboptimal concentration of PPD

Freshly isolated PBMC from 4 healthy PPD-positive donors were cultured for 7 days in the presence or absence of various concentrations of PPD antigen (0-30 µg/ml) at the final cell concentration of $1x10^6$ cells/ml. [^{3}H]thymidine (0.4 µCi/well) was pulsed 18 hrs. before harvesting and the [^{3}H]thymidine uptake was determined. Each point represents the mean counts per minute of incorporated [^{3}H]thymidine from triplicate cultures.

Table 11. Effect of 1B2 mAb on proliferation of activated PBMC

Donor number	Condition	Stimulators		
		PHA	ConA	PPD
1	without 1B2	19096*	23720	12631
	with 1B2	19310	22432	12914
2	without 1B2	16573	25778	20352
	with 1B2	16575	26060	23450

* mean of counts per minute (c.p.m.) from triplicate cultures

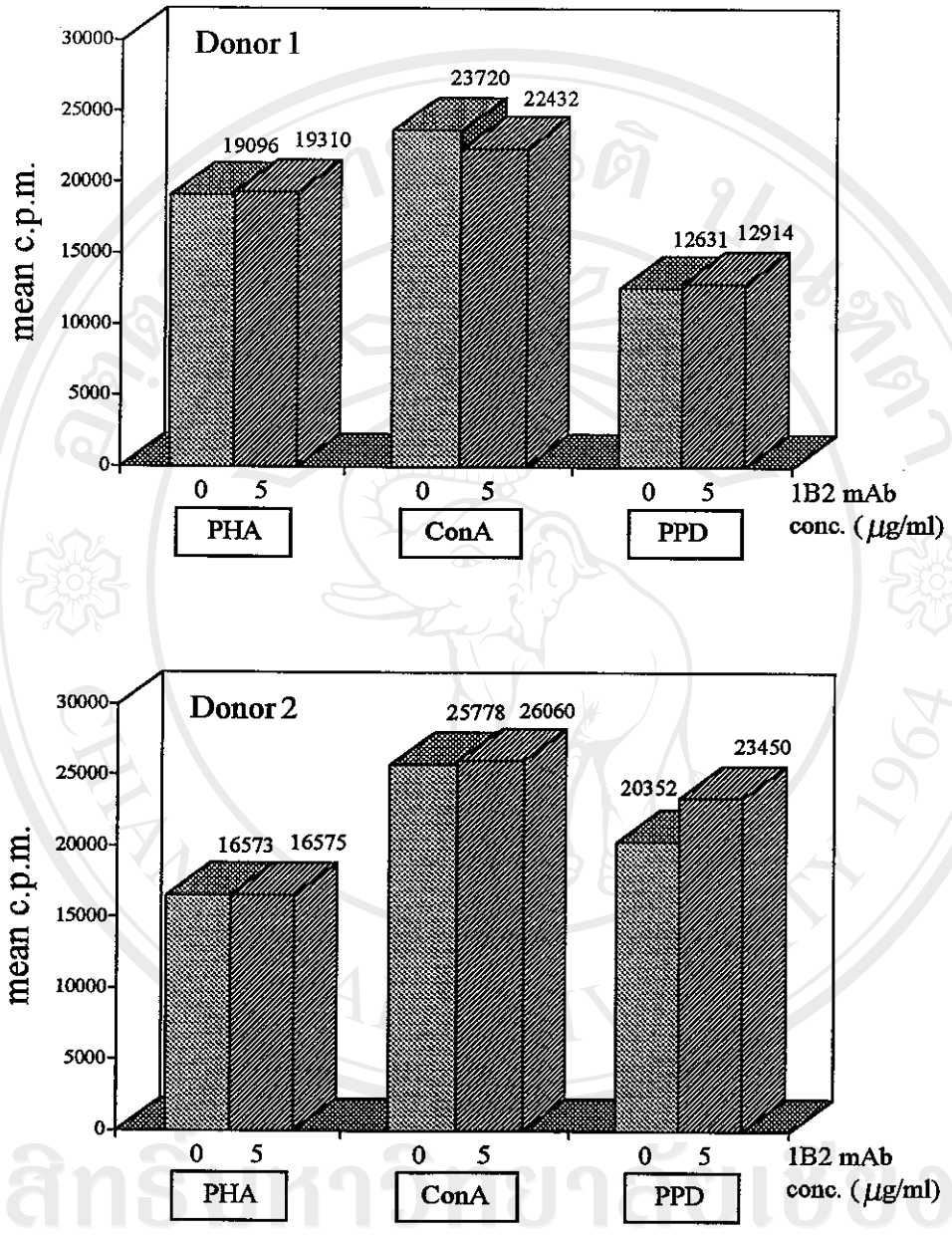

Figure 12. Effect of 1B2 mAb on proliferation of activated PBMC

PBMC of 2 PPD-positive donors were activated with PHA (0.03125 μg/ml), ConA (2.5 μg/ml) or PPD (1.25 μg/ml) in the presence or absence of 1B2 mAb (5 μg/ml). The cells were cultured for 3 days for PHA and ConA, and 7 days for PPD. Radioactive incorporation was determined by mean counts per minute from triplicate cultures.

expressed on both cell lines, control mAb were performed at the time of the experiments. Four control mAb were chosen including MEM18 (anti-CD14), which is specific to a cell surface molecule expressed on monocytes, but not on U-937 and K-562; 9C8 mAb that reacted to a molecule, expressed on both U-937 and K-562 as well as IgG2bκ, which is an isotype matched control.

The effect of various concentrations of 1B2 mAb on both cell lines was performed, at two different cell concentrations i.e. $1x10^5$ and $1x10^6$ cells/ml, and at 3 and 5 hr-incubation periods. It was found that, in all three experiments performed, 10-20 μg/ml of 1B2 mAb could inhibit the proliferation of both cell lines in any condition, compared to the condition in the absence of 1B2 mAb ($p<0.05$), (Tables 12 and 13). The percentage of the inhibition effect on both cell lines was determined, and proved that, they were significantly different from control mAb used ($p<0.05$). Figures 13-16 and Figures 17-20, portray the inhibitory effects of various concentrations of 1B2 mAb and control mAb on both cell lines, respectively. Control mAb, 9C8, inhibited cell pro-liferation slightly (Figures 17-20), however, the inhibitory effect was significantly lower than that of 1B2 mAb ($p<0.05$).

The experiments mentioned above suggested that 1B2 molecules might be involved in cellular proliferation, since 1B2 mAb decreased the [^{3}H]thymidine uptake of the cell lines tested. The question arose that such an effect might be due to the cytotoxicity of 1B2 mAb present in the cultures. To address this question, cytotoxicity assay of 1B2 mAb was evaluated. U-937 and K-562, were at a final concentration of $1x10^6$cells/ml when individually cultured in the presence or absence of 1B2 mAb or mouse IgG2bκ isotype matched control, at a final concentration of 20 μg/ml. The cultures were incubated for 5 hrs. and the percentage viability determined by the dye-exclusion test. The results of the two experiments are shown in Table 16. It was indicated that the percentage viability of either U-937 or K-562 in the presence or absence of 1B2 mAb or IgG2bκ isotype matched control, was more than 97%.

Table 12. Inhibitory effect of 1B2 mAb on the proliferation of U-937

Experiment number	1B2 conc. (µg/ml)	Conditions			
		3 hours*		5 hours	
		$1x10^{6}$**	$1x10^{5}$	$1x10^{6}$	$1x10^{5}$
1	0	9145#	958	17364	1818
	5	8253 (10%)♠	936 (2%)	17245 (0.7%)	1780 (2%)
	10	7603 (17%)	873 (9%)	12030 (31%)	1309 (28%)
2	0	5381	790	14015	1231
	10	2659 (51%)	367 (54%)	8134 (42%)	365 (71%)
	20	2114 (61%)	163 (80%)	6329 (55%)	213 (83%)
3	0	16156	1381	19742	1650
	10	12779 (21%)	1156 (17%)	11341 (43%)	934 (44%)
	20	8930 (45%)	766 (45%)	9454 (53%)	536 (68%)

* incubation period
** cell concentration (cells/ml)
# mean of counts per minute (c.p.m.) from triplicate cultures
♠ percentage of proliferation inhibition

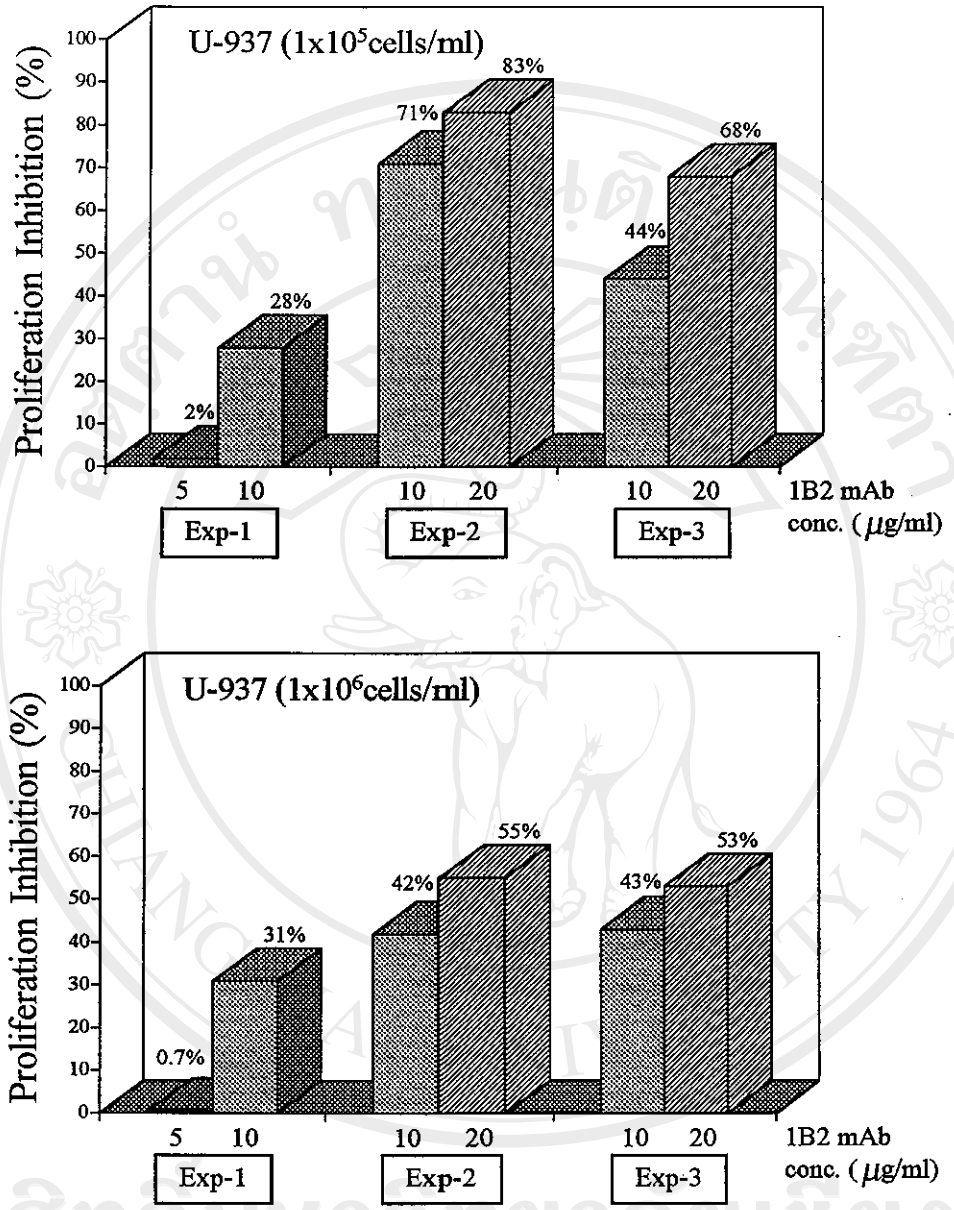

Figure 13. Inhibitory effect of 1B2 mAb on the proliferation of U-937 at 5 hours incubation.

U-937 was cultured in the final concentration of $1x10^5$ and $1x10^6$ cells/ml in the presence or absence of various concentrastions of 1B2 mAb (5-20 μg/ml) and 0.2 μCi/well of [^{3}H]thymidine for 5 hrs. The radioactive incorporation was determined and calculated for the percentage of proliferation inhibition.

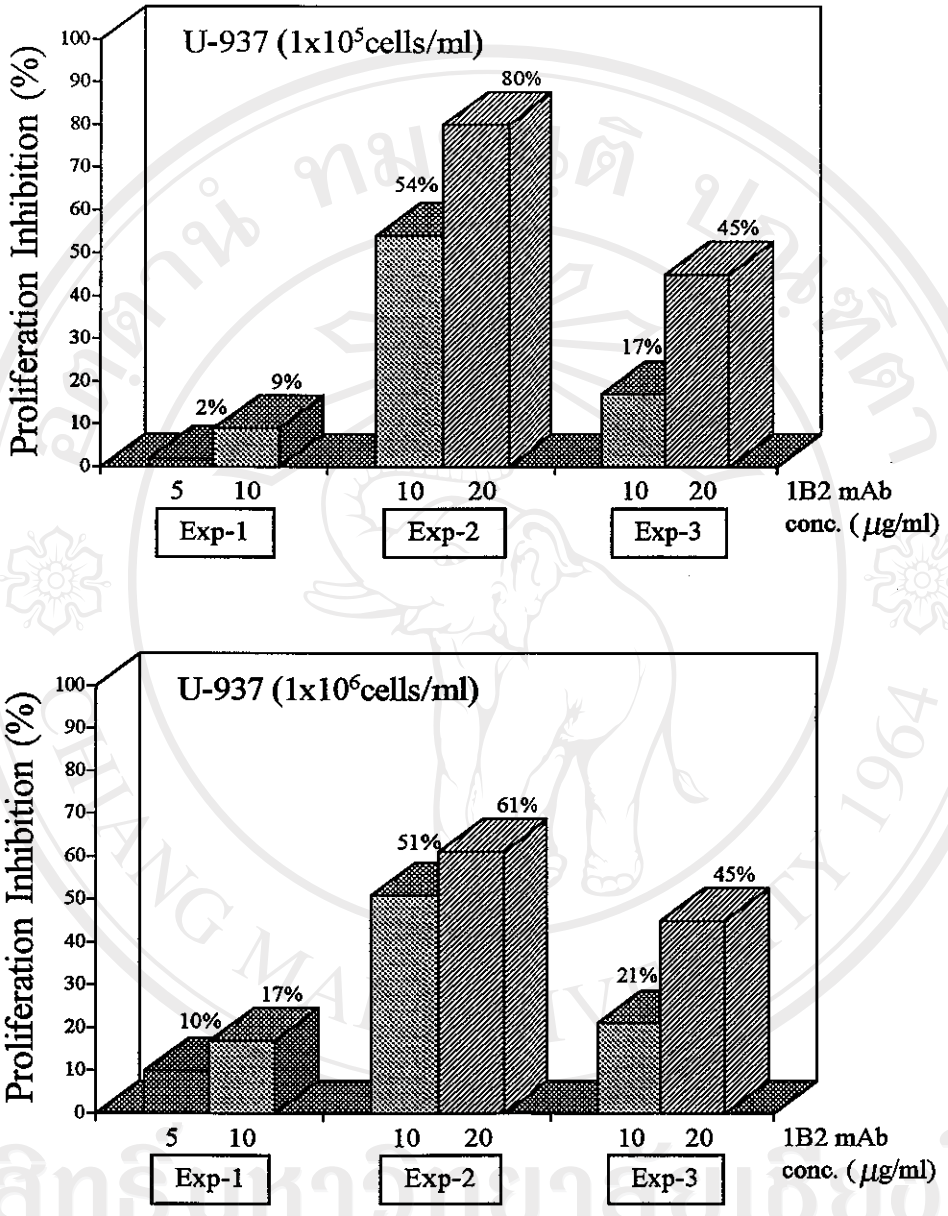

Figure 14. Inhibitory effect of 1B2 mAb on the proliferation of U-937 at 3 hours incubation.

U-937 was cultured in the final concentration of $1x10^5$ and $1x10^6$ cells/ml in the presence or absence of various concentrations of 1B2 mAb (5-20 μg/ml) and 0.2 μCi/well of [^{3}H]thymidine for 3 hrs. The radioactive incorporation was determined and calculated for the percentage of proliferation inhibition.

Table 13. Inhibitory effect of 1B2 mAb on the proliferation of K-562

Experiment number	1B2 conc. (µg/ml)	Conditions			
		3 hours*		5 hours	
		$1x10^{6}$**	$1x10^{5}$	$1x10^{6}$	$1x10^{5}$
1	0	11534#	1698	7660	798
	5	12316 (0%)♠	1562 (8%)	7090 (7%)	699 (13%)
	10	10401 (10%)	1342 (21%)	5401 (29%)	696 (13%)
2	0	8954	1119	14252	1921
	10	7945 (11%)	750 (33%)	13014 (9%)	961 (50%)
	20	6901 (23%)	273 (76%)	9587 (33%)	341 (82%)
3	0	42531	4860	49866	5874
	10	41262 (3%)	4600 (6%)	37597 (25%)	4380 (26%)
	20	18781 (56%)	1105 (78%)	11613 (77%)	344 (94%)

* incubation period
** cell concentration (cells/ml)
# mean of counts per minute (c.p.m.) from triplicate cultures
♠ percentage of proliferation inhibition

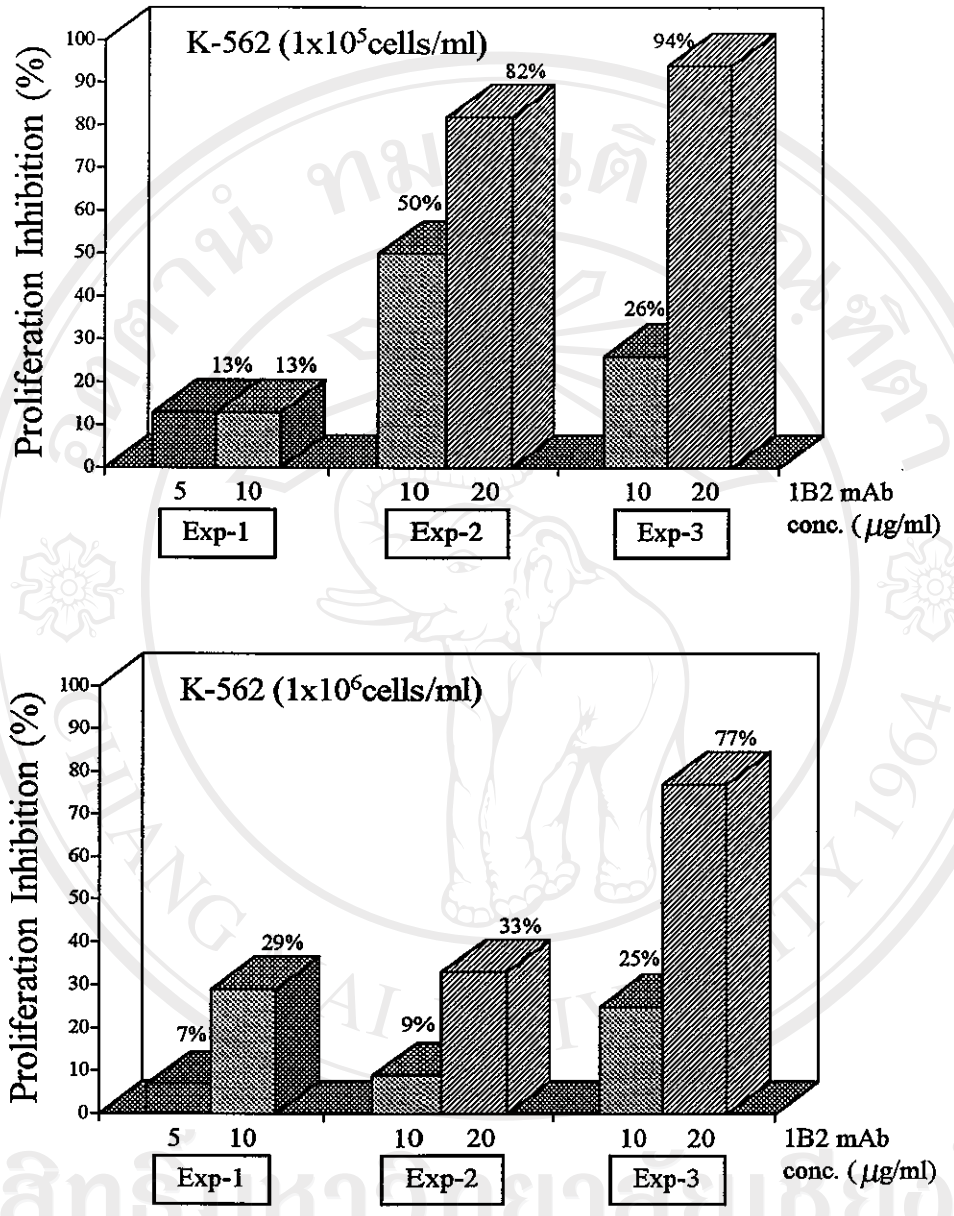

Figure 15. Inhibitory effect of 1B2 mAb on the proliferation of K-562 at 5 hours incubation.

K-562 was cultured in the presence or absence of various concentration of 1B2 mAb (5-20 μg/ml) and [^{3}H]thymidine (0.2 μCi/well) for 5 hrs. at the final cell concentration of $1x10^5$ and $1x10^6$ cells/ml. The [^{3}H]thymidine uptake was determined and calculated for the percentage of proliferation inhibition.

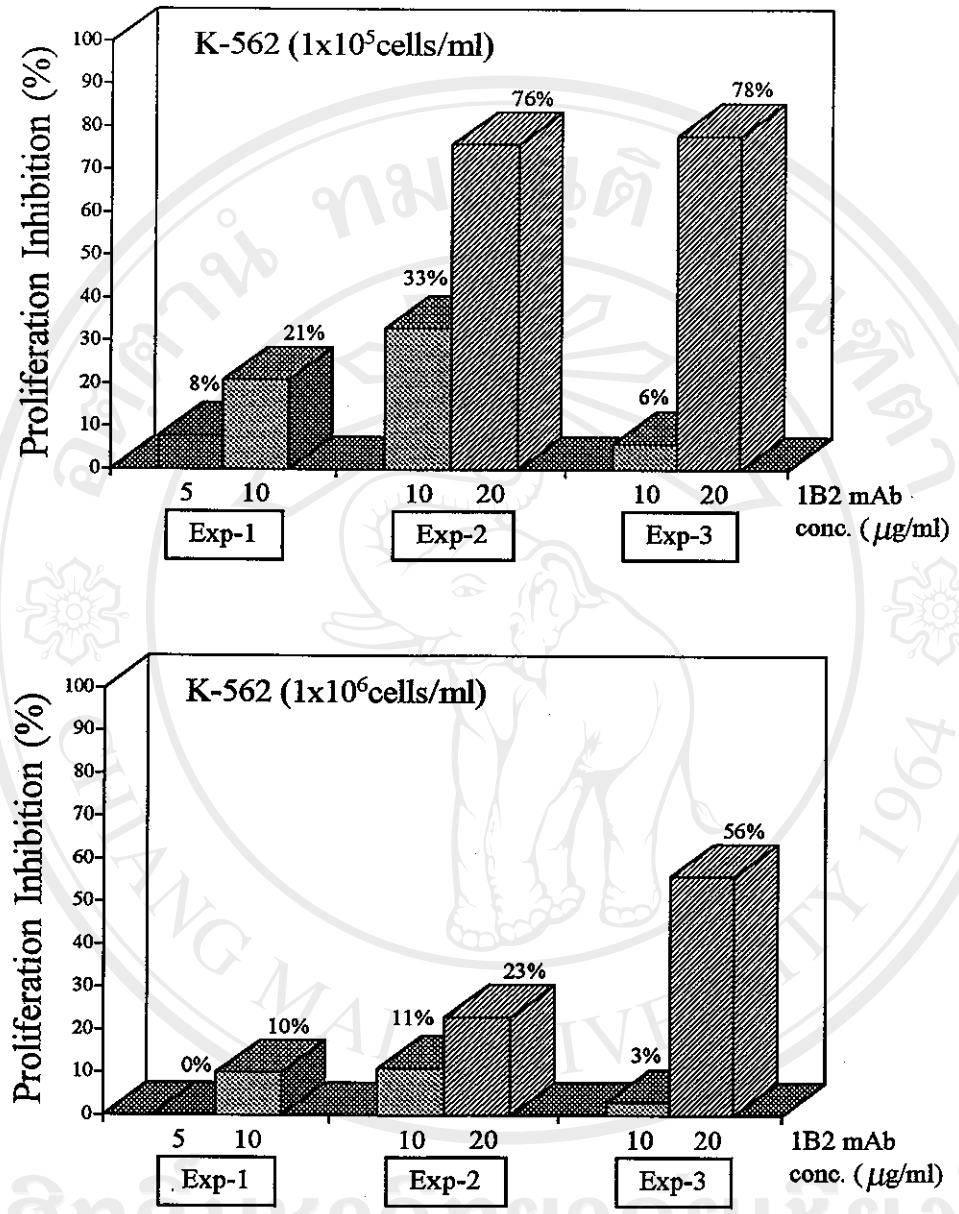

Figure 16. Inhibitory effect of 1B2 mAb on the proliferation of K-562 at 3 hours incubation.

K-562 was cultured in the presence or absence of various concentrations of 1B2 mAb (5-20 μg/ml) and [^{3}H]thymidine (0.2 μCi/well) for 3 hrs. at the final cell concentration of $1x10^5$ and $1x10^6$ cells/ml. The [^{3}H]thymidine uptake was determined and calculated for the percentage of proliferation inhibition.

Table 14. Inhibitory effect of control mAb on the proliferation of U-937

Experiment 1

mAb	Conc. (μg/ml)	Conditions			
		3 hours*		5 hours	
		$1x10^{6}$**	$1x10^{5}$	$1x10^{6}$	$1x10^{5}$
MEM18	0	4618#	508	6714	690
	10	4756 (0%)♠	502 (2%)	6476 (4%)	710 (0%)
	20	4401 (5%)	529 (0%)	6121 (9%)	723 (0%)
9C8	0	5077	583	7806	761
	10	4462 (12%)	551 (5%)	7385 (5%)	784 (0%)
	20	4489 (12%)	480 (18%)	7120 (9%)	667 (12%)
IgG2bκ	0	25648	2287	33159	2578
	5	26639 (0%)	2241 (2%)	32748 (2%)	2442 (6%)
	10	25066 (2%)	2229 (3%)	32605 (2%)	2559 (1%)
	20	24452 (5%)	1921 (16%)	30385 (9%)	2489 (4%)
	40	24102 (6%)	2266 (1%)	32497 (2%)	2439 (5%)

Experiment 2

mAb	Conc. (μg/ml)	Conditions			
		3 hours*		5 hours	
		$1x10^{6}$**	$1x10^{5}$	$1x10^{6}$	$1x10^{5}$
MEM18	0	16657#	1345	23637	1562
	10	17486 (0%)♠	1352 (0%)	23084 (3%)	1538 (2%)
	20	18062 (0%)	1362 (0%)	24287 (0%)	1601 (0%)
9C8	0	19640	1551	28050	1913
	10	17590 (10%)	1295 (16%)	25108 (10%)	1656 (13%)
	20	15576 (21%)	1169 (25%)	20637 (26%)	1479 (23%)
IgG2bκ	0	15020	1402	16412	1414
	5	14923 (1%)	1342 (4%)	16452 (0%)	1447 (0%)
	10	14823 (1%)	1342 (2%)	16320 (1%)	1410 (0%)
	20	14234 (5%)	1134 (19%)	16458 (0%)	1420 (0%)
	40	13952 (7%)	1263 (10%)	17065 (0%)	1419 (0%)

* incubation period

** cell concentration (cells/ml)

\# mean of counts per minute (c.p.m.) from triplicate cultures

♠ percentage of proliferation inhibition

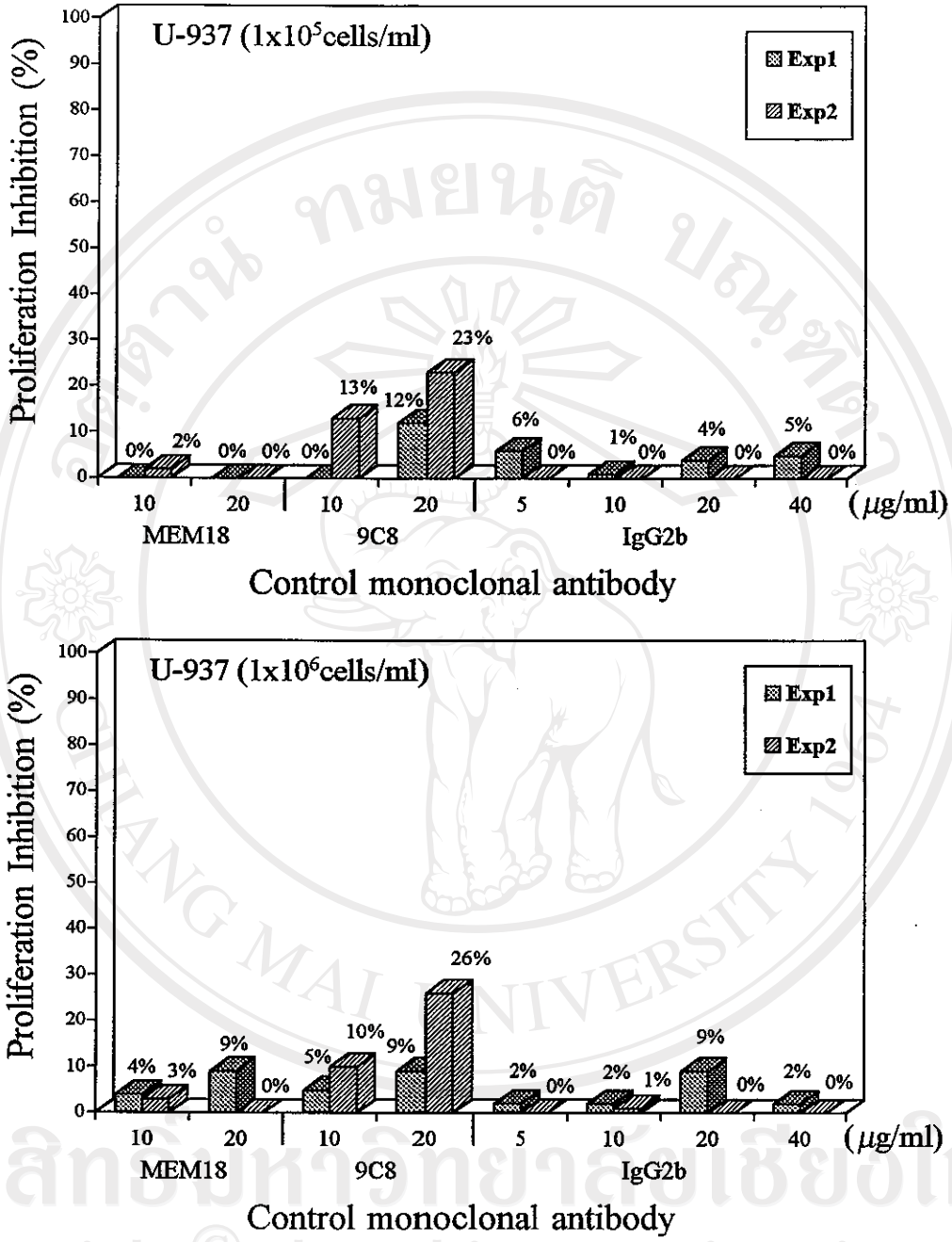

Figure 17. Effect of control mAb on the proliferation of U-937 at 5 hours incubation.

U-937 ($1x10^5$ and $1x10^6$ cells/ml) was cultured in the presence or absence of control mAb (MEM18, 9C8 and IgG2b isotype match, final concentration of 10 and 20 μg/ml) and 0.2 μCi/well of [^{3}H]thymidine for 5 hrs. The radioactive incorporation was determined and calculated for the percentage of proliferation inhibition.

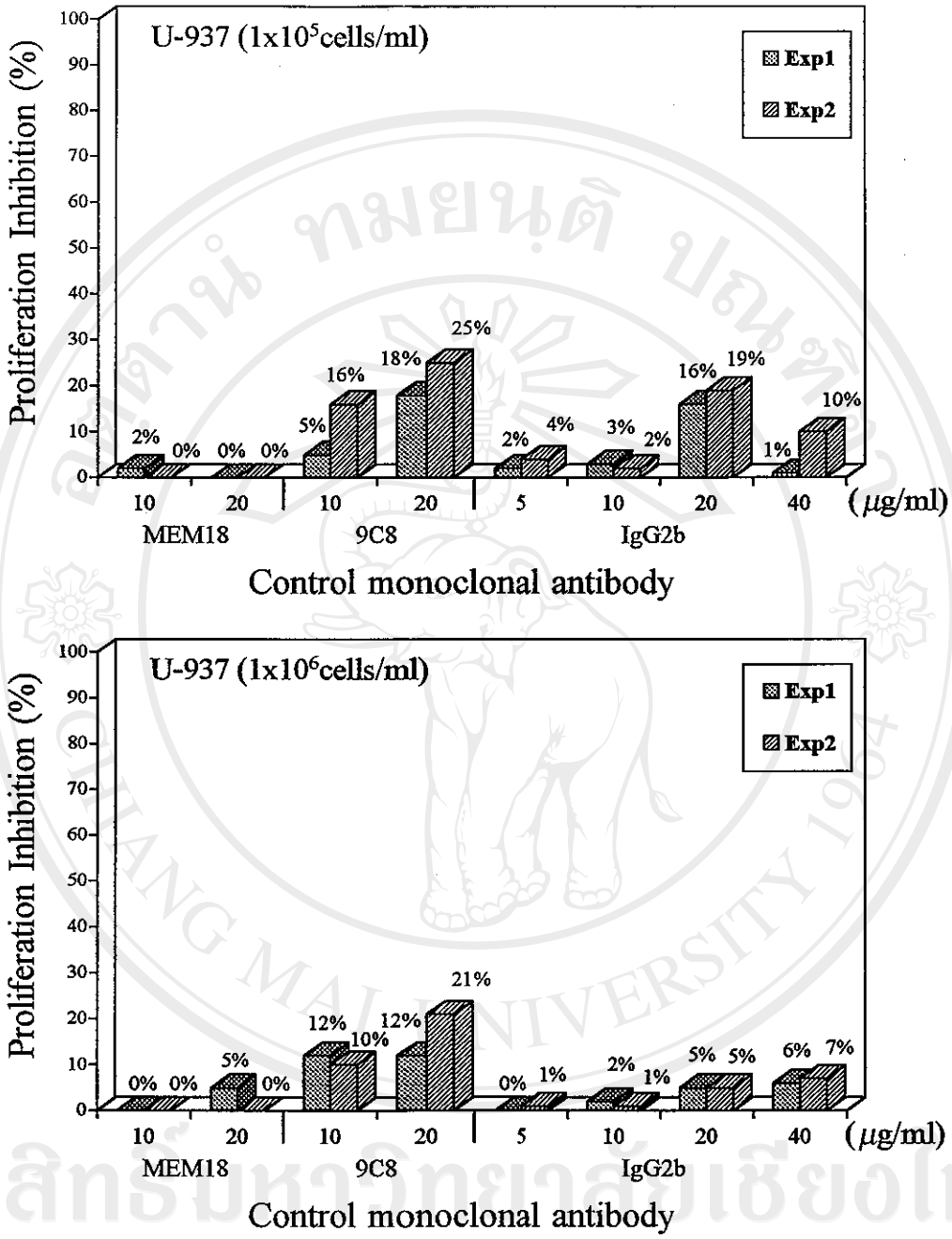

Figure 18. Effect of control mAb on the proliferation of U-937 at 3 hours incubation.

U-937 ($1x10^5$ and $1x10^6$ cells/ml) was cultured in the presence or absence of control mAb (MEM18, 9C8 and IgG2b isotype match, final concentration of 10 and 20 μg/ml) and 0.2 μCi/well of [^{3}H]thymidine for 3 hrs. The radioactive incorporation was determined and calculated for the percentage of proliferation inhibition.

Table 15 Inhibitory effect of control mAb on the proliferation of K-562

Experiment 1

mAb	Conc. (μg/ml)	Conditions			
		3 hours*		5 hours	
		$1x10^6$**	$1x10^5$	$1x10^6$	$1x10^5$
MEM18	0	14143#	2031	15242	2044
	10	13875 (2%)♠	2007 (2%)	13887 (9%)	1879 (9%)
	20	13387 (6%)	1758 (14%)	14080 (8%)	1862 (9%)
9C8	0	15937	2214	18601	2138
	10	14101 (12%)	2122 (5%)	15561 (17%)	1959 (9%)
	20	13556 (15%)	1928 (13%)	16392 (12%)	1818 (15%)
IgG2bκ	0	30867	3681	33239	3535
	5	30535 (1%)	3643 (1%)	32782 (1%)	3495 (1%)
	10	29034 (6%)	3536 (4%)	31493 (5%)	3552 (0%)
	20	28002 (9%)	3284 (11%)	30768 (7%)	2908 (17%)
	40	28950 (6%)	3422 (7%)	28265 (15%)	3201 (9%)

Experiment 2

mAb	Conc. (μg/ml)	Conditions			
		3 hours*		5 hours	
		$1x10^6$**	$1x10^5$	$1x10^6$	$1x10^5$
MEM18	0	36924#	3875	43597	5203
	10	35479 (4%)♠	3498 (10%)	43574 (0%)	4966 (5%)
	20	29831 (20%)	2968 (23%)	40154 (8%)	4690 (10%)
9C8	0	38449	4217	46757	5483
	10	36129 (6%)	3744 (11%)	43373 (7%)	4768 (13%)
	20	34197 (11%)	3476 (18%)	39435 (16%)	4624 (16%)
IgG2bκ	0	41071	4749	47709	5524
	5	39901 (3%)	4884 (0%)	45045 (6%)	5196 (6%)
	10	39326 (4%)	4611 (3%)	45928 (4%)	5330 (4%)
	20	38399 (7%)	4482 (6%)	43173 (10%)	5134 (7%)
	40	40505 (1%)	4364 (8%)	45254 (5%)	5516 (0%)

* incubation period

** cell concentration (cells/ml)

mean of counts per minute (c.p.m.) from triplicate cultures

♠ percentage of proliferation inhibition

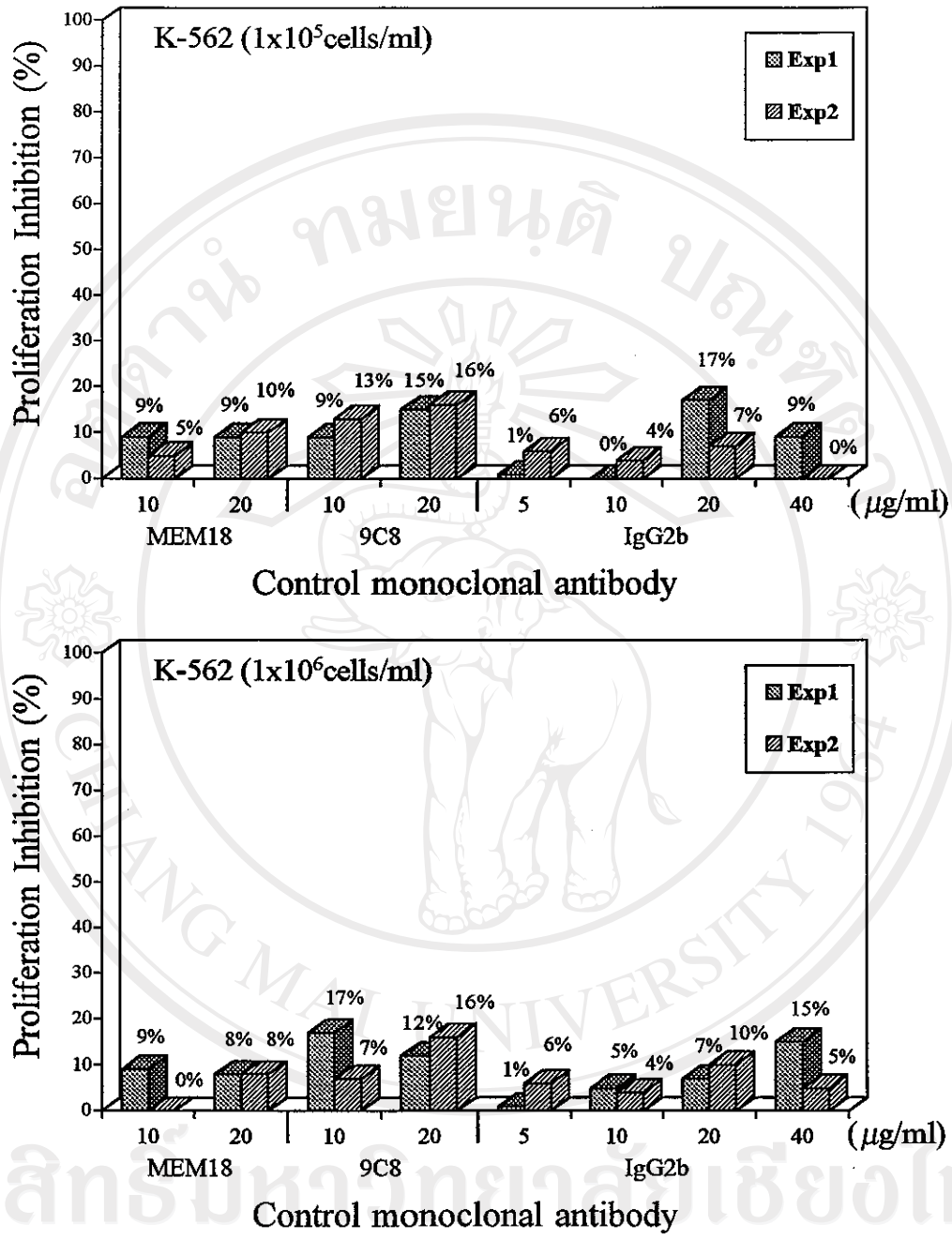

Figure 19. Effect of control mAb on the proliferation of K-562 at 5 hours incubation.

K-562 ($1x10^5$ and $1x10^6$ cells/ml) was cultured in the presence or absence of control mAb (MEM18, 9C8 and IgG2b isotype match, final concentration of 10 and 20 μg/ml) and 0.2 μCi/well of [^{3}H]thymidine for 5 hrs. The radioactive incorporation was determined and calculated for the percentage of proliferation inhibition.

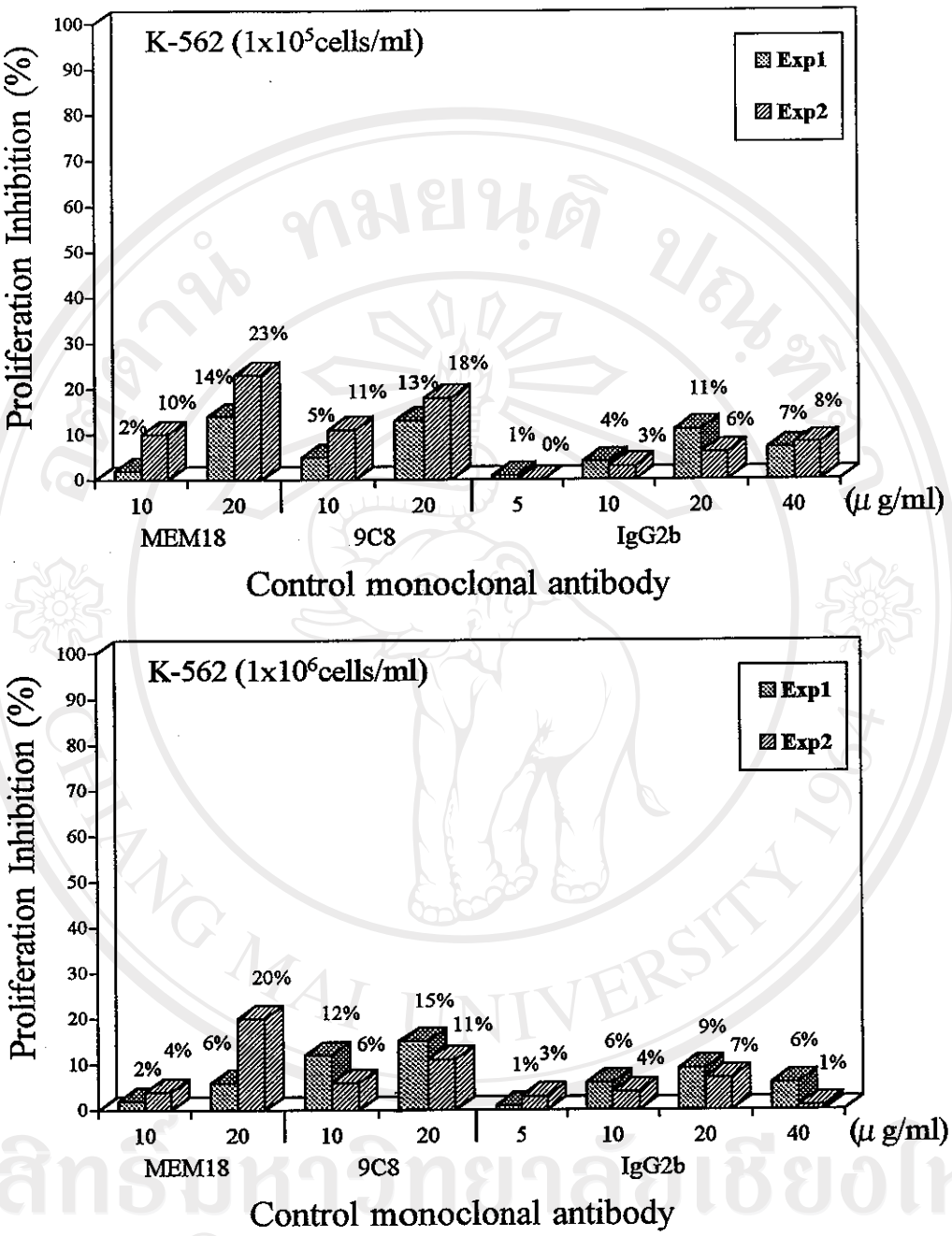

Figure 20. Effect of control mAb on the proliferation of K-562 at 3 hours incubation.

K-562 ($1x10^5$ and $1x10^6$ cells/ml) was cultured in the presence or absence of control mAb (MEM18, 9C8 and IgG2b isotype match, final concentration of 10 and 20 μg/ml) and 0.2 μCi/well of [^{3}H]thymidine for 3 hrs. The radioactive incorporation was determined and calculated for the percentage of proliferation inhibition.

Table 16. Assay for the cytotoxicity of 1B2 mAb

Experiment number	Cell type	Conditions		
		no mAb	1B2	mouse IgG2bκ
1	U937	99*	99	98
	K562	99	97	98
2	U937	98	99	98
	K562	99	97	98

* % Viability

7. Optimization of conditions for immunoblotting

To obtain the highest possible immunoblotting detection by using the chemiluminescent technique, titration for optimal concentrations of biotinylated rabbit anti mouse Igs and peroxidase-conjugated avidin were performed. For this purpose, normal mouse serum was used as a sample. Normal mouse serum was diluted to 1:500, 1:2500 and 1:12500, and applied to SDS-PAGE, blotted onto nitrocellulose membranes and incubated with two different concentrations of biotinylated rabbit anti mouse Igs. It was then incubated with three different concentrations of peroxidase-conjugated avidin. As shown in Figure 21, it was found that the optimal concentrations of biotinylated rabbit anti mouse Igs and peroxidase-conjugated avidin was 1:5000 and 1:20000, respectively. In this optimal condition, the antigen-antibody reaction can be distinguished with a lower staining background.

8. Determination of the molecular weight of 1B2 molecules on U-937

To determine the molecular weight of a cell surface molecule recognized by 1B2 mAb, U-937 were lysed with Tris lysis buffer pH 8.2. The lysates were then performed by SDS-PAGE, immunoblotting and reacting with 1B2 mAb or control mAb, anti-CD31 mAb. The antigen-antibody reaction on the membrane was visualized by the enhanced chemiluminescence detection system, using titrated optimal condition. The result indicated that, 1B2 mAb recognized a protein with a molecular mass of 23 kDa under reducing conditions, as shown in Figure 22.

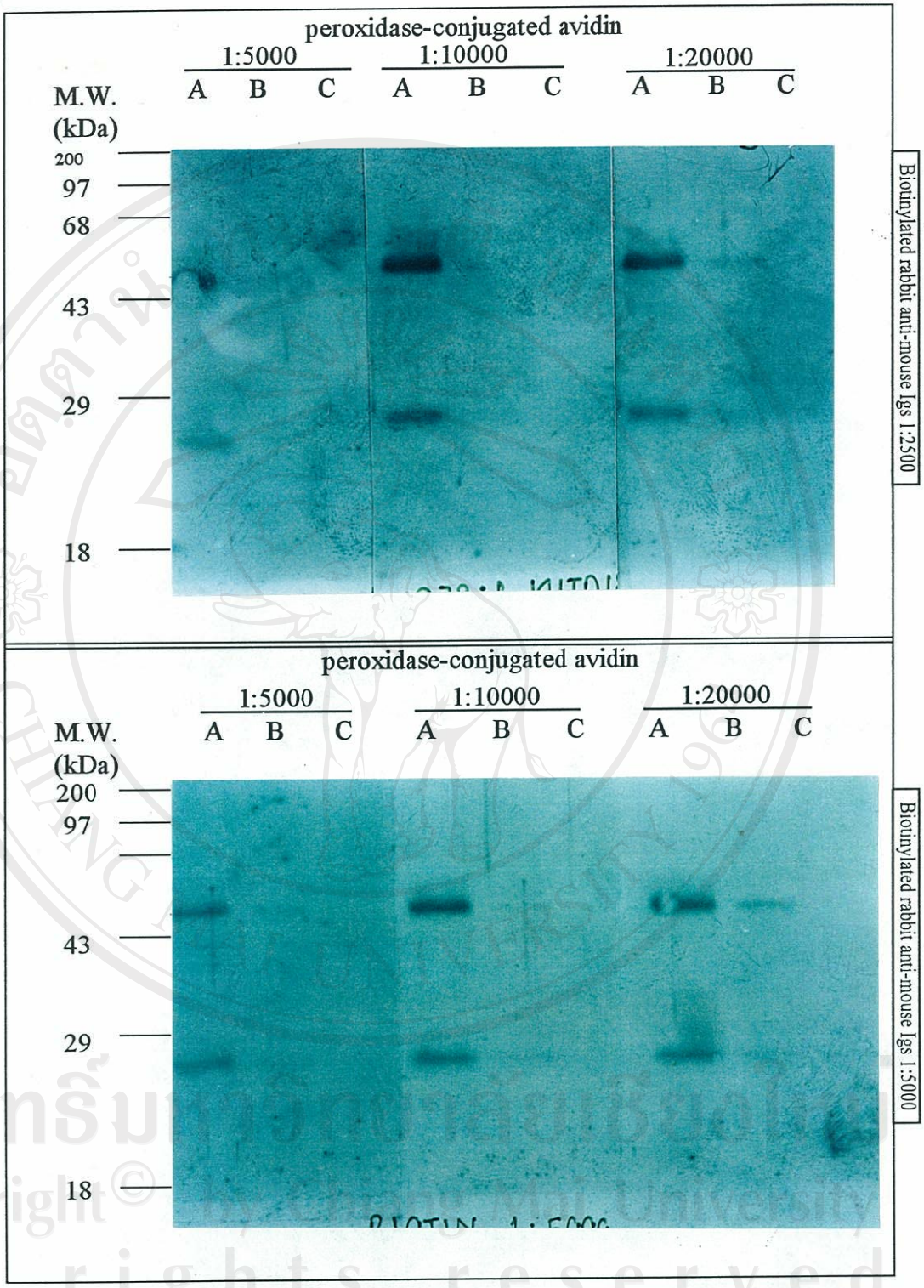

Figure 21. Optimization of conditions for immunoblotting

Normal mouse serum 1:500 (A), 1:2500 (B) and 1:12500 (C) were analyzed using reducing condition SDS-PAGE. Separated proteins were reacted with two different concentrations of biotinylated rabbit mouse Igs and three different concenrations of peroxidase-conjugated avidin. Molecular mass standards (in kDa) are indicated on the left.

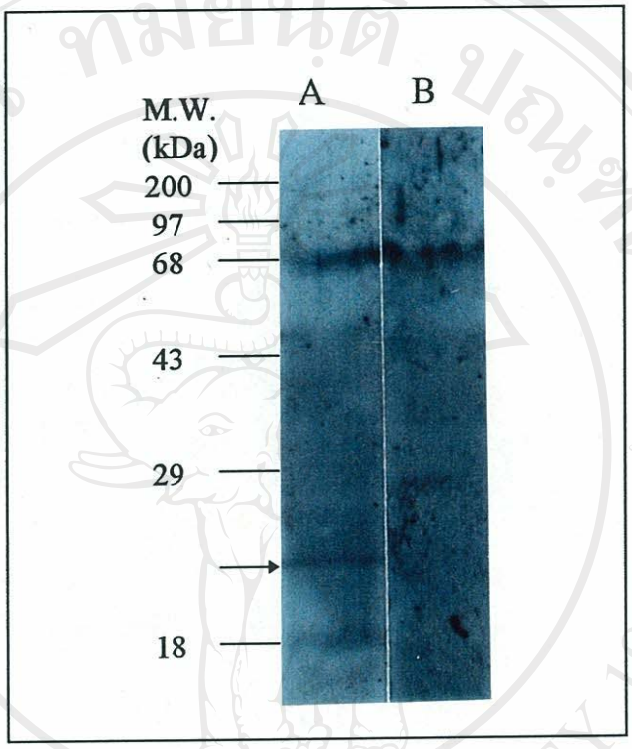

Figure 22. Determination of the molecular weight of 1B2 molecule on U-937

U-937 lysates were performed with SDS-PAGE in reducing condition. The immunoblots analyzed with 1B2 mAb (lane A) and anti-CD31 (lane B) were visualized by biotinylated rabbit anti mouse Igs and peroxidase-conjugated avidin, and an enhanced chemiluminescence detection system of Amersham. Molecular mass standards (in kDa) are indicated on the left.