IV. RESULTS

1. Production of Anti-M6 monoclonal antibodies

1.1 Preparation of M6 DNA and vector DNA

For screening the hybridoma which produced anti-M6 monoclonal antibodies, the M6 DNA transfected and mock transfected COS cells were needed. Therefore, the first step of this study was the preparation of M6 DNA and vector DNA lacking in any expressible gene insert.

Standard M6 DNA and vector DNA were transformed into competent *E. coli*. To obtain colonies that contained M6 DNA, 4 bacterial colonies were selected at random and plasmid DNA were isolated. The isolated plasmid DNA were digested with Xho1 restriction enzyme, followed by agarose gel electrophoresis. The DNA were stained with ethidium bromide and visualized with UV light. The fragments of DNA were compared with standard M6 DNA and standard DNA marker. All isolated plasmid DNA contained a fragment of 1.6 kb of cDNA encoding M6 protein and a 3.8 kb of vector (Figure 2). The DNA pattern was identical to standard M6 DNA, which was used as a control. All 4 colonies of M6 transformed *E. coli* were frozen at -70° C and kept for further studies.

For the preparation of vector DNA, one colony was selected for isolation of plasmid DNA. Restriction fragment analysis confirmed that the isolated DNA were vector without any gene insert and the pattern was the same as standard vector control (Figure 2). The transformed bacteria was then frozen at -70° C and kept for further studies.

The isolated M6 DNA were then tested for their capability in expressing M6 proteins. The M6 DNA were transfected into COS cells which were then stained with standard anti-M6 mAb (clone 1B2 obtained from Dr. O. Majdic, University of vienna) and anti-CD4 mAb as a negative control. These M6 DNA transfected COS cells reacted strongly to anti-M6 mAb (Figure 3), but were negative to the control antibody. In contrast, mock transfected COS cells were negative with both 1B2 and anti-CD4 mAb. The results indicated that the isolated M6 DNA could be transcribed and translated into M6 proteins in COS cells.

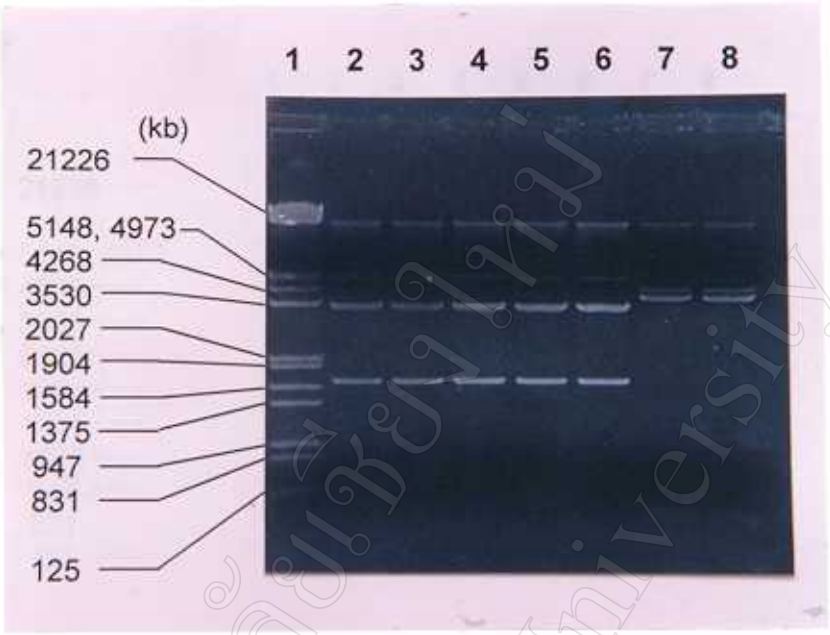


Figure 2. Restriction analysis of M6 DNA and vector DNA isolated from transformed *E. coli* by plasmid miniprep

Plasmid DNA were isolated from M6 DNA transformed *E.coli* and vector DNA transformed *E.coli*. The isolated plasmid DNA were cut by Xho1 restriction enzyme, followed by electrophoresis using 1% agarose gel. The fragments of DNA were visualized with a transiluminator and were compared to standard M6 DNA, standard vector DNA and standard DNA marker. Standard DNA marker (lane 1), standard M6 DNA (lane 2), plasmid DNA isolated from M6 DNA transformed *E. coli* colonies numbers 1-4 (lane 3-6) respectively, standard vector DNA (lane 7), and plasmid DNA isolated from vector DNA transformed *E. coli* colony (lane 8). Size of standard DNA marker (kb) are indicated on the left.

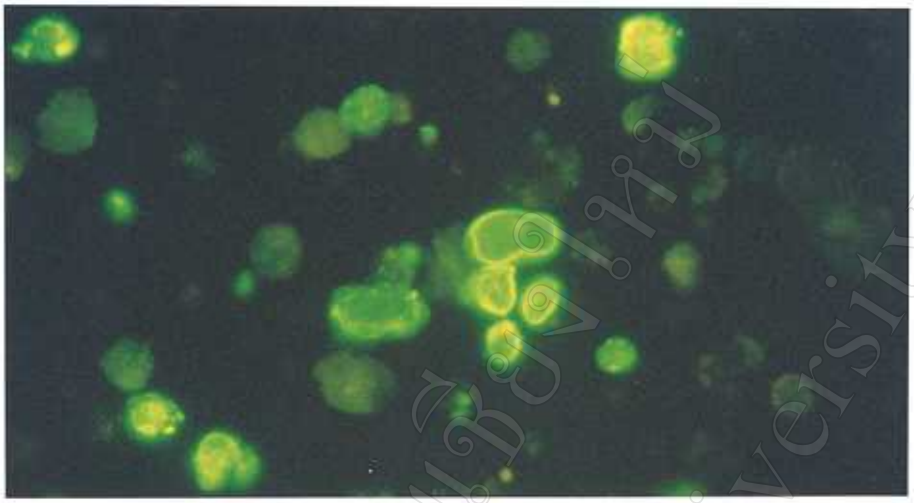


Figure 3. Photograph of M6 DNA transfected COS cells reacted with anti-M6 monoclonal antibody

M6 DNA isolated from M6 DNA transformed *E. coli* were transfected into COS cells. The cells were then stained with anti-M6 mAb, 1B2 and FITC-conjugated anti-mouse immunoglobulins by using the indirect immunofluorescence technique and photographed from a fluorescent microscope (Magnification 400x).

In order to obtain a large amount of M6 and vector DNA, colonies which have been proved to carry M6 and vector DNA, were used and performed on as described in the materials and methods section. Using Cesium Chloride-Ethidium bromide gradient ultracentrifugation, the yields of M6 DNA and vector DNA obtained were 4.4 mg and 2.7 mg per liter of bacteria, respectively. The OD_{260}/OD_{280} ratio obtained from both DNA preparations were between 1.8-2.0.

The isolated M6 and vector plasmid DNA were tested again for a content of M6 and vector DNA respectively by using the restriction fragment analysis. As shown in Figure 4, the resulting DNA fragment from M6 and vector DNA digestion were the same as standard M6 DNA and standard vector DNA, respectively.

M6 DNA were then tested for their capability in producing M6 proteins again in the COS cells expression system. The M6 transfected COS cells were strongly positive when stained with anti-M6 mAb 1B2. The vector DNA transfected COS cells, in contrast, were negative with anti-M6 mAb 1B2. These result indicated that isolated M6 and vector DNA can be used to prepare M6 transfected COS cells for the screening of anti-M6 mAb producing hybridoma clones.

1.2. Immunization and cell fusion

A female Balb/c mouse was immunized by means of a K-562 erythroid/ myeloid cell line with the immunization schedule as described in materials and methods. To check whether the animal was well immunized, its serum was analyzed for the presence of antibodies against the K-562 cells by using indirect immunofluorescence technique. After the second immunization, the mouse serum showed strong positive staining with K-562. Then spleen cells from the immunized mouse and X63-Ag8.653 myeloma cell line were fused. 511 out of 672 wells containing hybrid cells were obtained from this fusion.

1.3. Screening of hybridoma

The culture supernatant from 511 hybridoma positive wells were screened, firstly, for antibodies to K-562 by the indirect immunofluorescence technique. It was found that supernatants from 25 hybridoma containing wells were positive. Hybridoma from 25 wells, which showed positive staining with K-562, were then confirmed for anti-M6 mAb pro-

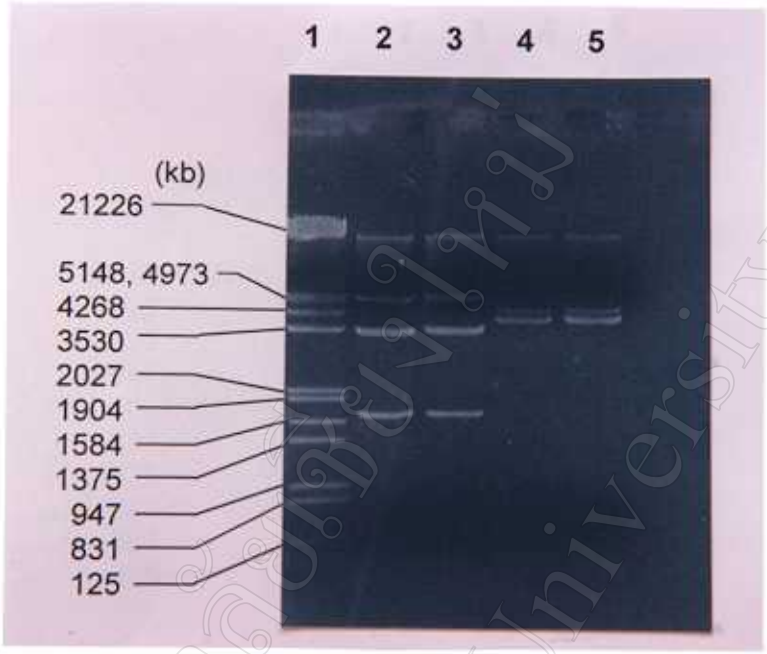


Figure 4. Restriction analysis of M6 DNA and vector DNA isolated from transformed *E. coli* by Cesium Chloride-Ethidium bromide gradient ultracentrifugation

Plasmid DNA were isolated from M6 DNA transformed *E.coli* and vector DNA transformed *E.coli* by Cesium Chloride-Ethidium bromide gradient ultracentrifugation. The isolated plasmid DNA were cut by Xho1 restriction enzyme, followed by running electrophoresis using 1% agarose gel. The fragments of DNA were visualized with a transilluminator and compared to standard M6 DNA, standard vector DNA and standard DNA marker. Standard DNA marker (lane 1), standard M6 DNA (lane 2), plasmid DNA isolated from M6 DNA transformed *E. coli* colony (lane 3), standard vector DNA (lane 4), plasmid DNA isolated from vector DNA transformed *E. coli* colony (lane 5). Size of standard DNA marker (kb) are indicated on the left.

duction using M6 DNA transfected COS cells as positive target cells and vector transfected COS cells as a negative control. Only 3 wells, 2D1, 5H5 and 7H11, contained anti-M6 mAb producing hybridoma cells, since these wells showed strong positive staining with M6 DNA transfected COS cells, but negative with vector DNA transfected COS cells. Four weeks later, hybrids in 5H5 well stopped producing anti-M6 mAb, whereas, those in 2D1 and 7H11 continued to produce. Hybrids in 2D1 and 7H11 wells were then expanded and cloned.

1.4. Cloning of positive hybridoma by limiting dilution

Hybridoma cells from 2D1 and 7H11 wells were cloned by limiting dilution in order to obtain a single-cell clone. Hybridoma cells from 2D1 well were cloned by one round of limiting dilution. The healthy single clone, named 2G11, was selected for further studies. Hybridoma cells from well 7H11 were cloned by 2 rounds of limiting dilution. The healthy single clone named 1B9 from the second round of limiting dilution was selected for further studies.

Supernatant from both 2G11 and 1B9 were tested again for anti-M6 antibody activity using M6 DNA transfected COS cells as the target, and found that supernatants from both clones strongly reacted with M6 transfected COS cells.

1.5. Production of culture supernatant and ascitic fluid

To produce anti-M6 mAb, 2G11 and 1B9 hybrid clones were cultured in IMDM supplemented with 10% FCS at 37°C in a CO_2 incubator. The individual cultures were allowed to grow until the hybridoma died, then the supernatants were collected and stored at -20°C for further use.

To produce ascitic fluid, which contains a high concentration of anti-M6 mAb, hybridomas from each clone were injected into pristane treated Balb/c mice peritoneum (2 mice per hybrid clone). Ascitic fluid was collected at days 11 to 19 after hybridoma inoculation. The ascitic fluid from 1B9 and 2G11 clones was tested for anti-M6 mAb by using the indirect immunofluorescence technique, with M6 DNA transfected COS cells as the target. It was found that both ascitic fluids were positive with M6 DNA transfected COS cells and negative with vector DNA transfected COS cells.

2. Determination of the isotype of monoclonal antibodies

The isotypes of anti-M6 mAb produced by 1B9 and 2G11 clones were determined by Capture ELISA. It was found that the isotypes of 1B9 and 2G11 were IgG3 and IgM, respectively (Figure 5).

3. Expression of M6 molecule on haematopoietic cell lines

To study the reactivity of haematopoietic cell lines with the generated anti-M6 mAb, erythroid/myeloid cell lines K-562, myeloid cell line U-937, T cell lines Sup T1 and Molt-4, and B cell lines Daudi were stained with anti-M6 mAb 1B9 and 2G11. As shown in the FACS profiles in Figure 6, all cell lines tested were positive with both anti-M6 mAb 1B9 and 2G11 and negative with myeloma induced ascitic fluid, which was used as a negative control. Photographs of positive staining are shown in Figure 7.

4. Expression of M6 molecule on peripheral blood leukocytes

Previously experiment indicated that anti-M6 mAb, 1B9 and 2G11, were strongly positive with all haematopoietic cell lines. M6 molecule expression on various types of white blood cell and activated lymphocytes were further characterized. The results obtained are as follows:

4.1 Peripheral blood mononuclear cells (PBMC)

Heparinized blood was collected from 9 healthy donors and PBMC were isolated by Ficoll-Hypaque density gradient centrifugation. PBMC were then stained with anti-M6 mAb 1B9 and 2G11, anti-CD3 (OKT3), anti-CD14 (MEM18) or myeloma induced ascitic fluid, and analysed by flow cytometry. To analyze the immunofluorescent reactivity of lymphocyte and monocyte populations, both cell types were gated according to their size and granularity. As shown in Table 3, lymphocytes from all donors reacted with anti-M6 mAb. However, they separated into 2 groups according to the immunofluorescent reactivity. The large population of cells were weaker than the small one (Figure 8). Lymphocytes from all samples showed up as strongly positive with anti-CD3 (OKT3) which was used as a positive control for the T lymphocyte population in

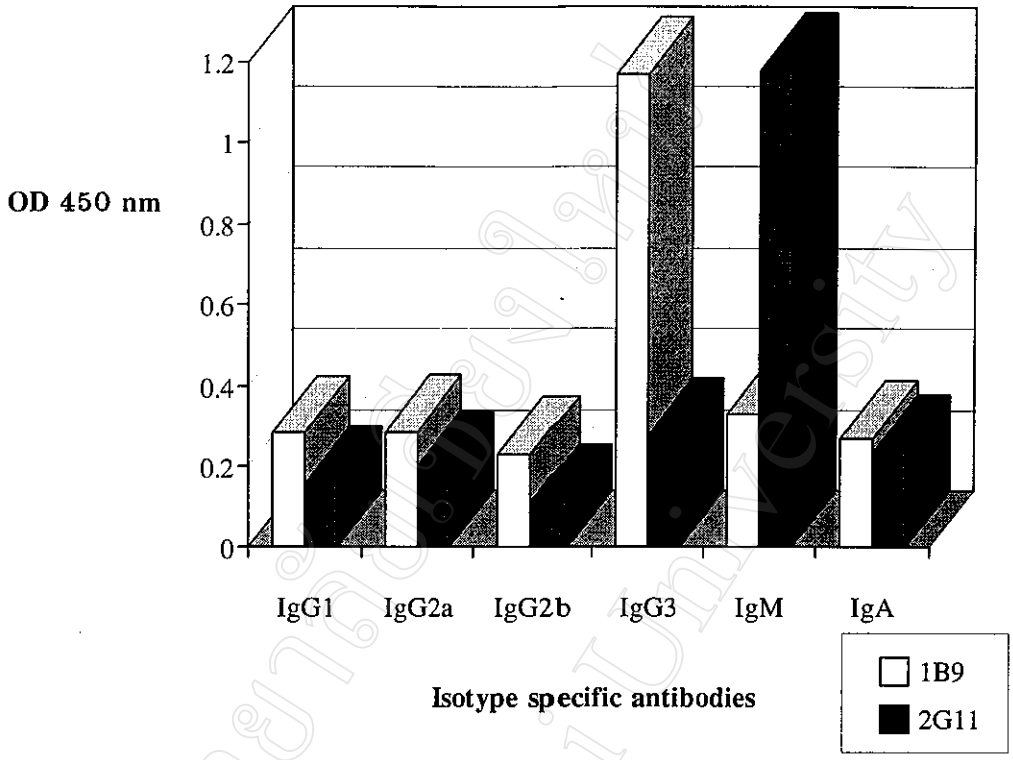


Figure 5. Determination of the isotype of anti-M6 mAb by capture ELISA

Tissue culture supernatants, which contained anti-M6 mAb from hybridoma clone 1B9 and 2G11 were added into each well of microtiter plate separately. Each well of microplate was previously coated with isotype specific antibodies (goat antimouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA), and the antigen-antibody reaction was visualized by using peroxidase labeled goat anti-mouse immunoglobulins and TMB substrate. The results were read at OD 450 nm.

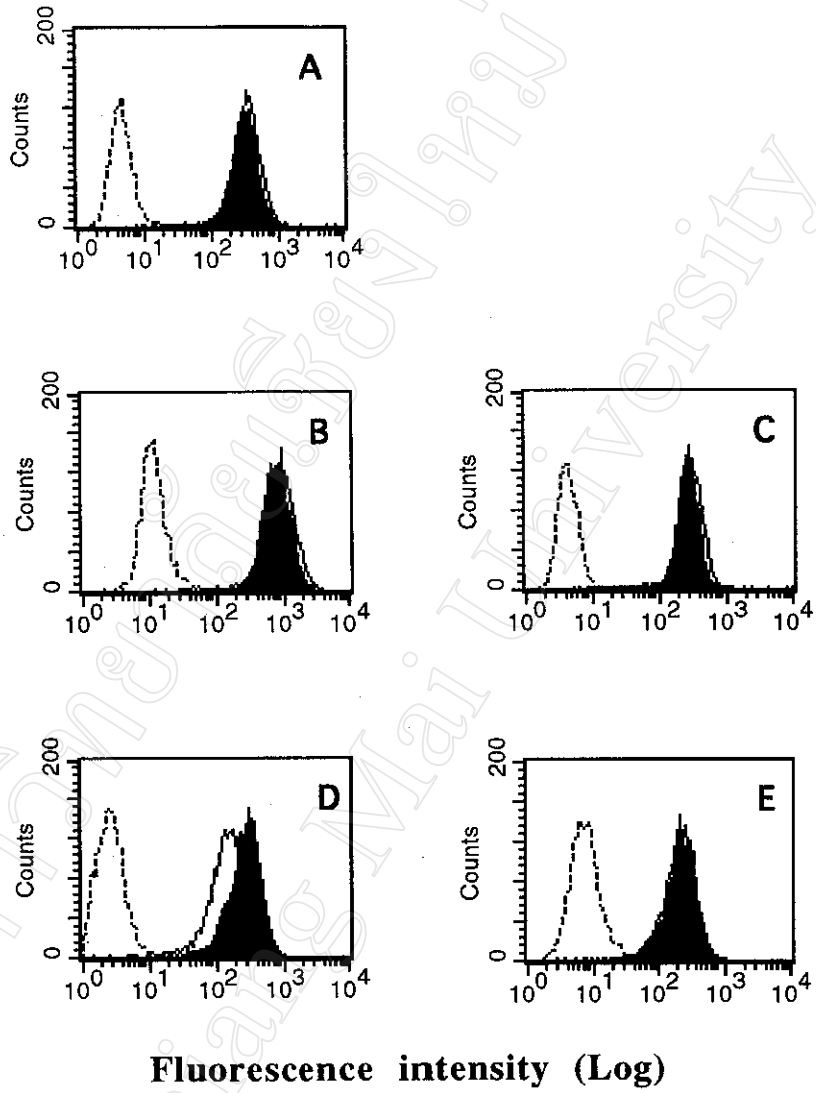


Figure 6. Expression of M6 molecule on haematopoietic cell lines

Haematopoietic cell lines including Sup-T1 (A), Daudi (B), Molt-4 (C), U-937 (D) and K-562 (E) were stained with anti-M6 mAb 1B9 and 2G11 or myeloma induced ascitic fluid. Shaded peaks represent the immunofluorescence profiles of cells stained with anti-M6 mAb 1B9; Solid line represent cells stained with anti-M6 mAb 2G11 and dashed lines represent the background fluorescence of myeloma induced ascitic fluid.

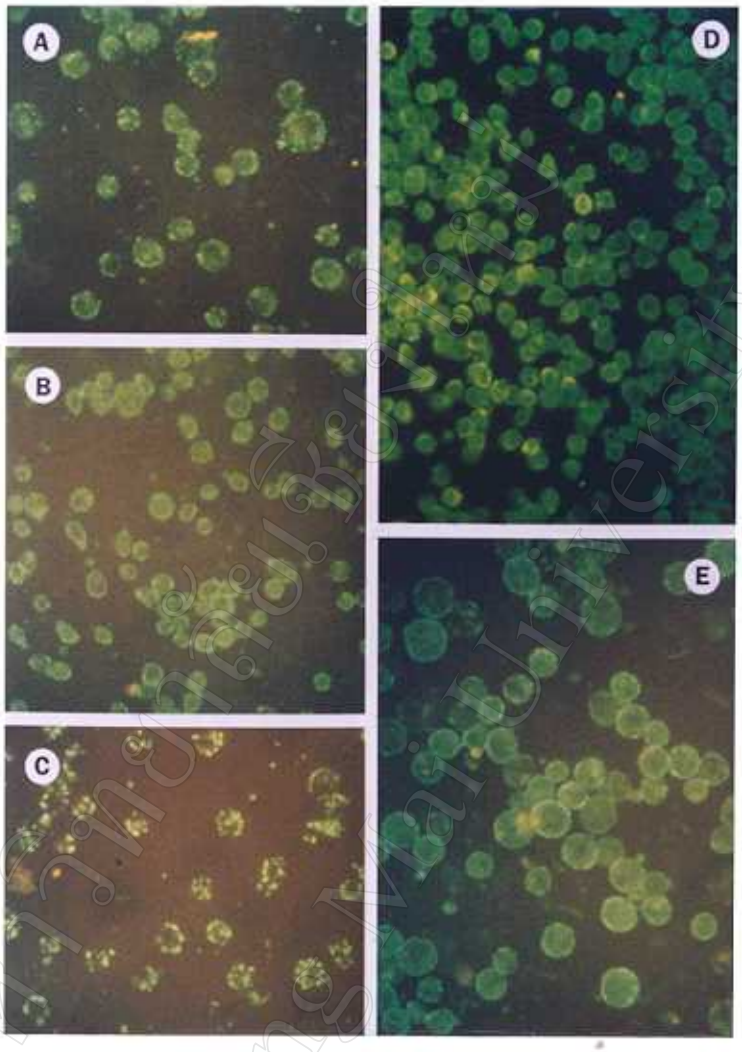


Figure 7. Photographs of cell lines given positive reaction with anti-M6 mAb

Human haematopoietic cell lines including Sup-T1 (A), Daudi (B), Molt 4 (C), U-937 (D) and K-562 (E) were stained with anti-M6 mAb (1B9) and FITC-conjugated anti-mouse immunoglobulins by using the indirect immunofluorescence technique. (Magnification 400x).

Table 3. Expression of M6 molecule on peripheral blood mononuclear cells

Sample number	Cell type	monoclonal antibody				myeloma induced ascitic fluid
		anti-M6		OKT3*	MEM 18**	
		1B9	2G11			
1	lymphocyte	+	+	+	-	-
	monocyte	+	+	-	+	-
2	lymphocyte	+	+	+	-	-
	monocyte	+	+	-	+	-
3	lymphocyte	+	+	+	-	-
	monocyte	+	+	-	+	-
4	lymphocyte	+	+	+	-	-
	monocyte	+	+	-	+	-
5	lymphocyte	+	+	+	-	-
	monocyte	+	+	-	+	-
6	lymphocyte	+	+	+	-	-
	monocyte	+	+	-	+	-
7	lymphocyte	+	+	+	-	-
	monocyte	+	+	-	+	-
8	lymphocyte	+	+	+	-	-
	monocyte	+	+	-	+	-
9	lymphocyte	+	+	+	-	-
	monocyte	+	+	-	+	-

* OKT 3 = anti-CD 3 mAb
** MEM 18 = anti-CD14 mAb

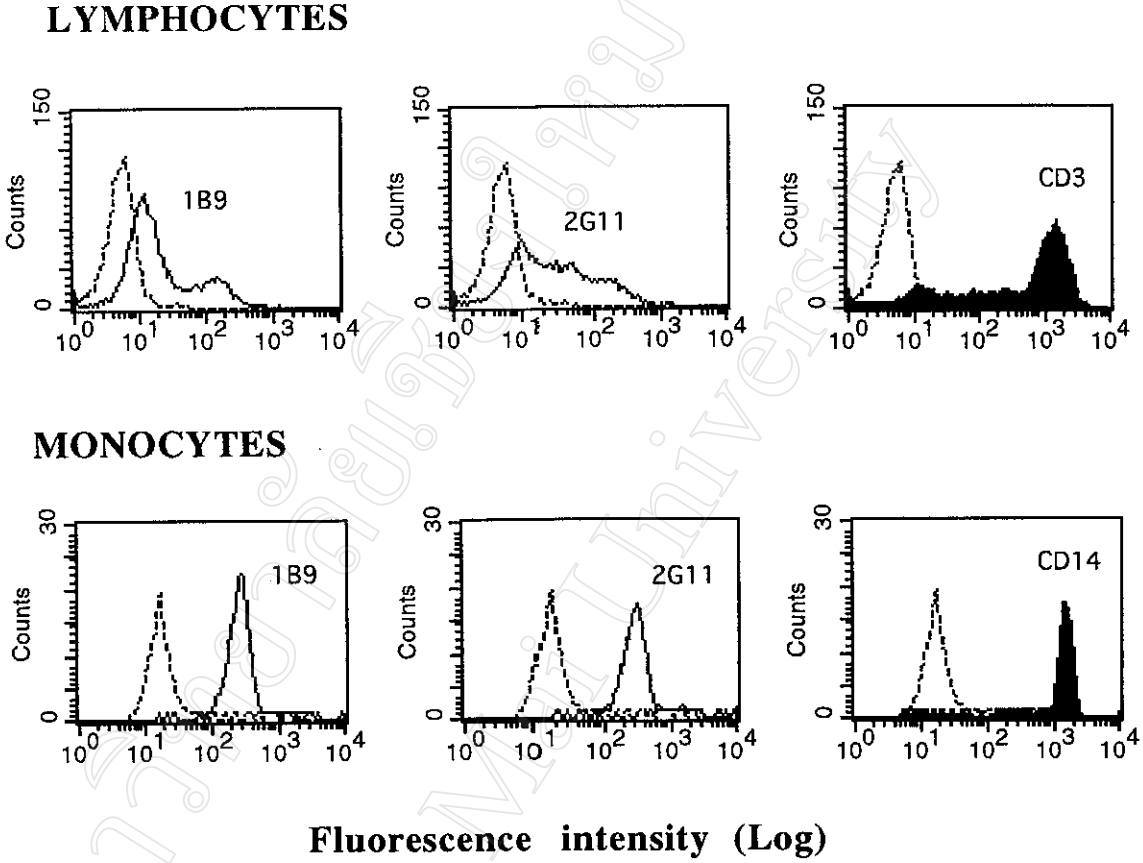


Figure 8. Expression of M6 molecule on peripheral blood mononuclear cells

Immunofluorescence analysis of the reactivity of anti-M6 mAb 1B9 and 2G11 with freshly isolated peripheral blood mononuclear cells was carried out. The lymphocytes and monocytes were gated according to size and granularity, and fluorescent intensity was analyzed on a FACS-can. Solid lines represent the immunofluorescent profiles of cells stained with indicated anti-M6 mAb 1B9 or 2G11; dashed lines represent the background fluorescence of negative control and the shaded peaks represent the fluorescence of indicated positive control monoclonal antibody.

the lymphogate. Monocytes from all samples were strongly positive with anti-M6 mAb, 1B9 and 2G11, as well as anti-CD14 (MEM18) which was used as a positive control for the monocyte population in monogate. Both lymphocytes and monocytes were negative with myeloma induced ascitic fluid, which is known not to react with human leukocyte and was used as a negative control in this study. The FACS profiles were similar for each donor tested and one of which is shown in Figure 8.

4.2 Granulocytes

To study the constitutive expression of M6 molecule on granulocytes, the granulocytes isolated from healthy donors were stained with anti-CD45, anti-CD14, 1B9 or 2G11 mAb. The granulocytes were then gated according to size and granularity during flow cytometric analysis. As shown in Table 4 and Figure 9, granulocytes from all donors tested showed weak expression of M6 molecule when stained with anti-M6 mAb from both 1B9 and 2G11 clones. However, they showed negative with myeloma induced ascitic fluid which was used as a negative control. To prove that these gated cells were granulocytes, they were stained with anti-CD45-FITC, which is react with CD45 molecules (common antigen) on every white blood cell, and also anti-CD14-PE which reacted with CD14 molecule on monocytes. The granulocyte population showed positive with anti-CD45 staining and negative with anti-CD14 staining. According to size and granularity and the staining pattern indicated that these gated cells were granulocytes without monocyte contamination. The staining reaction patterns were similar for each donor and one of which is demonstrated in Figure 9.

Table 4. Expression of M6 molecule on granulocytes

Sample number	Cell type	monoclonal antibody				Myeloma induced ascitic fluid
		anti-M6		anti-CD45	anti-CD14	
		1B9	2G11			
1	Granulocyte	w+ *	w+	+	-	-
2	Granulocyte	w+	w+	+	-	-
3	Granulocyte	w+	w+	+	-	-
4	Granulocyte	w+	w+	+	-	-

* w+ : weakly positive

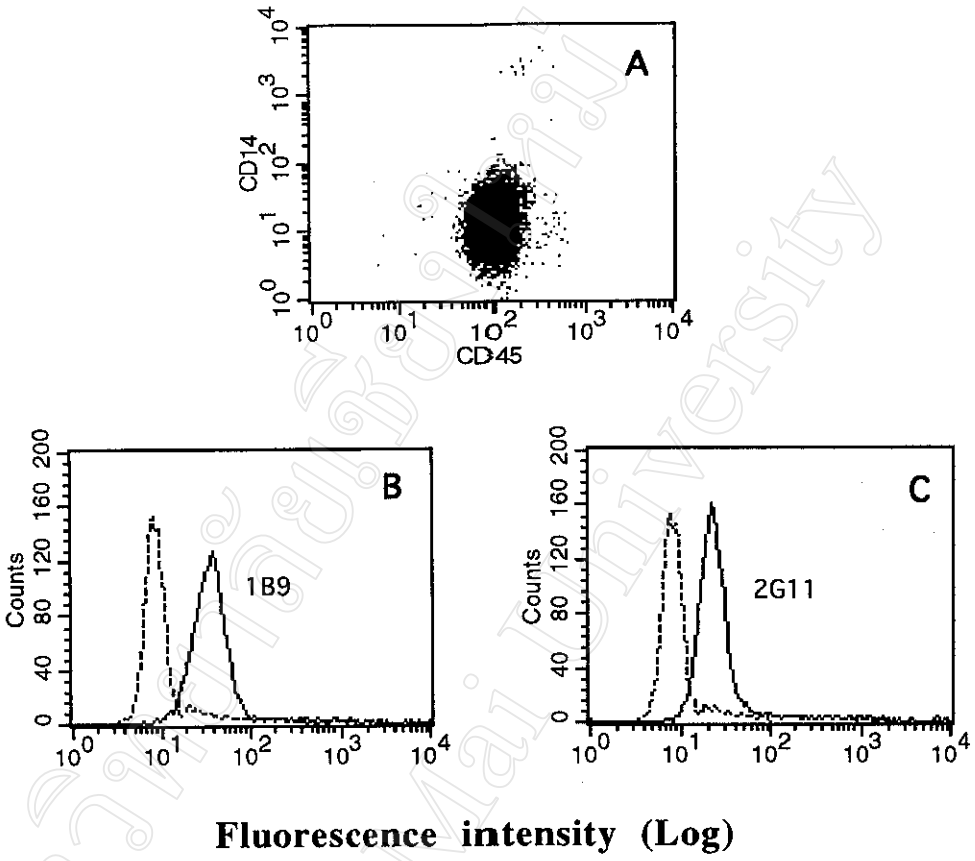


Figure 9. Expression of M6 molecule on granulocytes

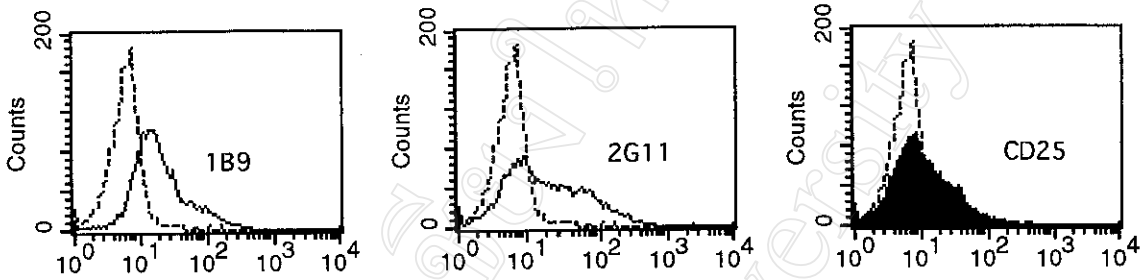
Immunofluorescence analysis of the reactivity of anti-M6 mAb 1B9 and 2G11 with freshly isolated granulocytes was carried out. The granulocytes were gated according to size and granularity, and fluorescent intensity was analyzed on a flow cytometer. The gated cells showed positive with anti-CD45-FITC staining and negative with anti-CD14-PE (A). Dashed lines represent the background fluorescence of negative control and solid lines represent the immunofluorescent profiles of cells stained with anti-M6 mAb 1B9 (B) and 2G11 (C).

5. Expression of M6 molecule on activated PBMC

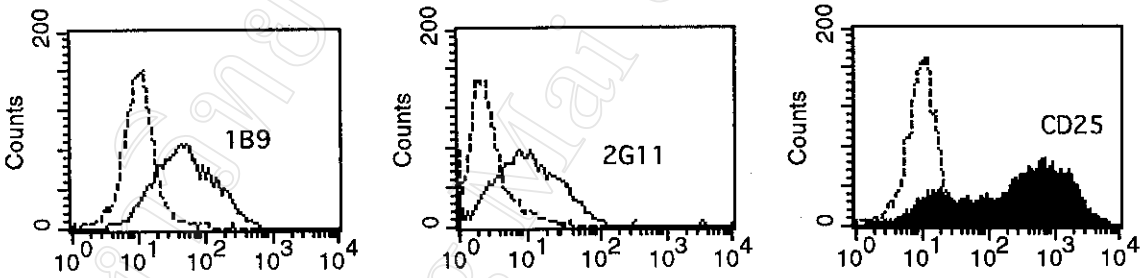
To study the possible induction of M6 molecule expression on the surface of mitogen or antigen activated PBMC, the PBMC isolated from healthy donors were cultured in the presence or absence of PHA or PPD. The activated PBMC were stained with anti-M6 mAb (1B9 and 2G11) and other controls mAb, i.e. anti-CD3 (OKT3), anti-CD25 (447), anti-CD14 (MEM 18) and myeloma induced ascitic fluid, followed by FITC-conjugated anti-mouse immunoglobulins. The stained cells were analyzed for immunofluorescent reaction with a flow cytometer.

In PHA activation, the expression of M6 molecule was increased on day 1 of PHA activation compared to freshly isolated lymphocytes (Figure 10). The expression, however, was up-regulated in day 3 of activation, which was as much as on day 5 of activation (Figure 10). The results indicated that the expression of M6 molecule on PHA activation was time dependent manner which has the maximum expression on day 3. In PPD activation, M6 molecule expression was slightly increased on day 5 of activation. On day 7, higher M6 expression was observed (Figure 11). In control staining, the activated lymphocytes were strongly positive with anti-CD25 (IL2 receptor), which was reported to express on activated lymphocytes. This result indicated that the activation system used in this study was successful. The lymphocyte population contained more than 90% $CD3^+$cells and very few $CD14^+$ cells, which demonstrated that most of the cells tested were T-lymphocytes. The reaction patterns of the mAb were similar for each experiment, one of which is demonstrated in Figures 10 and 11 for PHA activated PBMC and PPD activated PBMC, respectively.

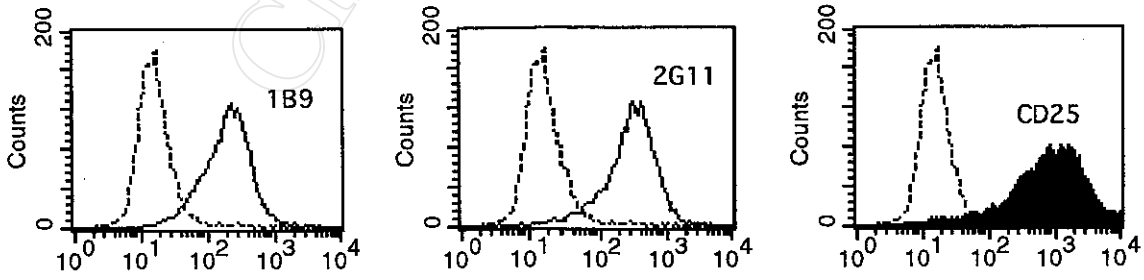
Fresh PBMC



Day 1



Day 3



Fluorescence intensity (Log)

Day 5

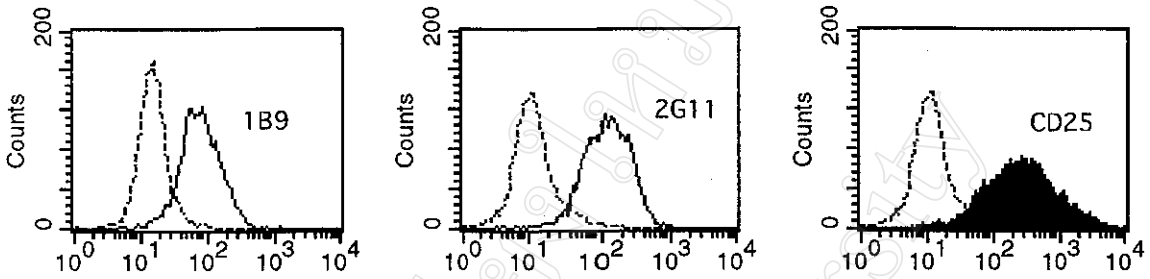


Figure 10. Expression of M6 molecule on PHA activated PBMC

PBMC were activated with PHA for 1, 3 and 5 days before staining with anti-M6 mAb. The fluorescent intensity was analyzed on a flow cytometer. Solid lines represent the immunofluorescent profiles of cells stained with indicated anti-M6 mAb 1B9 or 2G11; dashed lines represent the background fluorescence of negative control and shaded peaks represent the fluorescence of indicated positive control monoclonal antibody.

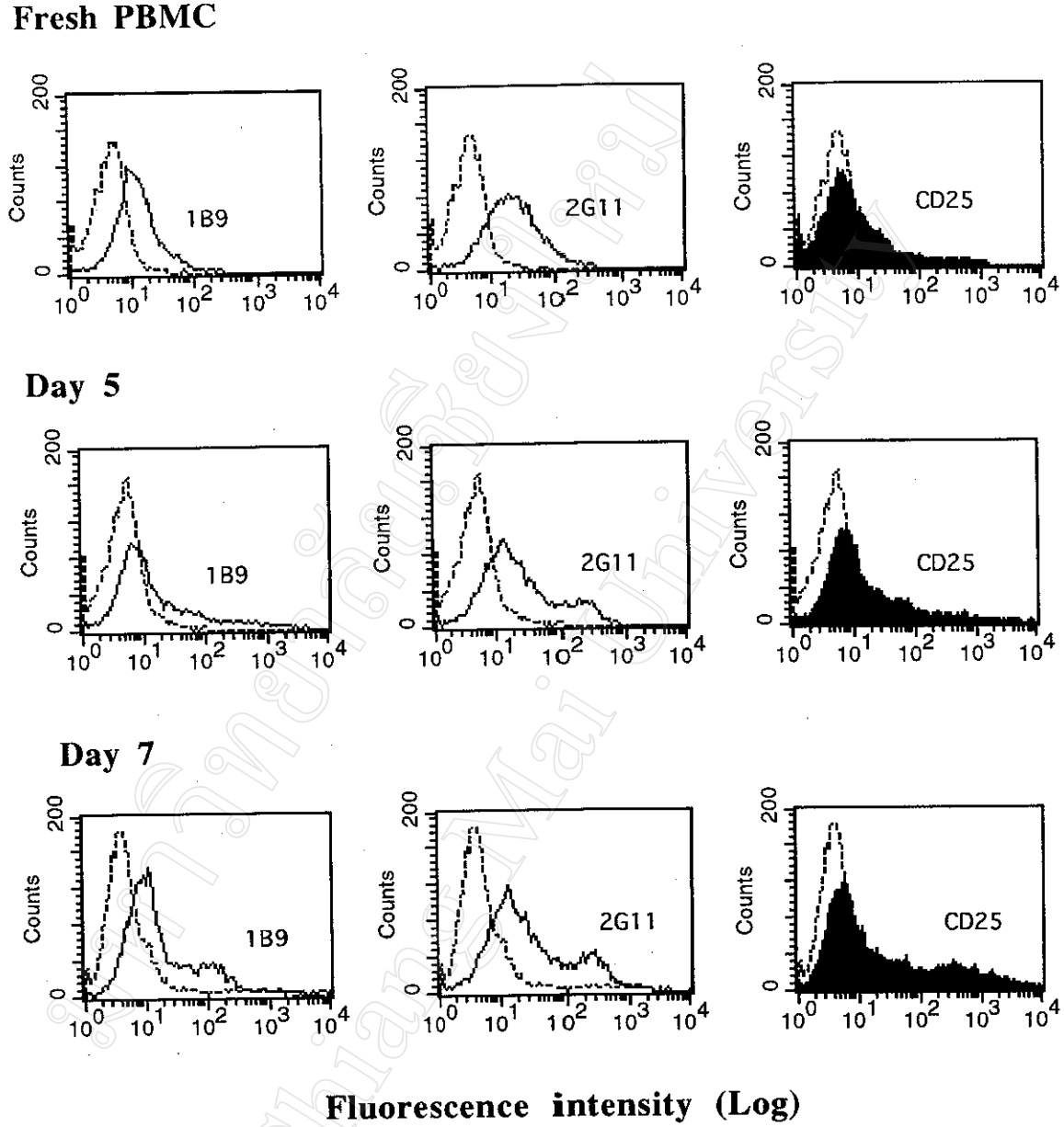


Figure 11. Expression of M6 molecule on PPD activated PBMC

PBMC were activated with PPD for 5 and 7 days before staining with anti-M6 mAb. The fluorescent intensity was analyzed on a flow cytometer. Solid lines represent the immunofluorescent profiles of cells stained with indicated anti-M6 mAb 1B9 or 2G11; dashed lines represent the background fluorescence of negative control and shaded peaks represent the fluorescence of indicated positive control monoclonal antibody.

6. The M6 molecule involved in cellular proliferation

6.1 Peripheral blood mononuclear cells (PBMC)

Result from previous experiments indicated that M6 molecule expressed on both activated T-lymphocytes and haematopoietic cell lines. Therefore, it was interesting to investigate whether the M6 molecule might be somehow involved in cellular proliferation. A study was carried out to find whether M6 molecule might inhibit or enhance the proliferation of activated PBMC. The optimal concentration of mitogen used to activate cells were, therefore, firstly titrated. PBMC from 3 healthy donors were cultured in the presence or absence of various concentrations of PHA. As shown in Figures 12, the suboptimal concentration of PHA were 0.03125 μg/ml. These concentration were used in all subsequent experiments.

The function of M6 molecule on activated PBMC was then studied. PBMC from three normal donors were stimulated in the presence or absence of anti-M6 mAb and PHA (0.03125 μg/ml). After 3 days activation, [^{3}H]thymidine incorporation was determined. As shown in Table 5 and Figure 13, both anti-M6 mAb, 1B9 and 2G11, at dilution 1:100, 1:200 and 1:400 inhibited the proliferation of PHA activated PBMC of all sample tested with the percentage of proliferation inhibition range from 50-10 %. The myeloma induced ascitic fluid, which was used as a negative control, at similar concentrations of anti-M6 mAb had a very slight inhibitory effect. The 2G11 mAb showed slightly more inhibitory effect than 1B9 mAb, as shown in Table 5. Statistic analysis indicated that there was a significant inhibition activity of both anti-M6 mAb at a dilution of 1:100 and 1:200 ($p < 0.05$; t test), however, there was no significant inhibition activity at a dilution of 1:400 ($p>0.05$; t test).

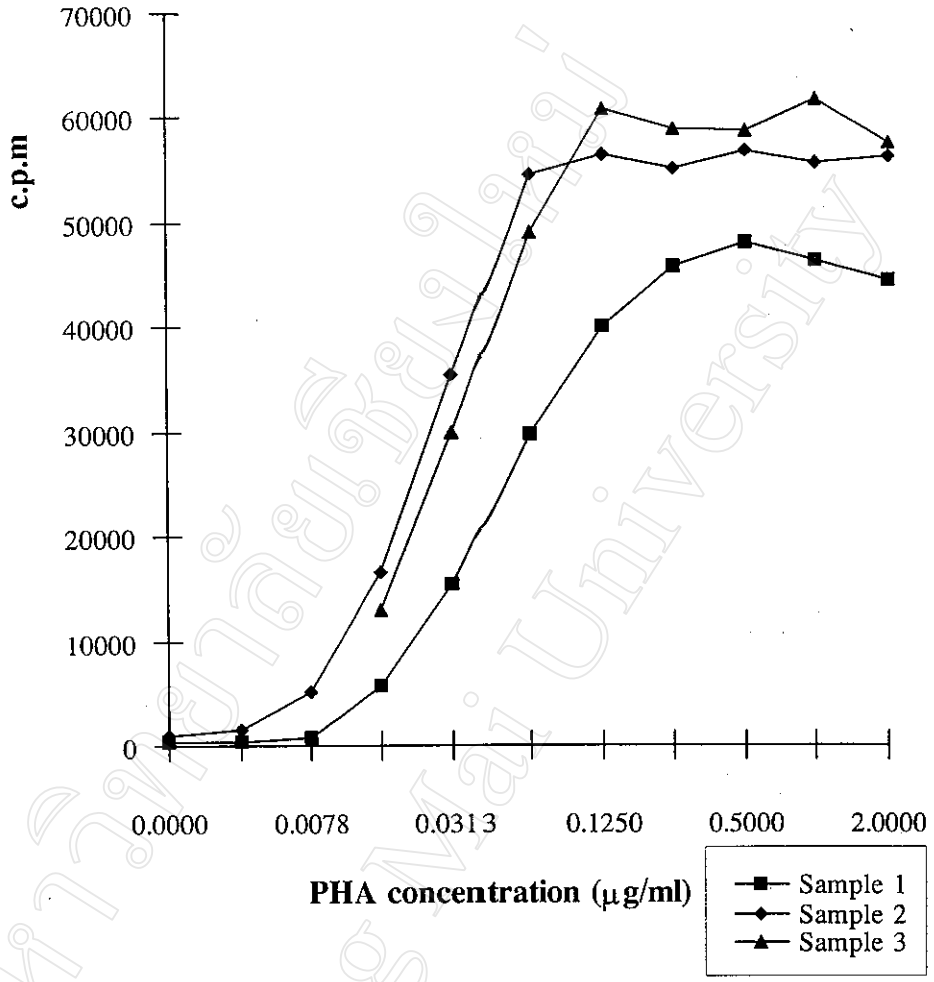


Figure 12. Determination of the suboptimal concentration of PHA for PBMC stimulation

PBMC from 3 healthy donors were cultured for 3 days in the presence or absence of various concentrations of PHA (0-2 μg/ml). [^{3}H]thymidine (0.4 μCi/well) was pulsed for 18 hours before harvesting, and the radioactive incorporation was determined. Each point represents the mean counts per minute of incorporated [^{3}H]thymidine from triplicate cultures.

Table 5. Effect of anti-M6 mAb (1B9 and 2G11) on proliferation of PHA activated PBMC

Sample number	dilution of antibody	monoclonal antibody anti-M6		Myeloma induced ascitic fluid
		1B9	2G11	
1	0*	18020**	18020	18020
	1:400	16326 (9.41)#	14802 (17.86)	17614 (2.25)
	1:200	12532 (30.46)	10774 (40.21)	17245 (4.30)
	1:100	12381 (31.29)	9685 (46.25)	18291 (-1.50)
2	0	11879	11879	11879
	1:400	9625 (18.97)	10215 (14.01)	11128 (6.32)
	1:200	8280 (30.30)	8252 (30.53)	11236 (5.41)
	1:100	8189 (31.06)	8199 (30.98)	11939 (-0.51)
3	0	14496	14496	14496
	1:400	8449 (41.71)	8293 (42.79)	14754 (-1.78)
	1:200	6796 (53.11)	7180 (50.47)	13859 (4.39)
	1:100	6963 (51.96)	7211 (50.25)	14062 (2.99)

* without anti-M6 mAb

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

\# percentage of proliferation inhibition

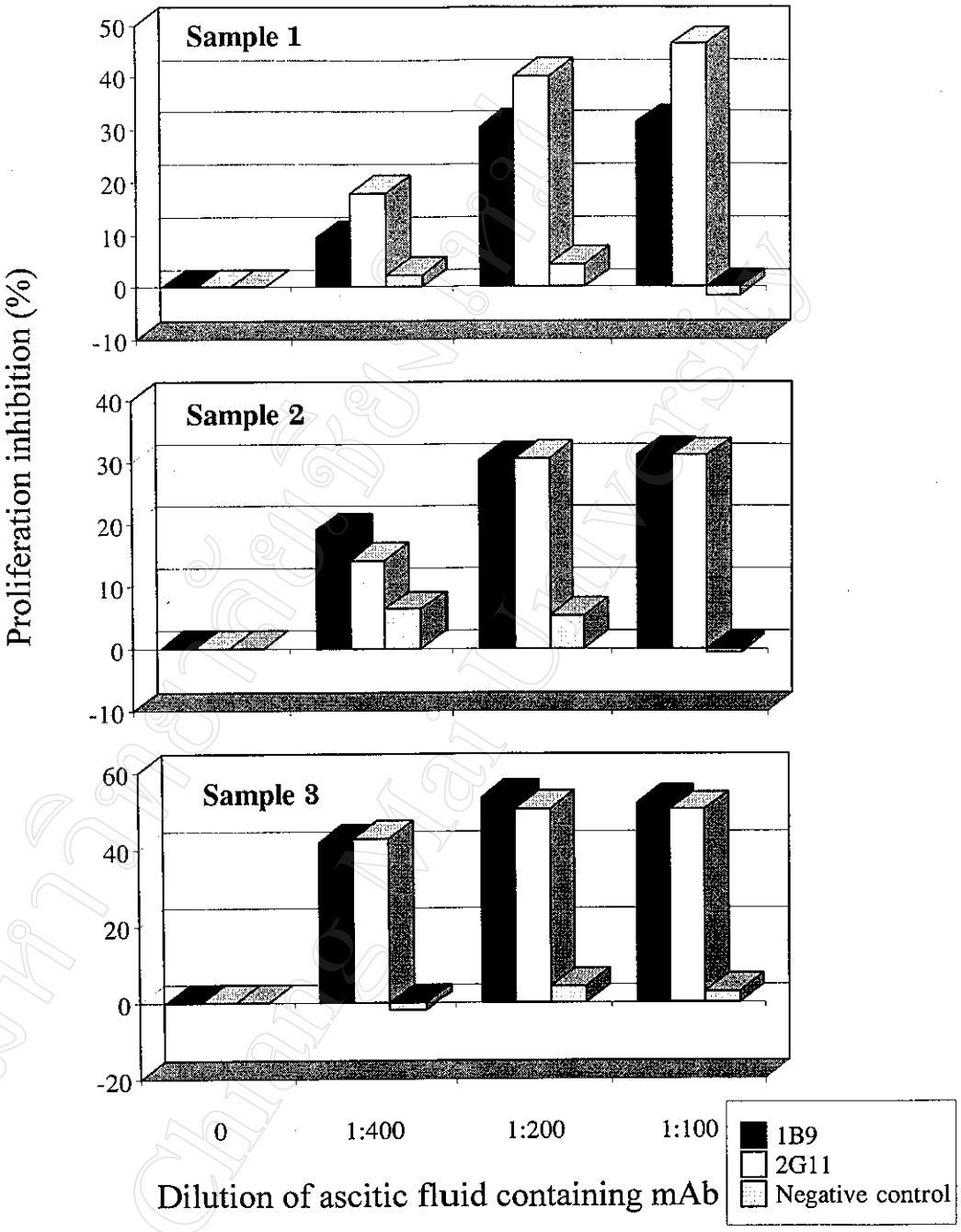


Figure 13. Inhibitory effect of anti-M6 mAb on the proliferation of PHA activated PBMC

PHA activated PBMC were cultured in the presence or absence of 0.03125 μg/ml of PHA and various concentrations of ascitic fluid containing anti-M6 mAb 1B9 or 2G11 (dilution 1:400-1:100) for 3 days and 0.4 μCi/well of [^{3}H]thymidine for 18 hours. The radioactive incorporation was determined and expressed in term of percent inhibition.

6.2 Haematopoietic cell lines

In addition, the proliferation function of M6 molecule on cell lines were studied. The haematopoietic cell lines K-562 and Molt-4, which express M6 molecule on their surface, were used for this study. To investigate the effect of time and cell concentration, K-562 cell line was cultured at the various cell concentration in the presence or absence of various concentrations of anti-M6 mAb and [^{3}H]thymidine at the incubation time of 3 and 5 hours. After each period, cells were harvested and the [^{3}H]thymidine uptake was compared between each condition. At the cell concentration of $1x10^5$ cells/ml and 5 hours-incubation time in the presence of anti-M6 mAb, the inhibition effect of anti-M6 mAb was higher than the other conditions (Table 6). The concentration of $1x10^5$ cells/ml and the incubation time of 5 hours were used in all subsequent experiments.

K-562 and Molt-4 were cultured individually in 96-well plates; in the presence or absence of anti-M6 mAb at a dilution of 1:100, 1:200 and 1:400 with a final cell concentration of $1x10^5$ cells/ml, together with [^{3}H] thymidine (0.2 μCi/well); and cultured for 5 hours. As shown in Table 7 and Figure 14 for K-562, and Table 8 and Figure 15 for Molt-4, both 1B9 and 2G11 have inhibition effect on the proliferation of both cell lines. The inhibition effect obtained from 1B9 mAb was slighly higher than those obtained from 2G11 mAb. In myeloma induce ascites control, very low inhibition effect on the proliferation of both cell lines were observed.

Table 6. Effects of incubation times and concentrations of the K-562 cell line in the proliferation assay

Condition		Antibody dilution	Anti-M6 mAb	
Cell concentration (cells/ml)	Incubation time (hours)		1B9	2G11
$1x10^5$	3	0*	2034**	2034
		1:200	2083 (-2.41)#	2012 (1.08)
		1:100	2109 (-3.69)	2031 (0.15)
$1x10^5$	5	0	5450	5450
		1:200	3620 (33.57)	3659 (32.86)
		1:100	3523 (35.36)	3335 (38.81)
$1x10^6$	3	0	5958	5958
		1:200	5842 (1.95)	5242 (12.02)
		1:100	5505 (7.60)	6418 (-7.72)
$1x10^6$	5	0	20873	20873
		1:200	18236 (12.63)	18787 (9.99)
		1:100	17225 (17.47)	19203 (8.00)

* without anti-M6 mAb
** mean of counts per minute (c.p.m) from triplicate culture
# percentage of proliferation inhibition

Table 7. Inhibitory effect of anti-M6 mAb on the proliferation of K-562 cell line

Antibody dilution	anti-M6 mAb		Myeloma induced ascitic fluid
	1B9	2G11	
0*	14590**	14590	14590
1:400	13687 (6.19)#	14220 (2.54)	15263 (-4.61)
1:200	12440 (14.74)	13403 (8.14)	14322 (1.84)
1:100	9273 (36.44)	12679 (13.09)	13753 (5.74)

* without anti-M6 mAb
** mean of counts per minute (c.p.m) from triplicate culture
# percentage of proliferation inhibition

Table 8. Inhibitory effect of anti-M6 mAb on the proliferation of Molt-4 cell line

Antibody dilution	anti-M6 mAb		Myeloma induced ascitic fluid
	1B9	2G11	
0*	9002**	9002	9002
1:400	8098 (10.04)#	8642 (3.99)	9347 (-3.83)
1:200	7264 (19.31)	7522 (16.44)	9138 (-1.51)
1:100	5947 (33.94)	6533 (27.43)	8794 (2.31)

* without anti-M6 mAb
** mean of counts per minute (c.p.m) from triplicate culture
# percentage of proliferation inhibition

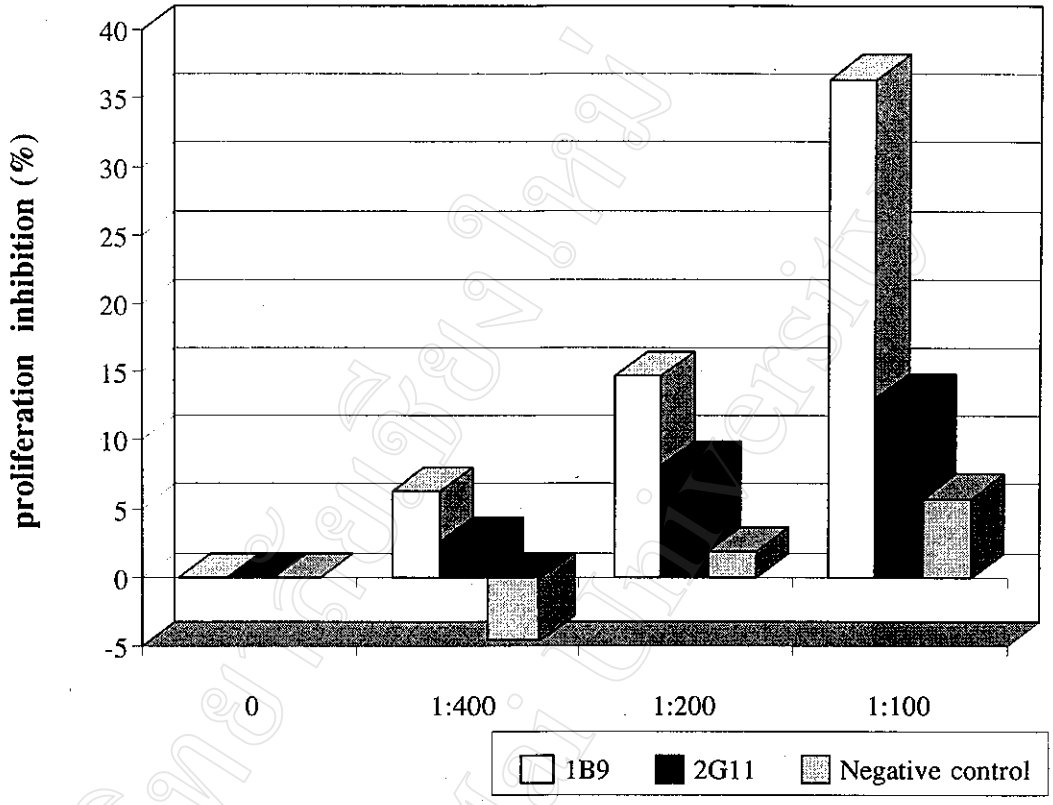


Figure 14. Inhibitory effect of anti-M6 mAb on the proliferation of K-562 cell line at 5 hours incubation

K-562, at the final concentration of $1x10^5$ cells/ml, were cultured in the presence or absence of various concentrations of ascitic fluid containing anti-M6 mAb 1B9 or 2G11 (dilution 1:100-1:400) and [^{3}H]thymidine (0.2 μCi/well) for 5 hours. The radioactive incorporation was determined and calculated for the percentage of proliferation inhibition.

Figure 15. Inhibitory effect of anti-M6 mAb on the proliferation of Molt-4 cell line at 5 hours incubation

Molt-4, at the final concentration of $1x10^5$ cells/ml, were cultured in the presence or absence of various concentrations of ascitic fluid containing anti-M6 mAb 1B9 or 2G11 (dilution 1:100-1:400) and [^{3}H]thymidine (0.2 μCi/well) for 5 hours. The radioactive incorporation was determined and calculated for the percentage of proliferation inhibition.