CHAPTER III

RESULTS

Part 1. Development of conditioned medium for hybridoma production

3.1 Generation of non-mitogen containing conditioned medium

3.1.1 Production and utilization of non-mitogen containing conditioned medium on hybridoma single cell cloning

In order to produce a conditioned medium for supporting of hybridoma growth, in this study, BW5147 mouse thymoma cell line was used as the conditioned medium source. BW5147 cells were cultured in the absence of any mitogen. The conditioned culture supernatants, named BW conditioned medium, were collected and tested for their ability to support hybridoma growth.

In the initiation phase, we determined whether the conditioned medium produced, BW conditioned media, could be used to support single-cell growth during hybridoma cloning. In this experiment, a stable hybridoma clone producing anti-hemoglobin mAb, Thal N/B, was used as a study model. The Thal N/B hybridoma cells were subjected to single cell cloning using basal medium (IMDM consisting of 10% FBS) supplemented with BW conditioned media obtained from 18-h and 40-h BW5147 cultivation and compared to the commercial conditioned medium BM-Condimed H1, or to no supplement. The numbers of well containing hybridoma single clone and clone size were determined at day 5, 7, 10, and 14 of cultivation. As shown in Table 3.1, in three individual experiments, the size of the hybridoma

single clone using BW conditioned media and BM-Condimed H1 supplementations were scored range from small to large, in relative to the period of cultivation. At day 14, more than 50% of the growing cells were graded as medium to large size. In contrast, without conditioned medium supplement the generated hybridomas were almost all arrested at the small size (Table 3.1). These results indicated that the BW conditioned medium could support single-cell growth during hybridoma single cell cloning as same as using the commercial BM-Condimed H1. At day 10 and day 14, however, the medium and large clone size was trended to be appeared in using of BM-Condimed H1 supplement compared to the BW conditioned medium (Table 3.1). Nevertheless, by using the produced BW conditioned medium, several single cells still could be achieved.

In comparing the BW conditioned media obtained from 18-h and 40-h of BW5147 cultivation, the 40-h BW conditioned medium provided slightly better growth of hybridomas than the 18-h conditioned medium (Table 3.1.). Using 40-h conditioned medium, more clones having medium and large clone size were observed compared to those using 18-h conditioned medium. At day 14 of cell cloning, the number of wells containing hybridoma single clone obtained by the generated BW conditioned medium at 40 hours and commercial BM-Condimed H1 were not significantly different (Table 3.1.). Our results suggest that the BW conditioned medium can be used as a supplement for hybridoma single cell cloning.

Table 3.1 Supporting of stable hybridoma single-cell growth by BW conditioned medium

Condition	**Size (Day5)			**Size (Day7)			**Size (Day10)			**Size (Day14)			***Single clone (day 14)
	S	M	L	S	M	L	S	M	L	S	M	L	
Exp. 1													
Medium only	30	-	-	39	-	-	26	-	-	21	2	-	23
BW conditioned medium (18 h)*	34	-	-	33	-	-	29	2	-	15	15	-	30
BW conditioned medium (40 h)*	25	-	-	25	-	-	19	6	-	3	16	6	25
BM-Condimed H1	26	-	-	28	-	-	15	11	-	2	13	11	26
Exp. 2													
Medium only	22	-	-	24	-	-	18	-	-	10	3	-	13
BW conditioned medium (18 h)*	22	-	-	22	-	-	17	2	-	11	6	1	18
BW conditioned medium (40 h)*	30	-	-	32	-	-	27	4	-	12	14	1	27
BM-Condimed H1	26	-	-	25	1	-	19	6	-	3	14	6	23
Exp. 3													
Medium only	29	-	-	29	-	-	29	-	-	28	1	-	29
BW conditioned medium (18 h)*	25	-	-	25	-	-	19	6	-	11	8	6	25
BW conditioned medium (40 h)*	24	-	-	24	-	-	12	12	-	3	17	4	24
BM-Condimed H1	40	-	-	40	-	-	15	22	3	8	16	15	39

A stable hybridoma clone Thal N/B was subjected to single cell cloning by limiting dilution using 10%FBS-IMDM supplemented with 50% BW conditioned medium or 10% BM-Condimed H1 or no supplement. Three independent experiments were performed. The clone size and number of hybridoma containing wells were determined at day 5, 7, 10, and 14 of cultivation by an inverted microscope.

*BW5147 thymoma cell line was cultured for 18 hours (18 h) or 40 hours (40 h). The culture supernatants were harvested and used as conditioned medium.

**Number of wells containing hybridoma single clone at various clone sizes. The size of a hybridoma single clone was scored as described in materials and methods. S; small, M; medium, L; large

*** Number of wells containing hybridoma single clone at day 14 of cultivation. There was no statistic different of wells containing hybridoma single clone at day 14 compare between BW conditioned medium (40 h) and BM-Condimed H1 by using Mann-Whitney U test T test (P=0.83).

We further determined the optimal concentration of BW conditioned medium for supporting hybridoma growth. The Thal N/B hybridoma cells were subjected to single cell cloning using various concentrations of 40-h BW conditioned medium. The 10%, 20% or 50% BW conditioned medium supplementation showed no much different in obtaining the single cells upon limiting dilution procedure (Table 3.2). BW conditioned medium at 20%, however, showed slight increase in the number single cell obtained. We, therefore, hand-picked 20% supplement as the appropriate dilution.

As the experiments described above were carried out using the stable hybridoma clone, we further confirmed whether BW conditioned medium could support the growth of newly generated hybridomas as well. A hybridoma clone named MT3, which was generated immediately after cell fusion, was subjected to single cell cloning using BW conditioned medium. BW conditioned medium could support the growth newly generated hybridoma. At day 14, the majority of the hybridoma clones scored medium to large in size (Table 3.3). The 20% BW conditioned medium supplementation was confirmed to be the optimal concentration. Taken together, our results indicated that BW conditioned medium can be used to support the growth of both stable and newly formed hybridomas. The BW conditioned medium can be employed in hybridoma single cell cloning in the hybridoma technique.

We then explored the question of whether the BW conditioned medium does have effect on the antibody production of the grown hybridomas. To address this question, after single cell cloning, culture supernatants of the obtained hybridoma MT3 using BW conditioned medium and commercial BM-Condimed H1 were

evaluated for antibody reactivity. Culture supernatants obtained from both conditioned media showed the same positivity patterns (Figure 3.1). The results indicated that the produced BW conditioned medium could be used to support the growth of hybridomas without altering the antibody production.

3.1.2 Employment of the BW conditioned medium in the hybridoma technique for production of monoclonal antibodies

Since the BW conditioned medium was demonstrated to support the growth of hybridomas, we studied the possibility of using the generated BW conditioned medium on hybridoma technique for production of mAbs. We studied the production of mAbs to 4 different antigens, including Hb A_2, platelet, CD99 and sugarcane crude leaf extract. Spleen cells from the antigen immunized mice were fused with myeloma cells. After cell fusion, HAT and HT medium prepared using BW conditioned medium or BM-Condimed H1 as supplements, were separately added to the fused cells. Cells were then spread into 96-well plates. The numbers of hybridoma containing wells obtained from both conditioned medium supplementations were determined. As shown in Figure 3.2A, for all antigens used, the % hybridoma containing wells obtained in BW conditioned medium and BM-Condimed H1 supplements were not different. Culture supernatants from hybridoma containing wells were screened for antibody activity. Hybridomas producing mAb of interest could be obtained using BW conditioned medium in amounts not different from the commercial BM-Condimed H1 (Figure 3.2B). It is important to note that in the post-fusion hybridoma wells, fibroblasts were markedly reduced when using BW conditioned medium compared to BM-Condimed H1. This is very advantageous as BW conditioned medium reduces the chance of fibroblast overgrowth the generated

hybridomas. We, therefore, concluded that the BW conditioned medium could be used as supplement for generation of hybridomas for mAb production.

Table 3.2 Supporting of hybridoma single-cell growth by various concentrations of BW conditioned medium

Condition	**Size (Day5)			**Size (Day7)			**Size (Day10)			**Size (Day14)			***Single clone (day 14)
	S	M	L	S	M	L	S	M	L	S	M	L	
10% BW conditioned medium*	25	-	-	28	-	-	23	8		11	15	4	30
20% BW conditioned medium*	34	-	-	34	-	-	17	18	2	11	10	13	34
50% BW conditioned medium*	25	-	-	26	-	-	12	12	3	4	18	5	27

A stable hybridoma clone Thal N/B was subjected to single cell cloning by limiting dilution using 10%FBS-IMDM supplemented with 10%, 20% or 50% BW conditioned medium. The clone size and number of hybridoma containing wells were determined at day 5, 7, 10, and 14 of cultivation by an inverted microscope.

*BW5147 thymoma cell line was cultured for 40 hours. The culture supernatants were harvested and used as conditioned medium.

**Number of wells containing hybridoma single clone at various clone sizes. The size of a hybridoma single clone was scored as described in materials and methods. S; small, M; medium, L; large

*** Number of wells containing hybridoma single clone at day 14 of cultivation.

Table 3.3 Supporting of newly formed hybridoma single cell growth by various concentrations of BW conditioned medium

Condition	**Size (Day5)			**Size (Day7)			**Size (Day10)			**Size (Day14)			***Single clone (day 14)
	S	M	L	S	M	L	S	M	L	S	M	L	
10% BW conditioned medium*	26	-	-	26	-	-	22	5	-	11	6	8	25
20% BW conditioned medium*	25	-	-	25	-	-	21	4	-	5	15	2	22
50% BW conditioned medium*	22	-	-	22	-	-	18	5	-	7	13	1	21

A newly formed hybridoma clone MT3 was subjected to single cell cloning by limiting dilution using 10%FBS-IMDM supplemented with 10%, 20% or 50% BW conditioned medium. The clone size and number of hybridoma containing wells were determined at day 5, 7, 10, and 14 of cultivation by an inverted microscope.

*BW5147 thymoma cell line was cultured for 40 hours. The culture supernatants were harvested and used as conditioned medium.

**Number of wells containing hybridoma single clone at various clone sizes. The size of a hybridoma single clone was scored as described in materials and methods. S; small, M; medium, L; large

*** Number of wells containing hybridoma single clone at day 14 of cultivation.

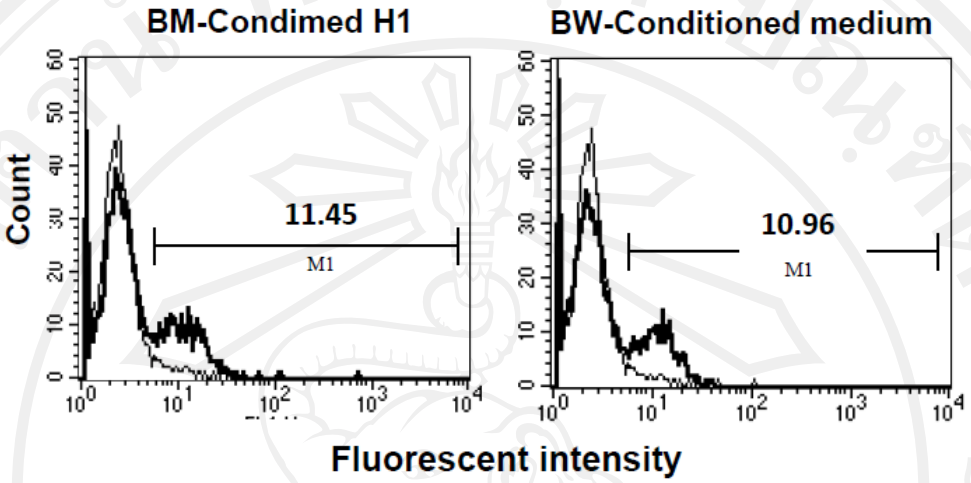


Figure 3.1 Activity of monoclonal antibody produced by hybridomas supplemented with BW conditioned medium and BM-Condimed H1. Culture supernatants from single cell cloning of hybridoma clone MT3 using 10% FBS-IMDM supplemented with BW conditioned medium or with BM-Condimed H1 were determined for antibody activity by lysed whole blood immunofluorescence staining. Thick lines represent the immunofluorescence profiles of the cells stained with hybridoma culture supernatants obtained from the indicated conditioned media. Thin lines represent background fluorescence of conjugate control. The mean fluorescent intensity (MFI) of the positive cells are indicated.

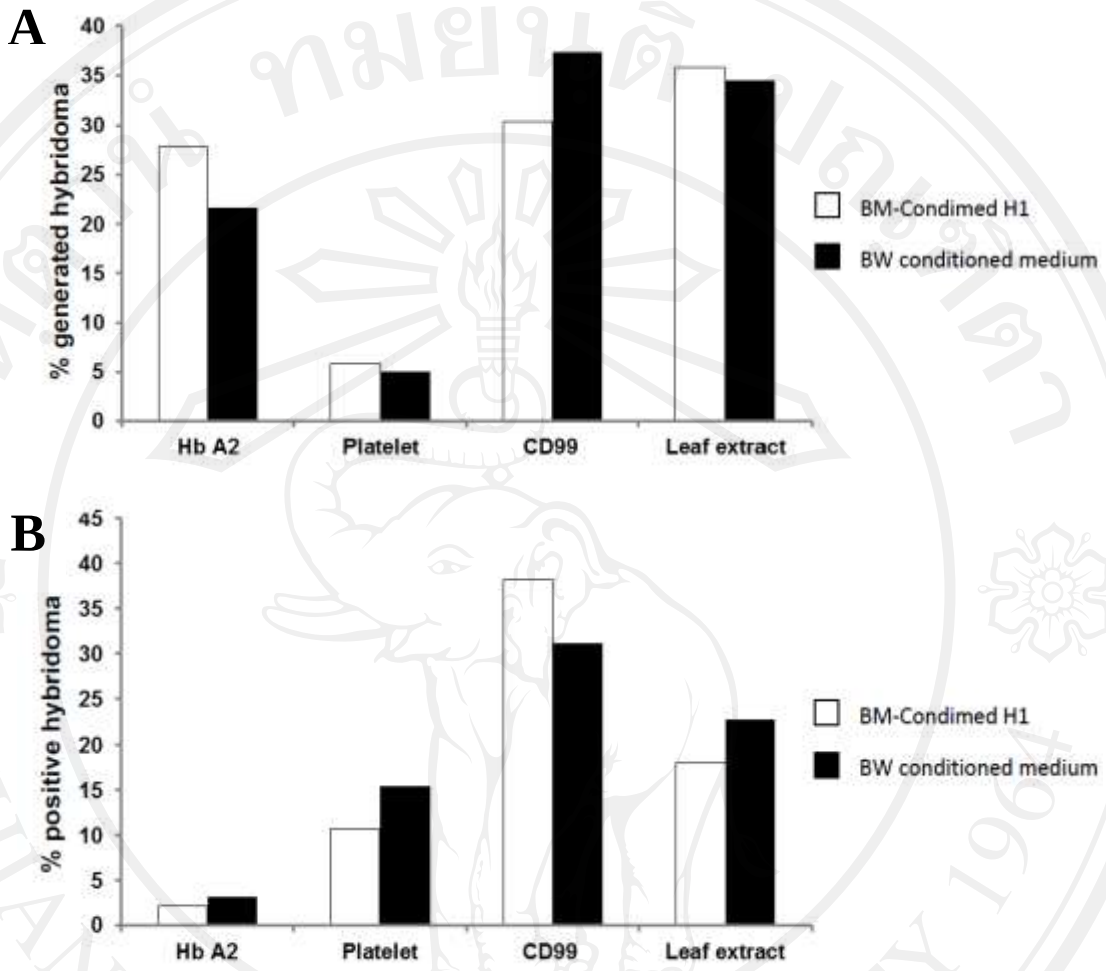


Figure 3.2 Comparison of BW conditioned medium and BM-Condimed H1 in the generation of hybridomas from mice immunized with Hb A_2, platelets, CD99 and sugarcane crude leaf extract. Total numbers of hybridoma containing wells were determined and converted to % hybridoma containing wells (A). Supernatants of the generated hybridomas were screened for the specific antibody. The numbers of antibody positive wells were determined and converted to % antibody positive wells (B).

3.2 Generation of mitogen (PMA) induced conditioned medium

3.2.1 Production and utilization of PMA-induced conditioned medium for hybridoma single cell cloning

From our results described above, a conditioned medium for hybridoma production was generated by culturing BW5147 cells in the absence of any stimulation. However, an effective conditioned medium was described by stimulation a mouse thymoma cell line EL4 with PMA (Grabstein *et al.* 1986) We, therefore, conducted a set of experiment for production of PMA-induced conditioned medium. BW 5147 thymoma cell line was cultured with 20 ng/ml PMA for 40 hours. The conditioned mediums were harvested (named PMA-induced conditioned medium) and tested for their ability in support hybridoma growth in hybridoma single cell cloning. In this experiment, two hybridoma clones MT99/2 and Thal M/B were used as the study model. The hybridoma clones were subjected for single cell cloning using 10% FBS-IMDM, 10% FBS-IMDM supplemented with 10% commercial BM-Condimed H1, 10% FBS-IMDM supplemented with 20% PMA-induced conditioned medium, and 10% FBS-IMDM supplemented with 20% BW conditioned medium (without PMA stimulation). The number and colony size of single hybridoma clones were determined after 7, 10, 13, 15, 18 and 20 days of cultivation. As shown in Table 3.4, at days 10-15, in either PMA-induced conditioned medium or BM-Condimed H1, the colony size of the growth hybridomas were scored from very small to medium. Whereas, in using of medium alone or supplement with BW conditioned medium (without PMA stimulation) (Table 3.4A), the colony size of hybridomas were scored very small to small. The results indicated that with PMA activation, the conditioned medium obtained provided more rapid growth of hybridoma cells. At days 18-20,

using PMA-induced conditioned medium or BM-Condimed H1, the hybridoma single clones developed to medium and large size. The growing of the hybridoma clones using PMA-induced conditioned medium and BM Condimed H1 were not obvious different (Table 3.4). Moreover, as shown in Table 3.4, PMA-induced conditioned medium lot.1 and lot.2 which were separately produced showed almost the same results. This result indicated that the production of PMA-induced conditioned medium is reproducible.

These results indicated that supernatants obtained from PMA stimulated BW5147 cells could be effectively used in hybridoma single cell cloning, with no different from using of the expensive commercial conditioned medium.

3.2.2 Employment of PMA-induced conditioned medium in the hybridoma technique for production of monoclonal antibodies

Since the PMA-induced conditioned medium was demonstrated to provide growth factors that promote growth of hybridomas in single cell cloning. We, therefore, determined whether this conditioned medium can be used as supplement for generation of hybridomas in hybridoma technique. To verify this postulation, generation of hybridomas secreting anti-Ag85B and anti-Hb E monoclonal antibodies were used as study models.

3.2.2.1 Mice immunization and antibody responses

BALB/c mice were immunized with recombinant Ag85 protein (Ag85B-BCCP) and Hb E. After the third immunization, the antibody responses were determined by indirect ELISA technique. The antibodies titers of Ag85B and Hb E in the immunized mice sera were pretty high after the third immunization (Figure 3.3A

and 3.3B, respectively). Both mice were, therefore, ready for further used for monoclonal antibody production.

Table 3.4 Supporting of hybridoma single-cell growth by PMA-induced conditioned medium

A: Hybridoma clone: MT99/2

Conditioned medium	Size (day 7)				Size (day 10)				Size (day 13)				Size (day 15)				Size (day 18)				Size (day 20)			
	VS	S	M	L	VS	S	M	L	VS	S	M	L	VS	S	M	L	VS	S	M	L	VS	S	M	L
10% FBS-IMDM	24				23				24				22	2			19	5			19	1	4	
BM-Condimed H1	31				19	6	6		10	8	12		5	1	15		3	2	15	1	2	2	16	1
PMA-BW lot 1	27				14	11	2		11	6	9	1	6	4	14	3	4	3	16	4	4	2	16	5
PMA-BW lot 2	32				29	2	1		19	10	3		10	5	17		8	4	19	1	6	4	21	1
BW without PMA	26				25	1			21	4	1		14	5	7		11	6	9		9	5	12	

B: Hybridoma clone: Thal M/B

Thal M/B	Size (day 7)			Size (day 10)			Size (day 13)			Size (day 15)			Size (day 18)			Size (day 20)		
	VS	S	M	VS	S	M	VS	S	M	VS	S	M	VS	S	M	VS	S	M
10% FBS-IMDM	57			57			57			55	2		51	4	2	48	3	6
BM-Condimed H1	68			67	1		51	16	1	33	10	25	23	8	37	21	7	40
PMA-BW lot 1	57			57			55			52	2	1	44	3	8	37	5	13
PMA-BW lot 2	70			70			70			66	2	2	53	5	12	46	6	18

Results of MT99/2 and Thal M/B single cell cloning using 10%FBS-IMDM supplemented with or without the indicated condition medium.

Size of hybridoma single clone at the indicated days was scored as described in materials and methods. VS; very small, S; small, M; medium, and L; large.

10% FBS-IMDM=10% FBS-IMDM without supplement; BM-Condimed H1=10% FBS-IMDM+BM-Condimed H1 medium; PMA-BW lot 1=10% FBS-IMDM+PMA-induced conditioned medium lot no. 1; PMA-BW lot 1=10% FBS-IMDM+PMA-induced conditioned medium lot no. 2; BW without PMA=10% FBS-IMDM+ non-PMA-induced conditioned medium.

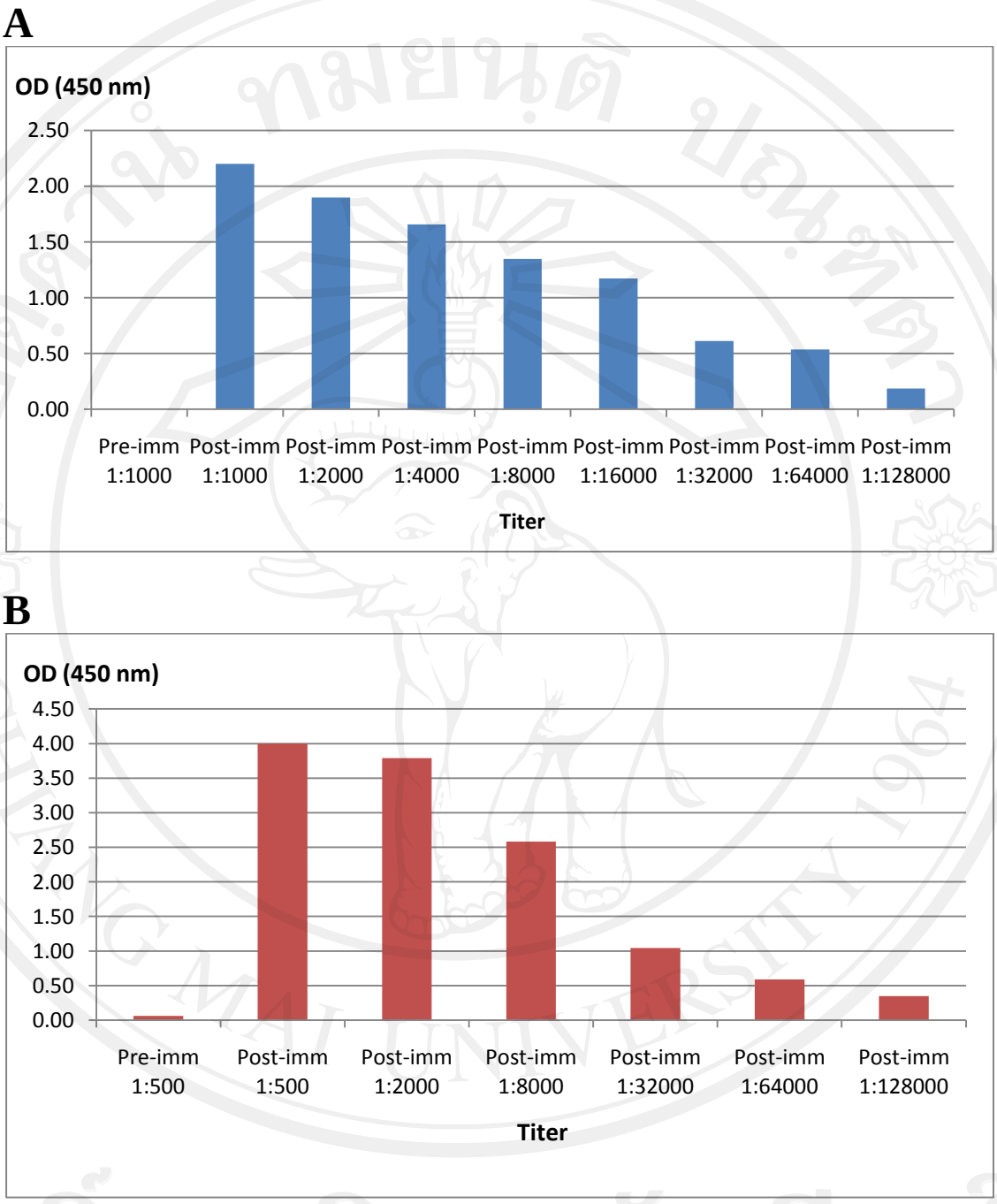


Figure 3.3 Antibody responses in BALB/c mice after immunizations with Ag85B-BCCP and Hb E. BALB/c mice were immunized with purified Ag85B-BCCP (A) or Hb E (B). Sera were collected at pre-immunization (pre-imm) and after the third immunization (post-imm) and determined for antibody to Ag85B protein and Hb E by indirect ELISA.

3.2.2.2 Hybridoma production

After the third immunization, spleen cells from the immunized mice were fused with myeloma cells using PEG as fusogen. In this study, after cell fusion, HAT and HT medium were prepared using 20% PMA-induced conditioned medium or 10% BM-Condimed H1 as supplement. Cells were resuspended in these HAT mediums and spread to 96-well plates for 5 plates. The numbers of hybridoma containing wells obtained from both conditioned medium supplementations were determined. The results were shown in Table 3.5.

In Ag85B-BCCP experiment, the total hydridoma containing wells obtained using BM-Condimed H1, PMA-induced conditioned medium or without supplement were 77, 82, and 23 wells, respectively. Culture supernatants from hybridoma containing wells were screened for the antibody activity by indirect ELISA. Total 6, 7 and 1 positive wells were obtained in using BM-Condimed H1, PMA-induced conditioned medium or without supplement, respectively. These results indicated that the produced PMA-induced conditioned medium could be used as supplement for generation of hybridoma in hybridoma technique.

In Hb E experiment, the total hydridoma containing wells obtained in BM-Condimed H1, PMA-induced conditioned medium or without supplement were 61, 89, and 65 wells, respectively. In this experiment, however, many fibroblasts were present in the fusion plate, especially in BM-Condimed H1 and PMA-induced conditioned medium. In the fusion using BM-Condimed H1 supplement, fibroblast overgrow were observed in almost every wells. Only 3 culture supernatants from hybridoma containing wells in BM-Condimed H1 supplement, therefore, were collected and screened for the antibody activity and 1 of those 3 wells showed

positive reactivity. The fibroblast overgrowth was lesser in using of PMA-induced conditioned medium. Thus, 50 culture supernatants from hybridoma containing wells were screened and 4 wells showed positive reactivity. In the fusion using medium without conditioned medium supplement, fewer fibroblasts were observed compare to BM-Condimed H1 and PMA-induced condition medium supplements. Total, 52 culture supernatants from hybridoma containing wells in without supplement condition were screened and 1 well showed positive reactivity (Table 3.5).

From our results, this is worthy to note that upon hybridoma production, by using conditioned medium contained mitogen, the presented mitogen will induce fibroblasts in the spleen cells to proliferate (Sivak *et al.* 1972; Goldberg *et al.* 1974; Estensen *et al.* 1978) and finally overgrow and kill the newly formed hybridomas. To overcome these problems, conditioned medium containing no mitogen, for supporting growth of freshly fused hybridomas and of single-cell during cloning, would be required. According to our results in section 3.1, the BW conditioned medium, a non-mitogen induced conditioned medium, was suggested to be the appropriate condition medium.

Table 3.5 Comparison of using PMA-induced conditioned medium and BM-Condimed H1 in generation of hybridomas producing anti-Ag85 and Hb E monoclonal antibodies

Fusion	Total[a]			Ab-screen[b]			Ab Positive[c]		
	BM	PMA-BW	None	BM	PMA-BW	None	BM	PMA-BW2	None
Ag85	77	82	23	77	71	23	6	7	1
Hb E	61	89	65	3	55	52	1	4	1

Mice was immunized with Ag85B-BCCP and Hb E for three immunizations and spleen cells were used to generate hybridomas using 20% PMA-induced conditioned medium (PMA-BW) and 10% BM-Condimed H1 (BM) supplement or no supplement (None).

[a] Total number of hybridoma containing wells.

[b] Number of hybridoma containing wells for antibody screening.

[c] Number of hybridoma positive wells.

3.2.2.3 Analysis of proteins in the BW conditioned medium, PMA-induced conditioned medium by sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The produced BW conditioned medium and PMA-induced conditioned medium, BM-Condimed H1, and 10% FBS-IMDM medium were separated by SDS-PAGE. After electrophoresis, the gels were stained with Coomassie Blue. The protein bands from all produced conditioned media, BM-Condimed H1 and 10% FBS-IMDM medium were not different (Figure 3.4). This may be because of that the produced conditioned media and the BM-Condimed H1 contained 10% fetal bovine serum. Therefore, the major protein appeared on the gel were serum proteins. From the SDS-PAGE results, we therefore could not conclude whether the produced conditioned media contains different or the same proteins compare to the commercial BM-Condimed H1.

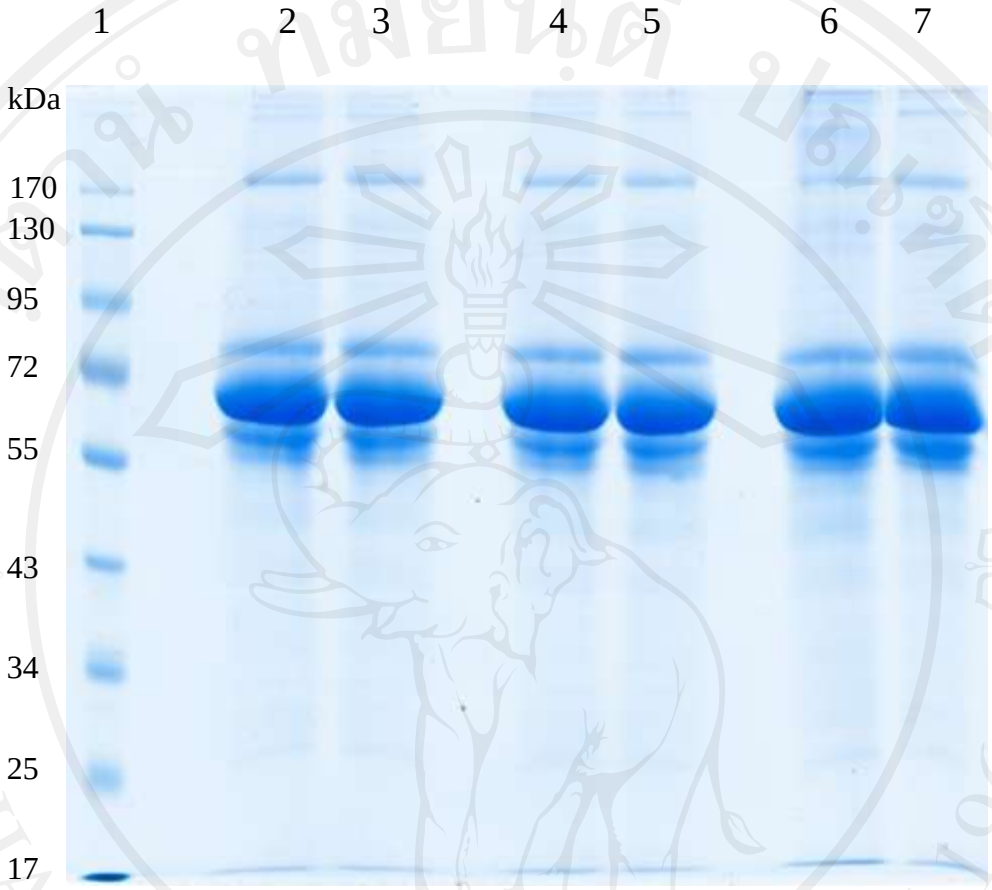


Figure 3.4 Proteins analysis of PMA-induced conditioned medium, BM-Condimed H1 and 10%FBS-IMDM medium using 10% SDS-PAGE. Lane 1: Standard protein markers (kDa), Lane 2: PMA-induced conditioned media lot 1, Lane 3: PMA-induced conditioned media lot 2, Lane 4: BW conditioned medium lot 1, Lane 5: BW conditioned medium lot 2, Lane 6: BM-Condimed H1, Lane 7: 10%FBS-IMDM medium.

Part 2. The development of high efficiency hybridoma technology for production of monoclonal antibody

3.3 Pre-B cell isolation strategy

In production of mAb using conventional hybridoma technique, antigen of interest is first immunized into mice. After having high antibody titer, spleen cells of the immunized mouse will be fused with myeloma cells to develop hybridoma producing mAb of interest. By this technique, as spleen cells contain several cell types, fusion between spleen cells and myeloma cells may occurred by chance. This will then reduce the opportunity of the fusion between B cells and myeloma cells, thus, reduce the efficacy of the hybridoma technique. In addition, the fibroblasts containing in the spleen cells will still be present after cell fusion and always overgrow during cell culturing. To increase the hybridoma technique efficiency, pre-isolation of B cells from other cells presented in the splenocytes and use, instead, to fuse with myeloma may increase the chance of obtaining hybridoma producing monoclonal antibody of interest. In this study, we aimed to develop the pre-B cell isolation strategy for hybridoma technique. We carried out the experiments by using the production of anti-Hb Bart's mAb as the study model.

3.3.1 Mice immunization and antibody responses

BALB/c mouse was immunized with Hb Bart's. After the third immunization, the antibody response was determined by indirect ELISA technique. The anti-Hb Bart's titers in the immunized mouse sera was more than 64,000 after the third immunization (Figure 3.5). Mouse was, therefore, used for further monoclonal antibody production using the pre-B cell isolation strategy.

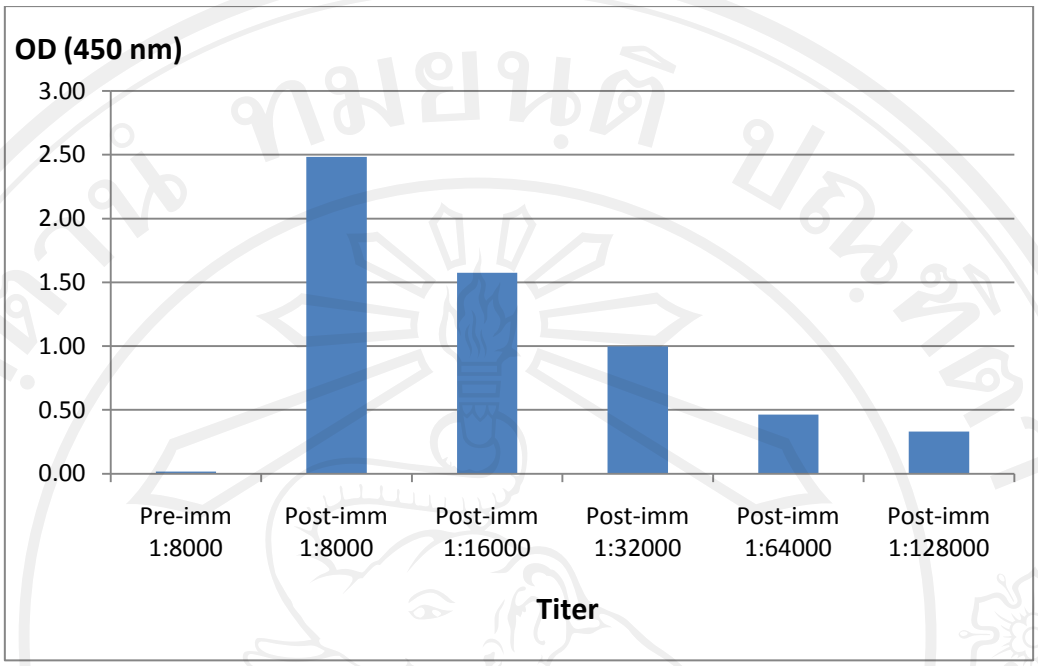


Figure 3.5 Antibody responses of a BALB/c mouse after immunizations with hemoglobin Bart's. A BALB/c mouse was immunized with 100 µg hemoglobin Bart's. Sera were collected at pre-immunization (pre-imm) and after the third immunization (post-imm) and determined for antibody to hemoglobin Bart's by indirect ELISA.

3.3.2 Hybridoma production

After the third immunization, as control, total spleen cells of the immunized mouse were fused with myeloma cells using standard hybridoma technique. A part of the spleen cells were subjected for isolation of B cells. Spleen cells expressing immunoglobulins were isolated using the MACS system. The immunoglobulin positive cell fractions (B cells) and negative cell fraction (non-B cells) were separately fused with myeloma cells for generation of hybridomas.

After cell fusions, the numbers of hybridoma containing wells obtained from total spleen cells fusion, B cell fusion and non-B cell fusion were determined. By using of total spleen cells (standard cell fusion method), cells were seeded into a total of 480 wells. After cultivation in HAT selective medium, 220 wells (45.8%) of the seeded wells contained hybridomas (Table 3.6). Total 132 culture supernatants were screened for their antibody activity. All 132 culture supernatants contained immunoglobulins and 15 out of 132 supernatants (11.4%) were specific to Hb Bart's (Table 3.6). In B cell fusion, cells were seeded into a total of 960 wells. After cultivation in HAT selective medium, 276 wells (28.8%) of the seeded wells contained hybridomas (Table 3.6). 192 culture supernatants were screened for their antibody activity. All 192 supernatants contained immunoglobulins and 16 supernatants (8.3%) were anti-Hb Bart's antibody (Table 3.6). By using non-B cells, cells were seeded into a total of 480 wells. After cultivation in HAT selective medium, 107 wells (22.3%) of the seeded wells contained hybridomas (Table 3.6). 87 culture supernatants were screened for their antibody activity and all of them were not produce antibody (0%) (Table 3.6). From this experiment, the result indicated that pre-isolated B cells can be used as cell fusion target and hybridoma producing

specific antibody can be generated. However, the hybridomas of interest were not increased compared to the conventional hybridoma method. Nevertheless, the fibroblasts in the seeded wells were pretty low in using B cells as fusion partner compare to the using of total spleen cells. This, therefore, is only the advantage of using pre-isolation B cell strategy.

To confirm whether B cell fusion was effectively used to produce mAbs, the pre-B cell isolation strategy was employed in several mAb productions. It was found that B cell fusion strategy was successfully used for production of mAbs against HDL, LDL, and TFF3 protein (Table 3.7).

Table 3.6 Comparison of Standard fusion method and pre-B cell isolation fusion strategy in generation of hybridomas produced anti-Hb Bart's monoclonal antibody

Fusion method	Seeded[a]	Total[b]	% of total[c]	Ab-screen[d]	% of Ab-screen[e]	Ab producing[f]	Ab specific[g]	% of Ab specific[h]
Standard method fusion	480	220	45.8	132	60.0	132	15	11.4
B cell isolation fusion	960	276	28.8	192	69.6	192	16	8.3
Negative cell fusion	480	107	22.3	85	79.4	0	0	0

Mouse was immunized with Hb Bart's for three immunizations and spleen cells were used to generate hybridomas using standard fusion method (total spleen cells) or B cell isolation fusion (B cells) and negative cell fusion (non-B cells).

[a] Total number of seeded wells.

[b] Total number of hybridoma containing wells.

[c] % of hybridoma containing wells.

[d] Number of hybridoma containing wells for antibody screening.

[e] % of hybridoma containing wells for antibody screening

[f] Number of wells containing hybridoma producing antibody.

[g] Total number of wells containing hybridoma producing specific antibody.

[h] % of hybridoma producing specific antibody containing wells.

Table 3.7 Using of pre-B cell isolation strategy in generation of hybridomas produce anti- LDL, HDL, and TFF3 monoclonal antibodies

Antigen	Seeded[a]	Total[b]	Ab-screen[c]	Ab specific[d]
HDL fusion	1056	471	322	50
LDL fusion	359	142	68	22
TFF3 Dimer fusion	1024	399	338	8

Mice were immunized with interested antigen for three immunizations and spleen cells were used to generate hybridomas using modified pre-B cell selection fusion method.

[a] Total number of seeded wells.

[b] Total number of hybridoma containing wells.

[c] Number of hybridoma containing wells for antibody screening.

[d] Number of hybridoma producing antibody against antigen containing wells.

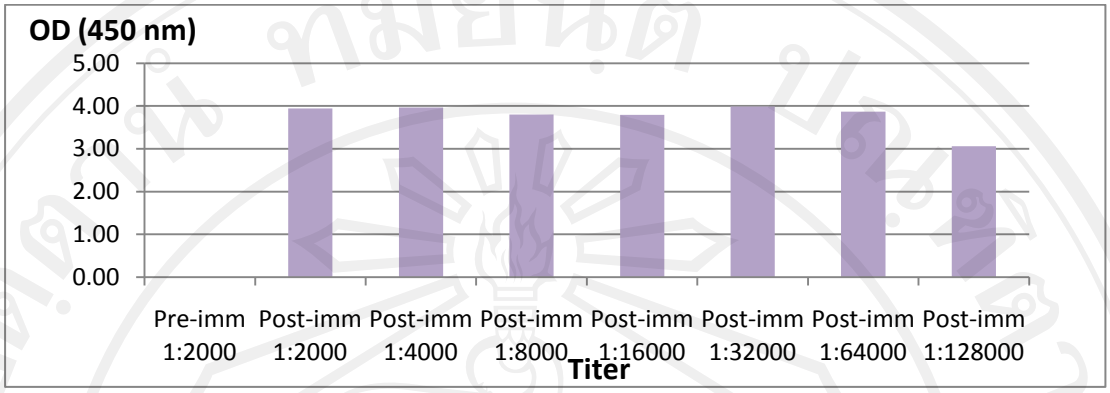
3.4 Pre-isolation of antigen specific B cell strategy

From our previous experiments, B cells isolated from spleen of the antigen immunized mice and used as the fusion partner was demonstrated to be a strategy that can reduce the displeased fibroblast in the fusion wells. Even though, this strategy can increase the possibility in obtaining of hybridoma producing mAb of interest, several hybridoma supernatants are required to be tediously screened. We, therefore, decided to develop a new strategy where specific B cells are isolated prior to cell fusion. The isolated B cells were then used as the fusion partner. We speculate that by this strategy, mAbs of interest are simply obtained. To verify this strategy, in this study, the production monoclonal antibody to Ag85 protein was used the study model.

3.4.1 Mice immunization and antibody responses

Three BALB/c mice were immunized with Ag85B-BCCP recombinant protein. After the third immunization, the antibody responses were determined by indirect ELISA technique. As shown in Figure 3.6, the antibodies responses after immunizations were very high. All mice were therefore could be used for further study.

Mouse no.1



Mouse no.2

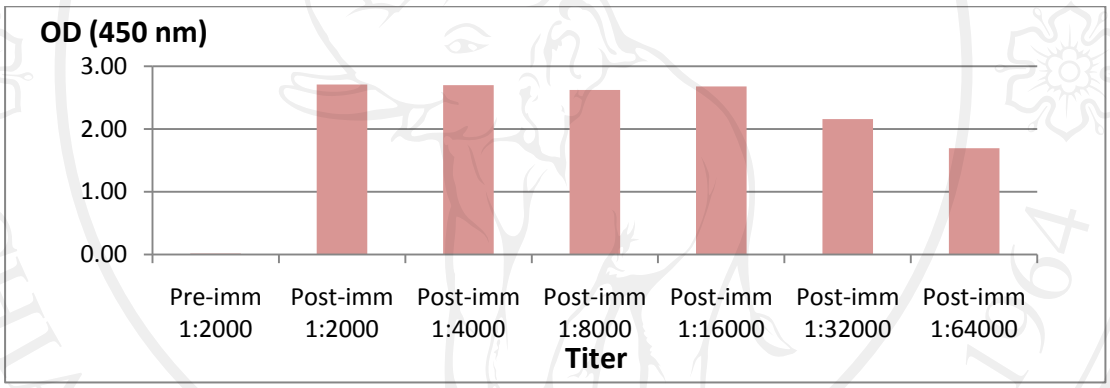


Figure 3.6 Antibody responses in BALB/c mice after immunizations with Ag85B-BCCP. BALB/c mice were immunized with Ag85B-BCCP. Sera were collected at pre-immunization (pre-imm) and after the third immunization (post-imm) and determined for antibody to Ag85-BBCP protein by indirect ELISA.

3.4.2 Hybridoma production

After the third immunization, as a control, total spleen cells were fused with myeloma cells using standard hybridoma technique. Spleen cells that express surface antibodies against Ag85B-BCCP were isolated using the MACS system. Cells from positive fraction (specific B cells) and negative fraction were obtained and then fused with myeloma cells for generation of hybridomas. In this study, two experiments were carried out.

In experiment 1, by using of total spleen cells (standard cell fusion method), the fused cells were seeded into a total of 456 wells. After cultivation in HAT selective medium, 56 wells (12.2%) of the seeded wells contained hybridomas (Table 3.8). As some wells were overgrown with fibroblasts, 37 culture supernatants (66.07%) were collected and screened for their antibody activity and 17 wells (45.95%) showed positive reactivity (Table 5). In Ag85B-BCCP specific B cell fusion, as expected a small number of cells was obtained upon specific B cell isolation, the fused cells could be seeded into only of 87 wells. After cultivation in HAT selective medium, 28 wells (32.2%) of the seeded wells contained hybridomas (Table 3.8). 27 culture supernatants (96.43%) were screened for their antibody activity testing and 24 wells (88.89%) showed positive reactivity (Table 3.8). The results indicated that almost of all hybridomas obtained from pre-isolation of antigen specific B cell strategy produce antibody specific to the immunized antigen.

Moreover, by using of negative-fractionated spleen cells, cells were seeded into a total of 876 wells. After cultivation in HAT selective medium, 211 wells (24%) of the seeded wells contained hybridomas (Table 3.8). 159 culture supernatants (75.36%) were screened for their antibody activity and 97 wells (67.01%) showed

positive reactivity (Table 3.8). This result indicated that some Ag85 specific B cells were still remained in negative cell population after isolation.

Experiment number 2 was then performed, in this experiment, unfortunately, the fusions were not well achieved. After cell fusion, the number of hybriodma generated in the seeded wells was very low. However, the results still have somehow remarkable. In standard fusion using total spleen cells, cells were seeded into a total of 480 wells. After cultivation in HAT selective medium, only 6 culture supernatants (1.25%) could be harvested and screened for their antibody activity. None of the tested supernatants showed positive reactivity (Table 3.9). In Ag85B-BCCP specific B cell fusion, 128 wells were seeded and 7 well culture supernatants (5.5%) could be harvested and screened for their antibody activity. Interestingly, 2 out of 7 wells (28.57%) showed positive reactivity (Table 3.9). In contrast, 1344 wells were seeded in negative-fractionated spleen cell fusion, 17 culture supernatants (1.3%) were screened but all of them showed negative reactivity (Table 3.9).

Taken together, the results indicated that using pre-isolation of B cell expressing the antibody of interest as target cell for myeloma fusion would be an effective strategy for production of monoclonal antibody. This technique will lead to the reduction of abundant work in the screening hybridoma process.

Table 3.8 Hybridomas generated from total spleen cells, Ag85B-BCCP specific B cells and negative cells: Experiment 1

cell type	Seeded[a]	Total[b]	% of total[c]	Ab-screen[d]	% of Ab-screen[e]	Ab positive[f]	% of Ab positive[g]
Total spleen cells	456	56	12.28	37	66.07	17	45.95
Specific B cells	87	28	32.18	27	96.43	24	88.89
Negative cells	876	211	24.09	159	75.36	97	61.01

Spleen cells from immunized mice were fractionated into Ag85B-BCCP specific B cells and negative cells or total spleen cells. Each cell fraction was fused with myeloma cells, adjusted to the same concentration and seeded into 96 well plates as indicated. The generated hybridomas were counted under an inverted microscope.

[a] Total number of seeded wells.

[b] Total number of hybridoma containing wells.

[c] % of hybridoma containing wells.

[d] Number of hybridoma containing wells for antibody screening.

[e] % of hybridoma containing wells for antibody screening

[f] Total number of hybridoma producing antibody against antigen containing wells.

[g] % of hybridoma producing antibody against antigen containing wells.

.

Table 3.9 Hybridomas generated from total spleen cells, Ag85B-BCCP specific B cells and negative cells: Experiment 2

cell type	Seeded[a]	Ab-screen[b]	% of Ab-screen[c]	Ab positive[d]	% of Ab positive[e]
Total spleen cells	480	6	1.25	0	0
Specific B cells	128	7	5.47	2	28.57
Negative cells	1344	17	1.26	0	0

Spleen cells from immunized mice were fractionated into Ag85B-BCCP specific B cells and negative cells and total spleen cells. Each cell fraction was fused with myeloma cells, adjusted to the same concentration and seeded into 96 well plates as indicated. The generated hybridomas were determined under an inverted microscope.

[a] Total number of seeded wells.

[b] Number of hybridoma containing wells for antibody screening.

[c] % of hybridoma containing wells for antibody screening

[d] Total number of hybridoma producing antibody against antigen containing wells.

[e] % of hybridoma producing antibody against antigen containing wells.

Part 3: Development of hybridoma techniques for production of monoclonal antibody having a desired isotype

Using the standard hybridoma technique, monoclonal antibodies specific to proteins of interest can be produced. However, it is not always straightforward to obtain monoclonal antibodies that have a specific isotype. As different antibody isotypes have different properties and utilizations, this uncertainty is a drawback of some mAbs applications. To overcome the uncertainty of obtaining a desired isotype monoclonal antibodies, we modified the conventional hybridoma technique for generation of monoclonal antibodies which have a specific isotype.

3.5 Generation of monoclonal antibodies which have a specific isotype.

In this study, production of mAbs to a protein antigen, Hb F, was used as the study model. Mice were immunized three times with purified Hb F. After the third immunizations, a suitably high anti-Hb F antibody titer could be detected in all immunized mice (Figure 3.7) The appearance of a high antibody titer to the immunized antigen indicated that the antigen specific B cells were activated and differentiated into effector cells that actively produce antibodies of different isotypes.

Spleen cells were then segregated from the immunized mice. Total spleen cells were fused with myeloma cells using standard hybridoma technique. According to surface immunoglobulin expression, B cells expressing IgM and IgG were isolated from the spleen cells using the MACS system. The isolated cell fractions were then fused with myeloma cells for generation of hybridomas. By this approach, which is different from the standard hybridoma technique, the IgG and IgM expressing cells were isolated prior to the cell fusion. The isolated cells were then used as fusion partners for generation of hybridomas.

In this study, by using of total spleen cells, after fusion, cells were seeded into a total of 960 wells. After cultivation in HAT selective medium, 359 wells (37%) of the seeded wells contained hybrodomas (Table 3.10). All 359 hybridoma culture supernatants were screened for their antibody isotypes. 80% of the tested hybridomas produced IgM isotype antibody and 11% produced IgG isotype antibody (Table 3.10). 4% of the tested hybridomas were non-IgG or IgM producing cells and 6% produced both IgM and IgG isotypes (Table 3.10). Wells containing both IgM and IgG isotypes can be presumed to have multi-hybridoma clones which produce different antibody isotypes. The anti-Hb F activity of the obtained hybridoma culture supernatants was also determined by ELISA. 7% of the generated hybridomas were positive to Hb F. Among of these, 5% were IgM and 2% were IgG isotype. We compared the obtained results to our previous un-published observation using Antigen85, a *Mycobacterium tuberculosis* secreted protein, as the immunogen. Using Antigen85 immunization, the percentages of hybridomas producing IgM and IgG were 26 and 43, respectively. Comparison between Hb F and Antigen85 immunizations suggested that different antigens induced different antibody isotype responses (Yamashita et al., 2002; Ernst et al., 1999; Schmitz et al., 1996; Yamada et al. 1993). The Hb F, immunogen used in this study, induced a preference for the IgM isotype.

Total spleen cells of the Hb F immunized mouse were also stained for IgM and IgG expression and analyzed by flow cytometry. Within the spleen cells, percentages of IgM and IgG expressing cells were 38% and 29%, respectively. These results suggested that both IgM and IgG positive B cells existed in the segregated spleen cells. Some of them might produce antibodies specific to the immunized antigen, Hb F.

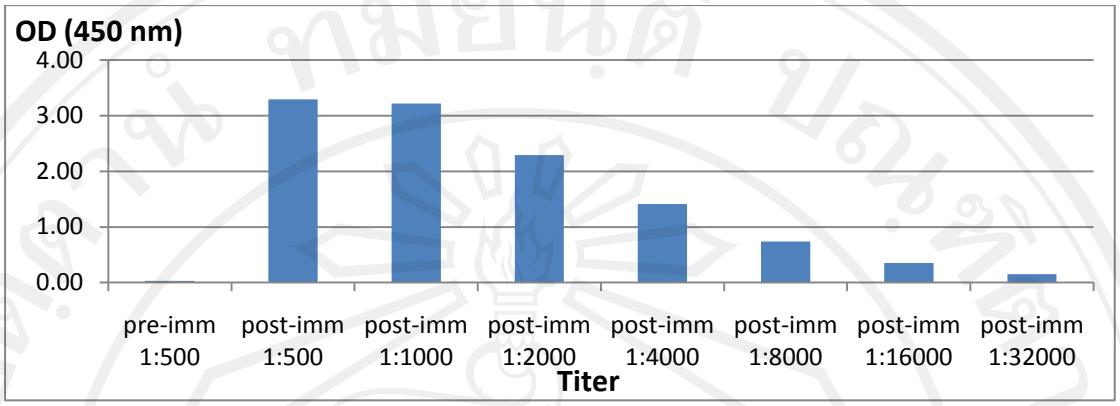
The IgG and IgM expressing cells were isolated from spleen cells prior to the cell fusion. By this cell fractionation, the spleen cells were separated into 3 populations, i.e., IgM expressing cells (IgM^+ cells), IgG expressing cells (IgG^+ cells) and IgM and IgG negative cells (IgM^-/IgG^- cells). All cell populations were separately fused with myeloma cells. After fusion, cells at the same cell concentration were spread into 96 well plates. Generated hybridomas were determined and culture supernatants from hybridoma containing wells were screened for antibody isotypes (IgM or IgG) and anti-Hb F activity by indirect ELISA. The results are shown in Tables 3.11 and 3.12.

In three independent experiments, using each cell fraction in cell fusion, resulted in percentage yields of 32 ± 11%, 35 ± 23% and 29 ± 17% (mean ± SD) of seeded wells contained hybridomas obtained from IgM^+, IgG^+ and IgM^-/IgG^- cell populations (Table 3.11). These results indicated that the isolated IgM and IgG expressing cells could be used as fusion partners and that there was no difference in fusion efficiency.

All hybridoma culture supernatants obtained were screened for their antibody isotypes. By using IgG^+ cells, in three independent experiments, the percentages of hybridomas producing IgG isotype antibody ranged from 86%-11% (41 ± 40%; mean ± SD)(Table 3.12). Interestingly, none of the tested hybridomas produced IgM antibody. A large number of hybridomas, however, were non-IgG or IgM secreting cells (Table 3.12). By this fusion, more than 50 hybridoma clones producing Hb F specific IgG mAbs were obtained. In contrast, when IgM^+ cells were used for cell fusion, the majority of the obtained hybridomas produced the IgM isotype antibody (88 ± 12%)(Table 3.12). Only 3 ± 4% of the obtained hybridomas produced the IgG

antibody and 2 ± 3% were classified as non-IgG or IgM producing cells (Table 3.12). For the small number of the obtained hybridomas producing IgG isotypes it was presumed that during magnetic cell sorting for IgM^+ cells, a small amount of IgG^+ cells was contaminated in the IgM^+ sorted cells. When such IgG^+ cells are placed into any wells, the wells will contain IgG producing hybridoma. Therefore, we could expect to obtain some wells containing IgG isotype. 7 ± 5% of tested hybridomas produced antibodies that were determined as IgG and IgM isotypes (Table 3.12). We assumed that, in these wells, there was more than one hybridoma clone which produced IgG and IgM in the same wells. From the IgM^+ cell fusion, a total of more than 50 hybridoma clones producing Hb F specific mAbs and having the IgM isotype were obtained. When the negative cells (IgM^-/IgG^- cells) were used, more than 90% of the generated hybridoma clones were non-IgM or IgG producing cells (Table 3.12). Our results indicated that spleen cells expressing IgM and IgG antibody can be used to generate hybridomas producing IgM or IgG antibodies, respectively.

A



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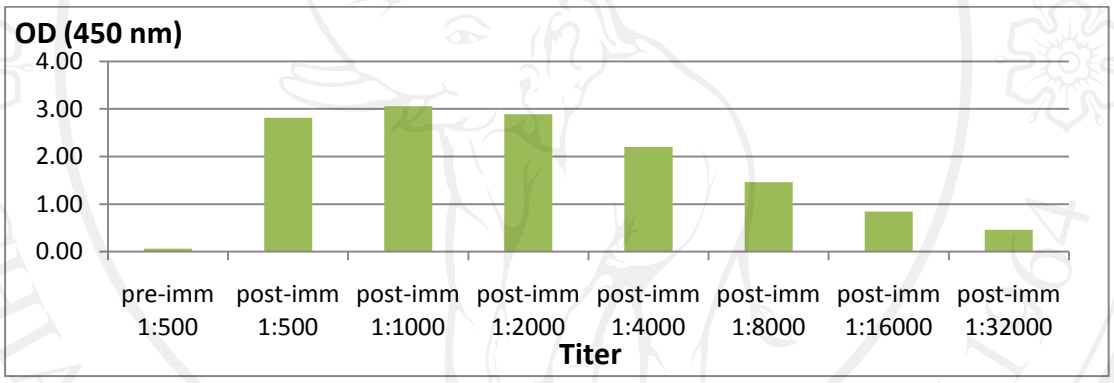

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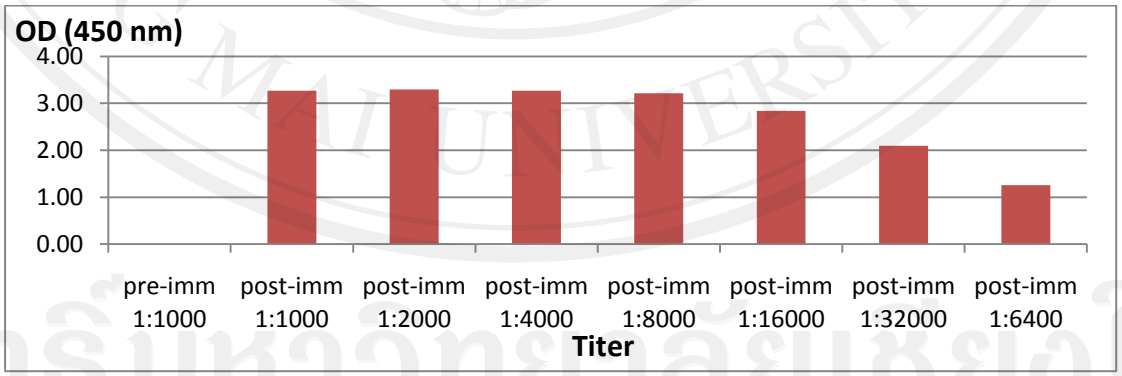

Figure 3.7 Antibody responses in BALB/c mice after immunizations with Hb F. BALB/c mice were immunized with Hb F. Sera were collected at pre-immunization (pre-imm) and after the third immunization (post-imm) and determined for antibody to Hb F by indirect ELISA.

Table 3.10 Antibody isotypes produced by the hybridomas generated from total spleen cells

Hybridomas obtained	Number of hybridoma containing wells[a]	% of total hybridoma containing wells[b]	Number of Ab against Ag wells[c]	%of Ab against Ag wells[d]
Total hybridomas	359	—	26	7.24
Hybridoma producing IgM antibody	287	80	17	4.74
Hybridoma producing IgG antibody	38	11	7	1.95
Hybridoma producing IgM and IgG antibody	20	6	2	0.56
Hybridoma producing none antibody	14	4	0	0

Spleen cells from Hb F immunized mouse were fused with myeloma cells using standard hybridoma technique. After fusion, cells were seeded into 960 wells

[a] Number of hybridoma containing wells was determined. Culture supernatants were taken from the hybridoma containing wells and determined for antibody isotypes by ELISA

[b] % of hybridoma producing IgG and IgM antibody was calculated from the total hybridomas obtained

[c] Number of hybridoma producing antibody against antigen containing wells

[d] % of hybridoma producing antibody against antigen containing wells was calculated from the total hybridomas obtained

Table 3.11 Hybridomas generated from IgM^+ cells, IgG^+ cells and IgM^- and IgG^- cells

Cell fractions	Seeded wells	Number of hybridoma containing wells	% of hybridoma containing wells
IgM^+ cells			
Exp. 1	960	418	44
Exp. 2	576	177	31
Exp. 3	768	161	21
IgG^+ cells			
Exp. 1	288	154	54
Exp. 2	136	57	42
Exp. 3	328	28	9
IgG^- or IgM^- cells			
Exp. 1	384	48	13
Exp. 2	960	447	47
Exp. 3	960	255	27

Spleen cells from immunized mice were fractionated into IgM+ cells, IgG+ cells and IgM^- and IgG^- cells. Each cell fraction was fused with myeloma cells, adjusted to the same concentration and seeded into 96 well plates. The generated hybridomas were observed with an inverted microscope. Three independent experiments (Exp. 1, Exp. 2 and Exp. 3) were carried out.

Table 3.12 Antibody isotypes produced by the hybridomas generated from IgG^+ cells, IgM^+ cells and IgM^- and IgG^- cells

Cell fraction	Antibody isotype	Experiment 1		Experiment 2		Experiment 3	
		Number of positive wells/total hybridoma containing wells	% of total hybridoma containing wells	Number of positive wells/total hybridoma containing wells	% of total hybridoma containing wells	Number of positive wells/total hybridoma containing wells	% of total hybridoma containing wells
IgG^+ Cells	IgG	133/154	86	14/57	25	3/28	11
	IgM	0/154	0	0/57	0	0/28	0
	IgG and IgM	0/154	0	0/57	0	0/28	0
	None	21/154	14	43/57	75	25/28	89
IgM^+ cells	IgG	35/418	8	0/177	0	4/161	2.49
	IgM	312/418	75	173/177	97.7	146/161	90.68
	IgG and IgM	46/418	11	3/177	1.7	11/161	6.83
	None	25/418	6	1/177	0.6	0/161	0
IgM^- and IgG^- cells	IgG	5/48	10	0/477	0	0/255	0
	IgM	0/48	0	0/477	0	0/255	0
	IgG and IgM	0/48	0	0/477	0	0/255	0
	None	43/48	90	477/477	100	255/255	100

Spleen cells from immunized mice were fractionated into IgG^+ cells, IgM^+ cells and IgM^- or IgG^- cells. Each cell fraction was fused with myeloma cells, adjusted to the same concentration and seeded into 96 well plates. Culture supernatants were taken from the hybridoma containing wells of each cell fraction and determined for antibody isotypes by ELISA. Results of the three independent experiments are shown.

Part 4: *In vitro* immunization for monoclonal antibody production

For either polyclonal or monoclonal antibody production, immunization of antigen into animal (*in vivo* immunization) is the necessity procedure. It is of great advantage if one can develop the *in vitro* immunization strategy for production of antibody. In this study, we aimed to establish *in vitro* immunization for production of mouse antibody in our laboratory.

3.6 Optimization of the conditions for *in vitro* immunization

Spleen cells from non-immunized BALB/c mice were harvested and red blood cells were lysed with a hypotonic solution. The spleen cells were then activated *in vitro* with 5, 10, and 20 µg/ml of Ag85B-BCCP. The cultures were incubated in a CO_2 incubator for a period of time. Two culture conditions, non-refed and refed conditions, were performed. In non-refed condition, 200 µl culture supernatant from each wells were collected at day 3, 5, 7, and 12 for determination of antibody activity and then re-added with the same amount of 20%FBS-RPMI medium. In refed condition, 800 µl culture supernatant from each wells were collected for determinant of antibody activity and then re-added with 800 µl 20%FBS-RPMI medium. Two independent experiments were carried out. The antibody activity in the collected culture supernatants were shown in Figure 3.8. Spleen cells produced anti-Ag85B-BCCP antibody in the presence of adjuvant MDP (Figure 3.8). High titer of anti-Ag85B-BCCP antibody could be observed on day 7 in both non-refed and refed conditions. However, cells in the refed condition look healthier than those in the non-refed condition. These results indicated that polyclonal anti-Ag85 protein could be produced by *in vitro* stimulation. Activation with 5µg/ml Ag85B-BCCP and refed condition were chosen as the optimal condition for *in vitro* immunization.

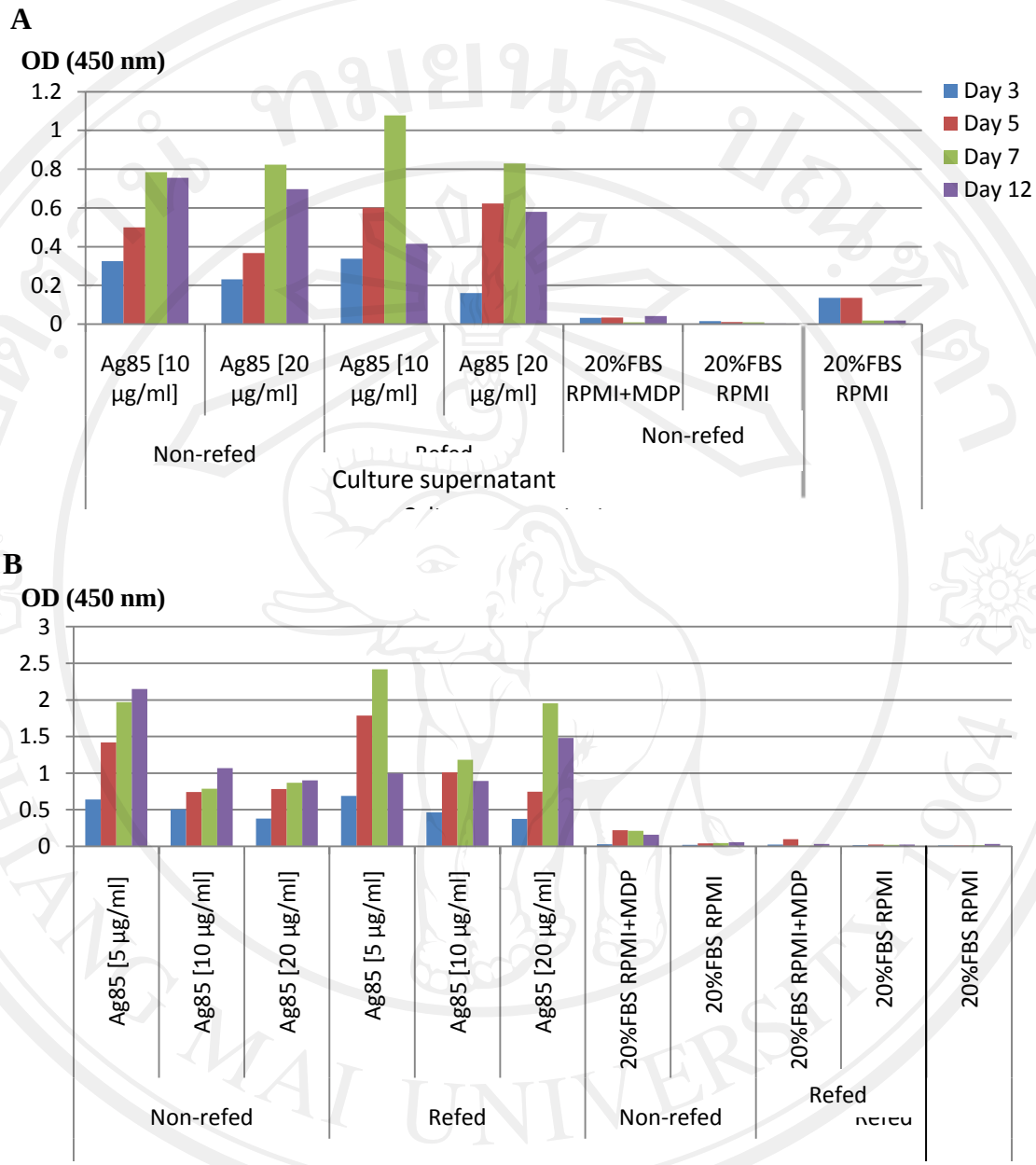

Figure 3.8 Antibody responses of spleen cells by *in vitro* immunizations with Ag85B-BCCP. Spleen cell from non-immunized BALB/c mouse were activated with indicated concentration of Ag85B-BCCP. Two culture conditions, non-refed and refed conditions, were performed. The antibody activity in the collected culture supernatants were determined by indirect ELISA. (A: experiment 1 and B: experiment 2)

3.7 Production of monoclonal antibody by *in vitro* immunization

We then pursued the production of monoclonal anti-Ag85 antibody by *in vitro* stimulation. Spleen cells were immunized with 5µg/ml Ag85B-BCCP by *in vitro* immunization. The anti-Ag85 antibodies could be detected in the culture supernatants at day 5 and 7 upon activation (Figure 3.9 and 3.12). In addition, after *in vitro* immunization, cells were determined for surface antibody specific to Ag85B-BCCP by immunofluorescence staining on day 3, 5, and 7. Two independent experiments were performed. Spleen cells produced surface anti-Ag85 antibody at day 5 and 7 (Figure 3.10 and 3.13). Surface immunoglobulins were also determined. Immunoglobulins were detected on cell surface of a population of spleen cells at day 0 (Figure 3.13), day 3, 5, and 7 (Figure 3.10 and 3.13). While, spleen cells expressed antibody against Ag85 on cell surface at day 3, 5, and 7 (Figure 3.11 and 3.14) but was not express on day 0.

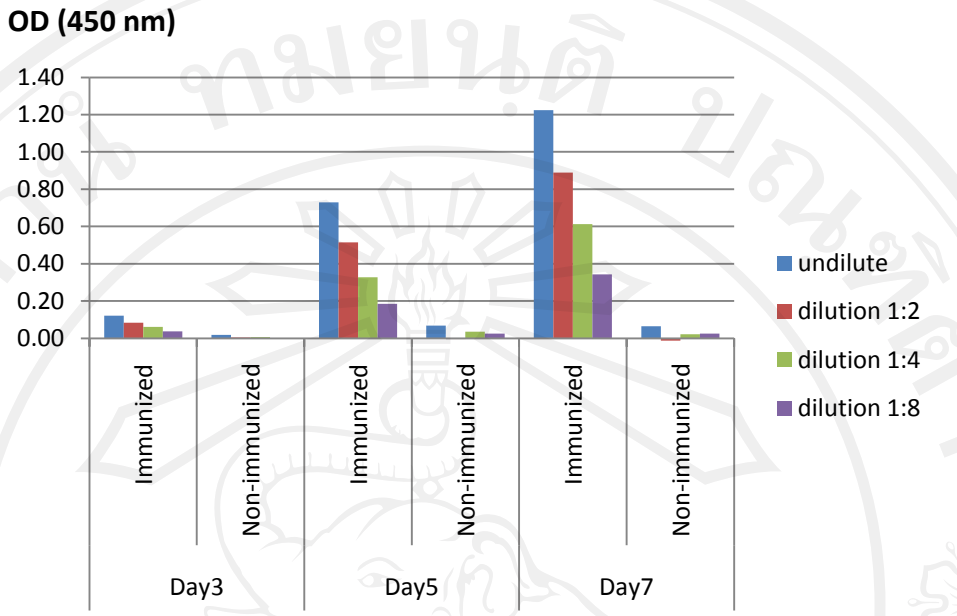

Figure 3.9 Antibody responses in culture supernatant after *in vitro* immunizations with Ag85B-BCCP (experiment 1). Spleen cells were immunized with Ag85B-BCCP by *in vitro* immunization using the optimal conditions. Culture supernatants were collected at day 3, 5, and 7 of cultivation and determined for antibody to Ag85B-BCCP by indirect ELISA.

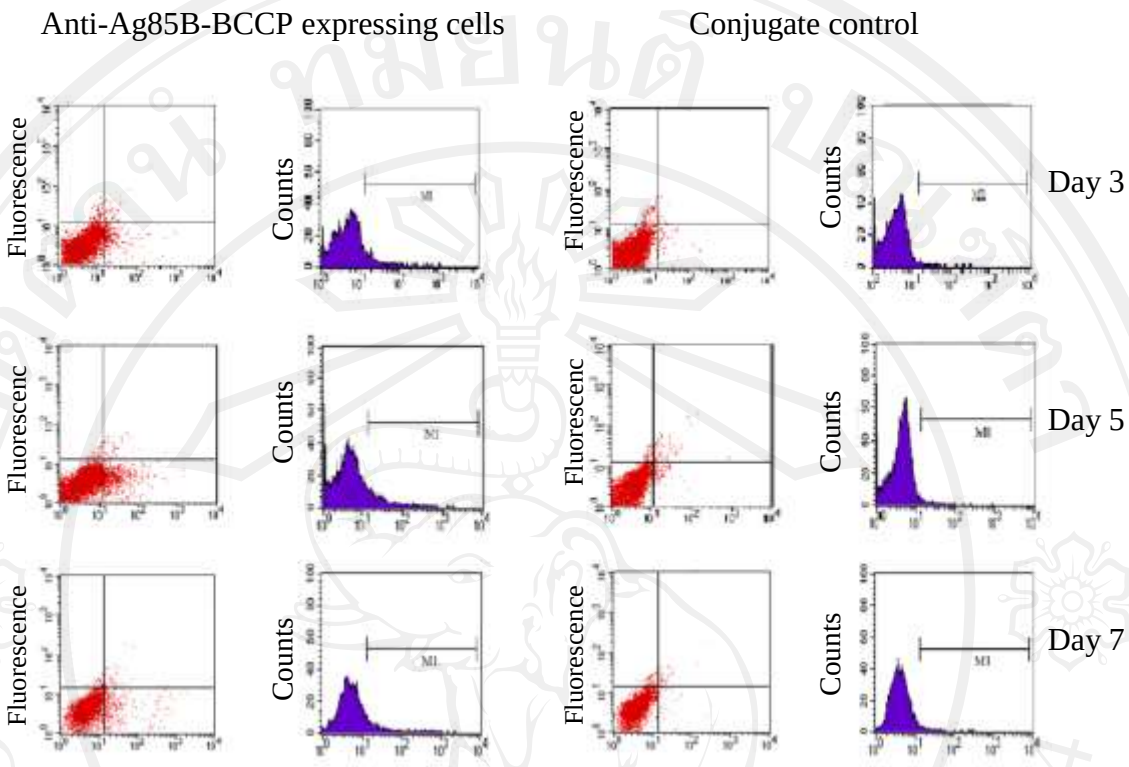

Figure 3.10 Specific antibody expression on spleen cells after *in vitro* immunizations with Ag85B-BCCP (experiment 1). Spleen cell were immunized with Ag85B-BCCP by *in vitro* immunization for 3, 5, and 7 days. Then, the immunized spleen cells were analyzed for antibody against Ag85 on cell surface by flow cytometry.

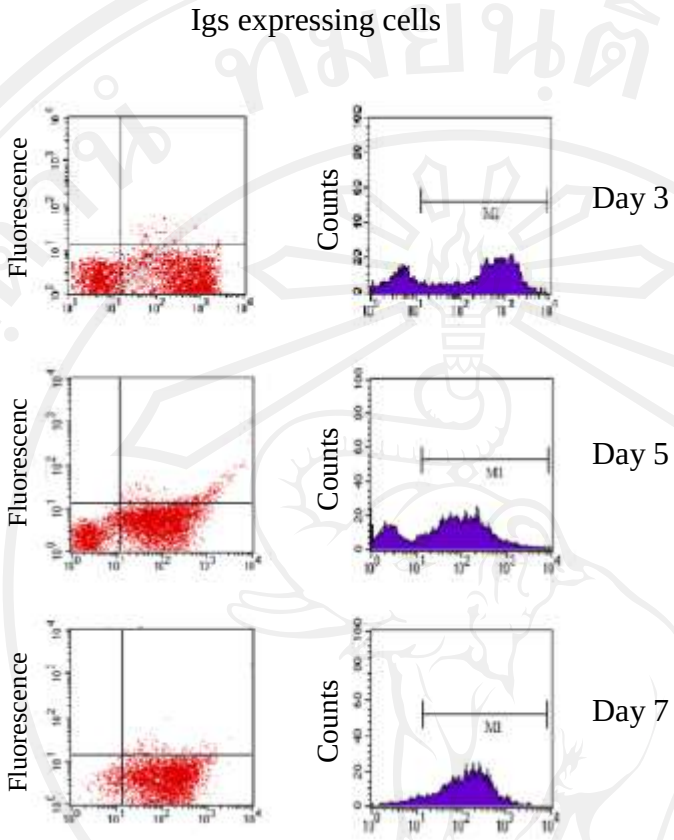

Figure 3.11 Antibody expression on spleen cells after *in vitro* immunizations with Ag85B-BCCP (experiment 1). Spleen cells were immunized with Ag85B-BCCP by *in vitro* immunization for 3, 5, and 7 days. Then, the immunized spleen cells were analyzed for antibody on cell surface by flow cytometry.

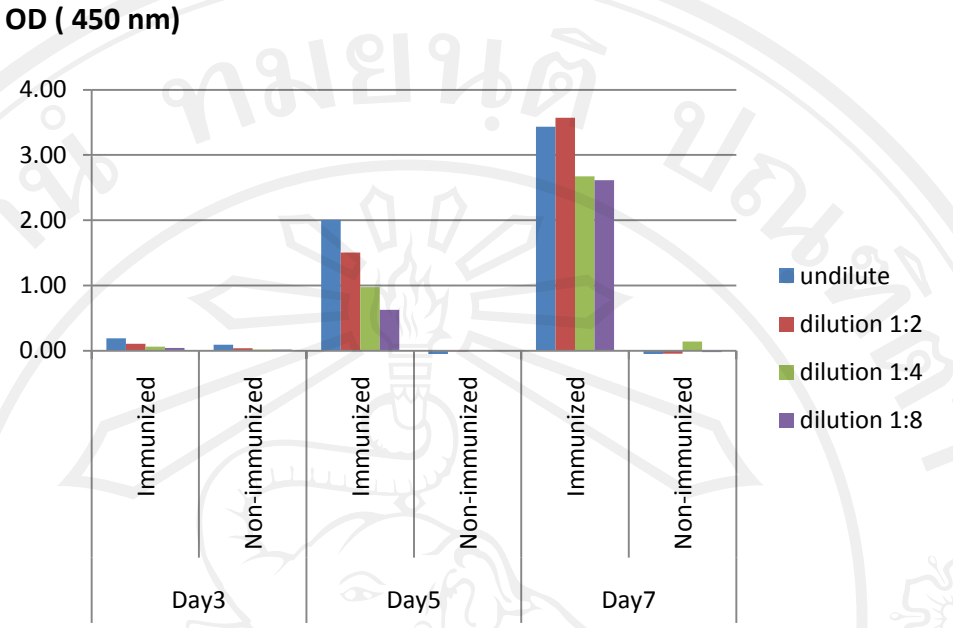

Figure 3.12 Antibody responses in culture supernatant after *in vitro* immunizations with Ag85-BCCP (experiment 2). Spleen cells were immunized with Ag85B-BCCP by *in vitro* immunization using the optimal conditions. Culture supernatants were collected at day 3, 5, and 7 of cultivation and determined for antibody to Ag85 by indirect ELISA.

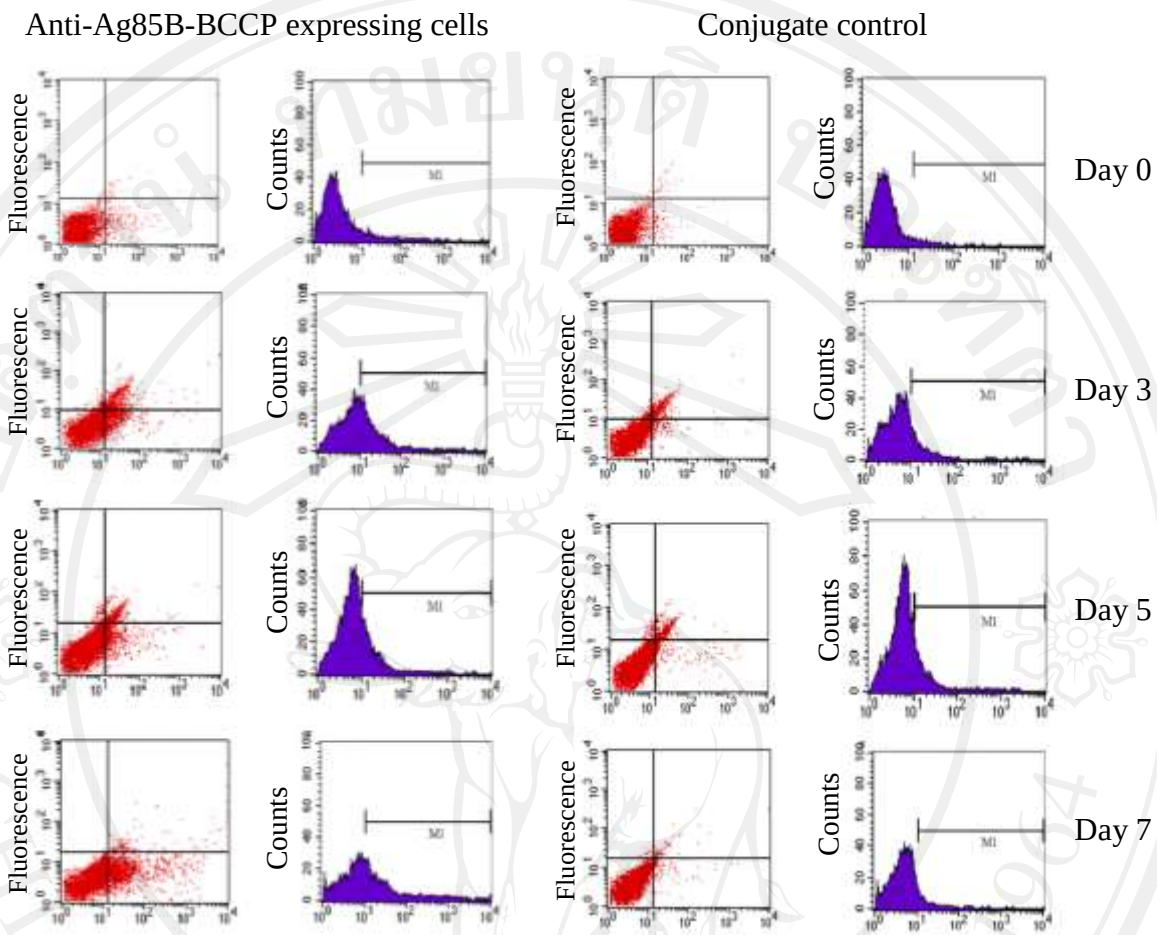

Figure 3.13 Specific antibody expression on spleen cells after *in vitro* immunizations with Ag85B-BCCP (experiment 2). Spleen cell were immunized with Ag85 by *in vitro* immunization for 0, 3, 5, and 7 days. Then, the immunized spleen cells were analyzed for antibody against Ag85 on cell surface by flow cytometry.

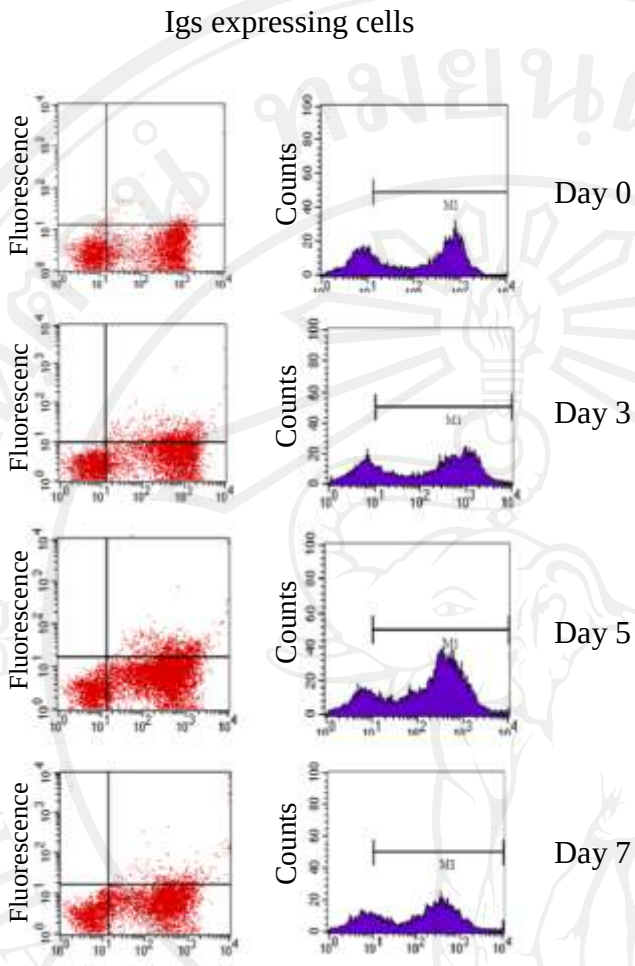

Figure 3.14 Antibody expression on spleen cells after *in vitro* immunizations with Ag85B-BCCP (experiment 2). Spleen cell were immunized with Ag85 by *in vitro* immunization for 0, 3, 5, and 7 days. Then, the immunized spleen cells were analyzed for antibody on cell surface by flow cytometry.

The *in vitro* immunized spleen cells were subjected for hybridoma production. After *in vitro* immunization for 3, 5, and 7 days, spleen cells were fused with myeloma cells by a standard hybridoma technique. After cell fusion, cells were resuspended in HAT selective medium and distributed in 96-well plates. The plates were incubated at 37°C in a 5% CO_2 incubator for hybridoma generation. The generated hybridomas were monitored by an inverted light microscope. Culture supernatants were collected from the hybridoma containing wells and tested for specific antibody by ELISA. In experiment 1; 3, 8, and 15 hybridomas containing wells were found in seeded wells at day 3, 5, and 7 after cell fusion, respectively (Table 3.13). All hybridoma culture supernatants were screened for antibody against Ag85. Two hybridoma containing wells from day 7 after fusion produced antibody against Ag85 (Table 3.13). For experiment 2; 30, 19, and 23 hybridomas containing wells were found in seeded wells at day 3, 5, and 7 after fusion, respectively (Table 3.13). All hybridoma culture supernatants were screened for antibody against Ag85. One hybridoma containing well from day 3 fusion produced antibody against Ag85 and 2 hybridoma containing wells from day 7 produced antibody against Ag85 (Table 3.13).

Our results indicated that *in vitro* immunization can be used to generate hybridomas producing monoclonal antibodies of interest.

Table 3.13 Hybridomas generated from *in vitro* immunization

Day of fusion	Number of seeded wells	Number of hybridoma containing wells	% of hybridoma containing wells	Number of antibody positive wells
Experiment 1				
Day3	254	3	1.18	0
Day5	336	8	2.38	0
Day7	344	15	4.36	2
Experiment 2				
Day3	192	30	15.63	1
Day5	196	19	9.69	0
Day7	185	23	12.43	2

Spleen cells were immunized with Ag85 by *in vitro* immunization for 3, 5, and 7 days. Spleen cells were fused with myeloma cells by a standard hybridoma technique. Culture supernatants were taken from the hybridoma containing wells and determined for antibody activity by ELISA. Results of the two independent experiments are shown.