CHAPTER IV

DISCUSSION AND CONCLUSION

Antibodies are the products of the immune system in response to the foreign molecules entering in the body. Specific binding property of the antibody to its recognized antigen leads to the use of antibodies in biomedical research, characterization of antigen, development of reagents for diagnosis and treatments (Yelton and Scharff 1981; Berger and Edelson 1982; Pollock *et al.* 1984; Spira *et al.* 1985; Valentino *et al.* 1985; Birch and Lennox 1995; von Mehren *et al.* 2003). In 1975, Kohler and Milstein developed hybridoma technique that allows the growth of clonal populations of cell secreting antibody with defined specificity. The hybridoma technique has, recently, become an important tool for production of monospecific antibodies, monoclonal antibodies (mAbs).

Since its discovery by Kohler and Milstein in 1975, hybridoma technology has become widely adopted for production of mAbs (Kohler and Milstein 1975). To produce mAb by the standard hybridoma technique, spleen cells of an antigen immunized mouse are fused with myeloma cells. The fused cells are then seeded into tissue culture plates. The hybridomas are generated and screened for their secreted antibodies. Each original antibody positive well often contains more than one hybridoma clone, only one of which produces the desired antibody. The non-producers, however, tend to overgrow the producer, leading to failure of production of

the focal mAb. To minimize the overgrowth by the non-antibody secreting cells, hyrbidoma single cell cloning is recommended to be performed as soon as the desired hybridomas are identified. However, post-fusion newly generated hybridomas and hybridomas that are grown at low density often grow poorly or die. This is due to the fact that growing mammalian cells, including hybridomas, requires various bioactive autocrine and paracrine growth factors (Murakami *et al.* 1981; Murakami *et al.* 1982; Lee *et al.* 1992). Supplements of such factors can thus support cell growth. Use of feeder layer cells to provide factors that enhance hybridoma growth has been widely used in the past (Hammerling *et al.* 1978; Kennett 1979; de StGroth and Scheidegger 1980; Oi and Herenberg 1980; Galfre and Milstein 1981). However, feeder cells in hybridoma culture have several serious drawbacks. Preparation of feeder cells is labor intensive and imposes time constraints on hybridoma preparation. During cultivation, feeder cells metabolize nutrients, necessitating frequent changes of medium. Dividing cells in the feeder culture may overgrow and kill the desired hybridomas. In addition, feeder cells also present a potential source of microbial contamination. During the last two decades, various substitutes for feeder layers have been considered. Conditioned media produced from various cell types and containing the necessary factors for supporting hybridoma growth have been described (Sugasawara *et al.* 1985; Rathjen and Geczy 1986; Walker *et al.* 1986; Micklem *et al.* 1987; Zhu *et al.* 1993; Hoffmann *et al.* 1996). Some of the conditioned media have become commercial products. Although the commercial conditioned media can be used as growth supplements for murine hybridomas, they are very expensive and reflect the high cost of monoclonal antibody production. In this study, we aimed to study and establish the method for preparations of conditioned medium for promote

growth of the hybridomas after cell fusion and during single cell cloning. We expected that the produce conditioned medium can replace the expensive imported conditioned medium. This replacement will lead to the reduction of the expense of mAb production.

A mouse thymoma cell line, EL4, produced and released several cytokines that support hybridoma growth upon PMA activation (Howard *et al.* 1982; Grabstein *et al.* 1986). The EL4 cell line has therefore been employed in the production of commercial hybridoma conditioned media, e.g. BM-Condimed H1. In this study, a mouse thymoma line BW5147, which has been demonstrated to constitutively produce various cytokines upon culturing without mitogen stimulation (Chu *et al.* 1987), were used for production of conditioned medium for hybridoma production. BW5147 cells were cultured for 18 and 40 hours and culture supernatants (BW conditioned medium) were harvested and tested for their effect on single cell cloning of both stable and newly formed hybridomas. IMDM medium consisting of 10% FBS and conditioned with supernatant from BW5147 cells could be successfully employed in hybridoma single cell cloning, but showed slightly less effectiveness compare to the commercial BM-Condimed H1 supplement. The 40-h culture-conditioned medium was demonstrated to be optimal for supporting single-cell growth of both freshly formed hybridomas and stable hybridoma clones. Supplementation at 20% was demonstrated to be the most effective concentration. It was also shown that 20% BW conditioned medium supports growth of hybridomas without altering the antibody reactivity. As observed for other thymoma cells (Grabstein *et al.* 1986), supporting hybridoma growth with the BW conditioned medium is likely due to the production of various cytokines which support B cell differentiation and growth of the BW5147

cells (Chu *et al.* 1987). As the BW conditioned medium was produced in the absence of any mitogen, enhancing of hybridoma growth appears to be BW5147 cell mediated. We therefore conclude that the prepared BW conditioned medium can be substituted for the relatively expensive commercial conditioned medium in hybridoma single cell cloning. Since the BW conditioned medium was demonstrated to provide growth factors that promote growth of hybridomas cultured at low cell density, we investigated whether this conditioned medium could be used as a supplement for production of mAb in the hybridoma technique. The produced BW conditioned medium was successfully used as growth supplement for production of mAbs in all cell fusions. The results, as predicted, indicate that BW conditioned medium contains growth factors that not only promote growing of hybridoma cultured at low cell densities but also promote newly formed hybridoma growth.

PMA was used to activate EL4 thymoma cells for production of BM Condimed H1 conditioned medium (Farrar *et al.* 1980; Grabstein *et al.* 1986). This mitogen is often used to stimulate lymphocytes for production of cytokines (Lagoo *et al.* 1990; Aarden and van Kooten 1992; Birch and Lennox 1995; Herrera *et al.* 1998; Caraher *et al.* 2000). In this study, instead of culturing cells in the absence of mitogen, we investigated the use of PMA as stimulant for production of conditioned medium. BW 5147 thymoma cell line were activated with PMA and the conditioned medium was collected. The produced conditioned medium (PMA-induced conditioned medium) were then verified whether it could support the growth in hybridoma cells. We found that the PMA-induced conditioned medium could effectively support the growth of hybridoma cells in single cell cloning as well as using commercial BM-Condimed H1. In addition, the PMA-induced conditioned medium could be utilized in

hybridoma production. As was described in our BW conditioned medium, the PMA-induced conditioned medium may contain growth factors that support the growth of hybridoma cells. We then analyzed the proteins contained in the produced conditioned medium by SDS-PAGE comparison to the BM-Condimed H1. However, by SDS-PAGE, we could not observe the different protein bands of the basal medium and the conditioned mediums. This is because of that the medium used in each condition contain 10% fetal bovine serum which comprising large number of proteins. The serum proteins therefore cover the small amount of cytokines produced by BW5147 cells. To really identify the cytokines contained in conditioned media, the gradient gel electrophoresis and silver staining are suggested to be performed. In addition, Western immunoblotting using specific antibodies is also suggested to be performed to determine the cytokine in produced conditioned medium.

In our experiments for the production of mAbs using BM-Condimed H1, together with the experiment of using PMA-induced conditioned medium, we always observed overgrowth of fibroblasts in post-fusion wells. However, the fibroblast overgrowth was reduced when using the BW conditioned medium (without PMA stimulation). Although the reason for this is still not well understood, we speculate that PMA is the cause (Grabstein *et al.* 1986; Zhu *et al.* 1993). As BW conditioned medium was prepared in the absence of any mitogen, the BW conditioned culture supernatants lacked PMA for induction of fibroblast proliferation. In addition to the reduction of possibility of fibroblast overgrow, another benefit of using the non-PMA BW conditioned medium is that the mAb produced by hybridomas using this supplementation method can be used for biological characterization without concern for the presence of mitogen.

As the purpose of this study was to produce relatively inexpensive conditioned medium, we analyzed and compared the cost of BW conditioned medium and BM-Condimed H1. The cost for 100 ml of BW conditioned medium was approximately 1,500 Baht. In contrast, 100 ml BM-Condimed H1 purchased from Roche is 20,000 Baht. The method for production of the BW conditioned medium is quite simple and needs no special techniques or sophisticated equipment. We have now implemented the BW conditioned medium in our routine work and are convinced that the BW conditioned medium can be used as a supplement for mAb production. The use of this home-made conditioned medium can lower the cost of the production of monoclonal antibodies.

By standard hybridoma technique, hybridomas is generated by fusing together an antibody secreting cell from spleen cells of an immunized animal with myeloma cells (Kohler and Milstein 1975). By the conventional hybridoma techniques, only about 1% of the starting spleen cells are fused with myeloma cells (Harlow and Lane 1988). This, therefore, affect the yield of the required hybridomas and results in very low number of hybridoma positive clones because of the failure to control cell fusion. To overcome these problems, in this thesis, we develop new fusion strategies by using B cells or antigen specific B cells as fusion partners instead of using total spleen cells. We believed that this approach can enhance fusion efficiency and increase the numbers of hybridoma produced antibody of interest. For pre-B cells isolation strategy, B cells were isolated from spleen cells prior to cell fusion. As B lymphocytes are the only cells that synthesize antibody, using B cells as fusion partner will increase the possibility of obtaining hybridomas producing antibody of interest. B lymphocytes express membrane Ig on their surface (Abbas *et al.* 2012). To separate B

cells from the spleen cells, we used membrane Ig as marker for isolation. By this strategy, total spleen cells from immunized mouse were mixed with rat anti-mouse IgM MicroBeads and goat anti-mouse IgG MicroBeads that can cross reaction to also other isotypes of antibodies. Only B cells were labeled with these magnetic beads and isolated prior to the cell fusion. The B cell fraction was then used as fusion partners. All hybridomas obtained from pre-B cell selected fusion generated antibody and some of them produced antibody against interested antigen. While, by using of negative-fractionated spleen cells (non-B cells fraction) as fusion partners, all of culture supernatants from hybridoma containing well were not produce antibody. This result indicates that pre-B cell isolation strategy can decrease the chance of mismatch cell fusion between non-B cell and myeloma cell. However, the hybridomas of interest were not increased compared to the conventional hybridoma method. It is interesting to note that by using the pre-B cell isolation technique fibroblasts overgrow in the post fusion wells was rarely observed. The reason for this is that the fibroblasts in the spleen cells have been discards during the B cell isolation process. The generated hybridomas were therefore increasing obtained. To confirm the above finding, pre-B cell isolation fusion was used to produce several monoclonal antibodies in our laboratory. As expected, pre-B cell isolation fusion was successfully used for production of several mAbsincluding mAbs to HDL, LDL, and TFF3 protein.

B cells express antibody in two forms: membrane-bound antibodies on the surface of B cells and secreted antibodies. The secreted antibodies have the same antigen-binding site as the cell surface antibodies (Abbas et al. 2012). Therefore, surface antibodies on specific B cells can also bind to the specific antigen. According to this fact, we developed a technique called pre-antigen specific B cells selection

strategy. By this technique, B cells express surface antibody of interest were first isolated from the spleen cells. The isolated B cells were then used as fusion partner for generation of hybridomas. In this approach, Ag85B-BCCP was used as the study model. This Ag85B was biotinylated at the BCCP motif in *E. coli* Origami B strain (Tayapiwatana *et al.* 2006). Ag85B-BCCP specific B cells were isolated from Ag85B-BCCP immunized mice prior to the cell fusion by using immunomagnetic separation. Spleen cells were first incubated with purified Ag85B-BCCP. B cells that express anti-Ag85B-BCCP bound to Ag85B-BCCP. After washing step, these cells were mixed with Streptavidin MicroBeads. These beads bound to biotinylated Ag85B that captured by specific Ig of B cell and separated by MACS. The Ag85B specific B cell were contained in the positive fraction and used as fusion partner. Ag85B-BCCP monoclonal antibodies were produced by using this pre-antigen specific B cells strategy. However, we also obtained hybridomas producing Ag85B mAbs from negative cell fusion. This result indicates that some of Ag85B specific B cells were not fully separated out from spleen cells. Some Ag85B specific B cells were still remained in negative fraction. The cause of this may be that the interaction of antigen-surface antibody and antigen-Streptavidin MicroBeads were not sufficient. Optimization process is required for isolation of B cell expressing antibody of interest. The developed antigen-specific B cell strategy is, however, potentially valuable for enhance efficiency of monoclonal antibody production and reduce abundant work in the screening hybridoma process.

By the standard hybridoma technique, mAbs specific to protein antigen of interest can be produced. However, it is not always straightforward to obtain mAbs that have a specific isotype. This uncertainty is a drawback of some mAb

applications, as different antibody isotypes have different properties and utilizations (Lund *et al.* 1991; Janeway *et al.* 1999 ; Isaacs 2009). To overcome this problem, we modified the hybridoma technique to generate the mAbs which have a specific isotype. Resting B cells express two classes of membrane bound immunoglobulins, IgM and IgD that function as the receptors for antigens. Binding of specific antigens to the antigen receptors, in the presence of appropriate cytokines from helper T cells, induces B cell proliferation and differentiation into antibody producing cells (Abbas and Lichtman 2007; Lanzavecchia and Sallusto 2009). During B cell differentiation, some activated B cells can switch class of heavy chain and results in a change of production immunoglobulin from IgM isotype to other isotypes. As the spleen is the major site for the production of antibodies, after appropriate antigen stimulation, B cells carrying various isotypes of membrane immunoglobulins contain in the spleen. In an attempt to develop new hybridoma technique for production of mAb having a desired isotype, in this study, the isolated B cells carrying IgM or IgG were used to generate hybridoma cells by the conventional hybridoma technique. In our experiment, production of mAb to Hb F was used as model. B cells were isolated according to desired surface immunoglobulin expression from spleen cells of the immunized mice. Firstly, B cells expressing IgM were isolated using rat anti-mouse IgM MicroBeads by MACS system. And then, B cells expressing IgG were isolated from the negative fraction of the first isolation using goat anti-mouse IgG MicroBeads. 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 IgM and IgG expressing cells could be used as fusion partners and that there was no difference in fusion efficiency compare to the use of total spleen cells. By

using IgG^+ cells, in three independent experiments, the generated hybridomas produced IgG isotype antibody and Hb F specific IgG mAbs were obtained. A large number of hybridomas, however, were non-IgG or IgM secreting cells. Interestingly, none of the tested hybridomas produced IgM antibody. In contrast, when IgM^+ cells were used for cell fusion, the majority of the obtained hybridomas produced the IgM isotype antibody. Hybridomas from IgM^+ cells selected fusion produced Hb F specific mAbs and obtain the IgM isotype. There are some contaminated hybridomas produced the IgG antibody and non-IgG or IgM producing cells. 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 or IgG and IgM producing hybridoma. Therefore, we could expect to obtain some wells containing IgG isotype. Moreover, there were reported that B cell can express two isotypes simultaneously, Cμ with Cγ, Cε or Cα, without class switch recombination. This would presumably be achieved by the differential termination of transcription and differential splicing (Esser and Radbruch 1990). From this reason, by cell sorting, we can isolate B cells that express IgM and IgG on the surface from the spleen and these B cell can produce IgM and IgG antibody. When the negative cells (IgM^-/IgG^- cells) were used as fusion partner, Most of the generated hybridoma clones were non-IgM or IgG producing cells. By modifying the hybridoma technique to generate the mAbs which have a specific isotype, the isolated IgM and IgG expressing spleen cells can be used to generate hybridomas producing IgM or IgG antibodies, respectively. The obtained hybridomas produced antibody isotypes corresponding to isotype of surface antibody of their

starting cells. The developed technique is potentially valuable for purposes that require a specific isotype of mAbs.

In vivo immunization still is a routine laboratory procedure for conventional hybridoma technique. By *in vivo* immunization, however, relatively large quantities of the antigen are required for injection into the host animal and required long-term period for antibody responses. To overcome this problem, *in vitro* immunization strategies have been described several years ago. *In vitro* immunization is the method where the antibody response can be activated *in vitro,* instead of direct immunizing antigen into animals. This technique has been successfully achieved for production of polyclonal and monoclonal antibodies (Pardue *et al.* 1983; Halabi and McCullough 1995; Capehart *et al.* 1997; McMahon and O'Kennedy 2001; Tomita *et al.* 2006). In this study, we aim to establish the *in vitro* immunization method for production of polyclonal and monoclonal antibody in our laboratory. In this study, to optimize condition of *in vitro* immunization, spleen cells from non-immunized BALB/c mice were activated with various concentrations of tested antigens, TB Ag85 antigen, in medium containing muramyldipeptide (MDP) as adjuvant. Regular and re-fed culture conditions were performed. Antibody responses were observed and increased depends on time of cultivation. The highest antibody response was detected on day 7 with 5 µg/ml Ag85B activation. Spleen cells expressed antibody against Ag85B on their surface at day 3, 5 and 7 but did not express on day 0. This result indicated that, upon *in vitro* activation, B cells were activated by the antigen within 3 days. To the best of our knowledge, this is the first time describing the kinetics of antibody responses upon *in vitro* immunization. According to the information obtained, *in vitro* activation of spleen cells for 3, 5, and 7 days could be used to fuse with myelomas for

generation of hybridomas. Several anti-Ag85 mAbs were obtained indicating the successful of using *in vitro* immunization for mAb production. The results indicate that *in vitro* activation of spleen cells with 5 µg/ml Ag85B were demonstrated to be the optimal condition for induction of antibody response. We demonstrate the benefit of *in vitro* immunization for production of polyclonal and monoclonal antibody. This method is simple and less time consuming and quantities of antigen for production of antibody of interest. However, in this thesis, *in vitro* immunization was performed by using only Ag85B as a model. We wonder whether the *in vitro* immunization can be achieved with all antigens. In further study, one should study *in vitro* immunization using other antigens to ensure that this optimal condition of *in vitro* immunization is effectively procedure and can produce antibodies against every antigen.

Taken together, in this thesis, we have developed several techniques for effectively production of hybridomas and mAb. We have produced home-made conditioned medium for effectively support growth of hybridomas. We have modified hybridoma techniques designed as pre-isolation of B cell strategy and pre-antigen specific B cell strategy for enhancing efficiency of monoclonal antibody production. We have also established the method for production of mAbs having a desired isotype. Finally, we have optimized the conditions of *in vitro* immunization to produce polyclonal and monoclonal antibodies in our laboratory.