CHAPTER IV

DISCUSSION AND CONCLUSION

Antibodies are produced in response to the presence of foreign molecules in the body. Specific binding property of the antibody to its recognized antigen leads to the use of antibodies in various biomedical researches, in diagnosis of diseases 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). Antibodies are synthesized primarily by plasma cells, a type of terminally differentiated B lymphocyte. In 1975, Kohler and Milstein developed a technique, named hybridoma technique that allows the growth of clonal populations of cell secreting antibody with defined specificity. The hybridoma technique has become a tool for production of monospecific antibodies called monoclonal antibodies. An important step of hybridoma technique is to provide a growth supplement for hybridomas, especially during earlier after fusion and during single cell cloning step. This is due to that newly fused hybridomas and the hybridomas that are cultured at low cell density often grow poorly or die. Feeder cell layer is one of the growth supplement strategy for hybridomas. The feeder cells are believed to supply growth factors that promote growth of the hybridoma cells (Harlow and Lane, 1988; Goding, 1993). Several types of cells are reported to be used as feeder cells for hybridoma growing. Commonly used feeder cells include mouse thymocytes, spleen cells, peritoneal cells, murine bone marrow-derived macrophages and fibroblasts (McCullough *et al.*, 1983; Long *et al.*, 1986; Harlow and Lane, 1988; Hlinak *et al.*, 1988; Goding, 1993; Hoffmann *et al.*, 1996). Although feeder cells can support

myeloma growth, however, some disadvantages in using of feeder cells have been reported. The major disadvantages of using feeder cells in hybridoma technique are: (1) they may deplete media of nutrients required by growing hybridomas, (2) they sometimes overgrow and kill newly formed hybridomas, (3) they represent a possible source of contamination, (4) their preparation insufficient quantities and reproducible quality (Hoffmann *et al.*, 1996), and (5) more than one mouse was sacrificed for preparing of feeder cells in one fusion. To circumvent these difficulties, several researchers have studied alternatives for replacing of the feeder cells. As supporting of hybridoma growth by feeder cells was demonstrated to be due to its produced cytokines. Cell culture supernatants were, therefore, studied and used to replace feeder cells for promoting freshly fused hybridoma growth. These cell culture supernatants were termed “conditioned medium”. Conditioned media are normally prepared from homologous cells or different cell lines such as spleen cells, fibroblasts, endothelial cell, macrophages or thymoma cell lines (Sugasawara *et al.*, 1985; Rathjen and Geczy, 1986; Walker *et al.*, 1986; Micklem *et al.*, 1987; Harlow and Lane, 1988; Zhu *et al.*, 1993; Hoffmann *et al.*, 1996; Greferath *et al.*, 1997; Ian, 2000). To date, several types of condition media have been produced. Various cell types and different cultured conditions are employed for production of the condition media. To date, the techniques for preparation of conditioned media were transfer from researchers to private companies for commercial preparations and generally sale. Several condition media are now available as commercial products such as BM condimed H1 (Roche), Hybridoma Cloning Factor (PAA), Hybridoma Enhancing Supplement (SIGMA), Briclone (QED Bioscinece) and Nutridoma CS (Roche). However, the available

commercial conditioned media are very expensive. This, therefore, makes the cost for production of monoclonal antibody is high.

In Dr. Watchara's laboratory at the Faculty of Associated Medical Sciences, Chaing Mai University, several monoclonal antibodies are being produced for both research purpose and publicity service. To reduce the 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 monoclonal antibody production.

Several types of condition media have been produced and put up for sale in market as commercial products. The available condition media are culture supernatants prepared by different methods from different cell types. At the moment, at least five conditioned media are commercially available. The BM condimed H1, is a conditioned medium distributed by Roche. It is culture supernatant produced by 24 hours PMA stimulation of EL4 mouse thymoma cells (Farrar *et al.*, 1980; Grabstein *et al.*, 1986). Hybridoma Cloning Factor, a conditioned medium distributed by PAA, is derived from the medium used to cultivate a murine macrophage-like cell line. Briclone, a conditioned medium distributed by QED Bioscience. Briclone is culture supernatant collected from a proprietary human cell line that produces IL-6. Hybridoma Enhancing Supplement, conditioned media distributed by SIGMA. Hybridoma Enhancing Supplement composed of several types of conditioned media. They are prepared from several cells such as thymoma, lymphoma cell line and murine macrophage like cell line. In contrast, Nutridoma CS, a conditioned medium

distributed by Roche, is a biochemically defined serum-free medium supplement composed of albumin, insulin, transferrin, cytokines, a cholesterol, other defined organic and inorganic compounds, and human proteins. All conditioned media mentioned have been purposed to be used to increase hybridoma survival and clonal development.

In this study, two cell lines including mouse myeloma cells and BW5147 mouse thymoma were used for generation of culture supernatants for using as conditioned medium for hybridoma production. Mouse myeloma was selected in this study because we have observed that myeloma cells when culturing at high density, they grow rapidly (un-published observations). However, their growth rate was reduced or even died when cultured in low cell density. We speculated that myeloma cells might produce growth factor to support themselves for growing. As myeloma cells are used as fusion partner in hybridoma technique, the growth factor produced by myeloma might also support the growth of generated hybrid cells. In this study, the culture supernatants harvested from myeloma cells was then tested for their supporting hybridoma growth. BW5147 mouse thymoma cell was another cell line that was included in our studied. BW5147 is mouse thymomas that are developing T cells in the thymus. This cell line produces various types of cytokines upon activation (Ralph, 1973). As mouse thymomas can be effectively used for production of conditioned medium (Farrar *et al.*, 1980; Butterfield and Weiler, 1986; Grabstein *et al.*, 1986; Micklem *et al.*, 1987; Matthes *et al.*, 1993), the BW5147 cells was therefore exploited in this study.

In our experiments, mouse myeloma and BW5147 thymoma cell lines were stimulated with or without PMA for 18 and 40 hours. PMA is a mitogen that

encourages a cell to commence cell division and triggering mitosis. 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). PMA was reported to be used for activation of EL4 thymoma for production of BM condimed H1 (Farrar *et al.*, 1980; Grabstein *et al.*, 1986). In this study, we therefore used PMA as stimulant for production of conditioned medium. Culture supernatants were sterilely harvested from the cultured cells by centrifugation. To assure that all cells were removed from the culture supernatants, after centrifugation, all culture supernatant were filtrated through a 0.1 μm filter. The produced conditioned media were then verified whether it could support the growth in hybridoma cells. In the present study, the first step, we used two hybridoma types i.e. stable hybridoma and newly fused hybridoma for determining the utilization of produced conditioned media in single cell cloning. We first determined whether the produced conditioned media could support the growth of stable hybridoma cells at low cell density. As in hybridoma technique, newly fused hybridomas are the target cells for single cell cloning, hybridomas obtained after cell fusion were further used to confirm that the produced conditioned media could be really used in single cell cloning. The second step, we set up new cell fusion by hybridoma technique and determined whether the produced conditioned media could be used to generate new hybridomas and compared with the standard condition medium.

In our first experiment, to determine whether the produced conditioned media could be used in hybridoma single cell cloning by limiting dilution, a stable hybridoma clone Thal N/B was used. Stable hybridomas are hybridoma which have been cloned and constantly produced monoclonal antibody. Thal N/B is constantly

produces anti human hemoglobin monoclonal antibody (un-published observations). The stable hybridoma line was used in this validation as it was convenience for us to determine the positive hybridoma clones after limiting dilution. In this case, as Thal N/B produced anti- hemoglobin monoclonal antibody, indirect ELISA was established for determination of the secreted monoclonal antibodies in screening step. The Thal N/B hybridomas were subjected for limiting dilution using the produced conditioned culture supernatants and compared to the commercial conditioned medium BM condimed H1. The results indicated that culture supernatants obtained from myeloma cells either with or without PMA stimulation did not have any growth supporting property for hybridoma cells. The myeloma was therefore excluded from our further studies. In contrast, supernatants obtained from BW5147 cells showed growth supporting effect. Supernatant obtained from BW5147 cultured for 40 hours without PMA activation could support hybridoma growth similar to those using commercial conditioned medium BM condimed H1. The results indicated that BW5147 thymoma cells when cultured even without any stimulation, they secret cytokines into the culture supernatant. The secreted cytokines may support the growth of hybridomas. The culture supernatant obtained from BW5147 thymoma cells without PMA stimulation and incubation for 40 hours was therefore selected for further studies.

We further determined the optimal concentration of BW5147 condition medium for using in supporting hybridoma growth. Thal N/B hybridomas were subjected for single cell cloning by using various concentrations of BW5147 conditioned medium. It was found that 20% supplementation with culture supernatants of 40 hours un-stimulated BW5147 cells was the optimal concentration for using to support the growth of hybridoma cells in single cell cloning. Whereas

higher or lower concentration of the culture supernatants showed lower number of hybridoma clones. However, the produced condition medium has to be used at a higher amount compare to the commercial BM condimed H1, which is recommended to be used at 10% supplement, The reason for this may be because of the different cell types and different cultured condition used for production of conditioned media. The growth factors contain in both conditioned media may be different in both quantity and quality.

We then evaluated whether BW5147 conditioned medium was also suitable for single cell cloning of freshly isolated hybridoma cells. For this purpose, we set up new cell fusion using standard hybridoma technique. In this cell fusion, spleen cells of mouse immunized with OKT3 immunoprecipitated beads were fused with myeloma cells. Total 313 hybridoma clones were obtained indicating the successful of cell fusion. The supernatants of the obtained hybridomas were screened for antibody reactive to lymphocyte sub-population using indirect immunofluorescence. By this screening, we found a positive hybridoma clone and named MT3. The newly generated hybridoma MT3 was subjected for single cell cloning using 20% BW5147 conditioned medium as supplement. It was found that, in addition to support the stable hybridoma, 20% BW5147 conditioned medium could also support newly generated unstable hybridoma growth in single cell cloning. The results indicated that 20% BW5147 conditioned medium can be used for hybridoma single cell cloning of both freshly fused hybridomas and stable hybridoma clones. Moreover, the culture supernatants of cloned cells obtained using 10% FCS-IMDM supplemented with 20% BW5147 conditioned medium and 10% BM condimed H1 (commercial conditioned medium) were evaluated for antibodies reactivity. It was found that the produced

conditioned medium has no effect on antibody activity. The results indicated that the produced BW5147 conditioned medium could be used to support the growth of hybridomas without altering the antibody reactivity.

Since the BW5147 conditioned medium was demonstrated to provide growth factors that promote growth of both newly established and stable hybridomas. We speculated that this conditioned medium also could be used as supplement for generated hybridoma in hybridoma technique. To verify this speculation, generation of hybridomas secreting anti-Hb Portland and anti-Hb A_2 monoclonal antibodies was used as study models. Mice were immunized with Hb Portland and Hb A_2. Splenocytes of the immunized mice were fused with myeloma cells. The hybrid cells were selected by HAT selection medium using BW5147 condition medium as supplement and compared with those using BM condimed H1 as supplement. After antibody screening, in Hb Portland experiment, by 23 and 7 hybridomas producing anti-Hb Portland antibodies were obtained from using standard BM condimed H1 and BW5147 conditioned medium, respectively. In Hb A_2 experiment, 8 and 9 hybridomas producing anti-HbA_2 were obtained from using standard BM condimed H1 and BW5147 conditioned medium, respectively. From these results, the numbers of hybridomas producing antibody of interest obtained from BW5147 conditioned medium as supplement was lower than those of BM condimed H1 as supplement in Hb Portland experiment, but was higher in Hb A_2 experiment. Therefore, it was controversial to conclude whether the BW5147 conditioned medium has different or same quality as the commercial conditioned medium for generation of hybridomas. The obtained positive hybridoma clones were then cloned by limiting dilution. As predicted, we found that the

produced BW5147 conditioned medium could be used to support the growth of hybridomas in single cell cloning as similar as the BM condimed H1.

To determine that the produced conditioned medium really can be used in hybridoma technique, we then explored the use of BW5147 conditioned medium in routine hybridoma production and single cell cloning. By this employment, the produced conditioned medium was successfully used for production of monoclonal antibodies against human Hb E/A_2, activated platelets and CD99 associated molecules. From these results, it is encourage that the home-made BW5147 conditioned medium can be utilized in hybridoma technology.

The hybridoma growth supporting effect of BW5147 conditioned medium is probably due to the cytokines produced and released into culture supernatant during culturing. We then analyzed the proteins contained in the produced conditioned medium by sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), compared with BM condimed H1 and 10%FBS-IMDM. The results indicated that a protein band of 26 kDa was observed in both BM condimed H1 and BW5147 conditioned medium, but not in 10%FCS-IMDM. However, this 26 kDa protein was quantitative higher in BM condimed H1 than in BW5147 conditioned medium. Although there is no direct proven, the 26 kDa protein is probably IL-6 (Bhardwaj *et al.*, 1989; Helfgott *et al.*, 1989; Romero *et al.*, 1990; May *et al.*, 1991) which was reported to be presented in several conditioned media (Goding, 1993; Zhu *et al.*, 1993). IL-6, a cytokine which is produced by macrophage, T cell, endothelial cells, fibroblast and many other cell types, was reported to stimulate the growth of antibody-producing B lymphocyte (Goding, 1993; Liu *et al.*, 1994; Abbas *et al.*, 2000). Moreover, the dependence of plasmacytomas and hybridomas on this cytokine

was demonstrated (Bazin and Lemieux, 1987; Bazin and Lemieux, 1989; Van Snick *et al.*, 1989; Goding, 1993). The different amount of 26 kDa protein presented in BM condimed H1 and BW5147 conditioned medium may due to its produced in different cell types. BM condimed H1 was produced from EL-4 cell lines whereas BW5147 conditioned medium was produced form BW5147 cell line. In addition, the culture condition used for preparation of both conditioned medium was different. PMA was used as activation in BM condimed H1 (Farrar *et al.*, 1980; Grabstein *et al.*, 1986), but not for the BW5147 conditioned medium in this study. In addition, IL-6 was also added in BM condimed H1 during commercially preparation for promoting growth efficacy. .

In addition to the 26 kDa protein, a protein with molecular weight of 17 kDa protein was observed in BM condimed H1, but not in 10%FCS-IMDM and BW5147 conditioned medium. It is still un-clear what this protein is. The 17 kDa protein is probable a growth factor for hybridomas that produced by EL-4 after PMA activation, but not produce in un-stimulated BW5147 cells. This factor may involve in the enhancement of the growth rate of hybridoma cells when supplement with BM condimed H1 compare to BW5147 conditioned medium.

As the purpose of this study is to replace the produced conditioned medium to the expensive imported conditioned medium, we analyzed and compared the cost of BW5147 conditioned medium and BM condimed H1. It was found that the cost for 100 ml of BW5147 conditioned medium was approximately 1,600 baht. In contrast, 100 ml BM condimed H1 purchased from Roche was 21,000 baht. Although, the preparation of BW5147 conditioned medium requires time and workforce, but the production method is so simple and need no special technology and sophisticated

equipment. As it was having used in routine work, we convince that BW5147 conditioned medium can be used as supplement for production hybridomas. The use of this home-made conditioned medium will bring about to the low cost of the production of monoclonal antibody. However, we realize a major problem on the production of this conditioned medium. The produced conditioned medium may has a lot to lot variation. Different lots of conditioned media may have different quality. The variation of conditioned medium may come from various factors such as quality of started cell line and fetal calf serum used in conditioned medium preparation.

In conclusion, in this study, culture supernatant harvested from BW5147 mouse thymoma after culturing for 40 hours without any stimulant can be used as conditioned medium for supporting hybridoma growth. Twenty percent supplementation is the optimal concentration for using in both generations of hybridoma growth and single cell cloning. The BW5147 conditioned medium was, therefore, recommended for replacement of very expensive commercial reagent in resource-limited countries including Thailand.