CHAPTER I

INTRODUCTION

1.1 Statement of the problem

Antibody is a glycoprotein that produced by B lymphocytes in response to antigens. Antibodies have a special property, it specifically binds to its induced antigen. This antigen-specific binding property of the antibody leads to the employment of antibody in many aspects. During the last several decades, antibodies against various molecules were produced and used as a tool for identification and characterization of its recognized molecules. In addition, antibodies have been employed as a key reagent for various biological researches, as well as for the development of diagnostic kits and therapeutic drugs. (Kohler and Milstein 1975; Yelton and Scharff 1981; Berger and Edelson 1982; Pollock *et al.* 1984; Spira *et al.* 1985; Valentino *et al.* 1985; Goding 1993; Birch and Lennox 1995; von Mehren *et al.* 2003).

Antibodies are synthesized by a terminally differentiated B lymphocytes, plasma cells. Since, plasma cells cannot grow *in vitro*, they cannot be used as an *in vitro* source of long term antibodies production. In 1975, Kohler and Milstein developed hybridoma technique that allows the growth of clonal populations of cell secreting antibodies against defined specific antigen. By the hybridoma technique, mouse is immunized with antigen of interest. Spleen cells from the immunized mouse are fused with immortal myeloma cancer cells (Kohler and Milstein 1975). After cell

fusion, the immortalized antibody-producing cell lines or hybridomas, are generated. By this procedure, as the hybrid cells were distributed in 96 well plates after cell fusion, each original positive well will often contain more than one hybridoma clone. Single cell cloning is then performed to ensure that cells produce the antibody of interest are truly monoclonal and that the secretion of this antibody can be stably maintained. The cloned hybridomas can be maintained *in vitro* and will continuously secrete antibodies with a define specificity. The produced antibody from hybridoma clone is termed monoclonal antibodies (mAbs) (Kohler and Milstein 1975; Masouredis 1981; Sikora and Smedley 1984; Catty 1988; Harlow and Lane 1988; Goding 1993; Birch and Lennox 1995; Abbas *et al.* 2012).

By this standard hybridoma method, however, production of any mAb is not always straightforward. Several problems those should be considered such as:

1) Newly fused hybridomas and hybridoma cells that are grown at low density often grow poorly or die. The reasons for this are still not well understood, but may relate to the requirements of growth factors. So, hybridoma cells may culture together with cells which secrete factors promoting growth of the hybridomas. These cells called “feeders”. A major difficulty for using these feeder cells is that their preparation always insufficient quantities and quality, and increase risk of contamination. To circumvent these difficulties, several researchers have used cell culture supernatants to replace the feeder cells. These cell conditioned culture supernatants usually termed “conditioned medium”. Conditioned media can be prepared from culturing of mouse thymocytes, fibroblast, spleen cells, macrophages and various 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; Ian 2000).

2) By the standard cell fusion procedure, 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.

3) The conventional hybridoma technique always results in low number of hybridoma producing antibody of interest. This may be due to the failure to control cell fusion, and the obtained hybridomas often stop to produce antibody, especially with the mAbs against molecule with low antigenicity (Schmidt *et al.* 2001; Tomita *et al.* 2006).

From the stated problems, it is already agreed that by the conventional hybridoma technique, production of any hybridoma clones are not simple. To overcome these problems, many researchers tried to develop new techniques for enhancing the production of mAb. Isolation of B cells from the spleen cells prior to cell fusion has been introduced (Tomita *et al.* 2006; Lin *et al.* 2010). Tomita and his colleagues developed a novel technique, named antigen specific B-cell targeting method, that was claimed as an advanced hybridoma technology. The established method was recommended for rapid and simple production of mAbs. Lin and her colleagues have developed a simplified procedure for efficient generation and selection of antibody-producing hybridomas, called cytoflow reactor-based cell sorter (CBCS) system. By this method, the antigen specific B cells were enriched and subsequent fusion of B cells with myelomas was performed in the bioreactor.

By conventional hybridoma technique, *in vivo* immunization of mouse still is a routine laboratory procedure. 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. *In vitro* immunization, the method where the antibody response can be activated *in vitro* instead of direct immunizing antigen into animals has been introduced for induction of antibody response. Several researchers have introduced the used of an *in vitro* immunization protocol for production of mAb (Pardue *et al.* 1983; Wohlleben *et al.* 1996; McMahon and O'Kennedy 2001). However, the success is still uncertain.

To overcome the hybridoma production difficulties, in this thesis, several approaches are designed and studied in order to obtain the alternative hybridoma techniques for effectively production of mAb. In this study, we aim to study in several aspects as follows:

1. Production of the conditioned medium containing growth factors for supporting the growth of hybridomas. This conditioned medium will be used to augment the growing of newly synthesis hybridoma cells and also in the hybridoma single cell cloning step.

2. Development of new fusion strategies by using B cells, antigen specific B cells or B cells carrying desired isotype of antibody as fusion partners, instead of using total spleen cells. This approach will enhance fusion efficiency and give rise to high numbers of hybridomas produced antibody of interest.

3. Establishment of the *in vitro* immunization method for induction of antibody responses. Instead of direct immunization of antigen into animals, technique for stimulation of B cells *in vitro* will be developed. This technique is invaluable for the production of polyclonal and monoclonal antibodies to the animal-harmful antigen and for production of human mAbs.

From this thesis, we expect that several knowledge and techniques involving in the production of mAbs will be obtained. Combination of the obtained knowledge, the high-efficiency hybridoma technology for production of mAbs will be set up and applied as a routine procedure in the Biomedical Technology Research Center at the Faculty of Associated Medical Sciences, Chiang Mai University.

1.2 Literature reviews

1.2.1 Antibody

In generally, our bodies have ability to recognized foreign molecules entering. When the body is challenged with a pathogen or antigen, the immune system responds by making an immune response to specifically eliminate the pathogen or antigen. One arm of the immune response, called humoral mediated immunity (HMI). HMI is mediated by a family of glycoproteins produced by activated B cells called antibodies. Antibodies have affinity for and specific to the antigenic determinant on the antigen which induced its production. In the body, antibody is a functional molecule combatting with the pathogens. In addition, antibody specifically reacts to recognized molecule *in vitro*. The specific binding properties of antibody in both *in vivo* and *in vitro* bring to several applications of antibody. In exploiting the specific binding properties of antibody, polyclonal antibodies and monoclonal antibodies are two very different forms of reagent. Both type of antibody are appropriate for different applications. (Sikora and Smedley 1984; Goding 1993; Abbas *et al.* 2012).

1.2.1.1 Polyclonal antibody

In nature, the antibody response in a body is polyclonal, that is a mixture of many different antibody specificities to the various epitopes of the structurally complex antigen. This polyclonal response to antigen is a result from a large number

of different clones of B cells activation. Each of B cell clone encodes a single immunoglobulin type with a single specificity for antigen, interacts and cooperates together with other cells in immune system, including T lymphocytes and antigen-presenting cells. After immunization of antigen into the body, polyclonal antibodies against the immunized antigen are induced and contained in circulation and body fluids. Polyclonal antibodies are, therefore, the conventional serum that collected from an antigen immunized animal that usually a rabbit, sheep or goat. The major advantage of polyclonal antibodies in vitro applications is their ability to form large insoluble immune complexes with antigen, or to agglutinate cells readily. So, the reactions can be seen and measured visually or determined photometrically. Because of their polyclonal, multi-specific nature, conventional antisera cannot be prepared easily or routinely to the degree of specificity needed to determine fine structural and antigenic differences between molecules at the individual epitope level. The use of mixed populations of antibodies creates a variety of different problems in immunochemical techniques. Therefore, the preparation of homogeneous antibodies with a defined specificity was a long-standing goal of immunochemical research. This goal was achieved with the development of the technology for hybridoma production (Masouredis 1981; Sikora and Smedley 1984; Birch and Lennox 1995; Abbas *et al.* 2012).

1.2.1.2 Monoclonal antibody

The first isolation of a homogeneous population of antibodies came from studies of B cell tumors. These tumor cells can be propagated as tumors in animals or grow in tissue culture *in vitro*. So, these tumor cells provide source of antibody production. Unfortunately, B cell tumors secreting antibodies of a predefined

specificity cannot be isolated conveniently. In vertebrates, antibodies are synthesized by plasma cells, a type of terminally differentiated B lymphocyte. Because plasma cells cannot be grown in tissue culture, they cannot be used as an *in vitro* source of antibody production. In 1975 Köhler and Milstein described a technique for the specific immortalization of the individual B cells that are responsible for the production of antibodies. These immortalized B cells can be grown as single clones of cells to secret a monoclonal antibody (Kohler and Milstein 1975). The monoclonal antibody is homogeneous in specificity, affinity and isotype and each monoclonal product is specific to a single antigen determinant on the immunogen (monospecific) (Sikora and Smedley 1984; Goding 1993; Birch and Lennox 1995; Abbas *et al.* 2012).

1.2.2 Hybridoma technology

As mention earlier, in 1975, Köhler and Milstein who first generates immortalized antibody-secreting lymphocyte by fusing them with a continuously growing cell line and then cloned individual hybrid cells to produce lines of cells that secret one particular antibody molecule (Kohler and Milstein 1975). By the method originated by Köhler and Milstein, an antibody secreting cell line is generated by fusing together an antibody secreting cell from lymphoid tissue of an immunized animal with a cell from a plasmacytoma cell line named myeloma cells. The immortalized antibody-producing cell lines are called hybridomas and the antibodies they produce are termed monoclonal antibodies. The generated hybrid cells have inherited some characteristics from both parents. They produce antibody and grow as rapidly as malignant cells. The immortalized antibody-producing cell lines could be established routinely and maintained *in vitro*. The characteristics required of hybridomas are immortality from the malignant myeloma cell and antibody

production from the B-lymphocyte (Masouredis 1981; Sikora and Smedley 1984; Catty 1988; Harlow and Lane 1988; Goding 1993; Birch and Lennox 1995; Abbas *et al.* 2012).

For hybridoma technique, early research suggested that myelomas from BALB/c mouse are appropriate fusion partners cells. Myelomas for fusion partners can be induced in a few strains of mice by injecting mineral oil into the peritoneum. As myelomas are plasmacytoma, they have all the cellular machinery necessary for the secretion of antibodies, and many secrete these proteins. To avoid the production of hybridomas that secrete more than one type of antibody, myelomas that are used a fusion partner in hybridoma technique have been selected for the lack of production of functional antibodies by itself.

The fusion between the myeloma cell and the antibody-secreting cell can be effected by any fusogen. In the practice, hybridoma fusions routinely use poly-ethylene glycol (PEG). PEG fused the plasma membranes of adjacent myeloma and/or antibody-secreting cell, forming a single cell with two or more nuclei called heterokaryons. This heterokaryon retains these nuclei until the nuclear membranes dissolve prior to mitosis. During mitosis and further rounds of division, the individual chromosomes are segregated into daughter cells. Because of the abnormal number of chromosomes, segregation does not always deliver identical sets of chromosomes to daughter cells, and chromosomes may be lost. If one of the chromosomes that carried a functional, rearranged immunoglobulin heavy-or light-chain gene is lost, production of the antibody will stop. In culture of hybridoma cells, this will be seen phenotypically as a decrease in antibody titer and will result unstable line (Figure 1.1).

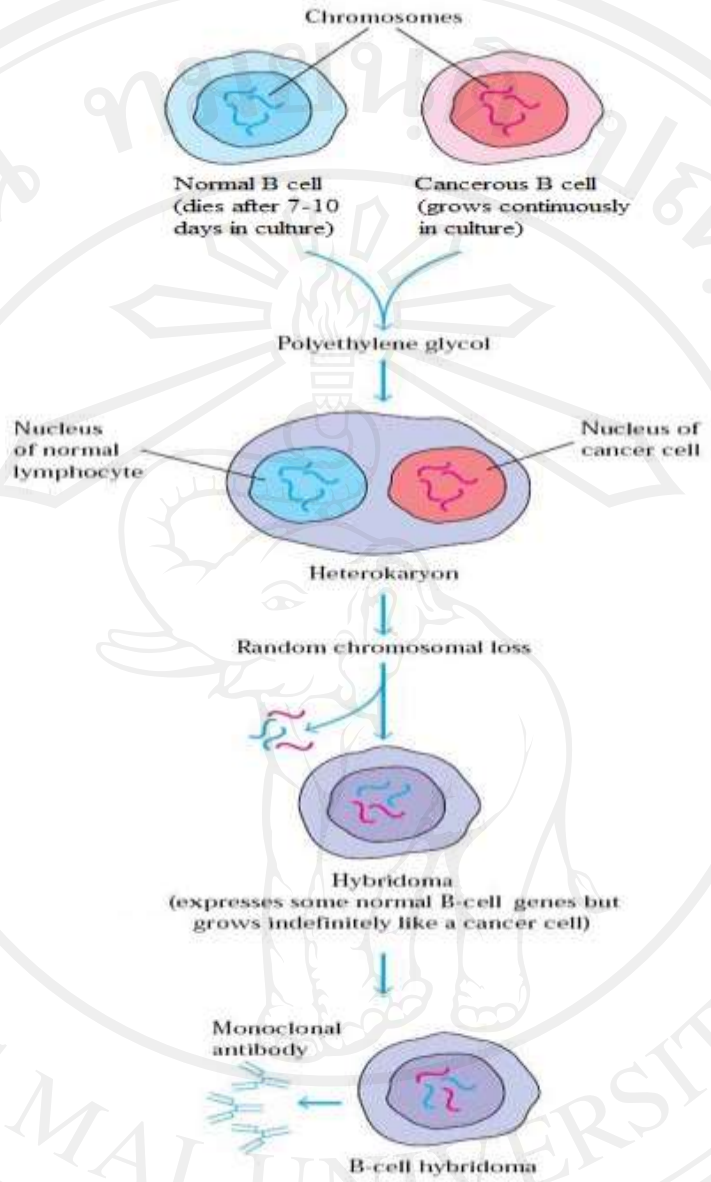

Figure 1.1 The cancer B cell (myeloma) and an antibody-producing cell (B cell) are fused by polyethylene glycol (PEG) to result in an immortal antibody-producing hybridoma. Cytoplasmic membrane of two cells is fused to form heterokaryon. Then the two nucleuses combined to form hybrid cells. Hybrid cells contain combination of both cell nuclear materials but maintaining species usual chromosomal number by loss of extra chromosome *(modified from http://biosiva.50webs.org/mab2.jpg and accessed on 28 February 2007).*

After cell fusion, it is always that not all the myeloma cells will have fused. Indeed, the majority will remain growing healthily in an unfused state. So, an appropriate system to select for hybrid cells against the background of unfused cells is very important. As these unfused myeloma cells grow faster than the hybridomas. Therefore, unfused myeloma cells need to be eliminated and usually by drug selection. Myeloma cell line that are used a fusion partner in hybridoma technique will grow in normal culture medium but will not grow in a defined "selection" medium because they lack functional genes required for DNA synthesis. In normal condition of DNA synthesis, both precursors of DNA, purine nucleotides and thymidylate, are synthesized by a *de novo* pathway requiring tetrahydrofolate. Antifolate drugs, such as aminopterin, can block activation of tetrahydrofolate, thereby inhibiting the synthesis of purine and therefore preventing DNA synthesis via the *de novo* pathway. Aminopterin-treated cells can use a salvage pathway in which purine is synthesized from exogenously supplied hypoxanthine using the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT), and thymidylate is synthesized from thymidine using the enzyme thymidine kinase (TK). Therefore, cells grow normally in the presence of aminopterin if the culture medium is supplemented with hypoxanthine and thymidine. This medium is called HAT medium (Figure 1.2). In contrast, myeloma cells that used as fusion partner in hybridoma technique are defected in enzyme hypoxanthine phosphoribosyltransferase, HPRT or HGPRT, an enzyme of a salvage pathway. Therefore, these myeloma cells cannot use the salvage pathway for nucleotide synthesis. Myeloma cells, which defecting in a salvage pathway, cannot survive in the presence of antifolate drug. In contrast, normal cells can still survive by producing DNA precursors from a salvage pathway (Figure 1.3).

Hybrid that generated between myelomas with defecting HGPRT and normal cells with functional HGPRT will be able to grow in HAT medium (Figure 1.4). Myeloma cell lines can be made defective in HGPRT or TK by mutagenesis. Selection of $HGPRT^-$ cells is performed by use of the toxic base analogues 8-azaguanine or 6-thioguanine, which are incorporated into DNA via HGPRT. Because the salvage pathway is not normally essential for cell survival, mutants that lack HGPRT will continue growing, while cells that possess HPGRT will die (Sikora and Smedley 1984; Catty 1988; Harlow and Lane 1988; Goding 1993).

The production of monoclonal antibody specific for a defined antigen is performed by hybridoma technique. The splenocytes from immunized mouse are fused with myeloma cells by PEG as fusogen. After cell fusion, unfused B cells, unfused myeloma cells and hybrid cells are randomly generated. Hybridoma cells are selected for growth in a medium containing hypoxanthine, thymidine, and an antifolate drug, aminopterin (HAT medium) (Figure 1.4). Under these conditions, unfused myeloma cells and myeloma-myeloma hydrids die because they are deficient in HGPRT enzyme required in the salvage pathway of nucleotide synthesis. In HAT medium, these cells are die because the *de novo* pathway of nucleotide synthesis is blocked by aminopterin and the utilization of hypoxanthine or thymidine in the salvage pathway are blocked by the HGPRT enzyme deficiency . So that only hybrid cells between myeloma cells fused to normal cells survive in HAT medium. The outcome hybridomas can survive indefinitely in HAT medium because the normal cells supply the HGPRT enzyme required in the salvage pathway of nucleotide synthesis for selection in HAT medium and the myeloma cells immortalize the hybrid cells. In generally, unfused normal lymphocytes can survive in culture medium for

approximately 1 week and then they die because they cannot grow *in vitro*. Therefore, after long-term culture, only normal-myeloma hybrid cells grow in the selective HAT medium. Fortunately, hybridomas generated from B lymphocyte and myeloma cell fusion can produce antibody. (Sikora and Smedley 1984; Catty 1988; Harlow and Lane 1988; Goding 1993; Abbas *et al.* 2012).

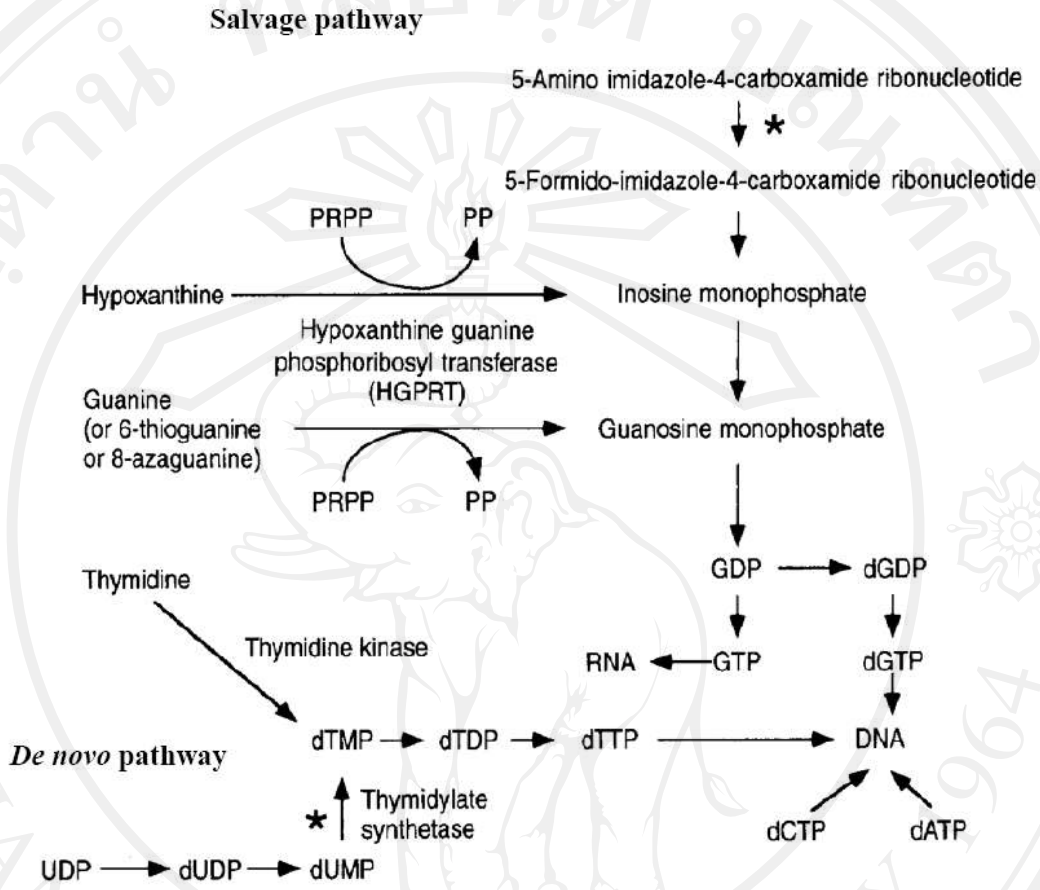

Figure 1.2 Metabolic pathways relevant to hybrid selection in medium containing hypoxanthine, aminopterin and thymidine (HAT medium). The *de novo* pathway is blocked with folic acid analogue aminopterin (*), cell must use the salvage pathway to synthesize DNA (Goding, 1996).

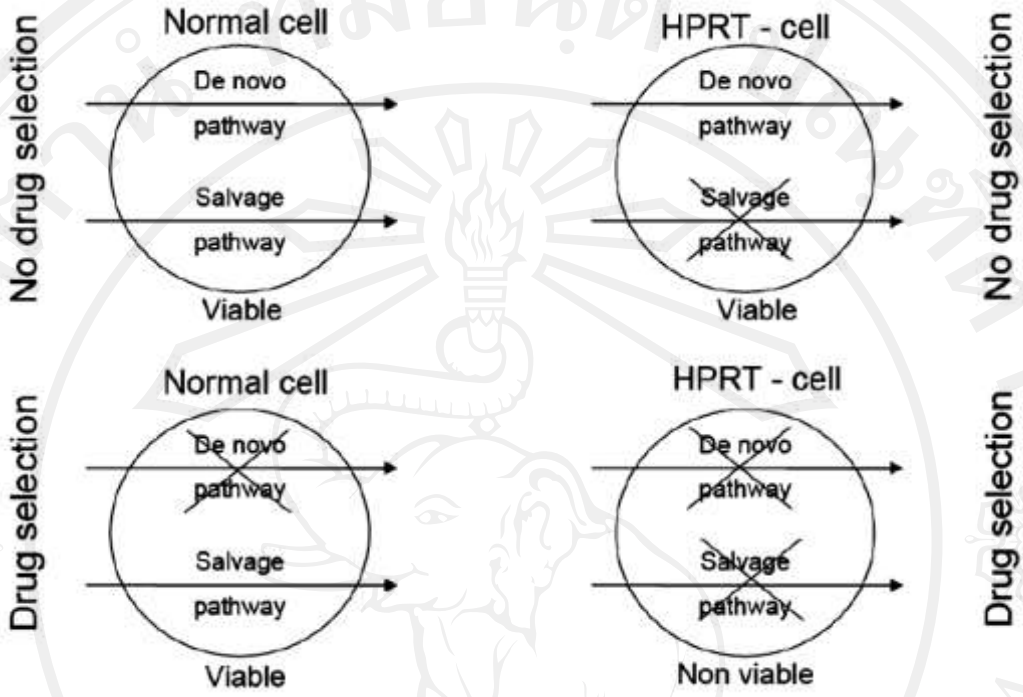

Figure 1.3 Pathway of nucleotide synthesis in antifolate drug selection, aminopterin, blocks activation of tetrahydrofolate, thereby inhibiting the synthesis of purine and therefore preventing DNA synthesis via the *de novo* pathway (Harlow and Lane 1988).

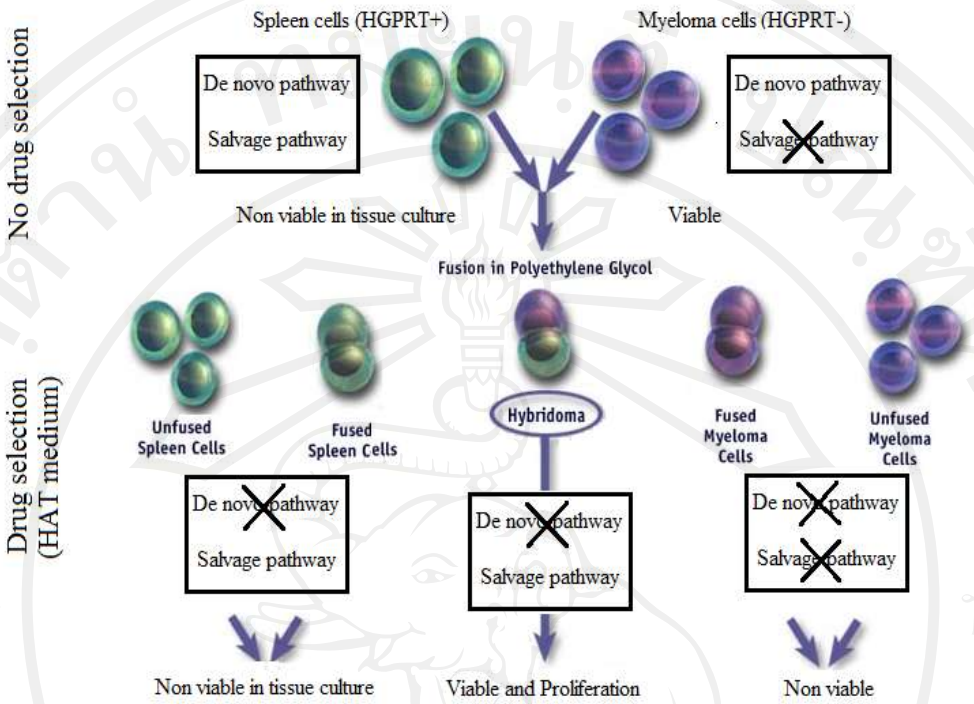

Figure 1.4 Drug selections for viable hybridomas. Hybrid cell between myelomas with nonfunctional HGPRT and spleen cells with functional HGPRT is able to grow in the selection medium containing antifolate drug, such as aminopterin *(modified from http://www.hopkins-arthritis.org/physician-corner/education/biomedical-science/mono_anti.html and accessed on 28 February 2007).*

The technology of hybridoma production steps involved in the production of mAbs including 1) the immunization protocol, 2) developing the screening procedure, and 3) producing hybridomas (Harlow and Lane 1988). Outlines the procedure of monoclonal antibody production by hybridoma technique is shown in figure 1.5. The details of hybridoma production procedure are as follows:

1.2.2.1 *In vivo* immunization

Protocols for immunization of animals, usually BALB/C mouse, vary widely. The choice of injection route is shaped to some extent by the adjuvant used and by the character, quantity, and volume of the immunogens. To enhance antibody response, at the same time of antigen immunization, the immune system of the animal may be stimulated by injecting a mixture of powerful immune stimulants or adjuvants. The most commonly used is Freund's adjuvant. This mixture of dead tuberculosis organisms in a fatty base has the effect of increasing the immune system to recognize any antigen injection with the mixture. Animals are normally injected on several occasions before immune response in serum is determined. Three boosters may be given at intervals of 2-8 weeks, to ensure good stimulation. Each immunization increase stimulation of the B-lymphocyte clones within the animal responding to the antigen. After high immune response of immunized mouse is present in serum, the final boost of antigen is injected 5 days before removal of the spleen for cell fusion, and is often given in aqueous form rather than in adjuvant.

The immunization schedule used will depend upon the nature of the immunogen just as it does in the preparation of a polyclonal antiserum. The mice that used for fusion experiments have to determined antibody response to the immunogen. A test bleed is taken from tail vein of mice usually 1-2 weeks after finishing the

immunization protocol. Antibody titer can be determined with various immunological techniques including ELISA, immunoblotting, Western blotting and immunofluorescence staining. The selected method depends on the properties and characteristics of the antigen. If an adequate titer of antibody is present in the serum, a final boost is given 5 days before fusion. (Sikora and Smedley 1984; Catty 1988; Harlow and Lane 1988; Goding 1993).

1.2.2.2 Developing the screening procedure

The tissue culture supernatants from all of the fusion wells usually are ready to screen within a few days. Depending on the fusion, individual wells will become ready to screen over 2- to 6-day period. Typically, the first wells would be ready to screen on day 7 or 8, and most of the wells will need to be screened within the next 4 or 5 days.

A good screening procedure must reduce the number of maintained cultures to a reasonable level. The procedure can identify positive supernatants or clones in 48 hour or less and be easily performed for all the needed wells. The selection of hybridomas which produce antibodies of interest usually involves several screening steps. Firstly, culture supernatants are assayed for activity against the immunogen. Positive supernatants are then screened against a test panel of antigen to reveal whether the antibody binds specifically to the antigen of interest. The most commonly used are direct or indirect ELISA, whole cell ELISA, immunofluorescence staining, immunoprecipitation, or immunoblot (Sikora and Smedley 1984; Catty 1988; Harlow and Lane 1988; Goding 1993).

1.2.2.3 Producing hybridomas

After an adequate immune response has developed in the mouse and an appropriate screening procedure has been developed, the production of hybridomas is ready to start. Spleen cell from immunized mouse are isolated, mixed with myeloma cells, and centrifuged to generate cell-to-cell contacts. Spleen cell and myeloma cells are fused with polyethylene glycol (PEG). The fused cells are then removed from the PEG solution, diluted into selective HAT medium, and plated in 96-well tissue culture plates. The hybridomas can be selected in HAT medium. Sample of the tissue culture supernatants are collected from wells that contain growing hybridomas and detected the presence of the produced antibodies against antigen by appropriate screening procedure.

Since an original positive well will often contain more than one hybridoma clones. So, single cell cloning is required for isolation the mixed clones and is essential to confirm monoclonality of the antibody production. The positive well is performed the single cell cloning by cloning in either soft agar or limiting dilution to isolate a single hybridoma cell from mixed hybridomas. Once hybridoma cells are successfully cloned, the cells are expanded for freezing and generating of stock solutions of monoclonal antibody (Sikora and Smedley 1984; Catty 1988; Harlow and Lane 1988; Goding 1993).

1.2.2.3.1 Single cell cloning

As soon as positive wells are identified, single cell cloning is important to reduce the risk of overgrowth by non antibody producing cells, and to ensure that the antibodies are truly monoclonal. There are two strategies used for single cell cloning. The first method of cloning is by growth in soft agar such as agarose gel. Typically,

two layers are used. A first under layer is consisting of 0.5 % agar in culture medium. A second upper layer is soft agar layer (0.3%agar) which contains the cell to be cloned is added. Cells divide to form clusters in soft agar that look like tiny spheres. The cluster can be picked by a fine pasture pipette and plated out into a microwell containing liquid medium for reculturing before antibody production. (Sikora and Smedley 1984; Catty 1988; Harlow and Lane 1988; Goding 1993).

The second method of cloning is limiting dilution. The hybrid suspension is diluted and distributed into a 96-well tissue culture plate. The dilution is calculated to contain on average a single in each well. Of cause some wells will receive no cells and no growth will occur, and some wells have more than one cell so that oligoclonal antibodies will result. After growing up, the initial cloning process can be repeated several times to ensure true monoclonality (Sikora and Smedley 1984; Catty 1988; Harlow and Lane 1988; Goding 1993)

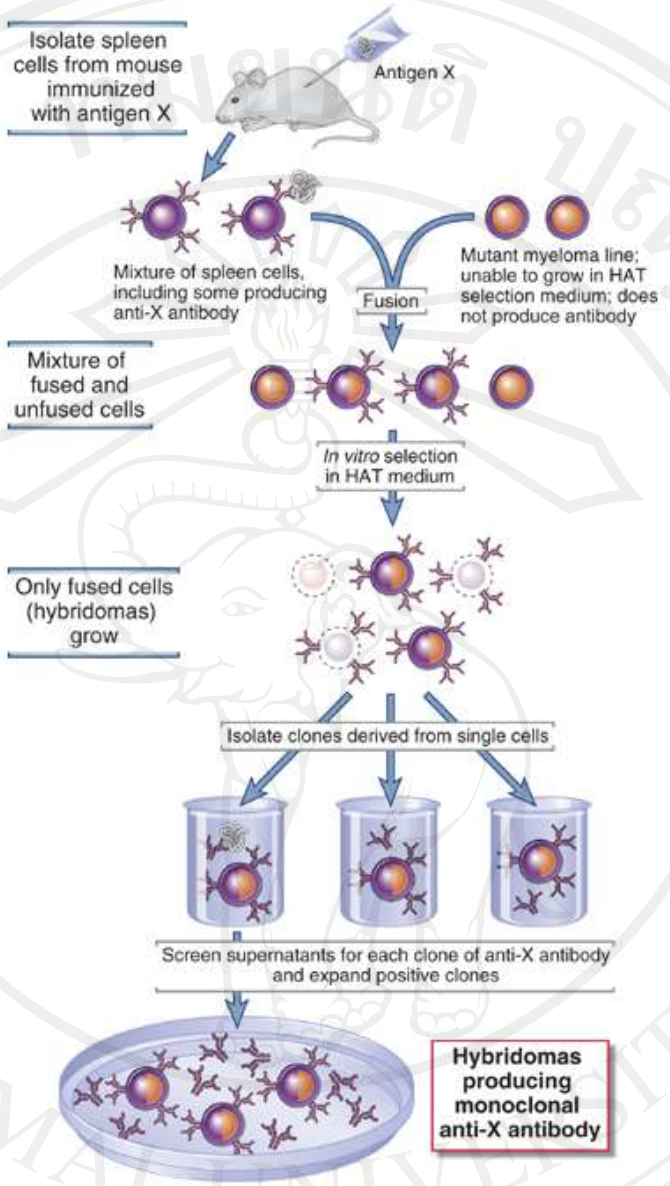

Figure 1.5 The generation of monoclonal antibodies by hybridoma technique (Abbas *et al.* 2012).

1.2.3 Feeder cells

Newly fused hybridomas and the hybridoma cells when growth at low density is often growth poorly or die. The reasons for these are still not well understood. Several reports suggested that hybridoma growing requires some growth factors. Therefore, to overcome the slowly grow or cell dead problems of hybridomas when culture at low cell density, the hybridomas have to culture together with a slow-growing or non-growing cells which provide hybridoma's growth factors. The co-culture cells are usually termed "feeder cells". These feeder cells release the growth factors necessary for growth of hybridomas. Feeder cells in hybridoma technique commonly are mouse thymocytes, mouse spleen cells, peritoneal cells, fibroblasts or murine bone marrow-derived macrophages (Galfre and Milstein 1981; McCullough *et al.* 1983; Long *et al.* 1986; Harlow and Lane 1988; Hlinak *et al.* 1988; Goding 1993; Hoffmann *et al.* 1996), (Hoffmann *et al.* 1996). One of the popular used feeder cells is peritoneal cells. In using of peritoneal cells as a feeder cell, peritoneal cells are harvested by using a syringe and needle wash out the peritoneal cavity with sterile saline. This technique has to avoid puncturing the gut because it risk to contamination. Roughly half are lymphocytes and half are macrophages. If the mouse is from specific pathogen-free colonies, yields will be $3\text{-}5x10^6$ cells per mouse. Conventional mice will yield up to 10 times as many cells (Goding 1993). The obtained peritoneal cells are washed and cultured together with the fused cells in the culture wells. The peritoneal cells in the culture will release growth factors which support the hybdidoma growth. As peritoneal cells are non-dividing cells, so the cells will be not interfere hybridoma cell growing and will die within a week.

The using of feeder cells in hybridoma technique has several disadvantages. Co-culture of feeder cells may deplete media of nutrients required by growing hybridomas. They sometimes overgrow and kill newly formed hybridomas. They also represent a possible source of contamination. Their preparation insufficient quantities and reproducible quality (Hoffmann *et al.* 1996). Finally, more than one mouse was satisfied to prepare feeder cells in one fusion. To circumvent these problems, several research groups have studied and developed alternatives for replacing of the feeder cells. Cell conditioned culture supernatants containing hybridoma growth factors were established and used to replace feeder cells for promoting newly fused hybridoma growth and hybridoma growth at low cell density. These cell conditioned culture supernatants usually termed "conditioned medium".

1.2.4 Conditioned medium

B-cell growth is affected by many factors or cytokines produced by several cell types (Kishimoto 1985; Oliver *et al.* 1985; Abbas *et al.* 2012). As hybridomas are derived from B cells, to support growth of hybridomas, these factors or cytokine should be presented in the hybridoma cultured. Over the past decades, several researchers have developed conditioned medium for supporting hybridoma growth (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; Ian 2000). The developed conditioned medium was demonstrated to contain some cytokines. For example, IL2 and BCGF are found in conditioned medium obtained from EL4 thymoma cells stimulated with phorbol myristate acetate (Howard and Paul 1983; Nakanishi et al. 1984; Kishimoto 1985). IL-1-α, IL-6, TNF-α and TNF-β are presented in the conditioned medium derived from fibroblast cell line (Ling 2000).

As conditioned media are produced in an order to use as an alternative to feeder cells, it is normally prepared from culturing of homologous cells or cell line such as thymocytes, fibroblast, normal spleen cells, endothelial cells, macrophages and T 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). For example, in the EL4-culture system, the murine thymoma cell line can produce factors support the growth and differentiation of B lymphocyte. So, a thymoma cell line, EL4, was used for preparation of conditioned media for support hybridoma growth. In this system, EL4 thymoma cells were cultured at a cell concentration of $1x10^6$ cells/ml and activated with 10 ng/ml of phorbol myristic acetate (PMA). The cells were cultured in tissue culture flasks for 40 hours (Hoffmann *et al.* 1996). After incubation, the culture supernatants were harvested by centrifugation and filtration to avoid the carrying cell over from the conditioning cells. This condition medium is used as a supplement in selective HAT medium in hybriboma technique or culture medium in the case of single cell cloning. The EL4 conditioned medium was demonstrated to substitute feeder cells in supporting of hybridoma growth.

Nowadays, several types of condition medium have been produced using different cell types. Some techniques of conditioned media preparation were transfer to private companies for the commercial preparations and generally sale. Several condition media are available as commercial products such as BM condimed H1 (Roche), Hybridoma Cloning Factor (PAA), Conditioned Media or Hybridoma Enhancing Supplement (SIGMA), Briclone (QED Bioscinece) and Nutridoma CS (Roche). One of conditioned medium which is widely used is BM condimed H1. The

detail of a condition medium, BM Condimed H1, is described below. BM Condimed H1 is prepared from the supernatant of an EL4 mouse thymoma cell line which has been stimulated with PMA for 24 hours (Farrar *et al.* 1980; Grabstein *et al.* 1986). The BM condimed H1 is supplied as a sterile filtered solution in RPMI 1640. The solution also contains 15% FCS (fetal calf serum) (v/v), 1 mM oxalacetate, 1 mM sodium pyruvate, 0.2 μg/ml insulin, 1 ng/ml hIL-6, 10 ng/ml PMA, and phenol red. The BM condimed H1, therefore, contains a complex mixture of growth factors and cytokines that stimulate growth of hybridomas after fusion and during single cell cloning.

Although, the commercial conditioned media can be suitable used as supplement for supporting hybridomas growth, the commercial conditioned media are very expensive. This, therefore, makes the high cost of monoclonal antibody production. In this thesis proposal, we are interesting in the study and preparation of a "home-made" conditioned medium. In our study, a BW5147 mouse thymoma cell line will be used to prepare conditioned medium for hybridoma technology in either the promoting growth of the hybridomas after cell fusion or during single cell cloning procedure. BW5147 is mouse thymomas that are developing T cells in the thymus. As mouse thymomas can be effectively used for production of conditioned medium (Farrar *et al.* 1980; Grabstein *et al.* 1986; Micklem *et al.* 1987), the BW5147 cells was therefore exploited in this study.

1.2.5 Technologies for enhancing the production of monoclonal antibody

As mentioned above, by the standard hybridoma technique, a major problem that should be considered is that very low number, perhaps none, of hybridoma

producing mAb of interest was always achieved. In some circumstance, after cell fusion, even many hybridoma clones are determined but hybridoma clones produced interesting mAbs are not or rarely obtained. To overcome this problem, many researchers had developed several strategies for enhancing the production of monoclonal antibody. Isolation of B cells from the splenocytes prior to cell fusion has been introduced (Tomita *et al.* 2001; Tomita *et al.* 2006; Lin *et al.* 2010). By this strategy, increasing probability for B-myeloma cell fusion was proposed. In addition, Tomita and his colleagues developed a novel technique, antigen specific B-cell targeting method that can yield mAbs against antigens with high efficiency and specificity (Tomita *et al.* 2001; Tomita *et al.* 2006). Theoretically, the technique comprises three critical steps. Firstly, antigen-based pre-selection of B lymphocytes, spleen cells of the immunized mouse were collected and mixed with antigen labeled with avidin. B-lymphocyte–antigen-avidin was formed to complexes. Secondary, formation of B-lymphocyte–myeloma cell complexes, spleen cell suspensions containing antigen-selected B lymphocytes were mixed with biotinylated myeloma cells to form biotin-avidin interactions. B-lymphocyte-antigen-avidin-biotinylated myeloma cell complexes were result. The last step is selective fusion of B-lymphocyte-myeloma cell complexes, B-lymphocyte-myeloma cell complexes were fused by PEG-mediated method or electrical pulses.

Recently, Lin and her colleagues have developed a simplified procedure for efficient generation and selection of antibody-producing hybridomas (Lin *et al.* 2010). A cytoflow reactor-based cell sorter (CBCS) system was developed in which cell fusion was performed on the newly designed bioreactor. Selection of the specific B-cells and the subsequent fusion of B-cells with myelomas can be accomplished in the

bioreactor within 1 h. Briefly, BALB/c mice were immunized using A549 cell that high express the surface marker, EGFR, as a model antigen. CBCS system contained the bamboo charcoal conjugated with purified human EGFR. The suspension of splenocytes was added into CBCS. The specific anti-human EGFR secreted B-cells would bind to the surface of the human EGFR-conjugated bamboo charcoals. Then non-specific B-cells were flushed out when the medium exceeded the tide line of CBCS. Thus, the specific anti-EGFR antibody bearing B-cells were retained on the human EGFR-conjugated bamboo charcoal. The myelomas/PEG mixture was incubated for 1 min at 37°C with gentle agitation. The mixture was then added to the CBCS over 4 min at 37°C with gentle agitation for cell fusion. Further, the fusion reagent and non-fusion myeloma cells were flushed out. After this, the fused cells on the bamboo charcoal were suspended in HAT selection medium. After 10 days, the medium was altered by changing the HAT selection medium to normal hybridoma culture medium. The specific hybridoma cells, which can produce mAbs, were left bound to the bamboo charcoal surface. CBCS provide an alternative for specific hybridoma production in a single step, consisting of the B-cell selection, the fusion of B-cell and myeloma, hybridoma cultivation and antibody secretion.

Moreover, the post-fusion selection method was also established (Ossendorp *et al.* 1989). By this method, magnetic beads coating with antigen were used to separate for antigen-specific B cell hybridomas. Briefly, BALB/c mice were immunized intraperitoneally with human thyroglobulin. The spleen cells from immunized mice were fused with myeloma cells. The fused cells were cultured in HAT medium. This culture was used for antigen-specific selection by using antigen-coated magnetic beads. The stable B cell hybridoma cell lines directed against human thyroglobulin

were incubated with thyroglobulin-coated beads for 2 hours of incubation at 4 °C. Rosette formation was isolated by strong magnet. The separated cells were plated in 96-well culture plates. After 10 days the culture supernatants were tested for anti-thyroglobulin antibodies using an ELISA. Antigen-coated magnetic beads were found to be suitable for the efficient selection of thyroglobulin specific hybridoma cells from bulk cultures shortly after fusion.

In this study, we also aim to develop high efficiency techniques for generation of hybridomas producing antibody of interested. Pre-isolation of B cells or antigen specific B cells will be carried out, prior to cell fusion, by employing the magnetic cell sorting system (MACS). Protocol for cell fusion involving the small cells number will be developed. These approaches are expected to enhance the cell fusion efficiency and give rise to high numbers of hybridoma produced antibody of interest. In addition, B cells carrying desired isotype of antibody will be isolated by MACS and fused with myeloma cells. This strategy will allow us to produce mAbs having the desired isotype.

1.2.6 *In vitro* immunization

In vitro immunization is the method where the antibody response can be activated in vitro, instead of direct immunizing antigen into animals. Although, *in vitro* immunization strategy have been described several years ago, by hybridoma technique, *in vivo* immunization still be a routine laboratory procedure. 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. Several researchers have introduced the used of an *in vitro* immunization protocol for production of mAb. Tomita and his colleagues confirmed this strategy and reported

that expression of surface immunoglobulin receptors on B lymphocytes was recognized even after immunization *in vitro*. The number of the antigen-selected B lymphocytes after *in vitro* immunization was in fact higher than that obtained after *in vivo* immunization, suggesting that such short-term immunization is applicable for B-cell targeting (Tomita et al. 2006). In this study, *in vitro* immunization method will develop and apply for mAb production the in our laboratory.

1.3 Objectives

1.3.1 To develop conditioned medium for hybridoma production

1.3.2 To develop hybridoma technique using pre-isolation of B cell strategy

1.3.3 To develop hybridoma technique using pre-isolation of antigen specific B cell strategy

1.3.4 To develop hybridoma technique for production of monoclonal antibody carrying a desired isotype

1.3.5 To optimize the conditions of *in vitro* immunization for polyclonal and monoclonal antibody production