CHAPTER I

INTRODUCTION

1.1 Statement and significance of the problem

Immunoglobulins or antibodies are glycoproteins produced by B lymphocytes in response to antigens. Specific binding property of the antibody to its recognized antigen leads to the use of antibodies in 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. Because plasma cells can not grow *in vitro*, they can not be used as an *in vitro* source of antibodies. In 1975, Kohler and Milstein developed a technique, named hybridoma technique, that allows the growth of clonal populations of cell secreting antibodies with a defined specificity. By hybridomas technique, mouse is immunized with antigen of interest. Splenocytes, isolated from the immunized animal, are fused with immortal myeloma cancer cells (Kohler and Milstein, 1975). After cell fusion, the immortalized antibody-producing cell lines, called hybridomas, are generated and the antibodies they produced are termed monoclonal antibodies (mAbs). By the hybridoma technique, after cell fusion the fused cells are usually plated into 96 well-plates. By this procedure, the 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

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cloned hybridomas can be maintained *in vitro* and will continuously secrete antibodies with a define specificity (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.*, 2000).

Newly fused hybridomas and 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 requirements of growth factors. To overcome these problems, hybridoma cells may culture together with a slow-growing or non-growing cells which secrete growth factors. These cells usually termed “feeders”. Feeder cells are believed to supply growth factors that promote growth of the hybridoma cells. Several types of cells are reported to be used as feeder cells for hybridoma growing. Commonly used feeder cells including thymocytes, spleen cells, peritoneal cells, murine bone marrow-derived macrophages and fibroblasts (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). A major difficulty for using these feeder cells is their preparation always insufficient quantities and reproducible quality. Moreover, preparation of feeder cells will increase risk of contamination and more than one mouse will be sacrificed. 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 are normally 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). For example, in the EL4-culture system,

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murine EL4 thymoma cells were used to produce conditioned medium for supporting the growth hybridomas. In this system, mouse EL4 thymoma cells are stimulated with phorbol myristate acetate (PMA) and the culture supernatant was harvested for the source of conditioned medium. This conditioned medium can support the growth of hybridomas, instead of feeder cells (Farrar *et al.*, 1980; Grabstein *et al.*, 1986). Nowadays, the technologies for preparation of conditioned media established by various groups of researchers were already transferred to private companies for commercial productions and generally sale. Although, the commercial conditioned media can be used as supplement for supporting hybridoma growth, the available commercial conditioned media however are very expensive. This, therefore, makes the high cost of monoclonal antibody production.

In this study, we have been interested in the study and preparation of conditioned medium for promoting growth of the hybridomas after cell fusion or during single cell cloning. The objectives of this study are to produce conditioned medium that can be replace expensive commercial conditioned medium. The produced conditioned mediums will be employed for generation of hybridoma cells in hybridoma technique and single cell cloning. The used of “home-made” conditioned medium will lead to the reduction of the expense of monoclonal antibody production.

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1.2 Literature reviews

1.2.1 Introduction to polyclonal and monoclonal antibody

All higher animals have the ability to recognized foreign and potentially harmful molecules entering their bodies. A substance capable of exciting such a reaction from the immune system is called an antigen. When challenged with a pathogen or antigen, the immune system of higher vertebrate responds by making an immune response that is aimed at the specific elimination of the pathogen or antigen from the body. One of the earliest experimental demonstrations of specific immunity was the induction of humoral immunity against microbial toxin. In the early 1900s, patients with life-threatening diphtheria infection were successfully treated by the administration of serum from horse immunized with diphtheria toxin. This form of immunity, called humoral immunity, is mediated by a family of structurally related glycoproteins called antibodies, which have affinity for and specific to the antigen determinant to which its binds. In exploiting the specific binding properties of antibody one has a choice between two very different forms of reagent, there are conventional polyclonal antisera and monoclonal antibodies (Sikora and Smedley, 1984; Goding, 1993; Abbas *et al.*, 2000).

The immune response in an animal is polyclonal in nature, and a mixture of many different antibody specificities to the various epitopes of the structurally complex immunogen. This polyclonal response to antigen is a result of the activation of a large number of different clones of B cells, each clone encoding a single immunoglobulin type with a single specificity for antigen, interacting and cooperating together with other cells of the immune system, including the thymus-derived T lymphocytes and antigen-presenting cells. A polyclonal antiserum is the conventional serum product of an immunized animal that usually a rabbit, sheep or goat. The major advantage of polyclonal antisera lies in their capacity to form large insoluble immune

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complexes with antigen, or to agglutinate cells readily so that the reactions can be seen and measured visually or determined photometrically. Because of their polyclonal, multispecific nature, conventional antisera can not 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.*, 2000).

1.2.2 Monoclonal antibody

The first isolation of a homogeneous population of antibodies came from studies of B cell tumors. Clonal populations of these cells can be propagated as tumors in animals or grow in tissue culture *in vitro*. Because all of the antibodies secreted by a B cell clone are identical, these tumor cells provide source homogeneous antibodies. Unfortunately, B cell tumors secreting antibodies of a predefined specificity can not be isolated conveniently. In vertebrates, antibodies are synthesized primarily by plasma cells, a type of terminally differentiated B lymphocyte. Because plasma cells can not be grown in tissue culture, they can not 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. During cultivation and propagation,

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these immortalized B cells can be grown as single clones of cells, each of which secrets 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.*, 2000).

1.2.2.1 Hybridoma technology

As mention earlier, in 1975, Köhler and Milstein who first immortalized antibody-secreting lymphocyte by fusing them with cells from a continuously growing cell line and then cloned individual hybrid cells to produce lines of cells each of which 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 or 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 (Figure 1.1). The immortalized antibody-producing cell lines could be established routinely and maintained *in vitro*. The characteristics required, which are actively selected for, 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.*, 2000).

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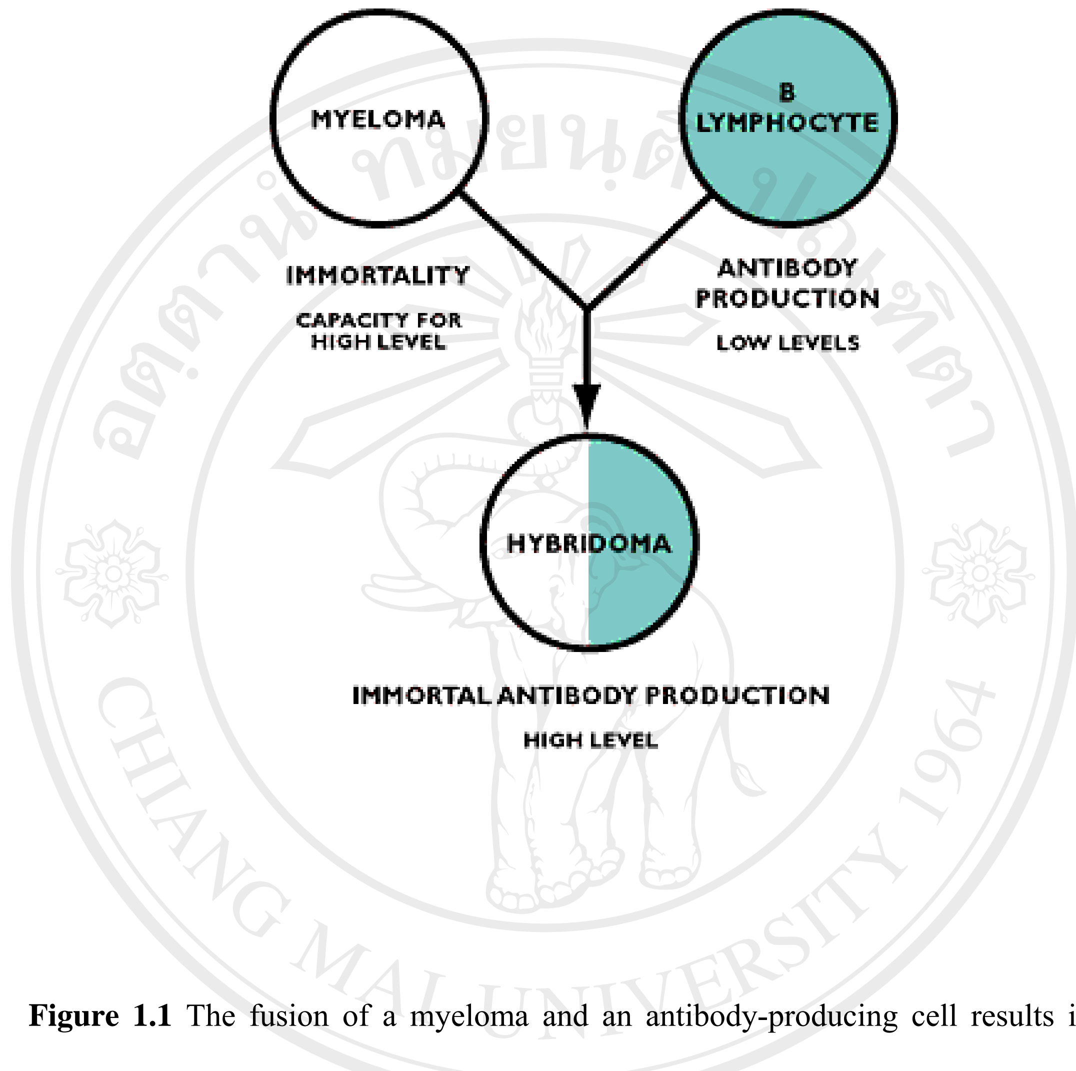

Figure 1.1 The fusion of a myeloma and an antibody-producing cell results in an immortal antibody-producing hybridoma. *(http--www2_mrc-lmb_cam_ac_uk-archive-Milstein-MONOCLONAL_ANTIBODIES_gif accessed 24 August 2007)*

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Early work solved the three technical problems for achieving a successful fusion.

(1) Finding appropriate fusion partners: Myelomas from BALB/c mouse are good cells for fusion. Myelomas 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.

(2) Defining conditions for efficient fusion: The fusion between the myeloma cell and the antibody-secreting cell can be effected by any fusogen. In the practice, hybridoma fusions became routine after the introduction of the use of 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.

(3) Choosing an appropriate system to select for hybrid cells against the background of unfuse cells: After cell fusion, it is always that not all the myeloma

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cells will have fused. Indeed, the majority will remain growing healthily in an unfused state. As these cells grow faster than the hybridomas they would rapidly out grow them. Therefore, unfused myeloma cells need to be eliminated and usually by drug selection. Hybridomas technique requires cultured myeloma cell line that 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, animal cells synthesize purine nucleotides and thymidylate, both precursors of DNA, by a *de novo* pathway requiring tetrahydrofolate. Antifolate drugs, such as aminopterin, 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, these cells grow normally in the presence of aminopterin if the culture medium is also supplemented with hypoxanthine and thymidine (called HAT medium) (Figure 1.2). In contrast, myeloma cells that used as fusion partner are defected in enzyme hypoxanthine phosphoribosyltransferase, HPRT or HGPRT, an enzyme of a salvage pathway. Therefore, these cells can not use the salvage pathway for nucleotide synthesis. Myeloma cells, which defecting in a salvage pathway, can not 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 generated between myelomas with nonfunctional HGPRT and cells with functional HGPRT will be able to grow (Figure 1.4). Myeloma cell lines can be made defective in HGPRT or TK by mutagenesis. Selection of $HGPRT^-$ cells

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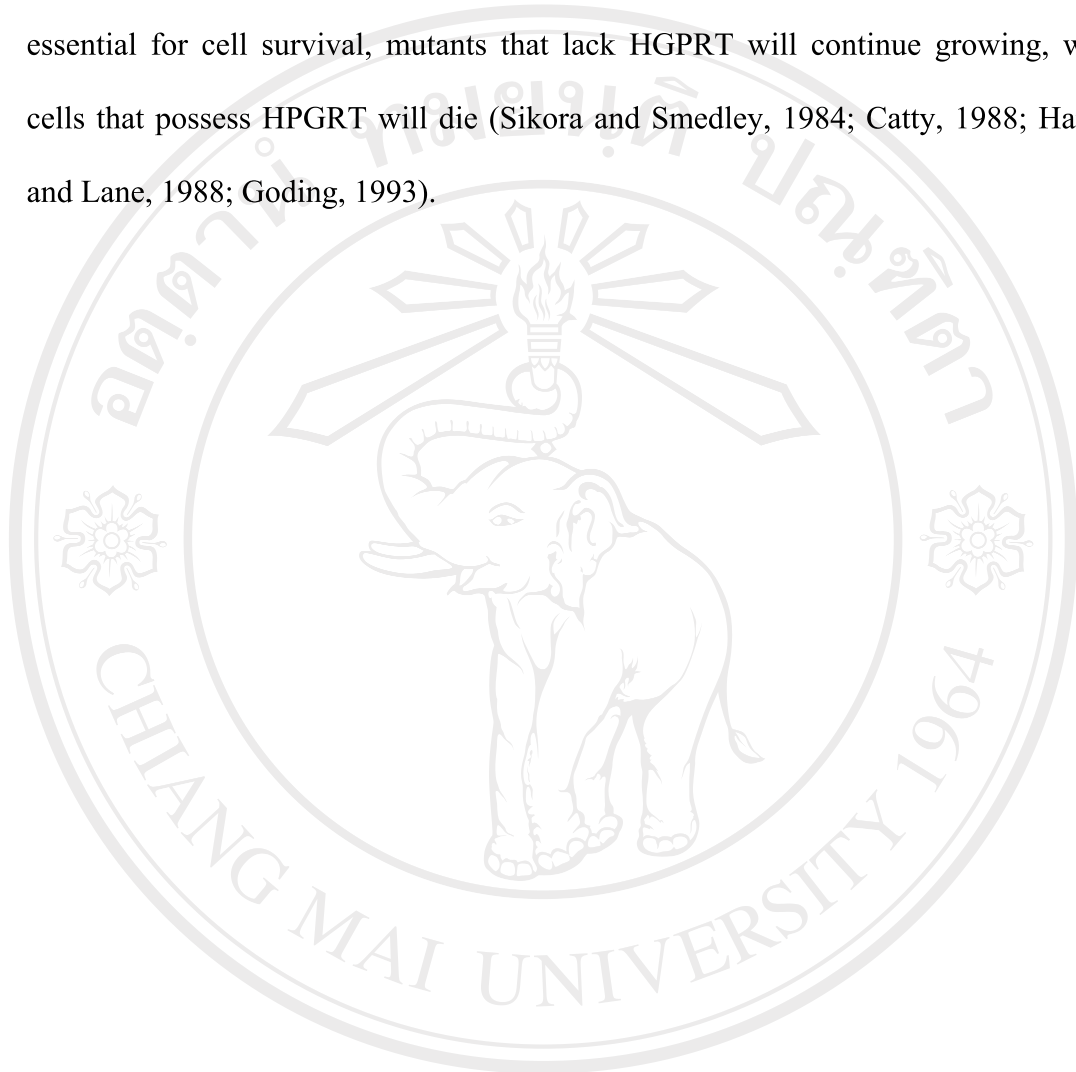
is performed by use of the toxic base analogues 8-azaguanine or 6-thioguanine, which are incorporated into DNA via HGPRT. Because the salvage pathway is not normally essential for cell survival, mutants that lack HGPRT will continue growing, while cells that possess HPGRT will die (Sikora and Smedley, 1984; Catty, 1988; Harlow and Lane, 1988; Goding, 1993).



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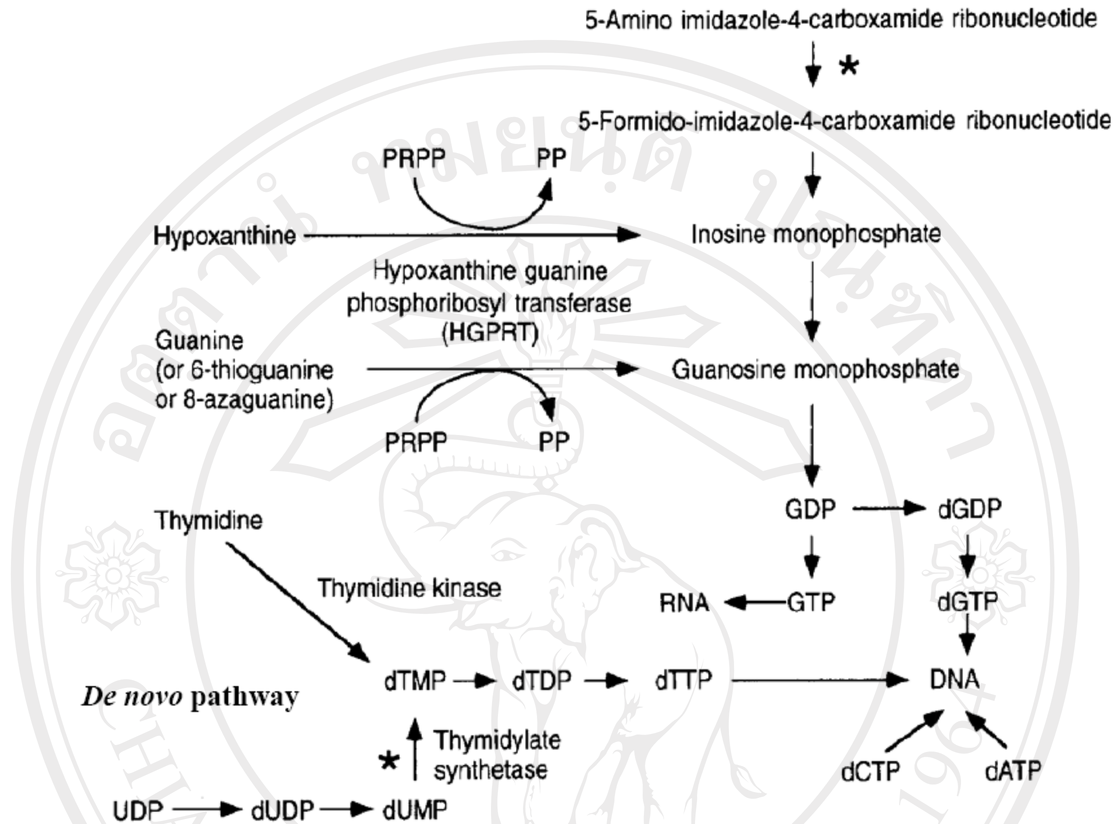

Figure 1.2 Metabolic pathways relevant to hybrid selection in medium containing hypoxanthine, aminopterin and thymidine (HAT medium). When the *de novo* pathway are blocked with folic acid analogue aminopterin (*), cell must depend on the salvage pathway (Goding, 1996).

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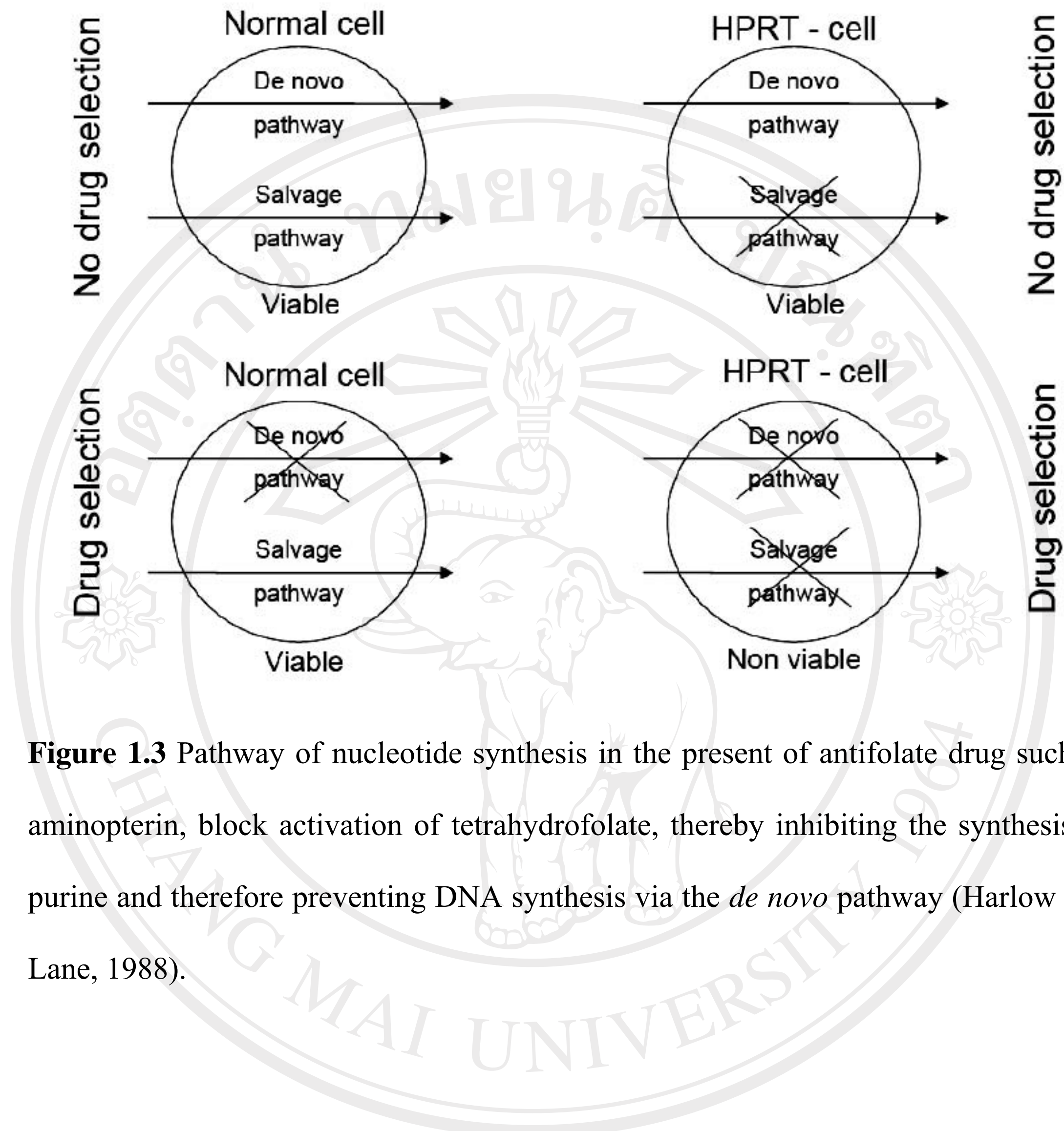

Figure 1.3 Pathway of nucleotide synthesis in the present of antifolate drug such as aminopterin, block activation of tetrahydrofolate, thereby inhibiting the synthesis of purine and therefore preventing DNA synthesis via the *de novo* pathway (Harlow and Lane, 1988).

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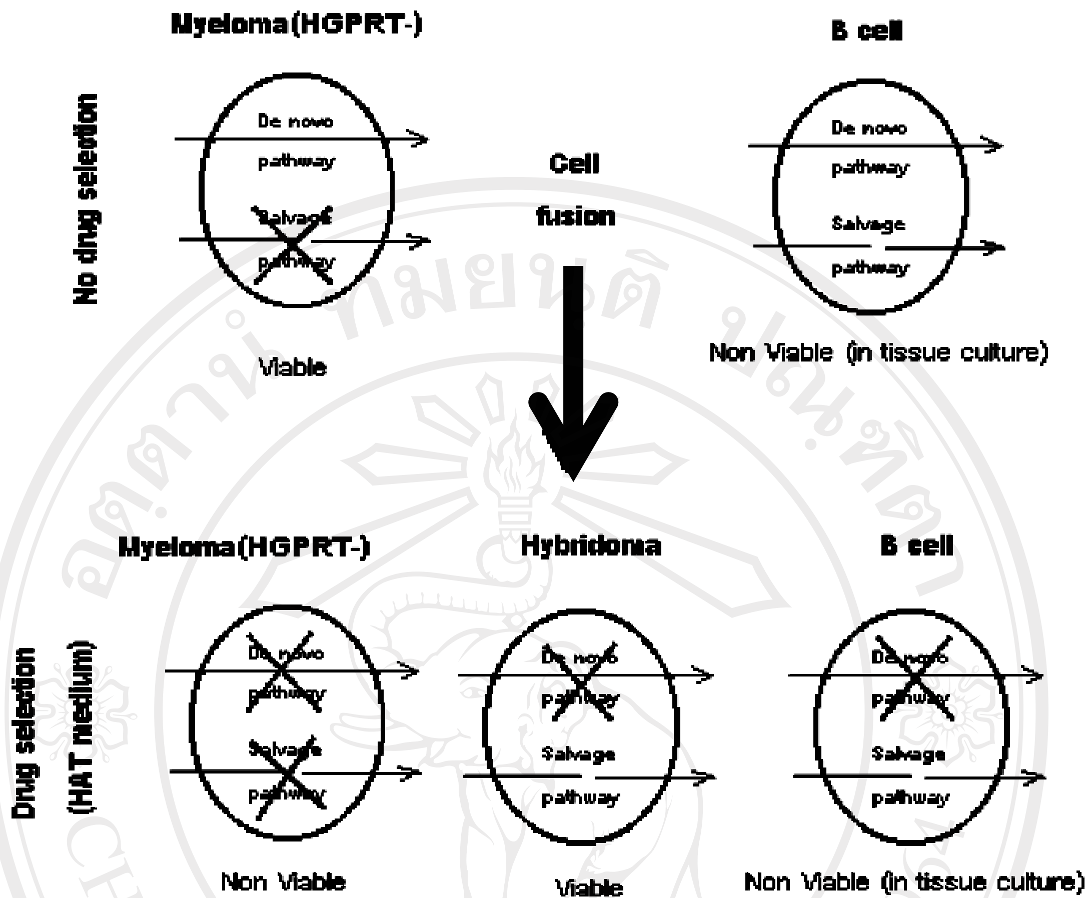

Figure 1.4 Drug selections for viable hybridomas. Hybrid between myelomas with nonfunctional HGPRT and cells with functional HGPRT will be able to grow in the present of antifolate drug, such as aminopterin (Harlow and Lane, 1988).

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In hybridoma technique, to produce a monoclonal antibody specific for a defined antigen, a mouse is firstly immunized with that antigen. After appearing of the antibody response, splenocytes are isolated from the spleen or lymph node of the immunized mouse. These splenocyte are then fused with myeloma cells by a fusogen, usually PEG. After cell fusion, un-fused B cells, un-fused 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); under these conditions, un-fused myeloma cells and myeloma-myeloma hydrids die because they are deficient in an enzyme required for the salvage pathway of nucleotide synthesis. In HAT medium, these cells are died because aminopterin blocks normal nucleotide synthesis and the enzyme deficiency blocks utilization of hypoxanthine or thymidine in the salvage pathway. So that only hybrid cells between myeloma cells fused to normal cells survive. The outcome hybridomas can survive indefinitely in culture medium because the normal cells supply the missing enzyme for selection in HAT medium and the myeloma cells immortalize the hybrid cells. In generally, un-fused normal lymphocytes can survive in culture medium for approximately 1 week then they die because they are not immortalized. Therefore, after long-term culture, only hydridomas of normal and myeloma cells grow in the selective HAT medium. Fortunately, hybrid cells generated from B lymphocyte and myeloma cell fusion can produce antibody. Wells containing the desired antibody produced by growing-hybridomas can be identified by a number of immunoassays such as enzyme-linked immunosorbent assay (ELISA), immunoblot or immunofluorescence assay.

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Since an original positive well will often contain more than one hybridoma clones. So, single cell cloning is required for isolation the mix up clones and is essential to confirm monoclonality of the antibody preparation. Isolation of a single hybridoma cell from a positive well is performed by cloning in either soft agar or limiting dilution. 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; Abbas *et al.*, 2000). Outlines the procedure of monoclonal antibody production by hybridoma technique is shown in figure 1.5.

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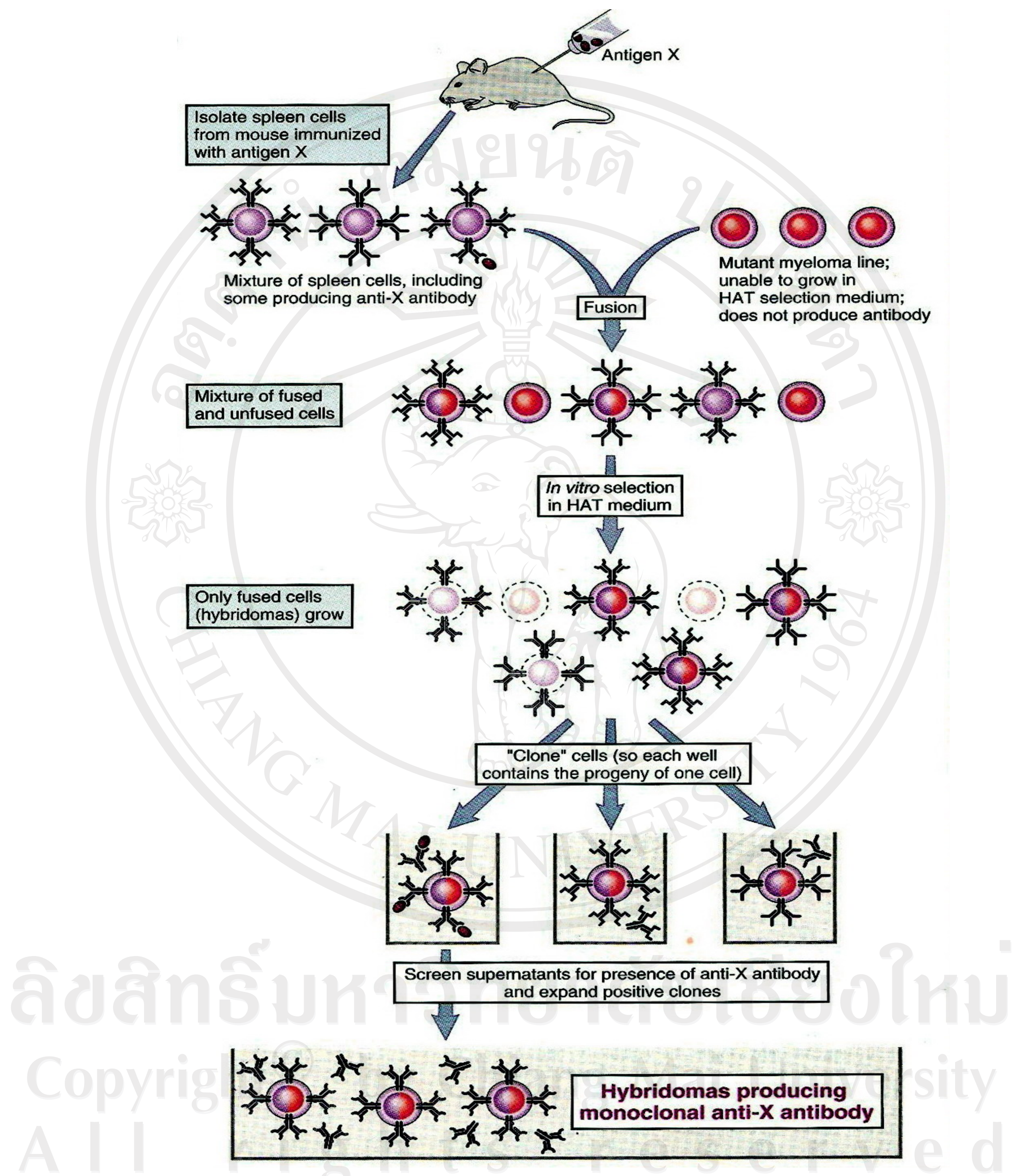

Figure 1.5 Outlines the procedure of monoclonal antibody production by hybridoma technique (Abbas *et al.*, 2000).

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The technology of hybridoma production is firmly established, steps involved in the production of mAbs include the following: (1) immunization protocol, (2) developing the screening procedure, and (3) producing hybridomas (Harlow and Lane, 1988).

1.2.2.1.1 Immunization protocol

Protocols for immunization of animals 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. Possible and usual immunization routes are intravenous (i.v.); intramuscular (i.m.); subcutaneous (s.c.); intraperitoneal (i.p.); and intradermal (i.d.), which is also called intracutaneous. At the same time the immune system of the animal may be stimulated by injecting a mixture of powerful immune stimulants. The most commonly used is Freund's adjuvant this mixture of dead tuberculosis organisms in a fatty base has the effect of priming the immune system to recognize avidly any antigen injection with the mixture. Animals are normally injected on several occasions, 1-3 boosters may be given at intervals of 2-8 weeks, to ensure good stimulation. With each successive immunization there is increased stimulation of the B-lymphocyte clones within the animal responding to the antigen. The final boost of antigen is given 2-5 days before removal of the spleen for cell fusion, and is often given in aqueous form rather than in adjuvant. Some authors recommend that the final boost be given intravenously. But intraperitoneal boosting is safer, and nearly as effective.

The immunization schedule used will depend upon the nature of the immunogen just as it does in the preparation of a polyclonal antiserum. It is importance to determine that mice used for fusion experiments have responded to the

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immunogen. A test bleed is taken from tail vein of mice usually 1 week 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 2-5 days before fusion. It is sometimes difficult to interpret the test bleed result because such a complex mixture of antibodies is present in the serum. However, the test bleed does indicate whether or not the right sort of antibodies is present (Sikora and Smedley, 1984; Catty, 1988; Harlow and Lane, 1988; Goding, 1993).

1.2.2.1.2 Developing the screening procedure

Most hybridoma cells grow at approximately the same rate, the tissue culture supernatants from all of the fusion wells usually are ready to screen within a few days of one another. 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: (1) reduce the number of cultures that must be maintained to a reasonable level, (2) identify positive supernatants or clones in 48 hour or less, and (3) be easy enough to perform for all the needed wells. The selection of hybridomas which produce antibodies of interest usually involves several screening steps. Culture supernatants are first assayed for activity against the immunogen. Positive supernatants are then screened against a test panel of antigen to reveal whether the antibody binds selectively to the antigen of interest. There are three

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classes of screening methods, antibody capture assays, antigen capture assays, and functional screens. 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).

1.2.2.1.3 Producing hybridomas

Once a good immune response has developed in an animal and an appropriate screening procedure has been developed, the construction of hybridomas is ready to begin. For the actual fusion, antibody-secreting cells are isolated from the appropriate lymphoid tissue and mixed with myeloma cells, centrifuged to generate cell-to-cell contacts, and fused with polyethylene glycol (PEG). The fused cells are then removed from the PEG solution, diluted into selective HAT medium, and plated in multiwell tissue culture plates. The hybridomas can be selected in HAT-containing medium. Beginning approximately 1 week later, sample of the tissue culture supernatants are removed from wells that contain growing hybridomas and tested for the presence of the appropriate antibodies. Cells from positive wells are grown, single cell cloned, and frozen. After single cell cloning, hybridomas producing antibody of interest will be obtained. Finally, by the generated hybridomas, the monoclonal antibodies are produced for further use (Sikora and Smedley, 1984; Catty, 1988; Harlow and Lane, 1988; Goding, 1993).

1.2.2.1.3.1 Single cell cloning

As soon as positive wells are identified, the hybrid cells should be cloned. Cloning is important to reduce the risk of overgrowth by non antibody producer cells, and to ensure that the antibodies are truly monoclonal. There are two strategies used for cloning cell in practice. The first method of cloning is by growth in

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soft agar such as agarose gel (Figure 1.6). Typically, two layers are used. A firm under layer, consisting of 0.5 % (w/v) agar in culture medium, is allowing to gel. A second soft agar layer (0.3%agar) which contains the cell to be cloned is added. Cells divide to form clusters that look like tiny spheres. The cluster can be picked by a fine pasture pipette and plated out into a microwell for future culture. Cloning in soft agar usually requires the additional step of reculturing in liquid medium before antibody production can be assessed (Sikora and Smedley, 1984; Catty, 1988; Harlow and Lane, 1988; Goding, 1993).

The second method of cloning is the technique of limiting dilution (Figure 1.6). The hybrid suspension is diluted and distributed into a series of sterile wells. The dilution is calculated so that the volume of fluid being placed in each well will contain on average a single cell. 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).

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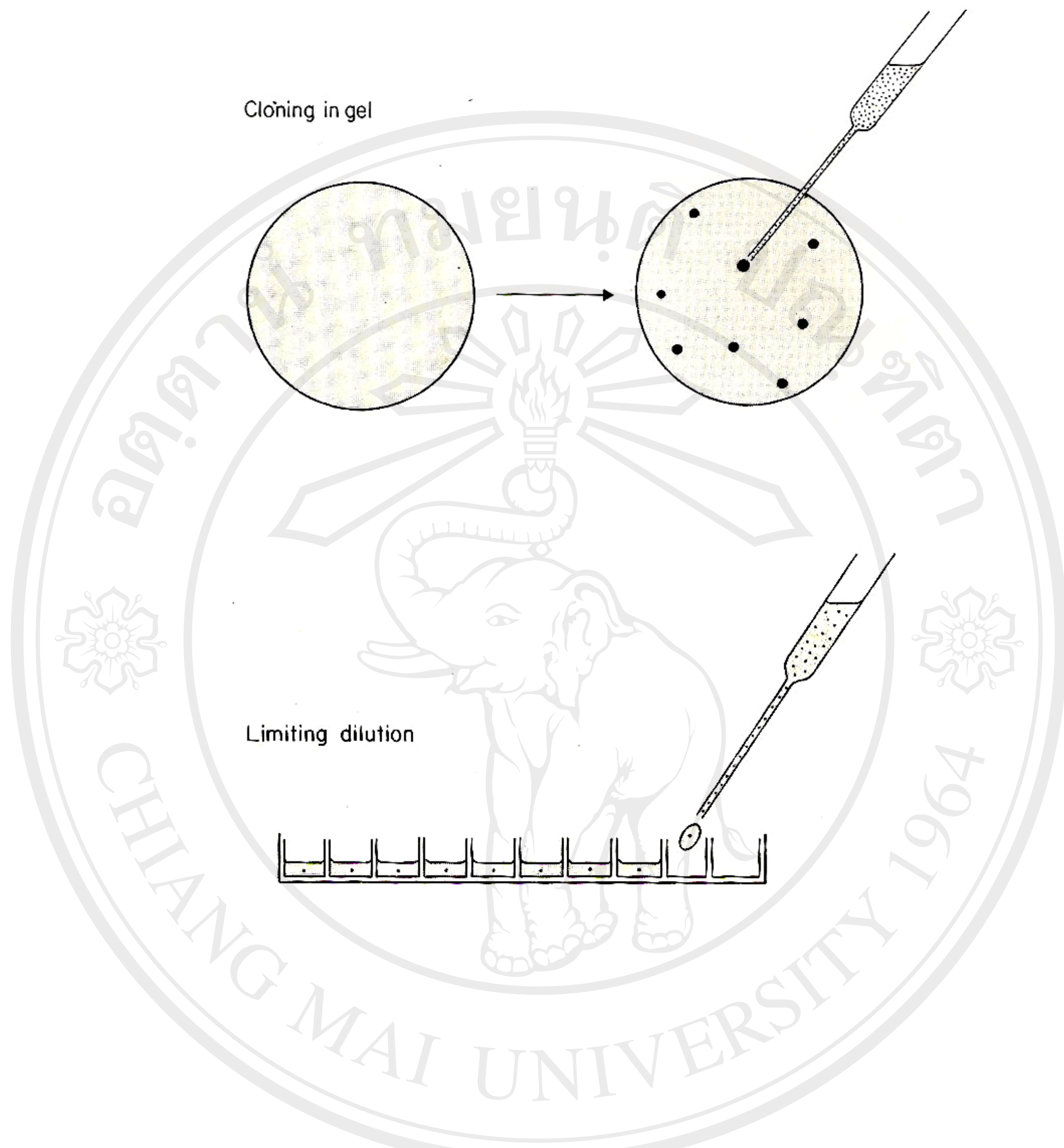

Figure 1.6 Cloning techniques to obtain cloned hybridomas. Uper panel and lower panel are single cell cloning by soft agar cloning and limiting dilution, respectively. (Sikora and Smedley, 1984).

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1.2.3 Feeder cells

Because newly fused hybridomas and the cell when growth at low density often grow poorly or die. The reasons for these are still not well understood. Several reports suggested that hybridoma growing requires some growth factors. Therefore when culturing of hybridomas at low cell density is demand, culturing hybridoma together with a slow-growing or non-growing cells is needed to overcome the slowly grow or cell dead problems. The cells used in co-culture are usually termed "feeder cells". In the culture, feeder cells release the growth factors necessary for growth of hybridomas. Commonly used feeder cells in hybridoma technique include mosue thymocytes, mouse spleen cells, peritoneal cells, fibroblasts (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), or murine bone marrow-derived macrophages (Hoffmann *et al.*, 1996). Example for using feeder cells in hybtidoma technique, in the case of using peritoneal cells as a feeder cell, peritoneal cells are harvested by washing out the peritoneal cavity with sterile saline, using a syringe and needle and taking care to avoid puncturing the gut. Roughly half are lymphocytes and half are macrophages if the mice are from specific pathogen-free colonies, yields will be 3-5 x 10^6 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-devding cells, the cells will not interfere hybridoma cell growing and will dye within a week.

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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 were satisfied for preparing of feeder cells in one fusion. To circumvent these difficulties, several research groups have studied an alternative for replacing of the feeder cells. As the supporting of hybridoma growth by feeder cells, it was demonstrated to be due to its produced growth factors. Cell conditioned culture supernatants were then used to replace feeder cells for promoting freshly fused hybridoma growth. These cell conditioned culture supernatants usually termed "conditioned medium".

1.2.4 Conditioned medium

As mentioned previously, condition media are produced in an order to use as an alternative to feeder cells. Conditioned media are normally prepared from homologous cells or a different cell line such as thymocytes, fibroblast, normal spleen cells, endothelial cell or macrophages (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, where murine thymoma cell line can support the growth and differentiation of B lymphocyte. A thymona cell line, EL4, was used for preparation of conditioned media for support hybridoma growth. In this system, EL4 thymoma cells were resuspended to a cell concentration of $1x10^6$ cells/ml in culture medium. The cells were cultured in tissue culture flasks for 40 hours in the presence of 10

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ng/ml of PMA (Hoffmann *et al.*, 1996). At the end of the incubation period, the culture supernatants were harvested by centrifugation and filtration to avoid the risk of carrying any cell over from the conditioning cells. The obtained culture supernatants were then frozen at -70°C until used. 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.

Nowadays, several types of condition medium have been produced. Various cell types and different cultured conditions are employed for production of the condition media. The techniques for preparation of conditioned media were transfer from researchers to private companies for the commercial preparations and generally sale. At the presence, 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). The detail of a condition medium, BM Condimed H1, is described below as an example. 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 cloning.

As same as BM condimed H1, all conditioned media contain a mixture of growth factors and cytokines that stimulate growth of hybridomas after fusion and

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during cloning. The product description, instruction for use and applications of the mentioned conditioned media are enclosed in the appendix D.

In this study, the researcher has interested on the study and preparation of conditioned medium to promote growth of the hybridomas after fusion or during single cell cloning. The objectives of this study are to produce conditioned media for replacing the expensive commercial conditioned media. The conditioned media were produced in our laboratory, low cost and can be reduced import of commercial reagents.

1.3 Objectives

1.3.1 To prepare the conditioned medium for hybridoma production for monoclonal production.

1.3.2 To prepare the conditioned medium for hybridoma single cell cloning

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