CHAPTER I

INTRODUCTION

1.1 Statement of problems

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, diagnosis and treatment of diseases (Coakham et al., 1984; Cole et al., 1984; James et al., 1984; Clark, 1986). In 1975, Kohler and Milstein devised a method of growing large numbers of antibody producing cells from a single B-cell by fusing B-cells from the immunized mouse with immortal myeloma cancer cells (Kohler and Milstein, 1975). The immortalized antibody-producing cell lines are called hybridomas and the antibodies they produced are termed monoclonal antibodies (mAbs). In hybridoma technique, mouse is firstly immunized with an immunogen. After antibody response is induced, splenocytes of the immunized mouse are fused with myeloma cells. Then the hybridoma producing mAb of interest are screened. The obtained hybridoma cells are maintained for using as the source of antibody production. In general, preparation of immunogen is one of the most important step involving in the production of monoclonal antibodies. Various techniques are employed for isolation and purification of immunogens of interest. However, these processes are laborious and can be expensive.

Recently, by advanced knowledge in molecular biology and biochemistry, various techniques have been developed for preparation of protein antigens. To

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overcome the difficulty of the preparation of immunogen process, in this study, we applied and compared three techniques for preparation of immunogen for monoclonal antibody production. The three techniques studied are immunoprecipitation technique, production of recombinant protein in bacterial expression system and in COS cell expression system. In this study, a leukocyte surface molecule, CD4 protein, was used as a model for reaching the research objectives.

Immunoprecipitation is the technique that widely used for analysis of interested proteins. This technique is involved in the interaction between a protein and its specific antibody. The generated immune complexes are then captured on a solid support such as protein A or protein G coated beads. To characterize the precipitated antigen, the antigen is eluted from the antibody coated beads and analyzed by biochemical techniques, e.g. SDS-PAGE (Harlow and Lane, 1988; Birch and Lennox, 1995; Luttman et al., 2006). By these techniques, as the antigen-antibody reaction is carried out under the native condition, antibodies that are directed against both linear and conformation epitopes can be used to precipitate the specific antigen. In this study, the immunoprecipiation technique was applied for isolation of protein of interest. The specific antibodies were coated on solid beads and incubated with protein mixtures containing protein of interest. After incubation, the interested proteins were captured on the antibody coated beads. By this strategy, the isolated proteins bound beads could be directly immunized into mouse for antibody production.

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Molecular biology and genetic engineering have provided powerful tools for recombinant protein production. The preferred host for recombinant protein expression has historically been *Escherichia coli* (*E. coli*) due to the simplicity and

low costs. Recombinant DNA technology enables the production of large quantities of proteins (Koths, 1995). Methods that will facilitate subsequent protein analysis and purification are of major interest during the initial design of the recombinant protein. Both detection and purification are greatly simplified by engineering the DNA construct so that the encoded protein is fused to a protein fusion partner (LaVallie and McCoy, 1995; Hannig and Makrides, 1998). The biotin carboxyl carrier protein (BCCP) is the post-translationally biotinylated protein in *E. coli* (Duffy et al., 1998). This domain can be fused to recombinant proteins and it can be biotinylated *in vivo* by the endogenous biotin ligase (Bir A) of *E. coli*. The BCCP domain fusion approach has previously been used for site-specific biotin labeling of various recombinant proteins (Smith et al., 1998; Santala and Lamminmaki, 2004). In this study, the *E. coli in vivo* biotinylation of recombinant protein system was applied. The biotinylated protein of interest were sorted by streptavidin beads and used as immunizing agent for antibody production.

In addition to the production of recombinant proteins in prokaryotic cells, eukaryotic cells were also used as hosts for protein production. Many mammalian cell lines have been used to express recombinant proteins via transfection of plasmids or infection of recombinant DNA or RNA viruses. In particular, COS cell expression system has been used successfully to generate recombinant proteins for structural and biochemical analysis and can also be used to search for their natural ligands (Edwards and Aruffo, 1993). COS cells expression system relies on SV40 large T antigen to drive the replication of plasmids containing the SV40 origin of replication (Elder et al., 1981). High-level of plasmid amplification and protein production is the advantage of this host vector system. In this study, the COS cell expression system

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were applied for preparation of immunogen. Plasmid DNA encoding protein of interest were transfected into COS cells. The transfected COS cells which transiently expressed the interested protein were used as immunogen for antibody production.

In this study, the three mentioned methods were used to generate CD4 protein. The prepared CD4 proteins were used as immunization agent for mouse immunization and production of monoclonal antibodies. In this study, the ability of monoclonal antibody production by immunization with each immunogen type will be obtained. The obtained information is very valuable for further production of monoclonal antibody against variety of protein antigens. The knowledge obtained can also be applied for production of antibodies to any interested proteins where the protein antigens are not available or difficult to prepare, but either the encoding cDNA or specific mAb is available.

1.2 Literature reviews

1.2.1 Immune system

The immune system is the system of specialized cells and organs that protect an organism from outside biological influences. When the immune system is functioning properly, it protects the body against infections, destroying cancer cells and foreign substances by making an immune response(Chinen and Shearer, 2005). The immune system consists of innate immunity, which mediates the initial protection against infections, and adaptive immunity, which develops more slowly and mediates the later, even more effective, defense against infections (Figure 1.1). Innate and adaptive immune responses are components of an integrated system of host defense in which numerous cells and molecules function cooperatively. The mechanisms of

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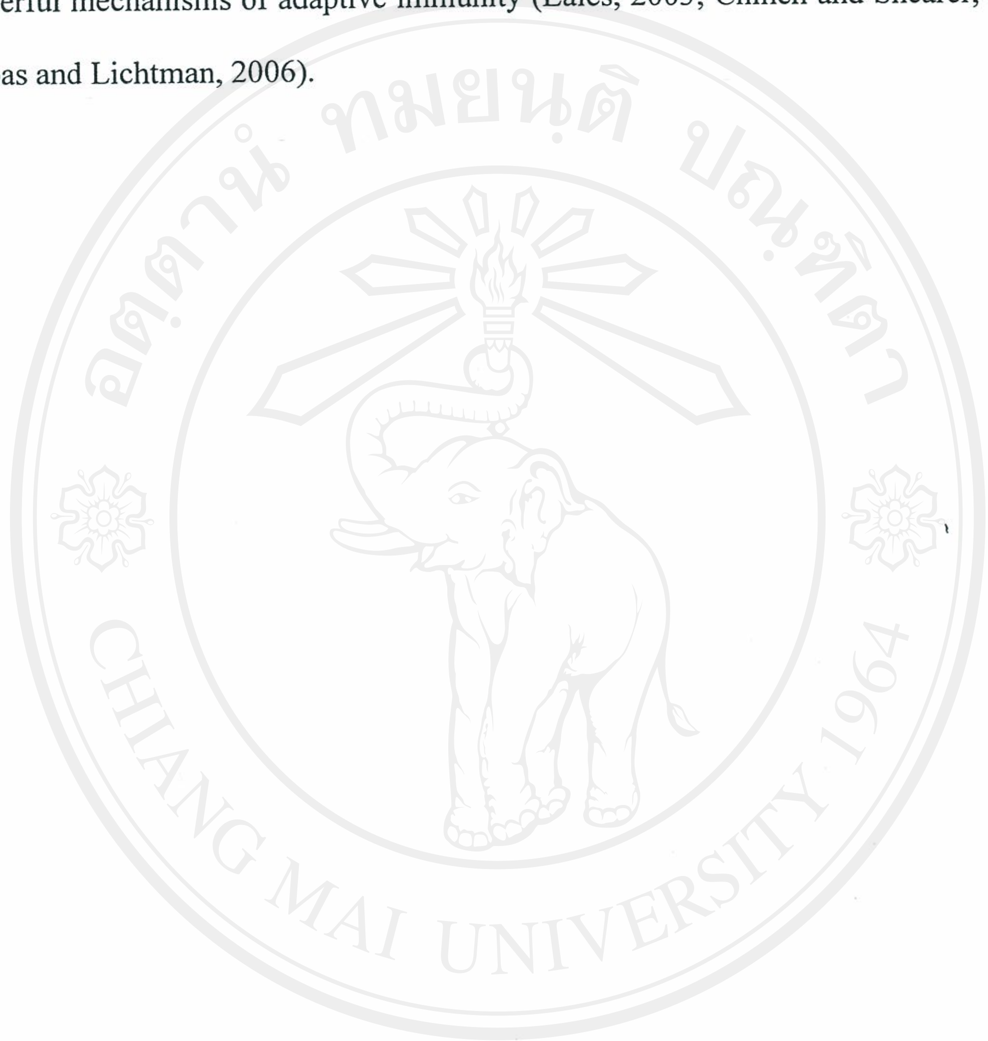
innate immunity provides effective defense against infections. However, many pathogenic evolved to resist innate immunity, and their elimination requires the powerful mechanisms of adaptive immunity (Eales, 2003; Chinen and Shearer, 2005; Abbas and Lichtman, 2006).



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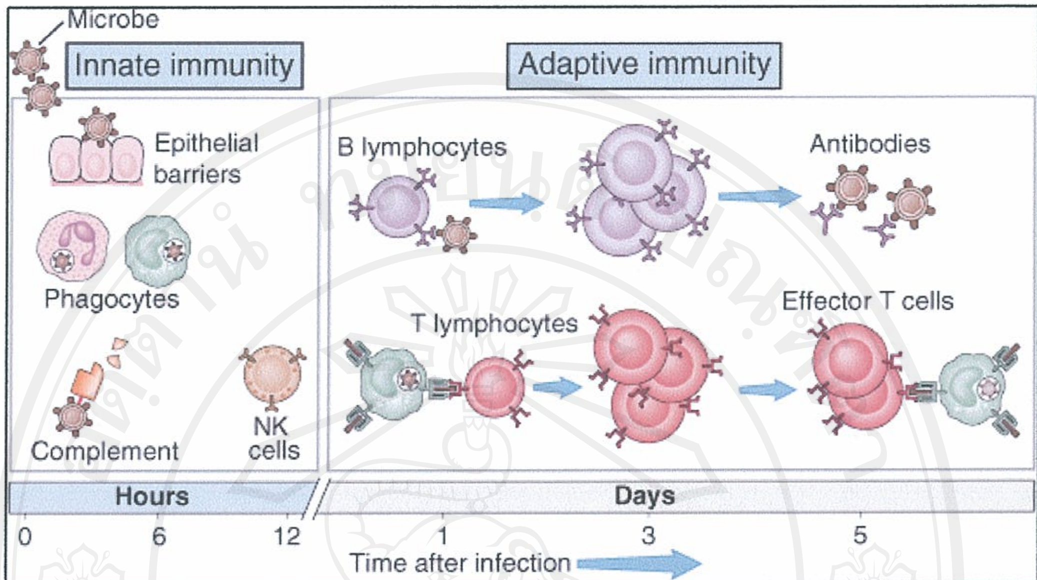

Figure 1.1 Innate and adaptive immunity. The mechanisms of innate immunity provide the initial defense against infections. Some of the mechanisms prevent infections (e.g., epithelial barriers) and others eliminate microbes (e.g., phagocytes, NK cells, and the complement system). Adaptive immune responses develop later and are mediated by lymphocytes and their products. Antibodies block infections and eliminate microbes, and T lymphocytes eradicate intracellular microbes (Abbas and Lichtman, 2006).

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1.2.1.1 Innate immunity

Innate immunity is the first line of host defense against microorganism. It refers to unsophisticated mechanism of host defense that plays a more important role in primitive life forms and host uses immediately or within several hours after exposure to an antigen. Contrasting adaptive immunity, innate immunity does not recognize every possible antigen. It is designed to recognize a few highly conserved structures present in many different microorganisms, which are called pathogen-associated molecular patterns such as Lipopolysacharide (LPS), peptidoglycan, lipotechoic acids, mannose, bacterial DNA, double-stranded RNA and glucans. The principal components of innate immunity are physical and chemical barriers (epithelia cells and normal flora), cells involved in body defense (phagocytic cells, natural killer cell and cells that release inflammatory mediators) and blood protein complements (complement proteins, acute phase protein, and cytokines). Although innate immunity can effectively combat many infections, microbes that are pathogenic, perhaps, have evolved to resist innate immunity. Defense against these infectious agents is the task of the adaptive immune responses (Abbas et al., 2000; Janeway et al., 2004; Chinen and Shearer, 2005).

For many years it was believed that innate immunity is nonspecific and weak and is not effective in combating most infections. It was now know that, in fact, innate immunity specifically targets microbes and is a powerful early defense mechanism capable of controlling and even eradicating infections before adaptive immunity becomes active. Innate immunity not only provides the early defense against infections but also instructs the adaptive immune system to respond to different microbes in ways that are effective at combating these microbes. Conversely, the

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adaptive immune response often uses mechanisms of innate immunity to eradicate infections. Thus, there is a constant bidirectional cross-talk between innate immunity and adaptive immunity. Without proper function of innate immunity, adaptive immunity can be induced (Abbas and Lichtman, 2006).

1.2.1.2 Adaptive immunity

Adaptive immunity is the next line of host defense after the innate immunity. Adaptive immune responses are trigger with microbes or substances called antigens after they pass through the innate immunity. This type of immunity refers to as antigen-specific defense mechanisms in which the process takes several days to cause microbial protection and elimination of a specific antigen. The adaptive immunity also plays an important role in elimination of tumor cells. Adaptive immune responses are mediated by lymphocytes and their products, such as antibodies and cytokines. There are two types of adaptive immunity, called humoral immunity (HMI) and cell-mediated immunity (CMI) that are mediated by different cells and molecules and are designed to provide defense against extracellular microbes and intracellular microbes, respectively (Abbas et al., 2000; Janeway et al., 2004). In HMI, B lymphocytes secrete antibodies that eliminate extra-cellular microbes. Whereas, T lymphocytes of the CMI either activate macrophages to destroy phagocytosed microbes or kill infected cells and tumor cells (Figure1.2).

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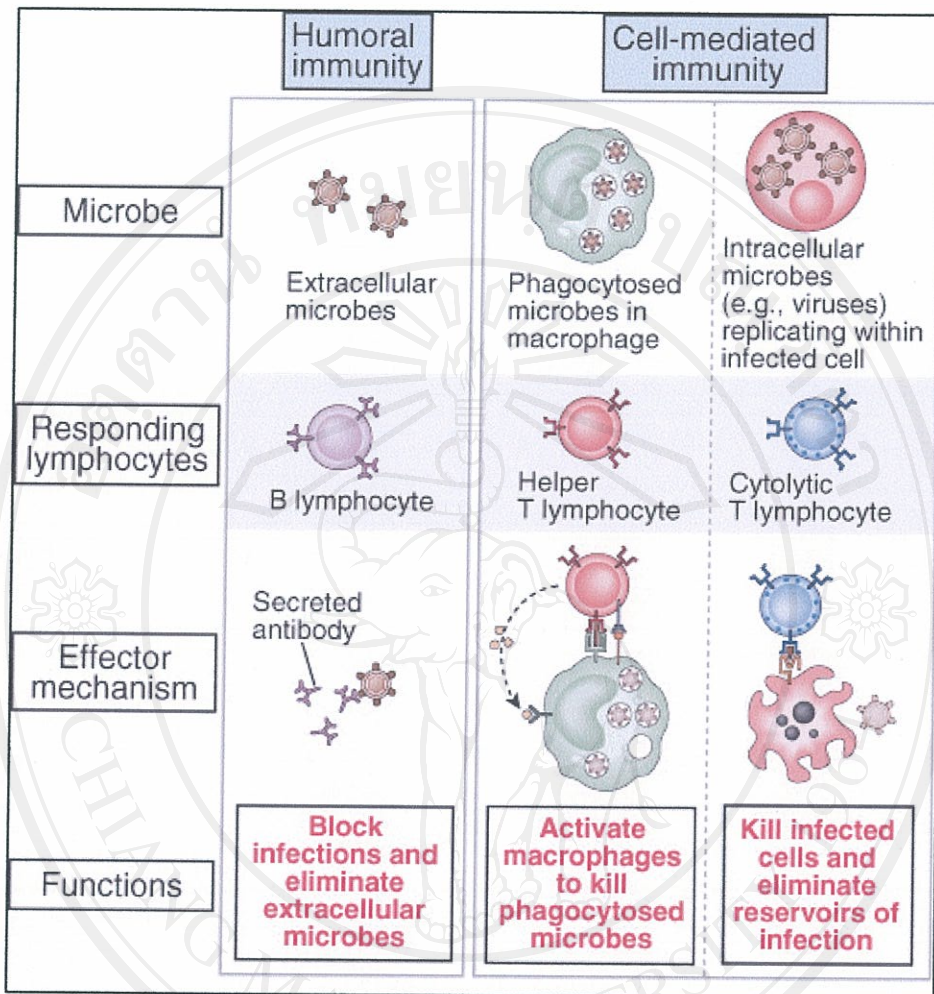

Figure 1.2 Types of adaptive immunity. In HMI, B lymphocytes secrete antibodies that block infections and eliminate extra-cellular microbes. In CMI, T lymphocytes eradicate intracellular microbes and altered cells (Abbas and Lichtman, 2006).

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1.2.1.2.1 Humoral mediated immunity (HMI)

The main function of the humoral immune response in the body is to destroy extracellular microorganisms and prevent the spread of intracellular infection. Humoral immunity is mediated by proteins called antibodies, which are produced by cells called B lymphocytes. B lymphocytes recognize and are activated by a wide variety of antigens, including proteins, polysaccharides, lipids, and small chemicals. The activation of B lymphocytes results in the proliferation of antigen specific cells, also called clonal expansion, and their differentiation into effector cells that actively secrete antibodies. Antibodies are secreted into the circulation and mucosal fluids, and they neutralize and eliminate microbes and microbial toxins that are present in the blood and in the lumens of mucosal organs, such as the gastrointestinal and respiratory tracts. One of the most important functions of antibodies is to stop microbes that are present at mucosal surfaces and in the blood from gaining access to and colonizing host cells and connective tissues. In this way, antibodies prevent infections from ever getting established. Antibodies use their antigen-binding (Fab) regions to bind and block, or neutralize the infectivity of microbes and the interactions of microbial toxins with host cells. Other functions of antibodies require the participation of various components of host defense, such as phagocytes and the complement system. Antibodies use their Fc regions, heavy chain constant regions which contain the binding sites for phagocytes and complement, to promote the phagocytosis or activate the complement system (Figure 1.3) (Janeway et al., 2004; Abbas and Lichtman, 2006).

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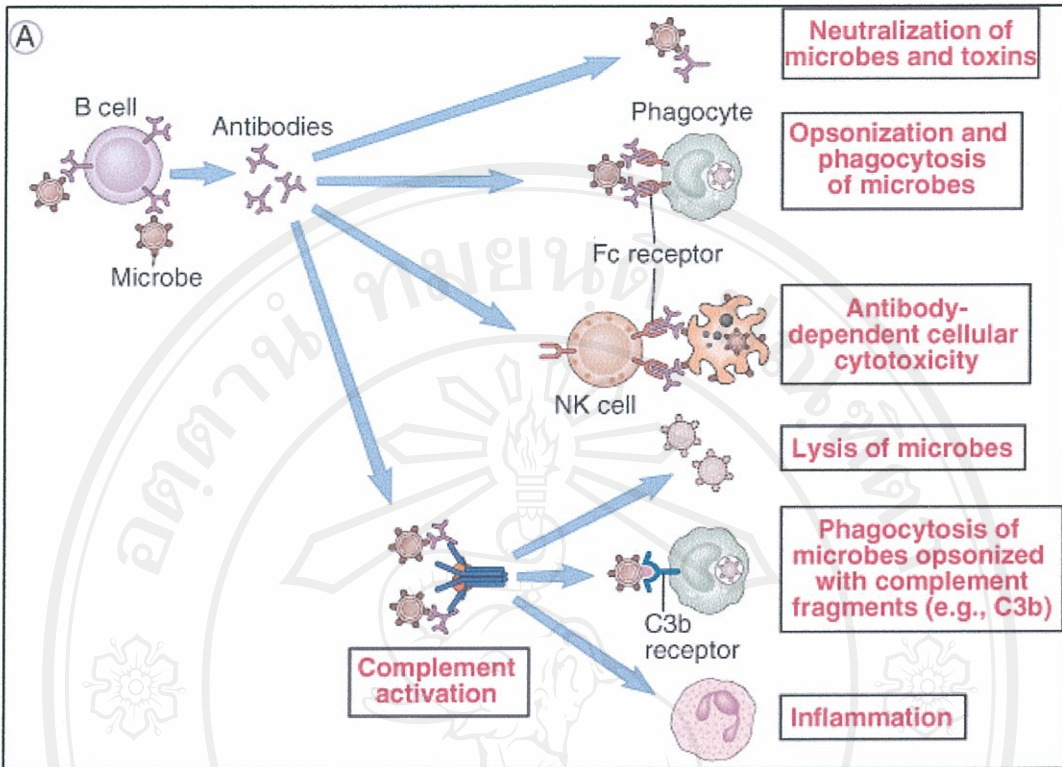

Figure 1.3 Effector functions of antibodies. Antibodies against microbes and their toxins neutralize these agents, opsonize them for phagocytosis and antibody-dependent cellular cytotoxicity, and activate the complement system (Abbas and Lichtman, 2006).

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1.2.1.2.2 Cell-mediated immunity (CMI)

Cell-mediated immunity is the results of effector function of T lymphocytes. The responses of T lymphocytes consist of sequential phases: recognition of cell-associated microbes by naive T cells, expansion of the antigen-specific clones by proliferation, differentiation of some of the progeny into effector cells and memory cells. The biochemical signals triggered in T cells by antigen recognition result in activation of various transcription factors that stimulate the expression of genes encoding cytokines, cytokine receptors, and other molecules involved in T cell responses. Consequently, the antigen-specific T cells secrete proteins called cytokines, some of which induce proliferation of the antigen-stimulated T cells and others mediate the effector functions of T cells (Figure 1.4)(Janeway et al., 2004; Chinen and Shearer, 2005; Abbas and Lichtman, 2006).

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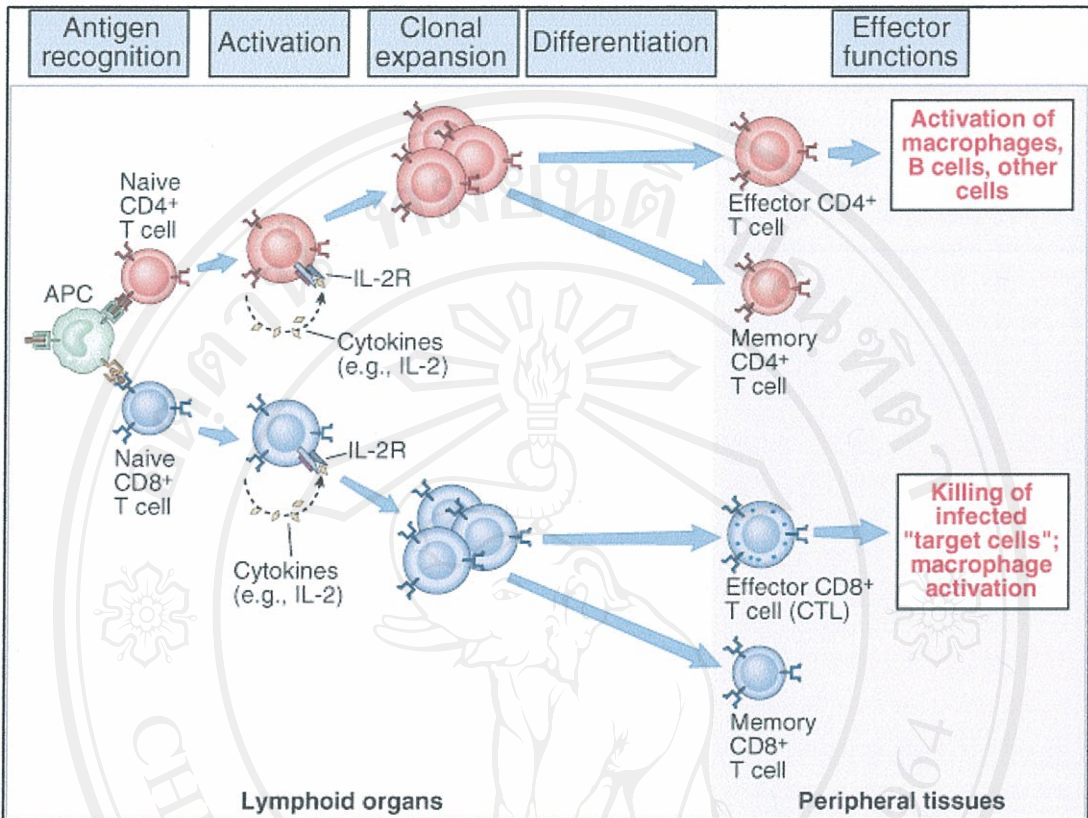

Figure 1.4 The induction and effector phases of cell-mediated immunity. The native T lymphocytes are stimulated to proliferate and differentiate into effector cells (Abbas and Lichtman, 2006).

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Two major types of T cells have been identified; $CD4^+$ helper T lymphocytes (T_H) and $CD8^+$ cytolytic T lymphocytes (CTLs) are bearing either CD4 or CD8 molecules on their surface, respectively. $CD4^+$ helper T cells differentiate into subsets of effector cells that produce restricted sets of cytokines and perform different functions. T_H1 cells, which produce IFN-γ, activate phagocytes to eliminate ingested microbes and stimulate the production of opsonizing and complement-binding antibodies. T_H2 cells, which produce IL-4 and IL-5, stimulate IgE production and activate eosinophils, which function mainly in defense against helminthes. Differentiated $CD8^+$ CTLs recognize class I MHC-peptide complexes on the surface of infected cells and kill these cells, thus eliminating the reservoir of infection. Antigen recognition by effector CTLs results in the activation of signal transduction pathways that lead to the exocytosis of the contents of the CTL's granules to the region of contact with the targets. CTLs kill target cells mainly as a result of delivery of granule proteins, granzymes and perforin, into the target cells. Granzymes are enzymes that cleave and thereby activate enzymes called caspases that are present in the cytoplasm of target cells, and the active caspases induce apoptosis. Perforin is necessary for delivery of granzymes into the cytoplasm of the target cell. Alternatively, both perforin and granzymes may enter the target cells by receptor-mediated endocytosis, both proteins bound to a sulfated glycoprotein called serglycine. Perforin may then insert into endosomal membranes and facilitate the movement of granzymes through these membranes and into the cytoplasm. $CD8^+$T lymphocytes also secrete the cytokine IFN-γ, which activates macrophages to destroy phagocytosed microbes and enhance the recruitment of additional leukocytes (Figure 1.5) (Janeway et al., 2004; Abbas and Lichtman, 2006).

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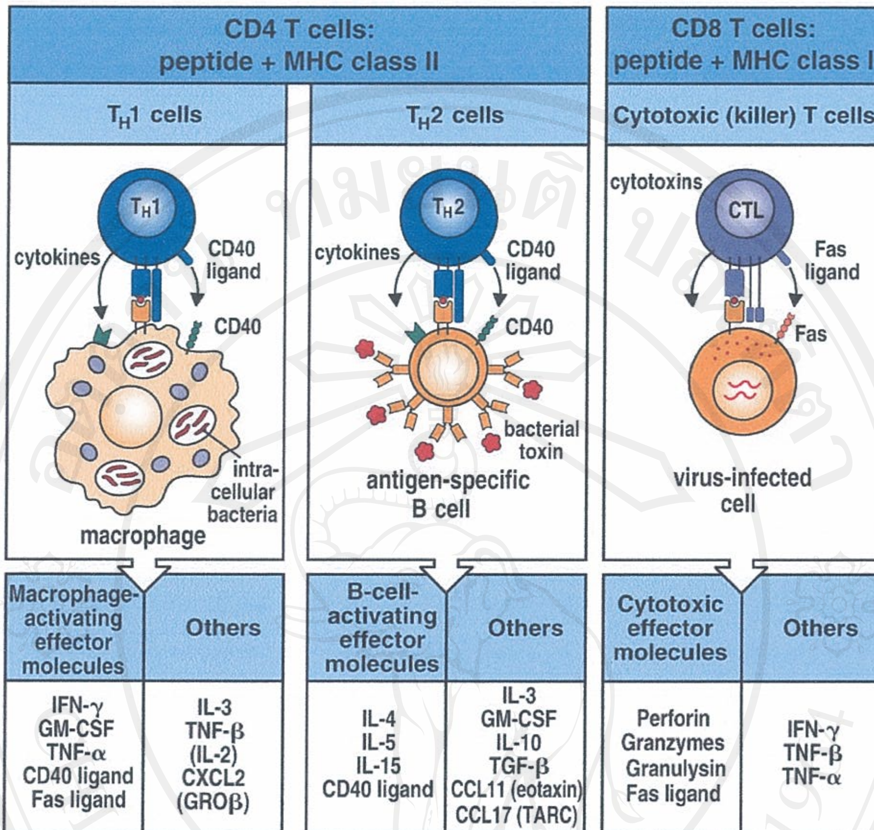

Figure 1.5 Effector functions of CMI. $CD4^+$ helper T cells differentiate into T_H1 cells or T_H2 cells secrete cytokines which act on macrophages to increase phagocytosis and killing of microbes and act on B lymphocytes to stimulate production of antibodies. $CD8^+$T lymphocytes directly destroy infected cells and secrete cytokines for phagocytosis activation (Janeway et al., 2004).

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1.2.2 Monoclonal antibody

1.2.2.1 Hybridoma technique

In 1975, Kohler and Milstein devised a method of growing large numbers of antibody producing cells from a single B-cell by fusing B-cells from the immunized animal with immortal myeloma cancer cells (Kohler and Milstein, 1975). The procedure yields an immortal cell line producing monospecific antibody. The immortalized antibody-producing cell lines are called hybridomas and the antibodies they produced are termed monoclonal antibodies. Myeloma cell lines that used as fusion partner are defected in hypoxanthine phosphoribosyltransferase, HPRT or HGPRT, an enzyme of a salvage pathway (Figure 1.6). Therefore, these cells can not use the salvage pathway for nucleotide synthesis. Normal animal cells synthesize DNA precursors, purine nucleotides and thymidylate, by de novo synthesis pathway which requires tetrahydrofolate (Figure 1.6). Hence, using of antifolate drugs, such as aminopterin, can block activation of tetrahydrofolate, thereby inhibits the synthesis of purine and rules out DNA synthesis by de novo pathway (Figure 1.6) (Yelton and Scharff, 1980; Goding, 1996; Abbas et al., 2000). However, normal cells can still survive by producing DNA precursors from a salvage pathway. In contrast, myeloma cells, which defecting in a salvage pathway, can not survive in the present of antifolate drugs (Yelton and Scharff, 1980; Harlow and Lane, 1988). (Figure 1.7)

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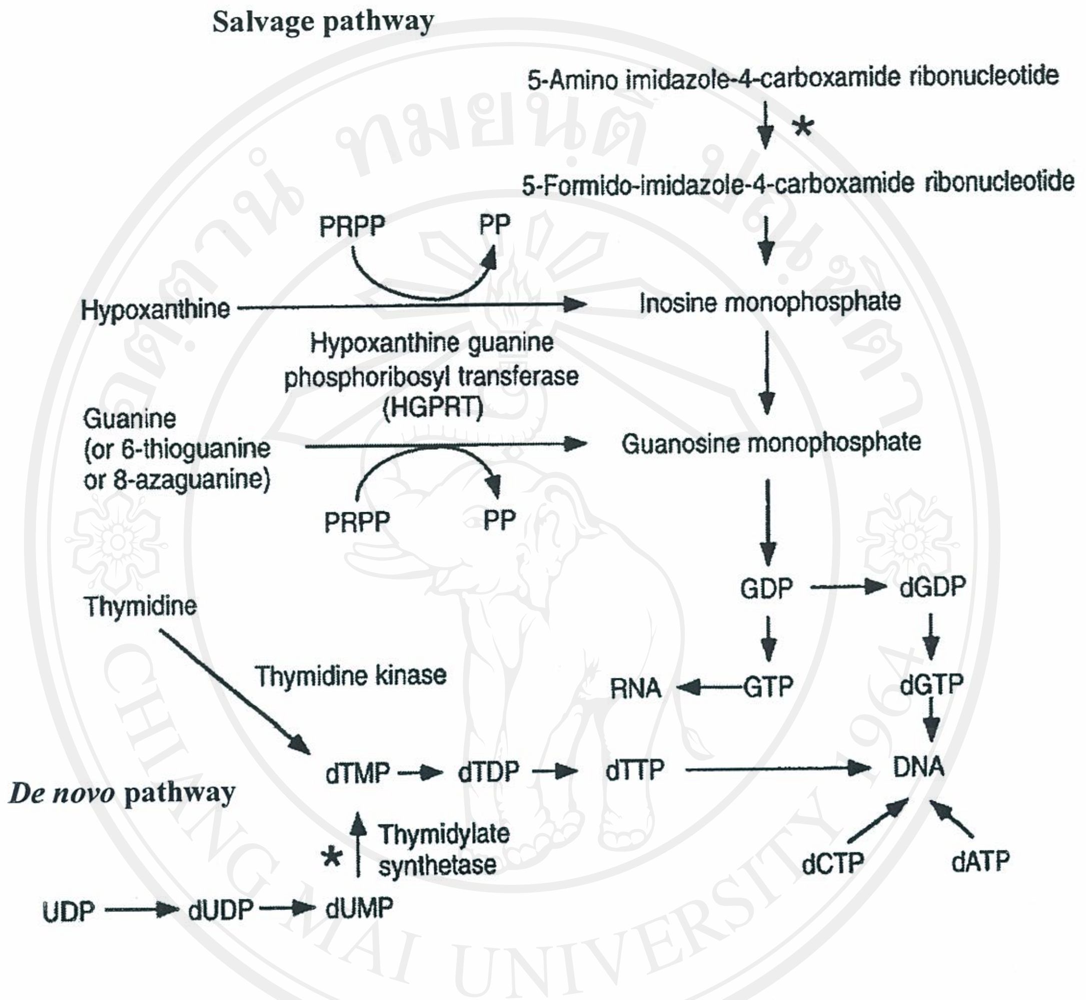

Figure 1.6 Metabolic pathways of DNA synthesis. When the *de novo* pathway are blocked with folic acid analogue (*), such as aminopterin, cell must depend on the salvage pathway (Goding, 1996).

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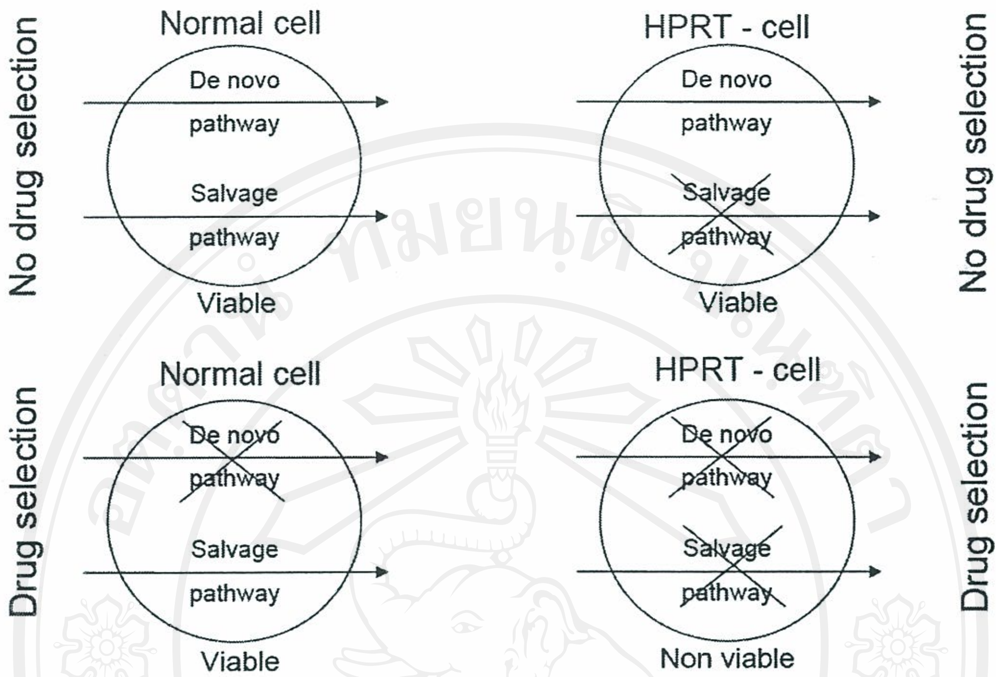

Figure 1.7 Pathway of nucleotide synthesis in the present of antifolate drug (Harlow and Lane, 1988).

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In hybridoma technique, mouse is firstly immunized by an antigen. After appearing of the antibody response, splenocytes of the immunized mouse are fused with myeloma cells. After cell fusion, un-fused B cells, un-fused myeloma cells and hybrid cells are randomly generated. Un-fused myeloma cells and myeloma-myeloma hydrids can be selected in a medium containing hypoxanthine, thymidine, and an antifolate drug, aminopterin (HAT medium). As myeloma fusion partners and myeloma-myeloma hydrids are deficient in an enzyme required for the salvage pathway of nucleotide synthesis, these cells are died in HAT medium because aminopterin blocks normal nucleotide synthesis and the enzyme deficiency blocks utilization of hypoxanthine or thymidine in the salvage pathway. By the HAT medium, thus non-fused myeloma cells and myeloma-myeloma hydrids are died and only those cells fused to normal cells survive. In the case of fusion of myeloma and normal cells, 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. Un-fused normal lymphocytes can survive in culture medium for approximately 1 week then they die. Therefore, after long-term culture, only hydridomas of normal and myeloma cells grow in the selective 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. Since a single well may contain multiple hybridoma clones, 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

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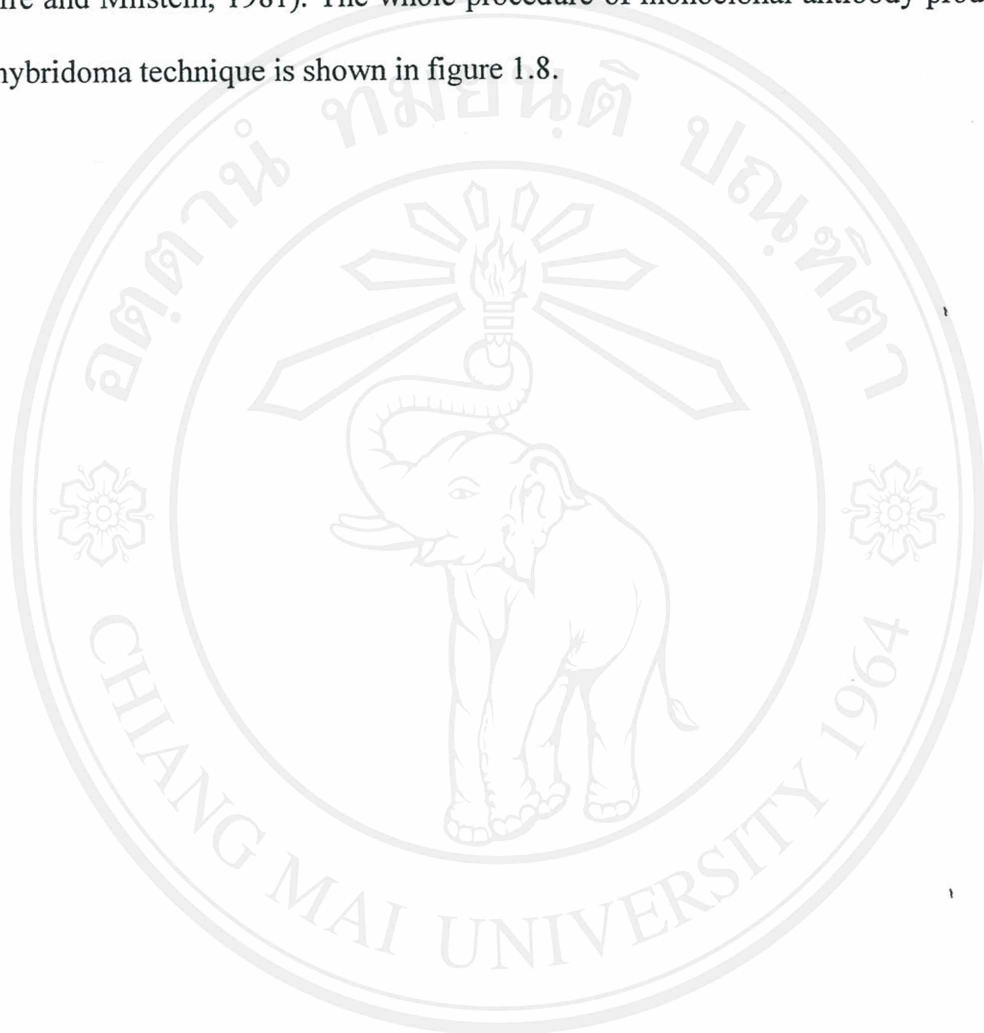
cloned, the cells are expanded for freezing and generating of stock solutions of monoclonal antibody (de StGroth and Scheidegger, 1980; Yelton and Scharff, 1980; Galfre and Milstein, 1981). The whole procedure of monoclonal antibody production by hybridoma technique is shown in figure 1.8.



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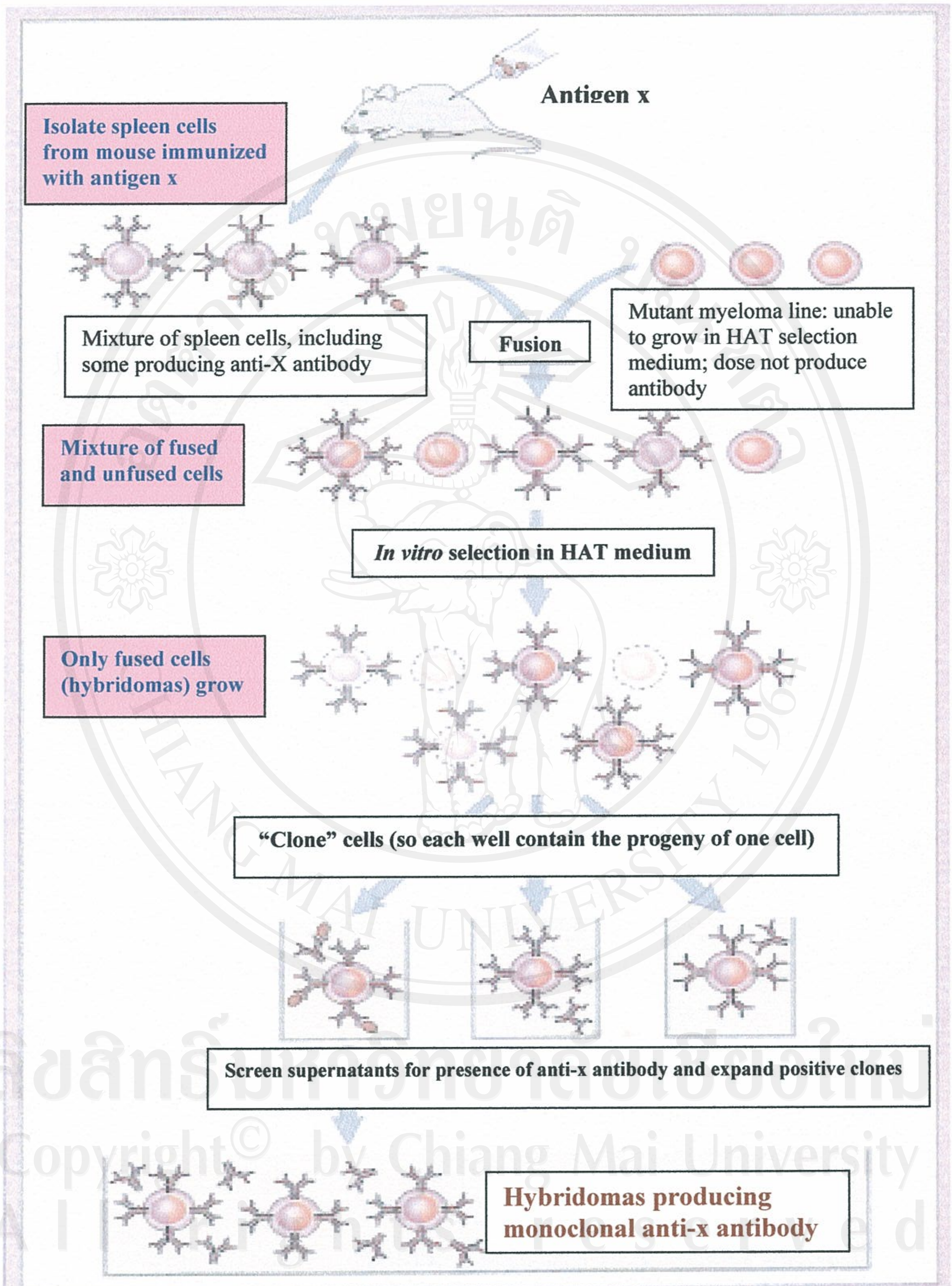

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Figure 1.8 Monoclonal antibody production(Abbas et al., 2000) .

As was mention, the generation of monoclonal antibodies by the hybridoma technology is dependent on immortalization of B-cell clones by fusion with myeloma cancer cells. A key feature in the success of this procedure is the immunization and expansion of antigen-specific B-cell clones to high enough numbers such that the frequency of targeting events for successful immortalization will be increased. The expansion and isolation of B-cell clones producing antibody molecules of a desired antigen-specificity and affinity is modulated by the immunogenicity of the antigen used for immunization. At the present, the technology of hybridoma production is firmly established, steps involved in the production of mAbs include the following: (1) preparation of the immunogen, (2) immunization protocol, (3), determined for antibody induction, (4) cell fusion, and 5) screening of hybridomas producing mAb of interest (de StGroth and Scheidegger, 1980; Galfre and Milstein, 1981; Liu, 1986; Rousseau and Forest, 1986; Tami et al., 1986; Bessler and Baron, 1988; Harlow and Lane, 1988; Birch and Lennox, 1995; Goding, 1996; Gosling, 2000; Andersen and Reilly, 2004; Leenaars and Hendriksen, 2005).

1.2.2.2 Preparation of Immunogens

Immunogen is defined as molecule that can elicit an immune response after immunization into the body. Features that contribute to the immunogenicity of any molecule include size, complexity, and non-self or foreignness to the host. The imunogen can be various forms. It can be soluble or insoluble proteins, polysaccharides, lipids or nucleic acids. In general, protein must have a molecular weight greater than 10,000 Daltons for induction antibody response. The proteins which molecular weight less than 10,000 Daltons are always non-immunogen and require some modifications for induction of antibody response. Globular proteins are

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usually more immunogenic than carbohydrates, lipids, or nucleic acids (Harlow and Lane, 1988; Vetterlein, 1989; Luttman et al., 2006).

Particulate antigens such as bacteria, viruses or mammalian cells are usually good immunogens because of its quantity, complexity, and foreignness particularly in case the immunogen and immunizing host are of different species. By hybridoma technique, the success of obtaining monoclonal antibodies to a specific cellular protein depends on the abundance and immunogenicity of the protein of interest. For cellular proteins which are expressed in low abundance on the cell surface, by immunization of whole cells the chance of obtaining specific mAb is low. A methodology that has successfully proven in the production of monoclonal antibodies specific for cell surface proteins of low abundance is the use of recombinant protein or synthetic peptides as immunizing immunogen (Lerner, 1982; Walter, 1986; Harlow and Lane, 1988; Claassen et al., 1993; Luttman et al., 2006). Peptides are normally synthesized *in vitro* using solid-phase technique (Woolley and Merrifield, 1963). The synthetic peptides are purified and couple to carrier proteins such as bovine serum albumin and Keyhole limpet hemocyanin (KLH).

The use of soluble or cellular proteins for immunization is advantageous but may not be possible or provide the best results due to insufficient quantity, low immunogenicity, or protein-protein interactions that may mask important antigenic determinants. Recent advanced molecular biology and biochemical technologies provide a powerful approach to prepare and isolate the interested antigens that are in low abundance, difficult to purify, weakly immunogenic, or even unknown (Koths, 1995). If partial or complete sequences of known or unknown proteins are identified, *in vitro* protein expression systems using both prokaryotic and eukaryotic expression

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vectors can be used to produce recombinant proteins that then can be used as immunogens (Harlow and Lane, 1988; Luttman et al., 2006).

For animal immunization, ideally, immunogen used should contain only pure immunogen. A wide range of techniques are available for purification of proteins, peptide and other immunogens from any given sources including affinity chromatography, ion exchange chromatography and gel filtration. The design of the purification scheme and method used depends on the physical and chemical properties of the immunogen. The abundance of immunogen in the particular source also needs to be concerned for selecting of the methods.

In some case, immunogenicity of the immunogens needs to be enhanced. Small proteins or peptides, by itself, do not induce antibody response. These molecules require conjugation to a carrier protein such as bovine serum albumin and Keyhole limpet hemocyanin (KLH) before immunization. In general, adjuvant is also need for better induction of the immune responses. The immunogens are emulsified in adjuvants, e.g Freund's adjuvant or RIBI Adjuvant System®, before immunization. The adjuvants create reservoirs of antigen that are released slowly over time and induce inflammatory responses resulting in the generation of nonspecific cytokines that activate immune cells (Harlow and Lane, 1988; Gosling, 2000; Luttman et al., 2006). In addition, conjugation of soluble antigens to particulate molecules, such as agarose beads, has demonstrated to stimulate phagocytosis of the complex resulting in enhance processing and presentation of antigenic peptides to T-lymphocytes (Posnett et al., 1988)

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1.2.2.3 Immunization protocol

Several immunization protocols are developed for hybridoma technique. 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 immuniztion routes are intravenous (i.v.); intramuscular (i.m.); subcutaneous (s.c.); intraperitoneal (i.p.); and intradermal (i.d.), which is also called intracutaneous (Harlow and Lane, 1988; Gosling, 2000). These routes are listed in probable order of increasing effect for soluble immunogen administered without adjuvant. The order listed may change with particulate immunogen and with the use of adjuvants. In addition to the above routes, intranodal or intrasplenic injection routes have been successfully used in cases of limited quantity of immunogen (Moonsom et al., 2001; Kasinrerk et al., 2002). Footpad and intra-articular routes have also been successfully used, but suitability of these as well as intranodal and intrasplenic routes is usually assessed today on an individual basis in terms of the welfare of the animal when adjuvants are used. The effectiveness of the various routes apparently relates to the efficiency of delivery of immunogen to lymphoid tissues. The distribution of Ag to the lymphoid tissue depends on the route of injection as well as the character and quantity of Ag and adjuvant.

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The optimal dose of immunogen is needed to generate a strong immune response. Immunization of too high or too low amount of immunogen may induce immunotolerance and no antibody production may occur (Leenaars and Hendriksen, 2005). The time between two immunization steps can affect both the induction of B memory cells and the class switch of B cells. Generally, the mice are immunized every two to three weeks but the immunization protocols vary among investigators.

The suggested dose and route of various immunogen for mouse immunization is shown in Table 1.1

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Table1.1 Suggested dose of immunogens for mice (Harlow and Lane, 1988)

Form of immunogens	Examples	Primary injections and boosts			Final boosts		
		Possible routes	Dose	Adjuvant	Possible routes	Dose	Adjuvant
Soluble proteins	Enzymes, carrier proteins conjugated with peptides, immune complexes	i.p*,s.c**	5-50 μg	+	i.v***	5-50 μg	-
Particular proteins	Virus (killed), yeast (killed), bacteria (killed), structural proteins	i.p,s.c	5-50 μg	+	i.v	5-50 μg	-
Insoluble proteins	Bacterially produced from inclusion bodies, immunopurified proteins bound to beads	i.p,s.c	5-50 μg	+	i.p	5-50 μg	-
Live cells	Mammalian cells	i.p	10^5-10^7 cells	-	i.v	10^6 cells	-
Live tumorigenic cells	Oncogenic mammalians cells	i.p,s.c	10^4-10^6 cells	-	i.v	10^6 cells	-
Carbohydrates	polysacharides	i.p,s.c	10-50 μg	+/-	i.v	10-50 μg	-
Nucleic acids	Carrier proteins conjugated with N.A	i.p	10-50 μg	+	i.v	10-50 μg	-

* Intraperitoneal

** Subcutaneous

***Intravenous

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1.2.2.4 Screening for antibody production

After immunogen immunization, immunized mice have to be determined for their specific antibody response before cell fusion. 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. When a sufficient antibody titer is reached in the serum, immunized mice are sacrificed and the spleen is removed and used as a source of cells for fusing with myeloma cells(Catty, 1988; William, 1995; Leenaars and Hendriksen, 2005).

1.2.2.5 Fusion and screening of hybridomas

The goal of the fusion procedure is to generate hybridoma cell lines from immunized B-lymphocytes that produce the desired monoclonal antibody and to immortalize these cells by fusing them to myeloma tumor cells. Fusion is carried out by co-centrifuging freshly harvested spleen cells and myeloma cells in polyethylene glycol (PEG), a substance that causes cell membranes to fuse (Pontecorvo, 1975) . PEG fuses the plasema membranes of adjacent myeloma and antibody-secreting cells, forming a single cell with two or more nuclei. 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. As mentioned, the hybridomas can be selected in HAT-containing medium. In further approximately 2-4 weeks hybrids grow to sufficient size so that the culture medium can be assayed for antibodies. A number of rapid, simple, and sensitive assays have been developed for screening hybridomas of which the most commonly used are

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direct or ELISA, whole cell ELISA, immunoprecipitation, or immunoblot (Sharon et al., 1979; Harlow and Lane, 1988; Vetterlein, 1989; William, 1995). Since a single well may contain multiple hybridoma clones, 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 antibodies (Yelton and Scharff, 1980; Galfre and Milstein, 1981; Harlow and Lane, 1988; Goding, 1996).

1.2.3 Application of monoclonal antibodies

The ability of antibodies to selectively bind a specific epitope present on a chemical, carbohydrate, protein, or nucleic acid has been thoroughly exploited through the years, as evidenced by the broad spectrum of research and clinical applications in which they are utilized. Applications include simple qualitative or quantitative analyses to ascertain the following: (1) whether an epitope is present within a solution, cell, tissue, or organism, and if so, where; (2) methods to facilitate purification of an antigen, antigen-associated molecules, or cells expressing an antigen; and (3) techniques that use antibodies to mediate and modulate physiological effects for research, diagnostic, or therapeutic purposes (Yelton and Scharff, 1981; James et al., 1984; Clark, 1986; Rousseau and Forest, 1986; Bessler and Baron, 1988; Voigt, 1989; Baldwin and Byers, 1991; Campobasso and Dammacco, 1991; Birch and Lennox, 1995). Applications of the monoclonal antibodies are as follows:

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1.2.3.1 Research reagent

Antibodies, especially mAbs, are used as a key reagent for biochemical characterizations, functional studies and immunological identification of antigens using various techniques, including Western blot, immunoprocipitation, enzyme-linked immunospot assay (ELISPOT), ELISA. In these assays, antibodies are used to detect an antigen or related molecule presented in the tested samples such as serum, cells or tissue lysates. Furthermore, antibodies are also very useful in immunofluorescence staining and immunohistochemistry which detect antigen in the context of cells, either as a single cell suspension or as immobilized cells and tissue sections (kennett et al., 1980; Luttman et al., 2006).

In biomedical research, mAbs that bind to cell surface molecules and either stimulate or inhibit particular cellular function are invaluable tools for defining the function of surface molecules (Kasinrerk et al., 2000; Khunkeawla et al., 2001; Chiampanichayakul et al., 2002; Chiampanichayakul et al., 2006). Antibodies that bind and neutralize cytokines are routinely used for detection the present and functional roles of these protein hormones *in vitro* and *in vivo*.

Antibodies are also used in the purification or enrichment of antigens, antigen-associated molecules, or cells expressing the antigen. For soluble proteins and associated molecules, antibodies are used to purify a substance with techniques called affinity chromatography. Subpopulations of cell suspensions can also be positively (enrichment) or negatively (depletion) selected by a fluorescent-activated cell sorter (FACSort) or magnetic-activated cell sorting (MACS) using antibodies against specific cell surface antigens (Voigt, 1989; Vaccaro, 1990; Luttman et al., 2006).

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Moreover, recently approach for high throughput proteomic analysis has been developed utilizing an antibody microarray platform.

At the present day, mAbs are as a very important tool for biological researches. A large number of antibodies are produced and commercially available for research purposes.

1.2.3.2 Diagnosis

Since antibodies can be raised against all molecules with immunologically distinctive characteristics, the application of mAbs in diagnostic is particularly wide-spread. Antibodies are used to detect the present of substance in specimens, which exploit some of the principles learned from their used in research laboratories. The diagnosis of many infectious and systemic diseases relies on the detection of particular antigens or antibodies in the circulation or in tissue by use of mAbs in immunoassay (Yelton and Scharff, 1981; Pekary and Hershman, 1984; Clark, 1986; Voigt, 1989; Gosling, 2000; Nelson et al., 2000). Tumor-specific mAbs are used for detection of tumor antigens for early diagnosis and prognosis (Coakham et al., 1984; Sikora and Smedley, 1984; Schmelter et al., 1986; Baldwin and Byers, 1991; Campobasso and Dammacco, 1991). The availability of mAbs produced in quantity allows for the antibody to be labeled and used in direct capture and binding to antigen. More simple, sensitive and reliable diagnostic kits are developed base on the available of specific mAb.

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1.2.3.3 Therapeutic applications

Antibody therapeutics can potentially treat diseases ranging from autoimmune disorders to cancer and viral or bacterial infections by neutralization of toxins *in vivo*,

passive immunization, delivery of radionuclides for imaging purposes, immunosuppression, and cancer therapy (Kenemans et al., 1988; Voigt, 1989; Birch and Lennox, 1995; Pelegrin et al., 2004). Antibodies are also being evaluated for use in the food and environmental industries as biosensors for routine monitoring to detect less than one part per billion of microbial contaminants or organic pesticides. Furthermore, antibodies offer the possibility of efficient removal of microorganisms and organic pollutants from contaminated water and land.

Antibody-based therapies have only recently begun to realize their potential as "magic bullets" in cancer therapeutics, and several promising treatments have recently gained FDA approval. Antibodies are useful in cancer therapy because they can recognize tumor-associated antigens that are expressed at higher density on malignant cells than on normal cells. Thus, the antibodies and any associated molecules become concentrated at the site of malignant tissue, 4–50 times relative to other tissues. Antibodies can be used as a single therapy, or in combination with traditional drug therapies, as some combinations are synergistic. Antibody binding is exploited to (a) specifically deliver toxic molecules to tumor cells, (b) recruit cytotoxic immune cells, and (c) activate apoptotic signaling pathways. Antibody-mediated therapy has been much more successful for circulating than for solid tumors, primarily because there are few diffusion boundaries, and targeted antigens are present at high density (Yelton and Scharff, 1981; Clark, 1986; Rousseau and Forest, 1986).

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1.2.4 Principle of immunoprecipitation technique

Immunoprecipitation is an immunogical method involving in the interaction between a protein and its specific antibody. The principle of immunoprecipitation is

very simple (Figure 1.8). The antigen-specific antibodies are coupled to the solid support, for instance sepharose and magnetic beads. The antibodies may directly bind to Protein A or Protein G which have been immobilized on the solid phase via their Fc part or can be covalently cross-linked by cross-linking reagent, such as disuccinimidyl suberate (DSS) or dithiobissulfosuccinimidylpropionate (DTSSP), to the solid pahse. The antibodies are then allowed to form immune complexes with its specific antigen. Hence, the immune complexes are captured on a solid support. The process of capturing these complexes from the solution is termed immunoprecipitation. Any proteins which are not precipitated will be washed away. Finally, components of the bound immune complex are eluted from the supports and analyzed (Harlow and Lane, 1988; Luttman et al., 2006).

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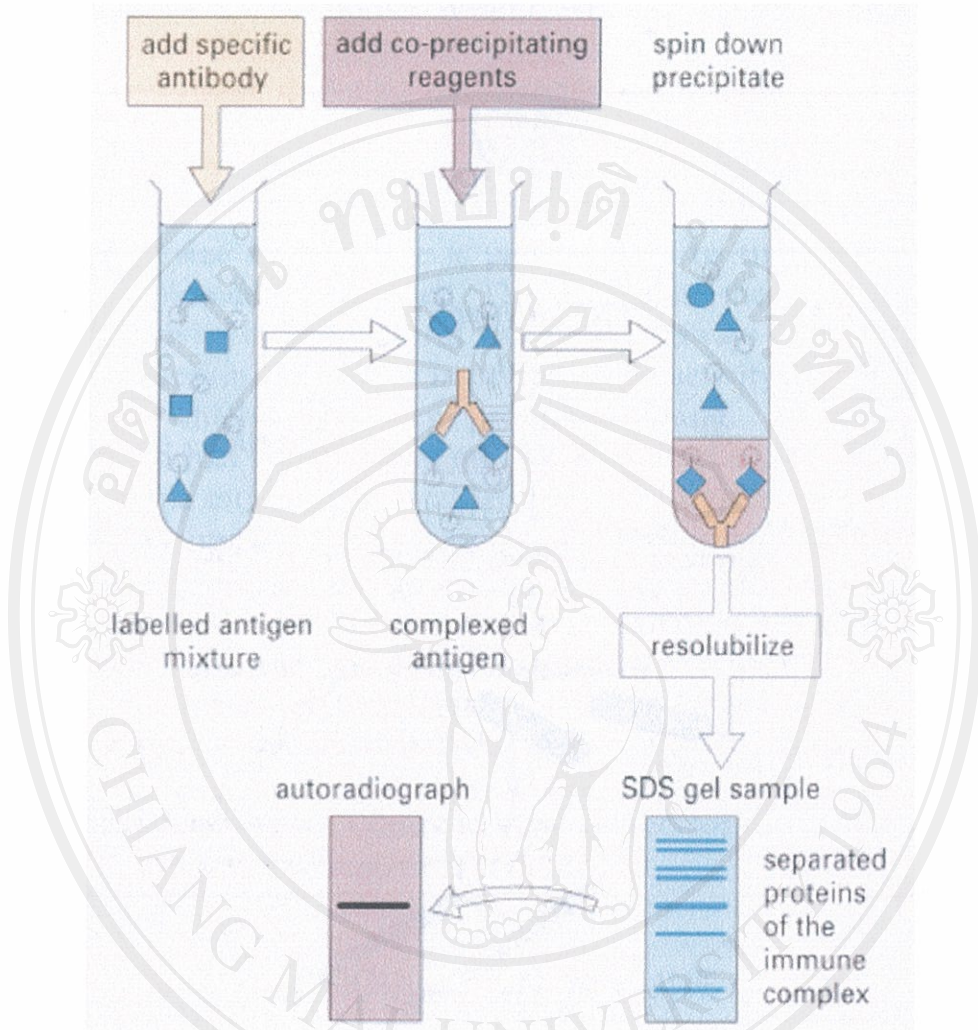

Figure 1.9 The test sequence of an immunoprecipitation technique (Roitt et al., 2001).

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Various techniques including SDS-PAGE, immunobloting, enzymatic studies, ligand binding and immunochemical methods are used to analyze the precipitated antigens. In most cases the precipitated antigens are analyzed by SDS-PAGE (Towbin et al., 1979), often followed by Western blot detection to verify the identity of the antigens (Burnette, 1981). For this analysis, the immune precipitate is solubilized in SDS buffer. This buffer usually contains reducing agents (mercaptoethanol or dithiotreithol) as well as SDS to reduce the disulphide bonds. Heating the sample (60-95°C) may be necessary in order to achieve a complete denaturing of the proteins. The solid support can now be separated by means of centrifugation. The supernatant contains only the antigen or antigen and antibody can be applied on the gel. Immunoprecopitation, nowadays, is predominantly used as an analytical method for interested molecules. These assays can determine the presence and quantity of the antigen, relative molecular weight of the polypeptide chain, rate of synthesis or degradation, presence of certain post-translational modifications, and interactions with proteins, nucleic acids, or ligands (Harlow and Lane, 1988; Luttman et al., 2006)

1.2.5 Principle of COS cell expression system

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A variety of eukaryotic expression systems have been used to produce recombinant proteins (Seed, 1995). The use of eukaryotic cell lines to produce proteins is advantageous because they have the ability to carry out normal post-translational modifications such as intra and inter-chain disulfide bond formation, signal peptide cleavage, and addition of O- and N-linked carbohydrates. Many mammalian cell lines have been used to express recombinant proteins via transfection

of plasmids or infection of recombinant DNA or RNA viruses. In particular, COS cell expression system has been used successfully to generate recombinant proteins that are used for structural and biochemical studies and can also be used to search for their natural ligands (Edwards and Aruffo, 1993).

COS cells expression system involves in the use of mammalian expression vector containing simian virus-40 (SV40) promoter (Elder et al., 1981). SV40 is a member of the papova group of mall and non-enveloped DNA viruses, which cause lytic infection of permissive monkey cells. Defective SV40 viruses and recombinant plasmids containing SV40 DNA segments have been widely used as amplification and expression vectors in mammalian cells. Some SV40 derived expression vectors lack large T-antigen gene and can only be amplified in simian cells, which constitutively express SV40 T-antigen, which is required to activated the SV40 origin of replication (Gluzman, 1981). The foreign DNA cloned into these vectors is transiently expressed, but no virus particles are produced.

There are several cell types used for transient gene expression including COS-7 cells. COS-7 cells are derived from simian CV-1 cells, which are transformed by an origin-defective mutant of SV40 genome. As a result, these cells constitutively express the wild-type of SV40 large T antigen and contain all the necessaries cellular factors required for replication of DNA. Upon transfection with an expression plasmid containing a functional SV40 origin of replication, the interaction between the SV40 origin of DNA replication and SV40 large T-antigen leads to extrachromosomal replication of the expression plasmid to high copy numbers. Hence high transcription and translation of the gene of interest from a suitable eukaryotic promotor will lead to remarkable product titers. Plasmid replication in COS cells peaks at around 48 hours

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post transfection. Thereafter the cells begin to slowly shed the high amount of plasmid copies, accompanied by signs of cytopathic effect and subsequently cell death, presumably, because they cannot tolerate the presence of high levels of extrachromosomally replicating DNA. As a consequence, the system is not suitable for large scale production over a prolonged period of time. Yet, recombinant protein expression in COS cells reaches its maximum after 72 hours posttransfection, and continues, despite the above described slow deterioration of cells, over a period of approximately 5–10 days. High-level of plasmid amplification and protein production is the advantage of this host vector system (Edwards and Aruffo, 1993; Trill et al., 1995).

Plasmid DNA can be introduced into mammalian cells by variety of techniques. One of the chemical reagents, which are used to transfer nucleic acids into cultured mammalian cells, is DEAE-dextran. DEAE-dextran is a cationic polymer that tightly associates with negative charge of nucleic acids. Excesses of positive charge are contributed by the polymer in the DNA:polymer complexes and then allowed the complexes to deposit with the negative charge of cell membrane. Uptaking of the complex is presumable by endocytosis. This method is successful for delivery of nucleic acids into cells for transient expression, which is useful for short-term expression studies of a few days in duration.

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1.2.6 Principle of the production of BCCP fusion protein

Nowadays, bacterial expression systems are commonly used to produce recombinant proteins. The production of recombinant proteins involves in the cloning of appropriate genes into a prokaryotic expression vector (Koths, 1995; Hannig and

Makrides, 1998; Duilio et al., 2004). A well-designed prokaryotic expression vector contains a set of optimally configured genetic elements that affect both transcriptional and translational aspects of protein production. The promoter is typically positioned approximately 10 to 100 bp upstream of the ribosome-binding site (RBS) and is under the control of a regulatory (repressor) gene, which may be present on the vector itself or integrated in the host chromosome. The RBS consists of the Shine-Dalgarno (SD) sequence followed by an AT rich translational space that has an optimal length of approximately 8 bases. The SD sequence interacts with the 3' end of the 16S rRNA during translation initiation. The transcription terminator serves to stabilise the mRNA and the vector. In addition, the inclusion of an antibiotic-resistance gene facilitates phenotypic selection of the vector and the origin of replication (Ori) determines the vector copy number. Multi copy plasmids have been extensively used as vectors for recombinant protein expression. Expression vectors commonly used for overexpression of foreign genes in *E. coli* can be categorised according to the type of promoter used. The systems commonly used are driven by IPTG-inducible, bacteriophage T7, or bacteriophage lambda pL promoters.

Gene fusion techniques permit the assembly of recombinant protein with a protein fusion partner that has been designed for purification or detection purpose (LaVallie and McCoy, 1995). Widely used fusion proteins include polyhistidine (His6), glutathione-S-transferase (GST), maltose-binding protein (MBP) and Biotin carboxyl carrier protein (BCCP). Biotin carboxyl carrier protein (BCCP), a subunit of acetyl-CoA carboxylase, is the only biotinylated protein in *E. coli* (Duffy et al., 1998). The biotinylated BCCP serves as a carrier of an activated carboxyl group in a metabolic carboxylate transfer process in the *E. coli* cells (Elborough et al., 1996).

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The biotinylation process which involves in the formation of an amide bond between the carboxyl group of biotin and the ε-amino group of the lysine residue is catalyzed by the enzyme biotin ligase (BirA) in an ATP-dependent reaction. The lysine residue, which attaches to the biotin, is located at the C-terminal region of the BCCP protein in an independent domain of about 80 amino acids. This domain can be fused to recombinant proteins and it can be biotinylated *in vivo* by the endogenous biotin ligase of *E. coli*. The BCCP domain fusion approach has previously been used for site-specific biotin labeling of various recombinant proteins (Sibler et al., 1999; Santala and Lamminmaki, 2004).

1.2.7 CD4 protein

CD4 is an integral membrane glycoprotein of 55 kDa. This molecule contains four external domains that show homology to members of the immunoglobulin super-family. The CD4 molecule is expressed on a subset of T lymphocytes utilizing MHC class II antigens as a restriction element for antigen recognition. T helper cells are also known as CD4 positive T cells (CD4+ T cells) which play an important role to organize the immune response. These cells alert B cells to start producing antibodies. Moreover, they also can activate other T cells and scavenger cells of the immune system called macrophages, which influence in the immune responses. T helper cells are activated after antigen presenting cells engulf and process an antigen and present fragments of that antigen in combination with a MHC class II protein on its surface membrane. This antigen-protein combination attracts a T helper cell and promotes its activation (Schnittman et al., 1989; Eiden and Lifson, 1992; Abbas et al., 2000; Janeway et al., 2004). In addition to its physiologic roles, CD4 is a receptor for the

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human immunodeficiency virus (HIV)(Camerini and Seed, 1990). Infection with HIV is caused of AIDS by massive dysfunction of the adaptive immune system. Most of the immunodeficiency in AIDS can be ascribed to the depletion of CD4+ T cells. Recently, the CD4+ T cell count has been used to assess prognosis for progression to AIDS or death, to formulate the differential diagnosis in symptomatic patients, and to make therapeutic decisions regarding antiviral treatment and prophylaxis for opportunistic pathogens (Camerini and Seed, 1990; Janeway et al., 2004). Therefore, monitoring of CD4+ T cell is very important tool used for successful HIV/AIDS management and care (1992; Stein et al., 1992; Phillips et al., 1994a; Phillips et al., 1994b). In this study, CD4 protein was used as a model for studying the antigen preparation techniques and for validating the antibody production purpose.

1.3 Ojectives

1.3.1 To develop the methods for preparation of immunogen

1.3.2 To develop and compare three immunization strategies for production of monoclonal antibodies to CD4 protein

1.3.3 To characterize the produced CD4 mAbs obtained from the three immunization strategies

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