CHAPTER IV

DISCUSSIONS AND CONCLUSION

Antibodies have become important tools for many applications. They have been widely used in the medical research and development, diagnostics, trade and, recently, for therapy. Antibodies are host proteins produced in response to the presence of foreign molecules in the body. They are synthesized primarily by plasma cells, a terminally differentiated cell of the B-lymphocyte lineage, and circulate throughout the blood and lymph where they bind to foreign antigens. An antibody response, however, is the culmination of a series of interactions between phagocytes, T lymphocytes and B lymphocytes, all reacting to the presence of a foreign antigen. Presence of an immunogenic molecule in an animal stimulates B-lymphocytes, which undergo proliferation, differentiation, and maturation such that numerous B cells produce antibodies. Each B-cell produces a single type of antibody molecule (monoclonal) so that the overall response of the body to an antigen involves different antibody molecules (polyclonal) from different B-cells (Abbas and Lichtman, 2006; Luttman et al., 2006). Because plasma cells cannot be grown in tissue culture, they cannot be used as an *in vitro* source of antibodies (Harlow and Lane, 1988). In 1975, Kohler and Milstein developed a technique that allows the growth of clonal populations of cells secreting antibodies with a defined specificity (Kohler and Milstein, 1975). In this technique an antibody-secreting cells, isolated from the immunized mouse, is fused with a myeloma cell, a type of B-cell tumor. These hybrid cells or hybridomas can be maintained *in vitro* and will continue to secrete antibodies with a defined specificity. Antibodies that are produce by hybridomas are known as

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monoclonal antibodies. The generation of monoclonal antibodies is dependent on immortalization of antigen-specific B-cell clones by fusion (Galfre and Milstein, 1981). A key feature in the success of this procedure is 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 of B-cell clones expressing antibody molecules of a desired antigen-specificity and affinity is modulated by the immunization with appropriate immunogen which can be soluble or insoluble proteins, polysaccharides, lipids or nucleic acids. Traditionally, the generation of mAbs has depended on the availability of immunogens for immunization. Generally, impure immunogens can be used to produce specific monoclonal antibodies. Because hybridomas are single-cell cloned prior to use, monospecific antibodies can produced after immunizations with complex mixtures of immunogens (Harlow and Lane, 1988; Luttman et al., 2006). However, when mixed populations of antigens are used for immunizations, an antibody response to several components of the preparation is expected. If all the compounds in a preparation are equally immunogenic and quantitative, the resulting antibody response will mimic this distribution. In the most case, the immunogenicity and quantity of the compounds varies, antibodies to some of the compounds may dominate the response. Hence, having pure and sufficient quantity immunogens provide the best case for production of antibodies (Harlow and Lane, 1988). In this study, immunoprecipitation technique, production of recombinant protein in bacterial expression system and COS cells expression system were applied to prepare immunogens. CD4 protein antigen was used as a model for immunogen preparations and antibody productions.

Immunoprecipitation technique is an immunological method involved in the interaction between a protein and its specific antibody. This technique was used in several approaches including the purification and enrich of protein of interest. In this study, the immunoprecipitation technique was applied in order to isolate CD4 protein from other cellular protein. By this technique, CD4 proteins were captured and separated from other proteins in PBMC lysates by using CD4 mAb immobilized on magnetic beads. After washed out the proteins that did not react with CD4 coated beads, the CD4 bound beads (CD4 immunoprecipitated-beads) were directly used as immunizing agent for antibody production, without eluting process. Conjugation of immunogens to particulate molecules stimulates phagocytic activity of antigen presenting cells resulting in enhancing the processing and presentation of antigenic peptides to T-lymphocytes (Unanue, 1984; Townsend and Bodmer, 1989). In addition, particulates antigens activates antigen presenting cells such as macrophages to secrete cytokines that enhance immune responses. Therefore, the use of protein immunoprecipitated beads as the source of immunogen for mouse immunization will increase the antibody induction compare to the immunization of soluble protein. In our study, before using this technique to prepare the CD4 immunogen, the success of CD4 protein precipitation was proved by using SDS-PAGE and Western blotting. It was found that, by using immunoprecipitation technique, commercialized CD4 mAbs L200 and VIT4 precipitated a protein with the molecular weight of 55-60 kDa which corresponding to CD4 protein. This result indicated that the immunoprecipitation technique could be used for CD4 immunogen preparation and the CD4 immunoprecipitated-beads can be used as immunizing agent. This technique provides

a powerful approach to isolate antigens that are in low abundance or difficult to purify in which the antibody is available.

In the case that natural immunogens are available, as was mention, the success of obtaining specific monoclonal antibodies depends on the abundance of the interested immunogen expression. In other hand, if natural immunogens are not available or expressed in low abundance, the problem in antibody production is occurred. Based on the available of gene sequence or partial gene sequence, advances in molecular biology and gene fusion technology have provided convenient means for recombinant protein production. By the recombinant DNA technology, it enables single proteins to be easily produced in various hosts, with multiple possibilities to purpose-design the protein product and also the production process (Koths, 1995). Expression of recombinant proteins or full-lengh polypeptide chains using both prokaryotic and eukaryotic vectors has become routine. These protein are often excellent antigens and can be produced in large quantities (Harlow and Lane, 1988; Luttman et al., 2006).

E. coli, the number one bacterium of recombinant DNA technology, has been extensively studied as production host for heterologous proteins. Since it is very well characterised, many strategies for optimising protein expression and protein quality, choice of E. coli strain, transcriptional and translational regulation, protein targeting to different cellular compartments and posttranslational modifications have been reported (Makrides, 1996; Murby et al., 1996). One potential drawback with prokaryotes as production hosts, however, is that they are unable to carry out some posttranslational modifications found in eukaryotic proteins but other beneficial properties, and in particular the cost-efficient production systems, make bacteria the

dominating hosts for production of recombinant proteins (Liljeqvist and Stahl, 1999). Several researchers employed this system to generated recombinant protein for animal immunization for production of antibodies.

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) . Recombinant fusion protein have been successfully used in production of monoclonal antibody (Sjolander et al., 1997; Tayapiwatana et al., 2006). Recently, Santala and Lamminmaki (2004) reported the use of biotin carboxyl carrier protein (BCCP) as a fusion partner of single chain Fv. In this study, we applied the BCCP-fusion protein strategy to produce the biotinylated CD4-BCCP fusion protein in *E. coli*. For this purpose, nucleotide sequence encoding CD4 protein was cloned into pAk400cb vector (Santala and Lamminmaki, 2004). In order to genetically link the nucleotide sequence to the BCCP biotin acceptor domain coding sequence. Transformation of the resulting vector, pAk400cb-CD4, into *E.coli* results in high level expression of a CD4 protein fused to BCCP and therefore this protein was biotinylated. To permit the formation of the disulfide bonds, we chose to produce recombinant proteins in *E. coli* Origami B (Prinz et al., 1997), the redox-modified *E. coli* strain with oxidizing cytoplasm where the formation of the disulfide bridge can occur. The biotinylation of recombinant proteins obtained in bacterial extract was firstly verified using indirect ELISA. Polyclonal CD4 antibodies and BCCP mAb BCCP-2 react to the BCCP fusion protein,indicating the presence of CD4-BCCP with biotin attached. The presence of biotinylated CD4-BCCP fusion protein was confirmed by Western blotting. Surprisingly, standard CD4 mAbs and polyclonal CD4 antibody did not react to the fusion protein. Nevertheless, a band of protein with

a molecular mass of 55 kDa, the size expected for CD4-BCCP protein was detected in Origami B-pAK400CB-CD4 extract when probed with BCCP mAb and HRP-conjugated streptavidin. According to the results of ELISA and Western blotting that the produced CD4-BCCP could not detected by CD4 mAbs. However, it could be detected by BCCP mAbs. We speculated that the CD4-BCCP was produced and could be used as immunogen for further antibody production processes.

As was mentioned, fusion proteins are generally designed for purification purpose. Herein, BCCP fusion can also simplify the purification by using streptavidin coated magnetic beads. The magnetic beads trapped the CD4-BCCP via streptavidin-biotin specific reaction. The presence of the fusion protein on the obtained beads, CD4-BCCP beads, was determined before use for immunization. The positive reactivity was observed in staining with polyclonal CD4 antibodies and BCCP mAbs BCCP-2. These indicate the immobilization of CD4-BCCP fusion protein on the magnetic beads, which were then used as an immunizing agent. The protein-coated beads serve as particulate antigens that are strongly immunogenic for induction of immune responses (Heinz et al., 1995; Telino et al., 2006). Based on the available of gene sequence or partial gene sequence, this technique can be used to produce and isolate proteins for used as immunogens. Besides overexpressing the protein and yielding sufficient quantities for immunization, the fusion proteins themselves are immunogenic and can potentially boost an immune response to the specific protein. Screening for specific monoclonal antibodies can be performed with the recombinant protein.

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. COS cell expression system is one of popular methods for production of recombinant proteins. In this study, COS cells expression system was used to prepare CD4 immunogen.

The CD4-DNA used in this study was cDNA encoding CD4 protein, which were cloned into eukaryotic expression vector, πH3M. The πH3M is a high efficiency expression vector (Aruffo and Seed, 1987), which was constructed for the expression of inserted cDNA in COS cells. This vector contains a simian virus 40(SV40) origin of replication for replication of cDNA in SV40 transfromed COS cells (Gluzman, 1981). Transcription of the inserted cDNA is driven by the human cytomegalovirus (CMV) promoter. Two features of this vector make it particularly suitable for this use: (1) the eukaryotic transcription unit allows a high-level expression in the COS cells of coding sequences placed under its control; (2) the small size and particular arrangement of sequences in the plasmid, permit high-level replication in COS cells.

To prepare CD4-DNA, plasmid DNA was firstly transformed into *E. coli* to increase the number. Then, plasmid DNA was isolated from the transformed bacterial colony. The obtained CD4-DNA was transfected into COS cells to test their capability in expressing CD4 proteins. The CD4-DNA transfected COS cells reacted strongly with standard CD4 mAb MT4, but were negative with the conjugate control. The CD4 mAb MT4, in contrast, were negative with untranfected COS cells. The result indicated that the CD4-DNA could be transcribe and translated into CD4 protein in

COS cells. This DNA can be used to prepare CD4-COS cells, and used as immunogen.

For the introduction of CD4-DNA into COS cells, the DEAE-dextran transfection method, which has been demonstrated as the best, was selected (Kasinrerk et al., 1992). In this method, a DEAE-dextran/CD4-DNA complex mixture was prepared and incubated with COS cells in culture. The complexes stuck to the cell surface, then, cells were exposed to the dimethyl sulfoxide to increase DNA uptake (Malienou-Ngassa et al., 1990). After transfection, transfected DNA are normally degraded by enzymes contained in lysosomes (Alberts et al., 1983). Inhibition of the lysosomal enzymes is required for the maintenance of transfected cDNA and, therefore, increases transfection efficiency. Chloroquine, which has been described as an inhibitor of lysosomal enzymes, is commonly used to increase transfection efficiency (Selden et al., 1987). However, it is generally agreed that chloroquine diphosphate is extremely cytotoxic if left on the cell for long period of time. Generally, most cells cannot survive exposure to choloroquine for more than 4 hours. The 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 (Edwards and Aruffo, 1993; Trill et al., 1995).

To prepare CD4-COS cells for using as immunogen, CD4-DNA was transfeced into COS cells by DEAE-dextran transfection method. The transfected COS cells were checked for CD4 protein expression by indirect immunofluorescence staining. It was found that, approximately 20 % of the transfected cells expressed CD4 protein. To obtain the high number of CD4 expressed cells, the CD4 expressed

COS cells were enriched by immunomagnetic beads sorting using standard CD4 mAb. In this technique, CD4 mAb coated magnetic beads was used to positive select CD4 expressing COS cells in transfected COS cells. By using magnetic particle concentrator, approximately 70% of CD4 positive cells were obtained. The obtained CD4-COS cells were used to immunize mouse for generation of monoclonal antibody. By this technique, mice were immunized with a population of cells that expressed protein of interest. The expressed proteins are in abundance and appeared in mostly cells. The recombinant proteins produced by COS cell expression system are post-translational modified and therefore they are similar to the native proteins.

To verify the effective of generated immunogens for monoclonal antibody production, mice were immunized with each generated immunogen. CD4 antibodies could be detected in all of immunized mice after immunizations with each type of immunogen. These results demonstrated the success of induction of antibody production by immunization with three different immunogens. Nevertheless, different imunogen showed different degree of antibody response. CD4-COS cells immunization induced highest titer of CD4 antibodies and CD4 immunoprecipitated-beads immunization induced higher titer than CD4-BCCP beads immunization. In contrast to CD4 specific antibody, in mice immunized with CD4-BCCP beads, the anti-BCCP-CD4 titer was very high. The titer was up to 312,500 after the third immunization of CD4-BCCP beads. This might due to that the protein antigen expressed in bacterial often folded incorrectly and lack of post-translational modifications therefore antibodies against bacterially expressed protein may fail to bind to native CD4 protein.

In an attempt to produce CD4 monoclonal antibodies, spleen cells from immunized mouse and X63Ag8.653 myeloma cells, which is a murine myeloma cell line (Kearney et al., 1979), were fuse using PEG as a fusing agent (Pontecorvo, 1975). All hybridoma production in this study, 60-90 % of the seed wells contained hybridomas demonstrating a very good fusion was performed.

For hybridoma production using CD4-BCCP beads immunization, the cultures supernatant from hybridoma containing wells were screened firstly by indirect ELISA using CD4-BCCP fusion protein as antigen. To eliminate any hybridomas specific to BCCP part, the culture supernatant were counter screened with irrelevant protein, CD147-BCCP. By this screening, hybridomas that indicated the difference of absorbance greater than 0.3 when tested with CD4-BCCP and CD147-BCCP were further screened for the specific CD4 antibody producing hybridomas.

To screen hybrids which produce CD4 antibody, supernatants from hybridoma containing were tested by indirect immunofluorescence technique. In the preliminary screening procedure, PBMCs were used as antigen. Because CD4 protein is expressed on a subset of T lymphocytes and weakly expressed on monocytes, hybridoma containing wells that showed positive with a lymphocyte subpopulation but negative or weakly positive with monocytes were further screened for specific antibody to CD4 protein by staining of CD4-DNA transfected COS cells. By this way, well containg CD4 antibody will positive with CD4 expressing COS but negative with untransfected COS cells.

In the screening of the hybridoma by using CD4-DNA transfected COS, one CD4 mAbs hybridoma producing cell, named MT4/4, derived from immunoprecipitated-beads immunization and two CD4 mAb hybridoma producing cells, named MT4/2

and MT4/3, derived from CD4-COS cells immunization were obtained. This results indicate the success of hybridoma productions by using immunoprecipitation and COS cell expression system for preparation of immunogen. The antibodies obtained by these two techniques always can react to native protein. In order to produce monoclonal antibodies for recognizing a native protein and particularly if anti-functional antibodies are desired, the immunprecipitated-beads technique and COS cells expression system should be employed.

For hybridoma production by CD4-BCCP beads immunization, only 1 hybridoma containing wells showed positive with a lymphocyte subpopulation but negative or weakly positive with monocytes whereas the other clones, which showed positive with CD4-BCCP, could not recognize the native antigen on cells. This might due to the native CD4 expressed on surface membrane protein has been post-translationally modified. Therefore, the mAbs which recognized the linear epitope can not react to the native CD4 molecule. Unfortunately, the only 1 clone that recognizes the native antigen on cells lost their activity during cultivation. However, 200 hybridomas producing CD4-BCCP antibodies were generated. This technique is suitable for generation of antibodies to unfolded or unmodified from of any protein. The resulting mAbs which recognized the linear epitope can be extremely useful for Western immunoblotting assay and indeed for any situation in which the antigen is denatured.

The generated CD4 mAbs, MT4/2, MT4/3 and MT4/4 were examined for their reactivity in different assays. All generated CD4 mAbs as well was standard CD4 mAb MT4 strongly reacted to CD4-DNA transfected COS cell but did not reacted to CD8-DNA transfected COS cells. Thus the specifically binding of all CD4 mAbs

generated in this study was confirmed. Furthermore, this indicates the ability of the generated CD4 mAbs in binding CD4 recombinant expressed by mammalian cells.

The reactivity of the generated CD4 mAbs to SDS-denatured antigen was tested by Western blotting. It was found that, all CD4 mAbs and standard CD4 mAb leu3a did not react to the denatured antigen under both reducing and non-reducing condition. This result may indicate that the generated CD4 mAbs are the mAbs that react to the conformational epitopes on the CD4 protein whose structure was destroyed by SDS treatment. By immunoprecipitation technique, in contrast to Western blotting, all generated CD4 mAbs precipitated a protein band at the molecular weight of approximately 55 kDa which is corresponding to the CD4 protein. These resutls indicates that the generated CD4 mAbs recognize the native CD4 protein. The produce CD4 monoclonal antibodies were then applied to enumerate CD4+ T cells in peripheral blood by immunofluorescence and flow cytometry. The percentage of CD4+ T cells obtained by using the generated CD4 monoclonal antibodies were similar to those obtained using the standard reagent. Therefore, the generated CD4 mAbs can be used as reagent for determination of CD4+ cells.

In conclusion, in this study, three methods were studied for preparation of CD4 protein for hybridoma productions. It was demonstrated that the studied methods can be used to produce CD4 mAbs. The developed methods are precious and can be applied for production of antibodies to other interested protein antigens where it is not available or difficult to prepare, but either the encoding cDNA or specific mAb is available.