CHAPTER IV

DISCUSSION AND CONCLUSION

In avian, IgY is an immunoglobulin of egg yolk that transfers from maternal circulation to confer passive immunity to embryos and neonate before the generation of their own humoral immunity (Rose *et al.*, 1974). The amount of IgY transported is known to be proportional to the maternal serum IgY concentration (Loeken and Roth, 1983). Previous studies demonstrated that, in chicken, it is very easy to induce humoral immune response. Only 20-30 μg of antigen is sufficient to induce an immune response. The specific antibodies appeared 20 days after immunization, reached a plateau after 30 days, and remained high until at least day 81 (Gassmann *et al.*, 1990). The induced antibodies were passed to egg yolk in a high quantity. In a single egg, approximately 75-100 mg of IgY is presence (Maya *et al.*, 2002). The IgY up to 1500 mg can be harvested each month from an egg (Schade *et al.*, 1994). Comparison of this amount of antibody to other animals, only larger mammals such as sheep can produce equal amounts of antibodies. When compared to rabbits, production of antibodies in chicken antibodies is ten times less expensive (Svendsen and Hau, 1996). Isolation of IgY antibody from egg is easy to perform and the collection of blood which is painful for animal could be omitted. From these advantages, the production of IgY antibody from egg yolk, IgY technology, has been developed. The IgY specific antibodies were then applied in both

immunodiagnostics and immunotherapy (Carlander *et al.*, 2000; Lindahl *et al.*, 1992; Gutierrez *et al.*, 2001; Ceuninck *et al.*, 2001)..

Indirect immunofluorescence is a technique for detection of antibodies and cellular antigens. For study cellular distribution of cell surface molecules, cells were stained with primary monoclonal antibody and counterstained with fluorochrome labeled anti-mouse immunoglobulins antibody (secondary antibody) and analyzed either with fluorescent microscope or flow cytometer. As the secondary antibody used in this technique is expensive, in this study, the generation of secondary antibody for using in the indirect immunofluorescence technique was proposed. Hen egg yolk antibodies (IgY) have been reported to offer many advantages over antibodies obtained from serum (Larsson *et al.*, 1993). In addition, mammal proteins, i.e. mouse immunoglobulins, are often more immunogenic in birds than in mammals (Gassmann *et al.*, 1990). In this study, the IgY technology was therefore chosen for production of anti-mouse immunoglobulins antibody.

To produce anti-mouse immunoglobulins antibody, mouse immunoglobulins for using as immunizing antigen were firstly prepared. In this study, mouse immunoglobulins were purified from normal mouse serum by affinity chromatography using Protein G Sepharose column. Protein G is an approximately 30 kDa polypeptide derived from cell wall of certain strains of β-hemolytic *Streptococci* (Frank, 2001). It binds Fc region of various immunoglobulins isotype and albumin from difference species, i.e. human, mouse, rat, rabbit and horse (Frank, 2001). After Protein G purification, in this study, the obtained immunoglobulins were verified by SDS-PAGE under both reduced and non-reduced conditions. In this experiment, under reducing conditions, 2 bands with molecular

weight of 51 and 26 kDa were observed. While under non-reducing conditions, a single high molecular weight band was observed on the top of the gel. As under reducing conditions the sample was treated with 2-mercaptoethanol that reduced disulfide bonds, the observed 2 bands were heavy and light chain of immunoglobulins respectively. On the SDS-PAGE, no other protein was observed. These results therefore indicated that mouse immunoglobulins obtained from the Protein G column was pure as was mentioned by Verdoliva *et al.*, 2002. The sandwich ELISA was applied to determine the immunoglobulin isotype contained in the isolated immunoglobulins. The isolated mouse immunoglobulins were demonstrated to contain all immunoglobulin isotypes. The IgA and IgM isotypes were suspected to be contaminated. However, the non-specific binding of IgA and IgM to Protein G Sepharose column take advantage for this preparation. Therefore, The isolated mouse immunoglobulins could be used as the immunizing antigen for production of specific antibodies.

To produce anti-mouse immunoglobulins antibody, two hens were immunized with the isolated immunoglobulins *via* two different routes, pectoralis and calf intramuscular immunizations. It was found that both hens produced specific antibody after immunization. Nevertheless, they showed different degree of immune response. Pectoralis intramuscular immunization induced higher titer of antibodies than calf intramuscular immunization. In addition, the antibodies could be detected longer period of time in pectoralis immunization compare to calf muscle immunization. This result was in correspondence to those which have been suggested by various reports (Gassmann *et al.*, 1990; Ceuninck *et al.*, 2001). In this study, only one hen was used for each route of

immunization. To clearly demonstrated that the pectoralis immunization route is really better than the calf immunization route, more hens need to be investigated.

Several methods have been reported for extraction of IgY from egg yolk (Jensenius *et al.*, 1981; Song *et al.*, 1985; Hassl and Aspock, 1988; Gassmann *et al.*, 1990; Polson, 1990; Akita and Nakai, 1992,1993, Polson *et al.*, 1980, 1985; Bhanushali *et al.*, 1994; Belew *et al.*, 1987). Water dilution with the combination of sodium sulfate precipitation is one of the methods demonstrated to be simple and reliable for isolation of chicken IgY (Akita and Nakai, 1993). The principle of water dilution and sodium sulfate precipitation is that water dilution will fractionate plasma proteins from the egg yolk lipids. Sodium sulfate will remove water molecules from the protein, resulting in precipitation of the protein. The majority plasma protein that precipitated at 19%(w/v) of sodium sulfate is immunoglobulins. In this study, the water dilution and sodium sulfate precipitation was selected for isolation of IgY from egg yolk. By using this method, from a single egg, approximately 40 mg of IgY could be extracted. The purity of extracted IgY was then determined by SDS-PAGE. It was found that, by the water dilution and sodium sulfate precipitation, the purity of extracted IgY was rather high. However, small amount of non-immunoglobulin protein was observed in the extracted IgY. This contaminated protein was likely to be albumin (Cook *et al.*, 2001). In this study, as just small amount, the contaminated protein was ignored. The extracted IgY were used in the further experiments. However, if the removal of the contaminated protein is necessary, more purification process such as ion-exchange chromatography, gel filtration, affinity

chromatography and thiophillic interaction chromatography were suggested (Cook *et al.*, 2001, Hassl and Aspoc, 1988; Gassmann *et al.*, 1990).

After mouse immunoglobulins immunization, the antibody titer in sera and extracted IgY were determined by ELISA. In this study, the optimization of antigen and conjugate used in the ELISA were first determined. It was found that 0.625 μg/ml of mouse immunoglobulins for coating plate and 1:6000 of HRP conjugated anti-chicken IgG (Zymed) were the optimized concentrations. The optimized ELISA was then used to determine the antibody titer in both sera and egg yolk IgY. In sera, by pectoralis intramuscular immunization, anti-mouse immunoglobulin antibody was detected after the first immunization. The antibody was increased after the second and the third immunizations. Up to titer of 1:64,000 was observed two weeks after the third immunization. The antibody reached a plateau at titer of 256,000 three weeks later (week 11) and maintained high titer at least 20 weeks after the first antigen immunization. This kinetics of antibodies responses is in corresponding to the previous study showing that specific antibodies appeared 20 days after immunization, reached a plateau after 30 days and remained high at least day 81 (Gassmann *et al.*, 1990). In egg yolk IgY, the antibodies reached a plateau after in serum seven days. Our results suggested that production of antibody in chicken is simple and the high quality of antibody can be obtained in both serum and egg.

In an attempt to produce FITC conjugated chicken anti-mouse immunoglobulins for using in indirect immunofluorescence assay, the extract IgY was then labeled with FITC fluorochrome. Several methods were described for FITC-antibody labeling

(Haugland, 1992; Brinkley, 1992; Khalfan *et al.*, 1986; Truneh and Machy, 1987; DeBiasio *et al.*, 1987; Catsinpoolas, 1979; Wong, 1991). Fluorescence modification of proteins is limited to three locations on the molecule: amines, thiols and carbohydrates. Labeling at the amine site is the most widely used method and gives the most strongly fluorescence derivative because of the large number of lysines present in antibodies. The isothiocyanate derivatives of fluorophores are relatively stable in aqueous solution and react with amines yielding thiourea bonds at pH 9-9.5. The labeled antibody was then separated from free FITC by dialysis or gel filtration. To obtain a useful degree of labeling, a molar incubation ratio of dye:antibody (R ratio) of 10-15 is generally used. However, it does not have an exact protocol for fluorescence labeling because the degree of labeling depend on the reactivity of dye or antibody, so trial conjugations with different molar ratios of dye:antibody should be titrated. To fluorochrome labeling, in this study, amine-reactive label method was used. The optimal conditions for FITC labeling was firstly studied. The purified IgY was labeled with various amount of FITC (R ratio). The activity of the FITC-labeled antibodies from various R ratio was then compared. It was found that the staining background was increased when R ratio was increased. While the positive fluorescence intensity was decreased when the R ratio was decreased. In these titration studies, the R ratio of 10 was demonstrated to be the optimal concentration for production conjugate using in indirect immunofluorescence assay. The R ratio of 10 was also demonstrated to be the optimal concentration for chicken IgY and other mammal immunoglobulin in other studies (Lindahl *et al.*, 1992).

The FITC labeled chicken anti-mouse immunoglobulins antibody was then tested for using as conjugate in the indirect immunofluorescence assay. In this study, white blood cells were stained with various isotypes of primary antibodies and the produced conjugate was used as secondary antibody. The stained cells were analyzed by flow cytometry in comparison to the commercial conjugate (Silenus). We found that the produced conjugate showed fluorescence intensity slightly lower than commercial one, especially when the primary antibody was IgM isotype. This may be because of the produced conjugate contains unlabeled antibodies which compete the binding of labeled antibodies to the antigen. However, when the percentages of T and B-lymphocytes in lymphocyte population were determined and compared between using the produced and commercial conjugate, the percentages of positive cells obtained from both conjugates were not difference (t test, $p>0.05$). These results indicated that the produced conjugate could be used in the indirect immunofluorescence assay.

As indirect immunofluorescnce was also used to determine the expression of protein of interest in COS cell expression system (Kasinrerk and Tokasinwit, 1999; Moonsom.and Kasinrerk, 2000), we therefore evaluated whether the produced conjugate can be used in this expression system. COS cells were transfected with cDNA encoding CD14 (Moonsom and Kasinrerk, 2000) and CD147 (Kasinrerk *et al.*, 1992; Khunkeawla *et al.*, 2001) proteins. The transfectants were then stained with various isotypes of CD147 and CD14 monoclonal antibodies and counterstained with the produced conjugate. By fluorescence microscope, the produced conjugate showed positive reactivity with all specific primary antibodies but showed negative reactivity with isotype matched control

antibodies. These results indicated that the IgY conjugate can be used not only in human but also in monkey system.

In conclusion, the use of IgY technology to produce anti-mouse immunoglobulins antibody has been described in this study. This technique is simple and large amounts of antibodies were obtained. The obtained IgY antibodies could be labeled with FITC fluorochrome. The IgY-FITC could be used to determine human lymphocyte sub-populations and protein expression in COS cell transfection system. The IgY technology has many advantages over other mammalian animals because egg collection is non-stressful to hens, antibodies purification is simple and very high concentration of the specific antibodies is obtained from egg yolk in short time after immunization and maintained long lasting titers. In addition, IgY has intrinsic biochemical advantages. It does not activate mammalian complement and show no interaction with mammalian Fc receptors, which avoid interference in immunoassays.