CHAPTER I

INTRODUCTION

1.1 Statement of problems

The immune system is an important system for the body, which is involved in protection the body from pathogens and from mutated and/or oncogenic cells. In avian, its immune responses are very similar to those of the mammals. Avian immune system can be divided into two parts, which are innate immunity and acquire (adaptive) immunity. Innate immunity represents the first line of defense against pathogens whereas the acquired immunity is specific immune responses for entering antigens.

In chicken, antibodies or immunoglobulins can be divided into 3 classes, IgA, IgM and IgY. Among of them, IgY is only the immunoglobulin that can be transported from maternal circulation to egg yolk to confer passive immunity to embryos and neonate before generation of its own humoral immune response (Rose *et al.*, 1974). In the previous studies demonstrated that very low quantities of antigen are required to induced high and long-lasting IgY titers in the yolk (Gassmann *et al.*, 1990). Recently, IgY antibodies isolated from egg yolk have been used in several aspects including immunotherapy and diagnostics applications. Antibody production from egg yolk, IgY technology, offers several advantages over conventional serum-based antibody production. Egg collection is easy, no blood bleeding is required, and the amount of immunoglobulins yielded from one egg is very high (Maya *et al.*, 2000).

Many immunological techniques have been employed in diagnostic applications such as agglutination, enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and immunofluorescence assay (IF). The techniques using labeled reagents for detecting antigens and antibodies are exquisitely sensitive and specificity. Immunofluorescence assay is one of the labeled immunoassay for detection of antibodies to tissue and cellular antigens and also specific antigens. Although this technique is more cumbersome than others if a quantitative measurement of antibody or antigen concentration is required, however, they do have advantages. By using tissue section (which contain a large number of antigen), antibodies to several different antigens can be identified on a single slide according to their distribution between cells or in different sub-cellular compartments. Furthermore, the immunofluorescence test can be used to identify particular cells in suspension, that is, to identify antigen on live cells. When stained-cell suspension is measured by a fluorescence-activated cell sorter (FACS), the fluorescence intensity of each cell is measured and then the cells are separated according to their particular fluorescence brightness. This technique permits the isolation of different cell populations with different antigens stained with different fluorescence antibodies. Immunofluorescence technique is divided into two types, direct and indirect immunofluorescence. Direct immunofluorescence is a technique for detection interested antigen by using the fluorochrome labeled antibody that specifically react to antigen on tissue or cell. However, when the primary antibody reacted firstly with antigen on cell or tissue, then counterstained with the fluorochrome labeled anti-immunoglobulin antibody (secondary antibody), this method is called indirect immunofluorescence. The indirect

immunofluorescence technique can be used for detection of either antibodies or antigens of interest. Several types of secondary antibodies are commercially available, however, it is expensive. In this study, therefore, the production of anti-mouse immunoglobulins for using as secondary antibody in the indirect immunofluorescence was proposed.

In an attempt to produce anti-mouse immunoglobulin antibody conjugate for using in indirect immunofluorescence technique, in this study, IgY technology was applied. Mouse immunoglobulins will be purified and used as antigen for immunization of chicken. The IgY will be then isolated from eggs of the immunized animals and conjugated with fluorescein isothiocyanate (FITC). The generated conjugate are then applied in indirect immunofluorescence assay. In this study, IgY technology will be developed in the Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University. The kinetics of antibodies production, both in serum and egg IgY, after antigen immunization will be obtained. The appropriate method for FITC labeling of IgY will be optimized. Finally, the produced FITC conjugate will be used in indirect immunofluorescence assay.

1.2 Literature reviews

Avian immune system

An immune system is present in all species in the animal kingdom. It functions as a defense against intruding organisms, molecules and malignant cells. The purpose is to protect its host from foreign substances. In a broad sense, the immune system in birds is no different from the immune system found in mammals. The immune system can be

divided into two parts: the innate and the acquired immune system. The innate response acts as a first line of defense infections whereas the adaptive immune response is highly specific for a particular antigen.

The innate immune system, skin and mucous membranes act as barriers to prevent invasion of microorganisms. Cilia, mucus and the cough reflex expel inhaled material from the respiratory tract. In the gastrointestinal tract the low pH in the stomach and the normal bacterial flora in the gut also have important protective roles. There are also several white blood cells involved in the innate immune system. Avian natural killer (NK) cells are large granular lymphocytes that are morphologically similar to mammalian NK cells. The NK cells selectively identify and kill virus-infected and tumor target cells and do not need prior antigenic exposure for target recognition (Gobel *et al.*, 1996; Telfer and Rothenberg, 2001). Unlike cytotoxic T-cells, the NK cells are not MHC-restricted (Telfer and Rothenberg, 2001). Another important component of avian innate immunity is the monocytes- macrophage system. Macrophage are the important part of the innate immune defense, operating immediately when a microorganism or foreign antigen enters the body, thereby limiting the growth of the pathogen (Qureshi *et al.*, 2000). Macrophages have microbicidal, phagocytic and tumoricidal functions but they also acts as regulatory cells through cytokines and other metabolites. Monocytes are the major phagocytic cells in chicken blood while tissue macrophages are present in almost all organs. Macrophage phagocytic function appears within the first two weeks of chicken embryonic development (Jeurissen and Janse, 1989). The non-specific mechanisms respond rapidly to a foreign

invasion but it does not have ability to respond with increasing strength to repeated challenges from the same organism.

The acquired immune system, specific immunity depends on the ability to recognize substances, respond to them and memorize the information in case of repeated exposure. The specific immune system functions through two interacting mechanisms, the humoral and cellular responses. The humoral response involves interaction of B cells with an antigen and subsequent proliferation and differentiation into antibody-secreting plasma cells with or without the help of T helper cells. Two basic types of lymphocytes are involved in an antigen-specific response. The B-lymphocytes express surface immunoglobulins that are specific to an epitope on the antigen and T-lymphocytes that recognize processed antigens on antigen-presenting cells. The antibody-secreting plasma cells produce soluble antibodies that are identical to the surface immunoglobulin on the original B-cell. The cellular immune response involves interaction of the T-cell receptor and processed antigen. There are two main pathways. The first is reaction of T-cells with antigen and lymphokine secretion that attracts phagocytes to the site, which will phagocyte the antigen. The second route is interaction of cytotoxic T-cells with processed antigen presented by MHC class I cells which eventually leads to cell lysis (Arstila *et al.*, 1994).

Immunoglobulins in the chicken

Three immunoglobulin classes, analogues to the mammalian immunoglobulin classes have been shown to exist in chicken, IgA, IgM and IgY (IgG). The presence of

antibodies homologous to mammalian IgE and IgD has also been proposed but has not been proven (Burns and Maxwell, 1981; Chen *et al.*, 1982). The molecular weights, morphology and immunoelectrophoretic mobility of chicken IgA and IgM are similar to mammalian IgA and IgM. IgY is the major low molecular weight serum immunoglobulin in oviparous (egg laying) animals. Chicken IgY is a systemic rather than a secretory antibody but IgY is also found in duodenal contents, tracheal washings and seminal plasma. It is called IgY rather than IgG to distinguish it from its mammalian counterpart (Leslie and Clem, 1969). The argument was that the heavy (H) chain of IgY molecule is larger and antigenically different from the mammalian heavy chain. There is no immunological similarity between chicken IgY and mammalian IgG, and the DNA sequence of chicken IgY resembles more the sequence of human IgE. There is a lower content of β sheet structures in IgY that may indicate that the conformation of the IgY domain is more disordered and less stabile compared to that of rabbit IgG domains (Shimizu *et al.*, 1992).

The overall structure of IgY is similar to mammalian IgG, with two light (L) and two heavy chains (Figure 1.1). The molecular mass has been reported to be 167,250 Da, slightly larger than IgG (~160 kDa) (Sun *et al.*, 2001). The H chain (MW 65,105), called upsilon (ν; capital letter Y), has one variable (V) region and four constant (C) regions. The light chain (MW 18,600 Da) is composed of one variable and one constant domain. The Cν3 and Cν4 of the IgY are most closely related to the Cγ2 and Cγ3 of IgG respectively and the Cν2 domain is absent in the γ chain. The Cν2 region was probably

condensed to form the hinge region of IgG as studies have shown that IgY is an ancestor to mammalian IgG and IgE and also to IgA (Warr *et al.*, 1995). The Fc region of IgY mediates most biological effector functions in the chicken, such as complement fixation and opsonization. IgY is a skin-sensitizing antibody that can mediate anaphylactic reactions, a function that is attributed to IgE in mammals (Faith and Clem, 1973). In many ways IgY combines the functions associated with mammalian IgG and IgE in the chicken.

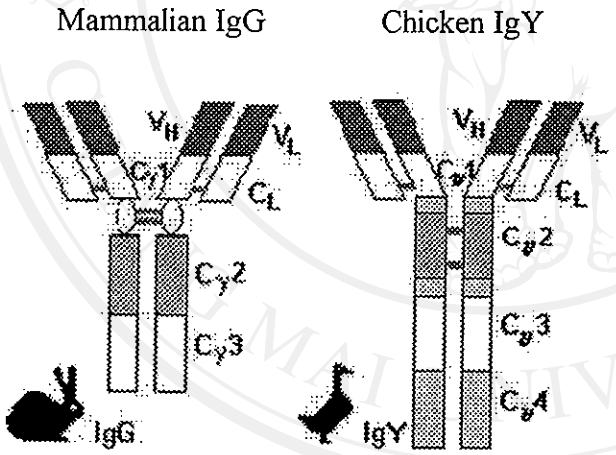

Figure 1.1 The structural differences between mammalian IgG and chicken IgY (Szabo *et al.*, 1998)

Immunoglobulin diversity in chickens

Studies of the chicken immune system have contributed substantially to the understanding of the immune response, including separation of the T- and B-cell lineages. The chicken immune system consists of the bursa of Fabricius, bone marrow, spleen,

thymus, the Harderian gland, lymph nodes, circulating lymphocytes and lymphoid tissue in the alimentary tract. The antibody-synthesizing cells (B -cell) are produced by the bursa of Fabricius. The chicken bone marrow is the source of bursal and thymic stem cells while the spleen is the center for plasma cell proliferation and memory B-cells (Toivanen *et al.*, 1974). Birds without spleen have a lower antibody production (Aitken, 1973). The thymus is a maturation center where stem cells differentiate into T-lymphocytes. The activities of chicken T-lymphocytes are similar to those in mammals.

The mechanism of antibody diversity in chicken differs from mammals and is mainly due to somatic hyper conversion. Rearrangement contributes little to the diversity as both the heavy and light chain loci consist of only one functional V (variable) gene (Reynaud *et al.*, 1985; Weill and Reynaud, 1987). There also seem to be a deficiency in the mechanism for selecting higher-affinity somatic mutants. The chicken has solved this deficiency by using three mechanisms that diversify the limited germ-line repertoire: gene hyper conversion (Weill and Reynaud, 1987; Reynaud *et al.*, 1987), V-J flexible joining (McCormack *et al.*, 1989) and somatic point mutations (Parvari *et al.*, 1990). Gene hyper conversion starts around day 15-17 of incubation after the immature B-cell progenitors migrate to the bursa of Fabricius. During the process blocks of DNA are transferred from pseudo-V genes to the recombined variable regions of the Ig genes resulting in the production of mature B-cells competent to form a functional humoral immune system (Masteller *et al.*, 1995).

The ν heavy chain gene is encoded by three exons separated by only two introns, as there is no intervening DNA sequence between the CH1 and CH2 alleles. The

immunoglobulins heavy-chain constant regions of IgY, IgA and IgM are all located on chromosome E18C15W15. The IgA gene is located upstream the IgY gene in an inverted transcriptional orientation. The distances between the IgA, IgY and IgM gene are about 18 and 15 kilobases, respectively. The size of the whole chicken IGHC locus is approximately 67 kilobases (Zhao *et al.*, 2000). Furthermore there are 16 alternative diversity (D) segments in the heavy chain locus. However, only V(D)J joining in the chicken can not produce the combinatorial diversity of the large numbers of V and joining (J) segments seen in mammals. Instead, an equivalent degree of diversity is achieved by successive partial conversions of the rearranged V(D) segments by templates in an upstream array of pseudo-V(D) genes (McCormack *et al.*, 1993). The variable and joining segments of both the heavy and light chain loci undergo V(D)J rearrangement. The entire naive B-cell repertoire of the adult chicken is produced in the Bursa of Fabricius of the young bird (Davies *et al.*, 1995).

Transport of IgY from maternal serum to the offspring

The transport of IgY from the hen serum to the offspring is a two-step process. First, IgY is transported from the serum to the egg yolk in analogy to the cross-placental transfer of antibodies in mammals. The second step is the transmission of IgY from the yolk sac to the developing embryo. The concentration of IgY in the yolk is essentially constant through the oocyte maturation, and at maturity the yolk will contain about 10-20 mg/ml IgY. Looking at the egg, IgY is not present in the white egg while IgA and IgM is not present in the yolk (Rose *et al.*, 1974). There is about 100-400 mg IgY packed in the

egg. Labeled IgY binds specifically to yolk sac tissue from day 7 up to at least day 18. This binding is saturable, Fc-specific, pH-dependent and reversible (Tressler and Roth, 1987). There is both a high and a low affinity receptor for IgY on the embryo. The low affinity receptor, $K_D 3.4x10^{-7}$, is present at day eight, whereas the high affinity receptor ($K_D 3.4x10^{-7}$) is detected at day 18. The low affinity receptor has a constant density as the total weight of the yolk sac increases, which implies that the rate change is due to an increase in tissue mass (Tressleer and Roth, 1987). The receptor binds some heterologous IgY such as pigeon IgY but in a less efficient manner. Molecules such as bovine serum albumin, phosvitin, conalbumin, chicken IgM and chicken Fab fragments does not bind (Tressleer and Roth, 1987). The IgY receptors on the oocyte bind and move all populations of IgY from the hen serum to the egg (Loeken and Roth, 1983). The populations of IgY are transported according to their concentration in the maternal serum. There is no selection nor destruction of IgY during transport and the yolk IgY has the same amount of sialic acid as the serum IgY.

The amount of IgY transported is independent of egg size and known to be proportional to the maternal serum IgY concentration (Loeken and Roth, 1983). A delay of three to four days is found between the appearance of IgY in the yolk is by a factor 1.23 to the serum concentration (Wooley and Landon, 1995). The density of yolk is about 1.1 g/ml. About 50% of the yolk is non-aqueous material. The total amount of IgY in the hatched chick has been estimated to be only 2-3 mg, compared to the 100-400 mg present in the yolk (Kowalczyk *et al.*, 1985). The major part of the IgY probably serves only as nutrition for the developing embryo. In the newly hatched chick the IgY concentration in

circulation is about 1-1.5 mg/ml and the circulating half-life of IgY is about 36 hours. IgY secreting cells in the offspring are not detectable until six days after hatching (Brambell, 1970).

Biochemical properties of IgY

There are two antigen binding sites for IgY molecule, same as for mammalian antibodies (Warr *et al.*,1995). In place of the hinge region of mammalian IgG, IgY has a sequence that is more rigid, giving IgY limited flexibility. This probably is the reason for many of the different properties of chicken IgY in comparison to mammalian IgG. The restricted mobility of the hinge region (Cv2) in IgY heavy chain makes the antibody more rigid. This affects the capability of the antibody to precipitate or agglutinate antigens. Only part of chicken antibodies is precipitated at physiological salt concentrations and approximately 25% of the antibodies remain in the supernatant at maximum precipitation. The precipitation curve resembles the curve obtained with horse antibodies with a rapid decline with antigen excess. The precipitation improves at 1.5 M NaCl. The poor precipitation properties might be due to steric hindrance of the Fab arms to crosslink epitopes of two large antigens. The conditions permitting precipitation might loosen the restricted movement of the Fab arms and give functional independence to the binding sites.

Orally given IgY is a generally not immunogenic but IgY injected intravenously is an immunogen and elicits a typical anti-IgY IgM and IgG response in mice (Walsh *et al.*, 2000). IgY applied to other endothelial surfaces then the gastrointestinal tract is probably immunogenic but not yet sufficiently tested. The stability of IgY under acidic conditions

and toward pepsin digestion is slightly lower than that of bovine IgG (Shimizu *et al.*, 1998). However, IgY is fairly stable against digestion by internal proteases such as trypsin and chymotrypsin, and heat stable. Most antibody activity remains after 15 minutes at 70° C. Incubation of IgY at pH above 4 is well tolerlated, but at pH 2 and 37°C the activity is rapidly decreased. The rapid activity loss is probably due to conformational changes, as the polypeptide is not broken down as observed by SDS-PAGE (Shimizu *et al.*, 1998). Incubation of IgY and Fab' fragment at pH 2 for 4 hours have led to a 16 fold decrease in neutralizing activity but there were still substantial neutralization titers retained (Akita *et al.*, 1998). The immunological activity of IgY is not affected by pasteurization at 60°C for 3.5 minutes (Hatta *et al.*, 1993).

Advantages of IgY in immunodiagnostic aspects

- Production of antibodies against conserved mammalian proteins

As the difference between the antigen and the immunized animal increases, the immune response usually increases. There is a greater phylogenetic difference between avian and mammalian species compared to the difference between two mammalian species. As a result, chicken is a better choice than e.g. rabbits for the production of antibodies against conserved mammalian proteins (Horton *et al.*, 1984). Due to this evolutionary difference, chicken antibodies will bind to more epitopes on a mammalian protein than the corresponding mammalian antibody. It has been shown that 3-5 times more chicken antibody than swine antibody will bind to rabbit IgG which will amplify the signal in an immunological assay (Horton *et al.*, 1984; Olovsson and Larsson, 1993). Chicken

antibodies also recognize other epitopes than mammalian antibodies (Song *et al.*, 1985). This gives access to a different antibody repertoire than the traditional mammalian antibodies.

- Non-invasive method and high productivity

The use of chicken egg yolk as a source for antibody production represents a reduction in animal use as chickens produce larger amounts of antibodies than laboratory rodents. The yield of IgY antibodies can be compare to that of IgG antibodies obtained by conventional immunization method; 200 mg of IgG can be obtained monthly whereas IgY can be harvested 1500 mg each month (Schade *et al.*, 1994). It also makes it possible to eliminate the collection of blood, which is painful for animal. The European Center for the Validation of Alternative Methods (ECVAM) recommends that yolk antibodies should be used instead of mammalian antibodies for animal welfare reasons (Schade *et al.*, 1996)

- Reduced cross-reactivity with mammalian IgG antibodies

A mammalian antibody against a mammalian immunoglobulin shows a high degree of cross-reactivity. For example, a rabbit anti-human IgG will cross-react with all other mammalian IgGs except with rabbit IgG. An increase background binding may result if a secondary anti-mammalian IgG antibody is used. The secondary antibody may cross-react with IgG that is present in a histological section or with bovine IgG in the bovine serum albumin solution often used for blocking purposes. Because chicken IgY is so different from mammalian IgG (Hadge and Ambrosius, 1984), no cross-reactivity occurs between

the two. A rabbit anti-chicken IgG will, consequently, not cross-react with other mammalian IgG. Therefore, the analytical background is reduced by using a chicken IgY antibody as the primary antibody and a rabbit anti-chicken IgY as the secondary antibody (Larsson and Lindahl, 1993).

- IgY avoid complement activation

In clinical laboratories, most analyses are performed on serum samples. A newly obtained serum sample contains an active complement system, but the activity declines during storage and handling. Accordingly, the complement activity may vary between different patients and also between different samples from the same patient. In many immunological tests, complement system in the standards and controls used in the tests was inactivated by heat-inactivation or adding EDTA. However, the serum samples to be tested still contain active complement system. This difference in activity between the samples and the standards or controls will cause erroneous results. Mammalian antibodies bound to a solid phase and antigen-antibody complexes containing mammalian antibodies will activate the complement system (Larsson and Sjoquist, 1989). Activated C4 bind to the Fab region of IgG and may interfere with the antigen binding (Campbell *et al.*, 1980). Complement components may also solubilize precipitated immune complexes and prevent soluble immune complexes from precipitating (Baatrup *et al.*, 1986; Miller and Nussenzweig, 1975). Such complement activation was shown to interfere in an immunometric TSH assay and depressed the TSH values by up to 40% (Kapyaho *et al.*, 1989). In case of chicken IgY, they do not activate the human complement system. IgY,

therefore, can be used to reduce interference by complement activation (Larsson *et al.*, 1992) and suitable for using in immunological assay.

- IgY avoid rheumatoid factor and human anti-mouse IgG antibodies interaction

Rheumatoid factor (RF) and human anti-mouse IgG antibodies (HAMA) are probably the most well known causes of false positive or false negative reactions in immunological assay (Boscato and Stuart, 1988). RF is an autoantibody that reacts with the Fc part of mammalian IgG. The disease most often associated with RF is rheumatoid arthritis, but RF can be found in serum from patients with many other diseases and also in 3-5% of healthy blood donors (Johnson and Faulk, 1976). An increasing number of patients are treated *in vivo* with mouse monoclonal antibodies. This treatment often evokes an antibody response in the patient resulting in production of HAMA. HAMA may also be found in serum from patients who have not been treated with antibodies. The increasing use of monoclonal and polyclonal antibodies *in vivo*, for therapeutic purposes, will increase the number of patient samples that contain HAMA.

RF or HAMA may react with both the capture antibody and the detection antibody in a sandwich assay, thereby mimicking antigen activity. A reaction with a detection antibody results in formation of an immune complex. This immune complex may influence the activity of the detection antibody. HAMA may also react with the antigen-binding epitopes and inhibit the antigen binding. The problem of RF and HAMA interference will increase as the sensitivity of the assay increase. Interference by anti-IgG antibodies and antibody-binding substances have been demonstrated in approximately 40% of serum

samples from healthy individuals in an immunoradiometric assay (Boscato and Stuart, 1986). RF and HAMA will also give erroneous results in nephelometry and turbidimetry as they change the size of antigen-antibody complex (Chamber *et al.*, 1987). Chicken IgY does not react with RF or HAMA. Therefore, IgY can be used to avoid interference due to these factors and reduced the erroneous results (Larsson *et al.*, 1991; Larsson and Mellstedt, 1992).

- Human Fc interaction

Intact mamalian IgG molecules contain the Fc portion of the antibody. Antibody Fc binds to Fc receptors, which are found on many types of blood cells (van de Winkel and Capel, 1993). Human FcγRI has a high affinity for monomeric mammalian IgG, while FcγRII and FcγRIII mainly bind mammalian IgG complexes. There is often some aggregated IgG formed during the purification of IgG or during the labeling procedures that will increase the binding to FcγRII and FcγRIII receptors. Interaction with Fc receptors may cause an increased background staining. When working with living cells, interaction with Fc receptors may cause cell activation and changes in the expression of surface proteins. It has been shown that mammalian antibodies used in flow cytometry form immune complexes that cause platelet activation and changes in the expression of the GpIIb-IIIa receptor (Lindahl *et al.*, 1992; Rubinstein *et al.*, 1991). No activation was observed when chicken antibodies were used (Lindahl *et al.*, 1992). Immune complexes containing mammalian IgG may also stimulate the production of cytokines (van de Winkel

and Capel, 1993). Chicken antibodies do not react with human Fc receptors and their use will avoid these problems (Lindahl *et al.*, 1992).

- Bacterial Fc-receptor interaction

Staphylococcal protein A and Streptococcal protein G are Fc-binding bacterial proteins which are widely used for their ability to bind to IgG. Bacteria of the *Staphylococcus aureus* Cowan I strain and group C Streptococcus sp. are also used as immunoadsorbent for mammalian IgG. Staphylococci and Streptococci are often found in bacteria samples. When present, they may bind detection antibodies with specificity for other bacteria and cause erroneous results. Chicken antibodies do not react with protein A or protein G. IgY, therefore, can be used to reduce interference problems due to bacterial Fc receptors (Guss *et al.*, 1986; Fischer and Hlinak, 2000; Hoffman *et al.*, 1996).

Antibody production in chicken

Chickens can be used for antibody production throughout their entire egg laying period. Animals that are used for antibody production for more than three months should be given booster immunizations every other month to assure that the antibody titer remain high. Chickens can produce high avidity antibodies already after one immunization, compared to sheep whose avidity becomes similar after four immunizations (Wcolley and Landon, 1995). In response to monthly re-immunizations sheep have been found to produce ten times more specific antibodies than chicken, probably due to the size difference of the animals. Another reason for this might be the differences in antibody

half-lives. In sheep the half- life is about 15 days, compared to 36 hours in the chicken. The species may therefore produce immunoglobulins at a comparable rate. The high catabolic rate of the chicken may prevent the accumulation of high titers. However, the avidity in both chickens and sheep after four immunizations was 10^9 to 10^{10} l/mol

Freund's complete adjuvant is quite well tolerated in chickens, as the characteristic local inflammatory response seen in mammals is often not observed (Gassmann *et al.*,1990). Other types of adjuvant than Freund's adjuvant can also be used, such as Specol, Huntes TiterMax and the lipopeptide Pam_3-Cys-Ser-$(Lys)_4$ (Hassl and Aspock, 1988). After immunization with human serum albumin the highest serum IgY titer is found seven to nine days after a single intravenous or intraperitoneal injection (Losch *et al.*, 1986). Intramuscular immunization shows a higher antibody level from day 28 after immunization and the specificity is almost more than 10 times higher compared to sub-cutaneous immunization (Losch *et al.*, 1986; Chang *et al.*, 1999). Non-reimmunized chickens investigated more than 200 days later showed a similar high IgY level of specific antibodies (Losch *et al.*, 1986). The presence of IgY in the egg yolk is detected four to seven days after the appearance in the serum.

The amount of antigen specific antibodies of the total pool of antibodies in an egg has been reported to be up to 10% (Thalley and Carroll, 1990; Akita and Li-Chan, 1998). However, the actual amount of specific antibodies probably varies depending on the individual animal, immunization procedures and the immunogenicity of the antigen itself. As a laying hen produces approximately 20 eggs per month, over 2 gram IgY per month can be isolated. The IgY concentration in chicken serum is approximately 5-7 mg/ml,

therefore 2 gram of egg yolk IgY corresponds approximately to the IgY content of 300 ml of serum or 600 ml of blood. Only larger mammals can produce equal amounts of serum antibodies and compared to rabbits, the chicken antibodies are ten times less expensive (Svendsen and Hau., 1996).

Isolation of IgY from egg yolk

After immunization of antigen of interest into chicken, the generated specific antibodies can be obtained either by bleeding of animal or collecting eggs. However, as the chicken has fragile veins, bleeding is often difficult and results in large haematoma formation. Poor clot retraction can also limit the amount of serum obtained. Sometimes, only 100 µl of serum is obtained from 2 ml of blood. Plasma is therefore more useful than serum. However, the best way to obtain antibodies is to purify them from the yolk.

In nature, approximately one-half of the material in chicken egg yolks is lipid including high contents of phospholipids and cholesterol (Landon *et al.*, 1995). The effects of these lipids in IgY preparations have yet to be carefully evaluated in immunoassays or in *in vivo* applications. Thus, in order to use egg yolks as antibody source for either therapeutic or diagnostic applications, lipid content should be minimized.

Several procedures for the isolation and purification of IgY from egg yolk have been described previously (Jensenius *et al.*, 1981; Song *et al.*, 1985; Hassl and Aspoc, 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). As the yolk consists of almost 50% non-aqueous material, the first step involves isolation of IgY in a water-soluble

fraction circumventing the tendency of IgY to fractionate with the yolk lipids. This step is called water dilution method. The next step generally involves the precipitation of IgY with precipitant, e.g., sodium sulfate, ammonium sulfate or polyethylene glycol (PEG). The general mode of action of the precipitant is the binding of water, thus decreasing protein solubility and resulting in protein precipitation. For highly purification, chromatographic procedures such as ion-exchange chromatography, gel filtration, affinity chromatography and thiophillic interaction chromatography are suggested. For ion-exchange chromatography, two strategies for purifying antibodies are involved. In the first method, the pH of the buffer is kept below the isoelectric point of antibodies to be purified (isoelectric point of IgY is 5.7-7.6) (Davalos *et al*, 2000), and therefore they will not bind to the anion exchange matrix such as the DEAE matrix. In the second approach, the pH is raised above the isoelectric point where the antibodies will bind to the DEAE groups. Antibodies are then eluted by increasing the salt concentration, which can be done either with continuous (gradient) or a discontinuous (step buffers) method. Gassman used PEG precipitation followed by DEAE-cellulose column to purified anti-proliferating cell nuclear antigen (PCNA) from egg yolk, as a result showed the purity was more than 90% (Gassman *et al*, 1990). Using gel filtration, protein is separated or fractionated on the basis of their molecular size. Large proteins, above the exclusion limit of the gel, will not enter the beads and so move with the advancing solute front while small molecules enter the beads and must traverse this space as well as the volume around the beads. Hassl and Aspock found the advantage from using two chromatographic processes, hydrophobic interaction chromatography (HIC) followed by gel filtration (GF), for IgY

isolation. The antibodies were completely separated from vitellin and lipids. (Hassl and Aspock, 1988). The separation by affinity chromatography requires that a biospecific ligand is available and that it can be covalently attach to a chromatographic to a bed material called matrix. It is important that the biospecific ligand still retain its specifics binding affinity for the substance of interest. Methods must also exist for removing bound material in active form with low pH, high pH or high salt. Fassina identified a new synthetic ligand, PAM (Protein A Mimertic, TG19318), which binds specifically and selectively to the constant portion of immunoglobulins (Fassina *et al*, 1996). By using this ligand, in a single chromatographic step, it led to an efficient capture of IgY directly from crude samples and showed a purity degree higher than 90% with full recovery of antibody activity (Verdoliva *et al.*, 2000). Another method for IgY purification is Thiophilic interaction chromatography. It was first described by Porath *et al*, 1985. The absorbent, consisting of 2-mercaptoethanol reacted with a di-vinylsulfone activated support, was named T-gel and showed selective binding of immunoglobulins in the presence of structure-forming salts. The T-gel has since been successfully used in the purification of antibodies from serum of human, rat, rabbit and bovine (Porath *et al.*, 1985, Porath and Belew, 1987, Lihme and Heegaard, 1991) and from egg yolk (Hansen *et al*, 1998). Thiophilic chromatography of ammonium sulfate-precipitated IgY preparations specific to several different antigens has previously demonstrated excellent enhancement of purity (Hansen *et al*, 1998). The purification of anti-α Galactose-trisaccharide IgY from egg yolk with several techniques was compared. It was found that ammonium sulfate precipitation contains the most impurity, T-gel column and affinity-purified sample show

the same high purity. The highly purified IgY can be obtained from egg yolk by the two-step procedure of ammonium sulfate precipitation followed by either T-gel or affinity chromatography (Cook *et al.*, 2001). As several methods are described for purification of IgY from egg yolk, the choice of method is a matter of yield and purity desired, final use of the IgY as well as material cost and labor skills.

Applications of IgY

IgY has a number of intrinsic biochemical properties that bring great advantages to the application of IgY technology in many medical areas, such as xenotransplantation (Fryer *et al.*,1999), diagnostics and antibiotic-alternative therapy (Carlander *et al.*,2000)

IgY in therapy: oral administration of antibodies specific to host pathogens is an attractive approach to establish protective immunity, especially against gastrointestinal pathogens both in humans and animals. The increasing number of antibiotic-resistant bacteria emphasizes the need to find alternatives that complement antibiotics. Immunotherapy may also be used as a complementary treatment against pathogens that are difficult to treat with traditional antibiotics. Eggs are normal dietary components and there is practically no risk of toxic side effects of IgY given orally. However, caution should be taken to give IgY to persons with known egg allergy. IgY has biochemical properties that make them attractive for peroral immunotherapy. As mentioned earlier, they neither activate mammalian complement nor interact with mammalian Fc-receptors that could mediate inflammatory response in the gastrointestinal tract. Activated complement components are potent inflammatory mediators. Theoretically, immune

complexes containing mammalian antibodies may also interact with Fc- and complement-receptors in the mammalian gastrointestinal tract, causing cell activation. Immune complexes containing IgY do not activate the mammalian complement system and do not interact with mammalian Fc- and complement-receptors. Moreover, IgY has acid resistant property. Orally administered antibodies are subjected to denaturation by the acidic pH of the stomach and degradation by protease, such as pepsin, trypsin, chymotrypsin, carboxypeptidase and elastase (Reilly *et al.*, 1997). Studies have shown that part of the antibodies remain intact in pepsin and trypsin digests but there is a considerable cleavage of the antibodies into Fab, Fab'2 and Fc fragments. However Fab'2 and Fab fragments still have the capability to bind to the antigen and exhibit neutralizing activity (Akita *et al.*, 1998). As a result, yolk IgY seems to be well suited for peroral immunotherapy.

There are many examples of the preparation and further use of specific antibodies isolated from egg yolk. These antibodies have been raised against different pathogen agents e.g. several bacteria: *Pseudomonas aeruginosa, Salmonella enteritidis and Staphylococcus aureus* (Sugita *et al.*, 1996). The IgY asserted a growth inhibition effect on *Pseudomonas aeruginosa,* but did not prevent growth. Staphylococcus enterotoxin A production was suppressed. Growth of *E. coli* is decreased in a presence of specific IgY against the bacteria, but no inhibition is seen with non-specific IgY (Shimizu *et al.*, 1998). Oral administration of spray dried yolk antibodies specific against *Salmonella typhimurium* or *S. dublin* was shown to prevent Salmonella infection in calves. The antibodies were administered three times a day for 7-10 days after inoculation with S. *typhimurium* or *S. dublin*. All calves in the control group died whereas only diarrhea and

fever was observed in the group treated with the highest antibody titer. These antibodies gave protection against fatal salmonellosis (Yokoyama *et al.*, 1998). In humans, a mouth rinse containing egg yolk antibodies to *Streptococcus mutans* has been used to reduce the establishment of these bacteria in dental plaque of humans. The antibodies inhibited S. *mutans* adherence to saliva-coated hydroxyapatite discs *in vitro* and decreased the percentage of S. *mutans* per total Streptococci *in vivo* (Hatta *et al.*, 1997).

Prevention of viral infections: a field trial with oral administration of chicken IgY specific for bovine rotavirus (BRV) resulted in a significantly increased mean body weight ($p<0.05$) and a decrease in number of infected calves ($p<0.01$)(Kuroki *et al.*, 1997). Passive oral immunization with yolk immunoglobulins has also been shown to protect mice against experimental bovine rotavirus-induced diarrhea (Kuroki *et al.*, 1993; Ebina, 1996; Yolken *et al.*, 1988). In a randomized, placebo controlled clinical trial, children with rotavirus diarrhea was treated with IgY from hens immunized with Wa, RV5, RV3 and ST3 rotavirus strains. There was a significant reduction in stool output (g/kg/day) and viral clearance in the group treated with specific IgY compared to placebo. However, there was no difference in diarrhea dilution (Sarker *et al.*, 2001). Oral treatment with bovine anti-rotavirus colostrum resulted in a reduction of rotavirus-associated diarrhea in infants (Ebina., 1996; Sarker and Casswall., 1998; Ebina *et al.*, 1990). It also reduced the infection rate in children (Davidson *et al.*, 1989).

Prevention of other infections or maladies: *In vitro* studies on mice have shown that IgY can be alternative to horse serum antivenoms to vipersnake, rattlesnake and scorpion toxin (Maya *et al.*, 2000; Thalley and Carroll, 1990; Almeida *et al.*, 1998).

Cryptosporidium infections are usually associated with immunosuppressed hosts but it may also occur in individuals with a normal immune system. The use of yolk preparations with high anti-Cryptosporidium activities caused a significant parasite reduction in a neonatal mouse model (Cama and Sterling, 1991).

IgY in diagnostics: It has been reported that in many serum samples endogenous heterophilic antibodies are present (Boscato and Stuart, 1988; Levinson, 1992,1997; Kricka, 1999). These antibodies interfere with two-site immunoassays involving murine antibodies by bridging labeled and immobilized antibodies, simulating the analyze and so evoking false positive signals (Hunter and Budd, 1980; Howanitz *et al.*, 1982; Hunter *et al.*, 1983; Bock *et al.*, 1985; Thomson *et al.*, 1986; Boscato and Stuart, 1986; Clark and Price, 1987). The interfering antibodies include human anti-animal antibodies (HAAAs) covering anti-mouse antibodies (HAMAs) and several other types of heterophilic antibodies including multivalent human autoantibodies, such as rheumatoid factors (RF). Replacement of the sheep coating antibody by duck antibodies completely prevent this interference (Grebenchtchikov *et al.*, 2002). Fluorescence activated flow cytometry (FACS) is an efficient method for studying membrane proteins. One problem with the use of mammalian antibodies is that immune complexes between human plasma proteins and mammalian antibodies induce platelet activation that cause a structural change of glycoprotein IIb/IIIa complexes that results in the exposure of the fibrinogen binding site (Shattil *et al.*, 1985), which may cause false positive reaction. No activation was observed when chicken antibodies were used, while immune complexes containing mouse or rabbit IgG cause platelet activation (Lindahl *et al.*, 1992). In haemagglutination assay, IgY was

used to determine human erythrocyte antigens. By IgY technology, the highest titers were observed only 4 weeks after the first immunization (Gutierrez *et al.*, 2001).

1.3 Objectives

1. To develop IgY technology at the Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University
2. To study the production antibody in chicken egg yolk by comparing two immunization routes: pectoralis and calf intramuscular immunization.
3. To study the kinetics of antibodies production in chicken after antigen immunization.
4. To produce chicken anti-mouse immunoglobulins antibody and study the appropriate method for FITC labeling of IgY.
5. To apply FITC conjugate in indirect immunofluorescence assay.