CHAPTER III

RESULTS

3.1 Preparation of mouse immunoglobulins for immunization

Mouse immunoglobulins were purified by affinity chromatography using Protein G Sepharose column. One milliliter of pooled normal mouse serum was applied into Protein G sepharose column. After elution process, the eluted immunoglobulin fraction was characterized in terms of immunoglobulin purity and isotype specificity by SDS-PAGE and ELISA, respectively.

The eluate was subjected for SDS-PAGE analysis under both non-reducing and reducing conditions. Under non-reducing conditions, a single high molecular weight band was appeared on the top of the gel (figure 3.1, lane 2). Whereas under reducing conditions, 2 bands with molecular weight of 51 and 26 kDa that corresponding to the heavy chain and light chain of immunoglobulins were obtained (figure 3.1, lane 3). The results indicated that by using Protein G Sepharose column, the immunoglobulins could be isolated. The concentration of isolated immunoglobulins was determined by measurement at O.D 280 nm. In this study, from one ml of the starting mouse serum by using Protein G Sepharose column, total amount of 2.6 mg of immunoglobulins was obtained.

To identify the isotypes of the isolated immunoglobulins, the eluate was subjected to sandwich ELISA using isotype specific antibody coating plate. As shown in Figure 3.2,

the eluate composed of IgA, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM isotypes. These results demonstrated that the isolated immunoglobulins contained IgA, all IgG and IgM isotypes. The isolated immunoglobulins therefore could be used to immunize chickens.

3.2 IgY extraction from egg yolk by water dilution and salt precipitation method

A common method for purifying IgY from egg yolk was the water dilution and salt precipitation method (Hansen *et al.*, 1998). In this study, the mention method was applied for isolation of IgY from chicken eggs. After purification process, the purity of the obtained IgY was assessed by SDS-PAGE and stained with Coomasie brilliant blue. As shown in Figure 3.3, under non-reducing conditions, a major high molecular weigh band of intact IgY was observed on the top of the gel. However, a faint band with molecular weight of approximately 33 kDa was also observed. Under reducing conditions, two bands at 69 and 26 kDa, which are attributed to heavy chain and light chain of IgY respectively, were observed (Figure 3.3, lane 1). The 2 faint bands at 39 and 45 kDa were also observed. The faint band observed in both non-reducing and reducing conditions were likely to be the contaminated ovalbumin that was a major component of chicken eggs. As the majority of extracted proteins were immunoglobulin, water dilution and sodium sulfate precipitation method were used to extract IgY from egg yolk in the further experiments.

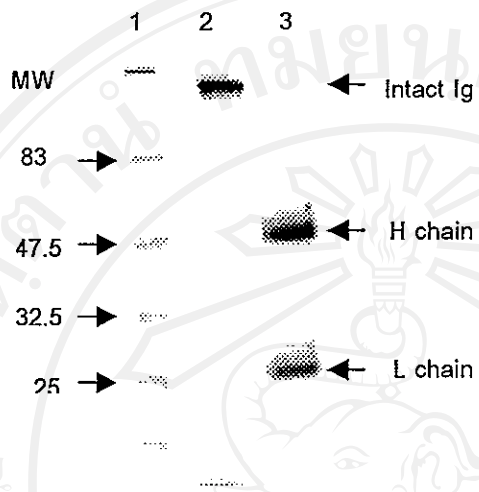

Figure 3.1 SDS-PAGE analysis of mouse immunoglobulins isolated by Protein G Sepharose chromatography. The eluate obtained from Protein G Sepharose column was subjected for 12.5% SDS-PAGE under reducing and non-reducing conditions and stained with Coomasie brilliant blue.

Lane 1: Molecular weight marker (kDa)

Lane 2: SDS-PAGE under non-reducing conditions

Lane 3: SDS-PAGE under reducing conditions

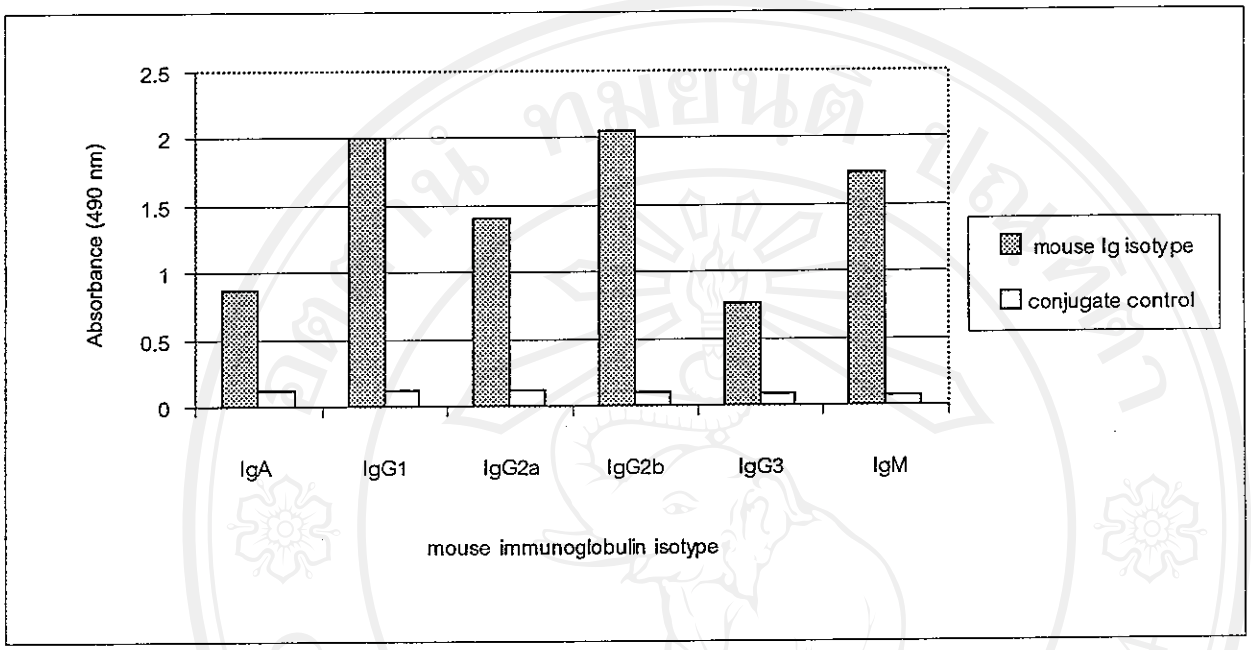

Figure 3.2 The absorbance of isolated immunoglobulin isotypes determined by sandwich ELISA using goat anti-mouse immunoglobulin isotypes coated plate and rabbit anti-mouse immunoglobulins-HRP as conjugate. O.D. of conjugate control are the O.D. obtained from wells contained coated antibody and conjugate.

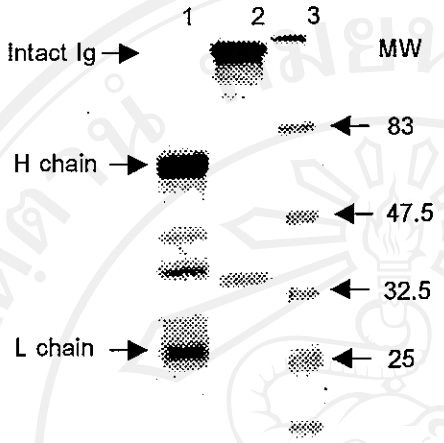

Figure 3.3 SDS-PAGE analysis of IgY extract from egg yolk by water dilution and salt precipitation method. The extracted IgY was subjected for 12.5% SDS-PAGE under reducing and non-reducing conditions and stained with Coomasie brilliant blue.

Lane 1: SDS-PAGE under reducing conditions

Lane 2: SDS-PAGE under non-reducing conditions

Lane 3: Molecular weight markers (kDa)

3.3 Optimization of ELISA conditions for measurement of chicken anti-mouse immunoglobulins in serum and IgY extracted from egg yolk.

Two set of experiments were performed to establish the best ELISA conditions for quantification of chicken anti-mouse immunoglobulins levels in serum and IgY isolated from egg yolk.

In the first experiment, the optimal concentration of mouse immunoglobulins for coating plate was studied. Various concentrations of mouse immunoglobulins were coated on ELISA plate and incubated with sera obtained from pre-immunized and six-week mouse immunoglobulins immunized chicken. As shown in figure 3.4, pre-immunized sera did not react to all concentrations of coated antigen. While the high positive reactivity was observed in the six-week immunized sera. The positive signals were correlated to the increasing of coated antigen. However, at antigen concentration of 2.5 and 1.25 μg/ml, the background signals were higher than others. The concentration of 0.625 μg/ml of mouse immunoglobulins was, therefore, selected as the optimal concentration for coating plate. With this concentration, pre-immune serum showed negative signal while the immunized serum showed high positive signal. No signal was observed in conjugate control, where 0.05% PBS-T was used instead of serum.

In the second experiment, the optimal concentration of HRP conjugated rabbit anti-chicken IgG was studied. Plate was coated with 0.625 μg/ml of mouse immunoglobulins. Various dilutions of normal chicken serum and six-week mouse immunoglobulins immunized chicken serum were used as primary antibodies and various dilution of HRP conjugated rabbit anti-chicken IgG were used as secondary antibodies. As shown in

Figure 3.5, the O.D. obtained from six-week immunized serum was increased in correlation to the increment of the concentrations of HRP conjugated rabbit anti-chicken IgG . Normal sera did not show positive reactivity at any concentrations of conjugate. The O.D. obtained from using conjugate dilutions range between 1:2000-1:6000 was satisfying high. However, HRP conjugated rabbit anti-chicken IgG at dilution of 1:6000 was the highest dilution that gave the satisfying O.D. with positive serum while showing low background with normal serum. This dilution was, therefore, selected for further studies.

From these studies, the optimal ELISA conditions for measurement chicken anti-mouse immunoglobulins in subsequence experiments were as follows. Plate was coated with mouse immunoglobulins at concentration of 0.625 μg/ml and HRP conjugated rabbit anti-chicken IgG at dilution of 1:6000 was used.

3.4 Production of chicken anti-mouse immunoglobulins antibodies

In an attempt to produce chicken anti-mouse immunoglobulins antibodies, two chicken, named SK1 and SK2, were used in this experiment. SK1 and SK2 chickens were immunized three times at two-week intervals with mouse immunoglobulins obtained from the Protein G Sepharose column by pectoralis muscle and calf muscle immunization, respectively. The presence of anti-mouse immunoglobulins in serum and IgY extract from egg yolk was determined by ELISA.

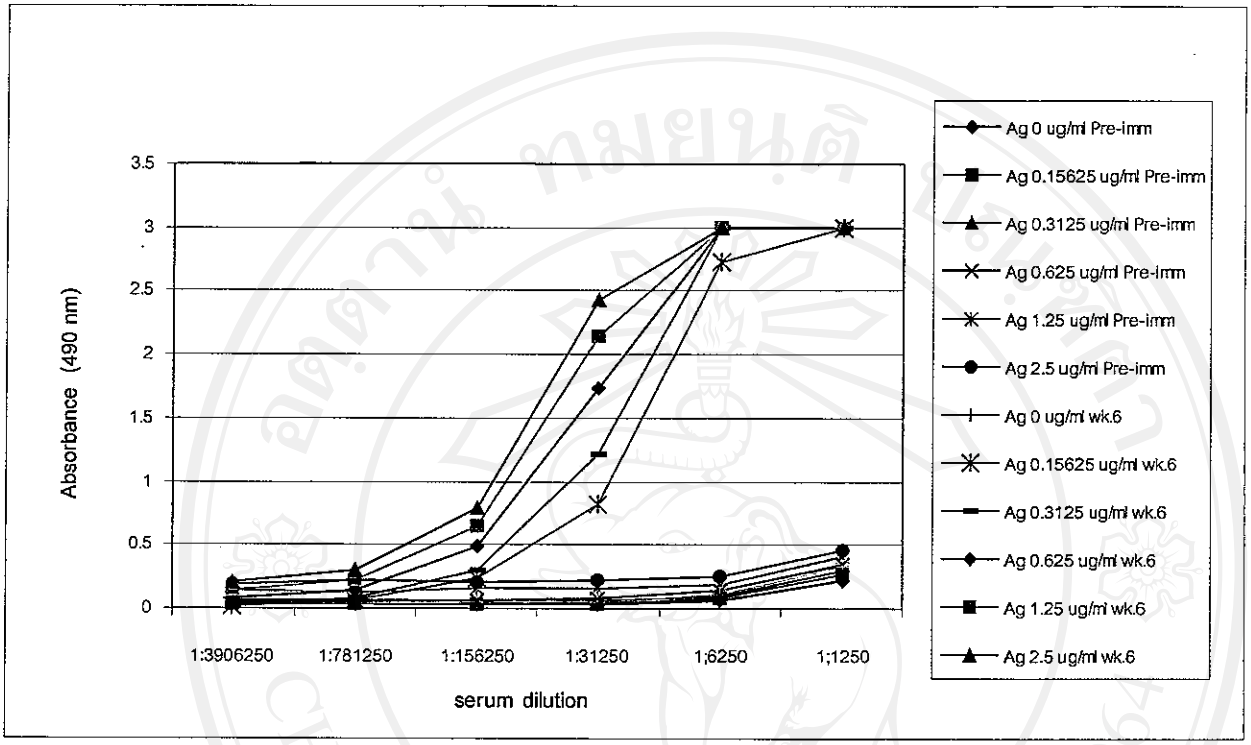

Figure 3.4 Titration of mouse immunoglobulins concentration for coating plate in sandwich ELISA for determination of chicken anti-mouse immunoglobulins. Plate was coated with various concentrations of mouse immunoglobulins. Various dilutions of normal and mouse immunoglobulins immunized serum were added. HRP conjugated rabbit anti-chicken IgG at dilution of 1: 4000 was used as conjugate.

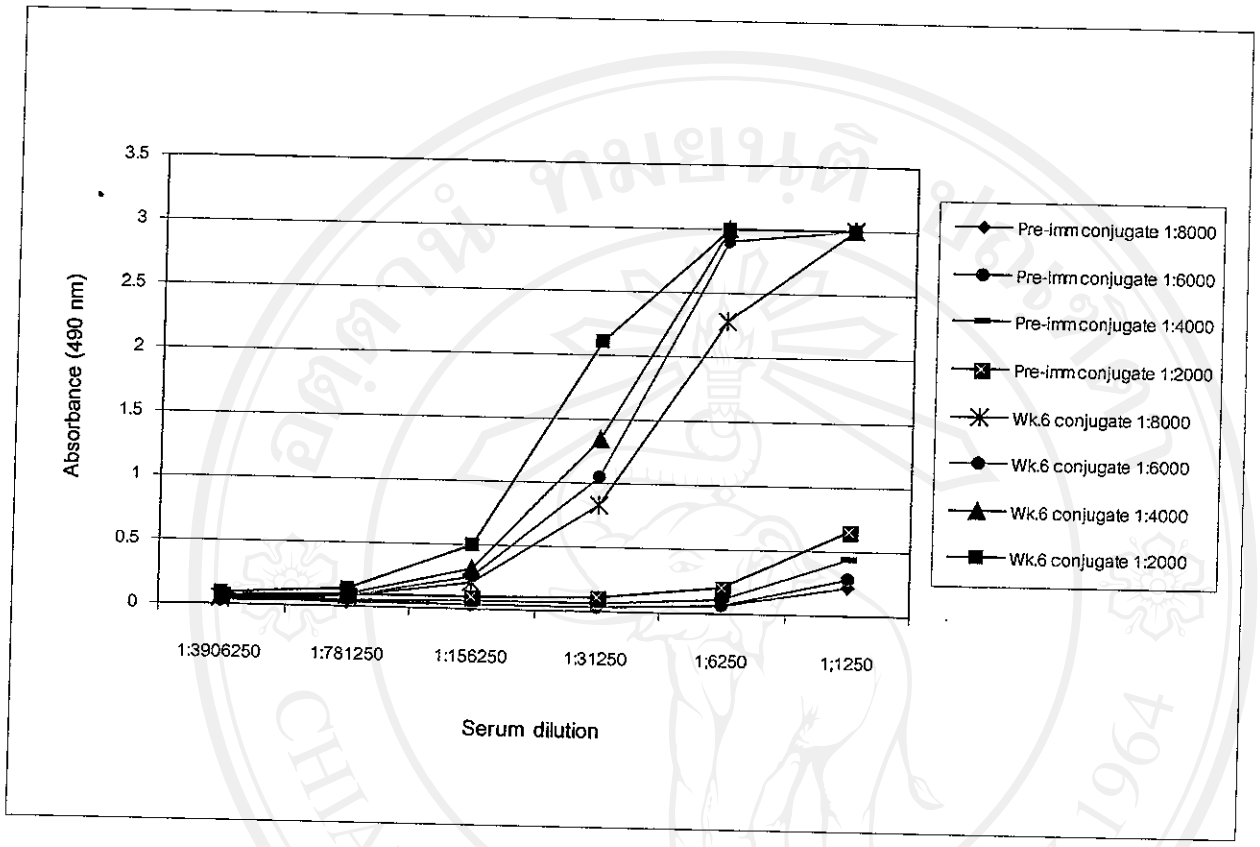

Figure 3.5 Titration of HRP conjugated rabbit anti-chicken IgG concentration for determination of chicken antibody by sandwich ELISA. In legends; pre-immune conjugate = sandwich ELISA using normal chicken serum as primary antibody and visualized with various dilutions of HRP conjugated rabbit anti-chicken IgG. Wk.6 conjugate = sandwich ELISA using 6 weeks mouse immunoglobulins immunized chicken serum as primary antibody and visualized with various dilutions of HRP conjugated rabbit anti-chicken IgG.

In serum, anti-mouse immunoglobulins antibody titer could be detected two weeks after antigen immunization in both chickens (Figure 3.6). The antibody levels were increased after the third immunizations. The antibody reached the maximum level at week 10 after the first antigen immunization. Three months after the last immunization, however, the antibody levels were decreased. Immunization at pectoralis muscle (SK1) showed higher antibody responses than calf muscle immunization (SK2) (Figure 3.6).

Eggs obtained from both chickens were collected every week. The IgY were extracted from the collected eggs and adjusted to 1 mg/ml. The extracted IgY (at concentration of 1 mg/ml) were diluted and subjected for determination of anti-mouse immnoglobulins titer. As was observed in serum, anti-mouse immunoglobulins antibody titer could be detected two week after antigen immunization and increased after the third immunizations (Figure 3.7). The antibody reached a plateau 11 weeks after antigen immunization, and maintained high titer at least 20 weeks after antigen immunization. As was demonstrated in serum, the pectoralis intramuscular immunization induced better antibody responses compared to calf intramuscular immunization (Figure 3.7).

These studies demonstrated that the appropriate immunization route for induction of antibody responses in chicken was pectoralis intramuscular immunization. The antibodies could be transferred from serum to egg yolk in high titer for several months.

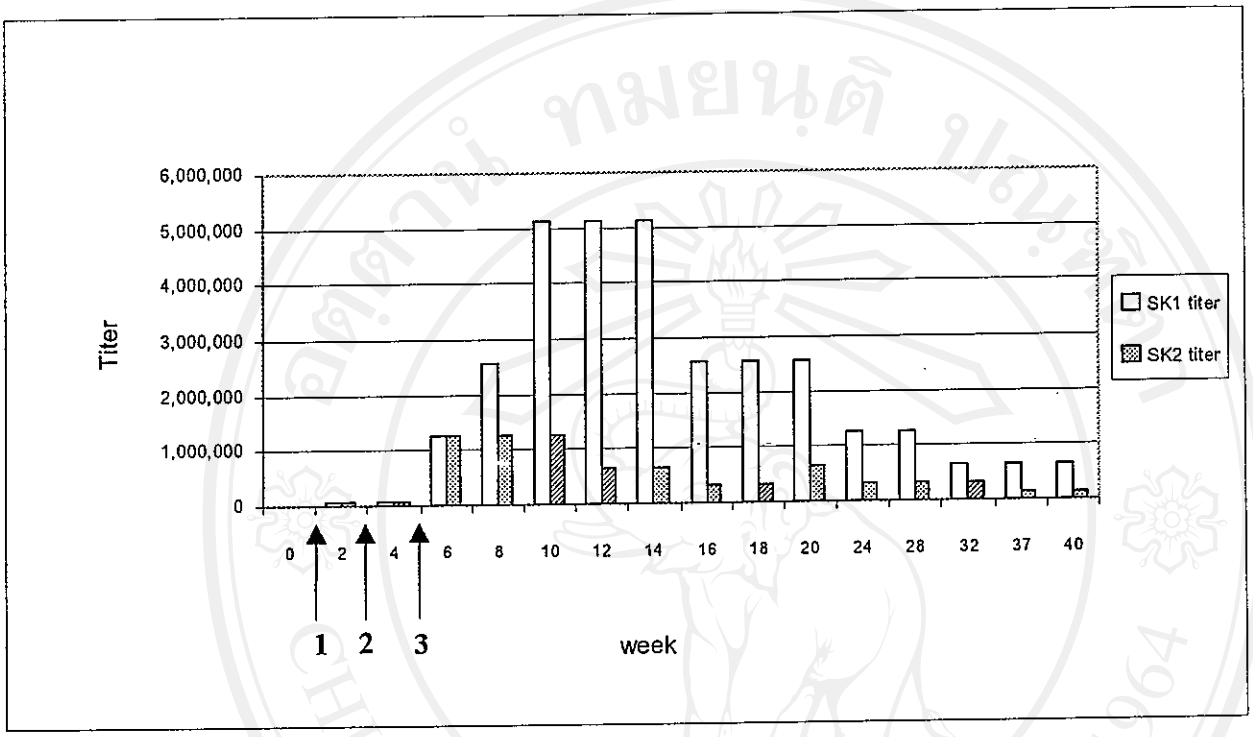

Figure 3.6 Anti-mouse immunoglobulins antibody responses of chicken sera after immunization with mouse immunoglobulins by pectoralis muscle (SK1) and calf muscle (SK2) immunization. Arrows indicate the first, second and third immunizations.

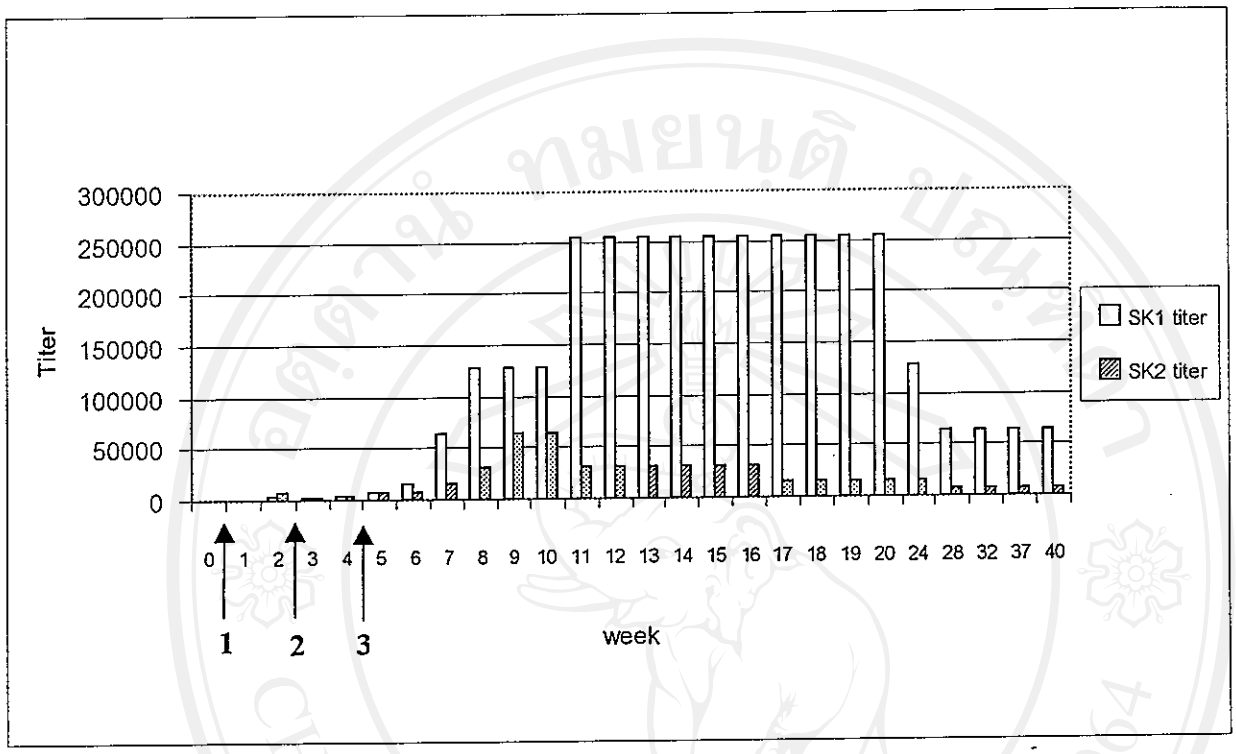

Figure 3.7 Anti-mouse immunoglobulins antibody responses of chicken IgY extracted from egg yolk after immunization with mouse immunoglobulins by pectoralis muscle (SK1) and calf muscle (SK2) immunization. Arrows indicate the first, second and third immunizations.

3.5 FITC conjugation and determination the activity of FITC conjugated chicken anti-mouse immunoglobulins

An egg was collected from chicken SK1 after reaching the maximum antibody response. By water dilution and salt precipitation method, 37 mg of IgY could be extracted from the collected egg. The extracted IgY was then labeled with various amount of FITC (R ratio). The activity of FITC conjugated chicken anti-mouse immunoglobulins was determined by indirect immunofluorescence and flow cytometer. In this study, U937 cells were stained with specific monoclonal antibodies, either IgM or IgG isotypes, and the prepared FITC-chicken anti-mouse immunoglobulins were used as conjugate. As demonstrated in Figure 3.8, at R ratio of 10, 20 and 40, the prepared IgY-FITC could react to all isotypes of primary antibodies with the same mean fluorescence intensity. The background of conjugate control, however, was increased in higher R ratio of FITC conjugate. Labeling of FITC to antibody at R ratio of 10 showed lower conjugate control background compared to those of 20 and 40 R ratio. Therefore, the R ratio of 10 was selected for FITC labeling of IgY in the further studies.

3.6 Titration of FITC conjugated chicken anti-mouse immunoglobulins for using in indirect immunofluorescence assay

To titrate the optimal concentration of the prepared chicken anti-mouse immunoglobulins-FITC for using in indirect immunofluorescence, peripheral blood mononuclear cells and U937 cells were stained with various isotypes mAbs and various

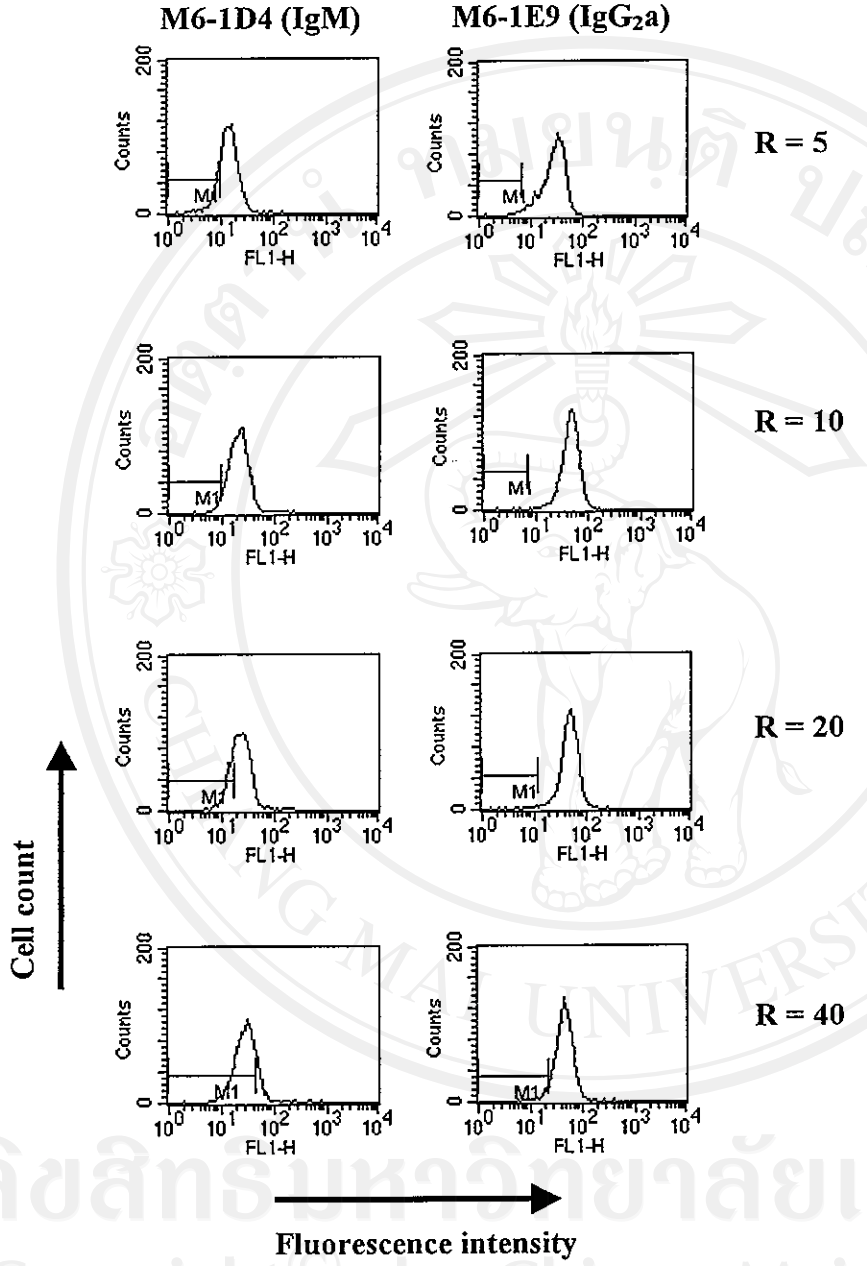

Figure 3.8 Comparison of the reactivity of FITC conjugated chicken anti-mouse immunoglobulins. U937 cells were stained with indicated primary mAbs and then counterstained with chicken anti-mouse immunoglobulins antibody conjugated with various amount of FITC (R ratio 5, 10, 20 and 40). The stained cells were analyzed by flow cytometry. Horizontal lines (M1) represent the conjugate control background.

concentrations of FITC conjugated chicken anti-mouse immunoglobulins (at R ratio of 10) were used as conjugate. As shown in Figures 3.9 and 3.10, FITC conjugated chicken anti-mouse immunoglobulins at concentration of 500 μg/ml showed high activity and low background with every isotype of primary antibodies. This concentration of conjugate was therefore selected as the optimal concentration for using in indirect immunofluorescence technique.

3.7 Determination of peripheral leukocyte populations by using the generated conjugate.

EDTA-whole blood samples was incubated with various primary antibodies that specific to surface molecules on T cells, B cells, monocytes and granulocytes. Then, the stained cells were counter-reacted with chicken anti-mouse immunoglobulins conjugated FITC (R ratio of 10) at the concentration of 500 μg/ml and analyzed by flow cytometry. As shown in Figures 3.11, with all primary antibodies, the generated conjugate showed similar FACS profiles as those obtained from using of commercial conjugate (Silenus, Boronia, Victoria, Australia). However, the fluorescence intensity obtained by the prepared conjugate was slightly lower than those of the commercial one (Figure 3.11). When percentages of T and B lymphocytes in lymphogate were determined, as was shown in table 3.1, the percentages of T and B lymphocytes obtained from the prepared and commercial conjugates were very similar (t test, $p > 0.50$). These results demonstrated that the generated conjugate could be used in indirect immunofluorescence assay for quantitation of lymphocyte sub-populations in blood samples.

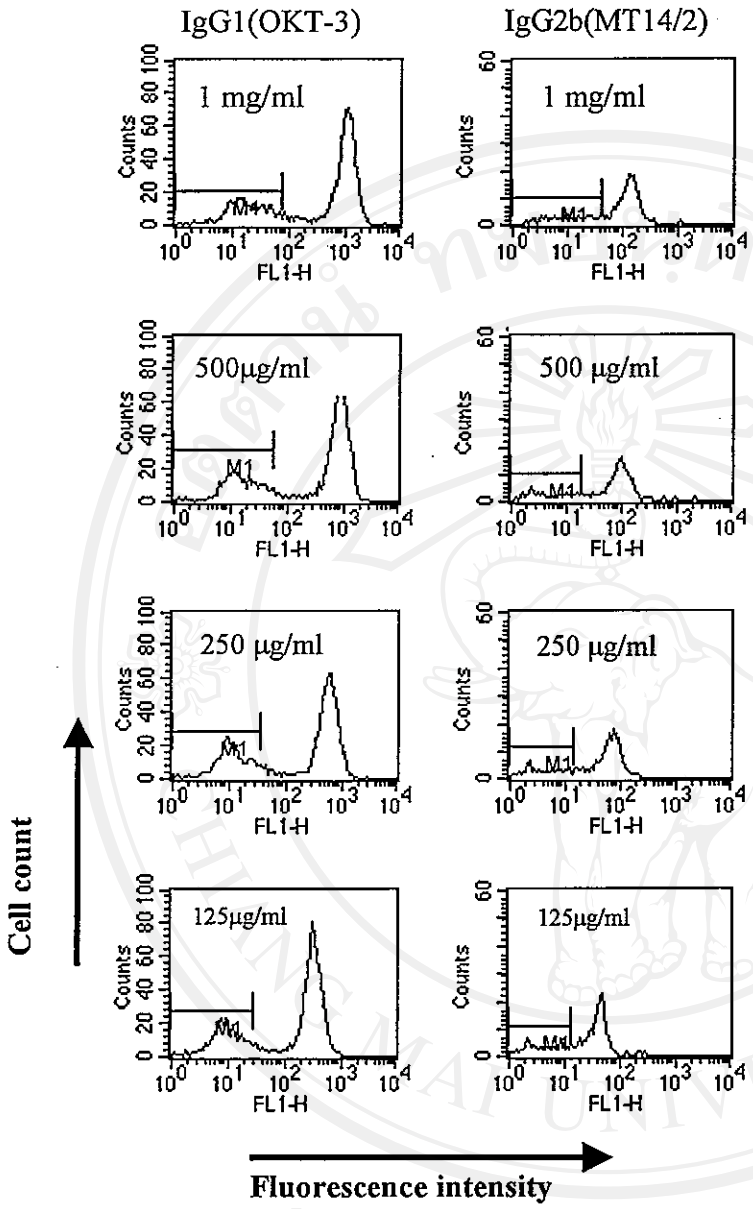

Figure 3.9 Immunofluorescence analysis of the reactivity of FITC conjugated chicken anti-mouse immunoglobulins (R ratio of 10) at various concentrations (1000, 500, 250 and 125 µg/ml) when IgG1 and IgG2b isotypes were used as primary monoclonal antibodies. PBMC were stained with anti CD3 mAb (OKT3) IgG1 isotype and anti CD14 mAb (MT14/2) IgG2b isotype and various concentrations of FITC conjugated chicken anti-mouse immunoglobulins were used as conjugate. Horizontal lines (M1) represent the conjugate control background.

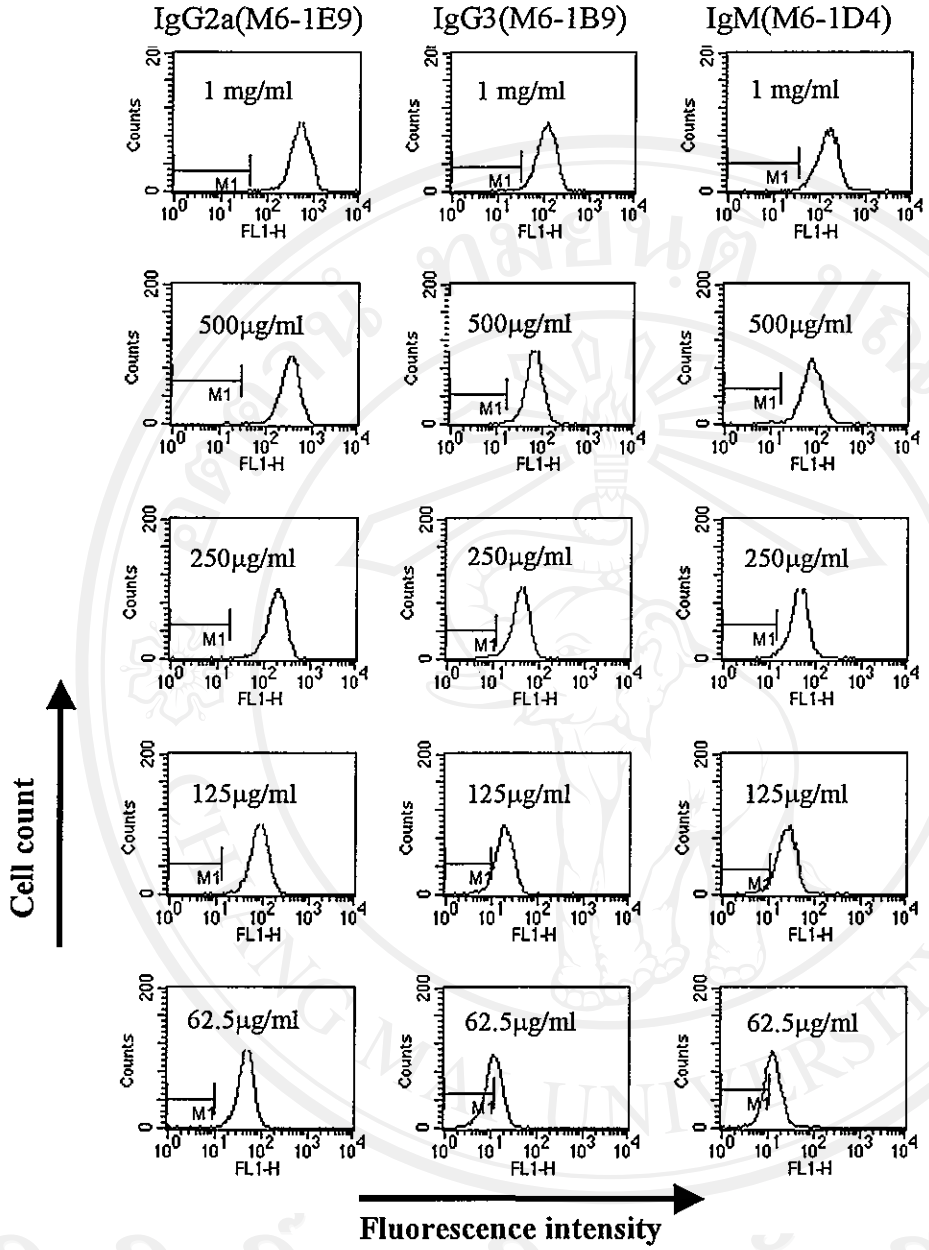

Figure 3.10 Immunofluorescence analysis of the reactivity of FITC conjugated chicken anti-mouse immunoglobulins (R ratio of 10) at various concentrations (1000, 500, 250, 125 and 62.5 µg/ml) when IgG2a, IgG3 and IgM isotypes were used as primary monoclonal antibodies. U937 cells were stained with anti CD147 mAb (M6-1E9) IgG2a isotype, anti CD147 mAb (M6-1B9) IgG3 isotype and anti CD147 mAb (M6-1D4) IgM isotype and various concentrations of FITC conjugated chicken anti-mouse immunoglobulins were used as conjugate. Horizontal lines (M1) represent the conjugate control background.

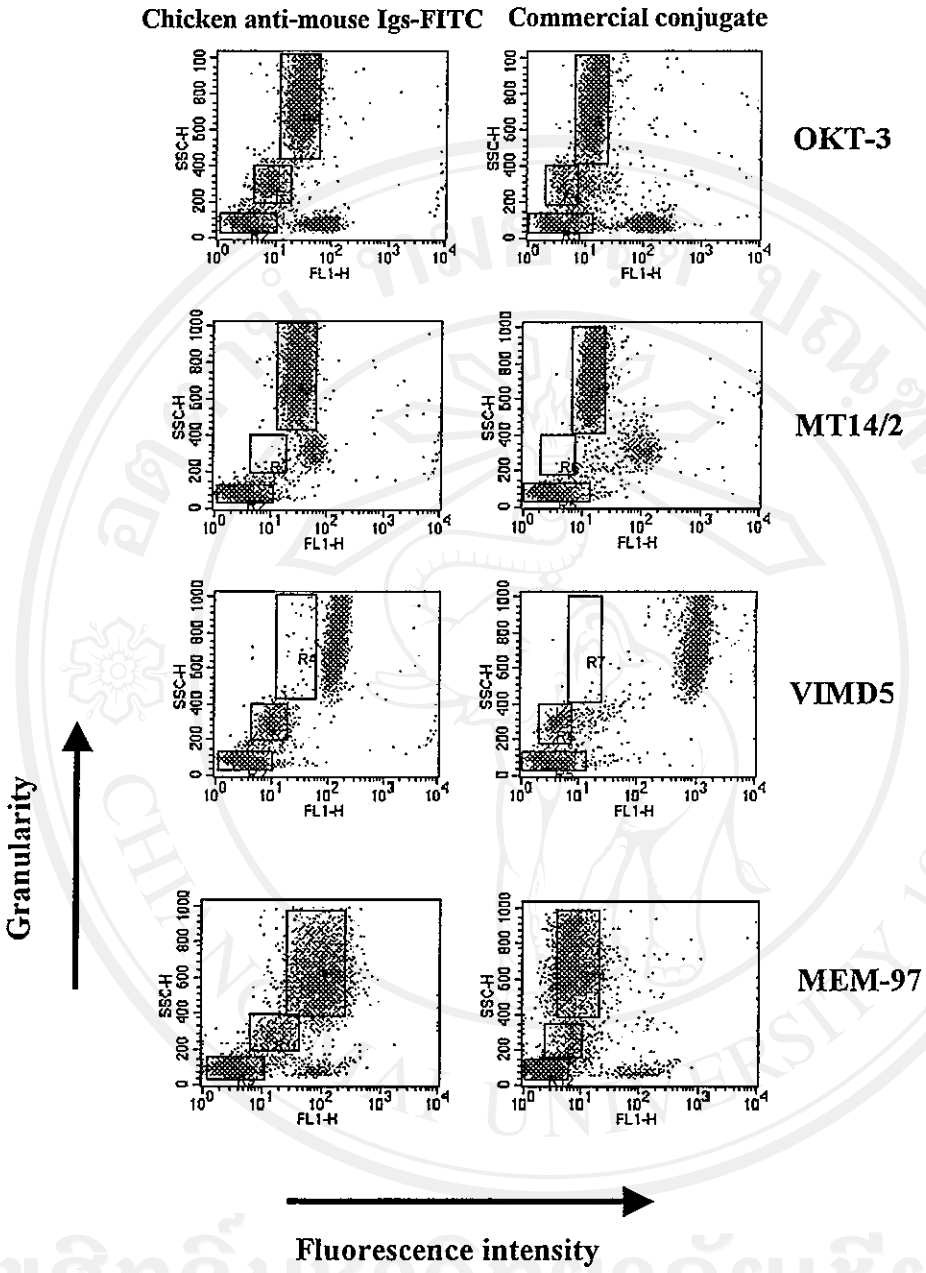

Figure 3.11 FACS profile of the determination of T lymphocytes, monocytes, granulocytes and B lymphocytes using prepared chicken anti-mouse immunoglobulins-FITC and commercial conjugate. Whole blood were stained with T lymphocyte specific mAb OKT3, monocyte specific mAb MT14/2, granulocyte specific mAb VIMD5 and B lymphocyte specific mAb MEM-97 and counterstained with generated and commercial conjugates. Data are representative of 5 independent experiments except data of B cell that representative of 3 independent experiments.

Table 3.1 Comparison of percent T and B lymphocyte sub-populations in lymphogate using generated conjugate and commercial conjugate.

Cell sup-population	Sample number	Percentage of positive cell	
		Generated conjugate	Commercial conjugate
T cells in lymphogate	1	67.4	66.4
	2	64.1	62.6
	3	70.1	69.9
	4	65.4	64.3
	5	73.8	73.4
B cells in lymphogate	1	7.1	6.5
	2	14.7	14.2
	3	11.6	12.2

Whole blood were stained with the mAb specific to T and B lymphocytes and counterstained with produced and commercial conjugates. After lysis of red blood cells, the stained cells were analyzed by a flow cytometer. Lymphocyte populations were gated and lymphocyte sub-populations were determined by flow cytometry.

3.8 Determination of protein expression in COS cell transfection system using the generated conjugate

In order to determine whether the produced conjugate can be used as secondary antibody in COS cell expression system, cDNA encoding CD14 and CD147 were transfected into COS cells. The transfected COS cells were stained with various isotypes of specific primary antibodies and the produced conjugate was used as secondary antibody. It was found that the produced chicken anti-mouse immunoglobulins conjugate showed positive reactivity with all specific primary antibodies but showed negative reactivity with non-specific primary antibodies (Table 3.2 and Figure 3.11). The results indicated that the generated conjugate did not directly react to COS cell surface proteins and can be used as secondary antibody in indirect immunofluorescence assay in COS cell expression system.

Table 3.2 The reactivity of chicken anti-mouse immunoglobulins-FITC with specific antibody against protein expressed on transfected COS cells.

Transfected COS cell	Primary Antibody (isotype)				
	M6-1D4 (IgM)	M6-1E9 (IgG2a)	M6-1B9 (IgG3)	MT14/2 (IgG2b)	MT14/3 (IgG1)
M6-DNA COS	+	+	+	-	-
CD14-DNA COS	-	-	-	+	+

COS cells were transfected with cDNA encoding CD147 protein (M6-DNA) and cDNA encoding CD14 protein (CD14-DNA). Transfected COS cells were stained with the indicated mAbs and counterstained with generated conjugate. Stained cells were analyzed under a fluorescence microscope.

+ : positive; -: negative

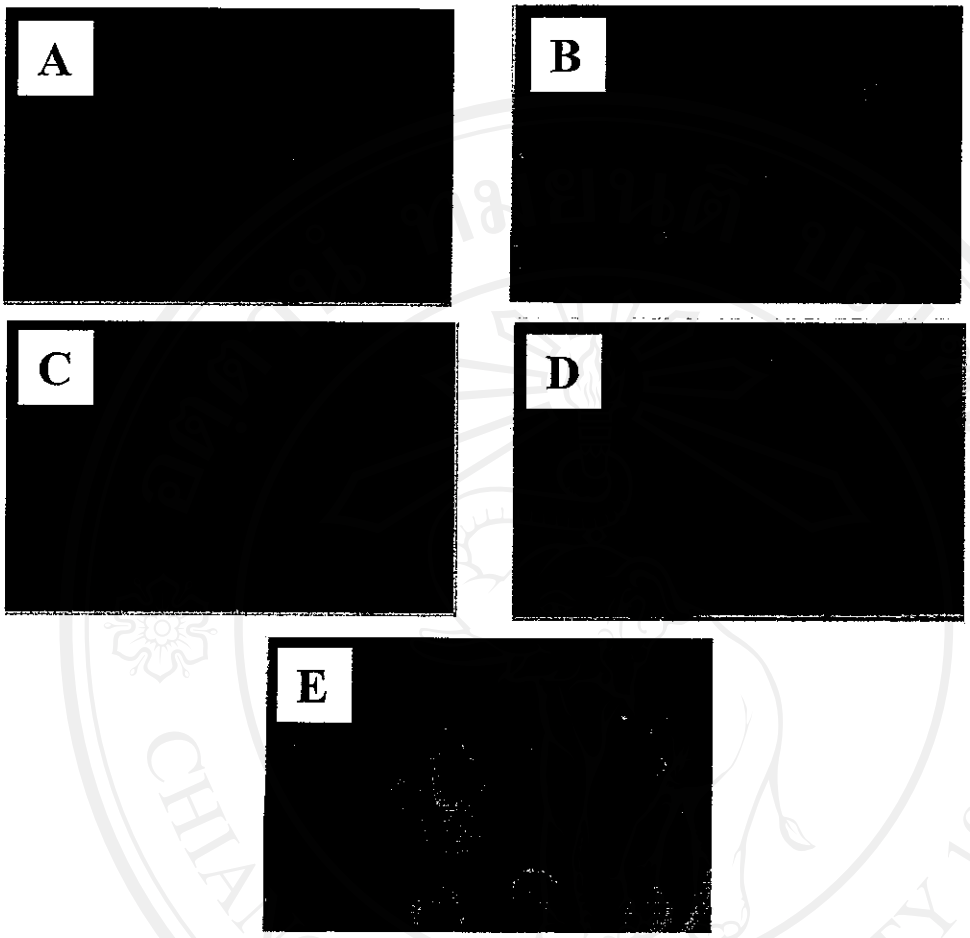

Figure 3.12 The reactivity of FITC conjugated chicken anti-mouse immunoglobulins with various isotypes of primary monoclonal antibodies specific to proteins expressed on COS cells. COS cells were transfected with cDNA encoding CD147 protein (M6-DNA) and cDNA encoding CD14 protein (CD14-DNA). M6-DNA transfected COS cells were stained with M6-1D4 (A), M6-1E9 (B), and M6-1B9 (C) mAbs. CD14-DNA transfected COS cells were stained with MT14/2 (D) and MT14/3 (E) mAbs. The stained cells were counter-stained with the produced conjugate.