CHAPTER III

RESULTS

3.1 Production and purification of CD147-hIgG

To produce CD147-hIgG, COS cells were transfected with CD147-Rg-DNA by DEAE-Dextran. The supernatant was collected three days after transfection. The CD147-hIgG present in the supernatant was purified by affinity chromatography. In this study, CD147-hIgG which is of IgG isotype was purified by Protein A coated Sepharose column. In this study, from 40 ml of starting supernatant, the yields of purified CD147-hIgG were 1.65 mg.

3.2 Determination of activity and specificity of the purified CD147-hIgG using indirect ELISA.

The purified CD147-hIgG was tested for their activity and specificity using indirect ELISA. The purified CD147-hIgG was determined with standard CD147 mAb, M6-1D4, and MT4 as a negative control. The CD147-hIgG strongly reacted with M6-1D4, but was negative control antibody (Figure 3.1). The results indicated that the purified CD147-hIgG can be used as antigen for the determination of the polyclonal anti-CD147 antibodies in mice sera.

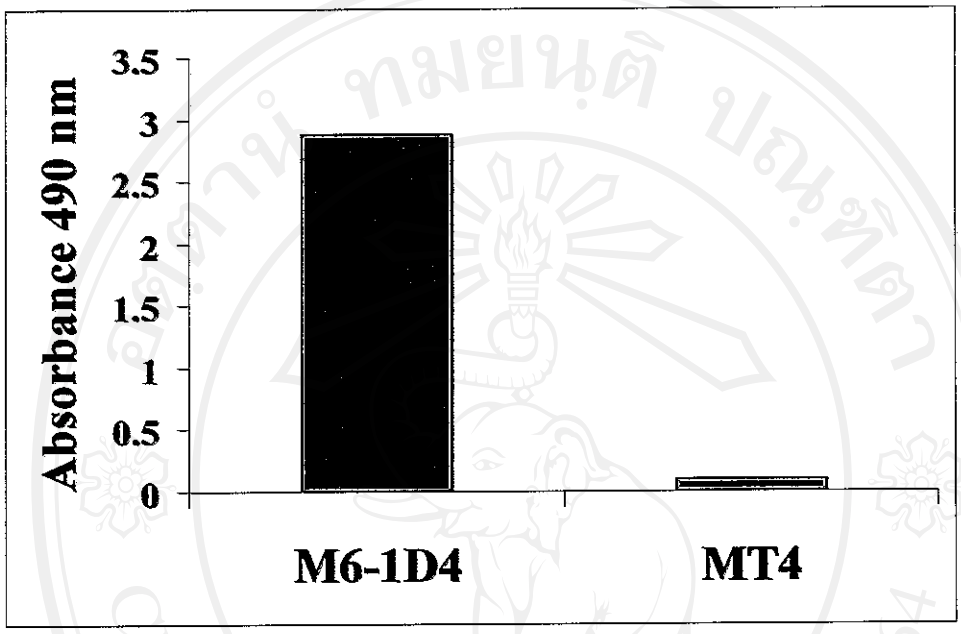

Figure 3.1. Determination of specificity of purified CD147-hIgG fusion protein by ELISA. Solid phase was coated with CD147 mAb, M6-1D4, or CD4 mAb, MT4. The binding of CD147-hIgG fusion protein was detected with peroxidase-conjugated rabbit anti-human immunoglobulin antibodies.

3.3 Preparation of pCDM8-CD147

The pCDM8-CD147 was transformed into competent *E. coli*. To obtain the *E. coli* containing pCDM8-CD147, two bacterial colonies were picked up by random and plasmid DNA were isolated. The plasmid DNA was digested with *Xba I* restriction enzyme, followed by agarose gel electrophoresis. The DNA were stained with ethidium bromide and visualized with UV light. The fragments of DNA were compared with standard pCDM8-CD147 and standard DNA marker. All isolated plasmid DNA contained a fragment of 1.8 kb of cDNA encoding CD147 protein and a 3.8 kb of vector (Figure 3.2). The DNA pattern was identical to standard pCDM8-CD147, which was used as a control. Both two colonies of CD147 transformed *E. coli* were frozen at -70°C and kept for further studies.

3.4 Expression of CD147 protein by COS cell expression system

The isolated pCDM8-CD147 was tested for their capability in expressing CD147 proteins. The COS cells were transfected with pCDM8-CD147 or plasmid DNA encoding CD8 molecule, which were then stained with M6-1D4 and MT8 as a negative control. These pCDM8-CD147 transfected COS cells strongly reacted with M6-1D4 (Figure 3.3), but were negative control antibody. In contrast, CD8 expressing COS cells showed positive reactivity only with MT8 (W. Kasinrerk, unpublished observations), but were not reacted with M6-1D4. The results indicated that the isolated pCDM8-CD147 could be transcribed and translated into CD147 proteins in COS cells

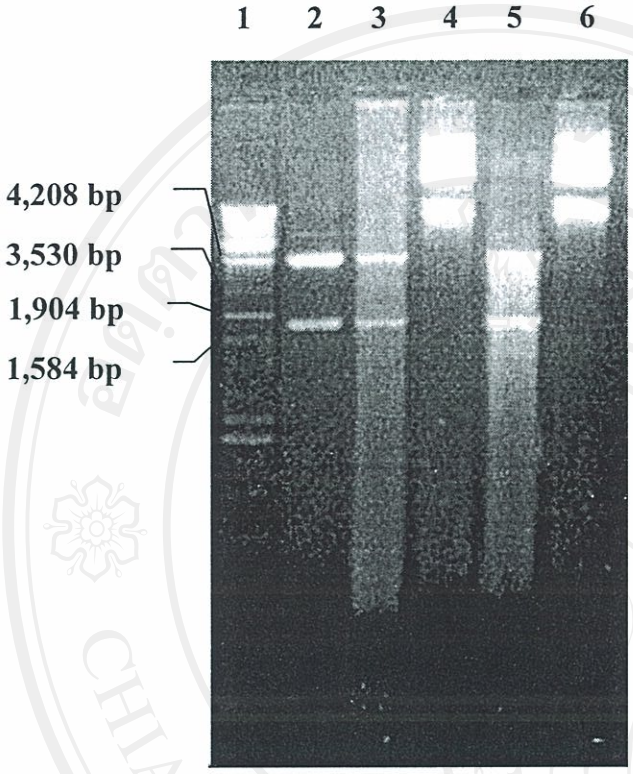

Figure 3.2. Restriction fragment analysis of pCDM8-CD147 isolated from transformed *E. coli* by plasmid miniprep using *Xba* I. Lane 1: Standard DNA marker. Lane 2: Standard pCDM8-CD147. Lane 3: plasmid DNA isolated from pCDM8-CD147 transformed *E. coli* colonies number 1 cut with *Xba I.* Lane 4: plasmid DNA isolated from pCDM8-CD147 transformed *E. coli* colonies number 1 without *Xba I* digestion. Lane 5: plasmid DNA isolated from pCDM8-CD147 transformed *E. coli* colonies number 2 cut with *Xba I.* Lane 6: plasmid DNA isolated from pCDM8-CD147 transformed *E. coli* colonies number 2 without *Xba I* digestion.

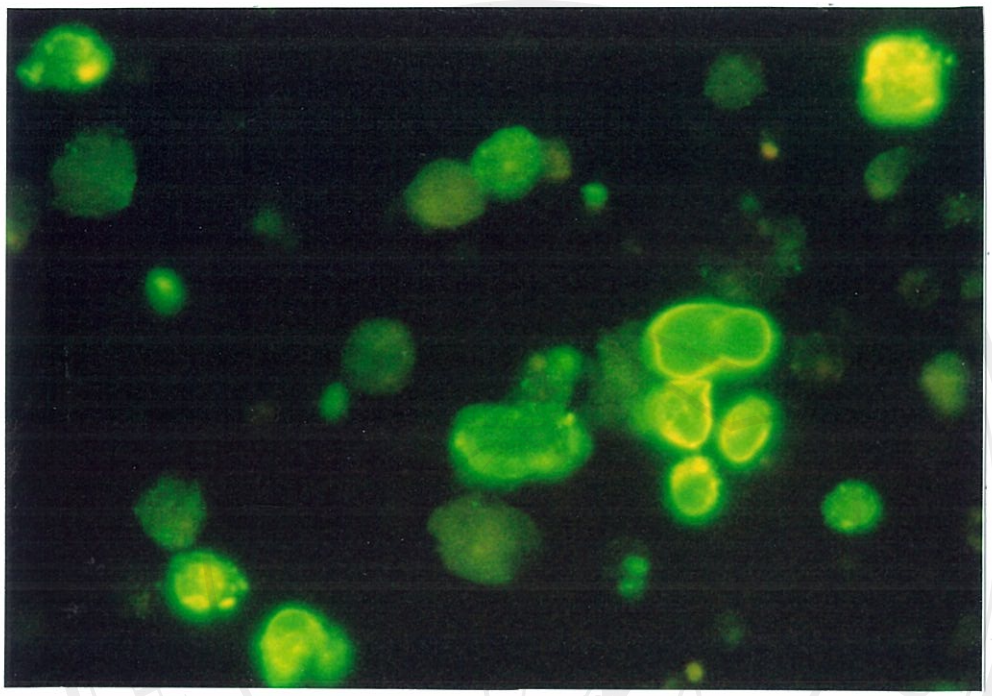

Figure 3.3. Photograph of pCDM8-CD147 transfected COS cells reacted to anti-CD147 monoclonal antibody, M6-1D4. COS cells were transfected with pCDM8-CD147 and stained with M6-1D4. The cells were counterstained with the FITC-conjugated anti-mouse immunoglobulins at dilution of 1:20. The stained cells were analyzed by a fluorescent microscope.

In order to obtain a large amount of pCDM8-CD147, *E. coli* colonies containing pCDM8-CD147, were used and performed as described in the materials and methods section. Using QIAGEN plasmid mega kit, the yields of pCDM8-CD147 obtained were 3.23 mg per liter of bacteria. The OD_{260}/OD_{280} ratio obtained from DNA preparation was between 1.8 - 2.0.

The purified pCDM8-CD147 plasmid was verified again by the restriction fragment analysis. As shown in Figure 3.4, the resulting DNA fragments of pCDM8-CD147 by *Xba I* digestion was the same as pCDM8-CD147 control DNA.

pCDM8-CD147 was then tested for their capability in producing CD147 proteins again in the COS cells expression system. The pCDM8-CD147 transfected COS cells were strongly positive when stained with M6-1D4, but were negative control antibody. The CD8 expressing COS cells, in contrast, were negative with CD147 mAb. The results indicated that the isolated pCDM8-CD147 can be used to prepare pCDM8-CD147 for the immunization of the mice producing polyclonal anti-CD147 antibodies.

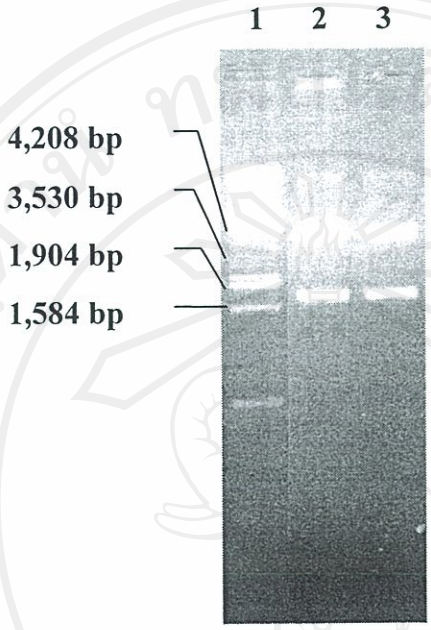

Figure 3.4. Restriction fragment analysis of pCDM8-CD147, isolated from transformed *E. coli* by QIAGEN plasmid mega kt, with *Xba* I. Lane 1: Standard DNA marker. Lane 2: plasmid DNA isolated from pCDM8-CD147 transformed *E. coli* colonies number 2. Lane 3: Control pCDM8-CD147.

3.5 Preparation of phage-displayed CD147

The pComb3H-CD147-transformed *E. coli* was infected by using VCSM13 helper phage to produce recombinant bacteriophages. During the assembly of progeny viruses, the CD147-gpIII fusion proteins were concomitantly incorporated into phage particles. The phage carrying CD147 molecule released into culture supernatant and was detected by sandwich ELISA. The CD147 mAb reacted against CD147-phage derived from *E. coli* TG-1 host when culturing in its standard growth condition (Figure 3.5). The results indicated that the phage-displayed CD147 can be used as immunogen to immunize mice for polyclonal anti-CD147 antibody production.

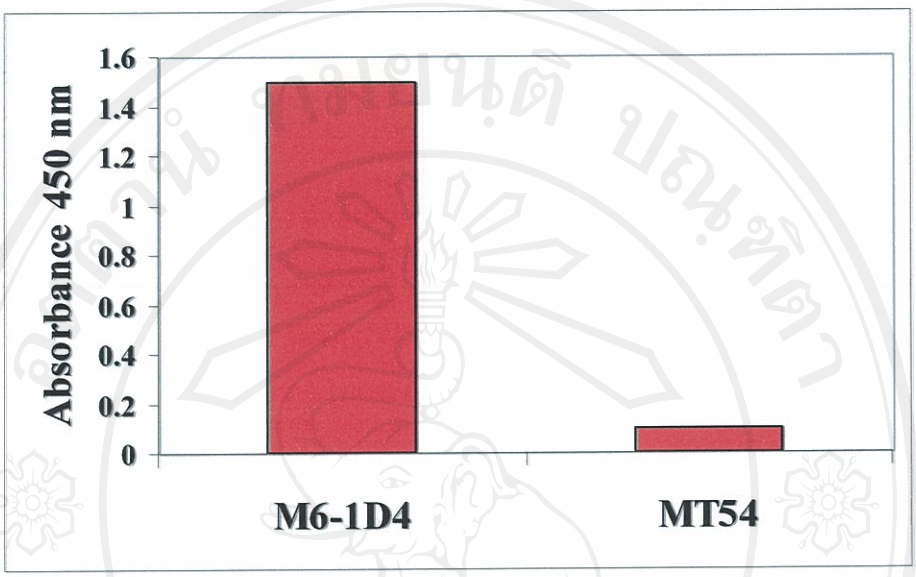

Figure 3.5. Sandwich ELISA for the detection of phage bearing CD147. Solid phase was coated with CD147 mAb, M6-1D4 or isotype matched CD54 mAb, MT54. The bound phages were traced with peroxidase conjugated sheep anti-M13 monoclonal antibody.

3.6 Production of polyclonal antibody in mice using pCDM8-CD147

In an attempt to produce polyclonal anti-CD147 antibodies, two female BALB/c mice were used in this experiment. They were immunized three times at two-week intervals with pCDM8-CD147 by intramuscular immunization. The presence of anti-CD147 antibodies in mouse serum was determined by indirect ELISA and indirect immunofluorescence analysis.

Using indirect ELISA, anti-CD147 antibody titer in immunized mouse sera could be detected four and six weeks after antigen immunization in both first and second BALB/c mice, respectively (Figure 3.6). The antibody levels were increased after the second and third immunization, respectively. The antibody reached the maximum level at week 4 and 8 after the first antigen immunization, respectively. Three months after the last immunization, however, the antibody levels were decreased.

In addition, the mouse sera were tested for CD147 antibody by using indirect immunofluorescence analysis. Immunofluorescence analysis of the reactivity of anti-CD147 antibody in mouse sera with B W and CD147 expressing BW5147 cell line was carried out. The fluorescent intensity was analyzed on a FACSCalibur flow cytometer. As shown in Figure 3.7. BW either CD147 expressing BW5147 cell line showed negative or positive with immunized mouse sera, respectively. The BW cell line were negative with mouse sera immunized with pCDM8-CD147, which is known not to react with BW cell line and used as a negative control in this study. In control staining, the BW and CD147 expressing BW5147 cell line were strongly positive with

anti-BW, which was reported to express on BW or CD147 expressing BW5147 cell line.

These studies demonstrated that pCDM8-CD147 was immunogenic during immunization and present in high CD147 antibody in mice sera have already been identified by indirect ELISA assay and indirect immunofluorescence analysis.

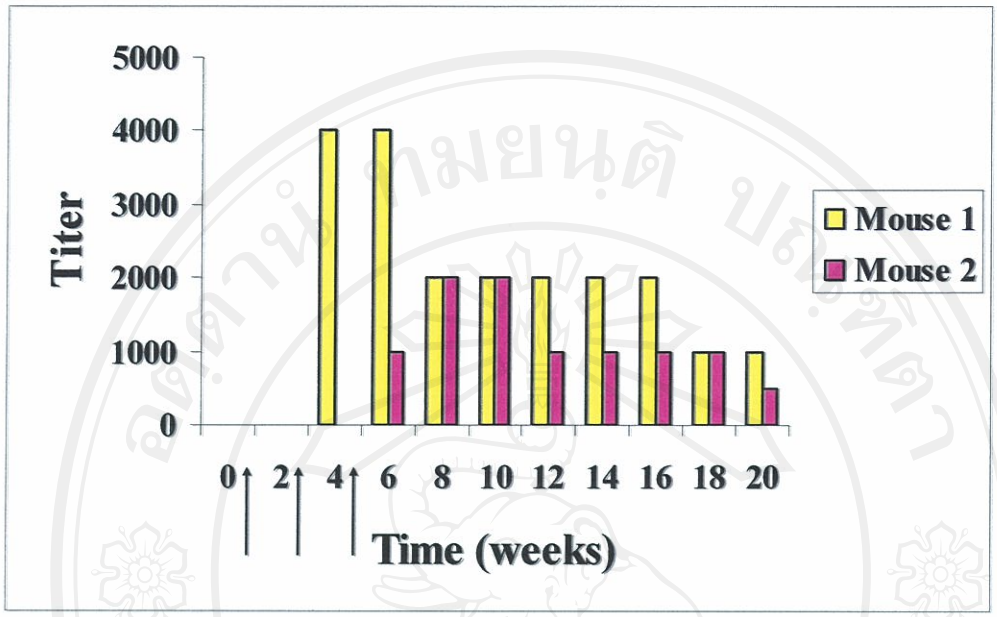

Figure 3.6. Anti-CD147 antibody responses of mice sera after immunization with pCDM8-CD147 determined by indirect ELISA. Arrows indicate the first, second and third immunizations.

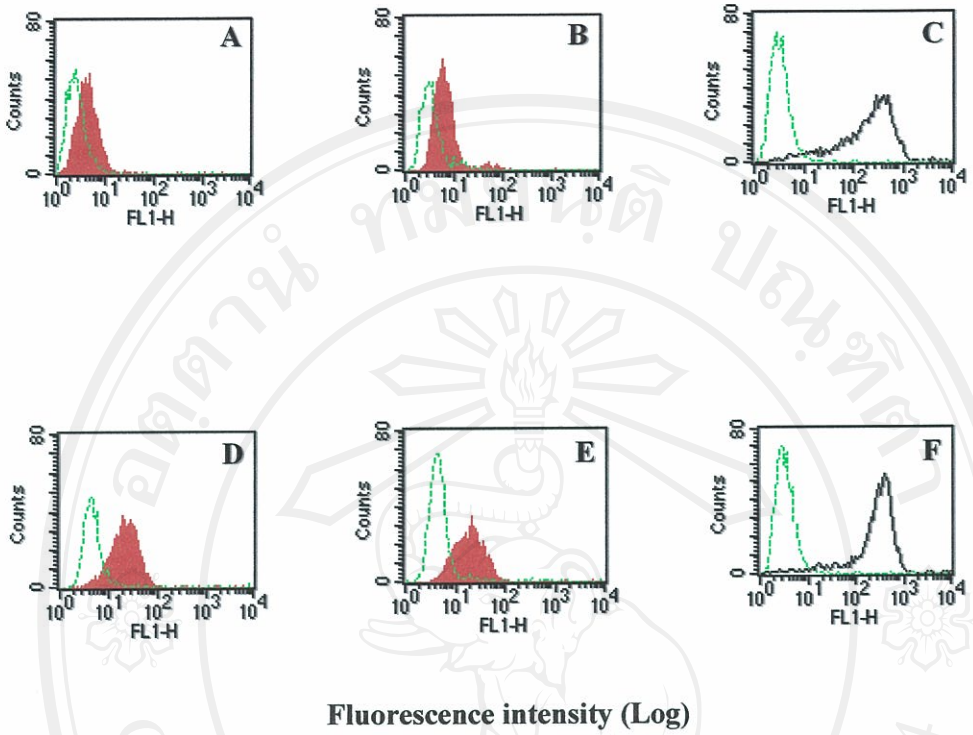

Figure 3.7. Immunofluorescence profiles of the reactivity of anti-CD147 antibody in mice sera after immunization with pCDM8-CD147. The BW cell line were stained with sera of BALB/c mice number 1 (A) and 2 (B) immunized with pCDM8-CD147 on day 70. CD147 expressing BW5147 cell line were stained with sera of BALB/c mice number 1 (D) and 2 (E) immunized with pCDM8-CD147 on day 70. As control, BW and CD147 expressing BW5147 cell line were stained with anti-BW (C) and M6-1D4 (F), Shaded peaks represent cells stained with immunized mouse sera on day 70; dashed lines represent the background fluorescence of negative control; and solid lines represent the fluorescence of indicated positive control monoclonal antibody.

3.7 Production of polyclonal antibody in mice using phage-displayed CD147

To produce polyclonal anti-CD147 antibodies, two female BALB/c mice were used in this experiment. They were immunized three times at two-week intervals with phage-displayed CD147 molecules by intraperitoneal immunization. The presence of anti-CD147 antibodies in serum was determined by indirect ELISA and indirect immunofluorescence analysis.

The mouse sera were determined for CD147 antibody by using indirect ELISA. In serum, anti-CD147 antibody titer could be detected four weeks after antigen immunization in both BALB/c mice (Figure 3.8). The antibody levels were increased after the second immunization. The antibody reached the maximum level at week 14 and 8 after the first antigen immunization, respectively. The antibody level was maintained for five months after phage immunization.

In addition, the mouse sera were determined for CD147 antibody by using indirect immunofluorescence analysis. Immunofluorescence analysis of the reactivity of anti-CD147 antibody in mouse sera with BW and CD147 expressing BW5147 cell line was carried out. The fluorescent intensity was analyzed on a FACSCalibur flow cytometer. As shown in Figure 3.9. BW either CD147 expressing BW5147 cell line showed negative or positive with immunized mouse sera, respectively. The BW cell line were negative with mouse sera immunized with phage-displayed CD147 molecules, which is known not to react with BW cell line and used as a negative control in this study. In control staining, the BW and CD147 expressing BW5147 cell line were strongly positive with anti-BW, which was reported to express on BW or CD147 expressing BW5147 cell line.

The specific antibody response was subsequently determined by ELISA and indirect immunofluorescence using flow cytometry. The results demonstrated that anti-CD147 antibodies were strongly induced in the immunized mice after second immunization and significantly increased after the third immunization. Both indirect ELISA assay and indirect immunofluorescence analysis demonstrated that mouse immunized with the phage-displayed CD147 response more potent than with pCDM8-CD147. These data supported the results from the indirect ELISA assay indicating a higher CD147 antibody in sera of mice immunized with phage-displayed CD147.

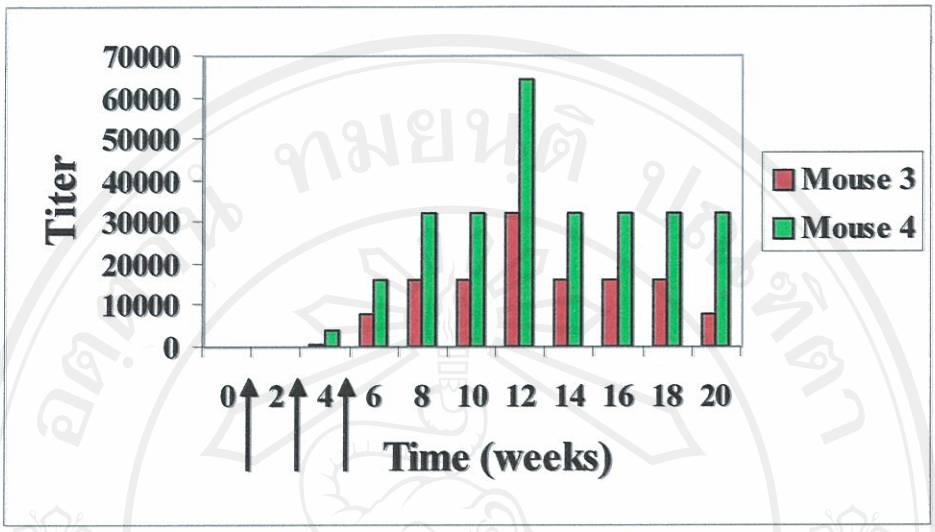

Figure 3.8. Anti-CD147 antibody responses of mice sera after immunization with phage-displayed CD147 determined by indirect ELISA. Arrows indicate the first, second and third immunizations.

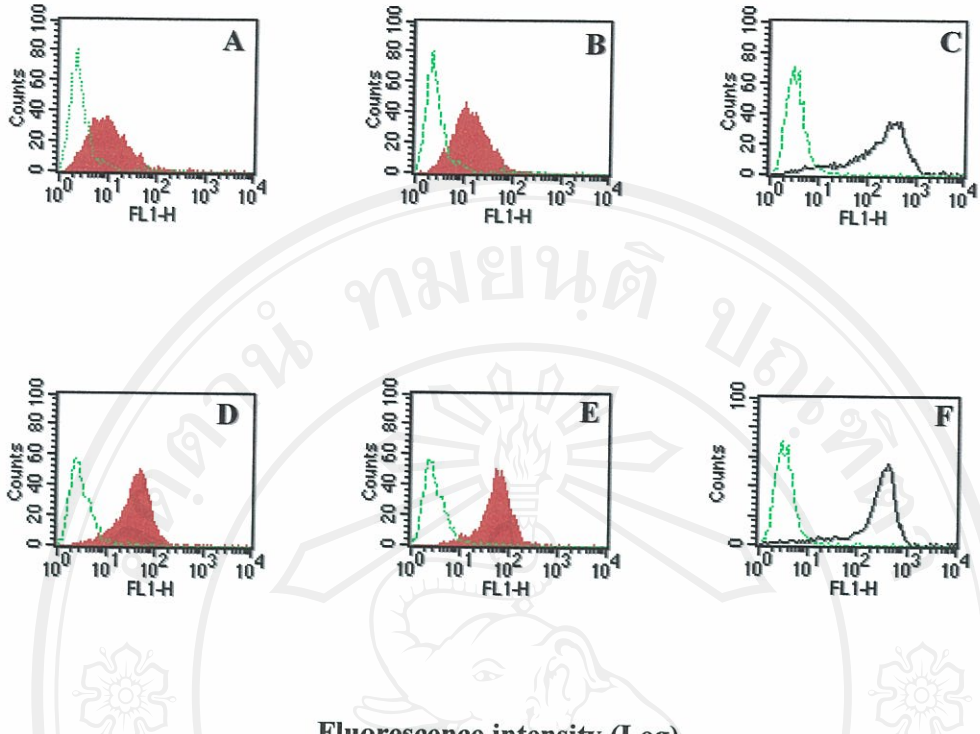

Figure 3.9. Immunofluorescence profiles of the reactivity of anti-CD147 antibody in mice sera after immunization with phage-displayed CD147. The BW cell line were stained with sera of a BALB/c mice number 3 (A) and 4 (B) that immunized with phage-displayed CD147 on day 70. CD147 expressing BW5147 cell line were stained with sera of a BALB/c mice number 3 (D) and 4 (E) that immunized with phage-displayed CD147 on day 70. As control, BW and CD147 expressing BW5147 cell line were stained with anti-BW (C) and M6-1D4 (F), respectively. Shaded peaks represent cells stained with immunized mouse sera on day 70; dashed lines represent the background fluorescence of negative control; and solid lines represent the fluorescence of indicated positive control monoclonal antibody.

3.8 Comparison of the antibody response induced by pCDM8-CD147 and phage-displayed CD147

Hyperimmune sera were produced by immunizing BALB/c mice with pCDM8-CD147 or phage-displayed CD147 *via* intramuscular or intraperitoneum route, respectively. The mouse sera were evaluated for the present of anti-CD147 antibodies by using indirect ELISA. It was found that the phage-displayed CD147 induced more potent anti-CD147 antibodies production than pCDM8-CD147. Even though the variation of antibody level among four mice was observed, anti-CD147 antibodies from mice immunized with phage-displayed CD147 were approximately 10 to 20 folds in comparison with pCDM8-CD147 (Figure 3.6 and 3.8). In addition the kinetic of immune response induced by phage-displayed CD147 was assumed to be better than using pCDM8-CD147. Anti-CD147 antibodies from mice immunized with phage-displayed CD147 or pCDM8-CD147 could be detected after the 2nd immunization and the responses continued to increase significantly after the 3rd boost. However, it was found that the antibody response induced by phage-displayed CD147 reached the maximum level more sooner and sustained in higher level than by pCDM8-CD147 (Figure 3.6 and 3.8). Demonstrating by the mean fluorescent signal, anti-CD147 antibodies from mouse immunized with the phage-displayed CD147 stained CD147 protein expressed on BW5147 cell line more stronger than with pCDM8-CD147 (Figure 3.7 and 3.9). These data supported the results from the indirect ELISA indicating the higher anti-CD147 antibody level in sera of mice immunized with phage-displayed CD147. However, the mouse immunized with the phage-displayed CD147 produced non-specific antibody, which was observed in the fluorescence pattern when BW cell line was used. The histogram showed the shift to

more right (Figure 3.10B) in comparison to the sera of pCDM8-CD147 immunized mouse (Figure 3.10A).

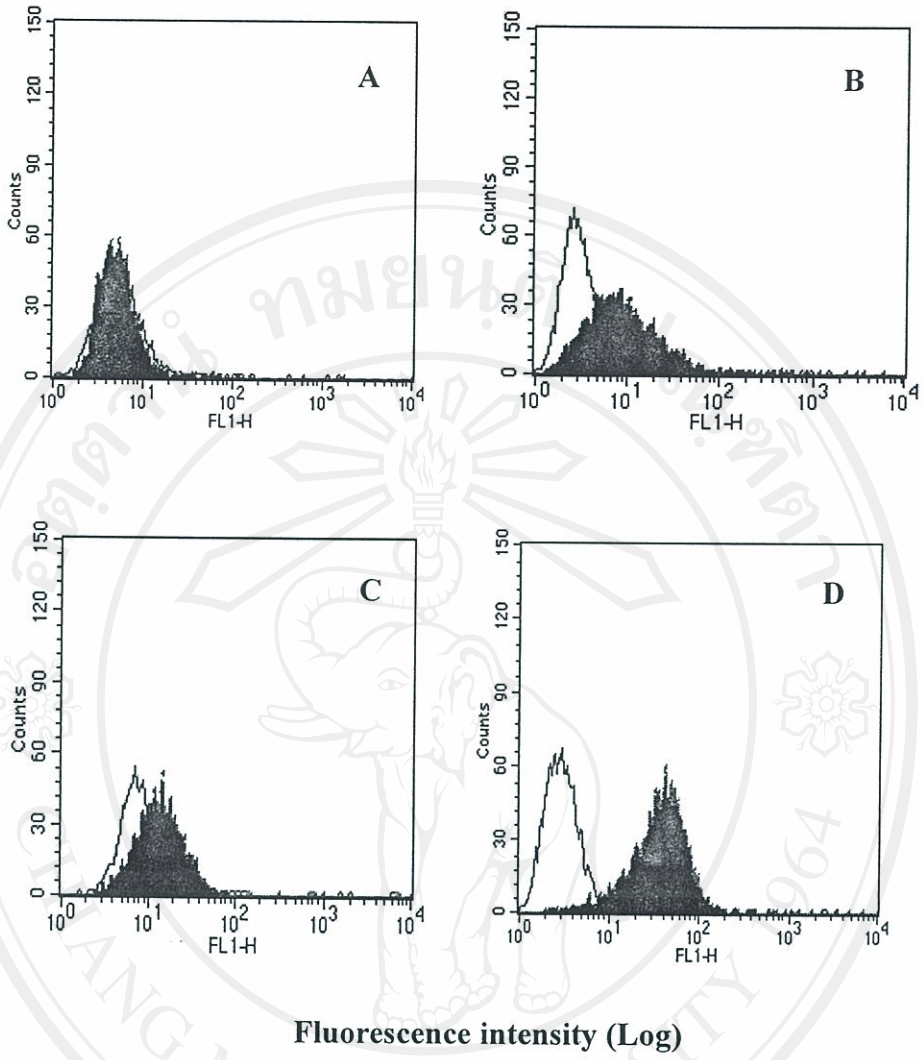

Figure 3.10. Immunofluorescence profiles of the reactivity of anti-CD147 antibody in mice sera after immunization with either pCDM8-CD147 or phage-displayed CD147. BW cell line were stained with sera of a BALB/c mouse immunized with pCDM8-CD147 (A) and phage-displayed CD147 on day 70 (B). CD147 protein expressed on BW5147 cell line were stained with sera of a BALB/c mouse immunized with pCDM8-CD147 (C) and phage-displayed CD147 on day 70 (D). Solid lines represent the fluorescent profiles of cells stained with preimmune mouse sera; shaded peaks represent cells stained with immunized mouse sera.