CHAPTER III

RESULTS

3.1 Production of monoclonal antibodies against surface molecules on thrombin-activated platelets

In an attempt to produce monoclonal antibodies against thrombin-activated platelets, the standard hybridoma technique was performed. Two female BALB/c mice, mouse A and mouse B, were intraperitoneally immunized with thrombin-activated platelets every week for 3 weeks. After immunization, blood was collected from mice by tail vein bleeding. After centrifugation, the obtained sera were screened for anti-activated platelets antibodies by indirect ELISA. The antibody levels from mouse sera increased after immunization. The antibody responses in mouse B were slightly higher than mouse A before the fusion (Figure 3.1). Therefore, mouse B was selected for monoclonal antibody production.

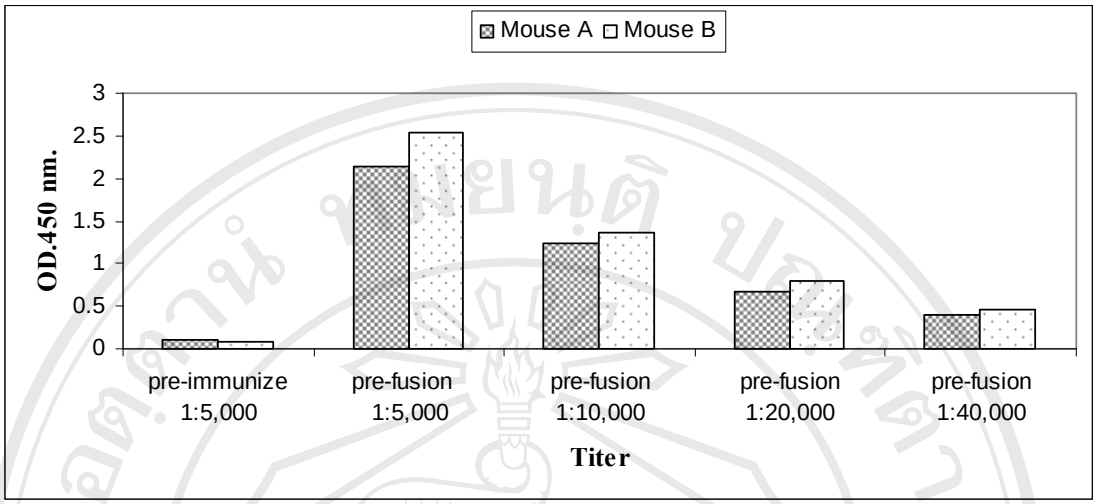


Figure 3.1 Antibody responses in BALB/c mice after immunization with thrombin-activated platelets. Mouse A and mouse B were immunized with 10^7-10^8 thrombin-activated platelets once a week for 3 weeks. Blood was collected at pre-immunization and after three immunization (pre-fusion). Then serum was determined for antibody response to activated platelets by indirect ELISA.

3.2 Production of monoclonal antibodies against thrombin-activated platelets

To generate hybridomas produced monoclonal antibody against thrombin-activated platelets, splenocytes from immunized mouse were carefully isolated and fused with myeloma cells using 50% polyethelene glycol. After HAT medium selection, 432 culture supernatants from the wells containing the hybridoma cells were collected and screened for the antibody against thrombin-activated platelets and resting platelets by indirect immunofluorescent staining and flow cytometry. From this experiment, culture supernatant from 13 hybridomas reacted to thrombin-activated platelets. These hybridomas were selected, subjected to limiting dilution.

To obtain single clone of interested hybridomas, the positive hybridoma cells were adjusted to single cell per well. Then, culture supernatant containing mAb from single clone was collected and confirmed the reactivity of mAb against thrombin-activated platelets and resting platelets by indirect immunofluorescent staining and flow cytometry. After screening, three strong reactivity hybridomas were selected and named as 138.7, 176.7 and 297.7**.**

3.3 Isotyping of monoclonal antibodies

After cell fusion experiments, three hybridoma clones, 138.7, 176.7 and 297.7, were obtained. The generated mAbs were isotyped using the Isostrip Mouse Monoclonal Antibody Isotyping Kit (Roche). All of generated mAbs include, 138.7, 176.7 and 297.7 were IgG1 isotype with kappa light chain (Figure 3.2).

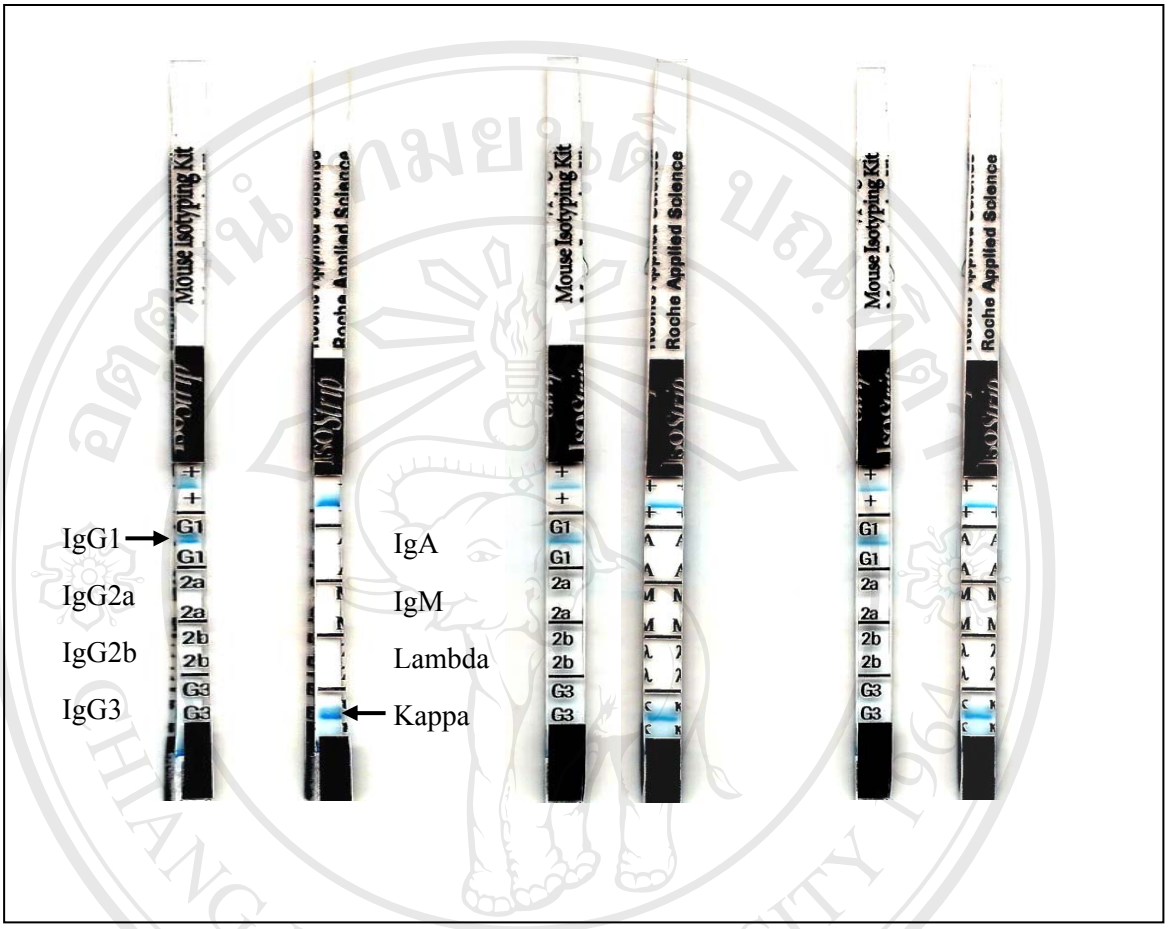


Figure 3.2 Isotyping of generated monoclonal antibodies. Interested mAbs were isotyped by an Isostrip Mouse Monoclonal Antibody Isotyping Kit. The result from mab clone 138.7, 176.7 and 297.7 were shown in A, B and C, respectively.

3.4 Large scale production and purification of monoclonal antibodies

The cellular expression, biochemical properties and function of mAbs clone 138.7, 176.7 and 297.7 were further studied. The purified mAbs were first prepared by *in vivo* and *in vitro* production.

For *in vivo* production, the hybridoma clones 138.7, 176.7 and 297.7 were injected into mouse peritoneal cavity for induction of ascitic fluid as described in the Materials and Methods. All of hybridoma clones successfully induced ascitic fluids and the ascitic fluid containing mAbs were then collected. Whereas, *in vitro* production, three hybridomas producing mAb were cultured in 100% SFM to avoid bovine immunoglobulin contamination. Then, ascetic fluid and culture supernatant were harvested, clarified and applied on protein G sepharose column. After elution process, the eluate containing mAb was checked for its activity by indirect immunofluorescent staining and flow cytometry. The purity of purified mAbs was determined using SDS-PAGE.

The immunoreactivity of all purified mAbs to thrombin-activated platelets is strongly positive, as shown in Figure 3.3. For the purity of all mAbs, SDS-PAGE was performed under reducing and non-reducing condition. Two protein bands correlated with heavy chain and light chain of immunoglobulin were observed under reducing condition (Figure 3.4). Under non-reducing condition, only one major protein band was observed on the gel (Figure 3.4).

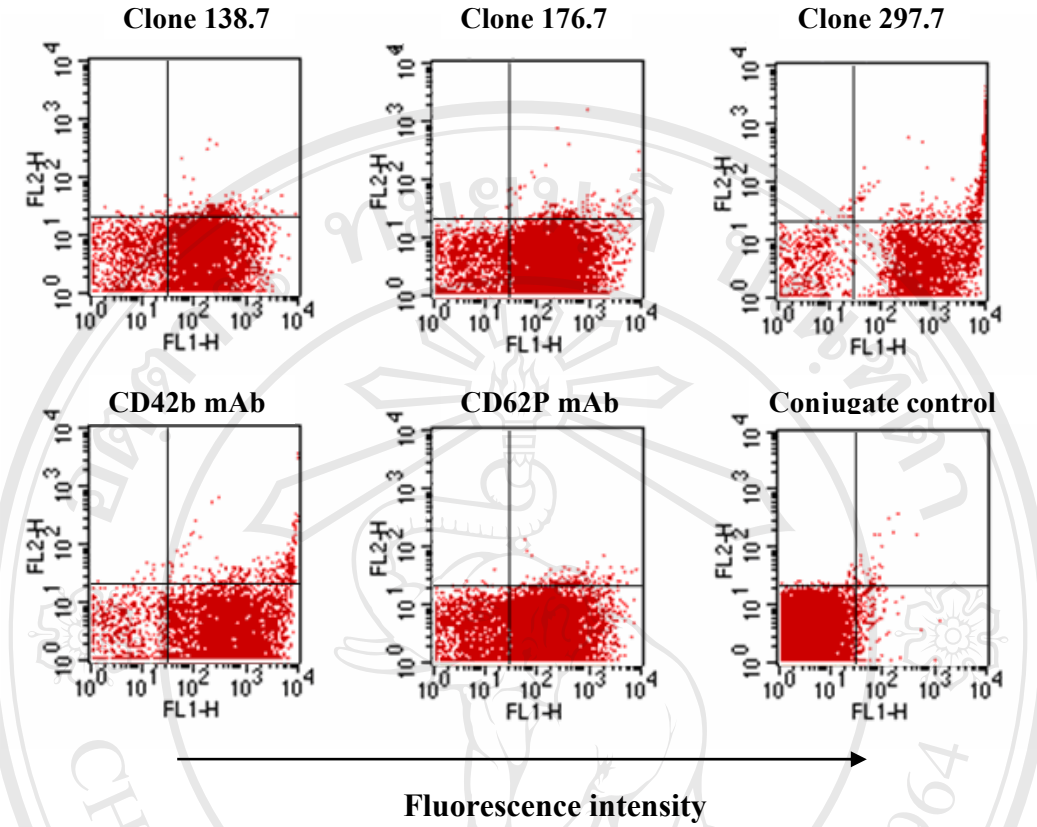


Figure 3.3 The immunoreactivity of purified monoclonal antibodies clone 138.7, 176.7 and 297.7. Thrombin-activated platelets were stained with purified mAbs 138.7, 176.7 and 297.7 by indirect immunofluorescent staining and analyzed by flow cytometry. CD42b mAb was used as platelet-specific mAb and CD62P mAb was used as activated platelet-specific mAb.

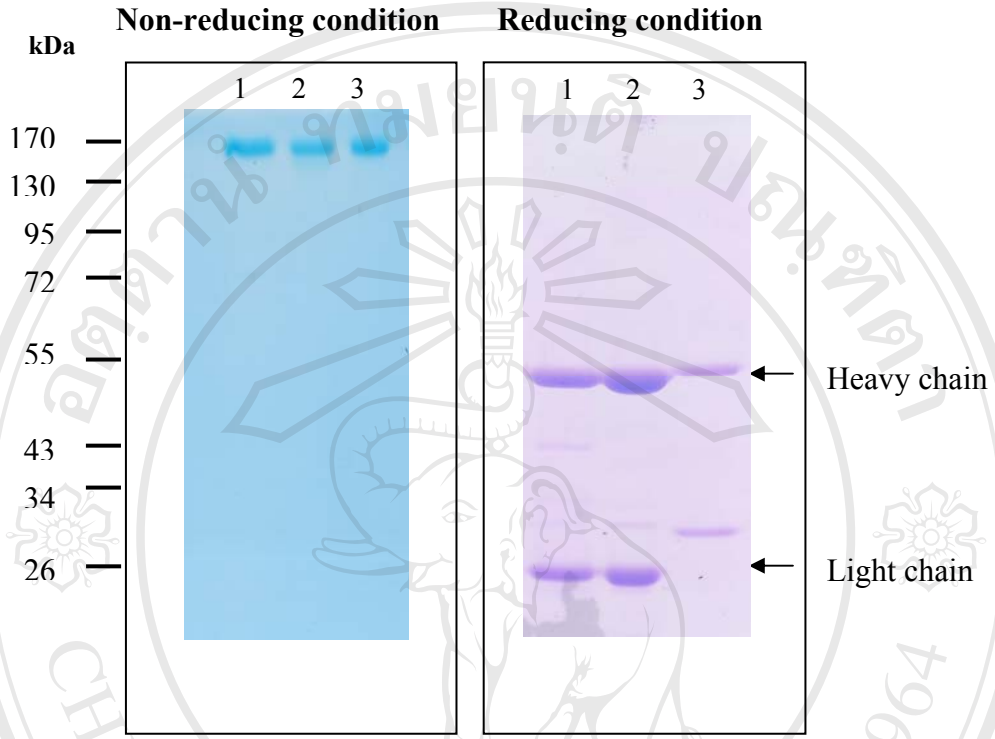


Figure 3.4 Analysis of the purified mAbs 138.7, 176.7 and 297.7 by SDS-PAGE. The purified mAb clone 138.7 (lane 1), 176.7 (lane 2) and 297.7 (lane 3) were separated onto 10% SDS-PAGE under non-reducing and reducing conditions. The gels were stained with coomassie brillant blue after electrophoresis. The molecular weight markers are indicated on the left (kDa).

3.5 Cellular distribution of the molecules recognized by monoclonal antibodies against thrombin-activated platelets

The cellular distribution of molecules recognized by monoclonal antibodies clone 138.7, 176.7 and 297.7, various hematopoietic cell lines (Daudi, SupT1, Jurkat, Molt4, HL60, K562, THP1 and U937) and peripheral blood cells include resting platelets, activated platelets, lymphocytes, monocytes, granulocytes and erythrocytes were stained with mAb clone 138.7, 176.7 and 297.7 and followed by flow cytometry.

3.5.1 Cellular distribution of the molecules recognized by mAb clone 138.7, 176.7 on peripheral blood cells

The resting platelets and activated platelets were stained with mAb clone 138.7, 176.7 and 297.7. As shown in Fig. 3.5, the mAbs clone 138.7 and 176.7 showed negative reactivity with resting platelets. In contrast, mAb 297.7 showed positive reactivity to membrane molecules on resting platelets. While, all of generated mAbs showed positive reactivity with membrane molecules on thrombin-activated platelets.

The peripheral blood cells include erythrocytes, lymphocytes, monocytes and granulocytes were stained with mAbs clone 138.7, 176.7 and 297.7. MAb clone WK5 (RBC mAb) and mAb clone MT45 (CD45 mAb specific to white blood cells) were used as positive control for erythrocytes and white blood cells, respectively. All of interested mAbs, clone 138.7, 176.7 and 297.7, showed negative reactivity with membrane molecules on erythrocytes and white blood cells tested. (Figure 3.6 and Figure 3.7)

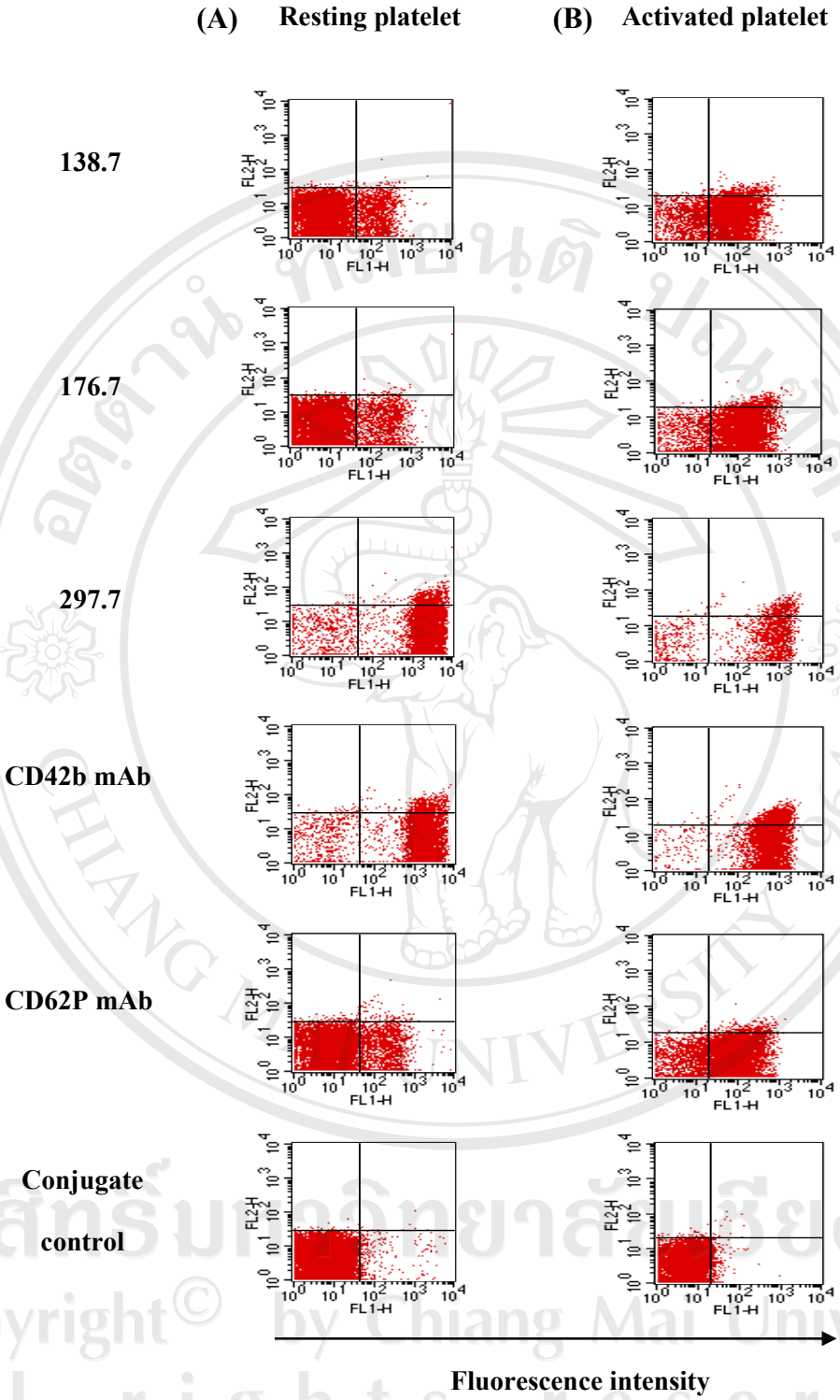


Figure 3.5 The cellular distribution of molecules recognized by mAbs clone 138.7, 176.7 and 297.7 on resting platelets and thrombin-activated platelets. The resting platelets (A) and thrombin-activated platelets (B) were stained with indicated monoclonal antibodies and determined by flow cytometry.

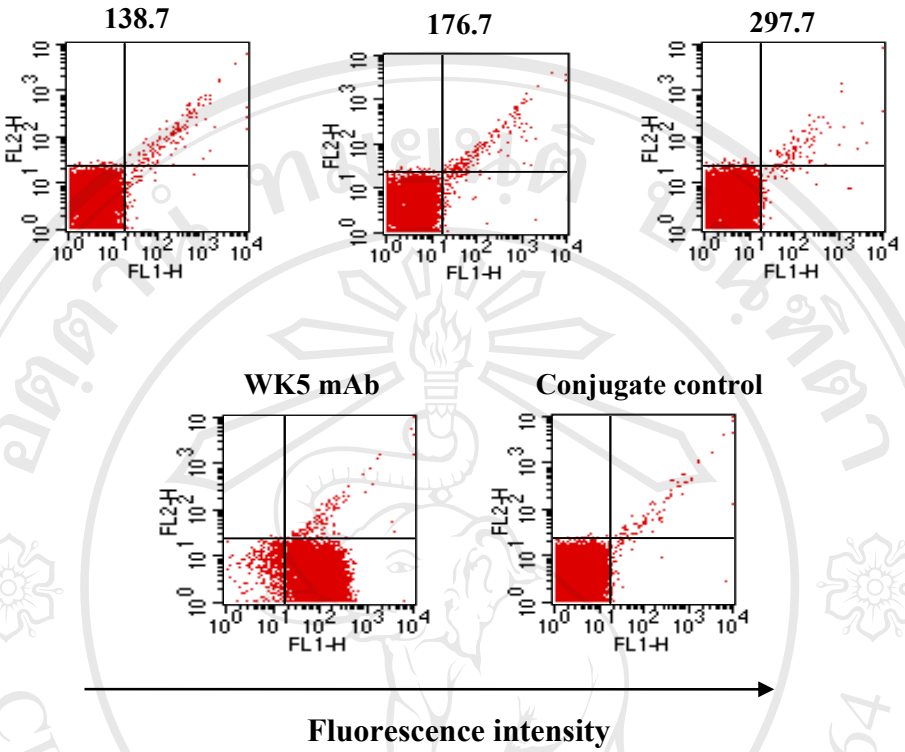


Figure 3.6 The cellular distribution of protein recognized by mAbs clone 138.7, 176.7 and 297.7 on red blood cells. Red blood cells were stained with indicated monoclonal antibodies and determined by flow cytometry. WK5 anti-RBC mAb was used as red blood cell positive control.

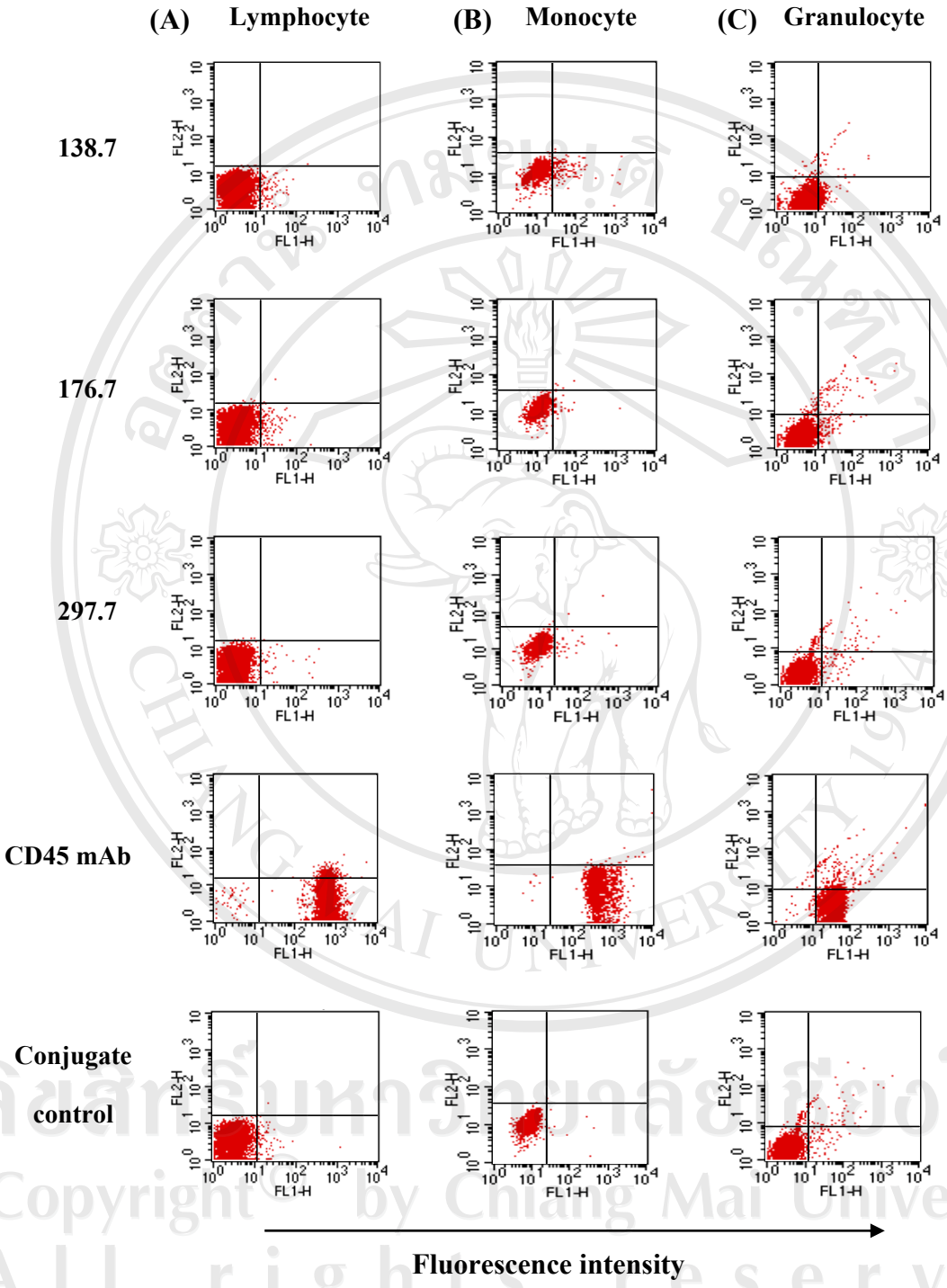


Figure 3.7 The cellular distribution of protein recognized by mAbs clone 138.7, 176.7 and 297.7 on white blood cells. Lymphocytes (A), monocytes (B) granulocytes (C) were stained with indicated monoclonal antibodies and determined by flow cytometry. CD45 mAb was used as white blood cell positive control.

3.5.2 The cellular distribution of protein recognized by mAb clone 138.7, 176.7 and 297.7 on hematopoietic cell lines

Eight types of hematopoietic cell lines include Daudi, SupT1, Jurkat, Molt4, HL60, K562, THP1 and U937 were stained with the produced monoclonal antibodies clone 138.7, 176.7 and 297.7. The CD298 mAb (anti-Na, K ATPase β3 subunit) was used for positive control of all cell lines and determined by flow cytometry.

All of selected mAbs, clone 138.7, 176.7 and 297.7, showed negative reactivity with membrane molecules on all cell lines tested (Figure 3.8 and Figure 3.9).

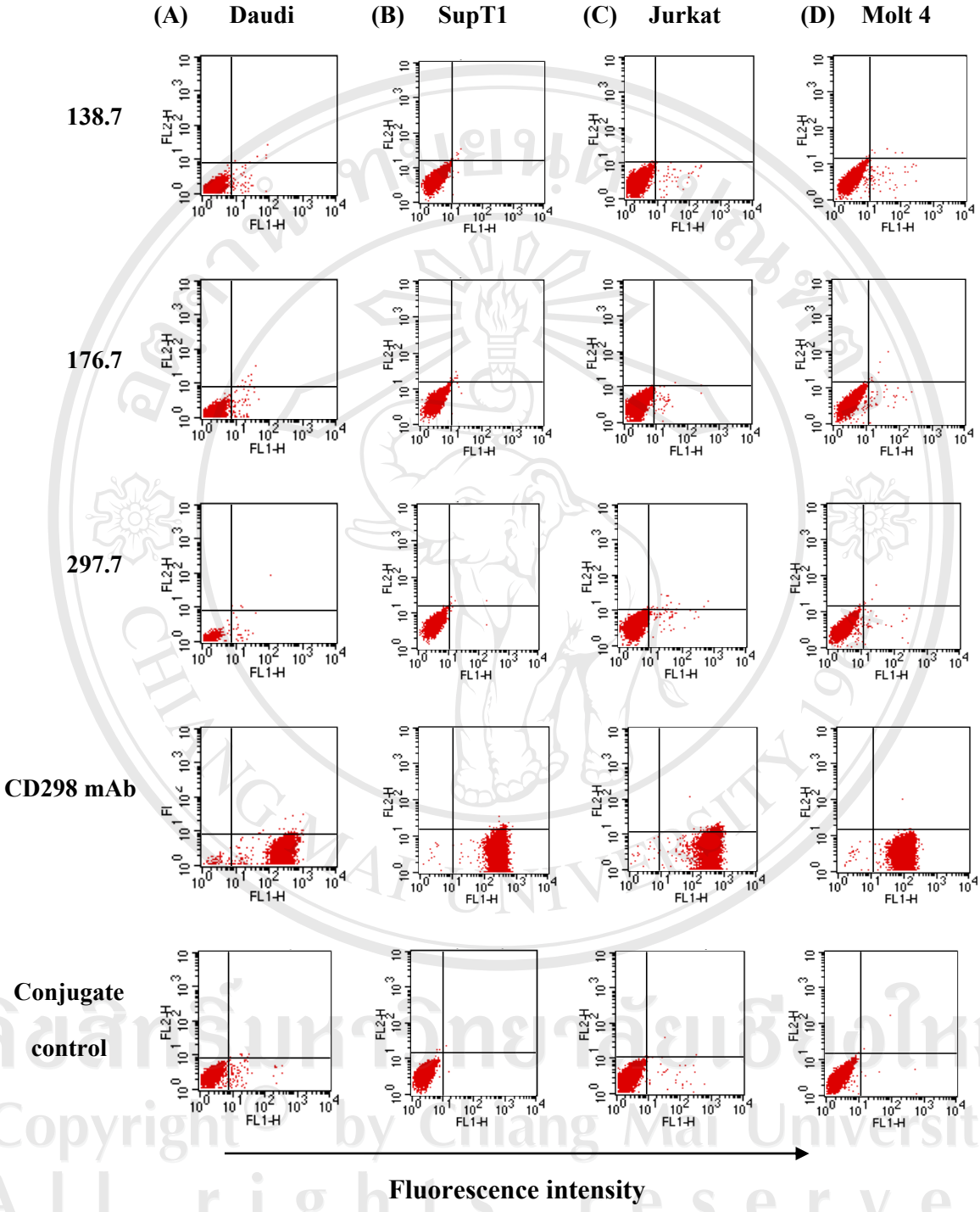


Figure 3.8 The cellular distribution of protein recognized by mAbs clone 138.7, 176.7 and 297.7 on hematopoietic cell lines. The cell lines include Daudi (A), SupT1 (B), Jurkat (C), Molt4 (D) were stained with indicated monoclonal antibodies and determined by flow cytometry.

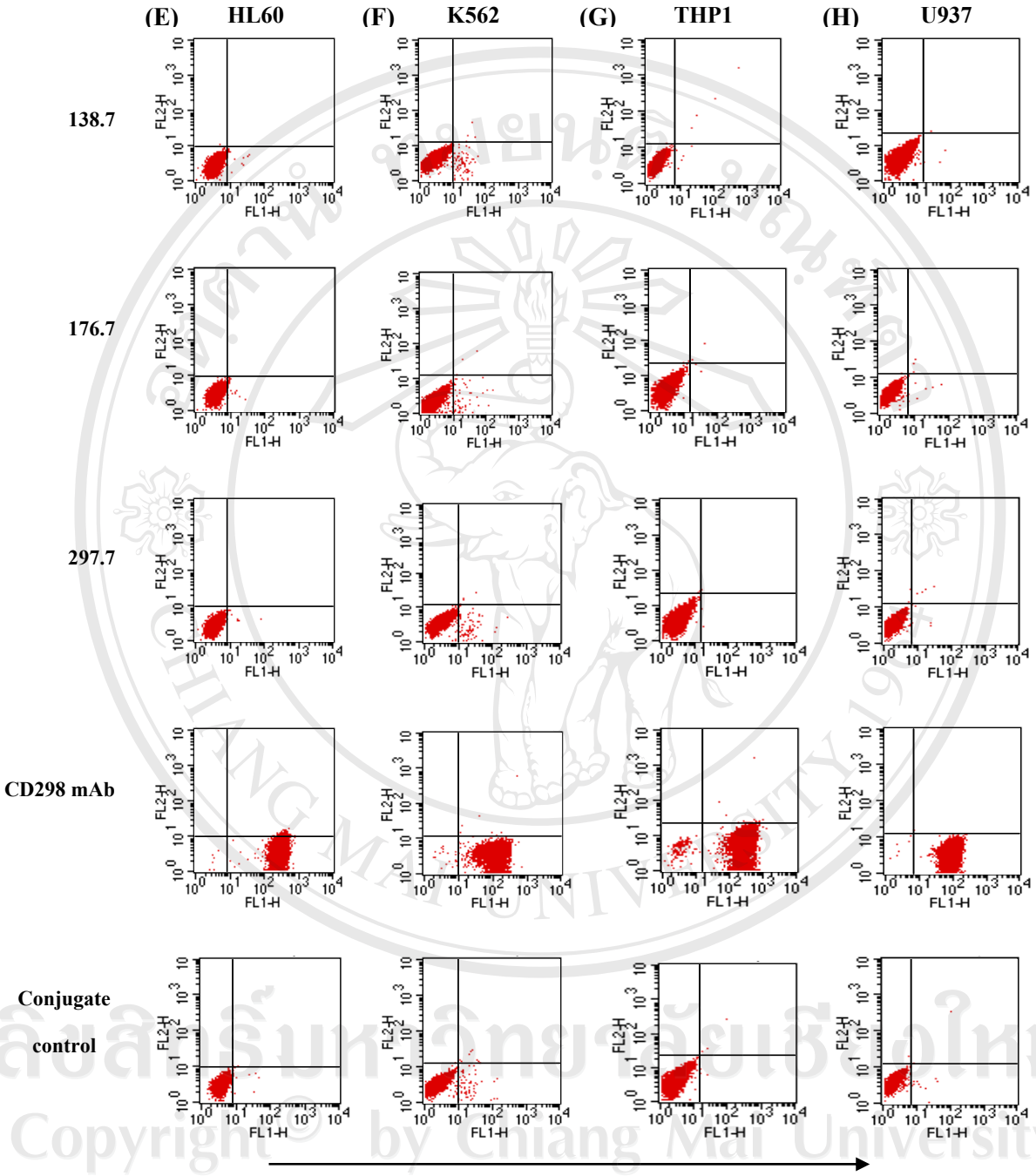


Figure 3.9 The cellular distribution of protein recognized by mAbs clone 138.7, 176.7 and 297.7 on hematopoietic cell lines. The cell lines include HL60 (E), K562 (F), THP1 (G) and U937 (H) were stained with indicated monoclonal antibodies and determined by flow cytometry.

3.6 Biochemical characterization of the molecules recognized by mAbs clone 138.7, 176.7 and 297.7 by SDS-PAGE and Western blotting

To determine the molecular weight of the molecules recognized by mAbs clone 138.7, 176.7 and 297.7, thrombin-activated platelets were lysed by using 1% NP-40 lysis buffer and separated onto 7.5% SDS-PAGE under non-reducing condition. After proteins were transferred to a polyvinylidene fluoride membrane (PVDF), the membrane was probed with the interested mAbs clone 138.7, 176.7 and 297.7. As shown in Figure 3.10, mAb clone 176.7 and 297.7 reacted to a protein band with the molecular weight of 120 kDa and 160 kDa, respectively. However, mAb clone 138.7 did not bind with any protein band. As a positive control, CD62P mAb reacted to a protein band at the molecular weight of 120 kDa that correlated with previously reported (Blann and Lip 1997).

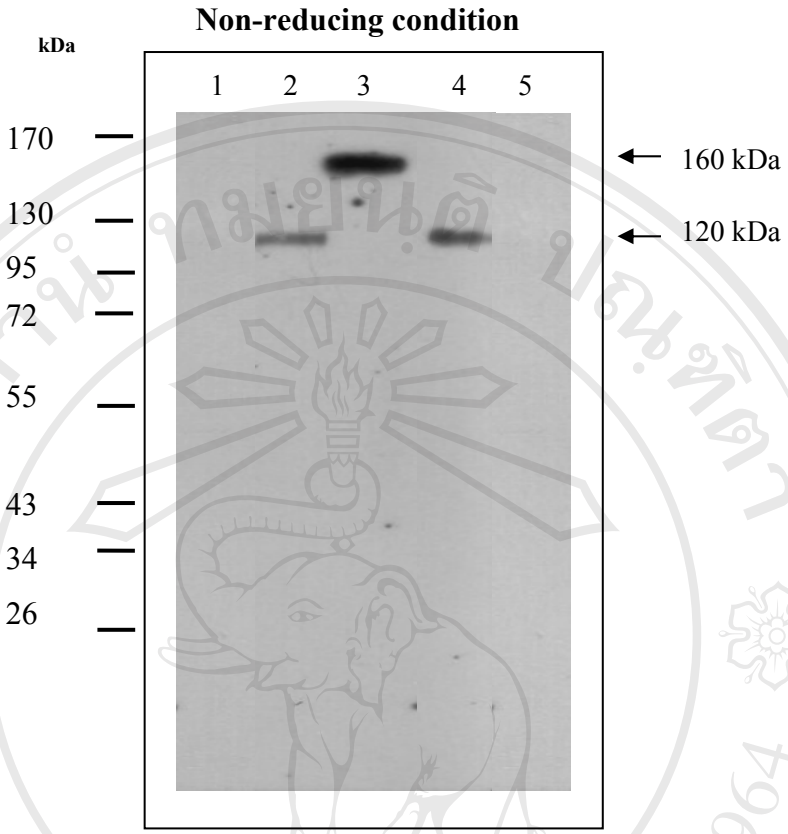


Figure 3.10 SDS-PAGE and Western immunobloting analysis of molecules recognized by mAbs clone 138.7, 176.7 and 297.7. Thrombin-activated platelets were separated under non-reducing condition by SDS-PAGE and transferred to PVDF membrane. The membranes were reacted to mAb clone 138.7 (lane 1), mAb clone 176.7 (lane 2), mAb clone 297.7 (lane 3), CD62P mAb as positive control (lane 4) and conjugated control (lane 5). The molecular weight markers are indicated on the left in kDa.

3.7 Effect of monoclonal antibodies on platelet adhesion by indirect ELISA

To investigate the effect of produced monoclonal antibodies on platelet adhesion by indirect ELISA. Thrombin-activated platelets were labeled with Sulfo-NHS-LC-biotin. Biotinylated platelets were incubated with mAbs clone 138.7, 176.7 and 297.7 at concentrations 2.5, 5, 10, 20 and 40 μg/ml, then, added into each well that coated with 100 μg/ml collagen or 2 mg/ml fibrinogen. After removing non-adherent platelets, the horseradish peroxidase-conjugated streptavidin were added into each well. After incubation, the color was developed by adding TMB substrate and the reaction was stopped by 4N HCl. The extent of adhesion at each concentration of mAbs were detected by ELISA reader and calculated the percentage of platelet adhesion comparison with the control (Table 3.1). The percentage of platelet adhesion was calculated with the following formula.

$$\text{Percentage of platelet adhesion} = \frac{(\text{sample - conjugate control})}{(\text{positive control - conjugate control})} \times 100$$

All of generated mAbs had no effect on platelet adhesion to immobilized collagen or fibrinogen (Figure.3.11A and B).

Table 3.1 The percentage of platelet adhesion to immobilized collagen and fibrinogen by indirect ELISA

Collagen

Monoclonal antibody	The percentage of platelet adhesion[a]				
	2.5 µg/ml[b]	**5 µg/ml**	**10 µg/ml**	**20 µg/ml**	**40 µg/ml**
Clone 138.7	85.88 ± 8.26 %	84.82 ± 6.50 %	88.04 ± 6.12 %	83.49 ± 7.66 %	88.98 ± 7.24 %
Clone 176.7	85.60 ± 6.80%	82.00 ± 8.28%	84.40 ± 7.33%	83.20 ± 8.87%	82.20 ± 9.18%
Clone 297.7	83.80 ± 7.19 %	83.80 ± 8.23 %	80.40 ± 10.36 %	81.00 ± 9.54 %	72.60 ± 9.37 %

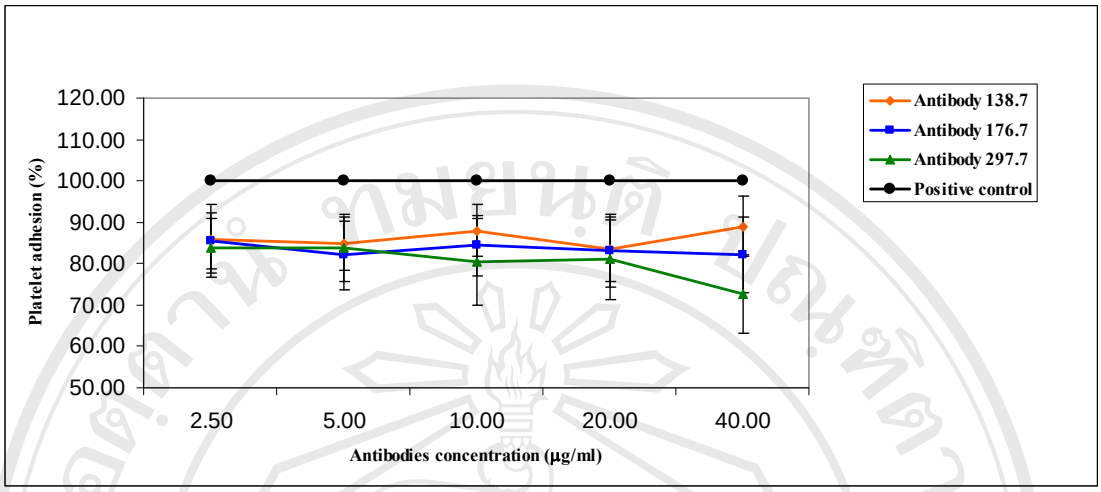
Fibrinogen

Monoclonal antibody	The percentage of platelet adhesion[a]				
	2.5 µg/ml[b]	**5 µg/ml**	**10 µg/ml**	**20 µg/ml**	**40 µg/ml**
Clone 138.7	99.8 ± 11.10 %	100.00 ± 11.60 %	98.80 ± 12.30 %	97.20 ± 11.60 %	97.20 ± 6.10 %
Clone 176.7	100.00 ± 8.60%	100.00 ± 12.90%	100.00 ± 9.90%	100.00 ± 12.10%	100.00 ± 14.70%
Clone 297.7	98.60 ± 7.40 %	97.00 ± 9.90%	97.60 ± 12.00 %	94.80 ± 11.90 %	96.60 ± 9.00 %

[a]Mean ± SD (n=5)

[b]Monoclonal antibodies concentration

(A)



(B)

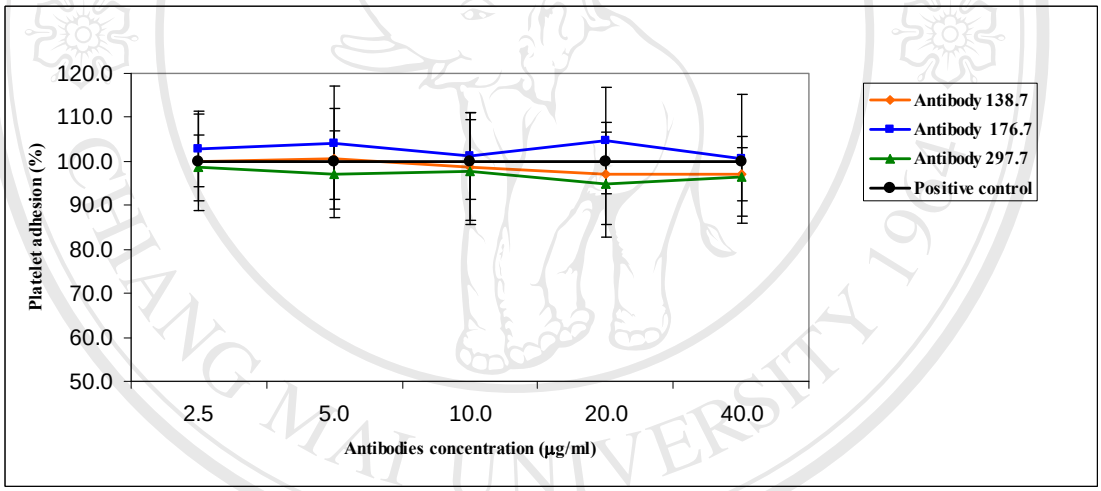


Figure 3.11 Effect of monoclonal antibodies on platelet adhesion by indirect ELISA. Biotinylated platelets were incubated with various concentrations of mAbs clone 138.7, 176.7 and 297.7 ,then, allowed to adhere to wells coated with 100 µg/ml collagen (A) or 2 mg/ml fibrinogen (B). After washing, adhered platelets were determined using horseradish peroxidase-conjugated streptavidin. The extent of adhesion was expressed as the percentage of control platelets adhered without mAbs.

3.8 Effect of monoclonal antibodies on platelet aggregation

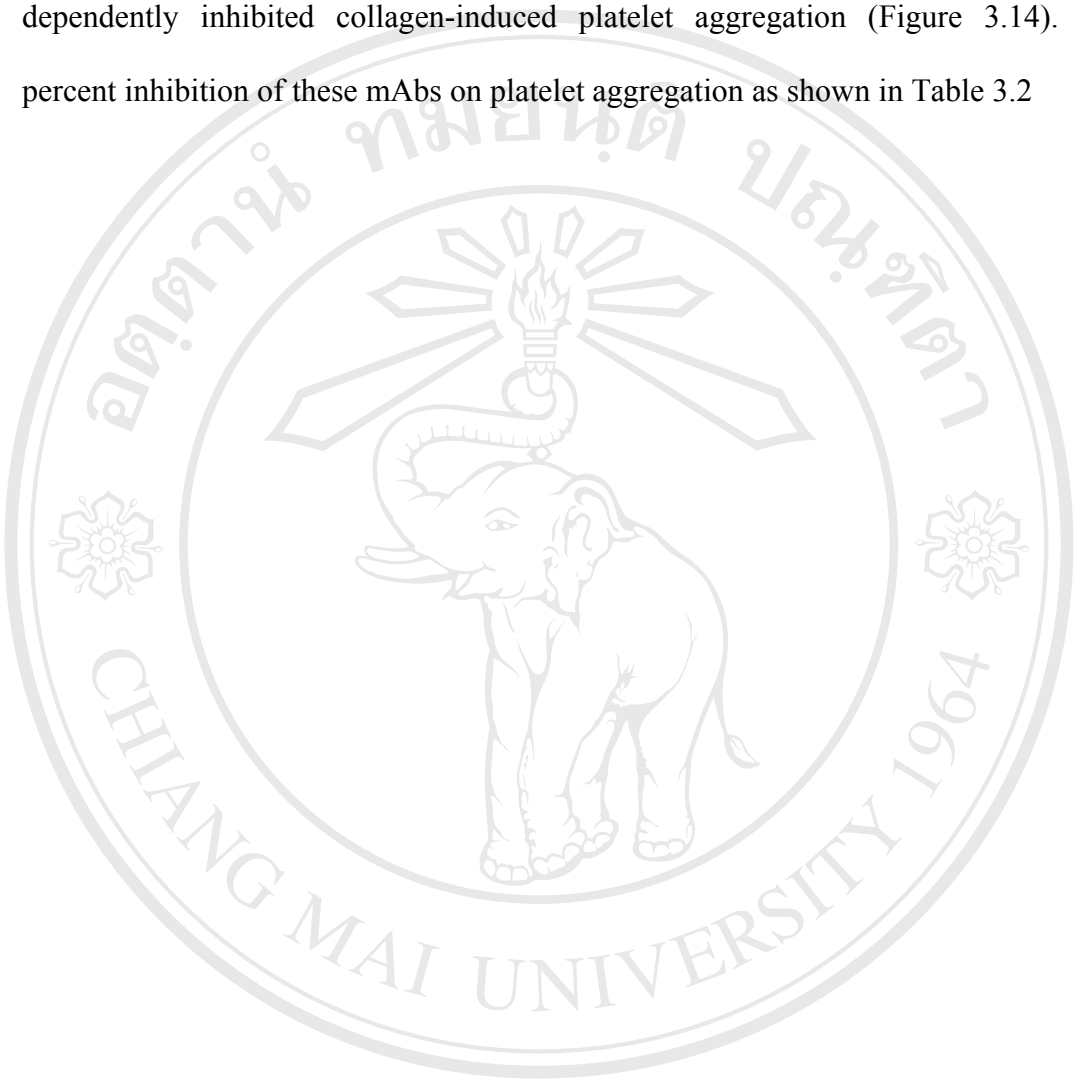
To study the effect of produced monoclonal antibodies to platelet aggregation, platelet aggregometry was performed using a Platelet aggrecorder II PA 3320. The platelet suspension was incubated with mAbs clone 138.7, 176.7 and 297.7 at concentration 5 and 20 μg/ml. The platelet suspension without mAb was used as a positive control and platelet suspension with neither mAb nor agonist was used for measuring spontaneous aggregation used.

For eight subjects, 200 μM ADP was used as an agonist, platelet rich plasma suspension was incubated with 5 and 20 μg/ml of generated mAb prior to add 200 μM ADP and the platelet aggregation was then measured using a Platelet aggrecorder II PA 3320. As shown in Figure 3.12, data was representative of one subject from ADP-induced platelet aggregation . In these experiment, pretreatment of platelets with the mAb clone 138.7, 176.7 and 297.7 for 5 minutes inhibited ADP- and collagen-induced platelet aggregation with statistically different from positive control (P<0.05) (Figure 3.14).

The percent inhibition of platelet aggregation by mAbs clone 138.7, 176.7 and 297.7 at 5 μg/ml were 27.46 %, 48.59 % and 21.27 %, respectively (Table 3.2). While, at concentration of 20 μg/ml, mAbs clone 138.7, 176.7 and 297.7 inhibited platelet aggregation at 30.28 %, 36.62 % and 19.72 %, respectively. (Table 3.2).

In addition, to examine the effect of produced mAbs on collagen-induced platelet aggregation, platelet rich plasma from three subjects were incubated with 5 and 20 μg/ml of produced mAbs before induced platelets with 20 μg/ml collagen. As shown in Figure 3.13, data was representative of one subject from collagen-induced platelet

aggregation. At 5 and 20 μg/ml of mAbs, mAb clone 138.7, 176.7 and 297.7 dose-dependently inhibited collagen-induced platelet aggregation (Figure 3.14). The percent inhibition of these mAbs on platelet aggregation as shown in Table 3.2



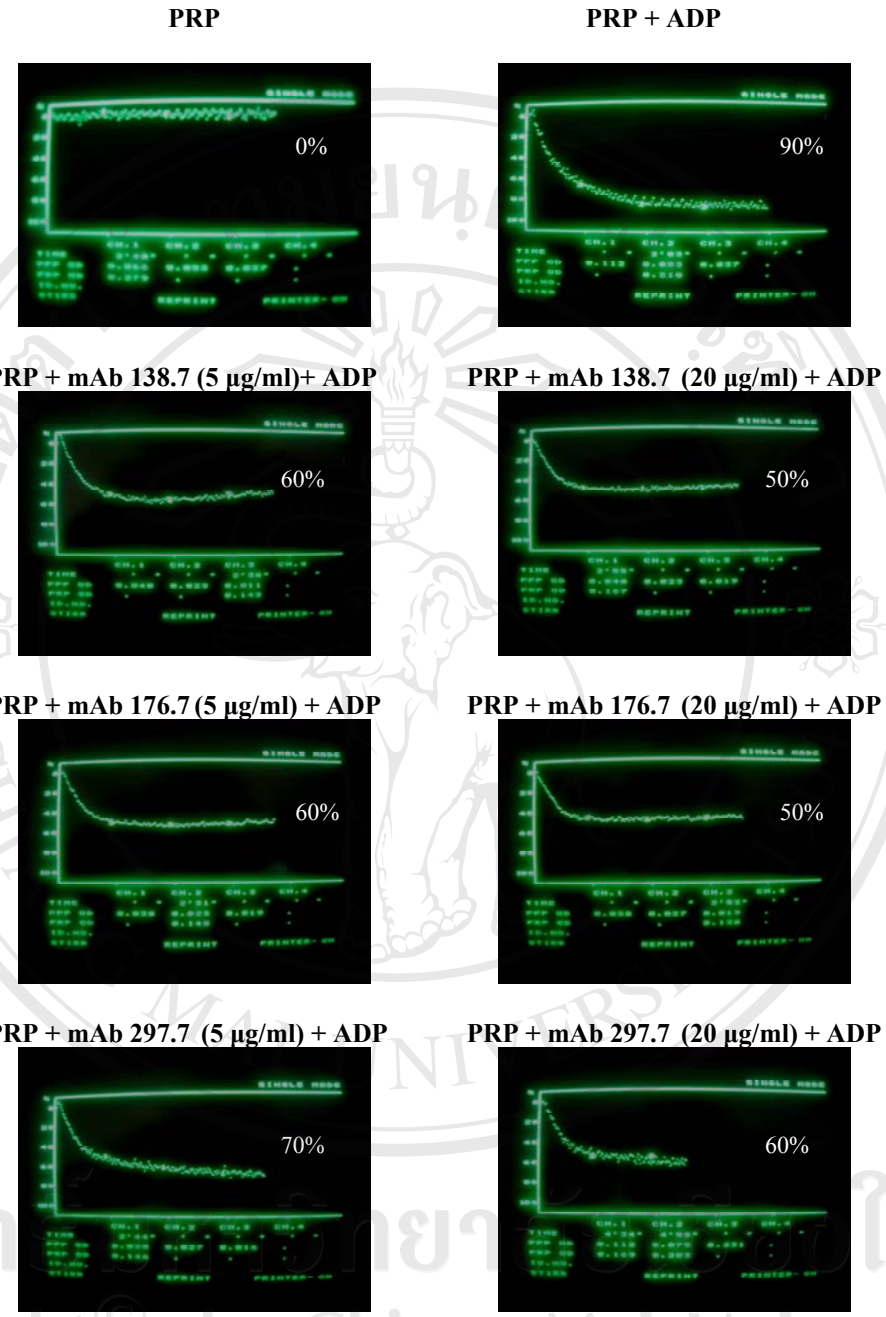


Figure 3.12 Effect of mAbs on ADP-induced platelet aggregation. PRP was incubated with 5 and 20 µg/ml of mAb clone 138.7, 176.7 and 297.7 at 37°C for 5 min. Platelet aggregation was induced by 200 µM ADP. The maximal platelet aggregation within 10 min was measured, and the aggregation at each concentration of mAb was expressed as the percentage of the maximal platelet aggregation.

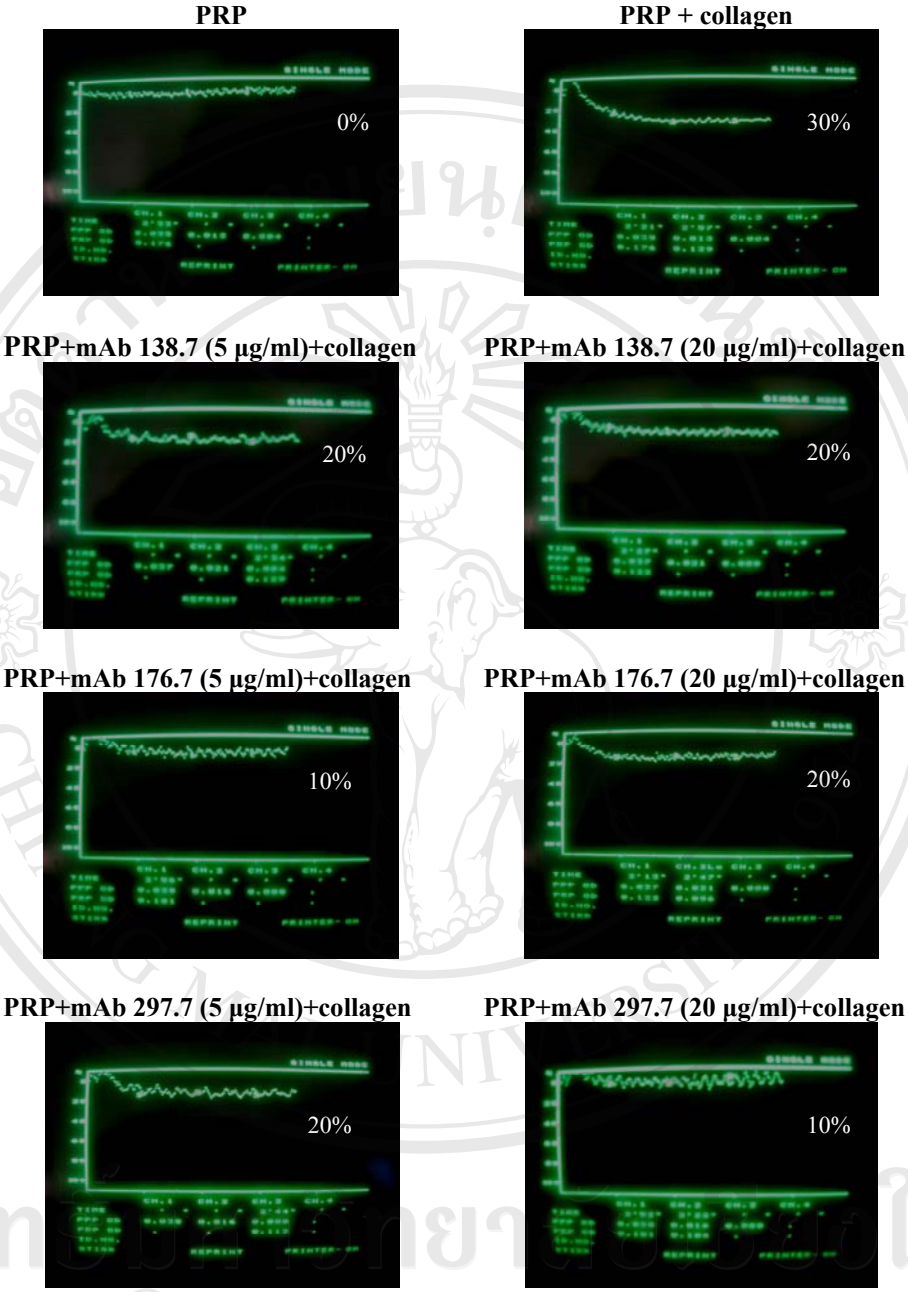
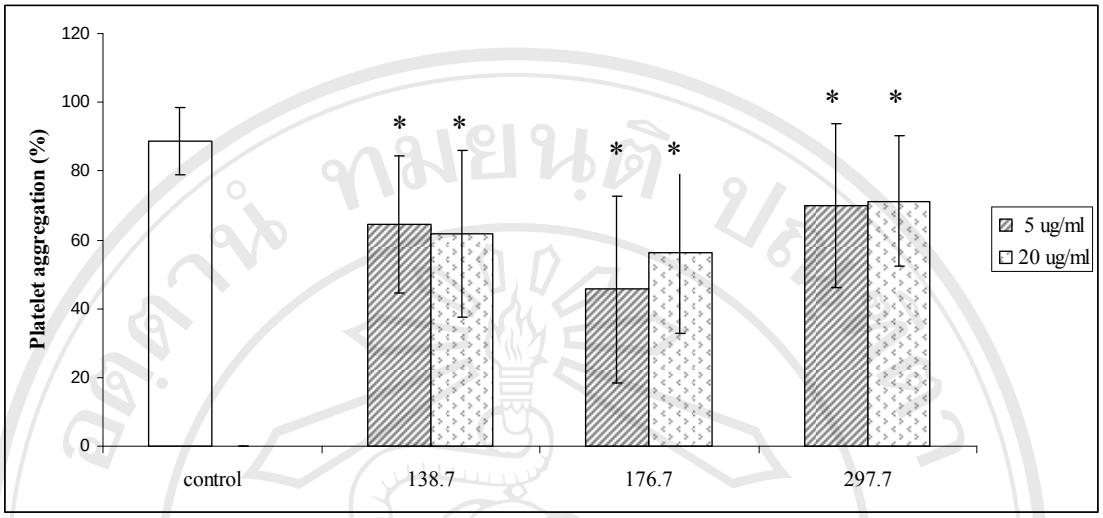


Figure 3.13 Effect of mAbs on collagen-induced platelet aggregation. PRP was incubated with 5 and 20 µg/ml of mAb clone 138.7, 176.7 and 297.7 at 37°C for 5 min. Platelet aggregation was induced by 20 µg/ml collagen. The maximal platelet aggregation within 10 min was measured, and the aggregation at each concentration of mAb was expressed as the percentage of the maximal platelet aggregation.

(A)



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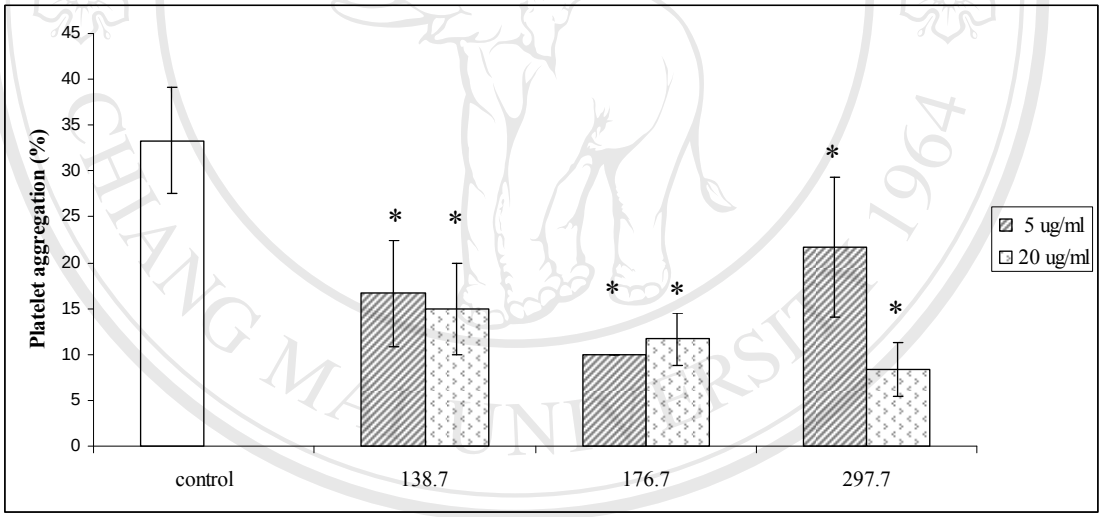

Figure 3.14 The effect of mAbs on platelet aggregation. Platelet aggregation initiated by ADP (n=8), (A) or collagen (n=3), (B) was blocked by 5 and 20 µg/ml of mAbs clone 138.7, 176.7 and 297.7 compared with platelet suspension in the absence of mAb (positive control). Data was presented as mean ± S.D. All values statistically different from positive control (* p<0.05).

Table 3.2 Effects of monoclonal antibodies on platelet aggregation induced by ADP and collagen

Sample	Aggregation (%)[a]		Inhibition[b]	
	ADP[c]	collagen[d]	ADP	collagen
control	88.75	33.33	0	0
138.7 (5 μg/ml)	64.38	16.67	27.46	49.98
176.7 (5 μg/ml)	45.63	10.00	48.59	70.00
297.7 (5 μg/ml)	70.00	21.67	21.27	34.98
138.7 (20 μg/ml)	61.88	15.00	30.28	55.00
176.7 (20 μg/ml)	56.25	11.67	36.62	64.99
297.7 (20 μg/ml)	71.25	8.33	19.72	75.00

[a]Aggregation is presented as mean±SD

[b]Inhibition (%) = [(X-Y) / (X)] x 100, where X is the % aggregation in the control and Y is the % aggregation in the sample

[c]ADP concentration = 200 μM

[d]collagen concentration = 20 μg/ml