III. RESULTS

1. General data of the subjects

There were two groups of studied blood samples. The first group consisted of 50 β-thalassemic patients collected from Department of Pediatrics (OPD 29), Maharaj Nakorn Chiang Mai Hospital. Among these, there were 38 β-thalassemia major and 12 β-thalassemia/Hb E, 22 male and 28 female patients, age ranging from 3 to 18 years old, 16 with and 34 without splenectomy. All were either, β-thalassemia major or β-thalassemia/Hb E. The second group consisted of 30 healthy blood donors, who were collected from blood blank of the same hospital or medical technology students from Faculty of Associated Medical Sciences, Chiang Mai University. Among these, there were 22 male and 8 female donors, age ranging from 15 to 28 years old.

2. Anti-platelet mixture

Anti-platelet mixture composed of aspirin 83 mM, caffeine 32 mM, theophylline 17 mM and NaN_3 15 mM, which was confirmed by platelet aggregation (platelet rich plasma) test using epinephrine as agonist on 2 healthy subjects each. The individual anti-platelet could inhibit platelet aggregation completely as shown in Figure 14. When these anti-platelets were mixed together to be an anti-platelet mixture, its ability to suppress platelet activation was confirm by platelet aggregation test using epinephrine as agonist on 10 healthy subjects. It could completely inhibit platelet aggregation as shown in Figure 15. The anti-platelet mixture was then incorporated into the anticoagulant (3.2% tri-sodium citrate) for collection of the blood samples for the detection of *in vivo* plasma β-TG and PF4 levels.

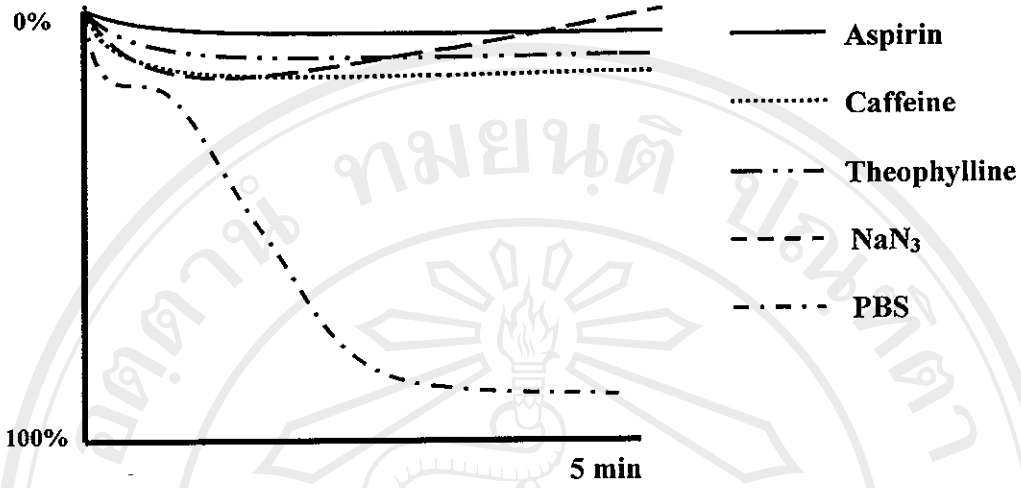


Figure 14 Effect of individual anti-platelet drugs on platelet aggregation

Only 1 healthy subject was shown as a representative figure.

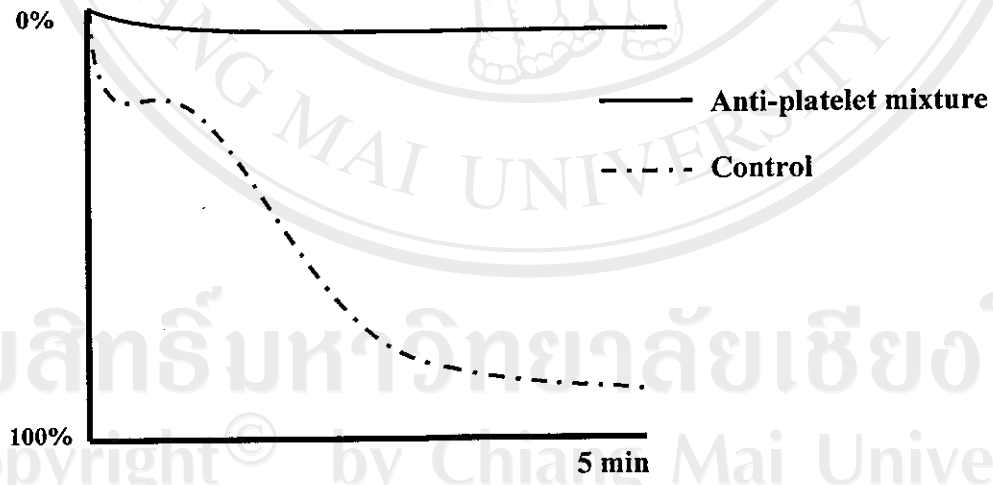


Figure 15 Effect of anti-platelet mixture on platelet aggregation test

Only 1 healthy subject was shown as a representative figure.

3. Complete blood counts (CBC) data

Complete blood counts (CBC) were performed in all samples by an automatic blood cell analyzer (Hemacel). All samples were collected into 3 mL EDTA vacuum tube.

White blood cell count (WBC) in β-thalassemia and healthy subjects were (mean ± SD) 7.39 ± 1.38 (n=50) and 6.53 ± 1.38 x 10^9/L (n=30) respectively.

Red blood cell count (RBC) in β-thalassemia and healthy subjects were (mean ± SD) 2.67 ± 0.53 (n=50) and 4.90 ± 0.42 x 10^{12}/L (n=30) respectively.

Hemoglobin (Hb) in β-thalassemia and healthy subjects were (mean ± SD) 6.39 ± 1.32 (n=50) and 13.80 ± 1.42 g/dL (n=30) respectively.

Hematocrit (Hct) in β-thalassemia and healthy subjects were (mean ± SD) 19.61 ± 3.73 (n=50) and 40.28 ± 3.31% (n=30) respectively.

Mean corpuscular volume (MCV) in β-thalassemia and healthy subjects were (mean ± SD) 73.95 ± 8.23 (n=50) and 82.45 ± 6.10 fL (n=30) respectively.

Mean corpuscular hemoglobin (MCH) in β-thalassemia and healthy subjects were (mean ± SD) 24.06 ± 2.69 (n=50) and 28.28 ± 2.46 pg (n=30) respectively.

Mean corpuscular hemoglobin concentration (MCHC) in β-thalassemia and healthy subjects were (mean ± SD) 32.52 ± 2.23 (n=50) and 34.28 ± 1.09% (n=30) respectively. These are shown in Table 4.

Platelet count in β-thalassemic and healthy subjects (mean ± SD) were 382.92 ± 179.50 (n=50) and 242.00 ± 39.03 x 10^9/L (n=30) respectively. In β-thalassemic patients with and without splenectomy were (mean ± SD) 551.75 ± 165.58 (n=16) and 303.47 ± 122.71 x 10^9/L (n=34) respectively. These are shown in Table 5.

Table 4 Complete blood counts data

Parameter	Group	Mean	SD
White blood cell count (x 10^9/L)	β-Thalassemic patients	7.39	1.38
	Healthy subjects	6.53	1.38
Red blood cell count (x 10^{12}/L)	β-Thalassemic patients	2.67	0.53
	Healthy subjects	4.90	0.42
Hemoglobin (g/dL)	β-Thalassemic patients	6.39	1.32
	Healthy subjects	13.80	1.42
Hematocrit (%)	β-Thalassemic patients	19.61	3.73
	Healthy subjects	40.28	3.31
MCV (fL)	β-Thalassemic patients	73.95	8.23
	Healthy subjects	82.45	6.10
MCH (pg)	β-Thalassemic patients	24.06	2.69
	Healthy subjects	28.28	2.46
MCHC (%)	β-Thalassemic patients	32.52	2.23
	Healthy subjects	34.28	1.09
Platelet count (x 10^9/L)	β-Thalassemic patients	382.92	179.50
	Healthy subjects	242.00	39.03

β-Thalassemic patients (n=50)

Healthy subjects (n=30)

Table 5 Platelet count in the patients with and without splenectomy

Parameter	Type	Number	Mean	SD
Platelet count (x 10^9/L)	With splenectomy	16	551.75	165.58
	Without splenectomy	34	303.47	122.71

3.1 Comparison of blood cell parameters between β-thalassemic patients and healthy subjects

The studied blood parameters were red blood cell count, hemoglobin, hematocrit and platelet count. The RBC of β-thalassemic patients and healthy subjects were (mean ± SD) 2.67 ± 0.53 and 4.90 ± 0.42 x 10^{12}/L respectively. The levels in the patients were significantly lower (p<0.05) than in the healthy subjects. The hemoglobin of β-thalassemic patients were significantly lower (p<0.05) than the healthy subjects (mean ± SD was 6.39 ± 1.32 and 13.80 ± 1.42 g/dL respectively). The hematocrit of β-thalassemic patients and healthy subjects were (mean ± SD) 19.61 ± 3.73 and 40.28 ± 3.31% respectively. The level in the patients were significantly lower (p<0.05) than those in healthy subjects. These are shown in Figure 16. The MCV, MCH and MCHC of β-thalassemic patients (mean ± SD; 73.95 ± 8.23 fL, 24.06 ± 2.69 pg and 32.52 ± 2.23% respectively) were significantly lower (p<0.05) than those in healthy subjects (mean ± SD; 82.45 ± 0.10 fL, 28.28 ± 2.46 pg, 34.28 ± 1.09% respectively).These are shown in Figure 17.

The platelet count in β-thalassemic patients were significantly higher (p<0.05) than those in healthy subjects (mean ± SD; 382.92 ± 179.50 and 242.00 ± 39.03 x 10^9/L respectively). The platelet count in patients with splenectomy were significantly higher (p<0.05) than those in patients without splenectomy (mean ± SD; 551.75 ± 165.58 and 303.47 ± 122.71 x 10^9/L respectively). These are shown in Figure 18.

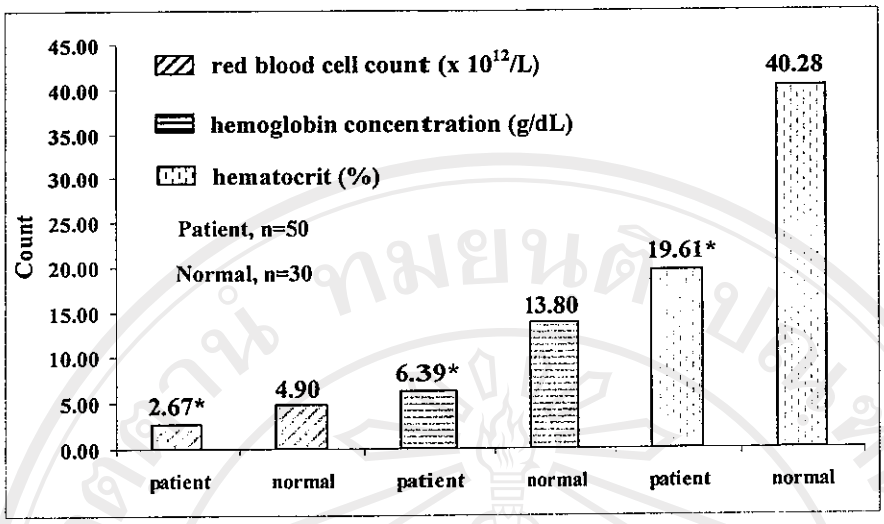


Figure 16 Comparison red blood cell count, hemoglobin and hematocrit, (*p<0.05)

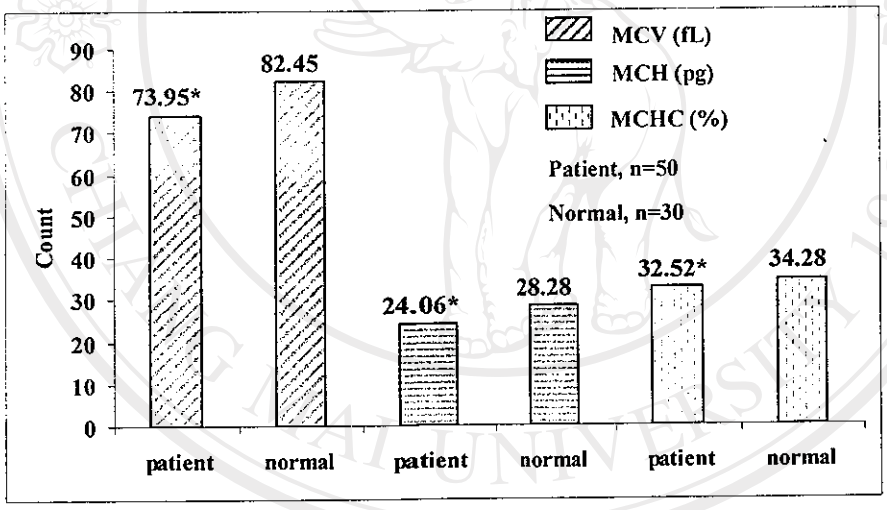


Figure 17 Comparison MCV, MCH and MCHC, (*p<0.05)

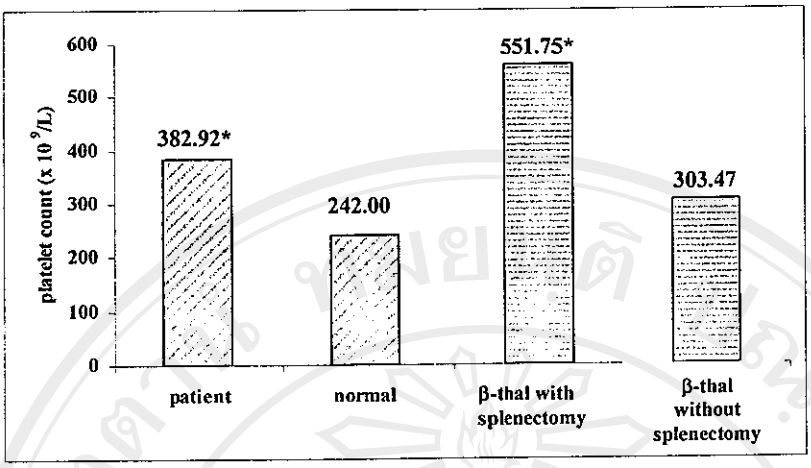


Figure 18 Comparison of platelet count

[Between β-thalassemic patients (patient, n=50) and healthy subjects (normal, n=30) and between β-thalassemic patients with splenectomy (n=16) and without splenectomy (n=34), (*p<0.05)]

4. Platelet aggregation test

Since there is no normal range of platelet aggregation test in Thais using platelet rich plasma for *in vitro* aggregation, therefore 30 healthy subjects were tested to establish the normal range.

The stimulators or agonists used were epinephrine, adenosine 5' diphosphate (ADP), collagen and ristocetin.

The normal range using epinephrine was 16-35%, ADP was 57-73%, collagen was 8-22% and ristocetin was 24-48% aggregation, respectively (95% confidence interval of the difference).

In the patients (n=50), using epinephrine as an agonist, there were 19 (50%) with hypo-aggregability (lower than 16% aggregation) in β-thalassemia major and 9 (75%) in β-thalassemia/Hb E. Normo-aggregability (in between 16-35% aggregation) were 9 (26.3%) in β-thalassemia major and 1 (8.3%) in β-thalassemia/Hb E. Hyper-aggregability

(higher than 35% aggregation) were 10 (16.7%) in β-thalassemia major and 2 (16.7%) in β-thalassemia/Hb E.

Using ADP as an agonist, there were 18 (47.4%) with hypo-aggregability (lower than 57% aggregation) in β-thalassemia major and 10 (83.3%) in β-thalassemia/Hb E. Normo-aggregability (in between 57-73% aggregation) were 14 (36.8%) in β-thalassemia major and 1 (8.3%) in β-thalassemia/Hb E. and Hyper-aggregability (higher than 73% aggregation) were 6 (15.8%) in β-thalassemia major and 1 (8.3%) in β-thalassemia/Hb E.

Using collagen as an agonist, there were 16 (42.1%) with hypo-aggregability (lower than 8% aggregation) in β-thalassemia major and 7 (58.3%) in β-thalassemia/Hb E. Normo-aggregability (in between 8-22% aggregation) were 11 (29%) in β-thalassemia major and 3 (25%) in β-thalassemia/Hb E. Hyper-aggregability (higher than 22% aggregation) were 11 (28.9%) in β-thalassemia major and 2 (16.7%) in β-thalassemia/Hb E.

Using ristocetin as an agonist, there were 14 (36.8%) with hypo-aggregability (lower than 24% aggregation) in β-thalassemia major and 8 (66.7%) in β-thalassemia/Hb E. Normo-aggregability (in between 24-48% aggregation) were 9 (23.7%) in β-thalassemia major and 3 (25%) in β-thalassemia/Hb E. Hyper-aggregability (higher than 48% aggregation) were 15 (39.5%) in β-thalassemia major and 1 (8.3%) in β-thalassemia/Hb E. When the aggregability data were averaged from all agonists used, the ratio of hypo-, normo- and hyper-aggregabilities were 4:3:3 in β-thalassemia major and 7:1.7:1.3 in β-thalassemia/Hb E. These are shown in Table 6.

Table 6 Platelet aggregability in β-thalassemia major and β-thalassemia/Hb E

Agonist	Patients	Percent of patients with (%)		
		Hypo-aggregability	Normo-aggregability	Hyper-aggregability
Epinephrine	β-Thal major	50	23.7	26.3
	β-Thal/Hb E	75	8.3	16.7
ADP	β-Thal major	47.4	36.8	15.8
	β-Thal/Hb E	83.3	8.3	8.4
Collagen	β-Thal major	42.1	29	28.9
	β-Thal/Hb E	58.3	25	16.7
Ristocetin	β-Thal major	36.8	23.7	39.5
	β-Thal/Hb E	66.7	25	8.3
All agonists	β-Thal major	44.1 (4)	28.3 (3)	27.6 (3)
	β-Thal/Hb E	70.8 (7)	16.7 (1.7)	12.5 (1.3)

β-Thalassemia major (β-Thal major), n = 38

β-Thalassemia/Hb E (β-Thal/Hb E), n = 12

The digits in parentheses were ratios of aggregability

5. Red cell membrane phosphatidylserine (PS) exposure

In order to enumerate red cells with PS exposure on their outer membrane in β-thalassemic patients and healthy subjects, staining with Annexin V conjugated FITC (AV-FITC) and monoclonal antibody to GPA conjugated RPE (MoAb-GPA-RPE) were employed before flow cytometric analysis. All samples were collected into 3.2% tri-sodium citrate.

Percentages of PS exposing RBCs (mean ± SD) in β-thalassemia and healthy subjects were 5.29 ± 1.84 (n=50) and 1.99 ± 0.44% (n=30) respectively. They were shown in Table 7.

Table 7 Percentages of PS exposing RBCs

Parameter	Group	Number	Mean	SD
PS exposing RBCs (%)	β-Thalassemic patients	50	5.29	1.84
	Healthy subjects	30	1.99	0.44

Percentages of PS exposing RBCs (mean ± SD) in β-thalassemic patients with and without splenectomy were 5.84 ± 1.56 (n=16) and 5.06 ± 1.92% (n=34) respectively. They are shown in Table 8.

Table 8 Percentages of PS exposing RBCs in β-thalassemia with and without splenectomy

Parameter	Group	Number	Mean	SD
PS exposing RBCs (%)	With splenectomy	16	5.84	1.56
	Without splenectomy	34	5.06	1.92

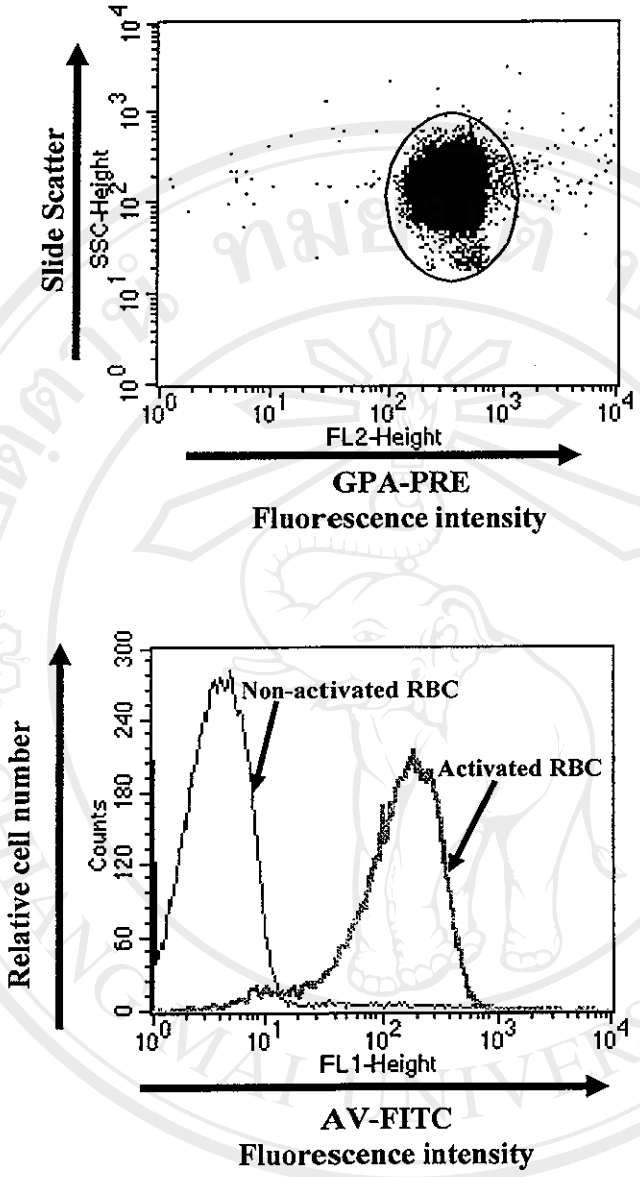


Figure 19 Flow cytometric histogram of non activated and activated RBC

Top; All GPA (FL2: RPE) positive cells were gated.

Bottom; Annexin V (FL1: FITC) bound (activated) red cells were shown overlaying on negative (non-activated) red cells.

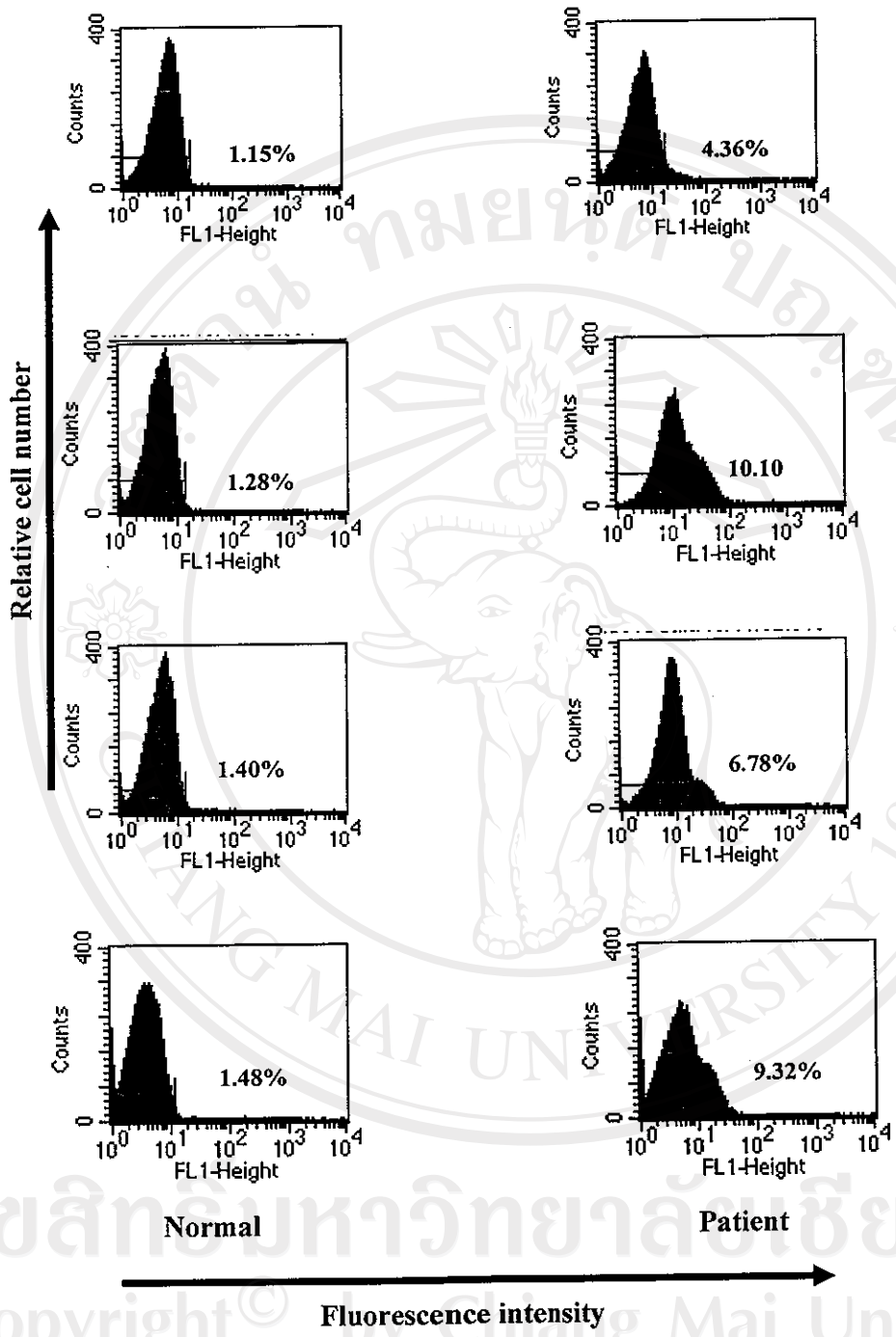


Figure 20 Flow cytometric histograms of %PS exposing RBCs

(Comparison of 4 pairs of normal individuals and patients)

5.1 Comparison of %PS exposing RBCs between β-thalassemic patients and healthy subjects

Percentages of PS exposing RBCs in β-thalassemic patients (mean ± SD; 5.29 ± 1.84%, n=50) were significantly higher (p<0.05) than the levels in healthy subjects (1.99 ± 0.44%, n=30).

Percentages of PS exposing RBCs in β-thalassemia with splenectomy (5.84 ± 1.56%, n=16) were significantly higher (p<0.05) than those in patients without splenectomy (5.06 ± 1.92%; n=34), as shown Figure 21.

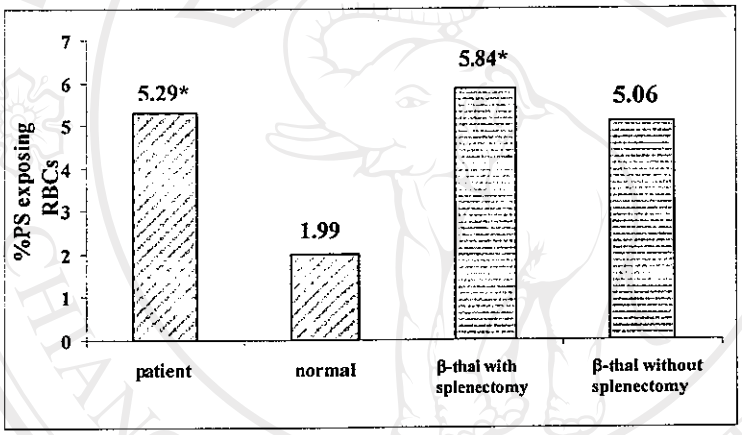


Figure 21 Comparison of %PS exposing RBCs

[Between β-thalassemic patients (patient, n=50) and healthy subjects (normal, n=30), β-thalassemia with splenectomy (n=16) and β-thalassemia without splenectomy (n=34), *(p<0.01)]

6. Platelet morphology by scanning electron microscopy (SEM)

Shape changed platelets are platelets with one or more pseudopods under SEM. Aggregated platelets are two or more platelets contacting each other under SEM. Activated platelets are combination of shape changed and aggregated platelets under SEM. Percentages of shape changed platelets in β-thalassemic patients and healthy subjects were 28.03 ± 7.09 and 14.76 ± 1.69% respectively. Percentages of aggregated platelets in β-thalassemic patients and healthy subjects were 8.62 ± 3.82 and 2.26 ± 0.56% respectively. Percentages of activated platelets (shape changed + aggregated) in β-thalassemic patients and healthy subjects were 37.15 ± 6.96 and 17 ± 2.21% respectively. Percentages of normal shape platelets in β-thalassemic patients and healthy subjects were (mean ± SD) 63.85 ± 8.92 and 82.98 ± 2.20% respectively. These are shown in Table 9.

Table 9 Platelet morphology by SEM

Platelet	Group	Mean	SD
Shape changes (%)	β-Thalassemic patients	28.03	7.09
	Healthy subjects	14.76	1.69
	β-Thal with splenectomy	28.94	4.88
	β-Thal without splenectomy	27.12	9.33
Aggregates (%)	β-Thalassemic patients	8.62	3.82
	Healthy subjects	2.26	0.56
	β-Thal with splenectomy	9.66	2.31
	β-Thal without splenectomy	7.58	4.97
Activation (%)	β-Thalassemic patients	36.65	6.96
	Healthy subjects	17.02	2.21
	β-Thal with splenectomy	38.60	5.67
	β-Thal without splenectomy	34.70	8.46
Normal shapes (%)	β-Thalassemic patients	63.85	8.92
	Healthy subjects	82.98	2.20
	β-Thal with splenectomy	61.40	5.67
	β-Thal without splenectomy	65.30	11.48

β-Thalassemic patients (n=10)

Healthy subjects (n=5)

β-Thalassemia (β-Thal) with splenectomy (n=5)

β-Thalassemia (β-Thal) without splenectomy (n=5)

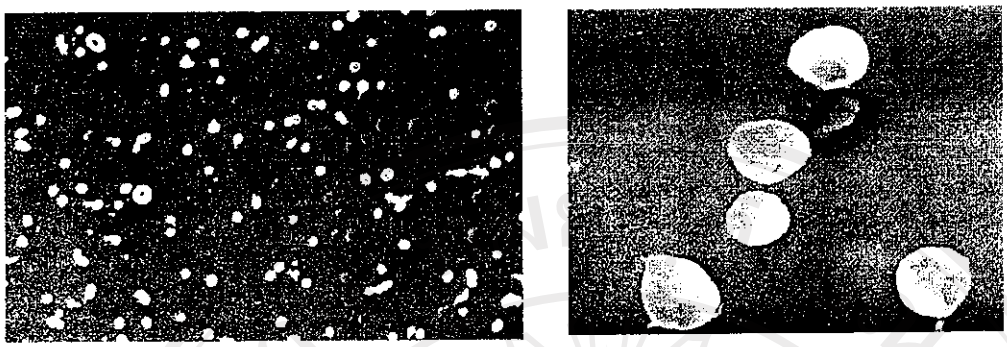


Figure 22 Scanning electron microscopic micrograph of healthy platelets

In this electron micrograph 13.5% shape changed platelets, 1.5% aggregated platelets and 85.0% normal shape platelets were demonstrated (left; x 550 and right; x 4,500).

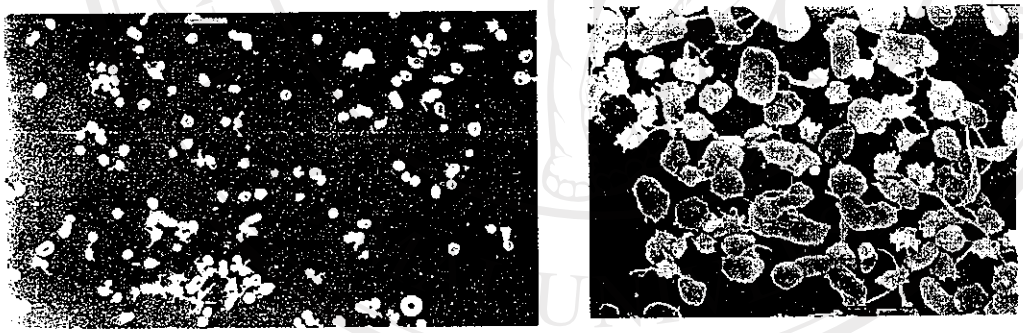


Figure 23 Scanning electron microscopic micrograph of β-thalassemic platelets

In this electron micrograph 23.4% shape changed platelets, 12.2% aggregated platelets and 64.4% normal shape platelets were demonstrated (left; x 550 and right; x 3,500).

6.1 Comparison of platelet morphology between β-thalassemic patients and healthy subjects using SEM

Normal shape platelets of healthy subjects (mean ± SD; 82.98 ± 2.20%) were significantly higher (p<0.05) than β-thalassemic patients (63.35 ± 8.92%). However in β-thalassemia with and without splenectomy, there were no statistically difference (61.40. ± 5.67 and 65.30 ± 11.48% respectively).

Shape changed platelets in β-thalassemic patients (mean ± SD; 28.03 ± 7.09%) were significantly higher (p<0.05) than those in healthy subjects (14.76 ± 1.69%). In contrast, between β-thalassemia with and without splenectomy, there were no statistically difference (28.94. ± 4.88 and 27.12 ± 9.33% respectively).

Aggregated platelets in β-thalassemic patients (mean ± SD; 8.62 ± 3.82%) were significantly higher (p<0.05) than healthy subjects (2.26 ± 0.56%). However in β-thalassemia with and without splenectomy, there were no statistically difference (9.66. ± 2.31 and 7.58 ± 4.97% respectively).

Total activated platelets in β-thalassemic patients (mean ± SD; 36.65 ± 6.69%) were significantly higher (p<0.05) than in healthy subjects (17.02 ± 2.21%). In contrast, between β-thalassemia with and without splenectomy, there were no statistically difference (38.60 ± 5.67 and 34.70 ± 8.46% respectively). These are shown in Figure 24-25.

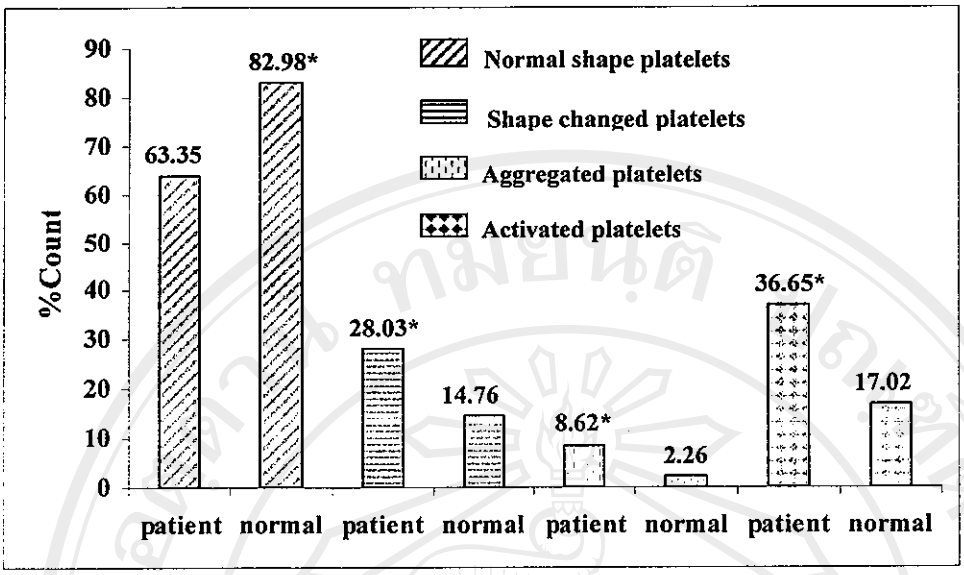


Figure 24 Comparison of platelet morphology by SEM

β-thalassemic patients (patient, n=10) and healthy subjects (normal, n=5), (*p<0.05)

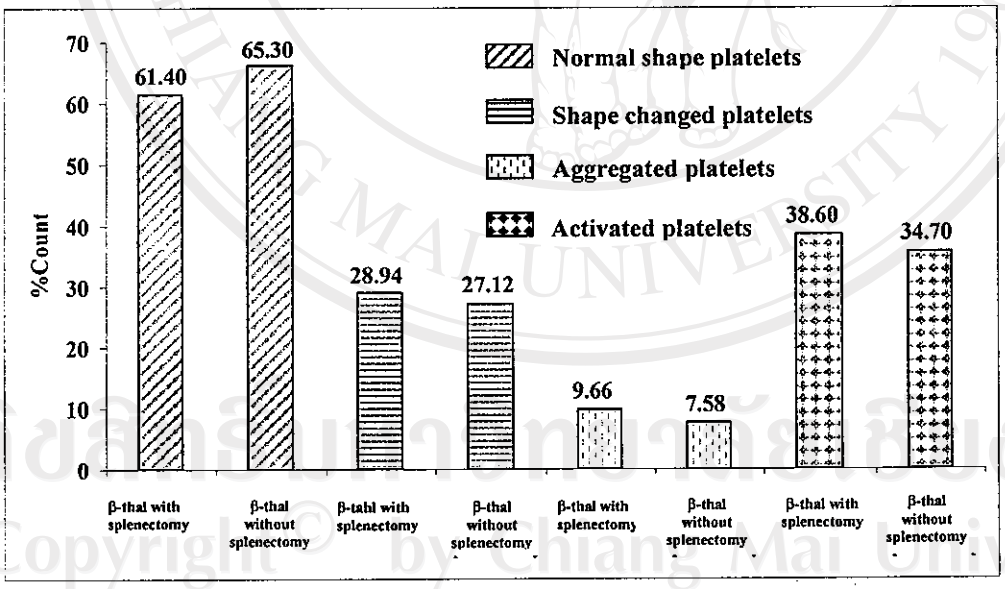


Figure 25 Comparison of platelet morphology by SEM in the patients

β-thalassemia with splenectomy (n=5) and β-thalassemia without splenectomy (n=5)

7. Plasma levels of β-thromboglobin (β-TG) and platelet factor 4 (PF4)

In order to measure β-TG and PF4 levels in the plasma using ELISA kit. All samples were collected into 3.2% tri-sodium citrate with anti-platelet mixture. Plasma β-TG calibration curve and plasma PF4 calibration curve were shown in Figure 26-27.

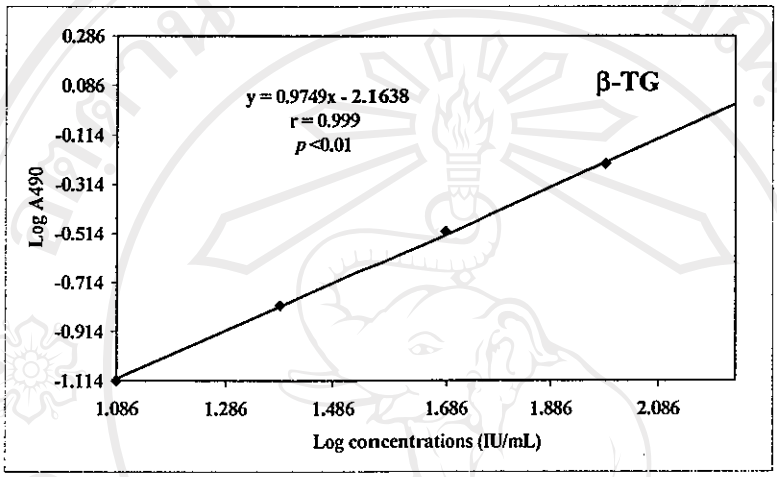


Figure 26 Calibration curve of plasma β-TG

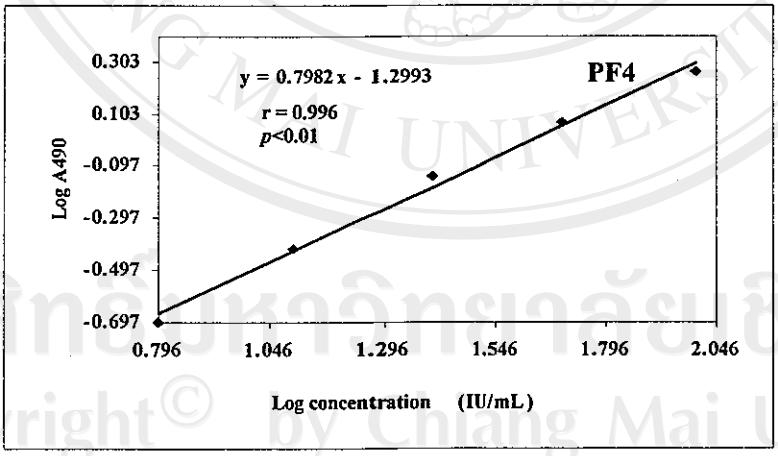


Figure 27 Calibration curve of plasma PF4

The correlation coefficients of plasma β-TG and PF 4 calibration curves were 0.999 and 0.996, respectively, which were very good correlation and high significance (p<0.01). Furthermore, the results of control samples were within the acceptable range instructed by the manufacturer.

Plasma β-TG concentration in β-thalassemic patients (n=50) and healthy subjects (n=30) were (mean ± SD) 263.10 ± 41.21 and 191.92 ± 52.99 IU/mL respectively. Plasma β-TG per 10^6 platelets (plt) in β-thalassemic patients and healthy subjects were (mean ± SD) 0.83 ± 0.41 and 0.82 ± 0.29 IU/10^6 plt respectively.

Plasma PF4 concentration in β-thalassemic patients and healthy subjects were (mean ± SD) 88.07 ± 13.73 and 60.53 ± 17.77 IU/mL respectively. Plasma PF4 per 10^6 plt in β-thalassemic patients and healthy subjects were (mean ± SD) 0.28 ± 0.14 and 0.26 ± 0.10 IU/106 plt respectively. These are shown in Table 10.

Table 10 Plasma β -TG and PF4 concentration in IU/mL and IU/10^6 plt

Parameter	Group	Mean	SD
β-TG (IU/mL)	β-Thalassemic patients	263.10	41.21
	Healthy subjects	191.92	52.99
β-TG (IU/10^6 plt)	β-Thalassemic patients	0.83	0.41
	Healthy subjects	0.82	0.29
PF4 (IU/mL)	β-Thalassemic patients	88.07	13.73
	Healthy subjects	60.53	17.77
PF4 (IU/10^6 plt)	β-Thalassemic patients	0.28	0.14
	Healthy subjects	0.26	0.10

β-Thalassemic patients (n=50)

Healthy subjects (n=30)

Plasma concentration of β-TG in β-thalassemic patients with and without splenectomy were (mean ± SD) 279.49± 39.57 (n=16) and 255.38 ± 40.23 IU/mL (n=34) respectively. Plasma concentration of β-TG per 10^6 plt in β-thalassemic patients with and without splenectomy were (mean ± SD) 0.56 ± 0.20 and 0.96 ± 0.42 IU/10^6 plt respectively.

Plasma concentration of PF4 in β-thalassemic patients with and without splenectomy were (mean ± SD) 93.09 ± 10.65 and 85.70 ± 14.50 IU/mL respectively. Plasma concentration of PF4 per 10^6 plt in β-thalassemic patients with and without splenectomy were (mean ± SD) 0.18 ± 0.06 and 0.32 ± 0.14 IU/10^6 plt respectively. These are shown in Table 11.

Table 11 Plasma β -TG and PF4 concentration in IU/mL and IU/10^6 plt of β-thalassemia with and without splenectomy

Parameter	Type	Mean	SD
β-TG (IU/mL)	With splenectomy	279.49	39.57
	Without splenectomy	255.38	40.23
β-TG (IU/10^6 plt)	With splenectomy	0.56	0.20
	Without splenectomy	0.95	0.42
PF4 (IU/mL)	With splenectomy	93.09	10.65
	Without splenectomy	85.70	14.50
PF4 (IU/10^6 plt)	With splenectomy	0.18	0.06
	Without splenectomy	0.32	0.14

With splenectomy (n=16)

Without splenectomy (n=34)

Table 12 Plasma levels of β-TG and PF4

No.	Group	Gender	Diagnosis	Molecular	Type	β -TG	β -TG/plt*	PF4	PF4/Plt*
1	N	M	Normal	NA	N	264.13	0.88	107.69	0.36
2	N	M	Normal	NA	N	256.89	1.64	90.53	0.58
3	N	M	Normal	NA	N	196.26	0.80	76.64	0.31
4	N	M	Normal	NA	N	150.21	0.78	49.46	0.26
5	N	M	Normal	NA	N	117.68	0.48	45.81	0.19
6	N	M	Normal	NA	N	174.73	0.62	36.98	0.13
7	N	M	Normal	NA	N	118.69	0.49	43.55	0.18
8	N	M	Normal	NA	N	98.13	0.37	35.44	0.13
9	N	M	Normal	NA	N	188.40	0.82	55.95	0.24
10	N	M	Normal	NA	N	152.08	0.55	45.00	0.16
11	N	M	Normal	NA	N	173.20	0.65	49.35	0.19
12	N	M	Normal	NA	N	168.25	0.88	61.06	0.31
13	N	M	Normal	NA	N	283.98	1.03	70.11	0.25
14	N	M	Normal	NA	N	166.04	0.89	46.08	0.25
15	N	M	Normal	NA	N	201.91	0.95	62.44	0.29
16	N	M	Normal	NA	N	206.03	0.72	53.05	0.18
17	N	M	Normal	NA	N	279.14	1.19	69.64	0.30
18	N	M	Normal	NA	N	185.32	0.79	75.15	0.32
19	N	M	Normal	NA	N	233.00	1.08	76.10	0.37
20	N	M	Normal	NA	N	180.54	0.86	51.44	0.25
21	N	M	Normal	NA	N	244.49	1.08	93.40	0.41
22	N	M	Normal	NA	N	269.23	1.45	62.61	0.34
23	N	F	Normal	NA	N	285.36	1.11	83.35	0.33
24	N	F	Normal	NA	N	143.76	0.52	43.82	0.16
25	N	F	Normal	NA	N	142.39	0.47	45.92	0.15
26	N	F	Normal	NA	N	204.48	0.70	66.43	0.23
27	N	F	Normal	NA	N	135.61	0.52	56.63	0.22
28	N	F	Normal	NA	N	204.14	0.79	64.57	0.25

Table 12 Plasma levels of β-TG and PF4 (Cont.)

No.	Group	Gender	Diagnosis	Molecular	Type	β -TG	β -TG/Plt*	PF4	PF4/Plt*
29	N	F	Normal	NA	N	197.12	0.76	57.76	0.22
30	N	F	Normal	NA	N	136.29	0.72	40.00	0.21
31	P	M	β-Thal major	NA	Nsp	275.34	0.51	99.92	0.19
32	P	M	β-Thal major	NA	Nsp	309.57	1.09	98.41	0.34
33	P	M	β-Thal major	cd 17 (A-T), cd 41/42	Nsp	224.38	1.01	82.07	0.37
34	P	M	β-Thal major	NA	Nsp	254.65	1.00	97.96	0.38
35	P	M	β-Thal major	cd 17 (A-T), cd 41/42	Nsp	203.45	0.97	79.41	0.38
36	P	M	β-Thal major	NA	Nsp	280.18	0.52	90.22	0.17
37	P	M	β-Thal major	cd 41/42, cd 41/42	Nsp	254.82	0.83	80.74	0.26
38	P	M	β-Thal major	cd 17 (A-T), cd 41/42	Nsp	287.78	0.59	94.33	0.19
39	P	M	β-Thal major	NA	Nsp	291.06	0.90	111.94	0.34
40	P	M	β-Thal major	NA	Sp	302.65	0.73	105.37	0.25
41	P	M	β-Thal major	NA	Sp	288.99	0.70	97.09	0.23
42	P	M	β-Thal major	NA	Sp	296.42	0.94	96.90	0.31
43	P	M	β-Thal major	NA	Sp	317.37	0.63	98.03	0.19
44	P	M	β-Thal major	NA	Sp	296.60	0.40	90.23	0.12
45	P	M	β-Thal major	NA	Sp	283.80	0.91	84.14	0.27
46	P	M	β-Thal major	cd 17 (A-T), cd 41/42	Sp	282.25	0.37	89.73	0.12
47	P	M	β-Thal/Hb E	NA	Nsp	309.57	0.89	103.22	0.30
48	P	M	β-Thal/Hb E	NA	Nsp	286.57	0.99	90.47	0.31
49	P	M	β-Thal/Hb E	NA	Nsp	233.66	1.03	51.77	0.23
50	P	M	β-Thal/Hb E	NA	Nsp	239.85	1.20	64.86	0.32
51	P	M	β-Thal/Hb E	NA	Sp	320.31	0.62	98.79	0.19
52	P	M	β-Thal/Hb E	NA	Sp	299.54	0.55	96.27	0.18
53	P	F	β-Thal major	NA	Nsp	263.27	1.61	96.08	0.56
54	P	F	β-Thal major	cd 17 (A-T), cd 41/42	Nsp	237.09	0.98	90.17	0.37
55	P	F	β-Thal major	IVSI nt1 (G-T), IVSI nt1 (G-T)	Nsp	285.53	1.33	99.54	0.46
56	P	F	β-Thal major	NA	Nsp	196.95	0.41	67.30	0.14

Table 12 Plasma levels of β-TG and PF4 (Cont.)

No.	Group	Gender	Diagnosis	Molecular	Type	β -TG	β -TG/Plt*	PF4	PF4/Plt*
57	P	F	β-Thal major	cd 17 (A-T), cd 41/42	Nsp	242.25	0.92	80.44	0.30
58	P	F	β-Thal major	NA	Nsp	275.86	0.79	86.10	0.25
59	P	F	β-Thal major	NA	Nsp	175.42	0.77	68.59	0.30
60	P	F	β-Thal major	NA	Nsp	143.08	0.61	50.67	0.22
61	P	F	β-Thal major	NA	Nsp	281.04	1.19	82.25	0.35
62	P	F	β-Thal major	cd 41/42, cd 41/42	Nsp	239.99	0.63	87.02	0.23
63	P	F	β-Thal major	NA	Nsp	276.89	1.02	92.21	0.34
64	P	F	β-Thal major	cd 41/42, cd 41/42	Nsp	206.37	0.81	65.21	0.26
65	P	F	β-Thal major	NA	Nsp	295.21	0.61	90.10	0.19
66	P	F	β-Thal major	NA	Nsp	310.27	1.06	85.73	0.29
67	P	F	β-Thal major	cd 41/42, cd 41/42	Nsp	235.55	0.84	87.57	0.32
68	P	F	β-Thal major	cd 17 (A-T), cd 17 (A-T)	Nsp	253.96	1.11	98.22	0.43
69	P	F	β-Thal major	cd 41/42, cd 41/42	Sp	296.42	0.73	97.59	0.24
70	P	F	β-Thal major	cd 17 (A-T), cd 41/42	Sp	191.82	0.48	70.94	0.18
71	P	F	β-Thal major	cd 71 (+A), cd 71 (+A)	Sp	302.65	0.44	99.23	0.14
72	P	F	β-Thal major	cd 17 (A-T), cd 41/42	Sp	281.56	0.39	89.49	0.12
73	P	F	β-Thal major	nt-28 (A-G), cd 17 (A-T)	Sp	266.16	0.38	108.34	0.15
74	P	F	β-Thal major	cd 41/42, cd41/42	Sp	180.20	0.32	69.88	0.12
75	P	F	β-Thal/Hb E	NA	Nsp	309.23	0.84	96.40	0.26
76	P	F	β-Thal/Hb E	NA	Nsp	206.54	1.10	64.22	0.34
77	P	F	β-Thal/Hb E	NA	Nsp	262.92	0.41	92.77	0.14
78	P	F	β-Thal/Hb E	NA	Nsp	258.61	1.36	97.71	0.51
79	P	F	β-Thal/Hb E	NA	Nsp	276.03	2.76	90.23	0.90
80	P	F	β-Thal/Hb E	NA	Sp	265.16	0.33	97.47	0.12

N = Normal healthy subjects, P = Thalassemic patients, M = Male, F = Female

NA = Not applicable, NSp = Without splenectomy, Sp = With splenectomy

β-TG/plt* = β-TG in IU/10^6 plt

PF4/plt* = PF4 in IU/10^6 plt

7.1 Comparison of plasma β-TG and PF4 concentration between β-thalassemic patients and healthy subjects

The β-TG concentration in plasma of β-thalassemic patients were significantly higher (p<0.05) than healthy subjects (mean ± SD; 263.10 ± 41.21 and 191.92 ± 52.99 IU/mL respectively). In β-thalassemic patients with splenectomy, the levels were significantly higher (p<0.05) than in the patients without splenectomy (mean ± SD; 279.49 ± 39.57 IU/mL, n=16 and 255.38 ± 40.23 IU/mL, n=34 respectively). These are shown in Figure 28.

The plasma concentration of β-TG per 10^6 plt in β-thalassemic patients (mean ± SD; 0.83 ± 0.41) were no statistically difference (p>0.05) from the healthy subjects (0.82 ± 0.29). In contrast, the levels in β-thalassemic patients without splenectomy (0.96 ± 0.42) were significantly higher (p<0.05) than with splenectomy (0.56 ± 0.20), as shown in Figure 29.

Mean ± SD of PF4 concentration in β-thalassemic patients (88.07 ± 13.73 IU/mL, n=50) were significantly higher (p<0.05) than healthy subjects (60.53 ± 17.77 IU/mL, n=30). In contrast, the levels in β-thalassemic patients with splenectomy were not statistically different from the patients without splenectomy, as shown in Figure 30.

Mean ± SD of PF4 concentration per 10^6 plt in β-thalassemic patients (0.28 ± 0.14, n=50) were not statistically different (p>0.05) from the healthy subjects (0.26 ± 0.10, n=30). In contrast, the levels in β-thalassemic patients without splenectomy (0.32 ± 0.14, n=34) were significantly higher (p<0.05) than with splenectomy (0.18 ± 0.06, n=16). These are shown Figure 31.

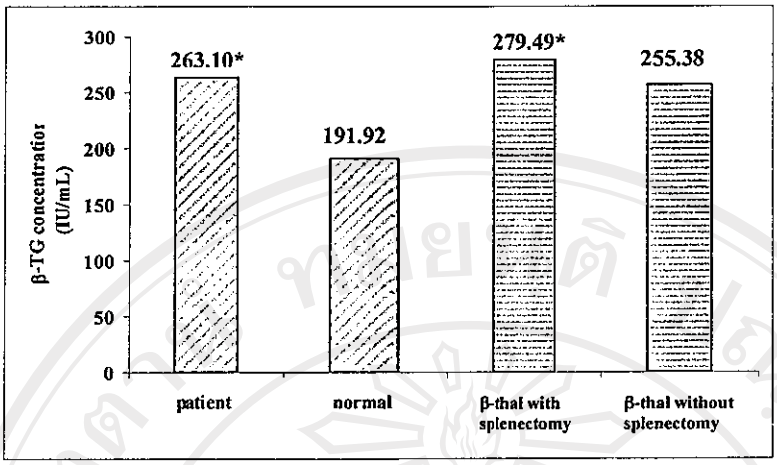


Figure 28 Comparison of plasma β-TG concentration (IU/mL)

[Between β-thalassemic patients (patient, n=50), healthy subjects (normal, n=30), β-thalassemic patients with splenectomy (n=16) and without splenectomy (n=34), (*p<0.05)]

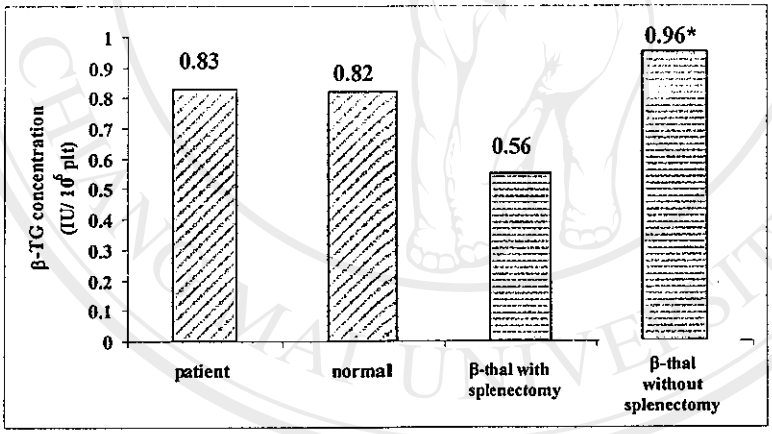


Figure 29 Comparison of plasma β-TG concentration (IU/10^6 plt)

[Between β-thalassemic patients (patient, n=50), healthy subjects (normal, n=30), β-thalassemic patients with splenectomy (n=16) and without splenectomy (n=34), (*p<0.05)]

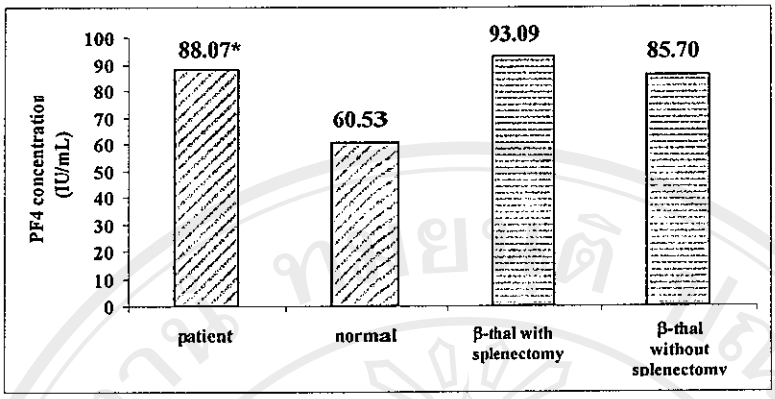


Figure 30 Comparison of plasma PF4 concentration (IU/mL)

[Between β-thalassemic patients (patient, n=50), healthy subjects (normal, n=30); β-thalassemic patients with splenectomy (n=16) and without splenectomy (n=34), (*p<0.05)]

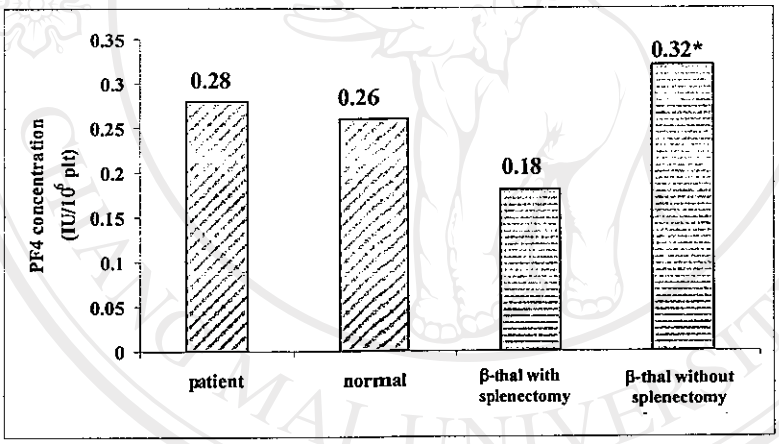


Figure 31 Comparison of plasma PF4 concentration (IU/10^6 plt)

[Between β-thalassemic patients (patient, n=50), healthy subjects (normal, n=30); β-thalassemic patients with splenectomy (n=16) and without splenectomy (n=34), (*p<0.05)]

7.2 Relationship between plasma β-TG or PF4 levels and platelet count

The correlation coefficient (r) between plasma β-TG with platelet count and plasma PF4 with platelet count of all samples were 0.475 and 0.431 respectively, which were significant (p<0.01), as shown Figure 32-33.

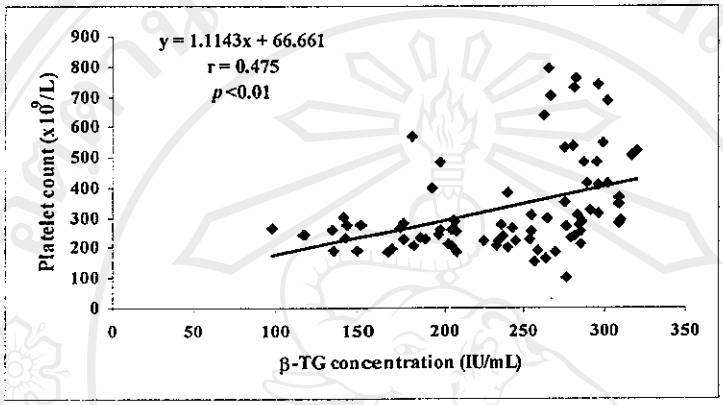


Figure 32 Relationship of plasma β-TG levels and platelet count

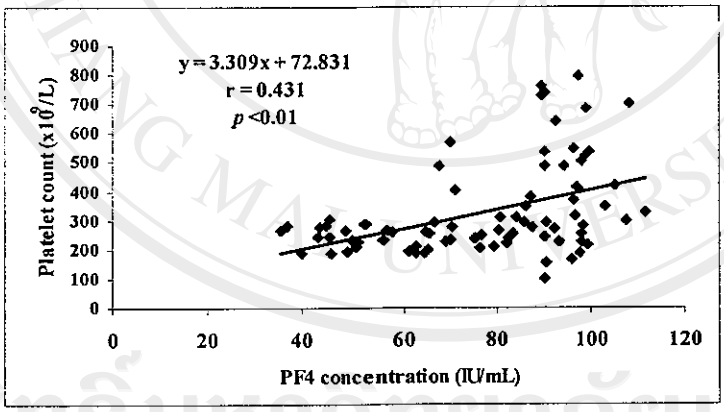


Figure 33 Relationship of plasma PF4 levels and platelet count

7.3 Relationship between plasma β-TG and PF 4 levels

The correlation coefficient (r) between plasma β-TG and PF 4 of all samples were 0.820, which were a significant correlation (p<0.01), as shown in Figure 34.

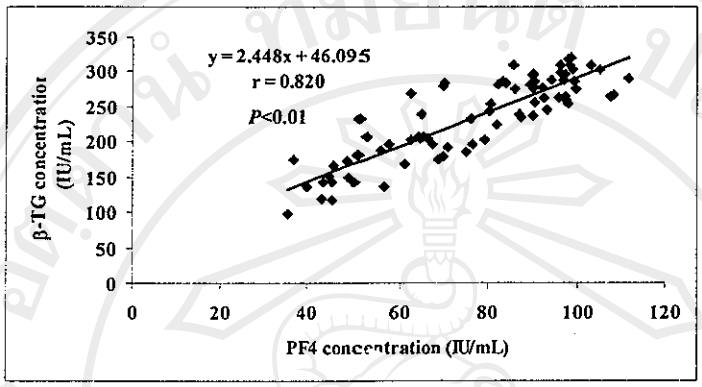


Figure 34 Relationship of plasma β-TG and PF4 levels

Table 13 Platelet activation markers and platelet morphology by SEM

No.	Group	Type	Platelet activation markers		Platelet morphology by SEM		
			β-TG (IU/mL)	PF4 (IU/mL)	%Shape changed platelets	% Aggregated platelets	% Activated platelets*
1	Normal	N	117.68	45.81	13.5	1.5	15
2	Normal	N	118.69	43.55	13.8	2.3	16
3	Normal	N	188.4	55.95	15.3	2.5	17.8
4	Normal	N	204.48	66.43	17.5	3	20.5
5	Normal	N	168.25	61.06	13.7	2	15.7
6	β-Thal major	Nsp	196.95	67.3	21.8	5.2	27
7	β-Thal major	Nsp	254.65	97.96	31.5	15.7	46.2
8	β-Thal major	Nsp	295.21	90.1	26.8	5.7	33.5
9	β-Thal major	Sp	317.37	98.03	33.1	9.5	42.6
10	β-Thal major	Sp	302.65	99.23	33.3	9.7	43
11	β-Thal major	Sp	180.2	69.88	24	6	30
12	β-Thal major	Sp	296.42	96.9	23.4	12.2	35.6
13	β-Thal/Hb E	Nsp	286.57	90.47	40	2.8	42.8
14	β-Thal/Hb E	Nsp	233.66	51.77	15.5	8.5	29
15	β-Thal/Hb E	Sp	265.16	97.47	30.9	10.9	41.8

N = Normal, NSp = Without splenectomy, Sp = With splenectomy

* Activated platelets = Shape changed platelets + Aggregated platelets

7.4 Relationship of %activated platelets (by SEM) with plasma β-TG and PF4 levels

The rather good and significant (p<0.01) correlation between plasma β-TG levels and %activated platelets by SEM was demonstrated (r = 0.836), as shown in Figure 35.

The rather good and significant (p<0.01) correlation between plasma PF4 levels and %activated platelets by SEM was also demonstrated (r = 0.907), as shown in Figure 36.

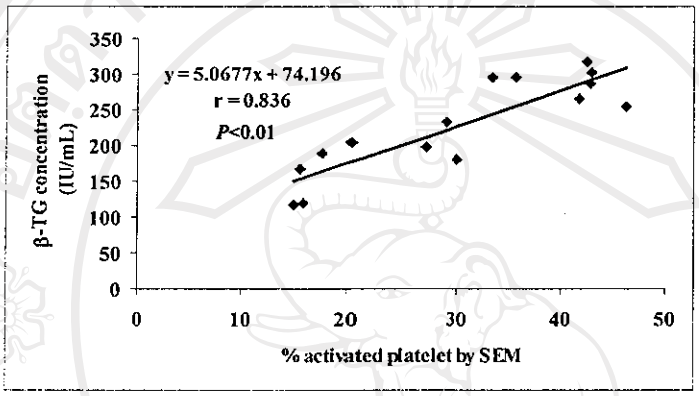


Figure 35 Relationship between %activated platelets by SEM and plasma β-TG levels

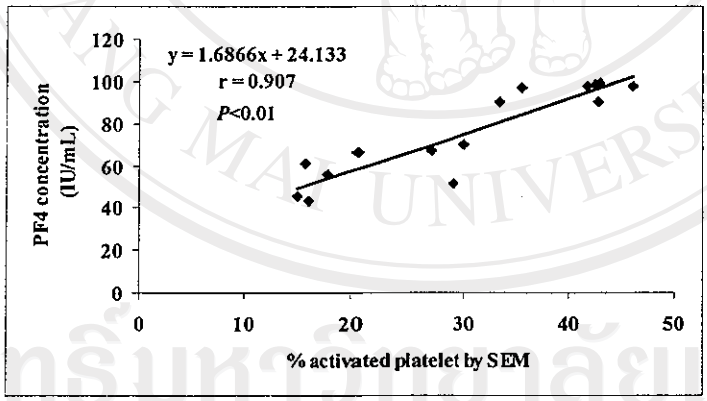


Figure 36 Relationship between %activated platelets by SEM and plasma PF4 levels

8. Activated platelets with CD63 expression ($CD63^+$) counted by flow cytometry

In order to count $CD63^+$ platelets in β-thalassemia and healthy subjects, staining with monoclonal antibody (MoAb) against CD63 (activated platelet marker) conjugated RPE (MoAb-CD63-RPE) and MoAb-CD42b-FITC (surface marker of platelet) was employed, before flow cytometry. All samples were collected into a mixture fixative solution (0.2% glycoxal and 0.4% paraformaldehyde).

Percentages of CD63 expressing platelets in β-thalassemic patients and normal subjects were (mean ± SD) 4.27 ± 1.29 (n=50) and 1.38 ± 0.29% (n=30) respectively (Table 14).

Table 14 Percentages of $CD63^+$ platelets

Parameter	Group	Number	Mean	SD
$CD63^+$ platelets (%)	β-Thalassemic patients	50	4.27	1.29
	Healthy subjects	30	1.38	0.29

Percentages of $CD63^+$ platelets in β-thalassemic patients with and without splenectomy are shown in Table 15. They were (mean ± SD) 4.70± 1.04 (n=16) and 4.06 ± 1.36% (n=34) respectively.

Table 15 Percentages of $CD63^+$ platelets in the patients with and without splenectomy

Parameter	Type	Number	Mean	SD
$CD63^+$ platelets (%)	With splenectomy	16	4.70	1.04
	Without splenectomy	34	4.06	1.36

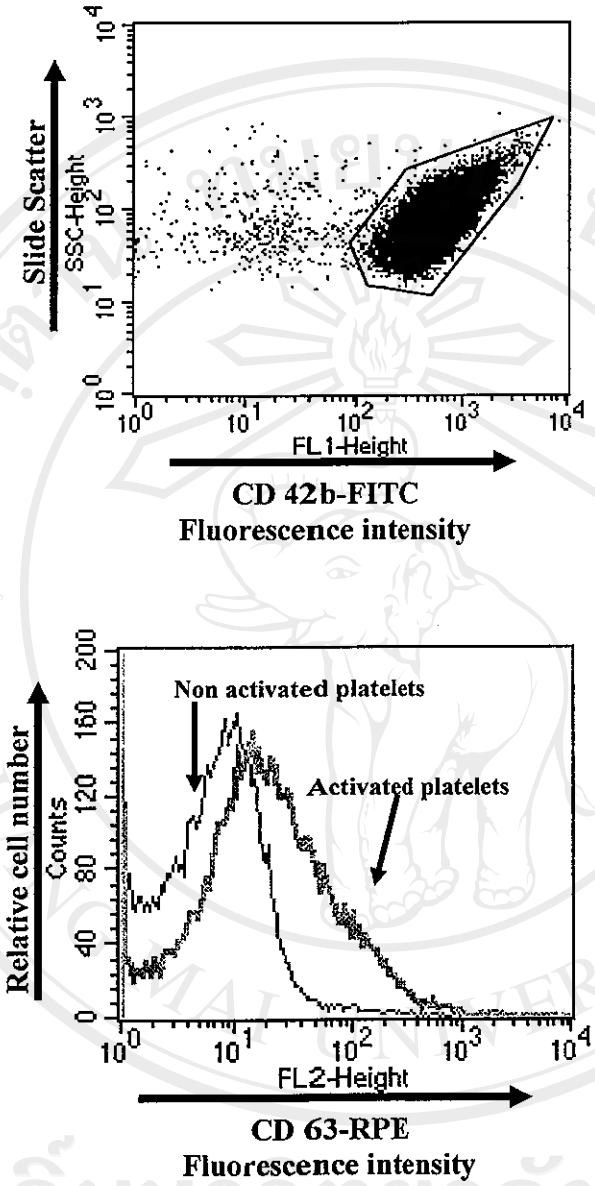


Figure 37 Overlay flow cytometric histogram of $CD63^+$ platelets

Top; All $CD42b^+$ (FL1: FITC) platelets were gated.

Bottom; $CD63^+$ (FL2: RPE) or activated platelets were shown overlaying on negative or non-activated platelets.

(Negative control = non-activated platelets, Positive control = activated platelets)

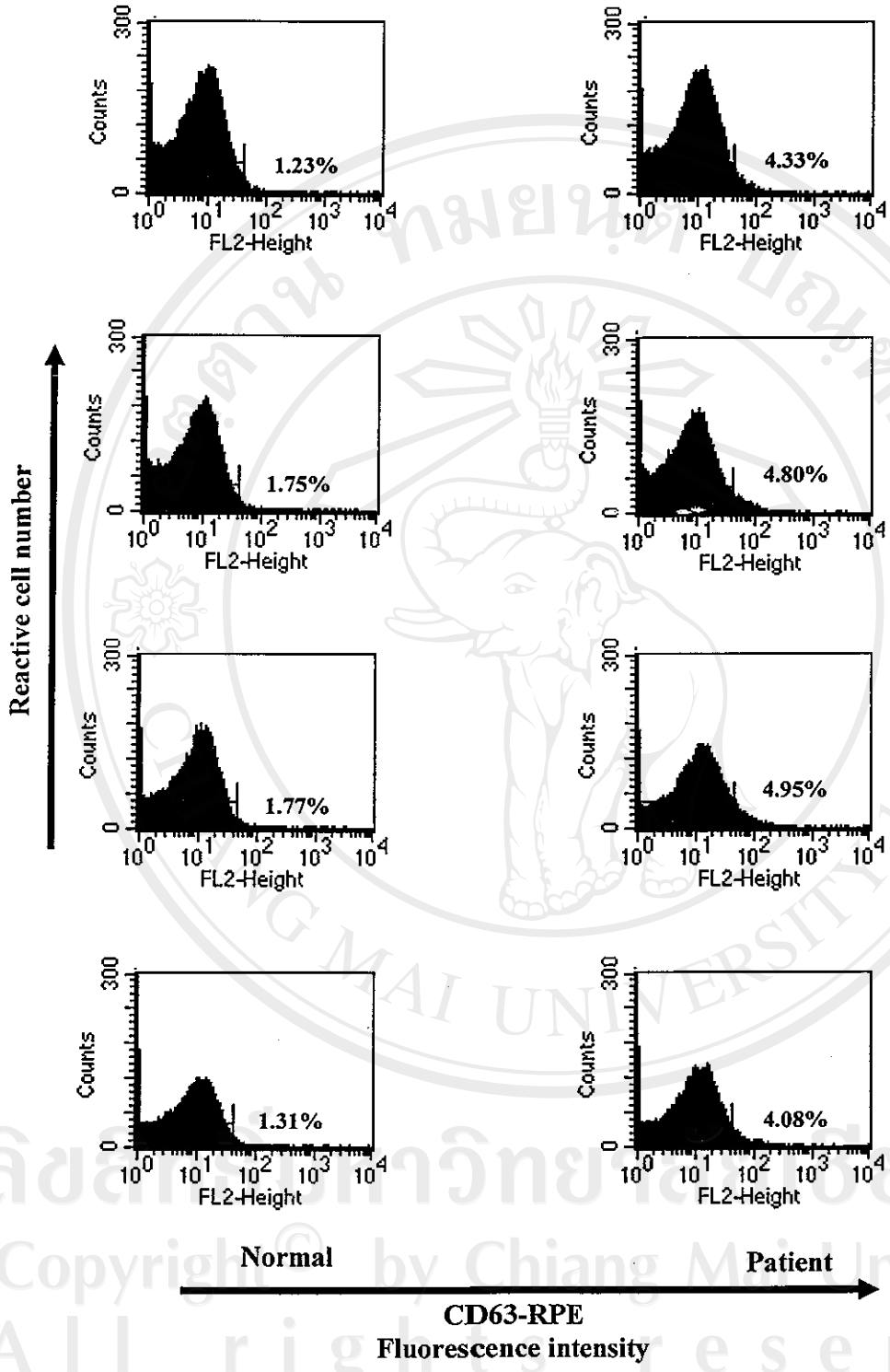


Figure 38 Flow cytometric histograms of %CD63$^+$ platelets

(In comparison between 4 pairs of normal individuals and patients)

8.1 Comparison of %CD63$^+$ platelets in β-thalassemic patients and healthy subjects

CD63$^+$ platelets in β-thalassemic patients was (mean ± SD) 4.27 ± 1.29% (n=50) which were significantly higher (p<0.05) than 1.38 ± 0.29% (n=30) in healthy subjects. Then the mean ± SD in β-thalassemic patients with or without splenectomy were 4.70 ± 1.04% (n=16) and 4.06 ± 1.36% (n=34) respectively. The levels in β-thalassemia with splenectomy were significantly higher (p<0.05) than without splenectomy, as shown in Figure 39.

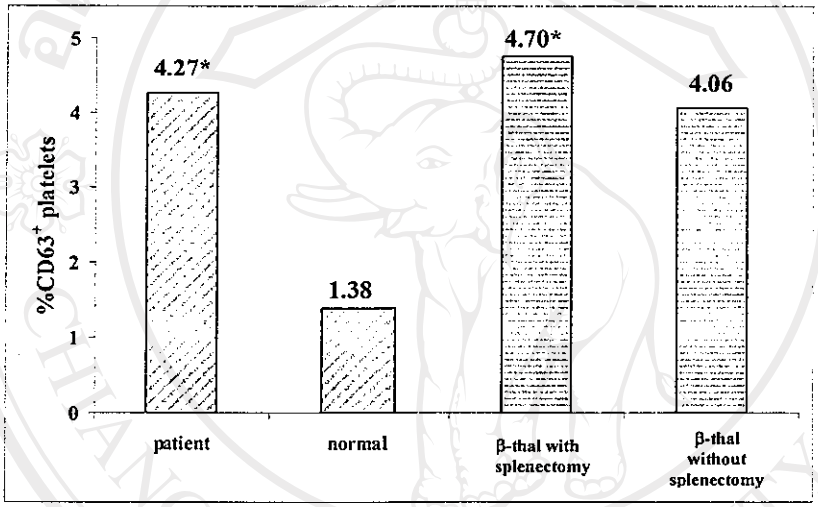


Figure 39 Comparison of %CD63$^+$ platelets

[Between β-thalassemic patients (patient, n=50), healthy subjects (normal, n=30), β-thalassemic patients with splenectomy (n=16) and without splenectomy (n=34), (*p<0.05)]

8.2 Relationship of %CD63$^+$ platelets and %activated platelets by SEM

The correlation coefficient (r) between the %CD63$^+$ platelets and %activated platelets by SEM was 0.785, which was a rather good and significant correlation (p<0.01), as shown in Figure 40.

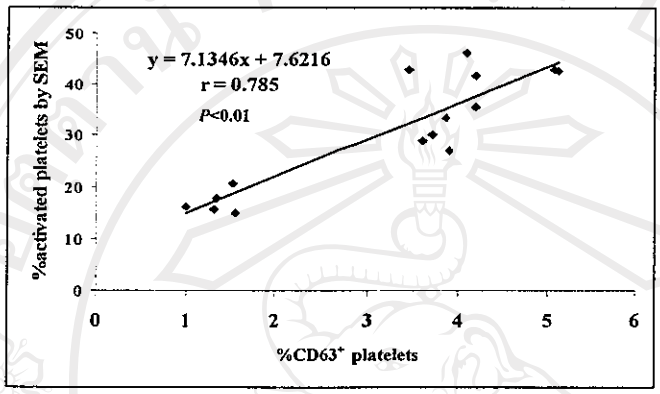


Figure 40 Relationship between %CD63$^+$ platelets and %activated platelets by SEM

8.3 Relationship of %CD63$^+$ platelets and plasma β-TG and PF4 levels

The correlation coefficient (r) between the %CD63$^+$ platelets and β-TG levels was 0.712, which was a rather good and significant correlation (p<0.01), as shown in Figure 41.

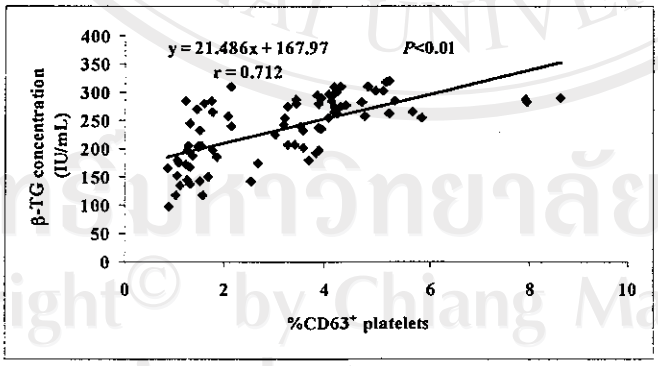


Figure 41 Relationship between %CD63$^+$ platelets and plasma β-TG levels

A rather good (r = 0.831) and significant correlation (p<0.01) between plasma PF4 levels and %CD63$^+$ platelets was also demonstrated, as shown in Figure 42.

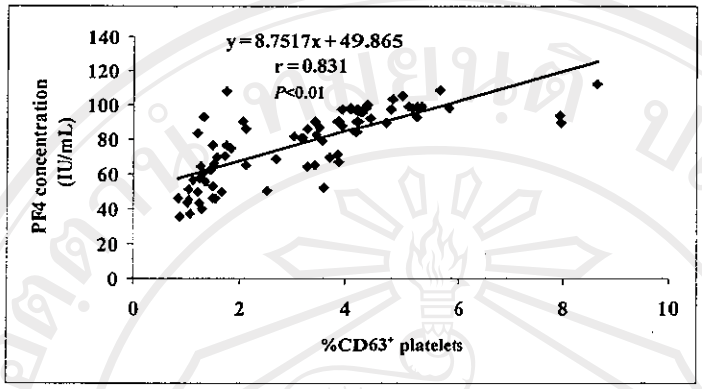


Figure 42 Relationship between %CD63$^+$ platelets and plasma PF4 levels

8.4 Relationship of %PS exposing RBCs and %CD63$^+$ platelets

The correlation coefficient (r) of the levels of %PS exposing RBCs and %CD63$^+$ platelets were 0.851. The correlation is significant (p<0.01), as shown in Figure 43.

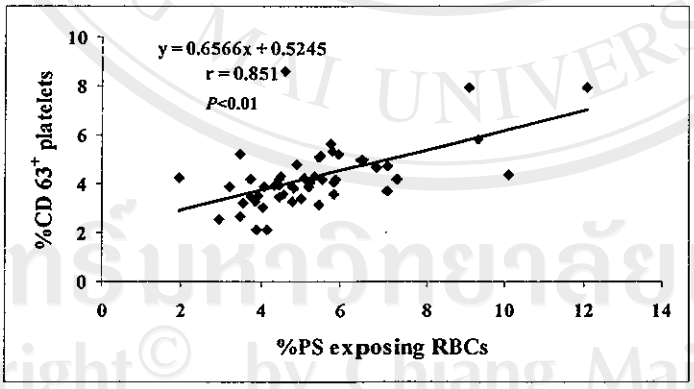


Figure 43 Relationship of %PS exposing RBCs and %CD63$^+$ platelets

Table 16 Percentages of PS exposing RBCs and $CD63^+$ platelets

No.	Group	Gender	Diagnosis	Type	%PS exposing RBCs	$\%CD63^+$ platelets
1	N	M	Normal	N	2.62	1.75
2	N	M	Normal	N	2.15	2.06
3	N	M	Normal	N	2.20	1.77
4	N	M	Normal	N	2.48	1.68
5	N	M	Normal	N	2.33	1.56
6	N	M	Normal	N	1.15	1.07
7	N	M	Normal	N	2.13	1.01
8	N	M	Normal	N	1.55	0.89
9	N	M	Normal	N	1.45	1.36
10	N	M	Normal	N	2.20	1.05
11	N	M	Normal	N	1.90	1.23
12	N	M	Normal	N	1.58	1.32
13	N	M	Normal	N	2.78	1.72
14	N	M	Normal	N	1.48	0.84
15	N	M	Normal	N	1.7	1.48
16	N	M	Normal	N	1.81	1.49
17	N	M	Normal	N	2.08	1.6
18	N	M	Normal	N	2.07	1.84
19	N	M	Normal	N	2.78	1.50
20	N	M	Normal	N	2.69	1.05
21	N	M	Normal	N	1.82	1.32
22	N	M	Normal	N	1.72	1.45
23	N	F	Normal	N	1.40	1.23
24	N	F	Normal	N	1.70	1.25
25	N	F	Normal	N	2.33	1.50
26	N	F	Normal	N	1.79	1.54
27	N	F	Normal	N	1.44	1.12
28	N	F	Normal	N	2.42	1.28

Table 16 Percentages of PS exposing RBCs and $CD63^+$ platelets (Cont.)

No.	Group	Gender	Diagnosis	Type	%PS exposing RBCs	$\%CD63^+$ platelets
29	N	F	Normal	N	1.75	1.25
30	N	F	Normal	N	2.10	1.31
31	P	M	β-Thal major	Nsp	5.36	4.33
32	P	M	β-Thal major	Nsp	4.49	4.31
33	P	M	β-Thal major	Nsp	4.07	3.02
34	P	M	β-Thal major	Nsp	5.24	4.09
35	P	M	β-Thal major	Nsp	5.87	3.58
36	P	M	β-Thal major	Nsp	4.82	3.89
37	P	M	β-Thal major	Nsp	3.57	3.2
38	P	M	β-Thal major	Nsp	12.1	7.94
39	P	M	β-Thal major	Nsp	4.61	8.63
40	P	M	β-Thal major	Sp	6.50	4.95
41	P	M	β-Thal major	Sp	4.45	3.95
42	P	M	β-Thal major	Sp	5.89	4.2
43	P	M	β-Thal major	Sp	5.50	5.13
44	P	M	β-Thal major	Sp	5.16	4.2
45	P	M	β-Thal major	Sp	4.45	4.16
46	P	M	β-Thal major	Sp	9.07	7.96
47	P	M	β-Thal/Hb E	Nsp	4.91	4.8
48	P	M	β-Thal/Hb E	Nsp	4.47	3.44
49	P	M	β-Thal/Hb E	Nsp	4.59	3.59
50	P	M	β-Thal/Hb E	Nsp	4.17	2.12
51	P	M	β-Thal/Hb E	Sp	5.98	5.21
52	P	M	β-Thal/Hb E	Sp	1.94	4.23
53	P	F	β-Thal major	Nsp	5.10	4.25
54	P	F	β-Thal major	Nsp	4.08	3.88
55	P	F	β-Thal major	Nsp	5.82	5.31
56	P	F	β-Thal major	Nsp	5.23	3.89

Table 16 Percentages of PS exposing RBCs and $CD63^+$ platelets (Cont.)

No.	Group	Gender	Diagnosis	Type	%PS exposing RBCs	$\%CD63^+$ platelets
57	P	F	β-Thal major	Nsp	5.47	3.18
58	P	F	β-Thal major	Nsp	4.80	3.28
59	P	F	β-Thal major	Nsp	3.50	2.68
60	P	F	β-Thal major	Nsp	2.98	2.52
61	P	F	β-Thal major	Nsp	3.75	3.45
62	P	F	β-Thal major	Nsp	3.95	3.51
63	P	F	β-Thal major	Nsp	10.10	4.39
64	P	F	β-Thal major	Nsp	5.02	3.42
65	P	F	β-Thal major	Nsp	3.22	3.85
66	P	F	β-Thal major	Nsp	3.89	2.13
67	P	F	β-Thal major	Nsp	4.36	3.95
68	P	F	β-Thal major	Nsp	9.32	5.81
69	P	F	β-Thal major	Sp	5.86	4.08
70	P	F	β-Thal major	Sp	4.83	3.84
71	P	F	β-Thal major	Sp	5.49	5.08
72	P	F	β-Thal major	Sp	6.78	4.68
73	P	F	β-Thal major	Sp	5.78	5.65
74	P	F	β-Thal major	Sp	7.05	3.7
75	P	F	β-Thal/Hb E	Nsp	5.54	4.2
76	P	F	β-Thal/Hb E	Nsp	3.88	3.28
77	P	F	β-Thal/Hb E	Nsp	3.48	5.21
78	P	F	β-Thal/Hb E	Nsp	7.05	4.75
79	P	F	β-Thal/Hb E	Nsp	3.75	4.2
80	P	F	β-Thal/Hb E	Sp	7.30	4.20

N = Normal healthy subjects, P = Thalassemic patients

M = Male, F = Female

Sp = With splenectomy, NSp = Without splenectomy

9. Effect of β-thalassemic RBC and plasma on normal platelets (co-culture)

To study the effect of β-thalassemic RBC on normal platelet, a co-culture was performed. Then activated ($CD63^+$) platelets were counted using flow cytometry. All samples were collected into CPDA-1. Normal platelet rich plasma of healthy subjects was used as responders to be co-cultured with RBC as stimulators. Whether the β-thalassemic RBC could or could not stimulate normal platelets to express CD63 on their membrane in comparison to the co-culture with normal RBC. In the similar way, whether the β-thalassemic plasma (both without and with platelet pre-absorption) could or could not stimulate normal platelets to express CD63 on their membrane in comparison to the co-culture with normal plasma. The overall results of 3 pairs of co-culture were shown in Table 17.

Table 17 The overall results of 3 pairs of co-culture

Sample ID	Lymphocyte Cross Matching	Time	Stimulator	%Activated platelets ($CD63^+$ platelets)					
				Auto	Control		Patient		Overall
				%	%	ratio	%	ratio	ratio
1	Negative or Compatible	0 min	Red blood cells	0.74	0.10	0.14	2.14	2.89	20.64
			Unabsorbed plasma	0.32	0.23	0.72	6.54	20.44	28.39
			Absorbed plasma	NA	0.10	0.31	6.31	19.91	64.23
		30 min	Red blood cells	0.83	1.01	1.22	6.45	7.77	6.37
			Unabsorbed plasma	0.29	0.57	1.97	7.15	24.66	12.52
			Absorbed plasma	NA	0.34	1.17	1.35	4.66	3.98
2	Positive or Incompatible	0 min	Red blood cells	1.30	0.7	0.54	1.84	1.42	2.63
			Unabsorbed plasma	1.34	0.50	0.37	1.36	1.01	2.73
			Absorbed plasma	NA	0.39	0.29	0.54	0.40	1.38
		30 min	Red blood cells	0.47	0.21	0.45	3.50	7.45	16.56
			Unabsorbed plasma	0.59	0.71	1.20	2.16	3.66	3.05
			Absorbed plasma	NA	0.67	1.14	0.50	0.85	0.75
3	Negative or Compatible	0 min	Red blood cells	1.4	1.10	0.78	3.75	2.68	3.46
			Unabsorbed plasma	2.08	1.23	0.59	1.32	0.63	1.07
			Absorbed plasma	NA	1.23	0.59	1.24	0.60	1.02
		30 min	Red blood cells	0.95	0.16	0.17	3.56	3.75	22.06
			Unabsorbed plasma	0.94	2.21	2.35	14.23	15.14	6.44
			Absorbed plasma	NA	1.45	1.54	2.57	2.73	1.77

NA = Not applicable

$$\text{Ratio of activated platelets} = \frac{\text{\% Activated platelets of control or patient}}{\text{\% Activated platelets of auto-control}}$$

$$\text{Overall ratio} = \frac{\text{Patient Ratio of activated platelets}}{\text{Control Ratio of activated platelets}}$$

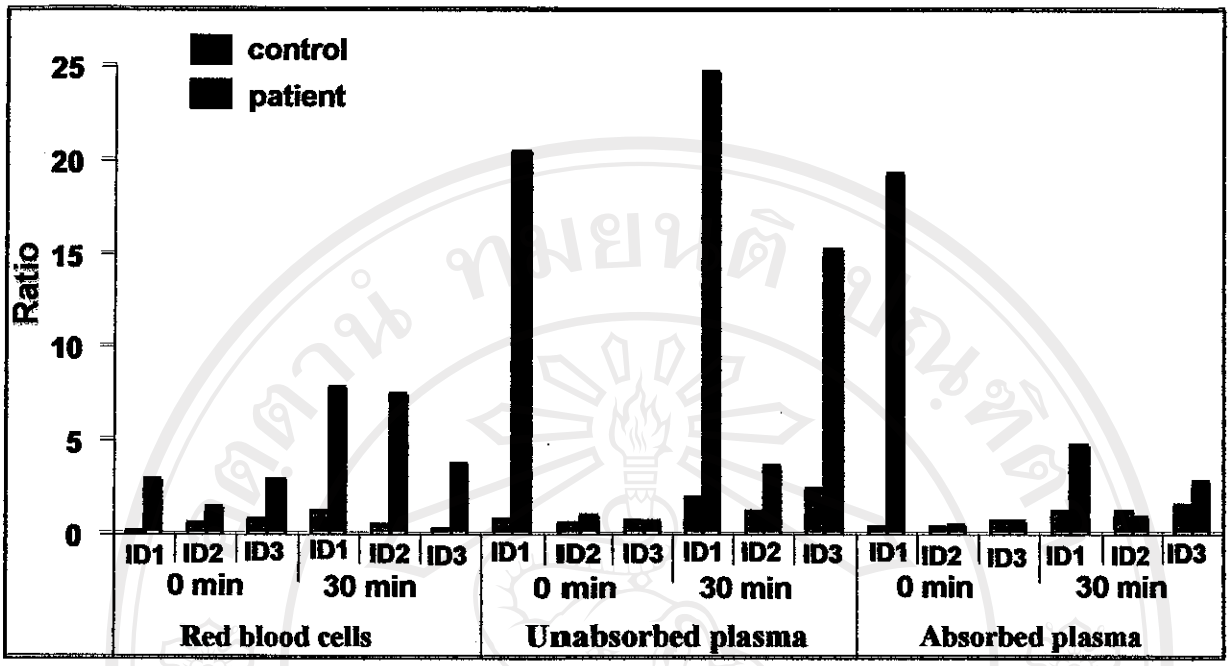


Figure 44 Comparison the ratio of activated platelets of 3 pairs of co-culture experiments

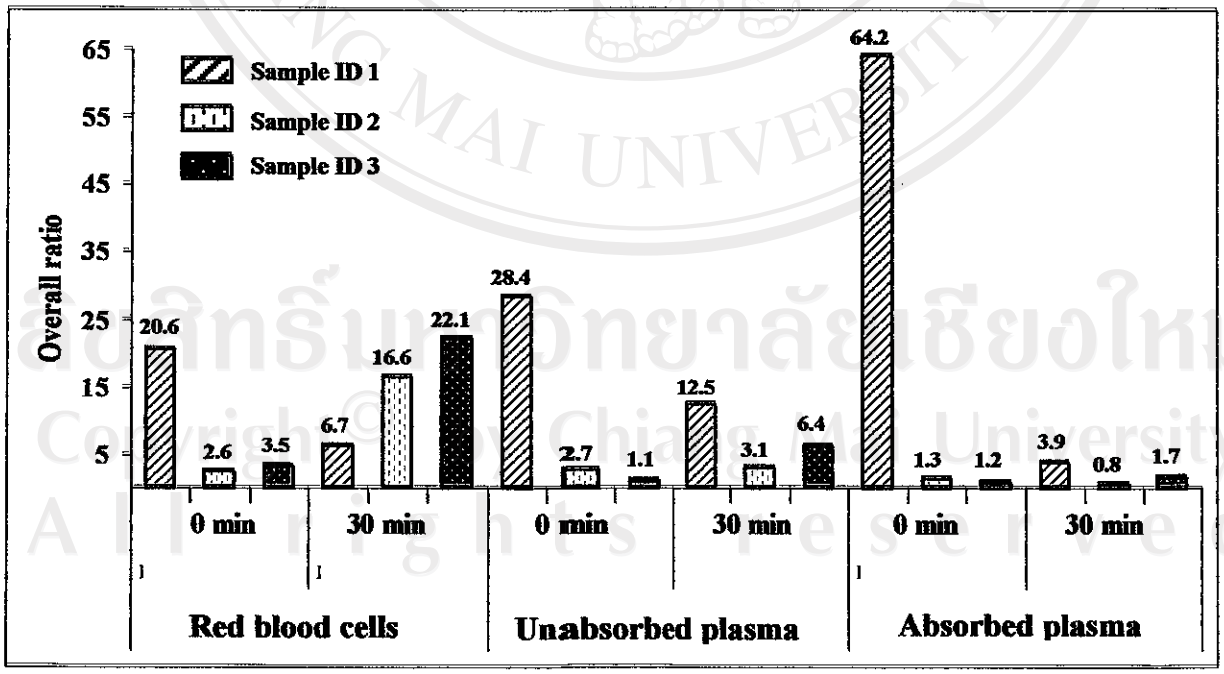
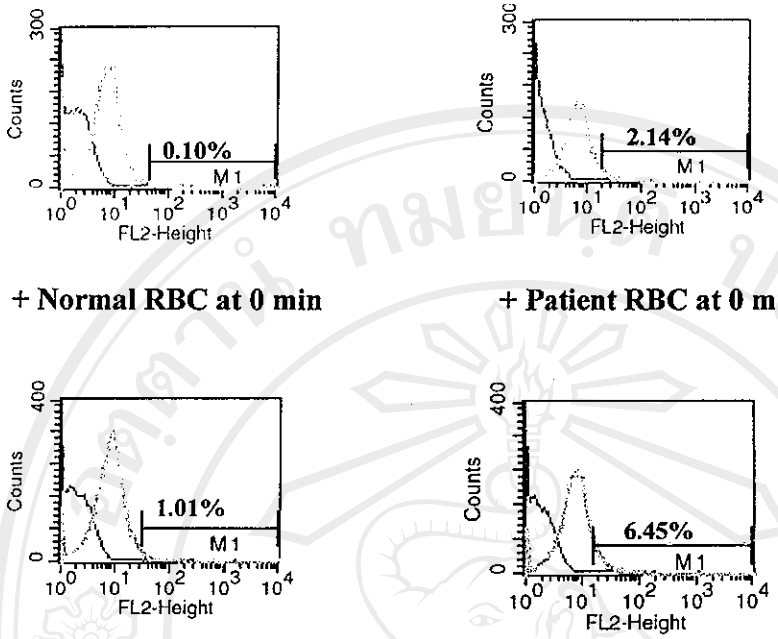


Figure 45 Overall ratio of 3 pairs of co-culture experiments

Normal platelet rich plasma



+ Normal RBC at 0 min **+ Patient RBC at 0 min**

+ Normal RBC at 30 min **+ Patient RBC at 30 min**

Reaction of normal platelet rich plasma and normal red blood cells

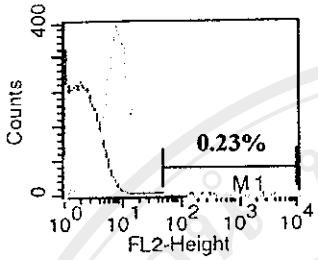
Reaction of normal platelet rich plasma and patient red blood cells

Figure 46 Comparison of %CD63$^+$ platelets after co-culture with RBC

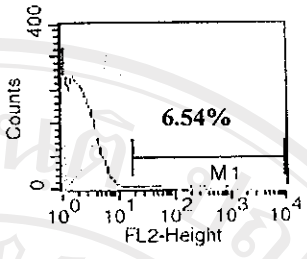
(*in vitro* co-culture between normal platelet rich plasma and normal red blood cells or patient red blood cells) patient sample identity number 1 (sample ID1)

The monoclonal anti-CD63 reacted platelet population (grey) was overlayed on isotype control reacted platelet population (black).

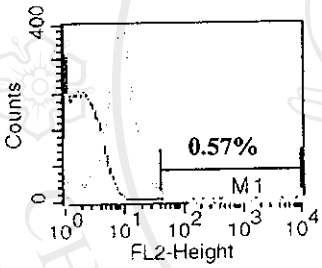
Normal platelet rich plasma



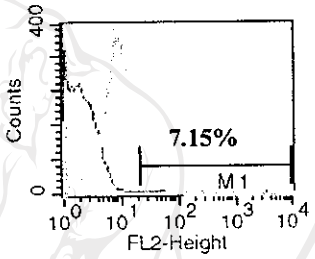
+ Normal plasma at 0 min



+ Patient plasma at 0 min



+ Normal plasma at 30 min



+ Patient plasma at 30 min

Reaction of normal platelet rich plasma and normal unabsorbed plasma

Reaction of normal platelet rich plasma and patient unabsorbed plasma

Figure 47 Comparison of %CD63$^+$ platelets after co-culture with unabsorbed plasma

(*in vitro* co-culture between normal platelet rich plasma and normal or patient unabsorbed plasma) sample ID1

The monoclonal anti-CD63 reacted platelet population (grey) was overlayed on isotype control reacted platelet population (black).

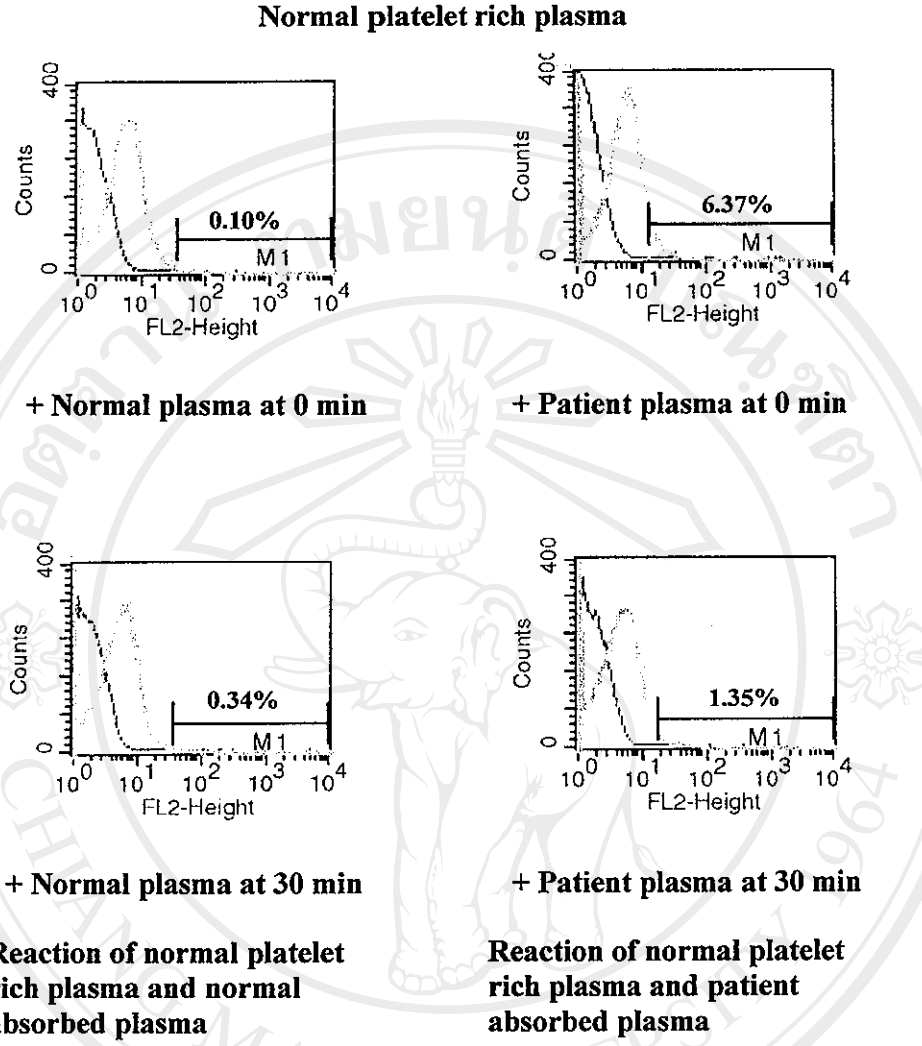


Figure 48 Comparison of %CD63$^+$platelets after co-culture with absorbed plasma (*in vitro* co-culture between normal platelet rich plasma and normal or patient absorbed plasma) sample ID1

The monoclonal anti-CD63 reacted platelet population (grey) was overlayed on isotype control reacted platelet population (black).

Normal platelet rich plasma

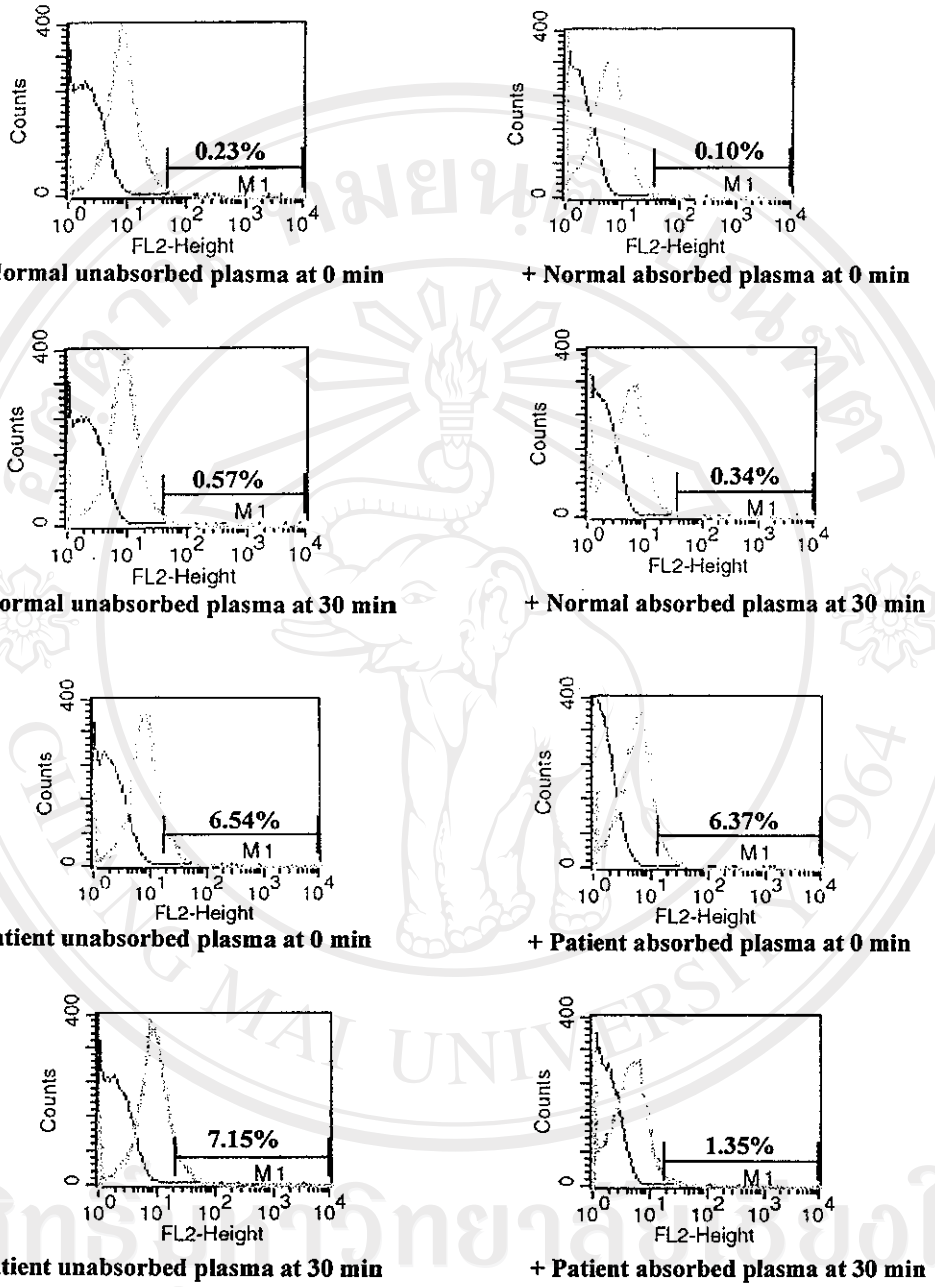


Figure 49 Comparison of %CD63$^+$ platelets by co-culture experiments
(*in vitro* co-culture between normal platelet rich plasma and normal or patient unabsorbed or absorbed plasma) sample ID1
The monoclonal anti-CD63 reacted platelet population (grey) was overlayed on isotype control reacted platelet population (black).