IV. DISCUSSION

The thromboembolic events in transfusion dependent β-thalassemic patients are usually associated with significant morbidity and mortality. These include recurrent and transient ischemic cerebral manifestations, stroke and a high frequency of thrombotic lesions in the pulmonary arteries. This leads to the recognition of a chronic hypercoagulable state in the particular patients.

In this study, all samples were tested for complete blood cell count (CBC) by automatic blood cell analyzer (Hemacel). The anemic markers of RBC, Hb, Hct in β-thalassemic patients were significantly lower ($p<0.05$) than healthy subjects. Since the bone marrow is exhausted from accelerated erythropoiesis due to the continuous response to the anemic state. Therefore RBC and Hb synthesis are decreased. In thalassemia, the hemolysis considerably causes by partial or complete deficiency of α- or β-globin chain synthesis due to their genes defects. These globin chains are the vital components of Hb, which is oxygen (O_2)/carbon dioxide (CO_2) carrying protein presenting in RBC. The low levels of Hb and the excess of globin chains induce an abnormal RBC membrane. This leads to an increased in phagocytosis by reticuloendothelial system such as spleen and liver. The MCV, MCH and MCHC of the patients were significantly lower ($p<0.05$) than the levels in healthy subjects. These confirmed the characteristic of such disease. However these blood indices does not represent only patients RBC but they also represent the combination of donor and patient RBC. Since all patients in this study are transfusion dependent. The platelet count of β-thalassemia with splenectomy was significantly higher ($p<0.05$) than both β-thalassemia without splenectomy and healthy subject. Since the spleen, a one-third storage site of platelets, were removed. Therefore all of platelets synthesized from bone marrow go directly to the circulation. This causes increasing in circulating

platelets in β-thalassemia with splenectomy. This finding was previously reported (Hathirat *et al.*, 1993).

In the present study, the *in vitro* platelet function represented by platelet aggregation, the hypo-, normo- and hyper-aggregabilities were demonstrated in all 4 agonists used. Percentage of β-thalassemia/Hb E with hypo-aggregability was higher than in β-thalassemia major. Additionally, percentage of β-thalassemia/Hb E with hyper-aggregability was lower than in β-thalassemia major. This is probably due to the less frequency of blood transfusion in β-thalassemia/Hb E. Since the blood transfusion may cause platelet activation. Then chronic persistent platelet activation may cause platelet exhaustion. These findings were concordant to the previous report (Eldor, 1978; Isarangkura *et al.*, 1987). The aggregabilities are likely to be related to many factors, particularly to the development of the severity. The hypo-aggregation may lead to a bleeding problem. A hemorrhagic tendency was observed in β-thalassemic patients. These included easy bruising, gingival bleeding and frequent epistaxis (Eldor, 1978). In most of the β-thalassemia major patients and in some with β-thalassemia minor, diminished platelet aggregation to ADP, collagen, ristocetin and epinephrine was found and could be responsible in part for the hemorrhagic phenomena (Eldor, 1978). Platelet hyper-aggregation may contribute to thrombosis, pulmonary embolism and arterial hypoxemia. A pulmonary functional defect was noted as one of a significant finding in thalassemic patients. These phenomena were noted in a very mild form even at an early age of life. This information will lead to a further exploration of the pathogenesis of pulmonary function defects as well as their role in the patients future health and prognosis (Isarangkura *et al.*, 1993). In addition, the finding of this study shows 2 possibilities of hypo-aggregability leading to bleeding event and the hyper-aggregability leading to thrombosis. These may be due to multifactorial involvement giving a difference in clinical symptoms even in the same disease.

In the present study, the percentages of PS exposing RBCs, measured by flow cytometric annexin V-FITC binding, in the β-thalassemic patients were significantly higher ($p<0.05$) than in the healthy subjects. Furthermore, the percentages in splenectomized patients were also significantly higher ($p<0.05$) than in non-splenectomized patients. This similar finding has been previously reported (Ruf *et al.*, 1997). This clearly shows evidence linking the abnormal red cell membrane to a procoagulating stage. Since the procoagulant activity of PS on outer RBC membrane can activate coagulation pathway that convert FVa, FXa to be a prothrombinase complex. Then the complex can further induce thrombin generation. The thrombin is known to be able to activate the platelet directly. Therefore the hypercoagulable state observed in the patients with β-thalassemia major and intermedia resulted from a procoagulant effect of the abnormal RBC. This has been confirmed again in this study. In addition, the RBC from β-thalassemia major or intermedia was shown to enhance thrombin generation in a prothrombinase assay (Borenstain-Ben Yashar *et al.*, 1993). Furthermore, the procoagulant effect of β-thalassemia major RBC determined by annexin V, which bound tightly to anionic membrane phospholipids such as PS, was also reported before (Helley *et al.*, 1996). These findings consistent with an abnormal membrane phospholipid asymmetry and PS exposure supported by the present results of a significant increase in the fraction of annexin V labeled RBC in β-thalassemia major and β-thalassemia/Hb E. It could be argued that the enhanced RBC binding of annexin V observed in β-thalassemia major may be modified by their frequent blood transfusion. However the evidence to support that idea is still to be elucidated. The translocation of both PS and PE operated by enzyme aminophospholipid translocase in such patients may be defect (Bitbol *et al.*, 1987; Helley *et al.*, 1996; Kuypers *et al.*, 1996; Kuypers *et al.*, 1996). Therefore the PS exposing RBCs were remained in the circulation especially in splenectomized patients. Aged RBC was shown a similar display of higher amounts of PS on the outer leaflet of their membranes as compared

to the young cells. This may serve as a signal for their recognition and removal by the reticuloendothelial system (Zwaal *et al.*, 1993; Connor *et al.*, 1994). Negatively charged PS-rich phospholipid surface are known to function as catalysts in the conversion of prothrombin to thrombin by assembly of activated coagulation factors (e.g. the prothrombinase complex) (Shattil *et al.*, 1987). The existence of a hypercoagulable state in β-thalassemia major and intermedia was recognized recently (Eldor, 1978; Del Principe *et al.*, 1993; Cappellini *et al.*, 2000). These findings are parallel with the results of the present study.

In the platelet morphology studied by SEM, the percentages of shape changed platelets, aggregated platelets and therefore activated platelets *in vivo* in β-thalassemic were significantly higher ($p<0.05$) than healthy subjects. In contrast, the percentages of normal discoid platelets in the patients were significantly lower ($p<0.05$) than healthy subjects. The decreased percentage of discoid platelets and the increased percentage of spherical platelets in thalassemic patients were previously reported by Bunyaratvej *et al.*, 1992. However in the methodology of that report, the platelets were not fixed immediately after the blood collection. This implied that the *in vitro* platelet activation may be present. Therefore the platelet activation *in vivo* in β-thalassemic patients was clearly shown in this study. This finding correlated with the lower platelet reversibility from pseudopods to smooth surface in β-thalassemic platelets comparing to normal platelets. In addition, splenectomized patients had a lower platelet pseudopods reversibility comparing to non-splenectomized patients. In this study the levels of activated platelets, shape changed platelets and aggregated platelets were no significantly difference between the patients with and without splenectomy. The shape change and impaired reversibility of platelet pseudopods may be associated with the high tendency of pulmonary thrombus in β-thalassemic patients (Bunyaratvej *et al.*, 1992). It was not possible to conduct platelet reversibility experiment in this study, because the platelet samples were fixed to death immediately

in order to examine the *in vivo* platelet activation only. However the findings of this study also imply that *in vivo* platelet activation may correlate with thrombosis and pulmonary embolism in β-thalassemic patients.

Evidence of *in vivo* platelet activation represented by plasma levels of 2 platelet activation markers, β-TG and PF4, was examined. They were released from α-granules of activated platelets. The high correlation coefficients (r) of calibration curves of both β-TG and PF4 and the control samples gave the levels within the acceptable ranges, indicated a high reliability of the 2 assays. The plasma concentration of β-TG and PF4 in IU/mL of β-thalassemic patients were significantly higher ($p<0.05$) than healthy subjects. These results showed a clear evidence of *in vivo* platelet activation in such patients. Furthermore, the plasma level of β-TG in splenectomized β-thalassemic patients was significantly higher ($p<0.05$) than the patients without splenectomy. This probably due to the absent of spleen, which act as a sequestrating site of defected blood cells especially PS exposing RBCs. Therefore the high levels of PS exposing RBCs were still presented in the circulation. They may induce coagulation pathway that converts FVa, FXa to be prothrombinase complex. They can then further induce thrombin generation. The thrombin itself can activate platelet directly. This had been discussed previously. Therefore plasma level of β-TG in the splenectomized patients was higher than the non-splenectomized patients as expected. However the plasma level of PF4 in these 2 groups of patients was not significantly different. Even the higher level was found in the splenectomized patients. This may be due to the lower actual levels of PF4 comparing to the β-TG. This may cause a lack of reaching the statistical significance. In addition, the circulating activated ($CD63^+$) platelets assayed by flow cytometry were higher in β-thalassemic patients comparing to the healthy subjects. These parameters were also higher in the patients with splenectomy comparing to the patients without splenectomy. Furthermore, the activated platelets with morphology changes (shape change and aggregation) counted by SEM showed a

similar results of higher level in the patients over the healthy subjects. The high levels of $CD63^+$ platelets and activated platelets (by SEM) indicated platelet activation *in vivo* clearly in such patients. Therefore the releasing of β-TG and PF4 from α-granules into the circulation is the consequence of the platelet activation. A high correlation between β-TG and PF4 was found as expected. Since these 2 markers were released from the same event and the same type of α-granules.

The plasma levels of β-TG and PF4 per 10^6 platelets of β-thalassemia were not statistically different from healthy subjects. This may be a result of elevated platelet count in the patients over the healthy subject. When the levels of these markers were averaged with the number of platelet count, they will be the new parameters of platelet activation marker per 10^6 (1 million) platelets. In the splenectomized patients both β-TG and PF4 per 10^6 platelets were significantly lower ($p<0.05$) than in non-splenectomized patients. This is possibly due to a higher platelet count in the group of splenectomized patients. Therefore a higher divider (platelet count) gives rise to a lower result (β-TG and PF4 per 10^6 platelets). In addition, the concentrations of these 2 markers between the 2 groups of patients were not much difference. While the difference of platelet counts between these 2 groups of patients were very wide. This may be a matter of arithmetic of just in part if not all.

In this study, the significantly higher levels ($p<0.05$) of circulating platelets expressing activation dependent neoantigen, the lysosomal glycoprotein CD63 ($CD63^+$ platelets), in β-thalassemic patients were found comparing to the healthy subjects. This similar finding has been reported before (Ruf *et al.*, 1997). Furthermore, the high correlation of %PS exposing RBCs and $\%CD63^+$ platelet was observed. An increased fraction of $CD63^+$ platelets was known to be related to chronic hypercoagulable state. Overt thromboembolic event occurs only rarely in thalassemic patients. However, laboratory tests have provided evidence for a chronic hypercoagulable state, which already exists even in an early childhood (Eldor *et al.*, 1991; Del Principe *et al.*, 1993).

Mild hypoxaemia and right ventricular heart failure are observed in many young and adult β-thalassemia major and intermedia (Cooper *et al.*, 1980; Grant *et al.*, 1986; Chantharaksri *et al.*, 1992; Fucharoen *et al.*, 1992; Gillis *et al.*, 1999). These conditions could result from microembolization in the lung as was shown by autopsies. That revealed a high frequency of thrombotic lesions in the pulmonary arteries and the development of cor-pulmonale. These are also consistent with a long–standing pulmonary vascular stenosis (Sonakul *et al.*, 1980; Sonakul *et al.*, 1987; Songkhla *et al.*, 1987; Sumiyoshi *et al.*, 1992; Taher *et al.*, 2002). In this study, the elevated $CD63^+$ platelets were observed in the splenectomized patients. These findings reply that removing of the spleen causes an absent of sequestrating organ for removing of the abnormal platelets and RBC. Therefore these kinds of cells still circulate in a high number comparing to the non-splenectomized patients. This is also applicable to higher levels of PS exposing RBCs in such patients too. However a non-significant difference of $\%CD63^+$ platelets between splenectomized and non-splenectomized β-thalassemic patients was previously reported (Ruf *et al.*, 1997). The discrepant results between these 2 studies may be due to a different group of patients, treatment protocols, race, age and the total number of participating patients.

For the co-culture experiment, the factors able to stimulate the responding platelets until reaching the CD63 expression were determined in both RBC and plasma (stimulators) of the patients. All 3 pairs of co-cultures showed the elevation of $\%CD63^+$ platelets on both stimulators (RBC and plasma). These indicated a present of platelet stimulating factors on patient RBC and plasma. These results may co-respond to the high levels of PS exposing RBCs in the patients that was previously discussed. Since the PS exposing RBCs are known to be able to generate thrombin, which can further activate the responding platelets directly. Thrombin is the most potent platelet agonist and its formation in β-thalassemia has been substantiated by elevated thrombin-anti-thrombin III (TAT) complex (Eldor and Rachmilewitz, 2002). However

the unknown mechanisms of such activation may be present. These are still waiting to be elucidated. The similar results were also found in the plasma co-culture experiments. All of the 3 pairs showed elevation of %$CD63^+$ platelets, which indicated the abilities of the patient plasma to activate the responding platelets. The unabsorbed and absorbed plasma (with pooled blood group "O" platelets) gave a similar trend of elevation of %$CD63^+$ platelets. These also imply that there are some platelets stimulating factors remained in the plasma, even after antibodies to human platelet and human leukocyte type1 (ABC) antigens were absorbed. The plasma factors able to activate the responding platelets may be atypical (human platelet or leukocyte antigens; HPA or HLA) antibodies, auto-antibodies and releasing substances from the previously activated platelets. These last substances are not able to be absorbed with pooled blood group "O" platelets. Therefore the absorption is unable to prevent the platelet activation by the thalassemic plasma

All of the results in this study point out to the genuine existence of *in vivo* platelet activation in β-thalassemic patients. The measure for preventing such activation should be considered, in order to give rise the higher quality of life of the patients. Prophylaxis with anti-platelet drugs such as low dose aspirin, dipyridamaol or others may benefit. However these measures are still waiting to be studies, before the rear implementation.