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INCREASED RELEASABILITY OF PLATELET PRODUCTS AND REDUCED  
HEPARIN-INDUCED PLATELET FACTOR 4 RELEASE FROM ENDOTHELIAL  
CELLS IN BRONCHIAL ASTHMA

(気管支喘息における血小板放出能亢進と血管内皮細胞からの  
ヘパリンによる血小板第4因子放出の低下)

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HEPARIN-INDUCED PLATELET FACTOR 4 RELEASE FROM ENDOTHELIAL  
CELLS IN BRONCHIAL ASTHMA

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## ABSTRACT

To determine whether or not platelet activation is involved in the mechanism of exacerbation of bronchial asthma, we evaluated adenosine triphosphate (ATP) release from thrombin-stimulated washed platelets, plasma levels of  $\beta$ -thromboglobulin ( $\beta$ -TG) and platelet factor 4 (PF4), and plasma  $\beta$ -TG/PF4 ratios during symptomatic and asymptomatic periods in 15 patients with bronchial asthma compared with 16 normal control subjects. We also measured these parameters during allergen provocation tests and acetylcholine inhalation tests in 6 allergic asthmatics. ATP release, plasma levels of  $\beta$ -TG and PF4 were significantly increased during symptomatic periods and after the allergen provocations but not after acetylcholine inhalations. However, these findings were not accompanied by the elevation of plasma  $\beta$ -TG/PF4 ratios. The heparin-induced PF4 release, which is reported to reflect release of PF4 attached on endothelial cells, was significantly reduced in 12 asymptomatic asthmatic patients compared with 11 normal subjects, and it was much more reduced in 7 symptomatic asthmatic patients, suggesting the possibility of the reduced PF4 binding on endothelial cell surface. This finding may represent the prolonged half life of PF4 in asthmatics. We concluded that 1) increased releasability of platelet products and *in vivo* release of the platelet granular contents are involved in the mechanism of the exacerbation of bronchial asthma, 2) some functional alteration in platelet-endothelial cell interaction may be involved in bronchial asthma, and 3) plasma  $\beta$ -TG/PF4 ratios are not elevated possibly because of both increased platelet releasability and prolonged half life of PF4 in the blood in asthmatic patients.

Key words: bronchial asthma, platelet, ATP release,  $\beta$ -thromboglobulin, platelet factor 4, heparin

Abbreviations used

- PF4 : platelet factor 4  
 $\beta$ -TG :  $\beta$ -thromboglobulin  
ATP : 5'-adenosine triphosphate  
PRP : platelet rich plasma  
PBS : phosphate buffered saline

## 1. INTRODUCTION

Several studies have suggested that platelet activation is involved in the pathogenesis of bronchial asthma. The findings of these studies include the involvement of the platelet activating factor (PAF) (Page, 1988), IgE-dependent activation of platelets (Cápron *et al.*, 1985), histamine release from human basophils stimulated with PF4 (Brindley *et al.*, 1983) and the activation of eosinophils by platelet factor 4 (PF4) (Chihara *et al.*, 1988).

On the other hand, in order to find out the evidence of platelet activation *in vivo*, several investigators have examined plasma levels of PF4 and/or  $\beta$ -thromboglobulin ( $\beta$ -TG) in asthmatic patients, but they have not arrived a consensus yet. Some investigators observed the elevations of these parameters during the exacerbation of asthma or after antigen challenge (Knauer *et al.*, 1981; Greselet *et al.*, 1982; Johnson *et al.*, 1986; Metzger *et al.*, 1983; Toga *et al.*, 1984), but some did not (Hemmendinger *et al.*, 1989; Shephard *et al.*, 1985; Greer *et al.*, 1985; Durham *et al.*, 1985). Furthermore, the plasma  $\beta$ -TG/PF4 ratio, which is reported to be the best index of platelet activation *in vivo* in case of its elevation (Kaplan and Owen, 1981), has been found to remain constant throughout the antigen provocation tests (Durham *et al.*, 1985). In addition to these findings, various studies determining other platelet functions such as platelet aggregation (Thompson *et al.*, 1983) and the kinetics of  $^{111}\text{In}$ -labelled platelets (Taytard *et al.*, 1986; Ind *et al.*, 1985) have been reported, but their results are still controversial.

Moreover, when platelet rich plasma (PRP) is used in the study, we can not eliminate the effects of various factors within the plasma which might affect the activity of the platelets. So, the true function of the platelet itself must be studied using washed platelets or gel-filtered platelets.

Therefore, we measured the ATP release from washed platelets to determine whether platelet activation occurs in the exacerbation of bronchial asthma. The plasma  $\beta$ -TG/PF4 ratio in addition to their own concentrations were also examined to confirm the existence of platelet degranulation in the pathogenesis of asthma.

Next, we tried to explain why the platelet activation in bronchial asthma was not associated with the elevation of  $\beta$ -TG/PF4 ratio. We considered that this might be explained by platelet hyperreactivity and also by altered PF4 behaviour *in vivo* such as the prolonged half life in the circulation. In order to investigate the possibility of prolonged half life of PF4 in the interaction between platelets and endothelial cells, we determined heparin-induced PF4 release *in vivo* which was reported to show the PF4 binding on the endothelial cell surface (Tsukamoto *et al.*, 1987) and might represent the half life of PF4 in the circulation.



## 2. MATERIAL AND METHODS

### 2.1 Subjects

Sixteen normal control subjects (19~60 years old, average age of 37.4 years) and fifteen patients with bronchial asthma as defined by the American Thoracic Society (1975) (19~63 years old, average age of 44.5) in our clinic were involved in this study. Eleven patients had allergic asthma, that is, a concentration of serum IgE > 400 IU/ml and IgE-RAST > class 2 against at least one of common air-borne allergens such as house dust mite, pollens and fungi.

ATP release from washed platelets, plasma PF4 levels, plasma  $\beta$ -TG levels, and plasma  $\beta$ -TG/PF4 ratios were determined in asthmatic patients between during their asymptomatic and symptomatic periods, and were also compared with those in normal subjects. None of them received medication with antipyretics or anti-platelet agents which suppressed platelet functions during the previous two weeks. The patients' drug histories are listed in Table 1. The interval between the two studies (the studies in Asymptomatic period and Symptomatic period) was 7~21 days. Medications during the 6 days preceding the two studies were the same in each patient as shown in Table 1. In the case of patients treated with triamcinolone acetonide (20-30mg/day), blood was collected just before its injection during a symptomatic period and 21 days after the injection during an asymptomatic period, when triamcinolone was not detected in serum. In 6 cases of allergic asthma, these parameters were measured during allergen provocation tests and acetylcholine inhalation tests.

Heparin-induced PF4 release was measured in 11 normal subjects, 12 asthmatic patients during asymptomatic periods and 7 patients during symptomatic periods. Twelve asymptomatic patients were divided into two groups, those with and those without medication, and the effect of medication was determined.

The symptomatic period was defined as the period when a patient had dyspnea and audible expiratory wheeze accompanied by a more than 20% reduction of FEV<sub>1.0</sub> compared with asymptomatic periods.

## 2.2 Blood collection

Twelve ml blood was drawn from the median cubital vein through a 21 G butterfly needle into a plastic syringe containing 1/10 volume of 3.2% sodium citrate between 11 A.M. and 3 P.M., avoiding the daily variation in platelet function which occurs early in the morning (Tofler *et al.*, 1987). After the needle was inserted, the tourniquet was quickly removed, and care was taken not to pull the syringe out too forcefully and not to re-insert the needle, avoiding platelet activation during blood collection.

## 2.3 Determination of plasma PF4 and $\beta$ -TG concentrations

Immediately after the collection, 2.5 ml of the blood sample were gently transferred to a pre-cooled sampling tube containing the solution of 0.66 mg EDTA and 1.08 mg theophylline (provided by Amersham Co.). The tube was inverted gently three times and put into crushed ice within two minutes after blood collection in order to minimize platelet release during handling. Fifty minutes later, the tube was centrifuged at  $\times 2000$  g, 4°C for 30 minutes and the platelet poor plasma was carefully collected and stored at -20°C until it was assayed by 'PF4 RIA kit' (ABBOT Co.) and ' $\beta$ -TG RIA Pack' (Amersham Co.) within one week after the blood collection. For PF4, the interassay coefficient of variation was 8.3% and the intra-assay coefficient of variation 5.5%. For  $\beta$ -TG, the values were 2.6% and 6.0%, respectively.

## 2.4 Preparation of washed platelets



PRP was obtained by centrifuging the remaining blood at  $\times 130$  g,  $22^{\circ}\text{C}$ , for 15 min.. PRP was laid on Histopaque (density 1.077, Sigma Co.) and centrifuged at  $\times 700$  g,  $22^{\circ}\text{C}$ , for 20 min.. Platelets were collected from a platelet-rich interface layer and washed once as follows. The platelet rich layer was suspended in 10 ml of Tyrode-gelatine-EGTA solution (8g NaCl, 0.2g KCl, 1g  $\text{NaHCO}_3$ , 0.05 g  $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$  and 1 g Glucose /L with 0.25% gelatine and 0.1 mM EGTA) pH 6.4, containing 10 U of apyrase (Sigma Co.). The suspension was incubated at  $37^{\circ}\text{C}$  for 15 min., and then centrifuged at  $\times 900$ g,  $22^{\circ}\text{C}$  for 15 min.. The pellet was resuspended gently in a Tyrode-gelatin-EGTA solution (pH 7.4). The number of platelets was counted on a Bürker-Türk cytometer after mixing a 1/10 dilution with an equal volume of Rees-Ecker's dye solution, and adjusted to  $1 \times 10^8$  cells/ml. The recovery rate of platelets was 20~25% and the absolute number of  $2 \sim 8 \times 10^8$  cells was obtained from 8 ml blood. Contamination of mononuclear cells in the platelet preparation was less than 0.05% throughout the study.

#### 2.5 Determination of ATP released from washed platelets

The amount of ATP released from thrombin-stimulated platelets was determined by a Bioluminescence assay with firefly enzyme (Detwiler and Feinmann, 1972) using a luminescence reader TD-4000 (Laboscience Co., Japan). Two hundred  $\mu\text{l}$  of standard ATP solution ( $0 \sim 5.0$   $\mu\text{M}$ ) in Tyrode-gelatine-EGTA pH 7.4 was mixed with 100  $\mu\text{l}$  of Ca, Mg(+) PBS (1mM  $\text{Ca}^{2+}$ , 12.5mM  $\text{Mg}^{2+}$ , pH7.4), 100  $\mu\text{l}$  of Ca, Mg(-) PBS (pH 7.4) and 100  $\mu\text{l}$  of firefly lantern extract (FLE-50, Sigma Co.) suspended in Ca, Mg(-) PBS. The mixture was set in the luminescence reader and the peak value of luminescence intensity (y) was recorded at a temperature  $25^{\circ}\text{C}$ . The concentration of ATP (x) and the luminescence intensity correlated well ( $r > 0.9980$ ). The standard regression line ( $y = ax$ ) was obtained by

plotting the peak value of luminescence intensity from each concentration of standard ATP.

Next, 200  $\mu\ell$  of the platelet suspension mixed with 100  $\mu\ell$  of Ca, Mg (+) PBS and 100  $\mu\ell$  of FLE-50 solution was stimulated by adding 100  $\mu\ell$  of bovine thrombin (Green Cross Co., Japan) suspended in Ca, Mg (-) PBS (4 U/ml final) and the peak value of luminescence intensity (Y) was measured in the luminescence reader. The amount of ATP released from  $1 \times 10^8$  platelets (X) was obtained from the standard regression line ( $y=ax$ ). We measured an average value obtained from three reactions on each sample.

Luminescence curve for the standard ATP solution and for the platelet suspensions reached their peak values during 3~5 and 30~60 seconds, respectively, after the start of the reaction, and slowly declined thereafter. No luminescence was detected in the case of medium alone or unstimulated platelets. The measurement was finished within 3 hours after blood collection. In our preliminary study platelet ATP release did not change significantly in the suspension within 2 to 6 hours after blood collection. For ATP release, the interassay coefficient of variation was 8.0% and the intra-assay coefficient of variation 5.8%. The peak value was employed in the following studies, because both the peak value and the integral value gave a strong correlation ( $r=0.9971$  for standard ATP solution,  $r=0.9930$  for stimulated platelet suspensions). Fig. 1 shows the dose response curve of thrombin for released ATP. Released ATP reached a plateau at more than 2 U/ml, and a saturation dose of 4 U/ml was employed in the study to avoid errors caused by the possible loss of thrombin activity. Four U/ml was a saturation dose in each subject.

## 2.6 Antigen provocation tests and acetylcholine inhalation tests

Antigen provocation tests and acetylcholine inhalation tests were carried out according to the standardized method of Japanese Society of Allergology (Miyamoto, 1982). The patients were stable in clinical condition and all medications (oral theophylline and/or  $\beta$ -stimulants) were withheld for 12 h before the test. Patients receiving steroids were excluded. Each patient gave informed consent and the study protocol was approved by the ethics committee of the Chest Disease Research Institute. The extract of antigen or acetylcholine was nebulized by a DeVilbiss No. 646 nebulizer with an airflow of 5 liters/min. by the tidal breathing method. Each test was preceded by saline inhalation, which caused no decrease of 5% or more in forced expiratory volume in one second (FEV<sub>1.0</sub>).

The antigens challenged were *Dermatophagoides farinae* (4 cases; case 1, 3, 4, and 5 in Fig. 6.), rabbit hair (case 2) and cat hair (case 6). The antigen provocation tests were carried out by 2 minutes inhalation of increasing dose of allergens initiated by 1/100 dose of skin test threshold. FEV<sub>1.0</sub> was measured 10 and 20 minutes after the end of inhalation of each allergen dose. Immediate asthmatic response was determined when FEV<sub>1.0</sub> fell down more than 20% from a baseline. Then FEV<sub>1.0</sub> was followed each half an hour for 10 hours. Blood was collected before the inhalation of allergens, and when IAR was detected, when patient was improved from IAR, and when LAR was detected.

The acetylcholine inhalation tests were carried out by 2 minutes inhalation of increasing doubling doses from 39  $\mu$ g/ml. FEV<sub>1.0</sub> was measured just after each inhalation. Blood was collected just before the inhalation of 39  $\mu$ g/ml and just after the maximal (at least 20%) fall of FEV<sub>1.0</sub>. The time intervals between the two blood collections were between 20 and 60 minutes.

## 2.7 Heparin-induced PF4 release in vivo

As previously reported (Tsukamoto *et al.*, 1987; Dawes *et al.*, 1982), 60 units/kg of heparin sodium (Novo Heparin; Novo Industry, Denmark) was injected into the median cubital vein with 4 times volume of saline after 10 ml blood collection for the measurement of the baseline PF4 (Pre. PF4) using a butterfly needle. Just 5 minutes later, 10 ml blood was withdrawn from the contralateral median cubital vein for the measurement of PF4 after heparin injection (Post. PF4). We calculated the increment of PF4 after heparin injection ( $\Delta$ PF4) which was expressed as the difference in plasma concentration of PF4 before and after heparin injection ( $\Delta$ PF4 = Post. PF4 - Pre. PF4). Informed consent was obtained from each subject and the study was approved by the ethics committee of the Chest Disease Research Institute. The maximum dose of heparin injected was 3800 units and no adverse reactions were observed.

### 3. RESULTS

#### 3.1 Platelet release functions in patients with bronchial asthma during symptomatic and asymptomatic periods, compared with normal control subjects

ATP release from washed platelets (Fig. 2): The average amount of ATP released from washed platelets (mean  $\pm$  S. D. / $10^8$  platelets) was  $1.47 \pm 0.25$  nmol for 16 normal control subjects and  $1.55 \pm 0.24$  nmol and  $2.03 \pm 0.43$  nmol for 12 asthmatic patients during asymptomatic and symptomatic periods, respectively. Released ATP increased significantly during symptomatic periods compared with that during asymptomatic periods or that of normal subjects ( $P < 0.01$ ). This tendency was observed in each of all asthmatic patients. There was no significant difference between asymptomatic periods and normal subjects.

Plasma concentrations of PF4 and  $\beta$ -TG (Fig. 3): The plasma concentrations of PF4 in 16 normal subjects and in 15 asthmatic patients during their asymptomatic periods and symptomatic periods were  $4.4 \pm 1.2$  ng/ml,  $3.9 \pm 1.1$  ng/ml,  $7.7 \pm 3.6$  ng/ml, respectively, and the plasma  $\beta$ -TG concentrations were  $19.3 \pm 6.7$  ng/ml,  $18.7 \pm 7.1$  ng/ml,  $29.4 \pm 8.9$  ng/ml, respectively. Both plasma PF4 and  $\beta$ -TG were significantly elevated during symptomatic periods compared with those of normal subjects or asymptomatic periods ( $P < 0.01$ ). This tendency was observed in each of all asthmatic patients. There was no significant difference between asymptomatic periods and normal subjects.

Plasma  $\beta$ -TG/PF4 ratio (Fig. 4): The plasma  $\beta$ -TG/PF4 ratios in 16 normal control subjects and in 15 asthmatic patients during their asymptomatic periods and symptomatic periods were  $4.45 \pm 1.64$ ,  $4.90 \pm 1.52$ ,  $4.11 \pm 1.09$ , respectively. This ratio did not increase in asthmatic patients during symptomatic periods, and in fact, it decreased significantly ( $P < 0.01$ ).



Comparison between patients with allergic and non-allergic asthma (Fig. 2, 3, 4): The type of asthma, allergic or non-allergic, had no influence on the parameters determined above. Namely, the same tendency was observed irrespective of whether or not the patients had allergies defined as before. For example, released ATP from washed platelets during symptomatic periods in 9 allergic and 3 non-allergic asthmatic patients were  $2.03 \pm 0.47$  and  $2.00 \pm 0.35$  nmol/ $10^8$  cells respectively, showing no difference between the two patient groups, and in addition, were all elevated compared with those during asymptomatic periods in both groups.

Correlation between ATP release and plasma PF4 or  $\beta$ -TG level (Fig. 5): In order to obtain one of the evidences for the possibility of *in vivo* platelet release, we investigated correlation between ATP release *in vitro* and plasma PF4 or  $\beta$ -TG level *in vivo*, which are measured at the same time. There were correlations between ATP release and plasma PF4 level ( $r=0.5537$ ,  $P<0.001$ ) or  $\beta$ -TG level ( $r=0.5435$ ,  $P<0.001$ ) in all 40 measurements.

### 3.2 Platelet release functions during antigen provocation tests (Fig. 6)

The types of provoked asthmatic response were immediate asthmatic response (IAR) alone in case 4, 5, and 6, late asthmatic response (LAR) alone in case 3 and biphasic response (IAR and LAR) in case 1 and 2. Released ATP from washed platelets that was determined in 5 cases and plasma PF4 and  $\beta$ -TG that were determined in 5 cases were increased during both IAR and LAR compared with the pre-challenge value. In contrast, there were no relationship between the changes in plasma  $\beta$ -TG/PF4 ratios and the asthmatic responses; the values were elevated in some cases (case 1 and 2) but decreased in other cases (case 3, 4, and 5).



### 3.3 Platelet release functions before and after acetylcholine inhalations (Fig. 7)

The doses of acetylcholine inducing more than 20% fall of FEV<sub>1.0</sub> from a baseline were 313~1250 µg/ml in the 6 patients. The range of the maximal fall of FEV<sub>1.0</sub> was 23 ~35%. Either released ATP, plasma PF4, plasma β-TG or plasma β-TG/PF4 ratio did not significantly change after acetylcholine inhalation, showing that the heightened platelet release functions in patients with bronchial asthma were not due to merely bronchospasm.

### 3.4 Heparin-induced PF4 release in patients with bronchial asthma and normal control subjects (Fig. 8)

To determine why the elevation of plasma β-TG/PF4 ratio did not accompany the heightened platelet release functions in asthmatic patients, we measured the heparin-induced PF4 release which was reported to demonstrate PF4 binding on endothelial cell surface (Dawes *et al.*, 1982). The plasma PF4 before and after heparin injection (Pre. and Post.) in 11 normal control subjects, 12 asymptomatic asthmatic patients and 7 symptomatic patients were  $5.7 \pm 1.6$  and  $117 \pm 31$  ng/ml;  $4.8 \pm 1.7$  and  $85 \pm 29$  ng/ml;  $7.7 \pm 3.5$  and  $59 \pm 18$  ng/ml, respectively. The mean increment of plasma PF4 ( $\Delta$ PF4) after the heparin injection was  $110 \pm 30$  ng/ml in normal control subjects,  $80 \pm 29$  ng/ml in asymptomatic asthmatic patients and  $52 \pm 19$  ng/ml in symptomatic asthmatic patients. Both plasma PF4 after heparin injection (Post.) and  $\Delta$ PF4 were significantly reduced in asymptomatic patients compared with normal subjects ( $P < 0.05$ ), and it was much further reduced in symptomatic patients ( $P < 0.05$ ). The reduced  $\Delta$ PF4 suggests that PF4 binding, namely, the amount of PF4 which bound on endothelial cell surface, was reduced in asthmatic patients.

In addition, the average  $\Delta$ PF4 in asymptomatic patients without medication was  $73 \pm 32$  ng/ml and lower than that in normal controls ( $P < 0.05$ ), but not significantly different from that in asymptomatic patients with medication ( $85 \pm 29$  ng/ml). This suggests that medication, such as theophylline,  $\beta$ -stimulants or steroids, had no effect on  $\Delta$ PF4.

Plasma  $\beta$ -TG did not change after heparin injection and heparin did not stimulate platelet release (data not shown).

#### 4. DISCUSSION

In this article we examined firstly ATP release from washed platelets in patients with bronchial asthma during symptomatic and asymptomatic periods compared with normal control subjects. ATP is stored in dense granules of platelets and released when platelets are activated. Since the absolute amount of ATP detected varies according to the system employed, choosing an adequate control group and preliminary studies must be performed for each system. Our system for measuring ATP release was carefully developed according to the system reported by other investigators (Detwiler and Feinmann, 1972). We employed washed platelets instead of PRP in this study in order to avoid the possible modification of platelet activity by plasma components. We employed thrombin as the stimulant of platelets because this agent is the most potent stimulant of washed platelets, and because there is an evidence that thrombin generation occurs in bronchial asthma, i. e., that plasma fibrinopeptide A is elevated after antigen provocation (Metzger *et al.*, 1983).

In all asthmatic patients tested, the amount of released ATP from washed platelets stimulated with thrombin was higher during symptomatic periods than during asymptomatic periods (Fig. 2), and was elevated after antigen provocations (Fig. 6), but not during bronchospasm provoked by acetylcholine (Fig. 7). These findings suggest the existence of platelet hyperreactivity in release reaction, i. e., an increased amount of released products during acute exacerbation of bronchial asthma, and also suggest that this is not due to merely bronchospasm. Although patients are receiving medication which may influence platelet functions slightly, we would like to emphasize that increased releasability is observed during symptomatic periods in all of the patients despite the variety of medications used.

Recent studies have shown that thrombin-induced ATP release is increased in hyperreactive platelets which are reported to have large size and have increased

intra-granular contents (Thompson *et al.*, 1988). Large-sized platelets may have increased ATP content and therefore release more ATP. If this is true, our data are consistent with the report which shows that mean platelet volume is increased during symptomatic periods in bronchial asthma (Mizumoto *et al.*, 1989). We intended to investigate platelet releasability directly because there are some evidences that platelet release products possibly play some role in the mechanism of bronchial asthma (Brindley *et al.*, 1983; Chihara *et al.*, 1988). The stimulation with thrombin causes not only release of dense granules but also release of  $\alpha$  granules containing PF4 and  $\beta$ -TG. Therefore, we considered that thrombin-induced ATP release is one of the methods which represent the platelet releasability.

In cases of DIC or massive thrombotic diseases, platelets in the peripheral blood may become 'exhausted' and their ATP release may be decreased, although plasma PF4 and  $\beta$ -TG levels may be markedly increased. But bronchial asthma is not considered to be accompanied by such a massive thrombotic state and our observations do not concern the exhausted and degranulated platelets which have already released their granule contents, showing defects in release reaction. Indeed, some investigators report the existense of the platelet hyperreactivity in release reaction in mild thrombotic disorders (Joseph *et al.*, 1989). These platelets show increased amounts of intragranular contents and increased size (Thompson *et al.*, 1988), and are different from 'exhausted' platelets.

The roles of platelet in pathogenesis of bronchial asthma are not yet clarified. However, we speculate that the hyperreactive platelets which possess increased intra-granular contents may be directly induced by various sub-threshold stimuli in the mechanism of bronchial asthma or may be produced from intra-pulmonary megakaryocytes (Slater *et al.*, 1985) stimulated by various stimulants in the lung. There are some reports identifying platelets in the bronchus in asthmatic patients

(Metzger *et al.*, 1987; Jeffery *et al.*, 1989). It is possible that hyperreactive platelets release their contents locally in the bronchus and their released products then stimulate other types of cells such as eosinophils (Chihara *et al.*, 1988) and mast cells (Brindley *et al.*, 1983) as one of the mechanisms of bronchial asthma.

In our study, platelet activation was observed in both allergic and non-allergic asthmatic patients. Various mechanisms are reported to be involved in platelet activation. Besides the postulated mechanism through the Fc $\epsilon$  receptor II on platelet surface membrane (Capron *et al.*, 1985) in allergic asthma, platelet activation may occur by cell-to-cell interactions in the early stage of inflammation during asthmatic reactions; PAF generated by eosinophil, monocyte and neutrophil (Page, 1988), procoagulant activity of activated monocyte (Geczy *et al.*, 1981) and/or immune complexes (Henson and Ginsberg, 1981), for example, may act together, resulting in platelet activation. Further study is necessary to determine the details of this mechanism together with the variations of platelet activation for each type of asthma.

Secondarily, we also examined whether these hyperreactive platelets may release their intra-granular proteins *in vivo*. The plasma levels of both PF4 and  $\beta$ -TG were increase during symptomatic periods in all asthmatic patients, and also after antigen challenges, but not after inhalation of acetylcholine (Fig. 3, 6, 7). However, we could not find any increase in  $\beta$ -TG/PF4 ratio during symptomatic periods nor after antigen provocations (Fig. 4, 6).

In 1981, Kaplan and Owen reported that the plasma  $\beta$ -TG/PF4 ratio was the most reliable index of platelet activation *in vivo*, in contrast to the measurement of PF4 or  $\beta$ -TG alone which might be affected by the *in vitro* activation of platelets after blood collection. They postulated that PF4 and  $\beta$ -TG are released from  $\alpha$ -granules of platelets in similar quantities, but, the half life of PF4 is immeasurably short

because PF4 immediately binds to the heparin-like substance (heparan sulphate) on the vascular endothelial cell surface, whereas the half life of  $\beta$ -TG is about 100 minutes. Thus, the release of intra-platelet granular proteins *in vivo* was considered to be associated with a disproportionate increase in plasma  $\beta$ -TG compared with PF4, hence increasing the  $\beta$ -TG/PF4 ratio. If PF4 and  $\beta$ -TG are released wholly after blood collection they are measured equally in quantity, then  $\beta$ -TG/PF4 ratio does not increase in such *in vitro* release.

From this point of view, our findings that both PF4 and  $\beta$ -TG were increased without an increase in  $\beta$ -TG/PF4 ratio during acute exacerbation of asthma may be explained by the following two possible mechanisms: (1) The half life of PF4 in circulating blood is prolonged in bronchial asthma because of reduced PF4 binding to the endothelial cell surface: and (2) During exacerbation of asthma, platelets become hyperreactive and then easily release intragranular contents *in vitro* after blood collection. The latter mechanism seems possible from our data that show the platelet hyperreactivity in release reaction during acute exacerbation of asthma, and this was also suggested by Durham *et al.* (1985). But this mechanism solely cannot explain all of the discrepancy that PF4 or  $\beta$ -TG is increased without an increase of  $\beta$ -TG/PF4 ratio, since these hyperreactive platelets seem also to easily release PF4 and  $\beta$ -TG *in vivo*, making it difficult for us to distinguish between *in vivo* and *in vitro* release. Thus, we must consider cooperation with the former mechanism.

To assess the possibility of a prolonged half life of PF4 in bronchial asthma, we examined heparin-induced PF4 release *in vivo*. Dawes and coworkers (1982) postulate as follows; when a certain amount of heparin is injected intravenously, PF4 is rapidly detached from the endothelial cells and bind to this extrinsic heparin. This results in the elevation of PF4 level ( $\Delta$ PF4) in circulating blood. This  $\Delta$ PF4



represents the amount of PF<sub>4</sub> that has bound to the endothelial cell surface just before heparin injection. In our findings,  $\Delta$ PF<sub>4</sub> was reduced in asthmatic patients during asymptomatic periods irrespectively with or without medications compared with normal control subjects, and it was much further reduced in patients during symptomatic periods (Fig. 8). From these findings and the hypothesis of Dawes and Tsukamoto we can suggest that the PF<sub>4</sub> binding on the endothelial cell surface is reduced in bronchial asthma. This may lead to the prolonged half life of PF<sub>4</sub> in asthmatic patients, because it is reported that PF<sub>4</sub> binding is the determinant of the half life of PF<sub>4</sub> in the blood (Kaplan and Owen, 1981).

$\Delta$ PF<sub>4</sub> may also be reduced when heparin-like substance is released from activated mast cell into the circulating blood in asthmatic patients, because PF<sub>4</sub> on the endothelial cell will bind to this substance and plasma PF<sub>4</sub> level before heparin injection will be elevated compared with normal subjects, resulting in decrement of PF<sub>4</sub> on the endothelial cell. However, our data show that plasma levels of PF<sub>4</sub> are not elevated in asymptomatic patients, though  $\Delta$ PF<sub>4</sub> is reduced. Therefore, we conclude that this mechanism can be neglected.  $\Delta$ PF<sub>4</sub> may be also reduced when the internalization of PF<sub>4</sub> into the endothelial cell or into the tissue through the endothelium is accelerated, or when PF<sub>4</sub> binds more tightly to endothelial cells. Although the role and metabolism of PF<sub>4</sub> must be much more clarified, we can suggest some functional alteration in platelet-endothelial cell interaction is involved in the mechanism of bronchial asthma.

We can suggest the following possibilities for the mechanisms of reduced PF<sub>4</sub> binding to the endothelial cells in asthmatics; 1) the amount of heparan sulphate on the endothelial cell surface may be decreased, 2) the amount of anti-thrombin III which binds to the heparan sulphate competitively with PF<sub>4</sub> is increased. Although it is not yet proved which is the true mechanism, some cytokine such as interleukin-

1 which is reported to influence the coagulant activity of the endothelial cells (Bevilacqua *et al.*, 1984) may be involved in this mechanism.

In conclusion, we believe that 1) increased platelet releasability is involved in the exacerbation of bronchial asthma, 2) some functional alteration in platelet-endothelial cell interaction is suspected as one of the inflammatory processes in the mechanism of bronchial asthma, 3) no increase in  $\beta$ -TG/PF4 ratio may be explained by both the increased platelet releasability and prolonged half life of PF4.

## REFERENCES

American Thoracic Society. Pulmonary terms and symbols. *Chest* 1975; 67: 583.

Bevilacqua MP, Pober JS, Majeau Gr, Cotran RS, Gimbrone Jr, MA. Interleukin 1 induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. *J Exp Med* 1984; 160: 618-623.

Brindley LL, Sweet JM, Goetzl EJ. Stimulation of histamine release from human basophils by human platelet factor 4. *J Clin Invest* 1983; 72: 1218-1223.

Capron A, Amiesen JC, Joseph M, Auriault C, Tonnel AB, Caen J. New functions for platelets and their pathological implications. *Int Arch Allergy Appl Immunol* 1985; 77: 107-114.

Chihara J, Fukuda K, Yasuba H, Kishigami N, Sugihara R, Kubo H, Nakajima S. Platelet factor 4 enhances eosinophil IgG and IgE Fc receptor and has eosinophil chemotactic activity [Abstract]. *Am Rev Resp Dis* 1988; 137: A421.

Dawes J, Pumphery CW, McLaren KM, Prowse CV, Pepper DS. The *in vivo* release of human platelet factor 4 by heparin. *Thromb Res* 1982; 27: 65-76.

Detwiler TC, Feinmann RD. Kinetics of the thrombin-induced release of adenosine triphosphate by platelets. Comparison with release of calcium. *Biochemistry* 1972, 12: 2462-2468.

Durham SR, Dawes J, Kay AB. Platelets in asthma. *Lancet* 1985; II: 36.

Geczy CL, Hopper KE. Lymphokines promote procoagulant activity of macrophages. In: Forster O, Landy M, ed. Heterogeneity of mononuclear phagocytes. London: Academic Press, 1981; 400-405.

Greer IA, Winter JII, Gaffney D, McLoughlin K, Belch JJF, Boyd G, Forbes CD. Platelet activation in allergic asthma. *Thromb Haemost* 1985; 53: 438.

Gresele P, Todisco T, Merate F, Nenci GG. Platelet activation and allergic asthma. *N Engl J Med* 1982; 306: 549.

Hemmendinger S, Pauli G, Tenabene A, Pujol JL, Bessot JC, DPharm ME, Cazenave JP. Platelet function: Aggregation by PAF or sequestration in lung is not modified during immediate or late allergen-induced bronchospasm in man. *J Allergy Clin Immunol* 1989; 83: 990-996.

Henson PM, Ginsberg MH. Immunological reactions of platelets. In: Gordon JL ed. *Platelets in biology and pathology 2*. Amsterdam: Elsevier, 1981; 265-308.

Ind PW, Peters AM, Malik F, Lavender JP, Dollery CT. Pulmonary platelet kinetics in asthma. *Thorax* 1985; 40: 412-417.

Jeffery PK, Wardlaw AJ, Nelson FC, Collins JV, Kay AB. Bronchial biopsy in asthma: An ultrastructural, quantitative study and correlation with hyperreactivity. *Am Rev Respir Dis* 1989; 140: 1745-1753.

Johnson CE, Belfield PW, Davis S, Cooke NJ, Spencer A, Davies JA. Platelet activation during exercise induced asthma: effect of prophylaxis with cromoglycate and salbutamol. *Thorax* 1986; 41: 290-294.

Joseph R, D'Andrea G, Oster SB, Welch KMA. Whole blood platelet function in acute ischemic stroke; Importance of dense body secretion and effect of antithrombotic agents. *Stroke* 1989; 20: 38-44.

Kaplan KL, Owen J. Plasma levels of  $\beta$ -thromboglobulin and platelet factor 4 as indices of platelet activation *in vivo*. *Blood* 1981; 57: 199-202.

Knauer KA, Lichtenstein LM, Adkinson NF, Fish JE. Platelet activation during antigen-induced airway reactions in asthmatic subjects. *N Engl J Med* 1981; 304: 1404-1407.

Metzger WJ, Henricksen RA, Zaleski T, Donnelly A. Evidence for platelet release and thrombin generation in early and late asthmatic responses [Abstract] Clin Res 1983; 31: A164.

Metzger WJ, Sjoerdsma K, Richerson HB, Moseley P, Zvala D, Monick M, Hunninghake GW. Platelets in bronchoalveolar lavage from asthmatic patients and allergic rabbits with allergen-induced late phase responses. In: Schmitz-Schumann M, Menz G, Page CP, eds. Agents and Actions Supplements vol. 21: PAF, platelets, and asthma. Basel: Birkhäuser Verlag, 1987: 151-159.

Mizumoto Y, Kunimatsu M, Ako H. Blood platelets activation and changes in the coagulation and fibrinolysis systems during bronchial asthmatic attacks (In Japanese). Jap J Chest Dis 1989; 48: 389-395.

Miyamoto A. Standardized methods of inhalation tests for bronchial asthma and hypersensitivity pneumonitis. (In Japanese) Jap J Allerg 1982; 31: 1074-1076.

Page, CP. The role of platelet-activating factor in asthma. J Allergy Clin Immunol 1988; 81: 144-152.

Shephard EG, Malan L, Macfarlane CM, Mouton W, Joubert JR. Lung function and plasma levels of thromboxane B<sub>2</sub>, 6-ketoprostaglandin F<sub>1α</sub> and β-thromboglobulin in antigen-induced asthma before and after indomethacin pretreatment. Br J Clin Pharmacol 1985; 19: 459-470.

Slater DN, Trowbridge EA, Martin JF. The platelet in asthma. Lancet 1985; i: 110.

Taytard A, Guenard H, Vuillemin L, Bouvot JL, Vergeret J, Ducassou D, Piquet Y, Treour P. Platelet kinetics in stable atopic asthmatic patients. Am Rev Respir Dis 1986; 134: 983-985.

Thompson PJ, Hanson JM, Bilani H, Turner-Warwick M, Morley J. Platelet, platelet activating factor and asthma [Abstract]. Am Rev Respir Dis 1983; 129: A3.

Thompson CB, Jakubowski JA. The pathophysiology and clinical relevance of platelet heterogeneity. *Blood* 1988; 72: 1-8.

Tofler GH, Brczinski D, Schafer AI, Czeisler CA, Rutherford JD, Willich SN, Gleason RE, Williams GH, Muller JE. Concurrent morning increase in platelet aggregability and the risk of myocardial infarction and sudden cardiac death. *N Eng J Med* 1987; 316: 1514-1518.

Toga H, Ohya N, Kitagawa S, Yoshida A. Clinical studies on plasma platelet factor 4 in patients with bronchial asthma. *Jap J Allerg* 1984; 33: 474-479.

Tsukasato T, Maruyama Y, Maruyama I, Shinmyozu K, Igata A. The heparin infusion test as a useful method for evaluating of endothelial antithrombogenic activity. (in Japanese) *J Clin Exp Med (Igaku No Ayumi)* 1987; 141: 107-108.



Asthmatic patients :

type	age sex	Medication			Asymptomatic period			Symptomatic period		
		Theophylline	Procaterol	Steroids	PF4	$\beta$ -TG	ATPr.	PF4	$\beta$ -TG	ATPr.
Allergic	46. F.	400mg/day	p. o.	Pred.	3	7	1.98	18	44	2.58
	63. F.	400	p. o.	Tr. a.	3	13	1.34	8	23	2.22
	50. F.	400	p. o.	Tr. a.	3	14	1.62	6	23	2.08
	39. M.	500	p. o.	no	4	14	1.48	9	31	1.86
	55. M.	400	p. o.	no	7	25	1.25	8	27	1.38
	30. M.	400	p. o.	no	4	10	—	5	18	—
	44. F.	400	p. o.	no	5	28	—	10	31	—
	48. M.	400	MDI	no	3	15	1.57	5	21	1.73
	37. F.	no	MDI	no	3	20	1.13	5	25	1.46
	39. M.	no	MDI	no	5	29	1.72	7	37	2.73
	57. M.	no	MDI	no	4	31	1.77	7	44	2.26
non- Allergic	50. F.	400	p. o.	Pred.	3	16	1.52	12	44	2.29
	44. F.	400	p. o.	Tr. a.	4	19	1.44	5	20	1.52
	47. M.	500	p. o.	no	4	20	—	5	27	—
	19. M.	no	no	no	3	20	1.79	5	26	2.10

Table 1. Medication during the 6 days preceding the study and the raw data of platelet function tests in each asthmatic patient.

p.o.; oral administration of procaterol 0.05~0.1 mg/day, MDI; administered by metered dose inhaler, no; no medication, Pred.; oral administration of prednisolone 20mg/day, Tr. a.; triamcinolone acetonide 20~30 mg intramuscularly administered once per one or two months, PF4; plasma PF4 level (ng/ml),  $\beta$ -TG; plasma  $\beta$ -TG level (ng/ml), ATPr.; ATP release from washed platelets (nmol/10<sup>8</sup> platelets).

Normal subjects :

age.	sex	PF4	$\beta$ -TG	ATPr.
60.	M.	4	16	1.45
59.	M.	4	25	1.18
52.	M.	4	8	1.47
34.	M.	3	9	1.79
30.	M.	4	25	1.57
28.	M.	6	24	1.27
27.	M.	5	21	1.41
25.	M.	5	15	1.72
23.	M.	5	19	1.71
19.	M.	5	23	1.69
60.	F.	7	22	1.24
59.	F.	6	26	1.41
35.	F.	3	7	1.06
30.	F.	3	20	1.04
25.	F.	3	19	1.79
21.	F.	4	29	1.47

Table 2. The raw data of platelet function tests in 16 normal control subjects.

PF4; plasma PF4 level (ng/ml),  $\beta$ -TG; plasma  $\beta$ -TG level (ng/ml), ATPr.; ATP release from washed platelets (nmol/10<sup>8</sup> platelets).

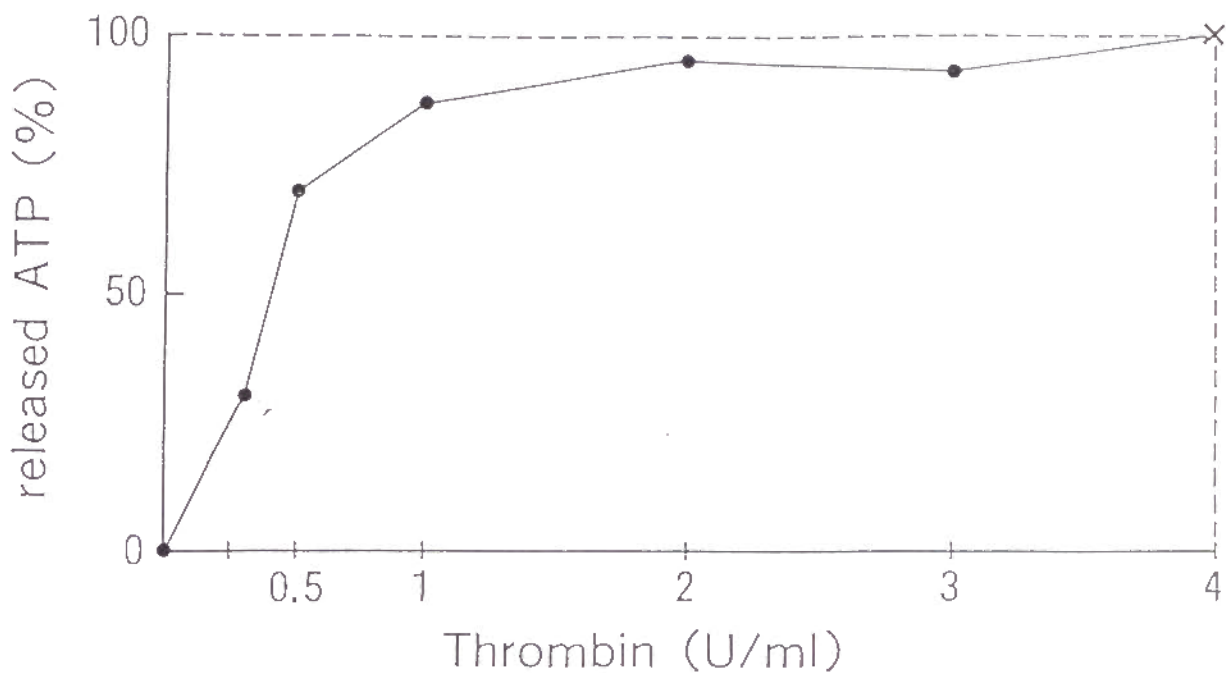


Fig. 1. The dose response curve of thrombin concentration for platelet ATP release. Mean value in two normal subjects is shown. The amount of released ATP is shown as a percentage of that released when stimulated with 4 U/ml thrombin.

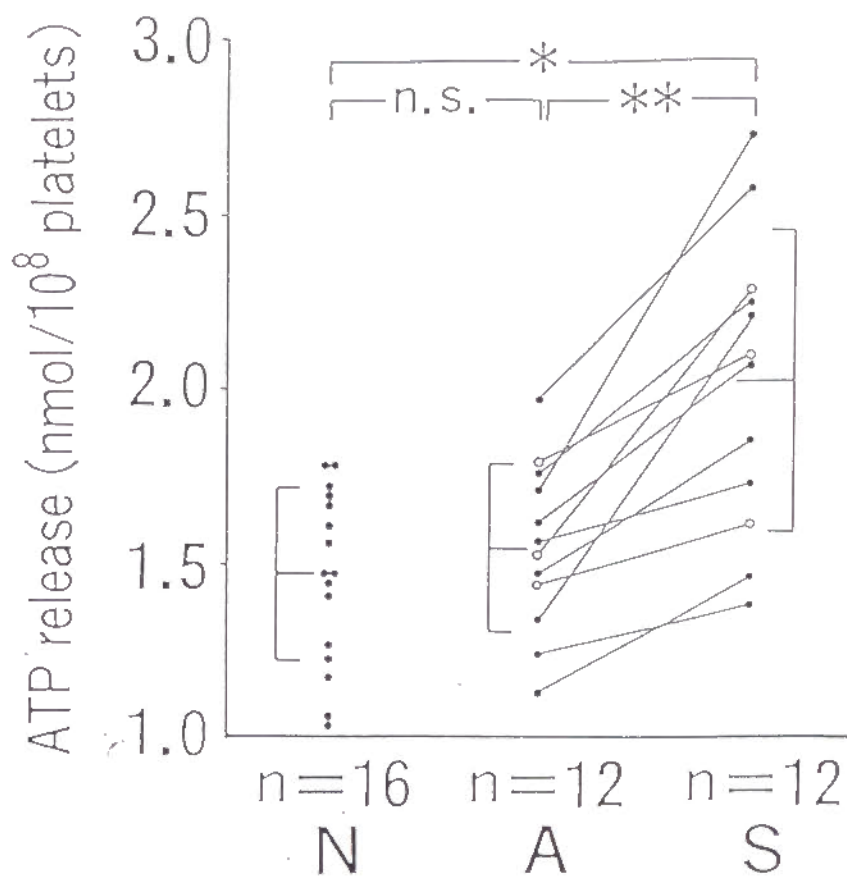


Fig. 2. Amount of ATP released from  $1 \times 10^8$  washed platelets stimulated by 4 U/ml thrombin. Comparisons were made among 16 normal control subjects (N), 12 asthmatic patients ( $\bullet - \bullet$  allergic,  $\circ - \circ$  non-allergic) during asymptomatic periods (A) and during symptomatic periods (S).

(\*  $p < 0.01$  by student t test; \*\*  $p < 0.01$  by paired t test)

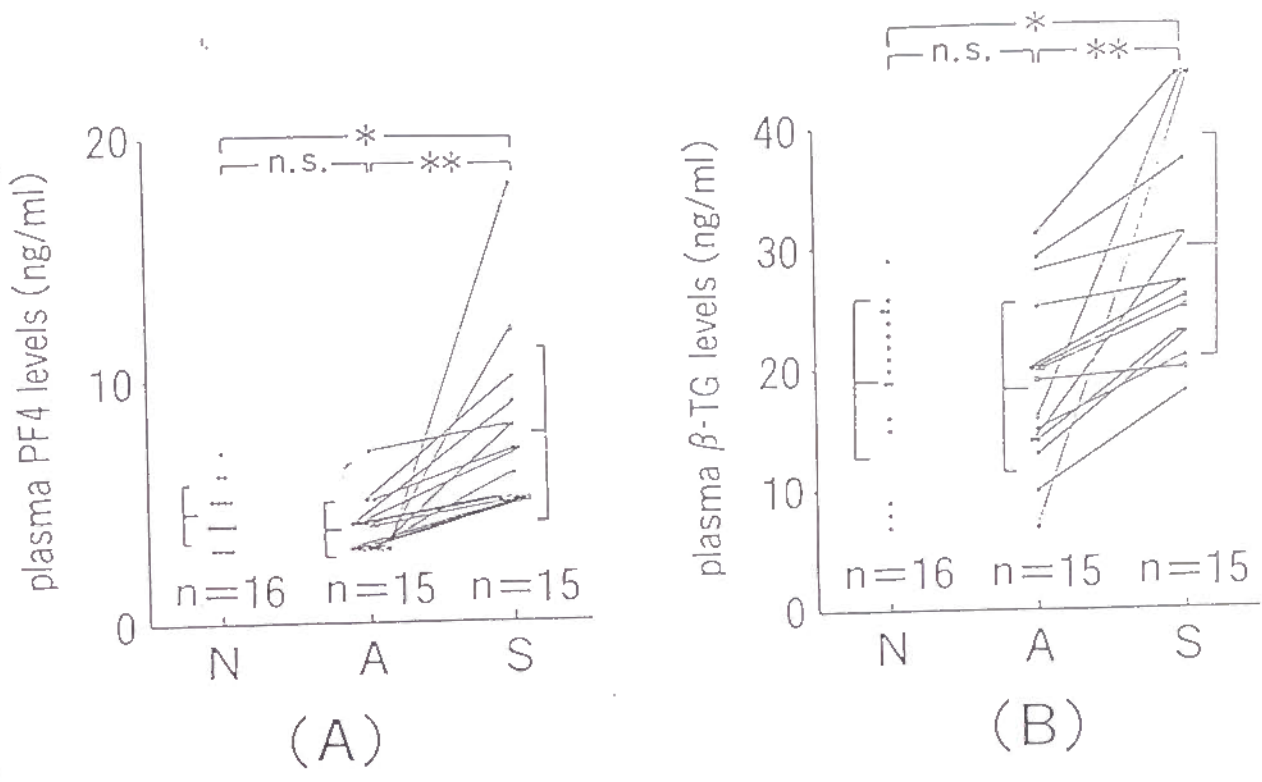


Fig. 3. (A) Plasma PF4 levels and (B) plasma  $\beta$ -TG levels in 16 normal subjects (N), 15 asthmatic patients ( $\bullet$  —  $\bullet$  allergic,  $\circ$  —  $\circ$  non-allergic) during asymptomatic periods (A) and during symptomatic periods (S). (\*  $p < 0.01$  by student t test; \*\*  $p < 0.01$  by paired t test)

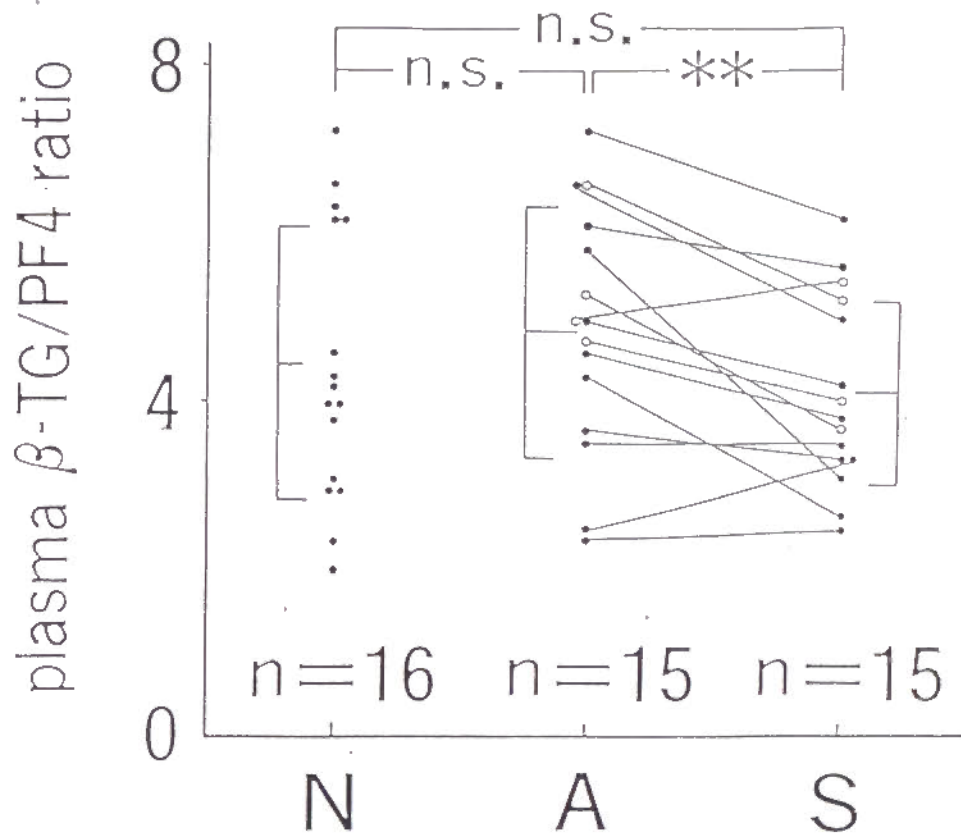


Fig. 4. Plasma  $\beta$ -TG/PF4 ratios in the same individuals shown in Fig. 4.  
 (\*\*  $p < 0.01$  by paired t test)



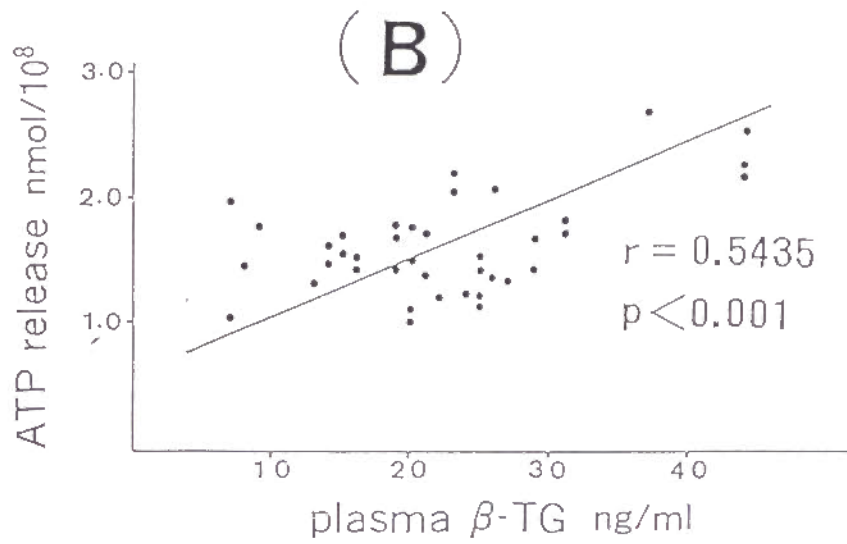
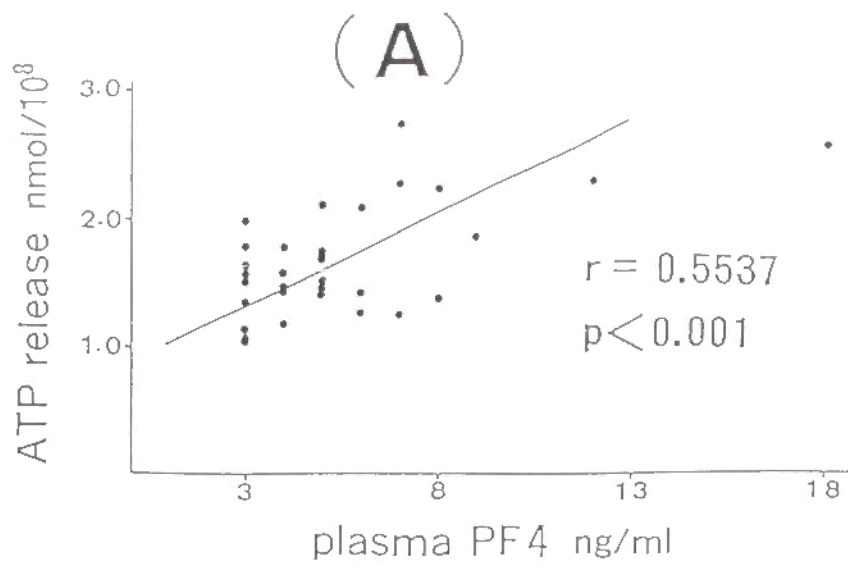


Fig. 5. Correlation between *in vivo* and *in vitro* release in normal subjects (16 measurements) and asthmatic patients (24 measurements);

(A) plasma PF4 levels and platelet ATP release,

(B) plasma  $\beta$ -TG level and platelet ATP release.

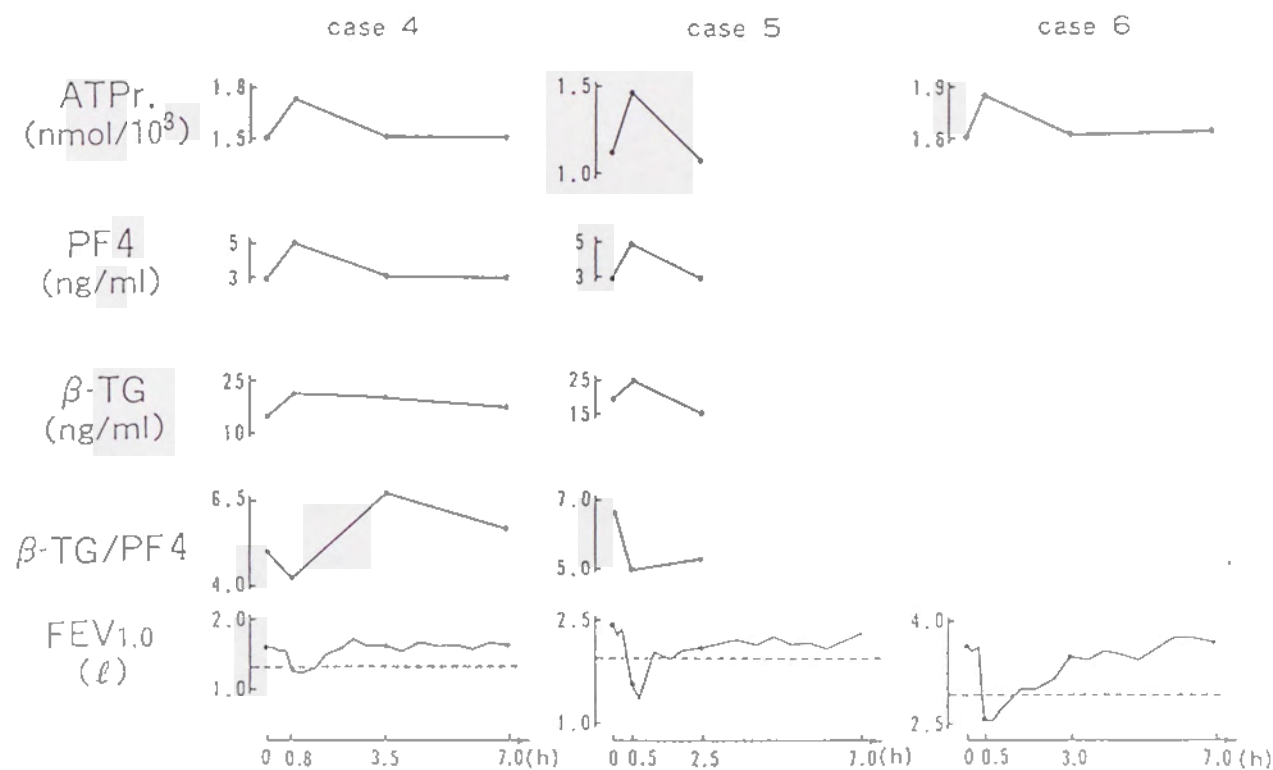
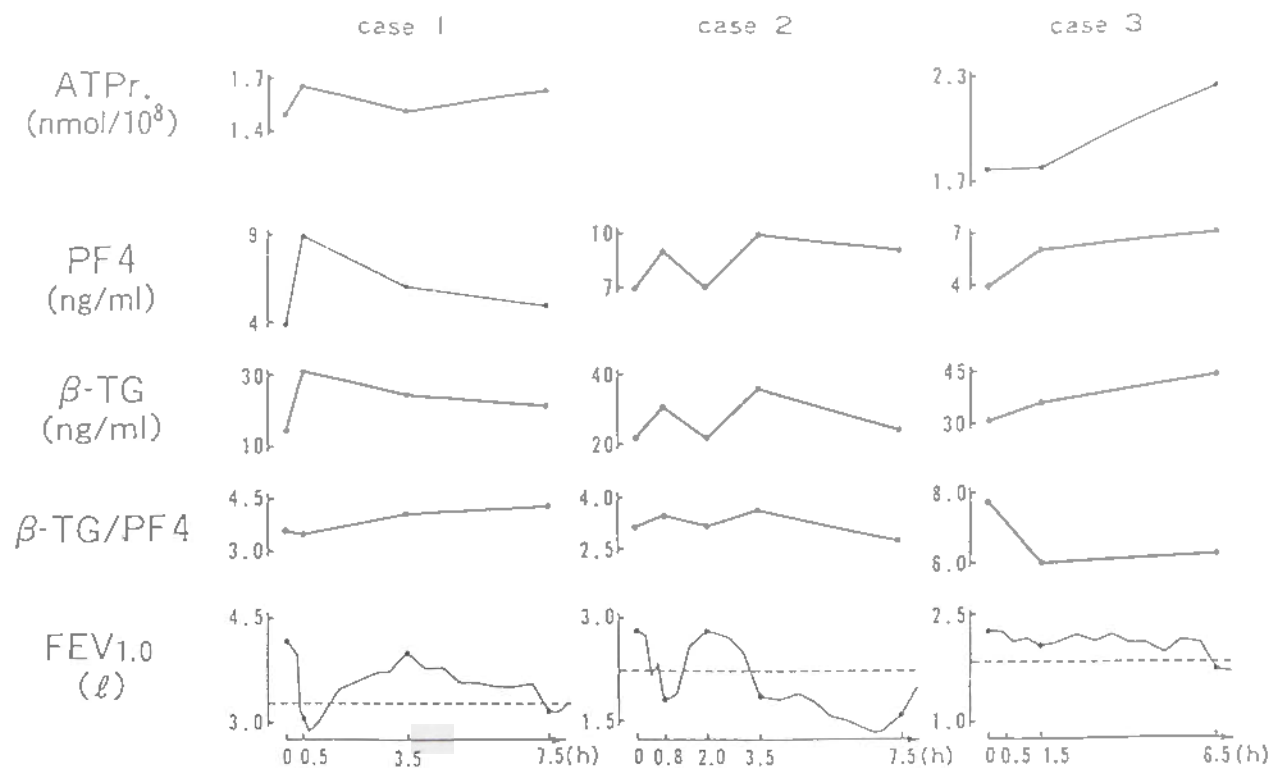


Fig. 6. The time courses (hours after the last inhalation of allergen) and the changes of platelet ATP release (ATPr.), plasma PF4 levels, plasma  $\beta$ -TG levels, plasma  $\beta$ -TG/PF4 ratios and FEV<sub>1.0</sub> (liter) during antigen provocation tests. 20% reduction of FEV<sub>1.0</sub> is expressed as the dotted line in each case.

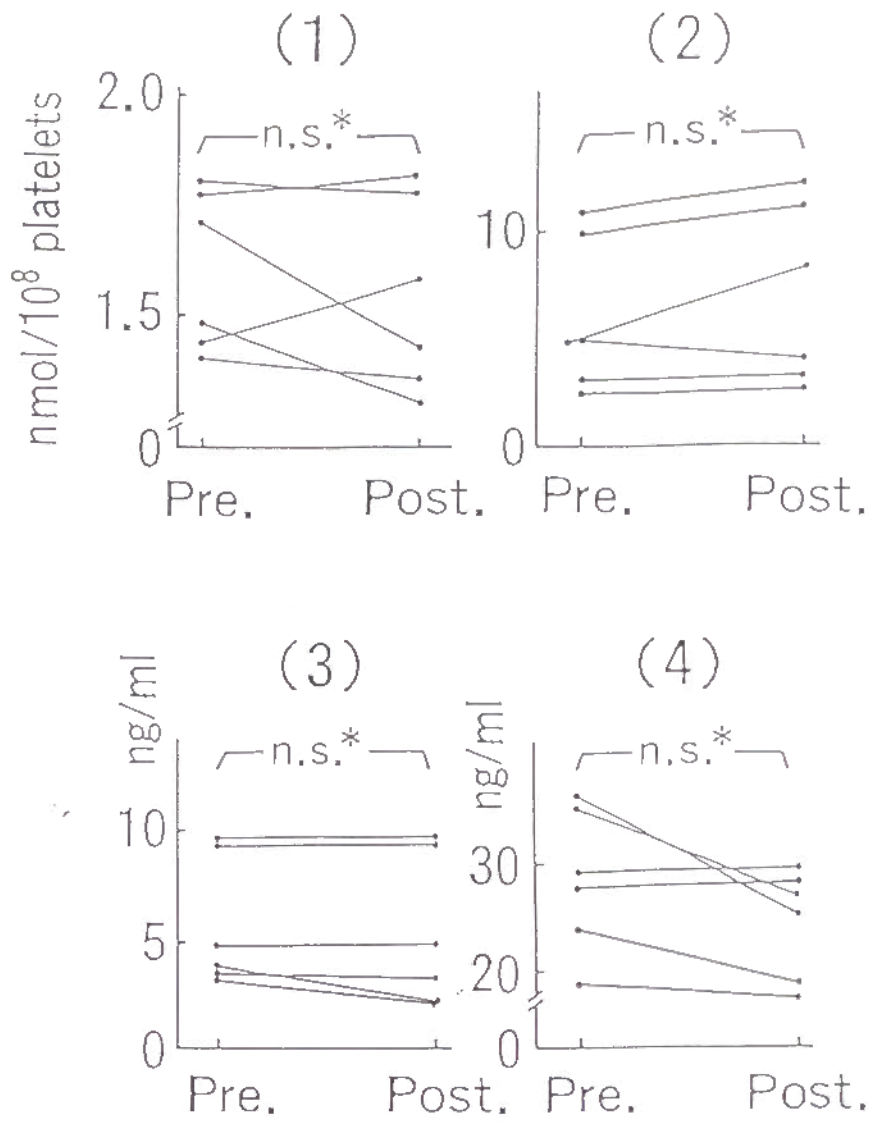


Fig. 7. Comparisons of (1) ATP release from washed platelets, (2) plasma  $\beta$ -TG/PF4 ratios, (3) plasma PF4 levels and (4) plasma  $\beta$ -TG levels before (Pre.) and after (Post.) acetylcholine inhalations. (\* not significant by paired t test)

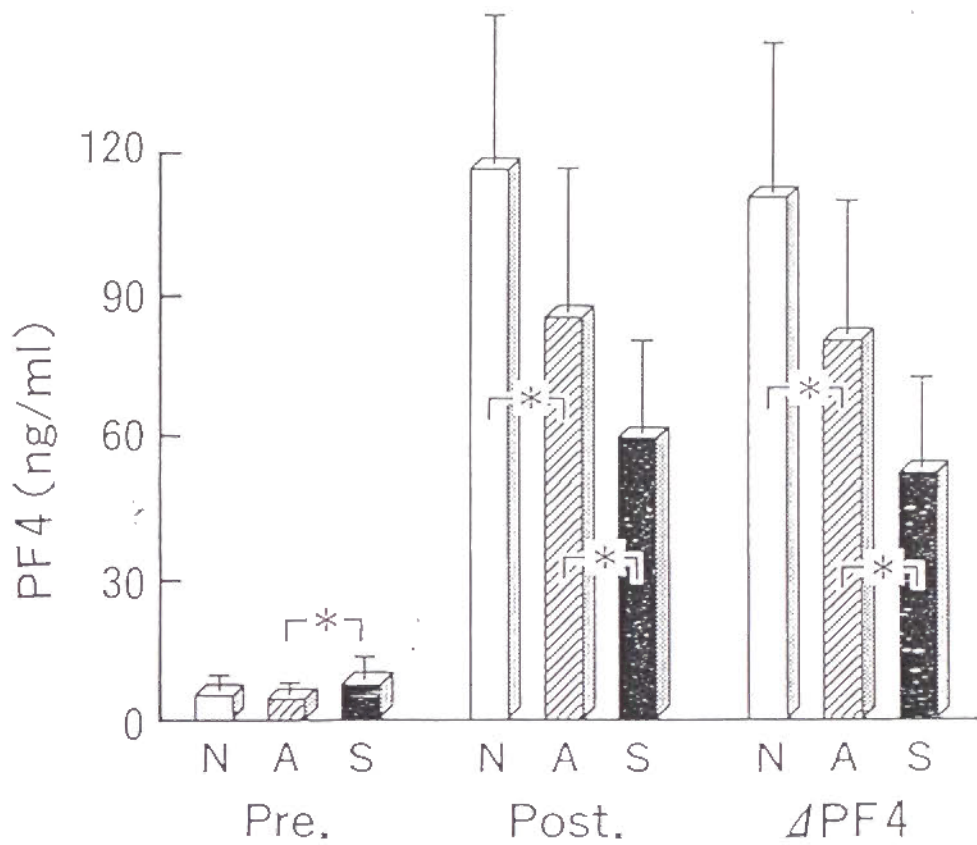


Fig. 8. Plasma PF4 before heparin injection (Pre.), 5 minutes after heparin injection (Post.) and  $\Delta$ PF4 in heparin-induced PF4 release. Comparisons were made among 11 normal control subjects (N), 12 asymptomatic asthmatic patients (A) and 7 symptomatic patients (S). (\*  $p < 0.05$  student t test)