

Failure of inhibitory effects by platelets on superoxide anion generation from stimulated neutrophils in a severe bronchial asthmatic

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Blood was drawn from healthy human volunteers and a 30-year-old woman with severe bronchial asthma (atopic and perennial type), whose peripheral blood platelets counts were continuously higher than $60 \times 10^4 / \text{mm}^3$. Neutrophils were purified on a Conray-Ficoll gradient and platelets were isolated by a gel filtration method with Sepharose 2B. Superoxide anion (O_2^-) generation from the cells was measured by 2-methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo [1,2-a] purazin-3-one (MCLA)-dependent luminescence. Addition of $0.5 \mu\text{M}$ MCLA and 10^{-8}M N-formyl-methionyl-leucyl-phenylalanine (fMLP) or 10 ng/ml phorbol myristate acetate (PMA) to a suspension of platelets obtained from the healthy controls and the patient caused no significant MCLA-dependent luminescence. O_2^- generation by neutrophils from healthy human volunteers, activated by 10^{-8}M fMLP or 10 ng/ml PMA, was inhibited by their platelets in a concentration-dependent manner. Platelets from the patient failed to inhibit O_2^- generation from neutrophils activated by 10^{-8}M fMLP. Cu-Zn superoxide dismutase concentrations in platelets from healthy human volunteers were not higher than the patient's. These data suggest that the absence of inhibition of superoxide generation from activated neutrophils by platelets might be involved in the pathogenesis of bronchial asthma.

Key words: bronchial asthma; neutrophil; platelet; superoxide anion; superoxide dismutase

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Airway hyperresponsiveness is a characteristic feature of bronchial asthma. Despite numerous clinical investigations, the precise causes of hyperresponsiveness are yet unknown. In recent years a number of studies have suggested that airway inflammation caused by polymorphonuclear cells, such as neutrophil, eosinophil and basophil, may play a role in its development. Recent animal models support neutrophil influx as critical to the development of airway hyperresponsiveness (19). Neutrophils may exert their effects in airway hyperresponsiveness through the generation of superoxide anion (O_2^-) and other oxygen-free radicals that induce lung tissue damage (24). Recent in-

vestigations have indicated that O_2^- generation from neutrophils in subjects with bronchial asthma correlated with the degree of airway hyperresponsiveness to inhaled methacholine (14).

Since Morley et al. (15) proposed that platelet-dependent events may determine exacerbation of bronchial asthma, a series of studies have addressed the problem of platelet participation in bronchial asthma. Much of the data to support this concept have been drawn from experiments performed on guinea pigs, rabbits and rats. However, the hypothesis of platelet involvement in human bronchial asthma is controversial. Some data (6,8) indicated that

platelets were activated during allergen-induced asthma after bronchial provocation tests and others (2,5) failed to detect evidence of its activation.

Luminol has often been used to amplify an emission by activated granulocytes even though it is not a specific luminescence compound for detecting any particular oxidant, because oxidation of luminol by any of a wide variety of oxidants leads to its characteristic luminescence (23). Recent investigations from Nakano's laboratory indicated that a chemiluminescent probe with a *Cypridina* luciferin analog, 2-methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo [1,2-a]pyrazin-3-one (MCLA) is useful for studying the ability of O_2^- generation by granulocytes, macrophages and eosinophils (9,16,25). This is a simple chemiluminescence method for estimating the rate of O_2^- formation from activated inflammatory cells *in vitro* serially as described (9). We investigated here the effects of platelets on O_2^- generation from neutrophils in a severe bronchial asthmatic patient, whose peripheral blood platelets counts were abnormally higher than $60 \times 10^4/\text{mm}^3$, with this unique chemiluminescence method to evaluate a possible platelet and neutrophil communication in allergic asthma.

Case presentation

A 30-year-old woman with bronchial asthma, free of bronchial asthmatic symptoms and any medications for the preceding 24 h, visited our hospital in September 1989 to undergo the study described below. At the age of 3 years the patient was diagnosed as having an atopic type bronchial asthma, based on the symptomatology, reversibility of airflow limitation by bronchodilator drugs, positive skin test to house dust antigen, and family history. She had had a series of admissions to our hospital, with severe bronchial asthma readily induced by house dust inhalation, infections, exercise and cold air. She had been controlled by medications, such as β -adrenergic bronchodilators, theophylline, inhaled cromolyn and prednisolone. Since the last admission in September

1981, she had been dependent on oral prednisolone therapy of 10 mg/day and was well controlled by medications of theophylline 700 mg/day, mabuterol hydrochloride 100–150 $\mu\text{g}/\text{day}$ and amlexanox 150 mg/day. Recent laboratory data, as summarized in Table 1, showed continuous elevations of peripheral blood platelet counts of more than $60 \times 10^4/\text{mm}^3$ since the last admission.

MATERIAL AND METHODS

Material

Dextran (Nacalai Tesque Inc., Kyoto, Japan); Ficoll 400, Sepharose 2B (Pharmacia Co., Uppsala, Sweden); Conray 400 (66.8% [w/v]) (Daiichi Seiyaku Co., Ltd., Tokyo, Japan); dimethyl sulfoxide (DMSO), phorbol myristate acetate (PMA), N-formyl-methionyl-leucyl-phenylalanine (fMLP), thrombin, xanthine oxidase (XOD, grade III), superoxide dismutase (SOD) (Sigma Chemical Co., St. Louis, MO, USA); hypoxanthine and others (Wako Pure Chemical Industries, Ltd., Osaka, Japan). MCLA was synthesized according to the method described (17).

Isolation of neutrophils

After informed consent was obtained, 10 ml of venous blood was drawn into a heparinized syringe (20 units/ml blood) from three healthy human volunteers, ranging in age from 28 to 32 years (mean, 30.0) and from the patient, and granulocytes were purified by the method of Boyum (1). Briefly, leukocytes were isolated by sedimentation in the presence of 6% (w/v) dextran followed by brief hypotonic lysis of contaminating erythrocytes (22). The resultant leukocytes were suspended in 10 ml Hanks' balanced salt solution (HBSS) (8.0 g/l NaCl, 0.4 g/l KCl, 0.14 g/l CaCl_2 , 0.2 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06 g/l $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.06 g/l KH_2PO_4 , 1.0 g/l glucose, pH 7.4). This was loaded on 15 ml Conray-Ficoll solution (16.7% [v/v] Conray 400, 72.0 g/l Ficoll 400, pH 7.4, density 1.0785) and centrifuged at 400 g for 15 min. Granulocytes were > 95%

pure and the purity of neutrophils was > 95 % by Wright staining. The viability of the cells was > 99 % in trypan blue exclusion test.

Isolation of platelets

Platelets were isolated by gel filtration (10) from the same venous blood. Briefly, 9 ml of venous blood obtained by venipuncture from the healthy donors and the patient was gently mixed with sodium citrate using a 9:1 ratio of blood and sodium citrate, yielding a final concentration of 0.38 % (v/v). Platelet-rich plasma was prepared by centrifugation of the blood at 200 *g* for 15 min and removal of the supernatant. Platelet-rich plasma was applied to siliconized columns (1.6 × 20 cm) containing Sepharose 2B equilibrated with HBSS. Platelets of > 99 % purity were eluted at the void volume and collected in plastic tubes. Functional integrity of the platelet preparations was routinely evaluated by 0.1–0.5 units /ml thrombin-induced aggregation as measured in a standard aggregometer and recorder (Chronolog Corporation, Haver Town, PA, USA).

Preparation of SOD samples from platelets

Platelets in HBSS were disrupted by sonication over ice using a Branson sonifier at a power

setting of 30 W in 30 consecutive 0.5-s bursts at 0.5-s intervals. This procedure was repeated three times at 1-min intervals. After obtaining the sonicated samples, platelet Cu-Zn SOD was separated by the procedure described by Oyanagui (20). Briefly, 2 ml of platelet sonicates were shaken well with 2 ml water, 1 ml ethanol and 0.6 ml chloroform for 15 min and centrifuged at 1,000 *g* for 10 min. Ethanol-water phase was aspirated as a sample.

Assay of O_2^- production

Unless otherwise indicated, the reaction mixtures contained the cells, one of the stimulatory agents, such as 10 ng/ml PMA, 10^{-8} M fMLP, and 0.5 μ M MCLA in 2.0 ml of continuously stirred buffer solutions. In some cases 0.5 μ M SOD was also present. All components, except for stimulatory agents and MCLA, were preincubated for 3 min at 37 °C and the reactions were started by the addition of MCLA and a stimulatory agent. Since PMA and fMLP have low solubility in water, each of them was dissolved to 1 mg/ml and 2 mM, respectively, in DMSO and diluted to a final concentration with the buffer solution immediately prior to the experiments (a final DMSO concentration was 0.01 % [v/v]). MCLA-dependent luminescence was monitored with the Aloka Company BLR-102 luminescence reader described previously (9, 16, 25). During luminescence measurement, the incubation mixture was rotatively agitated at 37 °C in the luminescence reader. Each experiment was repeated at least 3 times and each experiment of the same design gave similar results.

Assay of SOD activity

Cu-Zn SOD activities were determined by the MCLA chemiluminescence method as described (7). The standard reaction mixture contained 0.2 μ M MCLA, 50 μ M hypoxanthine, 6.5 units of XOD, 50 mM Tris-HCl buffer containing 0.6 mM EDTA at pH 7.8 and a sample, in a total volume of 3.0 ml. Chemiluminescence measurement was initiated

Table 1.

Main findings of laboratory examinations from the severe bronchial asthmatic patient

Peripheral blood cells counts		
RBC	508 × 10 ⁴ / mm ³	
Hb	8.4 g/dl	
Ht	26.2 %	
WBC	11000 /mm ³	
Neu	74.6 %	
Baso	0.2 %	
Eo	2.6 %	
Mo	6.8 %	
Lym	15.6 %	
Platelets	61 × 10 ⁴ /mm ³	
Serological allergic examinations		
IgE	307.8 U/ml	
RAST	HD	0.5 PRU/ml
	mite	4.1 PRU/ml

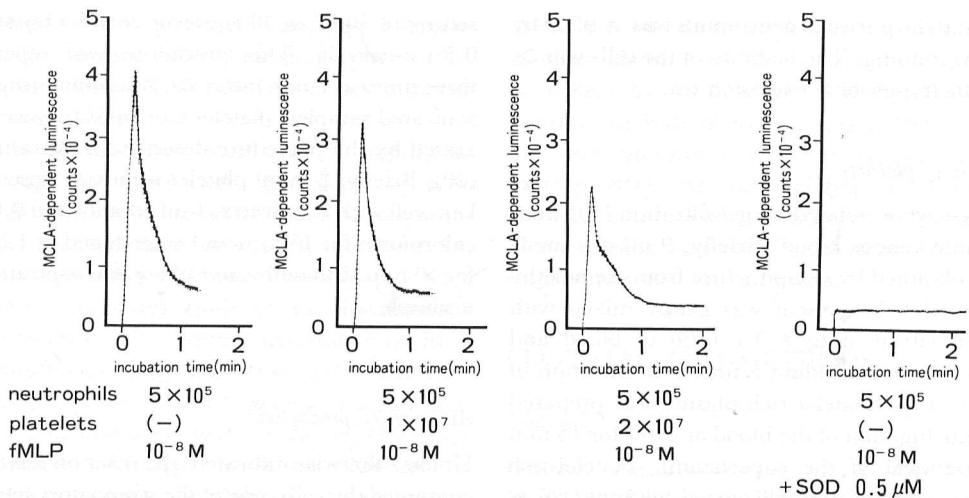


Fig. 1. Effects of platelets on MCLA-dependent luminescence by fMLP-stimulated peripheral blood neutrophils from healthy human volunteers. Two more experiments of the same design gave similar results.

by the addition of MCLA to the standard reaction mixture excluding XOD, continued for 2 min without additive and for an additional 4 min after the addition of XOD. Chemiluminescence was monitored with the Aloka Company BLR-102 luminescence reader at 25°C. The percent of SOD-dependent inhibition of XOD-induced luminescence was calculated and SOD concentration was obtained through the standard curve which was drawn using authentic SOD. The bovine erythrocyte SOD had a specific activity of 3200–3700 units/mg of protein in agreement

with the supplier's assay (Sigma Chemical CO.) of ≥ 3000 units/mg of protein.

RESULTS

MCLA-dependent luminescence by fMLP- or PMA-stimulated platelets from healthy subjects and the bronchial asthmatic

Platelets were isolated from the blood of healthy subjects and the bronchial asthmatic patient and MCLA-dependent luminescence from activated

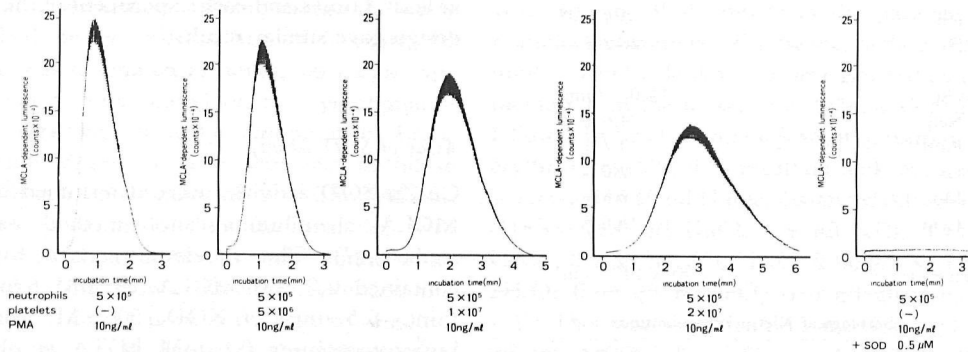


Fig. 2. Effects of platelets on MCLA-dependent luminescence by PMA-stimulated peripheral blood neutrophils from healthy human volunteers. Two more experiments of the same design gave similar results.

platelets were investigated. No detectable emission was obtained in HBSS containing platelets from healthy subjects or both the platelets and either of 10^{-8} M fMLP or 10 ng/ml PMA. Addition of 0.5 μ M MCLA in water and either of the stimulatory agents to a suspension of the platelets caused no detectable light. Also, addition of 0.5 μ M MCLA and 10^{-8} M fMLP to a suspension of platelets from the patient caused no significant MCLA-dependent luminescence.

Effects of platelets on MCLA-dependent luminescence by fMLP- or PMA-stimulated neutrophils from healthy subjects and the bronchial asthmatic

No detectable emission was obtained in HBSS containing neutrophils or both neutrophils and/or fMLP or PMA. However, as shown in Fig. 1, addition of MCLA and fMLP to a suspension of neutrophils purified from the blood of healthy controls caused a marked MCLA-dependent luminescence, reaching a maximum at 15 s and decreasing at 1 min, which was inhibited by SOD. The luminescence was decreased in the presence of platelets in a concentration-dependent manner. As shown in Fig. 2, addition of MCLA and PMA to a suspension of the neutrophils caused a higher MCLA-dependent luminescence than that by fMLP (9), reaching a maximum at 1 min and decreasing at 2.5 min, which was inhibited by SOD. The maximum light emission was also decreased in the presence of platelets in a concentration-dependent manner and a maximum light intensity was

prolonged in the presence of platelets. When MCLA and fMLP were added to a suspension of neutrophils purified from the blood of a bronchial asthmatic, a detectable light was emitted which reached a maximum at 15 s and decreased at 30 s as shown in Fig. 3. Platelets from the patient did not decrease the luminescence and a maximum light intensity was prolonged in the presence of platelets.

SOD activity in platelets

Cu-Zn SOD activities determined by the MCLA chemiluminescence method were expressed as nanograms of bovine erythrocyte SOD based upon a specific activity of 3500 units/mg of protein. SOD concentrations in 10^7 platelets from healthy human volunteers and the patient were 4.5, 4.8, 3.5, and 6.0 ng, respectively.

DISCUSSION

Addition of fMLP and MCLA to a suspension of platelets from healthy human volunteers and the bronchial asthmatic patient caused no detectable luminescence. The action mechanism of fMLP is thought to be via a specific receptor on the cell membranes (26) and this results in fMLP-stimulated platelets supports the evidence that human platelets do not possess fMLP receptor on the plasma membranes (4,21). PMA is known to stimulate platelets to secrete mediators and to aggregate through the activation of

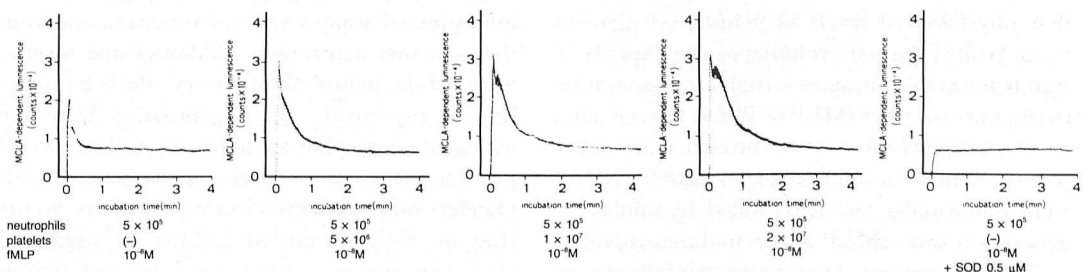


Fig. 3. Effects of platelets on MCLA-dependent luminescence by fMLP-stimulated peripheral blood neutrophils from the severe bronchial asthmatic patient. Two more experiments of the same design gave similar results.

so-called protein kinase C (18). Neither did the addition of PMA and MCLA to a suspension of normal human platelets emit detectable light. Marcus et al. (12) reported that the quantity of superoxide generation by platelets did not increase after aggregation by agents, such as collagen and thrombin, suggesting the aggregation process may not always result in a marked increase in O_2^- generation from platelets.

Addition of MCLA and either fMLP or PMA to a suspension of neutrophils from healthy human volunteers caused a marked MCLA-dependent luminescence which was inhibited by SOD. The MCLA-dependent luminescence from PMA-stimulated neutrophils was higher than that from fMLP-stimulated (9), indicating a higher O_2^- generation from PMA-activated neutrophils than that from neutrophils stimulated by fMLP. O_2^- generation from healthy controls, activated by fMLP or PMA, was inhibited by their platelets in a concentration-dependent manner. Ward et al. (27) reported the platelet-enhanced O_2^- generation in human neutrophils was stimulated by a variety of agonists. However, McGarrity et al. (13) demonstrated that platelets produced a concentration-dependent inhibition of O_2^- generation from fMLP- and PMA-stimulated neutrophils and suggested that methodological differences in the experiments could explain the observed differences. Namely, platelet rich plasma was used for the experiments by the former investigators, and the latter used a gel filtration for the isolation of human platelets. In the present study, platelets were isolated by gel filtration and our results support the data obtained by the latter investigators. Present data, together with the report of McGarrity et al. (13), indicate that physiological levels of gel-filtered platelets from healthy human volunteers are capable of significantly inhibiting neutrophil O_2^- generation in response to either fMLP or PMA. On the other hand, platelets from a severe bronchial asthmatic patient, whose peripheral blood platelet counts were abnormally elevated, failed to inhibit O_2^- generation from fMLP-stimulated neutrophils.

The mechanisms of the platelets inhibitory activity on O_2^- generation by the activated neutrophils from healthy human volunteers are as yet unknown. Since platelets contain SOD (12),

some of the inhibitory effect may be attributable to this enzyme in the platelets. However, addition of platelets to a cell-free system for O_2^- generation fails to affect the MCLA-dependent luminescence and thereby disputes a significant role for O_2^- scavenging as the primary mechanism for the inhibition of neutrophil responses by the platelets. Moreover, SOD concentrations in platelets from healthy human volunteers were no higher than the patient's. One possible explanation is adsorption of fMLP by platelets added to neutrophil reaction mixtures and reduction of the effective stimulus concentration. Using ^3H -fMLP, however, showed no specific binding or significant adsorption of this agent by platelets (13). The failure of platelets to adsorb fMLP suggests that direct physical interaction of platelets and neutrophils (21), and/or platelet mediator release evoked by activated neutrophils, may account for the observed inhibition by intact platelets (3). Platelet-derived growth factor has been demonstrated to block agonist-induced activation of the neutrophil oxidative burst, suggesting a significant role of platelets in the reduction of tissue damage induced by the neutrophil O_2^- generation and promotion of healing at sites of acute inflammation.

Airway inflammation with neutrophil infiltration may play a role in airway hyperreactivity (24) and neutrophils may exert their influence through the generation of superoxide anion. The most prominent finding of the laboratory data from the present bronchial asthmatic patient was the elevated peripheral blood platelets counts. Even though the functional integrity of the platelets measured by thrombin-induced aggregation was evaluated, the platelets failed to cause the inhibition of superoxide anion generation from the activated neutrophils. Although the mechanisms of the failure of inhibitory effects by platelets on superoxide anion generation from the activated neutrophils are at present unknown and possible medication effects can not be excluded, platelets may be altered during naturally occurring or antigen-induced asthma as suggested (11). The unique, continuously elevated peripheral blood platelet counts in the present caused our interest. Even though further investigation are required and more patients should be studied,

the present data might suggest the presence of abnormal platelet and neutrophil communications in some type of allergic asthma.

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