Deficient Induction of Leukotriene Synthesis in Human Neutrophils by Lipoxygenase-Deficient Platelets

By Kenji Kanaji, Minoru Okuma, and Haruto Uchino

The effect of human platelets with deficient lipoxygenase activities on leukotriene B4 (LTB4) synthesis by neutrophils was studied. When arachidonic acid (AA) metabolites obtained from the incubation of washed normal neutrophils and platelets with N-formylmethionylleucylphenylalanine (FMLP), cytochalasin B, and AA were analyzed by reversed-phase high-performance liquid chromatography, the synthesis of 5-lipoxygenase products, including LTB4, was remarkably stimulated by platelets, with their maximal effect at a ratio of platelets to neutrophils of 15:1. However, the use of lipoxygenase-deficient platelets obtained from MPD patients with myeloproliferative disorders instead of normal platelets showed the deficient production of 5-lipoxygenase-derived products, whereas platelets with normal lipoxygenase activities obtained from MPD patients stimulated the 5-lipoxygenase pathway similarly.

Arachidonic acid (AA) is converted via the 5-lipoxygenase pathway to eicosanoids, including leukotriene B4 (LTB4) and 5-hydroxyeicosatetraenoic acid (5-HETE), in polymorphonuclear neutrophils. LTB4 has potent chemotactic and chemokinetic activities and may be one of the major mediators of neutrophil function. On the other hand, platelets contain 12-lipoxygenase as well as cyclooxygenase. The former enzyme converts AA to 12-hydroxyeicosatetraenoic acid (12-HETE), which is reduced by peroxi-oxidase to 12-hydroxyeicosatetraenoic acid (12-HETE). Although the significance of the platelet 12-lipoxygenase pathway in platelet function is still obscure, recent studies have demonstrated the presence of biochemical interactions between platelets and neutrophils via lipoxygenase enzymes. Borgeat et al have shown that 5S,12S-dihydroxyeicosatetraenoic acid (5S,12S-diHETE) is a product of the successive reactions of AA with 5-lipoxygenase and 12-lipoxygenase. Marcus et al have demonstrated that AA and 12-HETE derived from platelets serve as substrates for eicosanoid formation by neutrophils and that both neutrophils and platelets need to be activated by a strong stimulus such as calcium ionophore A23187 for the production of significant amounts of 5S,12S-diHETE. Furthermore, Maclouf et al have reported that platelet-derived 12-HPETE stimulates LTB4 synthesis in human blood leukocytes, a finding that remains to be confirmed by other laboratories.

Patients with myeloproliferative disorders (MPD) are known to have a high incidence of infections, probably because of abnormal leukocyte function. Since LTB4 may regulate neutrophil function, it might be assumed that LTB4 synthesis is decreased in these patients. The deficient production of LTB4 may result not only from abnormalities of neutrophils themselves but also from abnormalities of platelets because platelets in some MPD patients are known to be deficient in 12-lipoxygenase activity.

In this study, we investigated the effects of platelets on AA metabolism by neutrophils, especially concerning LTB4 synthesis, when stimulated with N-formylmethionylleucylphenylalanine (FMLP) and AA in the presence of cytochalasin B. Lipoxygenase-deficient platelets from MPD patients showed a decreased activation of LTB4 synthesis in neutrophils, possibly because of deficient production of 12-HPETE by the platelets.

Materials and Methods

Patients and normal controls. Eight patients with MPD were investigated: three with chronic myeloid leukemia (CML) in the stable phase, two with polycythemia vera (PV), and three with essential thrombocythemia (ET). Diagnosis was determined by standard methods as described previously. Platelet AA metabolism was estimated by the thiobarbituric acid method by which deficient activities of the platelet lipoxygenase pathway were demonstrated in four of the eight patients. Seven normal volunteers were also studied as controls. Informed consent was obtained from all subjects.

Preparation of cell suspensions. Blood from normal donors and patients who had taken no drugs known to affect AA metabolism for at least 2 weeks before venipuncture was collected in 3.8% trisodium citrate solution (9 vol blood to 1 vol citrate) and centrifuged for the preparation of platelet-rich plasma as described previously. Neutrophils were prepared as described previously and suspended in the incubation buffer (pH 7.4) containing 50 mmol/L Tris-HCl, 101 mmol/L NaCl, and 5 mmol/L glucose. Neutrophil preparations from normal donors always contained more than 95% segmented neutrophils, and percentages of contaminating platelets were less than 30. Platelets were washed as described previously and finally suspended in the incubation buffer. Contamination of the platelet suspension by erythrocytes and leukocytes was negligible.

Preparation of 12-HPETE and 12-HETE. 12-HPETE and 12-HETE were prepared by a modification of the procedure reported by Pace-Asciak et al. In short, washed human platelets

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were resuspended in 50 mmol/L Tris-buffered saline, pH 7.4 (0.8 × 10⁷ platelets/mL), and disrupted sonically. After the lysate (8 mL) was incubated with 200 μmol/L AA in the presence of 100 μmol/L indomethacin for five minutes at 37 °C, the incubation mixture was placed on ice, immediately followed by the addition of ice-cold methanol (8 mL) and ice-cold diethyl ether (24 mL), and the mixture was acidified to pH 3.0 with 0.2 mol/L HCl. The ether phase was separated by rapid centrifugation at 4 °C and washed with 3 mL of ice-cold water. The ether phase was then dried over anhydrous sodium sulfate and evaporated to dryness under nitrogen. The residue was dissolved in hexane and purified by high-performance liquid chromatography (HPLC) using a column of Nucleosil 30-5 (4.6 × 250 mm, Macherey-Nagel, Düren, Germany) and a mobile phase consisting of hexane/isopropanol/acetic acid (98:2:0.1, vol/vol) at a flow rate of 1 mL/min. An Altex model 110A single-pump chromatograph (Altex Scientific, Berkeley, Calif) with a Soma S-310A variable-wavelength spectrophotometer detector (Soma Optics, Tokyo) was used. The identity of 12-HETE was confirmed by gas chromatography-mass spectrometry, and that of 12-HPETE was confirmed by reductive conversion to 12-HETE with stannous chloride. Quantitation was done by using UV absorption at 237 nm in ethanol. The purified material was dissolved in acetone, stored under nitrogen at −70 °C, and used within 2 weeks. The products used in the experiments were more than 97% pure, based on the HPLC analysis.

**Incubation and Extraction.** Unless otherwise specified, after cell suspensions containing 3 × 10⁷ neutrophils and 0 to 75 × 10⁶ (routinely 45 × 10⁶) platelets/mL were incubated with cytochalasin B (5 μg/mL) for five minutes at 37 °C, the mixtures were incubated with 10 μmol/L FMLP and 60 μmol/L AA for another five minutes at the same temperature. In experiments with 12-HPETE and 12-HETE, each was transferred to an assay tube, and the solvent was evaporated under nitrogen. Neutrophil suspensions (3 × 10⁷ cells/mL) preincubated with cytochalasin B (5 μg/mL) for five minutes at 37 °C were transferred to the assay tube without delay, immediately followed by the addition of 10 μmol/L FMLP and 60 μmol/L AA, and then the mixtures were incubated for another five minutes at the same temperature. When the calcium ionophore was used, neutrophils (3 × 10⁷ cells/mL) were incubated with 10 μmol/L A23187 and 60 μmol/L AA for five minutes at 37 °C. In all experiments with cell suspensions, 1 mmol/L CaCl₂ was added just before the incubation at 37 °C. The incubation was terminated by the addition of 4 vol ethyl acetate after acidification by 0.2 mol/L HCl to pH 3.0, and 450 ng of prostaglandin B₂ (PGB₂) was added as an internal standard. Extraction and evaporation were performed as described previously and evaporated residue dissolved in 30 μL methanol was subjected to chromatography as follows.

FMLP dissolved in the incubation buffer (1 mmol/L) and cytochalasin B in dimethyl sulfoxide (2 mg/mL) were stored at −20 °C and diluted with the incubation buffer when used. AA and A23187 were dissolved as described previously. Dimethyl sulfoxide was at a final concentration of less than 0.25%.

**HPLC Analysis.** Reversed-phase HPLC was routinely carried out using a column of Nucleosil C₁₈ (4.6 × 250 mm, Macherey-Nagel). The chromatograph and detector were the same as those used for preparation of 12-HPETE. A 10-μL aliquot of each sample was analyzed by the use of a mobile phase I consisting of methanol/water/acetic acid (75:25:0.01, vol/vol) at a flow rate of 1 mL/min. Another 10-μL aliquot was analyzed by a mobile phase II consisting of tetrahydrofuran/methanol/water/acetic acid (25:30:45:0.1, vol/vol) at a flow rate of 0.9 mL/min for the resolution of LTB₄ and SS,12S-diHETE. Column effluent was monitored at 270 nm for PGB₂, LTB₄, and SS,12S-diHETE and at 235 nm for 12-hydroxy-5,12-trans-diHETE (HHT), 12,15-trans-HETE, and 5-HETE. The amounts of 5-lipoxygenase-derived products were quantitated by comparing their peak areas with that of the internal standard (PGB₂). Two extinction coefficients used for the peaks of LTB₄, 12-HETE, and 12-HPETE were 48,700 for LTB₄ in the mobile phase (II), 27,900 for 5-HETE in the mobile phase (I), and 23,100 and 22,400 for PGB₂ in the mobile phase (I) and (II), respectively. The extinction coefficient of SS,12S-diHETE was assumed to be identical with that of LTB₄. Identification of LTB₄, 5-HETE and 12-HETE was confirmed by gas chromatography-mass spectrometry. SS,12S-diHETE and HHT were identified by comparisons of the chromatographic behaviors of each authentic sample.

**Materials.** FMLP and cytochalasin B were obtained from Sigma Chemical Co, St Louis; authentic SS,12S-diHETE was kindly donated by Dr Shozo Yamamoto, Department of Biochemistry, Tokushima University School of Medicine, Tokushima, Japan. Authentic HHT was prepared biosynthetically as previously described. The other agents were the same as described previously.

**RESULTS**

The incubation of neutrophils with FMLP, cytochalasin B, and AA led to the formation of small amounts of 5-lipoxygenase products, including LTB₄, SS,12S-diHETE, and 5-HETE, as illustrated in Fig 1A and 1C. However, the production of 5-lipoxygenase metabolites, especially LTB₄, was remarkably activated in the presence of platelets as shown in Figs 1B and 1D. Platelets (45 × 10⁷ cells/mL) incubated under these conditions in the absence of neutrophils produced significant amounts of 12-HETE and HHT, but no detectable amounts of 5-lipoxygenase products were obtained (data not shown).

The stimulating effect of added platelets on AA metabo-
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and 5-HETE was increased with this ratio up to 15:1, but a further increase to 25:1 reduced the production of these metabolites. SS,12S-diHETE generation peaked at a platelet:neutrophil ratio of 15:1. When the mixed cell suspension with the ratio of 15 to 1 was incubated with FMLP, cytochalasin B, and AA, the amount of LTB4 produced was similar to that obtained from the incubation of neutrophils with calcium ionophore A23187 and AA (Table 1).

In experiments using mixed cell suspensions incubated with cytochalasin B and AA in the presence of varying concentrations of FMLP, the amount of LTB4 produced was linearly increased with increasing FMLP concentrations up to 10 μmol/L, but the synthesis of 5-HETE and SS,12S-diHETE leveled off at its lower concentration (0.1 μmol/L) (Fig 2A). In experiments with similar cell suspensions incubated with FMLP and AA in the presence of varying concentrations of cytochalasin B, 1 μg/mL or higher concentrations of cytochalasin B reduced LTB4 synthesis but increased SS,12S-diHETE formation. No significant differences were observed in the 5-HETE formation (Fig 2B). In experiments with similar cell suspensions incubated with cytochalasin B and FMLP in the presence of various AA concentrations, the formation of LTB4 was greatest at AA concentrations from 60 μmol/L to 120 μmol/L. 5-HETE synthesis was greatest at AA concentrations higher than 90 μmol/L, but SS,12S-diHETE formation leveled off at 30 μmol/L. No 5-lipoxygenase products were consistently detectable without the addition of AA (Fig 2C).

When mixed cell suspensions were incubated with FMLP, cytochalasin B, and AA for varying periods of time, 5-HETE and LTB4 synthesis reached a maximum within three minutes and five minutes of incubation times, respectively, and decreased thereafter, whereas SS,12S-diHETE synthesis reached a steady level at one- to three-minute incubations and did not decrease thereafter (data not shown).

Fig 3 shows the effect of the addition of 12-HPETE instead of platelets on the production of 5-lipoxygenase metabolites by neutrophils incubated with FMLP and AA in the presence of cytochalasin B. This unstable product from platelet lipoxygenase stimulated the production of these metabolites in a concentration-dependent manner, and the effect of 10 μmol/L 12-HPETE was comparable to that obtained when platelets and neutrophils were incubated with FMLP, cytochalasin B, and AA. In contrast, stimulation was not observed when 12-HETE, a stable end product of the platelet lipoxygenase pathway, was added to the incubation mixture at the same concentrations (up to 10 μmol/L, data not shown).

Table 2 shows the effects of platelets from MPD patients on the production of 5-lipoxygenase metabolites by neutrophils from normal donors. Lipoxygenase-deficient platelets from four patients including two each with CML and ET (patients 1 to 4 in Table 2) brought about significantly reduced production of 5-lipoxygenase metabolites by neutrophils compared with normal platelets (P < .001, for LTB4, 5S,12S-diHETE, and 5-HETE, respectively), whereas platelets with normal lipoxygenase activities from four patients including two with PV and one each with ET and CML (patients 5 to 8 in Table 2) activated 5-lipoxygenase similarly to platelets from normal donors. Furthermore, it was demonstrated that the 5-lipoxygenase pathway in neutrophils from a patient with platelet lipoxygenase deficiency (patient 4 in Table 2) was normally stimulated by platelets from a normal donor, although

![Fig 2. Effects of stimulant concentrations on the synthesis of 5-lipoxygenase products by platelet-neutrophil interactions. Cell suspensions containing neutrophils and platelets (45 × 10⁷/mL) were incubated with varying concentrations of FMLP (A), cytochalasin B (B), or AA (C) as indicated in the presence of fixed concentrations of other two stimulants. The concentrations of FMLP, cytochalasin B, and AA were 10 μmol/L, 5 μg/mL, and 60 μmol/L, respectively. Products were analyzed as described in Materials and Methods. (A) and (B) were representatives of two experiments that yielded similar results, and (C) was that of three such experiments. Key: ○—○. LTB4; ∆—∆. 5-HETE; □—□. SS,12S-diHETE.](image-url)
the stimulation by this patient's platelets was reduced (Table 3).

**DISCUSSION**

Our experiments demonstrate that the synthesis of 5-lipoxygenase metabolites, including LTB₄, 5-HETE, and 5,12S-diHETE, by human neutrophils was enhanced by their incubation with human platelets in the presence of FMLP, cytochalasin B, and AA and that the effect of lipoxygenase-deficient platelets on AA metabolism was significantly decreased. FMLP is a formylated peptide similar to bacterial chemotaxins and activates various neutrophil functions. Stimulation of human neutrophils with the peptide in the presence or absence of cytochalasin B was reported to form a small amount of LTB₄ and its ω-carboxyl derivative. In our experimental conditions, FMLP was considered an effective stimulant of 5-lipoxygenase mediated by neutrophils and was considered a result of the conversion by the platelets themselves of AA to 5-HETE, an unstable intermediate of the 5-lipoxygenase pathway. Consumption of AA in the cell suspension medium by platelets via cyclo-oxygenase and 12-lipoxygenase pathways and liberation of AA from platelet phospholipids into the medium could occur, and they might alter the amount of this fatty acid available to neutrophils. Furthermore, platelet-derived AA has been reported to be transformed to LTB₄ and 5-HETE by stimulated neutrophils. However, these possible alterations in the AA concentration in the medium could not account for the stimulating effect of platelets on the LTB₄ production since LTB₄ produced by the incubation of neutrophils with the stimulants used in our experimental system was not significantly affected by the addition of AA from 30 μmol/L to 120 μmol/L (data not shown). In our incubations, time-course studies that demonstrated the transient increase of LTB₄ lessened the possibility that platelets reduced the transformation of LTB₄ via the ω-oxidation pathway in neutrophils, so that LTB₄ might accumulate in the incubation mixture with a five-minute incubation. Therefore, it was

**Table 2. Effects of Platelets From Patients With MPD on the Synthesis of 5-Lipoxygenase Products by Normal Neutrophils**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>PLO*</th>
<th>LTB₄†</th>
<th>5,12S-diHETE†</th>
<th>5-HETE†</th>
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<tr>
<td>1</td>
<td>51</td>
<td>F</td>
<td>ET</td>
<td>0.29</td>
<td>51.2</td>
<td>4.4</td>
<td>35.8</td>
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<td>2</td>
<td>27</td>
<td>F</td>
<td>ET</td>
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<td>29.8</td>
<td>3.1</td>
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</tr>
<tr>
<td>3</td>
<td>54</td>
<td>M</td>
<td>CML</td>
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<td>60.2</td>
</tr>
<tr>
<td>4</td>
<td>48</td>
<td>F</td>
<td>CML</td>
<td>0.06</td>
<td>52.7</td>
<td>2.2</td>
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</tr>
<tr>
<td>5</td>
<td>63</td>
<td>M</td>
<td>PV</td>
<td>1.18</td>
<td>88.8</td>
<td>30.7</td>
<td>111.4</td>
</tr>
<tr>
<td>6</td>
<td>55</td>
<td>M</td>
<td>PV</td>
<td>0.73</td>
<td>108.4</td>
<td>28.6</td>
<td>88.7</td>
</tr>
<tr>
<td>7</td>
<td>36</td>
<td>F</td>
<td>ET</td>
<td>1.00</td>
<td>104.9</td>
<td>23.6</td>
<td>124.6</td>
</tr>
<tr>
<td>8</td>
<td>45</td>
<td>M</td>
<td>CML</td>
<td>0.74</td>
<td>88.8</td>
<td>27.1</td>
<td>90.6</td>
</tr>
</tbody>
</table>

Normal ranges (mean ± 2 SD) (n): 1.17 ± 0.68 (31), 101.9 ± 20.0 (7), 30.6 ± 17.5 (7), 133.6 ± 78.1 (7)

Abbreviation: PLO, platelet lipoygenase.

*nmol malondialdehyde/10⁶ platelets.

†ng/10⁷ neutrophils.

‡Platelets of this patient showed a subnormal response to thromboxane A₂ similar to our previous patient (data not shown).
suggested that some platelet-derived factors other than AA per se contributed to activate the 5-lipoxygenase pathway of neutrophils.

Adenosine triphosphate and adenosine diphosphate, major constituents of platelet-dense granules, were demonstrated to activate the 5-lipoxygenase enzyme isolated from guinea pig peritoneal polymorphonuclear leukocytes. However, these granule contents seemed to be of minor importance for the platelet activation of LTB₄ production by neutrophils since supernatant from washed platelet suspension in which the release reaction had been completed by thrombin showed no significant effect on the production of 5-lipoxygenase metabolites (data not shown), although the supernatant might induce production of 12S,20-dihydroxyicosatetraenoic acid. Furthermore, the use of platelets with defective release mechanisms and with normal AA metabolism prepared from patient 6 in Table 2 showed normal stimulating effects on the 5-lipoxygenase metabolism (Table 2).

Since the consistent production of 5-lipoxygenase metabolites required exogenous AA (Fig 2C), it was suggested that AA was necessary as a substrate of the 5-lipoxygenase enzyme as well as for the conversion to a metabolite with the stimulating activity by platelets. However, metabolites via the platelet cyclo-oxygenase pathway seemed to be of little significance for the platelet-induced stimulation because the addition of 500 μmol/L aspirin to the incubation mixture did not reduce the production of 5-lipoxygenase metabolites (data not shown).

The addition of 1 to 10 μmol/L 12-HPETE to neutrophils incubated with FMLP, cytochalasin B and AA stimulated the synthesis of LTB₄, 5-HETE, and 5S,12S-diHETE, whereas its stable derivative, 12-HETE, had no stimulating effect. Similar data were reported by Maclouf et al., whereas its stable derivative, 12-HETE, had no stimulating activity by platelets. However, metabolites via AA was necessary as a substrate of the 5-lipoxygenase enzyme as well as for the conversion to a metabolite with the stimulating activity by platelets. However, metabolites via AA metabolism of the platelet lipoxygenase pathway, AA metabolism of the patients' neutrophils were stimulated by their own platelets in a fashion similar to that in the experiments using normal donors' platelets (data not shown). These findings also confirmed the role of 12-HPETE for the effect of platelets. Since even the lipoxygenase-deficient platelets stimulated the 5-lipoxygenase metabolism to some extent, potentiation of this metabolism by platelets may proceed through various mechanisms, only some of which involve 12-HPETE, which was studied here.

It has been suggested that LTB₄ synthesis plays an important role for some functions of neutrophils, including adhesion, chemotaxis, aggregation, lysosomal enzyme release, and generation of superoxide. Therefore, our results suggest that platelets may contribute to both potentiation of inflammatory reactions and the recruitment of neutrophils to vascular lesions. Thus, synthesis of LTB₄ may play a role in thrombosis and hemostasis. It is also possible that the deficient stimulation of LTB₄ synthesis by lipoxygenase-deficient platelets from MPD patients may contribute to the high incidence of infections and vascular complications of these patients.

REFERENCES

Deficient induction of leukotriene synthesis in human neutrophils by lipoxygenase-deficient platelets

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