Human polymorphonuclear leukocytes (PMN) activated by n-formyl-methionyl-leucyl-phenylalanine (fMLP), in the presence of cytochalasin B, are able to induce activation of coinubulated autologous platelets "via" cathepsin G released from the azurophlic granules. However, thromboxane (Tx) B2 were stimulated by supernatants from fMLP-activated PMN.

Indeed, the amount of TxB2 found in supernatants of platelet/PMN suspensions challenged with 1 μmol/L fMLP was twofold to fourfold higher than that measured when platelets were stimulated by supernatants from fMLP-activated PMN. In the present report, we analyzed the possibility that PMN-induced TxB2 production in this system is the result of transcellular cooperation between cells in the processing of reactive metabolites, potentiating cathepsin G-induced platelet activation.

The aim of this study was to investigate the possibility that the greater production of TxB2 observed when platelets and PMN are activated together forming mixed aggregates could be the result of a transfer of AA (or a metabolite) from PMN to platelets.

**MATERIALS AND METHODS**

**Chemicals.** fMLP, prostaglandin (PG) E1, PGE2, TxB2, leukotriene (LT) Ba, 12-hydroxy-eicosatetraenoic acid (HETE), cytochalasin B, N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES), and ethylene glycol-bis (b-aminethyl ether)-N,N,N'-N'-tetraacetie acid (EGTA) were purchased from Sigma Chemical Co (St Louis, MO). 6-trans-LTB4, 12-trans-LTB4, LTC4, LTE4, 15-HETE, PGD2, and 12-hydroxy-heptadecatrienoic acid (HHT) were purchased from Cayman Chemical Company (Ann Arbor, MI). Dextran T 500 and Ficoll Hypaque were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden); Triton X-100 was obtained from Aldrich Chimica S.r.l. (Milano, Italy); purified human fibrogenin was from Kabi Diagnostica (Stockholm, Sweden); eglin C (recombinant CGP 32968) was kindly provided by Ciba Geigy (Basel, Switzerland); 3H-PGE2, and 3H-TxB2, [5,6,8,9,11,12,14,15-3H (N)]-arachidonic acid (3H-AA), specific activity 180 to 240 Ci/mmol, were from Du Pont de Nemours (Firenze, Italy); thin-layer chromatography (TLC)-plastic sheets silica gel 60 were from Merck (Darmstadt, Germany). fMLP and cytochalasin B were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 50 mmol/L and 100 mmol/L, respectively, stored at -20°C, and diluted in isotonic saline just before use. Egin C was dissolved in saline solution.

**Preparation of platelets and PMN.** PMN and platelets were isolated from citrated human blood and washed as described. Aspirin-treated platelets were obtained by incubating platelet-rich
plasma (PRP) with 400 μmol/L aspirin (acetylsalicylate of lysine, Flectadol; Maggioni, Milan, Italy) for 30 minutes, washing in the presence of 200 μmol/L aspirin, and resuspending in HEPES-Tyrode aspirin-free. PMN suspensions contained more than 95% PMN and less than 1 to 2 platelets/100 PMN.

Experimental procedures. Platelets (10⁹/mL), PMN (0.5 × 10⁷/mL except for the dose-response experiments), or a mixture of the two cells were incubated in a final volume of 1 mL of HEPES-Tyrode (pH 7.4) in the presence of 1 mmol/L Ca²⁺, 0.38 mg/mL fibrinogen, and 2.5 μg/mL cytochalasin B in the aggregometer for 2 minutes at 37°C under constant stirring at 1,000 rpm. Where indicated, eglin C (1 mg/mL) was incubated with the cells 2 minutes before addition of the stimulus. fMLP (1 μmol/L) was added to the cell suspension and the reaction stopped after 3 minutes; samples were then cooled to 0°C for 15 minutes and centrifuged in an Eppendorf centrifuge, and supernatants were collected, and stored at −20°C until assayed for TxB₂. Platelet samples stimulated with PMN supernatants were obtained by addition of 800 μL of supernatants (20-second spin in an Eppendorf centrifuge) from PMN (0.25 to 2 × 10⁷/800 μL) stimulated for 1 minute with fMLP to 10⁸ platelets in a final volume of 1 mL. The production of TxB₂ in these samples is reported in Table 1.

In preliminary experiments, in which fMLP was added to the platelet/PMN mixture (10⁸ platelets/0.5 × 10⁷ PMN/mL) without 2 minutes of prestirring or platelets were added to PMN stimulated for 1 minute with fMLP, TxB₂ production was reduced to 55% ± 17% and 61% ± 17% (respectively) of TxB₂ production in mixed cell suspensions in the standard experimental conditions (mean ± SEM, n = 7). All together, these experiments indicated that platelet-PMN contact is important for the observed increase of TxB₂ production.

TxB₂ determination by radioimmunoassay. TxB₂ was quantitated by radioimmunoassay, using a specific antiserum kindly provided by Prof C. Patrono (Cardiac University, Rome, Italy), as described. The detection limit was 5 pg/mL. TxB₂ values are reported as nanograms per milliliter of incubate.

Preparation of ³H-AA-labeled PMN. PMN (3 × 10⁷/mL) were incubated (30 minutes at 37°C) with 0.25 μCi/mL of ³H-AA, washed twice, and suspended in Tyrode buffer. ³H-AA incorporation determined was 69% ± 4% (n = 12). Experimental conditions used with ³H-AA-labeled PMN were identical to those used with unlabeled cells.

Analysis of ³H-AA release. Aliquots of supernatants from the different samples were immediately counted by liquid scintillation to evaluate the total radioactivity released. To evaluate the release of unesterified ³H-AA in lipophilic membranes of stimulated PMN in the presence or in the absence of platelets, we terminated incubations by adding 3.5 mL of chloroform: methanol (2:5; vol:vol) directly to the cell(s) suspensions, according to Nigam et al., and extracted according to Walsh et al. Briefly, samples were acidified (pH 3.5) by dropwise addition of HCl and the organic and aqueous phases were separated by adding 1 mL chloroform and 1 mL distilled water. After centrifugation at 1,200 rpm for 3 minutes, the organic phases were washed with 1 mL distilled water and centrifuged again, before concentrating the organic phases under N₂. The dried residues were resuspended in 25 μL chloroform and spotted on silica gel plates and TLC developed by using petroleum ether/diethyl ether/acetic acid (70: 30: 1; vol:vol:vol). Unesterified AA was identified by comigration with authentic ³H-AA, which was chromatographed on each TLC plate (Rf: 0.42). The plates were cut from the origin to the solvent front in segments of 5 mm each and counted by liquid scintillation. ³H-AA was calculated and expressed as the percent of radioactivity recovered with the same Rf of authentic standard over total radioactivity recovered from the entire TLC lane.

Analysis of ³H-TxB₂ formation. After taking aliquots to determine total radioactivity released, supernatants of different samples were acidified to pH 4 with citric acid and lipid-extracted twice with chloroform as described, evaporated under nitrogen at 37°C, and stored (−70°C). These samples were analyzed either by TLC or by high performance liquid chromatography (HPLC) as follows. For TLC, dried residues were dissolved in 50 μL of chloroform, spotted on TLC silica gel plates, and developed by using chloroform: methanol: acetic acid/water (90:8:1.5:1.5; vol:vol:vol). ³H-TxB₂ (RF = 0.25), ³H-PGE₂ (RF = 0.60), 12-HETE (RF = 0.68), and ³H-AA (RF = 0.75) were used as standards. The plates were cut from the origin to the solvent front in segments of 5 mm each and counted by liquid scintillation. ³H-TxB₂ was calculated and expressed as the percent of radioactivity recovered with the same RF of authentic standard over total radioactivity recovered from the entire TLC lane. The radioactivity recovered from the samples through lipid extraction and TLC was 85% ± 9% (n = 10).

HPLC. Dried residues were dissolved in 100 μL of methanol: acetonitrile (1:1; vol:vol). The apparatus consisted of a liquid chromatograph (Beckman System Gold; Beckman, San Ramon, CA), equipped with a Diode Array Detector module 168 and a 5-μm reversed phase column (Nucleosil RP-8 25 cm × 4.6 mm i.d.; Chrompack, Mönchenglad, The Netherlands). The mobile phase consisted of two eluents: 50 mmol/L Na₂HP0₄:CH₃CN 62.5:37.5; vol:vol; pH 5.1 (solvent A) and CH₃OH: CH₃COOH 0.1%; 63: 37; vol:vol; pH 5.6 (solvent B). Elution was performed using a combination of the two solvents in a single run of 90 minutes with three major steps (flow-rate 1 mL/min). During the first step, solvent A was used for 20 minutes. In the second step, a 10-minute linear gradient with solvent B was performed from 0% to 100% B. The third step was isocratic at 100% B up to 90 minutes. Standards and samples were shown at 205 nm from 0 to 23 minutes, at 270 nm from 23 to 45 minutes, and at 236 nm from 43 to 90 minutes. TxB₂, PGE₂, LTC₄, LTE₄, 12-trans-LTB₄, 6-trans-LTB₄, LTB₄, 12-HHT, PGD₂, 15-HETE, and 12-HETE were used as authentic standards and showed a retention time of 14.2, 19.5, 25.3, 40, 41.4, 42.4, 43.6, 47.5, 53.1, 74.4, and 81.3 minutes, respectively. The eluate collected in fractions every 24 seconds was counted for radioactivity determination.

RESULTS

TxB₂ production was measured in PMN and platelets challenged with fMLP either separately or coincubated. Only when platelets and PMN were coincubated in mixed cell suspensions was TxB₂ production measurable (not shown). In these conditions, platelet activation induced by fMLP-activated PMN is essentially mediated by released

<table>
<thead>
<tr>
<th>Table 1. TxB₂ Production (ng/mL) in Samples of Platelet/PMN Suspensions or Platelets Activated by PMN-Derived Supernatants After Stimulation With 1 μmol/L fMLP</th>
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<tbody>
<tr>
<td>PMN (×10⁷/mL)</td>
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<td>----------------</td>
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<tr>
<td>0.25</td>
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<tr>
<td>0.50</td>
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<tr>
<td>1.00</td>
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Values are means ± SEM (n = 9 to 17).

*P < .05 significantly different from the corresponding sample of platelets activated by PMN-derived supernatants by ANOVA and Tukey test.

1P < .01 significantly different from the corresponding sample of platelets activated by PMN-derived supernatants by ANOVA and Tukey test.
PLATELET-PMN TRANSCELLULAR METABOLISM OF AA

The amount of TxB\(_2\) found in supernatants of mixed platelet/PMN suspensions challenged with 1 \(\mu\)mol/L fMLP was twofold to fourfold higher than that measured when platelets were stimulated by supernatants from fMLP-activated PMN (Table 1). These results suggest that TxB\(_2\) production in this system cannot be completely explained by cathepsin G-stimulated platelet arachidonate metabolism.

\([\text{3H}]\)-labeled PMN were used to test if a transfer of AA or metabolite(s) could occur from PMN to platelets. Radioactivity released by PMN stimulated with fMLP was 5.0% ± 0.4% (n = 7) of total radioactivity incorporated, and increased to 9.3% ± 2.7% (n = 10) in the presence of platelets (\(P < .05\)). When platelet/PMN samples were stimulated by fMLP, 15% ± 3% (n = 10) of total radioactivity recovered from TLC migrated with the same \(R_f\) of authentic TxB\(_2\). In contrast, no detectable \([\text{3H}]\)-TxB\(_2\) was observed when \([\text{3H}]\)-AA–PMN were activated in the absence of platelets or when platelets were activated by supernatants from fMLP-activated \([\text{3H}]\)-AA–PMN or when \([\text{3H}]\)-AA–PMN and platelets were incubated without fMLP (Table 2). In the same samples, no detectable radioactivity cochromatographed with standard \([\text{3H}]\)-AA (\(R_f\) 0.75).

Because no detectable \([\text{3H}]\)-AA was present in aqueous supernatants of fMLP-activated PMN alone or in the presence of platelets, the membrane content of unesterified \([\text{3H}]\)-AA was analyzed in the same experimental conditions. A time-course experiment (Fig 1) shows a maximal release of unesterified \([\text{3H}]\)-AA at 1 minute after fMLP activation. Measurable unesterified \([\text{3H}]\)-AA was reduced by the presence of platelets (\(P < .05\) only at 180 seconds). This finding is not in contrast with the increased total radioactivity measured in supernatants of activated platelet/PMN in respect to supernatants of activated PMN alone over reported, because an increased metabolism of PMN-derived \([\text{3H}]\)-AA by platelets or PMN could occur in the presence of platelets.

Figure 2 reports the HPLC analysis of radioactive AA metabolism by fMLP-activated PMN in the presence and in the absence of platelets. The most striking difference in the AA metabolites profiles obtained in the presence and in the absence of platelets was constituted by the appearance in the former condition of a large peak eluted with the same retention time of authentic TxB\(_2\). No detectable radioactivity was eluted with retention time corresponding to that of 12-HETE, while the presence of radioactivity with retention time corresponding to that of 12-HHT was detectable only in samples of PMN incubated in the presence of platelets.

\([\text{3H}]\)-TxB\(_2\) peak was abolished by aspirin treatment of platelets (Fig 3), a finding in agreement with TxB\(_2\) measurement by radioimmunoassay in comparable nonradioactive samples. Indeed, production of immunoreactive TxB\(_2\) in platelets/PMN suspensions challenged with fMLP was virtually abolished in samples of aspirin-treated platelets (from 44.2 ± 14.4 to 0.6 ± 0.3 ng/mL; \(n = 3\)). These results indicate that PMN contribute to TxB\(_2\) production by providing platelets with unmetabolized AA and suggest an essential role for platelet cyclooxygenase in TxB\(_2\) production by mixed cells.

To test if cathepsin G-induced platelet activation is an essential step for transcellular AA metabolism, we performed experiments in the presence of the specific inhibitor of cathepsin G, eglin C.11 Eglin C did not affect platelet TxB\(_2\) production induced by 0.05 to 10 \(\mu\)mol/L AA concentrations (not shown), but completely suppressed \([\text{3H}]\)-TxB\(_2\) production in \([\text{3H}]\)-AA–PMN–platelet system activated by fMLP (Fig 3). This finding suggests an essential role for cathepsin G-induced platelet activation in TxB\(_2\) synthesis from PMN-derived AA.

**DISCUSSION**

Human PMN activated with fMLP stimulate TxB\(_2\) production in coincubated autologous platelets. In these conditions, we observed platelet-leukocyte transcellular metabo-

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**Table 2. Formation of \([\text{3H}]\)-TxB\(_2\) From PMN-Derived \([\text{3H}]\)-AA by Platelets**

<table>
<thead>
<tr>
<th>Sample</th>
<th>([\text{3H}])-TxB(_2) (cpm) ± SEM</th>
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<tr>
<td>PMN + fMLP</td>
<td>38 ± 15</td>
</tr>
<tr>
<td>Platelets/PMN + fMLP</td>
<td>1,619 ± 227</td>
</tr>
<tr>
<td>Platelets + supernatant from fMLP-treated PMN</td>
<td>80 ± 20</td>
</tr>
<tr>
<td>Platelets/PMN</td>
<td>80 ± 20</td>
</tr>
<tr>
<td>Platelets + fMLP</td>
<td>30 ± 10</td>
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</tbody>
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Results (mean ± SEM from 10 different experiments) are expressed as cpm corrected to 100% of recovery from TLC plates and to 1 x 10\(^4\) cpm spotted. \([\text{3H}]\)-labeled PMN (0.5 x 10\(^3\)/mL) were stimulated with fMLP in presence of cytochalasin B and fibrinogen. Three minutes later, the reaction was stopped, lipids extracted, separated by TLC along with authentic standards, and identified areas were scraped and quantified by scintillation counting.
When PMN labeled with \(^3\)H-AA and unlabeled platelets were stimulated with fMLP, \(^3\)H-TxB\(_2\) was formed. This was evident both with TLC and HPLC. HPLC analysis of \(^3\)H-AA metabolites also evidenced radioactive material comigrating with 12-HHT only in fMLP-activated mixed cells samples. The presence in the chromatographic profiles of this metabolite strengthened the finding of platelet cyclooxygenase-dependent metabolism of PMN-derived \(^3\)H-AA. No detectable radioactivity was eluted with retention time corresponding to 12-HETE, indicating that platelet...

The background observation was the marked difference in TxB\(_2\) production between platelets activated by supernatants from fMLP-stimulated PMN and platelets activated by PMN in mixed cell suspensions. In both cases, cathepsin G released by activated PMN was responsible for platelet activation.\(^2,3\)

![HPLC analysis of \(^3\)H-AA metabolites](image-url)

Fig 2. HPLC analysis of \(^3\)H-AA metabolites. (A) Standards of different AA metabolites from 1 to 11: TxB\(_2\), PGE\(_2\), LTC\(_4\), LTE\(_4\), 12-trans-LTB\(_4\), 6-trans-LTB\(_4\), LTBr\(_2\), 12-HHT, PGD\(_2\), 15-HETE, and 12-HETE. (B) \(^3\)H-AA–labeled PMN/platelets mixed cell suspensions. (C) \(^3\)H-AA–labeled PMN. PMN (0.5 \times 10\(^7\)/mL) were stimulated by fMLP in the presence of cytochalasin B and fibrinogen, in the presence or in the absence of 1 \times 10\(^6\)/mL platelets. The reaction was stopped 3 minutes after stimulus, lipid extracted, and injected into RP-HPLC column, and radioactivity of fractions was quantified by scintillation counting (n = 5). For further details, see Materials and Methods.

![HPLC analysis of \(^3\)H-AA metabolites: effect of aspirin and eglin C](image-url)

Fig 3. HPLC analysis of \(^3\)H-AA metabolites: effect of aspirin and eglin C. (A) \(^3\)H-AA–labeled PMN/platelets mixed cell suspensions (control). (B) \(^3\)H-AA–labeled PMN/aspirin-treated platelets. (C) \(^3\)H-AA–labeled PMN/platelets preincubated with 1 mg/mL of eglin C. For (B), platelets in PRP were incubated with 400 \(\mu\)mol/L aspirin for 30 minutes, washed in the presence of 200 \(\mu\)mol/L, and finally resuspended in HEPES-Tyrode as in control samples. For (C), eglin C was added to the mixed cell suspension 2 minutes before the addition of fMLP (n = 3). For further details, see Materials and Methods and the legend to Fig 2.
lipoxygenase is not involved in using PMN-derived $^3$H-AA in these conditions. The release of total radioactivity measured in the supernatants of fMLP-activated PMN was significantly higher in the presence of platelets. Because the amount of unesterified $^3$H-AA was reduced in the presence of platelets, an increased metabolism of PMN-derived AA could explain the higher total radioactivity recovered in supernatants of mixed cell suspension. Our data indicate that platelets provide a new metabolic pathway for PMN-derived AA only through cyclooxygenase metabolism, as indicated by $^3$H-TxB$_2$ and 12-HETE production. On the other hand, previous reports indicate that platelets stimulate metabolism of AA by PMN. The experiments with aspirin-treated platelets clearly indicate that fMLP-activated PMN contribute to TxB$_2$ production in mixed cell suspensions by providing unmetabolized AA to activated platelets.

Moreover, inhibition of cathepsin G-induced platelet activation with the antiprotease eglin C blocked the formation of $^3$H-TxB$_2$ from PMN-derived $^3$H-AA. Indeed, platelet activation is required for the formation of mixed platelet/PMN aggregates, in which tight membrane to membrane contact, shown by electron microscopy, would favor unesterified AA transfer in a lipophilic environment.

REFERENCES


Other pathways of transcellular metabolism between platelets and PMN have also been reported. In particular, activated PMN can transform 12-HETE produced by activated platelets into 5S,12S di-HETE$^{12,13}$ or 12, 20 di-HETE from resting neutrophils.$^{14,15}$ On the other hand, platelets are able to transform LTA$_4$ derived from neutrophils into LTC$_4$.$^{16,17}$ or lipoxins.$^{6,16,18}$

To the best of our knowledge, this is the first description of a platelet-PMN transcellular interaction in which platelets use AA released from fMLP-activated PMN to synthesize TxB$_2$. In this experimental system, the activation of PMN not only results in activation of platelets "via" released cathepsin G, but also makes available AA to contiguous platelets, resulting in increased TxB$_2$ production.

Our results suggest a novel mechanism by which PMN might activate platelet function that could be relevant for thrombotic and inflammatory processes.

ACKNOWLEDGMENT

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